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DEVELOPMENT OF AN INITIATION/ PROMOTION ASSAY TO DETECT FOCI OF ENZYME-ALTERED HEPATOCYTES

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The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

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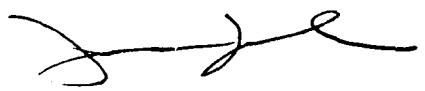

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Initiation with the potent carcinogen diethylnitrosamine followed by promotion with phenobarbital resulted in detectable foci at the end of six weeks. Quantitation of these foci by image analysis stereological techniques indicated that staining for gamma-glutamyltranspeptidase-positive foci was the best marker for the presence of enzyme-altered foci produced in response to a genotoxic carcinogen.

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PREFACE

This is one of a series of technical reports describing results of the experimental laboratory programs conducted at the Toxic Hazards Research Unit, NSI Technology Services Corporation-Environmental Sciences. This document serves as a final report on the development of procedures to conduct an initiation/promotion assay. The research described in this report began in August 1988 and was completed in December 1989 under U S. Air Force Contract No. F33615-85-C-0532. Melvin E. Andersen, Ph.D., served as Contract Technical Monitor for the U.S. Air Force, Harry G. Armstrong Aerospace Medical Research Laboratory.

The animals used in this study were handled in accordance with the principles stated in the *Guide for the Care and Use of Laboratory Animals*, prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council, Department of Health and Human Services, National Institute of Health Publication #86-23, 1985, and the Animal Welfare Act of 1966, as amended.

The opinions contained herein are those of the authors and are not to be construed as official or reflecting the views of the Department of the Air Force. The use of trade names in this report does not constitute an official endorsement or approval of the use of such commercial hardware or software. This report may not be cited for purposes of advertisement.

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ABBREVIATIONS

ATPase	Adenosine triphosphatase
cm	Centimeter
CTFE	Chlorotrifluoroethylene
DEN	Diethylnitrosamine
EAF	Enzyme-altered foci
g	Gram
GGT	Gamma-glutamyltransferase
G6Pase	Glucose-6-phosphatase
GST-P	Glutathione S-transferase (placental)
h	Hour
H&E	Hematoxylin and eosin
Kg	Kilogram
µm	micrometer
mg	Milligram
min	Minute
mL	Milliliter
mm	Millimeter
N	Normal
ODC	Ornithine decarboxylase
P	Probability
PAS	Periodic acid/Schiff
PB	Phenobarbital
SEM	Standard error of the mean
SOP	Standard operating procedure

SECTION 1

INTRODUCTION

The progression of biochemical and morphological changes that occur following the transformation of a normal cell to a malignant cell have been poorly understood. There have been nine well-documented experimental protocols that have been developed and used to assess hepatocarcinogens (Farber and Sarma, 1987). One of these models has described a two-stage development of hepatocarcinogenesis in which a limited exposure to a potential carcinogen (initiation) is followed by a prolonged exposure to a second agent (promotion)(Peraino et al., 1973). Evidence has indicated that stimulation of cellular proliferation is required before the initiated cell can be detected (Cayama et al., 1978; Columbano, 1981), resulting in the occurrence of enzyme-altered foci (EAF).

One method described by Pitot et al. (1978) and Sirica et al. (1978) has shown that, following partial hepatectomy to induce cell proliferation, initiation with a single dose of diethylnitrosamine (DEN) followed by several weeks of promotion with phenobarbital (PB) resulted in an increased prevalence of altered foci, usually measured as gamma-glutamyltransferase (GGT)-positive foci. Only those animals receiving both DEN and PB developed hepatocellular carcinomas, thereby clearly distinguishing between the initiation and promotion stages of hepatocarcinogenesis.

GGT-positive foci have been used widely as a marker for the detection of EAF and appear to be a dependable marker in the rat liver (Pugh and Goldfarb, 1978). While GGT-positive foci have been widely used, other enzyme markers have been used, such as adenosine triphosphatase (ATPase)-deficient foci, glucose-6-phosphatase (G6Pase)-deficient foci, and foci with decreased ability to accumulate iron. Recently, the placental form of rat glutathione S-transferase (GST-P) was shown to be elevated markedly in the liver following the administration of DEN (Sato et al., 1984), and more recently single cells containing elevated GST-P levels were detected within 48 h of the administration of DEN (Moore et al., 1987).

In addition to the histochemical markers discussed, changes in certain enzyme activities have been demonstrated following promotion. The induction of ornithine decarboxylase (ODC) has been associated with tumor promotion in the liver (Olson and Russell, 1980). Induction of ODC activity has been correlated with tumor promotion and appears to be a useful enzymatic marker. Changes in peroxisomal enzymes involved in fatty acid beta-oxidation have been shown to occur following exposure to such compounds as hypolipidemic agents and industrial plasticizers. Some of these compounds have been reported to produce liver tumors in rats (Lalwani et al., 1981; Reddy et al ,1980).

The U.S. Air Force has requested that the following study be designed to establish and verify an initiation/promotion model based on the techniques described by Parnell (1986), in which a partial hepatectomy was followed 24 h later by an injection of DEN (10 mg/Kg) and promotion with 0.05% PB for several weeks.

SECTION 2

MATERIALS

TEST AGENTS

DEN (purity >98%) was supplied by Sigma Chemical Company, St. Louis, MO. A 1 mg/mL solution of DEN in normal saline was prepared by adding 99 mL of normal saline directly into the sealed vial via a rubber septum. All animal doses were removed via syringe through the septum.

Pertinent physical characteristics:

Synonym	N-nitrosodiethylamine	
CAS Reg. No.	55-18-5	
Vapor Pressure, mmHg	20°C	0.81
	40°C	3.10
Specific Gravity (g/mL)	0.942	

PB (purity 99%) was supplied by Sigma Chemical Company, St. Louis, Mo. Solutions of PB (0.05% in the drinking water) were prepared by adding 4N sodium hydroxide until all PB dissolved. The pH of the solution was then adjusted to 7.4 by the addition of 4N hydrochloric acid.

Pertinent physical characteristics:

CAS Reg. No.	50-06-6
Melting Point	174-178°C
Solubility	water-soluble

ANIMALS

Male Sprague-Dawley rats (three weeks of age) were purchased from Charles River Breeding Laboratories, Kingston, NY. Upon receipt, the animals were quarantined in a single laminar-flow unit. The animals were group housed (four/cage) in plastic cages containing hardwood-chip bedding prior to surgical procedures and given a commercial diet (Purina Formula 5008) and water *ad libitum*.

Following surgery, animals were housed singly. The animal room temperatures were maintained at 21° to 25°C and the light/dark cycle was set at 12-h intervals.

SECTION 3

EXPERIMENTAL APPROACH

Surgery and Administration of Test Chemicals

At 4 weeks of age all animals were anesthetized with isoflurane vapor and subjected to a two-thirds partial hepatectomy using the procedure of Higgins and Anderson (1931) (See Appendix A, SOP #4300-937). Twenty-four hours later, five animals received intraperitoneal injections of DEN (10 mg/kg body weight) in saline, while the control group received injections of saline (1 mL/kg). Two weeks following this injection, animals treated with DEN were given PB (0.05% in the drinking water) for the next four consecutive weeks. At the conclusion of the study all animals were euthanatized by CO_2 asphyxiation and terminal whole animal and liver weights were obtained from each animal.

Histological and Histochemical Procedures

Immediately after death, the liver was excised, weighed, and the liver lobules bisected. Sections of the right anterior and posterior lobes were taken for fixation in the following fixatives: buffered neutral formalin for routine hematoxylin and eosin (H&E) staining, alcoholic buffered formalin for glycogen staining, and acetic acid/formalin containing sodium sulfate for iron staining. Following fixation, each portion of tissue was embedded in paraffin and a five-micron section was prepared from three distinct areas (front, middle and back) within each block of tissue and stained. Routine hematoxylin and eosin staining was performed on tissue submitted for fixation in buffered neutral formalin. Pieces of tissue fixed in alcoholic formalin were stained for the presence of glycogen using the periodic acid/Schiff (PAS) reaction described by Bedi and Horobin (1976) (See Appendix A, SOP #4300-4033). Iron staining was accomplished using the Prussian Blue procedure described by Hirota and Williams (1979) (Appendix A, SOP #4300-4034).

A separate portion of liver from each of the lobes described above was frozen and six serial frozen sections (10 μm thick) were prepared from three separate areas (front, middle and back) within each portion of liver. Adjacent sections cut from the three different areas within each of the blocks of tissue were stained according to the procedures listed below. One section was stained for the presence of GGT activity using the method described by Rutenburg et al. (1969) (Appendix A, SOP #4300-4032). An adjacent section was stained for the presence of ATPase activity according to the method described by Wachstein and Meisel (1957) (Appendix A, SOP #4300-4031). Another adjacent

section was also stained for the presence of G6Pase activity by the method described by Wachstein and Meisel (1958) (Appendix A, SOP #4300-4030)

Image Analysis

Following staining and coverslipping, all slides were examined for the presence of foci. All foci were counted directly using a HIPAD digitizing tablet (Houston Instruments, Austin, TX) coupled optically to the microscope, and were identified as those areas containing nine or more nuclei. The tissue area, number of foci, and foci area all were recorded. The numbers of foci per unit area and volume of liver, the percent foci volume, mean focus area, and mean focus volume were calculated by the stereological procedures of Campbell *et al*.,(1982) (Appendix A, SOPs #4300-4038, 4300-4039, 4300-4040).

Enzyme Studies

For the demonstration of ODC activity, a 20% homogenate of liver was prepared from a portion of liver removed from the right lobule of each animal. All enzyme activities were assayed using a cytosolic preparation (100,000 x g, 1 h) and normalized to protein content. ODC activity was determined using the procedure of Bethell and Pegg (1979) (Appendix A, SOP #4300-4027). Protein was determined by the method of Bradford (1976).

Another portion of liver was removed from the right lobule, weighed, and placed in ice-cold 0.25 M sucrose. The cyanide-insensitive peroxisomal beta-oxidation of the palmitoyl Coenzyme A procedure of Lazarow (1982) (Appendix A, SOP #4300-4036) was performed on a 1500 x g supernatant fraction of a 20% liver homogenate prepared in 0.25 M sucrose. The initial rate of oxidation was expressed as the amount of nicotinamide adenine dinucleotide formed per minute, and the rate was normalized to gram of liver.

Statistics

Body weights, relative liver weights, and enzyme data were compared by means of the two-sample independent Student's t-test ($p<0.05$). Foci and related parameters were compared by means of the one-factor Multivariate Analysis of Variance for Repeated Measures Test for missing data (Dixon 1985) ($p<0.05$).

SECTION 4

RESULTS

Body and Liver Weight

Mean terminal body weights of the animals in the treated group were not significantly different from those of control (389.4 ± 14 3 and 380 2 ± 16.3 grams, respectively) The relative liver weight of the animals receiving PB as the tumor promoter were significantly higher than control (6 85% and 5 11%, respectively; p = 0 0005)

Enzyme Data

A significant difference ($p < 0.05$) in the activity of ODC between test and control groups (4.77 ± 1 45 and 2 24 ± 1 09 pmol/0 5 h/mg protein, respectively) was observed. No significant differences were found in the rate of beta-oxidation between the animals in the test and control groups (1.75 ± 0 17 µmol/min/g and 1 77 ± 0 35 µmol/min/g.

Enzyme Altered Foci

No foci could be detected in liver sections stained with H&E or in sections stained by the PAS technique Quantitation of foci from sections stained with the four other methods was accomplished and the results of stereological analyses are presented in Table 1. The number of foci per cubic centimeter detected by each of the staining methods in the treated animals was significantly different from that of control animals Differences in the number of foci detected by the different staining methods in the animals comprising the treatment group were not statistically significant due to large standard errors Staining for the presence of G6Pase-deficient foci initially appeared to be the most sensitive marker of EAF, however, the value obtained for the number of foci was misleading because one of the animals in the study had a high number of G6Pase-deficient foci. The value obtained for iron-deficient foci per cubic centimeter also was misleading because two animals had very high numbers of foci while two had much lower numbers of foci. Quantitation of ATPase-deficient foci yielded consistently lower numbers of foci than the other three methods, which may have been due to the very weak staining observed with this method that caused difficulties with detection GGT-positive foci appeared to be the most sensitive marker for EAF in this study because the numbers of foci were consistently higher than those detected by other methods and the foci were easier to detect

Significant differences in the volume of the liver occupied by foci (percent foci volume) were found between animals in the treated group and those of the control group in the case of each of the different staining methods There were no significant differences in the percent foci volume in animals from the treated group when the different staining methods were compared.

There were significant differences in the size of foci in animals in the treated group that were detected by each method. The foci that were positive for GGT were significantly smaller in area and volume than foci detected by iron staining, but they were not significantly different than foci detected by staining for G6Pase or ATPase activity. The foci detected by iron staining were nearly twice the size of GGT-positive foci and occupied nearly three times the volume. The mean area and volume of the G6Pase-deficient foci were not significantly different than the mean area and volume of ATPase-deficient foci but were only half the size and volume of iron-deficient foci.

Table 1. Quantitation[a] of DEN-Initiated Enzyme-Altered Foci in Male Sprague Dawley Rats Stained for the Presence of GGTase-positive Foci, ATPase-Deficient Foci, G6Pase-Deficient Foci, and Iron-Deficient Foci.

STAIN	TREATMENT	N	FOCI/cm^3	%FOCI VOL	MEAN FOCUS AREA (mm2)	MEAN FOCUS VOLUME (mm3)
GGT	DEN PB	5	122.6 ± 15.2[b]	0.075 ± 0.017[b]	0.025 ± 0.005[b,d]	0.007 ± 0.002[b,d]
	CONTROL	5	19.3 ± 7.9	0.010 ± 0.005	0.017 ± 0.005	0.004 ± 0.002
IRON	DEN PB	4	101.7 ± 36.9[b]	0.168 ± 0.070[b]	0.049 ± 0.004[b]	0.017 ± 0.002[b]
	CONTROL	4	10.1[c]	0.020[c]	0.055[c]	0.02[c]
G6Pase	DEN PB	4	118.2 ± 56.4[b]	0.143 ± 0.093	0.027 ± 0.007[d]	0.008 ± 0.002[d]
	CONTROL	4	n.d.	n.d.	n.d.	n.d.
ATPase	DEN PB	4	51.8 ± 13.2[b]	0.063 ± 0.016	0.040 ± 0.006	0.015 ± 0.004
	CONTROL	4	n.d.	n.d.	n.d.	n.d.

[a] Values represent the mean of N determinations ± 1 SEM
[b] Significantly different than control at $p < 0.01$, as determined by the Multivariate Analysis of Variance for Repeated Measures Test
[c] Only one animal in the group had detectable foci
[d] Significantly different from iron group at $p < 0.01$, as determined by the Multivariate Analysis of Variance for Repeated Measures Test
n.d. = None detected

SECTION 5

DISCUSSION

The most commonly used markers for EAF have included the deficiency of G6Pase activity, the deficiency of membrane-bound ATPase activity, increased GGT activity (Farber and Cameron, 1980; Bannasch, 1986), resistance to accumulation of iron (Williams, 1982), and increased glycogen storage (Bannasch, 1986). GGT staining has been the most widely used for quantitation of foci (Hanigan and Pitot, 1985; Hendrich and Pitot, 1987) because of the ease with which the foci are visualized and because 90 to 95% of the EAF produced in various rat liver experimental carcinogenesis protocols models are GGT positive (Tatematsu et al., 1980; Goldfarb and Pugh, 1981). The large number of GGT-positive foci that have been observed in these studies have the advantage of increasing

sensitivity, but by themselves cannot predict tumor incidence. Before any predictions can be made, a quantitative relationship between the incidence of foci and tumor incidence should be determined for both the protocol and the class of potential carcinogen under investigation. GGT-positive foci are not produced in response to all initiators and promoters and this serves as a further limitation of this marker. WY-14,643, the most potent carcinogenic peroxisome proliferator, does not induce GGT in either EAF, nodules or tumors (Rao *et al.*, 1982). While PB has induced GGT activity preferentially in EAF in rats (Pereira *et al.*, 1984), other chemicals might also induce GGT activity in EAF without an increased tumor incidence.

Iron-deficient foci have been shown to be nearly as consistent a marker as GGT (Williams, 1982). The least prevalent of the markers within EAF has been shown to be the loss of G6Pase (Hirota and Williams, 1979; Ogawa et al., 1980). Deficiency of this enzyme almost always has been associated with either increased GGT or a deficiency of ATPase (Ogawa et al., 1980). Recently, Sato et al. (1984) reported a remarkable increase in the enzyme GST-P in the liver of rats treated with hepatocarcinogens. Foci containing this enzyme have been demonstrated by immunohistochemical methods and a comparison between foci staining positive for both GST-P and GGT has revealed GST-P to be the most effective marker for DEN-initiated lesions. The antibody to GST-P has not been widely available, and, therefore staining for this marker in the present study was not accomplished.

The present study has shown that GGT-positive foci are produced in response to DEN initiation and that they are the best choice for the demonstration of foci produced in response to DEN. The numbers of GGT-positive foci per square centimeter produced in response to DEN and PB in the present study (2.9 ± 0.36, data not shown) were similar to those reported by other investigators at the same time point and using a similar protocol (Tatematsu et al, 1979; Goldsworthy et al., 1984). Iron-deficient foci also were shown to be a good marker in the present study. However, quantitation of these foci was not consistent from animal to animal. Two animals had foci that were comparable in number to those detected in liver sections stained for GGT-positive foci, while two had substantially lower numbers of foci. In the present study ATPase-deficient foci were found to be the least detectable, which is not in agreement with the findings of Hirota and Williams (1979) and Ogawa et al. (1980), who observed that G6Pase-deficient foci were the least prevalent marker for EAF. The difficulty in quantitation of ATPase-deficient foci may have been due, in part, to the drying of slides during storage at -40°C prior to staining. Although foci characterized by excessive storage of glycogen have been reported to be one of the earliest detectable markers (Bannasch et al., 1980), foci showing an increase in glycogen were not detectable in the present study. This was most likely the result of masking of the glycogen by the counterstain.

The use of foci per cubic centimeter is a good estimate of the quantity of foci in a liver, but the quantitative stereological formulas used to derive this value assume that the foci are spherical in

shape. This is not the case in rats, because foci in this animal model vary widely and tend to be irregular. Therefore, the mathematical derivation of the number of foci per cubic centimeter is only an estimate of the true number. In addition to the value of foci per cubic centimeter other, values such as mean area, mean volume, and percent foci volume can also be mathematically derived. These derived values have been used in the determination of growth kinetics of foci. The use of these values has been recently employed to examine differences in promoting activity of PB and WY 14,643 in rat liver (Cattley and Popp, 1989). In that study, WY 14,643 was found to be more efficient than PB in promoting hepatocarcinogenesis in DEN-initiated rats because of the ability to cause the formation of larger foci occupying larger percentages of the liver.

The activities of two enzymes were evaluated in this study. The elevation of the enzyme ODC has served as a unique, early marker of the action of complete chemical carcinogens or tumor promoters (Olson and Russell, 1979;1980). DEN administered as a single dose has been shown to cause elevation in ODC activity within hours, but this elevation is not sustained unless a large dose of DEN is administered. DEN (200 mg/kg) resulted in a 8- to 14-fold elevation of liver ODC activity for only seven days following a single dose (Olson and Russell, 1979). Although PB has been shown to stimulate ODC induction (Pereira et al., 1982) a long-term study of dietary PB as a promoter of DEN-induced hepatic carcinogenesis failed to demonstrate elevated ODC activity at any point in the study (Farwell et al., 1978). In the present study significant differences were found in the activity of ODC in the two groups of animals, but the difference in ODC activity, while statistically significant, was probably not biologically significant because much greater increases in ODC have usually been observed with tumor promoters.

The second enzyme activity examined in this study was the peroxisomal beta-oxidation of palmitoyl CoA. Elevations of this enzyme activity have been correlated with an induction of peroxisomes (Stott, 1988). The chronic administration of chemicals causing peroxisomal proliferation has been associated with the development of malignant hepatic tumors (Reddy et al., 1980). Although DEN and PB have not been shown to be peroxisomal proliferators in this study and those of others (Cattley and Popp, 1989), the enzyme assay to detect this phenomenon was included in the study because future initiation/promotion studies will be conducted using compounds that may be peroxisomal proliferators. The ability to correlate the degree of enzyme induction with formation of detectable foci will be essential to the understanding of the mechanism of tumor formation by peroxisome proliferators.

The methods described by Parnell (1986) have been successfully employed in the development of a positive control for future initiation/promotion studies. In addition to staining of foci for GGT, several other staining methods were implemented. These methods have shown that initiation with DEN results in the formation of detectable EAF after four weeks of promotion with PB. The

procedures implemented in this study currently are being used to assess the carcinogenic potential of CTFE trimer acid.

SECTION 6

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SECTION 7

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APPENDIX A

NSI TECHNOLOGY SERVICES CORPORATION

ENVIRONMENTAL SCIENCES
TOXIC HAZARDS RESEARCH UNIT

STANDARD OPERATING PROCEDURE

PARTIAL HEPATECTOMY

AUTHOR: P. A. Deiser

OBJECTIVE/PURPOSE: The objective is to successfully surgically remove 60 to 70% of the liver of the rat.

1.0 MATERIALS:

1.1 Scalpel (#3) and blade (#15)
1.2 Tissue forceps
1.3 Blunt/sharp scissors
1.4 Needle holders
1.5 Single armed surgical chromic gut (4.0)
1.6 Unarmed surgical silk (0)
1.7 9mm wound clips
1.8 Wound clip applicator
1.9 48" surgical drape
1.10 3" eye drape
1.11 18" surgical drape for pedestal
1.12 Sterile 4x4's
1.13 5% Novalsan® or 3% Isosporin
1.14 Rodent heated pedestal
1.15 Heated floor pads with recovery cages
1.16 Heated recovery unit

2.0 PROCEDURES:

2.1 Prepare the animal for surgery (after light isoflurane anesthesia) by shaving the ventral abdominal area from approximately the xyphoid process to the pubic symphysis.

2.2 Wash the shaved area with 4x4 surgical sponge liberally saturated with Wescodyne®.

2.3 Place the animal in the anesthesia chamber at 5% isoflurane until a sufficient level of anesthesia has been reached (i.e., no toe pinch reflex).

2.4 Remove the animal from the chamber and position the anesthesia cone securely in place. While maintaining a flow rate of O_2 and isoflurane

equal to 5% isoflurane, place animal on the heated pedestal and apply leg restraints.

2.5 With the rat in dorsal recumbency (belly side up), locate the xyphoid process.

2.6 Make a caudal dermal incision approximately 2.5 cm long. Grasping the peritoneum with tissue forceps, lift upwards and make a horizontal incision with a pair of scissors. Return scissors to a vertical plane and incise along the lineae alba (midline) to just above or just below the xyphoid process, thus exposing the viscera.

2.7 Thus exposed, the median lobe of the liver and the left lobe are easily delivered. The connecting ligaments are snipped and the liver is securely ligated by 0 surgical silk and excised.

2.8 The peritoneum and abdominal muscles are closed by simple interrupted stitches with 4.0 chromic gut.

2.9 Upon closing the first layer cease to administer anesthesia and close integument with 9 mm wound clips.

2.10 All surgeries are carried out with careful asepsis throughout. Following surgery, rats are placed on heated floor pad with recovery cages. After 30 minutes-1 hour, place the cages in pre-warmed recovery unit with dextrose/saline solution given "ad libitum" and observed closely for 48 hours.

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ENVIRONMENTAL SCIENCES
TOXIC HAZARDS RESEARCH UNIT

STANDARD OPERATING PROCEDURE

ORNITHINE DECARBOXYLASE ASSAY

AUTHOR: C. S. Godin

PURPOSE: This standard operating procedure describes the procedure for the routine determination of the activity of ornithine decarboxylase in homogenates prepared from rat tissues.

1.0 PREPARATION OF STOCK BUFFER

1.1 Dissolve 2.84 g sodium phosphate dibasic in sufficient distilled water to make a final volume of 0.5 L. This results in a 40 mM stock solution. Refrigerate at 4° C.

1.2 Dissolve 1.09 g potassium phosphate monobasic in sufficient distilled water to make a final volume of 0.2 L. This results in a 40 mM stock solution. Refrigerate at 4° C.

1.3 Dissolve 7.6 g tetrasodium EDTA in suffucient distilled water to make a final volume of 0.1 L. This makes a 200 mM stock solution.
1.3.1 Adjust the pH to 7.2 with 1N HCl and refrigerate at 4° C.

1.4 Dissolve 2.1 g sodium fluoride in sufficient distilled water to make a final volume of 0.1 L. This results in a 500 mM stock solution. Refrigerate at 4° C.

1.5 Mix the above solutions in the following order.
1.5.1 337.5 mL of the 40 mM sodium phosphate dibasic solution.
1.5.2 162.5 mL of the 40 mM potassium phosphate monobasic stock solution.
1.5.3 0.5 mL of the 200 mM EDTA stock solution.
1.5.4 10.0 mL of the 500 mM sodium fluoride solution.
1.5.5 489.5 mL distilled water.
1.5.6 Measure the pH and adjust to 7.2 with 1N NaOH or 1N HCl as necessary.

2.0 PREPARATIONN OF HOMOGENIZING AND ASSAY BUFFER

2.1 Dissolve 0.154 g dithiothreitol in sufficient distilled water to make a final volume of 0.01 L. This results in a 100 mM stock solution.
2.1.1 Divide into 1 mL aliquots and freeze at -20° C.

2.2 Dissolve 0.124 g pyridoxal phosphate in sufficient distilled water to make a final volume of 0.25 L. This results in a 2 mM stock solution. Refrigerate at 4° C.

2.3 Dissolve 0.087 g phenylmethylsulfonylfluoride (PMSF) in 5 mL absolute ethanol. This results in a 0.5 mM stock solution.
2.3.1 Divide into 0.244 mL aliquots and freeze at -20° C.

2.4 Mix the above solutions in the following order.
2.4.1 Place 20 mL stock buffer in a beaker with a stirring bar.
2.4.2 Place beaker in an ice bath.
2.4.3 Add 1 mL of the dithiothreitol solution and 1 mL of the pyridoxal phosphate solution.
2.4.4 Add 0.244 mL of the PMSF dropwise while stirring the solution.
2.4.5 Add stock buffer solution to give a final volume of 0.05 L.

3.0 PREPARATION OF STOCK ORNITHINE SOLUTION

3.1 Dissolve 0.843 g DL-ornithine in sufficient distilled water to make a final volume of 0.1 L.

3.2 Add 0.1 mL of this stock to each 0.5 mL [^{14}C]-ornithine (providing that the amount of radioactivity is 1 microCurie/10 microliters). Also add 0.65 mL distilled water for each 0.5 mL radiolabeled ornithine.
3.2.1 The dilutions of the radiolabeled ornithine result in 1 microCurie of radioactivity and 1 micromole ornithine in each 0.02 mL.

4.0 PREPARATION OF MOLAR CITRIC ACID

4.1 Dissolve 19.212 g citric acid in sufficient distilled water to make a final volume of 0.1 L.

5.0 PREPARATION OF LIVER HOMOGENATE

5.1 Euthanize rats with carbon dioxide.

5.2 Remove the liver, blot dry and weigh.

5.3 Add 4 volumes of homogenizing buffer.

5.4 Mince the liver with scissors.

5.5 Homogenize with a mortar and pestle type homogenizer.

5.6 Dispense the homogenate into polyallomar ultracentrifuge tubes and weigh. This tube and its balance tube must weigh within 10 mg of each other.

5.7 Centrifuge at 48000 x g for 60 minutes at 4° C.

5.8 Remove supernatant from centrifuge tubes and store on ice.

6.0 ENZYME ASSAY PROCEDURE

6.1 Prespot 2.3 cm diameter filter paper disk (Fisher #09-820AA) with 20 microliters of 2N NaOH.

6.2 Fold disks of filter paper into thirds after drying.

6.3 Place disks into center wells (Kontes #882320-0000).

6.4 Place 0.125 mL assay buffer into a 16 x 100 mm culture tube cooled at 4° C.

6.5 Add 0.05 mL liver supernatant.

6.6 Add 0.025 mL ornithine stock solution.

6.7 Prepare a blank by adding 1 volume of 50% trichloracetic acid to 4 volumes of liver homogenate. Remove 0.05 mL of this and place in a tube with assay buffer and ornithine.

6.8 Stopper culture tubes (Kontes #882310-0000).

6.9 Remove tubes from ice bath and place in a water bath at 37° C for 30 minutes.

6.10 After 30 minutes, terminate the reaction by injecting 1 mL of 1.0 M citric acid through the stopper.

6.11 Allow the tubes to stand at 37° C for 30 additional minutes.

6.12 Uncap and remove filter paper disks and place each in a separate scintillation vial.

6.12.1 Spot 25 microliters of the stock ornithine solution on a piece of filter paper and place this in a scintillation vial.

6.13 Add 2 mL Scintiverse II scintillation cocktail to each vial and count on a liquid scintillation counter for 10 minutes.

7.0 QUANTITATION OF ACTIVITY

7.1 Subtract the disintegrations per minutes (dpm) of the background from the dpm's of the sample (Section 6.12.1).

7.2 Divide the total amount of ornithine by the total number of dpm's added to each assay tube (Section 6.12.1).

7.3 Multiply this number by the dpm value obtained in Section 7.1.
7.3.1 This number is the picomoles of CO_2 produced/30 minutes.

7.4 Determine the protein concentration of the tissue homogenate by the Bradford method (See SOP #4300-4019).
7.4.1 Calculate the amount of protein in 50 microliters.

7.5 Take the value obtain in Section 7.3 and divide by the value obtained in Section 7.4.1.
7.5.1 This value is the number of picomoles of CO_2 produced/30 min/mg protein.

GLUCOSE-6-PHOSPHATASE (G-6-Pase) STAINING PROCEDURE

AUTHOR: C. S. Godin

PURPOSE: This standard operating procedure describes the method to be used for the routine staining of frozen tissue for G-6-Pase activity.

1.0 PREPARATION OF 0.05 M TRIS-MALEATE BUFFER

1.1 Dissolve 6.05 g Tris-maleate in sufficient distilled water to make a total volume of 0.5 L.

1.2 Adjust pH of solution to 7.4 with 1N NaOH.

2.0 PREPARATION OF G-6-P SOLUTION

2.1 Dissolve 0.025 g glucose-6-phosphate (G-6-P) potassium salt in 20 mL distilled water.

2.2 Refrigerate any unused portion at 4°C.

3.0 PREPARATION OF 2% LEAD NITRATE

3.1 Dissolve 2.0 g lead nitrate in sufficient distilled water to make a final volume of 0.1 L.

4.0 PREPARATION OF 7.5% AMMONIUM SULFIDE SOLUTION

4.1 Add 30 mL of 20% ammonium sulfide (Aldrich) to 370 mL distilled water.

4.2 This solution must be made fresh daily.

5.0 PREPARATION OF SUBSTRATE SOLUTION

5.1 Add the following solution in the order listed:
- 5.1.1 20 mL G-6-P solution
- 5.1.2 7 mL distilled water
- 5.1.3 20 mL Tris-maleate buffer solution
- 5.1.4 3 mL 2% lead nitrate solution

5.2 Combine, mix and filter the solution.

6.0 STAINING PROCEDURE

6.1 Cut 10 micron thick frozen section on cryostat (See SOP #4300-933) and mount on subbed slides.

6.2 Keep sections frozen at -40°C until ready to stain.

6.3 Thaw frozen sections by warming with hand for 30 seconds.

6.4 Place a single slide in a single Petri dish.

6.5 Place substrate solution on slide by tracing the outline of the section with a Pasteur pipet and then fill the center with additional substrate solution.

6.6 Cover Petri dish and incubate for 45 minutes at 37°C.

6.7 After incubation place slides in the following solutions:
- 6.7.1 Distilled water, two changes
- 6.7.2 10% buffered neutral formalin for five minutes
- 6.7.3 Rinse briefly in distilled water
- 6.7.4 7.5% ammonium sulfide for 1 minute
- 6.7.5 Wash for five minutes in tap water
- 6.7.6 Stain with hematoxylin.
- 6.7.7 Rinse in tap water for five minutes.
- 6.7.8 Dehydrate in two changes of 95% ethanol and then two changes of 100% ethanol for 1 minute each.
- 6.7.9 Clear in three changes of xylene.
- 6.7.10 Coverslip with Permount® or other mounting medium.
- 6.7.11 Areas staining positive for G-6-Pase activity will be brown-black.

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ENVIRONMENTAL SCIENCES
TOXIC HAZARDS RESEARCH UNIT

STANDARD OPERATING PROCEDURE

ADENOSINE-5-TRIPHOSPHATASE (ATPase) STAINING PROCEDURE

AUTHOR: C. S. Godin

PURPOSE: This standard operating procedure describes the method to be used for the routine staining of frozen tissue sections for ATPase activity.

1.0 PREPARATION OF 0.5 M TRIS-MALEATE BUFFER

1.1 Dissolve 6.05 g Tris-maleate in sufficient distilled water to make a total volume of 0.5 L.

1.2 Adjust pH of solution to 7.4 with 1N NaOH.

2.0 PREPARATION OF ATP SOLUTION

2.1 Dissolve 0.025 g adenosine triphosphate (ATP) disodium salt in 20 mL distilled water.

2.2 Refrigerate any unused portion at 4°C.

3.0 PREPARATION OF 2% LEAD NITRATE

3.1 Dissolve 2.0 g lead nitrate in sufficient distilled water to make a final volume of 0.1 L.

4.0 PREPARATION OF 7.5% AMMONIUM SULFIDE SOLUTION

4.1 Add 30 mL of 20% ammonium sulfide (Aldrich) to 370 mL distilled water.

4.2 This solution must be made fresh daily.

5.0 PREPARATION OF SUBSTRATE SOLUTION

5.1 Add the following solution in the order listed:
- 5.1.1 20 mL ATP solution
- 5.1.2 7 mL distilled water
- 5.1.3 20 mL Tris-maleate buffer solution
- 5.1.4 3 mL 2% lead nitrate solution.

THRU APPROVAL: Henry A. Hall DATE 20 March 1990

QA COORDINATOR: M B Schneider Jr DATE 22 Mar 90

AUTHOR APPROVAL: C Steven Godin DATE 20 March 1990

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5.2 Combine, mix and filter the solution.

6.0 STAINING PROCEDURE

6.1 Cut 10 micron thick frozen section on cryostat (See SOP #4300-933) and mount on subbed slides.

6.2 Keep frozen at -40°C until ready to stain.

6.3 Thaw frozen sections by warming slides with hand for 30 seconds.

6.4 Place a single slide in a single Petri dish.

6.5 Place substrate solution on slide by tracing the outline of the section with a Pasteur pipet and then fill in the center with additional substrate solution.

6.6 Cover Petri dish and incubate for 45 minutes at 37°C.

6.7 After incubation place slides in the following solutions:
- 6.7.1 Distilled water, two changes
- 6.7.2 10% buffered neutral formalin for five minutes
- 6.7.3 Rinse briefly in distilled water.
- 6.7.4 7.5% ammonium sulfide for 1 minute
- 6.7.5 Wash for five minutes in tap water
- 6.7.6 Stain with hematoxylin.
- 6.7.7 Rinse in tap water for five minutes
- 6.7.8 Dehydrate in two changes of 95% ethanol and then two changes of 100% ethanol for 1 minute each
- 6.7.9 Clear in three changes of xylene
- 6.7.10 Coverslip with Permount® or other mounting medium.
- 6.7.11 Areas staining positive for ATPase activity will be brown-black.

GAMMA-GLUTAMYLTRANSPEPTIDASE (GGTase) STAINING PROCEDURE

AUTHOR: C. S. Godin

PURPOSE: This standard operating procedure describes the method to be used for the routine staining of frozen tissue sections for GGTase activity.

1.0 PREPARATION OF 0.1 M TRIS-HCl BUFFER

1.1 Dissolve 6.05 g Tris base in sufficient distilled water to make a total volume of 0.5 L.

1.2 Adjust the pH of the final solution to 7.4 with 1N HCl.

2.0 PREPARATION OF 0.85% SALINE

2.1 Dissolve 8.5 g NaCl in sufficient distilled water to make a total volume of 1L.

3.0 PREPARATION OF 1N SODIUM HYDROXIDE

3.1 Dissolve 4.0 g sodium hydroxide in sufficient distilled water to make a total volume of 1 L.

4.0 PREPARATION OF 0.1M COPPER SULFATE

4.1 Dissolve 24.97 g copper sulfate in sufficient distilled water to make a final volume of 1 L.

5.0 PREPARATION OF KAISER'S GLYCERIN JELLY

5.1 Weigh 8.0 g gelatin and place in 52.0 mL distilled water.

5.2 Soak gelatin for 2 hours, add 50 mL glycerin and 0.1 g phenol.

5.3 Heat to 70°C for 10-15 minutes while stirring.

5.4 Store at 4°C in refrigerator.

6.0 PREPARATION OF STOCK INCUBATION SOLUTION

6.1 Mix 50 mL of 0.1M Tris buffer and 140.0 mL of 0.85% saline and dissolve 1.0 g glycylglycine in this solution.

6.2 Store in refrigerator at 4°C.

7.0 PREPARATION OF WORKING INCUBATION SOLUTION

7.1 Preparation of GMNA solution.
- 7.1.1 Weigh 0.005 g gamma-L-glutamyl-4-methoxy-beta-napthylamide (GMNA).
- 7.1.2 Add to the GMNA 0.05 mL dimethylsulfoxide and stir with a glass rod until a paste forms.
- 7.1.3 Add 0.05 mL 1N NaOH and mix well.
- 7.1.4 Add 0.9 mL distilled water and mix well.

7.2 Fast Blue BB Salt solution
- 7.2.1 Weigh 0.016 g Fast Blue BB Salt and place in a 50 mL beaker.
- 7.2.2 Add 20 mL of the stock incubation solution.

7.3 Working solution
- 7.3.1 Add Fast Blue BB Salt solution to GMNA solution in this order only.
- 7.3.2 Shake or stir until the solution is a pale orange (5-10 minutes).
- 7.3.3 Solution must be refrigerated and used on the day it is made.

8.0 STAINING PROCEDURE

8.1 Cut 10 micron thick frozen section (see SOP #4300-933) and mount sections on subbed slides. Keep frozen until ready to stain.

8.2 Thaw frozen sections by warming with hand for 30 seconds.

8.3 Place a single slide in a single Petri dish.

8.4 Place a drop of the incubation solution prepared in Section 7.1 on the section by tracing around the border of the section with a Pasteur pipet and then fill in the center with more incubation solution.

8.5 Incubate at 37°C for 1 hour.
- 8.5.1 Do not allow the section to become dry at any time.

8.6 After incubation, place slides in a staining jar containing 0.85% saline for two minutes.

8.7 Transfer the slide to a staining jar containing 0.1M copper sulfate for two minutes.

8.8 Transfer slides to a staining jar containing fresh 0.85% saline for two minutes.

8.9 Place slide in a staining jar containing hematoxylin for 2 minutes.
8.9.1 Decolorize and blue in multiple rinses of tap water until no coloring drains from the slides and the water remains clear.

8.10 Coverslip slides with Kaiser's glycerin jelly and store in a freezer at -20°C.

8.11 A positive reaction is indicated by intense red staining.

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STANDARD OPERATING PROCEDURE

GYLCOGEN STAINING PROCEDURE

AUTHOR: C. S. Godin

PURPOSE: This standard operating procedure describes the procedure to follow when attempting to demonstrate glycogen-deficient foci in liver sections.

1.0 TREATMENT OF LIVER

1.1 Take a cross section of the right lateral and posterior lobes of the liver.
1.1.1 Prepare a ~1 cm section from each slice taken.
1.1.2 Place the section in a tissue cassette and place in 10% formalin for no more than 48 hours.

1.2 Place cassette directly into 70% ethanol and process as for routine hematoxylin and eosin sections and prepare paraffin sections (see SOP #4300-930).

2.0 STAINING PROCEDURE

2.1 Periodic acid solution
2.1.1 Dissolve 1.0 g periodic acid in 100 mL 90% ethanol.

2.2 Deparaffinize sections in three changes of xylene.

2.3 Place sections in two changes of absolute alcohol for two minutes each followed by two changes of 95% alcohol for two minutes each. Place in 70% alcohol for two minutes.

2.4 Briefly rinse in distilled water.

2.5 Place slides in periodic acid solution for 1 hour.

2.6 Rinse in distilled water briefly.

2.7 Treat with Schiff reagent (Sigma) until section turns reddish-pink (15 minutes).

2.8 Rinse in two changes of absolute alcohol for 5 minutes each.

2.9 Clear in three changes of xylene for three minutes each.

2.10 Mount with Permount or other mounting medium.

2.11 Areas containing glycogen will be stained reddish-pink.

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ENVIRONMENTAL SCIENCES
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STANDARD OPERATING PROCEDURE

IRON STAINING PROCEDURE

AUTHOR: C. S. Godin

PURPOSE: This standard operating procedure describes the procedure to follow when attempting to demonstrate the presence of iron deficient foci in liver sections.

1.0 IRON LOADING OF ANIMALS

1.1 Inject animals with iron dextran (Sigma) at a dose of 125 mg/kg body weight three times a week for two weeks.

1.2 Make injections subcutaneously in the inguinal area.
1.2.1 Alternate the injections in the right and left inguinal regions.

1.3 Sacrifice animal after two weeks by CO_2 asphyxiation.

2.0 TREATMENT OF LIVER

2.1 Take a cross section of the right lateral and posterior lobes of the liver.
2.1.1 Prepare a ~1 cm section from each slice taken.
2.1.2 Place the section in a tissue cassette and place in 10% formalin fixative.

2.2 Process the section as for routine hematoxylin and eosin sections and prepare paraffin section (see SOP #4300-930).

3.0 STAINING PROCEDURE

3.1 Ferrocyanide-hydrochloric acid solution
3.1.1 Dissolve 2.0 g potassium ferrocyanide in sufficient distilled water to make a final volume of 50.0 mL.
3.1.2 Mix 2.0 mL concentrated HCl in 48.0 mL distilled water.
3.1.3 Mix these solutions, warm slightly and filter.
3.1.4 Place solution in a Coplin jar and warm to 56°C.

THRU APPROVAL: Henry S. Hall DATE 20 March 1990

QA COORDINATOR: _____ DATE _____

AUTHOR APPROVAL: C. Steven Godin DATE 20 March 1990

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3.2 Counterstain solution
- 3.2.1 Dissolve 5.0 g aluminum sulfate in sufficient distilled water to make a final volume of 100 mL.
- 3.2.2 Dissolve 0.1 g nuclear fast red in the aluminum sulfate solution with heat, cool, filter and add a thymol crystal.

3.3 Deparaffinize slides and hydrate to water in graded series of alcohol.
- 3.3.1 Place slides in three changes of xylene for 2 minutes each, followed by 2 changes of absolute alcohol for 2 minutes each and 2 changes of 95% alcohol for two minutes.
- 3.3.2 Place slides in 70% alcohol for two minutes and then place in distilled water for 5 minutes.

3.4 Place in ferrocyanide-HCl solution for 10 minutes.

3.5 Rinse in distilled water for five minutes.

3.6 Counterstain with nuclear fast red for 10 minutes.

3.7 Rinse in 70% alcohol for 30 seconds.

3.8 Dehydrate in two changes of 95% ethanol followed by two changes of absolute ethanol for two minutes each.

3.9 Clear in three changes of xylene for two minutes each.

3.10 Iron pigment will be greenish-blue and nuclei will be red.

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ENVIRONMENTAL SCIENCES
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STANDARD OPERATING PROCEDURE

CYANIDE-INSENSITIVE ACYL-CoA OXIDASE ASSAY

AUTHOR: C. S. Godin

OBJECTIVE: This standard operating procedure describes the method to be routinely used to assay peroxisomal beta oxidation activity using either cell sonicates or tissue homogenate.

1.0 REAGENTS

1.1 Tris-Hydrochloride (pH 7.8) ..... 50 mM
- 1.1.1 Add 24.33 gm of Tris to 1L of distilled water (200 mM stock).
- 1.1.2 Make a 0.1N HCl stock solution by adding 0.82 mL of HCl to 99.18 mL of distilled water.
- 1.1.3 Add 25.0 mL of the 200 mM Tris to 33.7 mL of the 0.1N HCl and bring to a final volume of 100 mL with distilled water (final pH 7.8).
- 1.1.4 Adjust pH to 7.8 if necessary using 1N HCl or 1N NaOH. Refrigerate at 4°C.

1.2 Bovine Serum Albumin (BSA) ..... 3.0 gm/100 mL
- 1.2.1 Freeze at -20°C.

1.3 Triton-X 100 ..... 2.0 gm/100 mL
- 1.3.1 Store at 4°C.

1.4 Potassium Cyanide (KCN) ..... 10.0 mg/mL
- 1.4.1 Make fresh daily.

1.5 Dithiothreitol ..... 321.0 mg/mL
- 1.5.1 Store at -20°C.

1.6 Coenzyme A ..... 10.0 mg/mL
- 1.6.1 Store at -20°C.

1.7 Nicotinamide Adenine Dinucleotide (NAD) ..... 16.6 mg/mL
- 1.7.1 Store at -20°C.

1.8 Palmitoyl CoA ..... 25.2 mg/mL
- 1.8.1 Store at -20°C.

THRU APPROVAL: Henry B. Hall DATE 20 March 1990

QA COORDINATOR: M B Schaeidler DATE 20 Mar 90

AUTHOR APPROVAL: C. Steven Godin DATE 20 March 1990

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DATE: 2 March 1990
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2.0 PROCEDURE

2.1 For each assay cuvette add the following proportions of the above reagents. Prepare a reference and test cuvette for each assay.

2.1.1	Tris-Hydrochloride	950.0 µL
2.1.2	NAD	10.0 µL
2.1.3	Coenzyme A	10.0 µL
2.1.4	KCN	10.0 µL
2.1.5	BSA	5.0 µL
2.1.6	Triton-X 100	5.0 µL
2.1.7	Dithiothreitol	3.0 µL
2.1.8	Cell Sonicate/Homogenate (125-500 µg)	5.0 µL

2.1.8.1 Preparation of cell sonicate:

2.1.8.1.1 Sonicate a cell suspension, on ice, at a cell density of 2.5-10.0 x 10^7 cells/mL for 10 seconds at maximum output.

2.1.8.2 Preparation of tissue homogenate:

2.1.8.2.1 Euthanize rats with CO_2.

2.1.8.2.2 Remove the liver, blot dry and weigh.

2.1.8.2.3 Add 4 volumes 0.25M sucrose.

2.1.8.2.4 Mince the liver with scissors.

2.1.8.2.5 Homogenize with a mortar and pestle type homogenizer.

2.1.8.2.6 Dispense homogenates into centrifuge tubes and centrifuge at 1500 x g for ten minutes at 4°C.

2.1.8.2.7 Remove supernatant and store on ice.

2.1.8.3 Remove an aliquot (100 µl) for protein analysis (see SOP #4300-4019).

2.2 Place cuvettes containing the reaction mixture in a waterbath at 37°C.

2.3 When the temperature of the reaction mixture has reached 37°C (~30 min.), add 2.0 µl of palmitoyl CoA reagent to the test cuvette only and read immediately.

2.4 Record the change in absorbance in the test cuvette using a Gilford Response™ spectrophotometer at a wavelength of 340 nM for five minutes.

3.0 CALCULATIONS

3.1 Record the difference in absorbance of a linear portion of the rate curve over a one minute period of time.

3.2 Divide this difference by the molar extinction coefficient (6.22×10^{6}).

3.2.1 The value obtained is given in terms of nmoles of NAD reduced/minute.

3.3 This above value is normalized to protein by dividing this value by the amount of protein in the reaction mixture as determined by the Bradford protein assay (see SOP #4300-4019).

3.4 This value is normalized to gram of liver by multiplying the value by 1000.

3.4.2 The value obtained is given in terms of moles of NAD reduced/minute/gram

NSI TECHNOLOGY SERVICES CORPORATION

ENVIRONMENTAL SCIENCES
TOXIC HAZARDS RESEARCH UNIT **STANDARD OPERATING PROCEDURE**

OPERATION OF THE BIOQUANT IV IMAGE ANALYSIS SYSTEM

AUTHOR: C. S. Godin

PURPOSE: This SOP describes the routine procedure to follow when setting up the Bioquant IV Image Analysis System to record data.

1.0 COMPONENTS

1.1 IBM XT computer with keyboard

1.2 Black and white monitor

1.3 MTI video camera with microscope mounts

1.4 HIPAD digitizing tablet

1.5 Bioquant IV software

1.6 Olympus BH-2 microscope

1.7 Aus JENA Cytoval-2 stereomicroscope with light source

2.0 INITIAL OPERATION

2.1 Format diskettes if necessary.
2.1.1 Use only diskettes for IBM XT computers.

2.2 Turn on power strip to activate the system.

2.3 Choose Option #1 entitled "Bioquant Morphometry" when the main menu appears on the screen.
2.3.1 Enter current date and time.

2.4 When the Bioquant IV main menu appears, use the mouse arrow to select the option entitled "Templates and Extant Data." See Exhibit A.

2.5 When the menu bar at the top of the screen appears, select the choice entitled "Options." See Exhibit B.
2.5.1 While holding down the button of the mouse, move the arrow to the option entitled "Create New Data Volume." See Exhibit C.

2.5.2 When the next screen appears (Exhibit D) choose the appropriate disk drive by moving the mouse so that the vertical dog-bone pointer appears. Select the proper drive and press return.

2.5.3 Name the data volume by moving the mouse so that the vertical dog-bone pointer appears. Depress the mouse and type the name. Press return.

2.5.4 Move mouse arrow to "OK" and release button.

2.6 Select the option entitled "Create New Data Set" from the main menu bar. See Exhibit E, Figure 1.

2.6.1 When the next screen appears (Exhibit E, Figure 2) select choice entitled "Empty Plate."

2.6.2 Move mouse arrow to area marked "Sample Name for New Data Set." When vertical dog-bone pointer appears, type name and press return.

2.6.3 Move mouse arrow to "Select Template" and depress mouse button. See Exhibit F.

2.7 Move mouse arrow to "Create Data Set" when the next screen appears and depress button. See Exhibit G.

2.8 Select option entitled "Procedures" from main menu bar when next screen (Exhibit H) appears.

2.8.1 Select option entitled "Measurement."

3.0 MEASUREMENT OF DATA

3.1 Move mouse arrow to main menu bar and select option entitled "Select" when next screen appears. See Exhibit I.

3.1.1 Choose option entitled "Add/Assoc."

3.2 Choose either direct measurements (top) or derived measurements (bottom) by moving mouse arrow to choice and depressing button. See Exhibit J for choices.

3.2.1 Move mouse arrow to "Add" box and depress. Repeat as necessary for each array. See Exhibit K for measurement choices.

3.2.2 For associations, move mouse arrow to the measurement to be grouped under an array and depress mouse button. See Exhibit K.

3.2.2.1 Move mouse arrow to "Group" and depress button.

3.2.2.2 Move mouse arrow to array chosen and depress button.

3.3 Move mouse to "OK-Select" box when all arrays and associations have been made and depress button.

3.3.1 Move mouse arrow to "Select All" box and depress. See Exhibit L.

3.3.2 Name arrays.

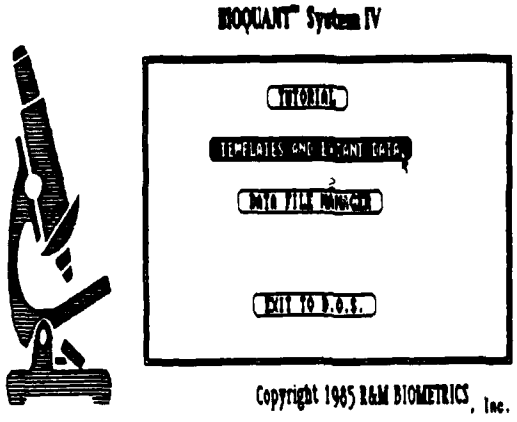
3.3.2.1 Move mouse arrow until array selected is highlighted and depress.

3.3.2.2 Move mouse arrow to "Name and Comment" section at bottom of screen and depress button when vertical dog-bone pointer appears.

3.3.2.3 Type in the name of the array.

3.3.3 Move mouse arrow to "OK" box and depress.

3.4 Proceed with measurements when next screen appears. See Exhibit M.

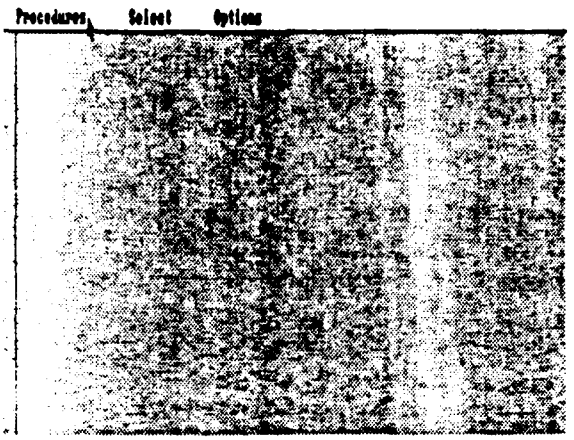


TEMPLATES & EXTANT DATA

This set of routines provides an entrance to the full research capabilities of the BQ System IV. They allow you to access data or templates from previous measurements, as well as to perform a number of configuration options.

For efficiency, the BQ System IV assumes when you click this box that you will continue using the most recent data volume and one of the templates saved on this Startup Volume. Therefore, you are presented with a screen similar to that on the next page.

If you wish to change data volumes or access data acquired previously (extant data), simply pull down the menu under **Select** as described on page Startup 9.

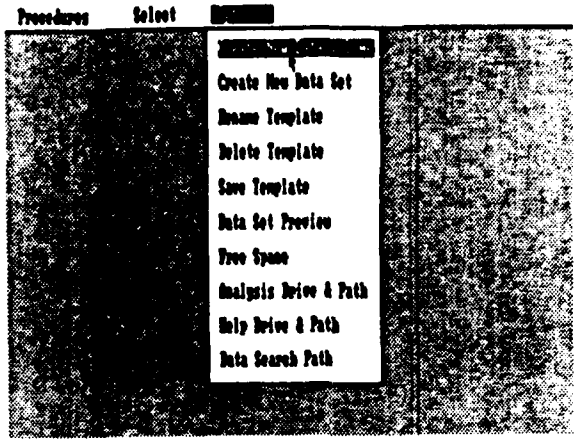


MENU BAR

The region at the top of the screen is called the **MENU BAR.** It provides immediate access to the most powerful menus of the system.

Although the BQ System IV displays a wide variety of different information on the video screen in the course of image analysis, the top of the screen will appear familiar to you throughout even the most complex operations.

The choices shown above, **Procedures, Select,** and **Options** are used throughout the programs, and you will soon regard them as good friends.

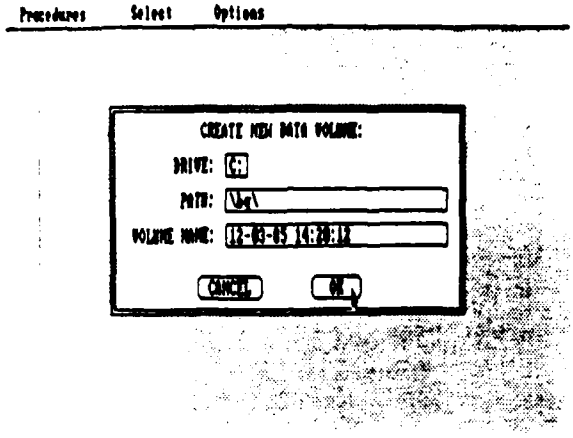


Create New Data Volume

As explained on page Introduction 17
Data Volumes are like lab notebooks.

You may wish to begin a new one in order to keep the data sets organized, or your diskette may be full.

You may wish to define some parameters as described on the next page.



VOLUME NAME

The date and time are provided as default name.

For editing rules see page Startup 5.

DRIVE

Any drive configured by your DOS may be used for data.

PATH

The default path to the data volume is \BQ\. If you do decide to change this parameter, it is recommended that you limit the paths and keep good records of your settings, since the computer will need this information periodically.

Note: for floppy drive data disks, data access time slows significantly if a diskette is used for more than one volume.

Edit the DRIVE and PATH according to MS-DOS or PC-DOS stipulations. Be sure to begin and end the Path: with backslash (\).

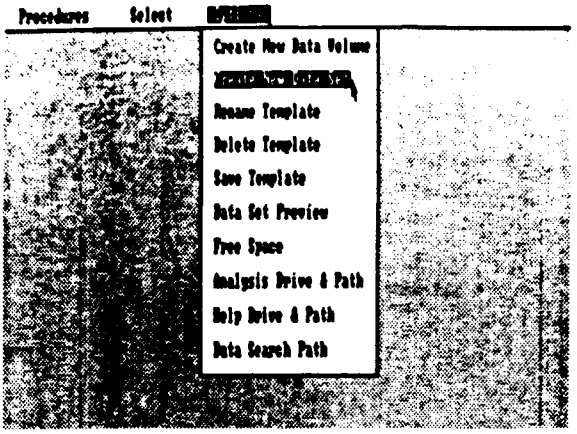


Figure 1

Create New Data Set

The process of creating a data set involves selecting its template and giving it a name exactly like **Select Template** (available from the **Select** pull down menu).

Choosing **Create New Data Set** from the **Options** menu leads to the **TEMPLATE** routines described on page Startup 3.

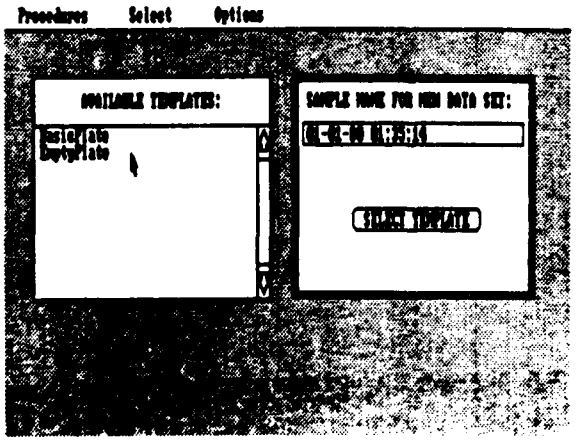
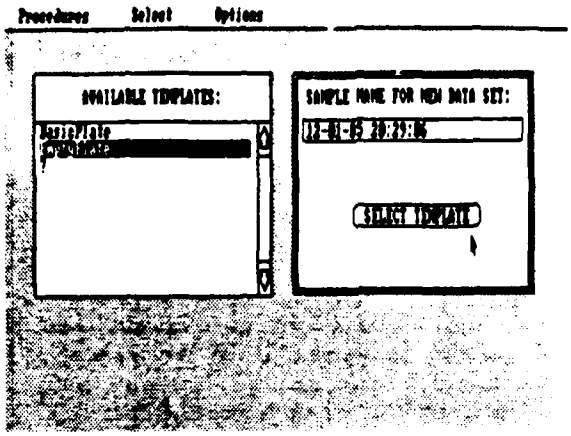


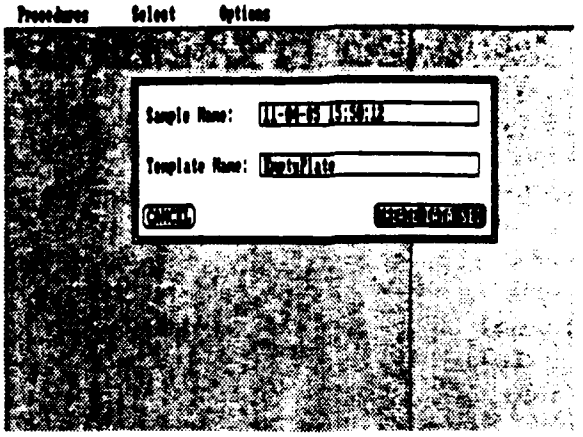
Figure 2



SELECT TEMPLATE

Once the name is acceptable and the desired template highlighted, clicking this box completes the selection and produces the screen on the next page.

NOTE: *The "double click" technique also may be used to effect the selection. Just press the button twice in rapid succession while pointing at the template name of your choice.*



CREATE DATA SET

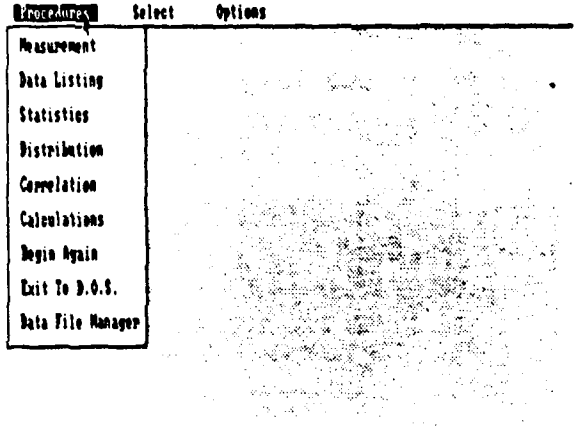
The BQ System IV provides a summary of the selected template and name for the new data set.

Assuming they are correct for this run, click the **CREATE DATA SET** box.

Obviously, the newly created data set contains no data values; however, the pattern of arrays and names corresponds to the template.

NOTE: "CREATE DATA SET" is not as final as it sounds. While you are still in the **Startup** routine, you may change the name or template for the current data set at any time. Just pull down the **Select** menu and go through the **Select Template** procedure again.

Also, once the data set has been "created", it is possible to use the **Data Set Preview** feature found under the **Options** menu as described on page Startup 24.

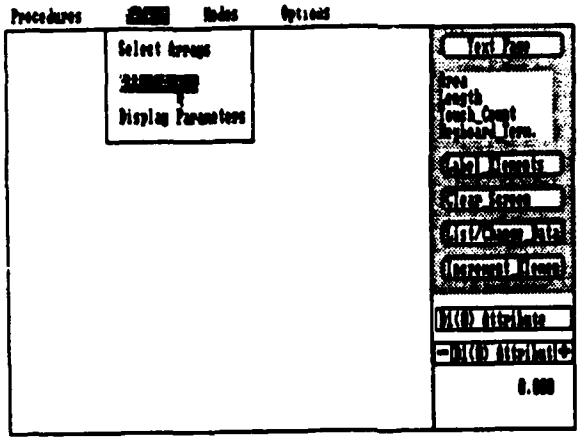


Procedures

This menu provides access to the major routines of the BQ System IV.

As illustrated, you normally will proceed to **Measurement** after selecting a template and creating a data set.

NOTE: The documentation proceeds from left to right along the menu bar (e.g., all of the choices under **Select** will be covered first, then those under **Options**).



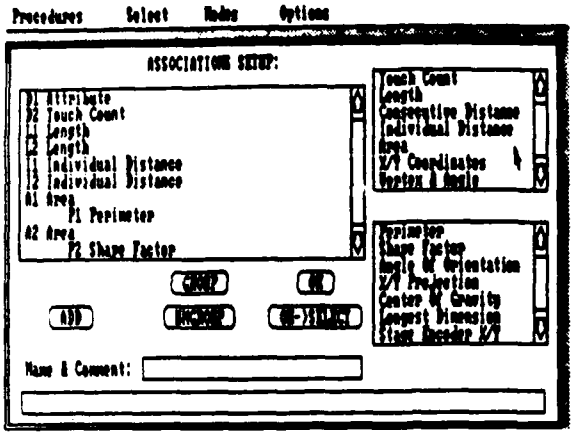
Select: Add / Assoc

This routine allows you to configure the data set to your own specifications; either by adding more arrays or associating the arrays into groups.

Add is easy to understand. In the simplest case, you can choose an "Empty Template" that contains only one array as a place marker (see page Startup 3). From that starting point, you can add as many area, length, distance, or other arrays as your morphometric inquiry requires. The Add operation is described on page Measurement 16.

Association enables you to stipulate that you wish to consider certain groups of arrays as units that will be measured together, like area and perimeter.

The next page illustrates the screen provided for both adding arrays and associating them.



Add / Assoc

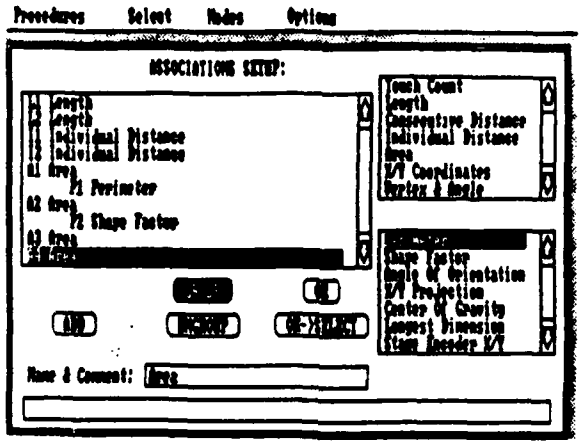
The ASSOCIATIONS SETUP: scroll window lists all the arrays available for grouping or ungrouping.

Arrays are elegible for grouping only when they are empty, i.e., they contain no values. If **Add / Assoc** is selected before any measurements have been made in a data set, then all arrays are listed because they all are empty.

The two scroll windows to the right provide lists of the two types of arrays available as described on the next page.

Briefly, the top list presents the **direct** measurement arrays; this type of measurement often is made independent of any others or as the primary measurement that others are associated with.

The bottom list has **derived** arrays, the ones that always are measured in association with others.



GROUP

The organization of the data sets may be clarified by grouping "associated arrays" (either derived or direct) with primary arrays. Grouping requires three simple steps:

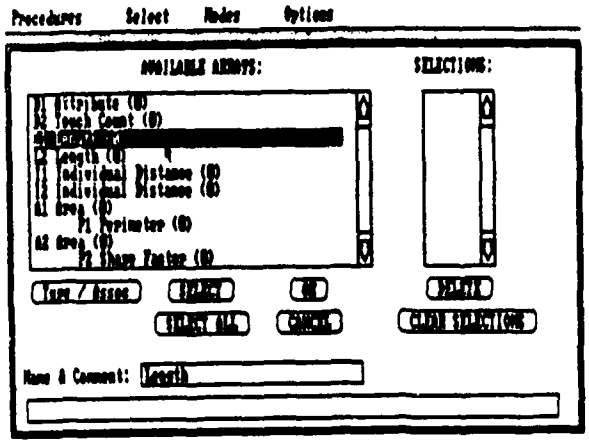
1. Click the source array you wish to group. Its name will become highlighted (**Perimeter** in the example above).

2. Click "GROUP".

3. Click the destination array you wish to have the source associated with (**A4 Area** in the example above).

The source array may be either a **Measurement TYPE** or one of the **ASSOCIATIONS SETUP** arrays. If it is a measurement type, a new array of that type will be added to the data set (see ADD , page Measurement 16).

The destination array must be one of the **ASSOCIATIONS SETUP** arrays.

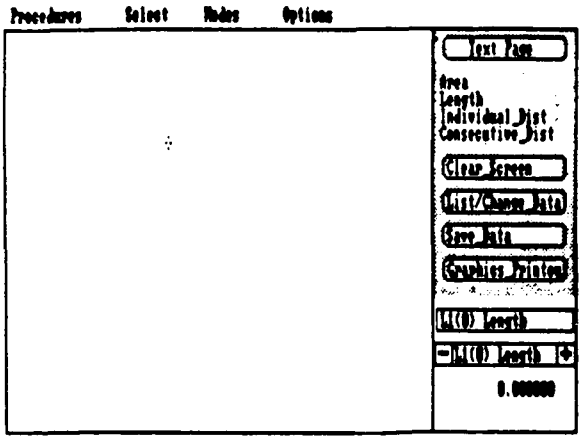


SELECT

To select an array, place the video cursor at the horizontal level of the choice and "click" (press the button). The choice name will become highlighted.

To effect a selection once a name is highlighted, simply click the **SELECT** box. The T# of the selected array appears on the list in the SELECTIONS window. To add the T# to the end of the list, simply click **SELECT**. If you wish to insert the T# somewhere above the end of the list, just click the place on the list that you want the new selection inserted above; then click **SELECT**.

Advanced operators may wish to use the "double click" technique for selection; simply position the cursor at the choice name and click the button twice in rapid succession.



Measurement Screen Organization

In addition to information on the values measured, the measurement screen provides access to functions integral to the measurement process.

The screen is divided into three regions:

MENU BAR - narrow strip across the top of the screen, as seen in all BQ System IV screens.

ACTIVE AREA - large rectangle on which the tracings of measurements are displayed.

TEXT/MENU AREA - the wide right border of the screen displays information on the measurements and offers easy access to **PREFERRED** options and modes. Because this area can be configured to meet your own requirements, your screen may not be identical to the one above.

The **Measurement** documentation on the following pages follows the choices on the menu bar from left to right (i.e., **Select, Modes, Options**).

NSI TECHNOLOGY SERVICES CORPORATION

ENVIRONMENTAL SCIENCES
TOXIC HAZARDS RESEARCH UNIT **STANDARD OPERATING PROCEDURE**

MEASUREMENT OF ENZYME-ALTERED FOCI

AUTHOR: C. S. Godin

PURPOSE: This SOP describes the routine measurement of enzyme-altered foci in liver sections from animals used in initiation/promotion assays.

1.0 INITIAL OPERATIONS

1.1 Set up the Bioquant system according to the procedures outlined in Sections 1.0 and 2.0 of SOP #4300-4038.
- 1.1.1 Make sure that two arrays have been added for each slide from a given animal.

1.2 Check magnification and units of measurement.
- 1.2.1 Choose "Modes" option from main menu bar.
- 1.2.2 Change "Units of Measurement" and "Magnification" depending on type of measurement.

2.0 MEASUREMENT OF TISSUE AREAS

2.1 Move camera and mount on Cytoval 2 stereomicroscope.

2.2 Change "Units of Measurement" to centimeters by moving mouse arrow to choice and depressing button. Move mouse arrow to "OK" and depress. See Exhibit A.

2.3 Change "Magnification" to choice labeled 0.63x JEN or manually enter 27x. Move mouse arrow to "OK" and depress. See Exhibit B.

2.4 Make sure that array #A1 appears at the bottom of the Text Page. See Exhibit C.
- 2.4.1 If array #A1 is not present use the mouse to move back or ahead by moving the arrow over the boxes designated "+" or "-".

2.5 Trace outline of tissue areas using the mouse and the digitizing tablet.
- 2.5.1 Hold button of mouse down while tracing.
- 2.5.2 When tracing is nearly complete, release button to join ends of the tracing. Value for this measurement will appear at the bottom of the Text Page.

2.6 Advance to array #A3 and trace the tissue section from the next slide. Repeat for all slides.

THRU APPROVAL: Mary G. Hall DATE 20 March 1990

QA COORDINATOR: M S Schneider DATE 22 Mar 90

AUTHOR APPROVAL: C Steven Godin DATE 20 March 1990

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DATE: 27 February 1990
REVISION NO.: 0

3.0 MEASUREMENT OF FOCI

3.1 Move video camera and mount on Olympus BH-2 microscope.

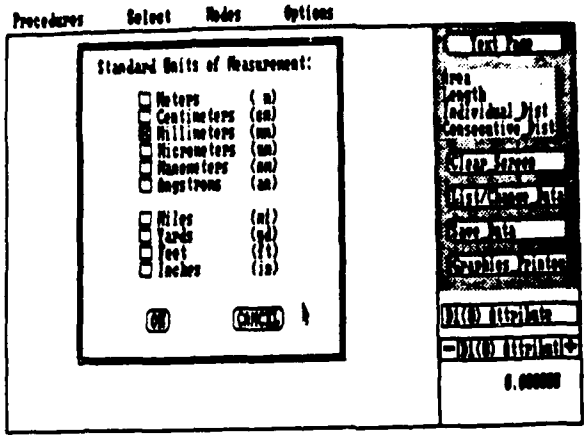
3.2 Change "Units of Measurement" to millimeters. Move mouse arrow to "OK" and depress. See Exhibit A.

3.3 Change "Magnification" to 10x or manually enter 605x. Move mouse arrow to "OK" and depress. See Exhibit B.

3.4 Make sure array #A2 is listed in box at the bottom of the Text Page.

3.5 Scan the entire slide.
- 3.5.1 When a focus is observed, decrease light intensity of microscope until image appears on the monitor.
- 3.5.2 Trace the area of the focus in the same way that tissue areas were measured.
- 3.5.3 Repeat for each focus on a given slide.
- 3.5.4 When the slide has been completely examined advance to array #A4 and read foci on the next slide.

3.6 When all tissue areas and foci from all slides have been completed save data to disk.
- 3.6.1 Move mouse arrow to "Save Data" box on the Text Page and depress the button.



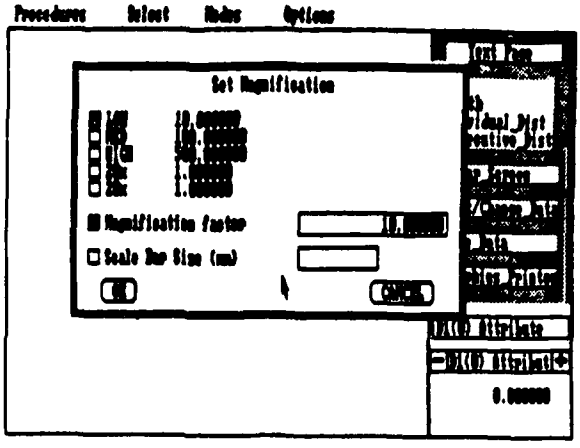
Units of Measurement

You may choose any of the standard units of measurement by simply clicking on the box to the left.

It is possible to change units at any time; however, you should remember that it may be difficult to interpret values in an array measured using more than one unit.

The Units you select are remembered by the BQ System IV **Analysis Volume**, so that the next time you begin the program it will not be necessary to select units unless you wish to change.

The default units are millimeters as illustrated above.



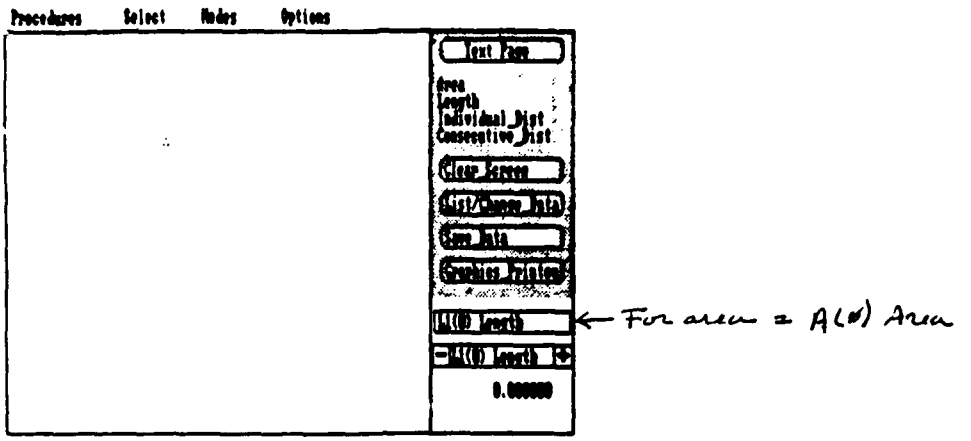
Magnification

You may either enter the numeric magnification factor using the editing rules found on page Startup 5, or measure a scale bar for which you first enter the length and then measure the bar.

(1) Click the check box to the left of "Scale bar size";
(2) Enter the length in the box to the right;
(3) The pointer changes to crosshairs to indicate that you are ready to measure the scale bar - just like an "individual distance"

At your discretion, the magnifications may be set to be "remembered" by the BQ System IV. This feature will be helpful if you are measuring direct from the microscope (video or camera lucida) or for any other circumstance where the same magnifications are used repeatedly. Simply click the "check box" to the left before entering a mag.

Each pre-set mag has space for an identifier, such as "10X" to identify the objective lens corresponding to that mag. Click on the space and then type in the identifier.



Measurement Screen Organization

In addition to information on the values measured, the measurement screen provides access to functions integral to the measurement process.

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NSI TECHNOLOGY SERVICES CORPORATION

ENVIRONMENTAL SCIENCES
TOXIC HAZARDS RESEARCH UNIT **STANDARD OPERATING PROCEDURE**

QUANTITATIVE STEREOLOGY OF ENZYME-ALTERED FOCI

AUTHOR: C. S. Godin

PURPOSE: This SOP describes the routine methods to be used to determine the number of foci per square centimeter of tissue, the number of foci per cubic centimeter of tissue, the percent of the tissue occupied by foci, the mean area of foci and the mean volume of foci.

1.0 INITIAL OPERATIONS

1.1 Add 16 Attribute arrays for each animal in the group.
1.1.1 Use "Add/Assoc" option under "Select" option of the main menu bar.

2.0 CALCULATIONS

2.1 Select "Procedures" option from main menu bar and then select "Calculations" option. See Exhibit A.

2.2 Select "Calculations" from main menu bar when next screen (Exhibit B) appears and select "Formulas" option.

2.3 Select "Edit Formulas" box when the next screen appears (see Exhibit C).
2.3.1 Enter formulas exactly as listed in Exhibit D (three animals) or Exhibit E (four animals).
2.3.2 When all formulas are entered, save to disk by selecting "Save Formulas" box.

2.4 Execute calculations by selecting box marked "Execute All". See Exhibit F.

2.5 Obtain a hard copy of the data.
2.5.1 Select "Procedure" option from main menu bar and choose the option entitled "Data Listing".
2.5.2 Select "Options" from the main menu bar and select "Hard Copy".

2.6 Back up file to diskette.

THRU APPROVAL: Henry A. Hill DATE 20 March 1990
QA COORDINATOR: M B Schneider Jr DATE 22 Mar 90
AUTHOR APPROVAL: C. Steven Godin DATE 20 March 1990

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DATE: 27 February 1990
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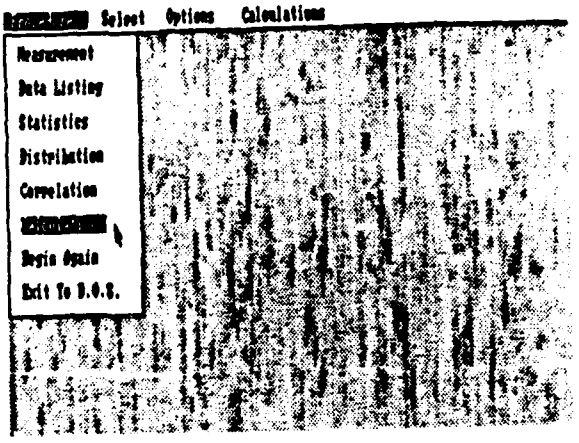


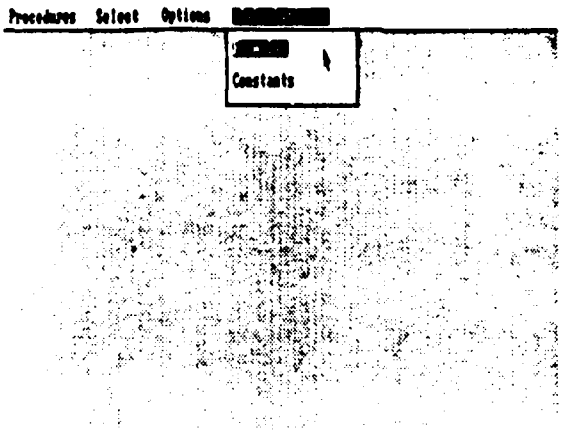
Calculations

Although standard programs of the BQ System IV will calculate many of the parameters your research requires, in some cases it may be necessary to test different mathematical relationships.

Toward that end, the BQ System IV **Calculations** procedure offers the tools for mathematical manipulations devised to your own specifications.

Furthermore, the formulas may be saved for retrieval and reutilization, and different sets of formulas can be saved for specific purposes.

To reach **Calculations**, choose appropriately from the **Procedures** menu as shown above.



Calculations

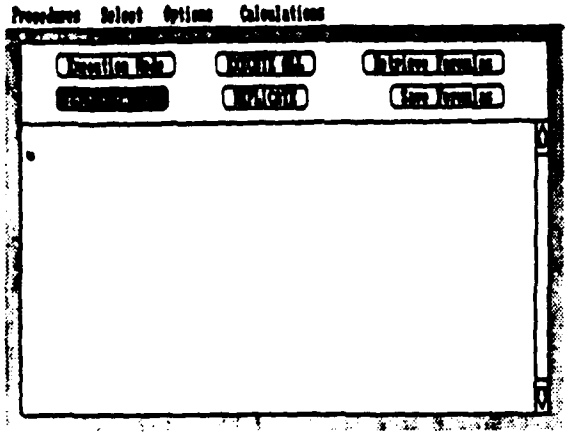
As shown above, two choices are available from the **Calculations** menu. To get to either choice, highlight it by moving the pointer while keeping the button pressed, and then release the button.

Formulas provides an **editing** mode and an **execution** mode, in addition to allowing storage and retrieval.

Formulas may be used to calculate both single values (constants) and arrays. The array resulting from a calculated formula has as many elements as the longest array addressed in the formula.

Constants provides a list of the current value for each of up to 100 constants. Each constant also may have an identifier of up to 40 characters. The constants are saved along with the formulas, allowing retrieval of the values and identifiers.

These choices are described more fully on the following pages.



Edit Formulas

When you arrive at this routine, the screen is blank and the **Edit Formulas** box is highlighted.

In the **Edit** mode, the pointer is a vertical dog bone that is moved to the point where editing is desired. Pressing the button at this point changes the pointer to the underline "entry prompt" and editing follows the rules on page Startup 5.

To enter new formulas, you would begin editing one of the blank lines following the rules on the next page; however, if you have not had any experience with **Calculations**, it would be best to go to the **Tutorial** first.

FORMULA:

```
d1=num(a1)+num(a3)+num(a5)+num(a7)+num(a9)+num(a11): # OF SLIDES
d2=num(a2)+num(a4)+num(a6)+num(a8)+num(a10)+num(a12): # OF FOCI
d3=d2/(sum(a1)+sum(a3)+sum(a5)+sum(a7)+sum(a9)+sum(a11)): FOCI/SQ CM.
d9=sqrt(3.141592654/a2): INVERSE AREAS
d10=sqrt(3.141592654/a4): INVERSE AREAS
d11=sqrt(3.141592654/a6): INVERSE AREAS
d12=sqrt(3.141592654/a8): INVERSE AREAS
d13=sqrt(3.141592654/a10): INVERSE AREAS
d14=sqrt(3.141592654/a12): INVERSE AREAS
d15=sum(a1)+sum(a3)+sum(a5)+sum(a7)+sum(a9)+sum(a11): TOTAL TISSUE AREA
d16=sum(a2)+sum(a4)+sum(a6)+sum(a8)+sum(a10)+sum(a12): TOTAL FOCI AREA
d4=(sum(d9)+sum(d10)+sum(d11)+sum(d12)+sum(d13)+sum(d14)):
d5=d4/(3.141592654*d15)*10: FOCI/CU. CM.
d6=d16/d15: % FOCI VOLUME
d7=d16/d2: MEAN FOCUS AREA
d8=d6/d5*10: MEAN FOCUS VOLUME
```

(1)

```
d17=num(a13)+num(a15)+num(a17)+num(a19)+num(a21)+num(a23):
d18=num(a14)+num(a16)+num(a18)+num(a20)+num(a22)+num(a24):
d19=d18/(sum(a13)+sum(a15)+sum(a17)+sum(a19)+sum(a21)+sum(a23)):
d25=sqrt(3.141592654/a14):
d26=sqrt(3.141592654/a16):
d27=sqrt(3.141592654/a18):
d28=sqrt(3.141592654/a20):
d29=sqrt(3.141592654/a22):
d30=sqrt(3.141592654/a24):
d31=sum(a13)+sum(a15)+sum(a17)+sum(a19)+sum(a21)+sum(a23):
d32=sum(a14)+sum(a16)+sum(a18)+sum(a20)+sum(a22)+sum(a24):
d20=(sum(d25)+sum(d26)+sum(d27)+sum(d28)+sum(d29)+sum(d30)):
d21=d20/(3.141592654*d31)*10:
d22=d32/d31:
d23=d32/d18:
d24=d22/d21*10:
```

(2)

```
d33=num(a25)+num(a27)+num(a29)+num(a31)+num(a33)+num(a35):
d34=num(a26)+num(a28)+num(a30)+num(a32)+num(a34)+num(a36):
d35=d34/(sum(a25)+sum(a27)+sum(a29)+sum(a31)+sum(a33)+sum(a35)):
d41=sqrt(3.141592654/a26):
d42=sqrt(3.141592654/a28):
d43=sqrt(3.141592654/a30):
d44=sqrt(3.141592654/a32):
d45=sqrt(3.141592654/a34):
d46=sqrt(3.141592654/a36):
d47=sum(a25)+sum(a27)+sum(a29)+sum(a31)+sum(a33)+sum(a35):
d48=sum(a26)+sum(a28)+sum(a30)+sum(a32)+sum(a34)+sum(a36):
d36=(sum(d41)+sum(d42)+sum(d43)+sum(d44)+sum(d45)+sum(d46)):
d37=d35/(3.141592654*d47)*10:
d38=d48/d47:
d39=d48/d34:
d40=d38/d37*10:
```

(3)

FORMULAS

```
d1=num(a1)+num(a3)+num(a5)+num(a7)+num(a9)+num(a11): # OF SLIDES
d2=num(a2)+num(a4)+num(a6)+num(a8)+num(a10)+num(a12): NUMBER OF FOCI
d3=d2/(sum(a1)+sum(a3)+sum(a5)+sum(a7)+sum(a9)+sum(a11)): FOCI/SQ CM
d9=sqrt(3.141592654/a2): INVERSE AREAS
d10=sqrt(3.141592654/a4): INVERSE AREAS
d11=sqrt(3.141592654/a6): INVERSE AREAS
d12=sqrt(3.141592654/a8): INVERSE AREAS
d13=sqrt(3.141592654/a10): INVERSE AREAS
d14=sqrt(3.141592654/a12): INVERSE AREAS
d15=sum(a1)+sum(a3)+sum(a5)+sum(a7)+sum(a9)+sum(a11): TOTAL TISSUE AREA
d16=sum(a2)+sum(a4)+sum(a6)+sum(a8)+sum(a10)+sum(a12): TOTAL FOCI AREA
d4=(sum(d9)+sum(d10)+sum(d11)+sum(d12)+sum(d13)+sum(d14)):
d5=d4/(3.141592654*d15)*10: FOCI/CU. CM.
d6=d16/d15: % FOCI VOLUME
d7=d16/d2: MEAN FOCI AREA
d8=d6/d5*10: MEAN FOCI VOLUME
```

(1)

```
d17=num(a13)+num(a15)+num(a17)+num(a19)+num(a21)+num(a23):
d18=num(a14)+num(a16)+num(a18)+num(a20)+num(a22)+num(a24):
d19=d18/(sum(a13)+sum(a15)+sum(a17)+sum(a19)+sum(a21)+sum(a23)):
d25=sqrt(3.141592654/a14):
d26=sqrt(3.141592654/a16):
d27=sqrt(3.141592654/a18):
d28=sqrt(3.141592654/a20):
d29=sqrt(3.141592654/a22):
d30=sqrt(3.141592654/a24):
d31=sum(a13)+sum(a15)+sum(a17)+sum(a19)+sum(a21)+sum(a23):
d32=sum(a14)+sum(a16)+sum(a18)+sum(a20)+sum(a22)+sum(a24):
d20=(sum(d25)+sum(d26)+sum(d27)+sum(d28)+sum(d29)+sum(d30)):
d21=d20/(3.141592654*d31)*10:
d22=d32/d31:
d23=d32/d18:
d24=d22/d21*10:
```

(2)

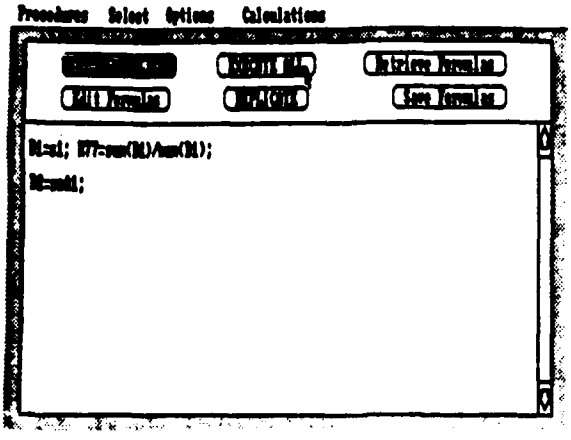
```
d33=num(a25)+num(a27)+num(a29)+num(a31)+num(a33)+num(a35):
d34=num(a26)+num(a28)+num(a30)+num(a32)+num(a34)+num(a36):
d35=d34/(sum(a25)+sum(a27)+sum(a29)+sum(a31)+sum(a33)+sum(a35)):
d41=sqrt(3.141592654/a26):
d42=sqrt(3.141592654/a28):
d43=sqrt(3.141592654/a30):
d44=sqrt(3.141592654/a32):
d45=sqrt(3.141592654/a34):
d46=sqrt(3.141592654/a36):
d47=sum(a25)+sum(a27)+sum(a29)+sum(a31)+sum(a33)+sum(a35):
d48=sum(a26)+sum(a28)+sum(a30)+sum(a32)+sum(a34)+sum(a36):
d36=(sum(d41)+sum(d42)+sum(d43)+sum(d44)+sum(d45)+sum(d46)):
d37=d36/(3.141592654*d47)*10:
d38=d48/d47:
d39=d48/d34:
d40=D38/D37*10:
```

(3)

```
d49=num(a37)+num(a39)+num(a41)+num(a43)+num(a45)+num(a47):
d50=num(a38)+num(a40)+num(a42)+num(a44)+num(a46)+num(a48):
d51=d50/(sum(a37)+sum(a39)+sum(a41)+sum(a43)+sum(a45)+sum(a47)):
d57=sqrt(3.141592654/a38):
d58=sqrt(3.141592654/a40):
d59=sqrt(3.141592654/a42):
d60=sqrt(3.141592654/a44):
d61=sqrt(3.141592654/a46):
d62=sqrt(3.141592654/a48):
d63=sum(a37)+sum(a39)+sum(a41)+sum(a43)+sum(a45)+sum(a47):
d64=sum(a38)+sum(a40)+sum(a42)+sum(a44)+sum(a46)+sum(a48):
d52=(sum(d57)+sum(d58)+sum(d59)+sum(d60)+sum(d61)+sum(d62)):
d53=d52/(3.141592654*d61)*10:
d54=d64/d63:
d55=d64/d50:
```

(4)

EXHIBIT E
SOP NO. 4300-4040
27 February 1990
REVISION NO.: 0



EXECUTE ALL

Pointing to the **EXECUTE ALL** button as illustrated above and clicking causes all formulas to be executed in the order they appear on the list.

Note that the list is displayed on a scroll window, and that up to 100 formulas can be generated.

To Examine Calculated Arrays

The newly calculated values may be viewed quickly by using the **List/Change** option (see page Data Listing 19-20). Press **S** or choose **Select Arrays** from the **Select** pull down menu to first select the new arrays. Of course, **Data Listing** is available from the **Procedures** menu for more thorough scrutiny.

☆U.S. Government Printing Office: 1990 — 748-002/00241

QUALITY ASSURANCE

The study, "Development of an Initiation/Promotion Assay to Detect Foci of Enzyme-Altered Hepatocytes," was conducted by the NSI Technology Services Corporation, Toxic Hazards Research Unit under the guidance of the Environmental Protection Agency's Good Laboratory Practices Guidelines, 40CFR PART 792. No claim will be made that this was a "GLP" study as no attempt was made to adhere to the strict requirements of these guidelines. The various phases of this study were inspected by members of the Quality Assurance Unit. Results of these inspections were reported directly to the Study Director at the close of each inspection.

DATE OF INSPECTION:	**ITEM INSPECTED:**
August 23, 1988	Liver hepatectomy
September 14, 1988	Initiation dose
September 27, 1988	Promotion dose
October 25, 1988	Terminal sacrifice, tissue preparations, and assays
January 30, 1989	Histology specimens
September 8, 1989	Image analysis
March 12-14, 1990	Final report audit

The Quality Assurance Unit has determined by review process that this report accurately describes those methods and standard operating procedures required by the protocol and that the reported results accurately reflect the raw data obtained during the course of the study. No discrepancies were found that would alter the interpretation presented in this Final Report.

M S Schneider Jr

M. G. Schneider
QA Coordinator
Toxic Hazards Research Unit

Date 15 Mar 90