

General Disclaimer

One or more of the Following Statements may affect this Document

- This document has been reproduced from the best copy furnished by the organizational source. It is being released in the interest of making available as much information as possible.
- This document may contain data, which exceeds the sheet parameters. It was furnished in this condition by the organizational source and is the best copy available.
- This document may contain tone-on-tone or color graphs, charts and/or pictures, which have been reproduced in black and white.
- This document is paginated as submitted by the original source.
- Portions of this document are not fully legible due to the historical nature of some of the material. However, it is the best reproduction available from the original submission.

ANNUAL REPORT
"SUPERIOR DIET FOR MAN IN SPACE"
October 1964 - October 1965
SCHWARZ BIORESEARCH, INC.

GPO PRICE \$ _____

CFSTI PRICE(S) \$ _____

Hard copy (HC) \$ 6.00

Microfiche (MF) 1.25

653 July 65

FACILITY FORM 602

N66-22377

(ACCESSION NUMBER)

220

(PAGES)

CR-71817

(NASA CR OR TMX OR AD NUMBER)

(THRU)

1

(CODE)

04

(CATEGORY)

SUPERIOR DIET

FOR

MAN IN SPACE

CONTRACT NASw-517

ANNUAL REPORT

10/64 : 10/65

Submitted

by

SCHWARZ BIORESEARCH, INC.
Mountain View Avenue
Orangeburg, New York

April 1966

Norman A. Rosenthal
Norman A. Rosenthal, Ph.D.
Principal Investigator

SCHWARZ BIORESEARCH, INC.

TABLE OF CONTENTS

	<u>Pages</u>
Index of Tables	I-IV
Index of Figures	V-VII
Abstract	a-c
Introduction	i-ii
Nature of Chemical Interactions Occurring Within Liquid Diet Solutions	
A. Non-enzymatic Browning	1-37
1. Nutritional Consequence of Browning	5-7
2. Role of Oxygen in Browning	8-13
3. Reactions of Model Compounds	14-37
B. Vitamin K--Ethyl Cysteinate Interaction	38-39
Stability Study, Vitamin Assays	40-54
Microbial Control in Liquid Defined Diets	55-68
I. The Use of 2,3 Epoxypropanol (Glycidol) as a Sterilant	55-56
II. Attempts at Sterilizing Liquid Diets	57
III. The Use of Methyl and Propyl Parabens as Liquid Diet Preservatives	57-65
Animal Experiments	66-162
Appendix	163-172
References	173
Cost Statement	174- 176

SCHWARZ BIORESEARCH, INC.

INDEX OF TABLES AND FIGURES

<u>Table Number</u>	<u>Title</u>	<u>Pages</u>
1	Comparison Between Extent of Amino Acid - Glucose Reaction and Ease of Amino Acid Deamination	24
2	Destruction of Amino Acid Residues in Casein on Reaction with Glucose at 37°C (70% Relative Humidity)	25
3	Significant Losses in Amino Acid Content of an 8% Solution of Bovine Serum Albumin Heated with 30% Glucose for 6 Days at 53°C	26
4	Vitamin Concentration and Diet Dilutions Used in Assays	42
5	Effect of Storage Temperature on Vitamin Stability in Liquid Diet	48
6	The Effect of Eight Months Storage at Various Temperatures on Vitamin Stability in Liquid Diet	49
7	Effect of Light on Vitamin Stability in Liquid Diet	50
8	Effect of Irradiation in the Presence and Absence of Ethyl Cysteinate-HCl	51
9	The Effect of Time on Vitamin Stability of Liquid Diets Stored at Room Temperature	52
10	The Effect of Time on Vitamin Stability of Liquid Diets Stored at Refrigerator Temperature	53
11	The Effect of Time on Vitamin Stability of Liquid Diets Stored at Freezer Temperature	54
12	The Effect of Glycidol on Airborne Contaminants in a Liquid Defined Diet	58

SCHWARZ BIORESEARCH, INC.

INDEX OF TABLES AND FIGURES

<u>Table Number</u>	<u>Title</u>	<u>Pages</u>
13	The Effect of a Fat Mix (Containing Polysorbate-80) on the Antimicrobial Effectiveness of Parabens	62
14	The Effectiveness of Parabens in the Presence of Various Levels of Microbial Contamination	60
15	Composition of Basal Diet	70
16	A Preliminary Bioassay on the Stability of Liquid Diets Refrigerated For 3 or 6 Months	71
17	Composition of Test Diets	73
18	Composition of Test Amino Acid Mixtures	74
19	Growth Rate, Diet Consumption and Water Consumption of Rats Fed a Chemically Defined Liquid Diet Exposed to Several Environmental Conditions	75
20	Amino Acid Composition of a Chemically Defined Liquid Diet After Exposure to Several Environmental Conditions	81
21	Assay of a Chemically Defined Liquid Diet After Exposure to Several Environmental Conditions	82
22	Growth Rate, Diet Consumption and Water Consumption of Rats Fed a Chemically Defined Liquid Diet Stored For Four Months	87
23	Amino Acid Composition of Diet Stored For Four Months	89
24	Assay of Diet Following Environmental Exposure For Four Months	90
25	Growth Rate, Diet and Water Consumption of Rats Fed a Chemically Defined Liquid Diet Stored For Eight Months	93

SCHWARZ BIORESEARCH, INC.

INDEX OF TABLES AND FIGURES

<u>Table Number</u>	<u>Title</u>	<u>Pages</u>
26	Amino Acid Composition of a Chemically Defined Liquid Diet After Storage at 4° and (-)6°	96
27	Assay of Liquid Diet After Eight Months Environmental Exposure	97
28	Growth Rate, Diet and Water Consumption of Rats Fed Liquid Diet Containing 0.5% Glutathione Stored Under Varied Environmental Conditions	100
29	Growth of Rats Fed a Supplemented Protein Hydrolysate	107
30	Comparison of Chemically Defined Liquid Diets Containing Synthetic Amino Acid Mixtures vs. a Protein Hydrolysate Supplemented to Simulate These Mixtures	111
31	Comparison of the Essential Amino Acid Composition of an Acid Hydrolysed Protein With Recommended Allowances	113
32	Growth, Diet and Water Consumption of Rats Fed a Liquid Diet Containing an Amino Acid Supplemented Protein Hydrolysate	115
33	The Effect of Agar or Cellulose Supplementation of Liquid Diets on the Growth Rate of Rats	118
34	Estimated Energy Content and Energy Consumption of Chemically Defined Diets Fed in Different Physical Forms	120
35	Growth, Diet and Water Consumption of Rats Fed Various Diets in Both Liquid and Gel Forms	123
36	Growth of Male Weanling Fisher Rats Fed Codelid Liquid Diets Differing in Amino Acid Patterns	129

SCHWARZ BIORESEARCH, INC.

INDEX OF TABLES AND FIGURES

<u>Table Number</u>	<u>Title</u>	<u>Pages</u>
37	Diet Consumption of Male Weanling Fisher Rats Fed Codelid Diets Differing in Amino Acid Patterns	130
38	Water Consumption of Male Weanling Fisher Rats Fed Codelid Diets Differing in Amino Acid Pattern	131
39	The Effect of Ad Libitum Water and Liquid Diet Concentration on the Growth Rate of Rats.	140
40	The Effect of Ad Libitum Water and Liquid Diet Concentration on Diet and Energy Intake of Rats	142
41	Partition of Water Intake of Rats Fed Various Concentrations of a Chemically Defined Liquid Diet With and Without Ad Libitum Water	145
42	Volume, Osmotic Pressure and Specific Gravity of Urine Obtained From Rats Fed Different Concentrations of Liquid Diet	147
43	The Effect of Ad Libitum Water and Liquid Diet Concentration on Diet and Energy Intake of Rats	151
44	Heavy Water Concentration in the Urine of Rats Fed Dietary D ₂ O or D ₂ O in Their Drinking Water	157
45	Heavy Water Intake of Rats Fed Dietary D ₂ O or D ₂ O in Their Drinking Water	158
46	Distribution of D ₂ O in the Organs and Tissues of Rats Fed D ₂ O in Either Their Diet or Drinking Water	159
47	Effect of Feeding D ₂ O Via Diet or Drinking Water on Growth and Organ Size Of Rats	160

SCHWARZ BIORESEARCH, INC.

INDEX OF TABLES AND FIGURES

<u>Figure Number</u>	<u>Title</u>	<u>Pages</u>
1	Effect of Environmental Exposure on Diet Amino Acid Composition	27
2	Effect of Temperature on Glucose (2M), Glycine (1M) Interaction at 296mp	28
3	Effect of Temperature on Glucose (2M), Glycine (1M) Interaction at 408mp	29
4	Effect of Temperature on Glucose (2M), Lysine (1M) Interaction at 296mp	30
5	Effect of Temperature on Glucose (2M), Lysine (1M) Interaction at 408mp	31
6	Inhibition by Glutathione (Reduced) of Browning in a Glucose-Amino Acid Solution Containing Peracetic Acid (2mM/l)	32
7	Thioglycerol Inhibition of Browning in a 2M Glucose -- 1M Lysine Solution at 60°C	33
8	Inhibition Time of Browning In a Glucose Glucose -- Lysine Solution at 60°C With Increasing Thioglycerol Concentration	34
9	Rate of Browning of a Glucose -- Amino Acid Solution Containing Varying Amounts of Glutathione (Reduced) In the Presence of Peracetic Acid (0.002M at 4°C)	35
10	Interaction of Lysine (1M) With α -Pyrrolaldehyde (2M) in a Water -- Cellosolve Solution at 60°C	36
11	Rate of Browning of Lysine (1M) -- α -Pyrrolaldehyde (2M) in a Water--Cello-solve Solution at 60°C Measured at 426mp	37
12	Effect of Glycidol Concentration on Rate of Sterilization	65a

INDEX OF TABLES AND FIGURES

<u>Figure Number</u>	<u>Title</u>	<u>Pages</u>
13	Effect of Initial Contamination Level on Antimicrobial Efficiency of Parabens	65b
14	Antimicrobial Efficiency of Parabens in a Liquid Diet Containing a Fat Mix	65c
15	Growth of Rats Fed Chemically Defined vs. Standard Diet	76
16	Growth of Rats Fed Liquid Diet Heat Aged 60°C for 12 Days	77
17	Growth of Rats Fed Liquid Diet Irradiated With 5×10^5 Roentgens	78
18	Growth of Rats Fed Liquid Diet Irradiated With 5×10^6 Roentgens	79
19	Effect of Environmental Exposure on Diet Essential Amino Acid Composition	83
20	Growth of Rats Fed a Chemically Defined Diet Stored Four Months	88
21	Effect of Environmental Exposure on Diet Essential Amino Acid Composition	91
22	Growth of Rats Fed Codelid Diet-14 Refrigerated or Frozen For 8 Months	94
23	Amino Acid Composition of Codelid Diet-14 at 4°C or (-)6° For 8 Months	98
24	Growth of Rats Fed Refrigerated Diet With and Without Glutathione	101
25	Growth of Rats Fed Liquid Diet Stored at Ambient Temperatures With and Without Glutathione	102
26	Growth of Rats Fed Chemically Defined Liquid Diet Stored at 37°C With or Without Glutathione	103

SCHWARZ BIORESEARCH, INC.

INDEX OF TABLES AND FIGURES

<u>Figure Number</u>	<u>Title</u>	<u>Pages</u>
27	Growth of Rats Fed Chemically Defined Diet Stored at 60°C With and Without Glutathione	104
28	Growth of Rats Fed Amino Acid Supplemented Protein Hydrolysate	108
29	Growth of Rats Fed Both Liquid Diets of Amino Acids and Hydrolysates	110
30	Growth of Rats Fed Supplemented Protein Hydrolysate	116
31	Growth of Rats Fed Codelid Diet-17	124
32	Growth of Rats Fed Codelid Diet-18	125
33	Richter Tube	136
34	Growth of Rats Fed Diets of Different Concentration	138
35	Growth of Rats Fed Diets of Different Concentration Without Ad Libitum Water	139
36	Partition of Water Intake of Rats	144
37	Osmotic Pressure of Urine From Rats Fed Liquid Diets	148
38	Calculated Osmotic Pressure in G.I. Tract of Rats Fed Liquid Diets	150

ABSTRACT

- I. Stability studies of liquid diet solutions maintained under a variety of environmental conditions reveal, based on both chemical and bioassay the following facts:
 1. Refrigerated diets continue to retain full utility for periods observed to be in excess of twelve months.
 2. Radiation exposure to Co_{60} gamma rays at dose levels of 5×10^6 Rad, and 5×10^5 Rad respectively, reveals that a slight loss in biological activity occurs only at the higher radiation level.
 3. Marked loss in biological activity occurs in diets stored at room or higher temperatures. Animals fail to grow when fed a diet held at room temperature for four months, while death occurs quite rapidly among rats fed diet solution previously held at 60°C for 12 days time.
 4. Loss of biological activity and the development of a toxic character in the diet solution are invariably accompanied by the appearance of "browning".
- II. Studies of the "browning phenomenon" reveal the hitherto unrecognized fact that the key step in this reaction is a diffusion controlled process involving molecular oxygen as the determining factor in the generation of peroxides within the diet media. By both simultaneous destruction of preformed peroxides and the prevention of their formation, the browning phenomenon can be arrested. On the basis of these findings, a new mechanism for the "browning reaction" has been proposed. Further, it is believed that the toxic character of heat aged diet solutions is the

consequence of both the formation of hydroxylated amino acid antagonists as well as linoleate hydroperoxides. Both products result from attack by peroxides upon the diet amino acids and unsaturated fatty esters.

- III. From the economy standpoint, liquid chemical diets have been prepared using acid protein hydrolysates supplemented by the addition of necessary quantities of amino acids. These solutions have further been shown to be completely comparable to diets prepared from pure individual amino acids.

From the efficiency standpoint, liquid chemical diet formulations have now been prepared with amino acid patterns which yield a growth rate of 6.0 gm/rat/day comparable to that obtained with protein diets.

- IV. Water balance studies in which "heavy water" was substituted in part for water present in the diet, reveal from determinations of the D_2O content in the urine that two thirds of the animal body water turns over. The remaining one third water represents water immobilized with the cell structures. The time required for water equilibrium to be established is of the order of one week.

- V. Study of total electrolyte distribution and diet osmolality indicates that diet concentrations which result in a kidney loading in excess of 1,000 milliosmoles/liter produce kidney failure and ultimately death. This was found to be the case in the use of diet concentration of 40% and 50% fed to animals which were denied ad libitum water. Diets of 30% concentration

are found to be optimal in terms of both osmolality and caloric content. Diets of concentrations lower than 30% while osmotically safe, fail to meet efficiently the caloric requirements of the rat, vis. 55/cal/rat/day.

INTRODUCTION

Earlier investigations on our part¹ as well as those of Greenstein and coworkers² with liquid diets, revealed that the best chemical nutrient solutions while adequate to promote the growth of weanling rats (3-4 gm/day) and to maintain adult animals in positive nitrogen balance, were not optimal nutritionally. Judged in comparison with standard animal chows, the liquid diet formulations were only between 50%-60% as effective in promoting growth. It was not known to what extent amino acid patterns were contributory or whether other features of the diet such as osmolality were factors in impeding the attainment of their ultimate nutritional potential. Further, it had been observed previously that liquid diet solutions "browned" when exposed to ambient or higher temperatures and that these changes were accompanied by loss in part of the nutritional content of the diet. The quantitative aspects of these nutrient losses and the related environmental conditions responsible for inducing these changes had still to be evaluated. Generally speaking, therefore, the earlier studies had solved successfully the short term problems associated with liquid diet formulation.

Those studies which at this stage still remained, were of longer range. These were related to defining the limits of utility of chemically constituted liquid nutrient media in terms of the ability of their component nutrients to withstand a variety of imposed environmental stresses. It was appreciated that in order to accomplish this objective of quantitatively determining the magnitude of the induced chemical changes, a number of analytical and bioassay techniques would be required to be developed. Once having defined the intrinsic limitations of liquid diet systems as presently constituted, an effort then

would need to be directed to understanding the nature of the chemical changes thus induced so that an attempt could be made to mitigate in part their full nutritional impact.

In this connection specifically, questions relating to vitamin or microbiological stability or of whether during browning the amino acids within the diet reacted with glucose with equal speed, all remained to be explored. Further, data as to whether a liquid diet could be formulated which was optimal in terms of satisfying both nutrient and water requirements of an individual were lacking.

Thus, it is with problems such as these, as well as with attempts at answering some of the questions posed by the indicated chemical changes occurring within the liquid diet that the major portion of this report is concerned in the sections that follow.

SCHWARZ BIORESEARCH, INC

NATURE OF CHEMICAL INTERACTIONS OCCURRING
WITHIN LIQUID DIET SOLUTIONS

A. NON-ENZYMATIC BROWNING

The stability studies performed on liquid diet preparations which had undergone a wide range of environmental exposures, in some instances for periods extending up to a year in duration, revealed that intrinsically solutions of this type were nutritionally adequate as well as chemically stable provided that adequate care was taken to insure that these solutions were maintained at 4°C or less. Visual and biological evidence of substantial interactions appear, however, as the temperature of the diet solutions is increased. Thus, diet solutions which had been allowed to stand at room temperature in sealed bottles for a period of 4 months, failed to support normal growth of weanling rats. The same formulation, when heated at 60°C for 12 days, caused the death of all test animals within a 10 day period.

The extensive "browning" noted in both these diet solutions was thought to be due to the Maillard reaction. This reaction, appropriately named after an early investigator³ (1912), occurs whenever a reducing sugar (aldose) and an amino acid or protein are permitted to interact. Brown bread crust, as well as the brown appearance of corn flakes, coffee, tobacco, ale etc. are familiar examples of browning as it is normally encountered in everyday living.

The classical picture of browning as it is known to occur in solutions of amino acids and reducing sugar is also observed with liquid diet formulations. The first indication of a reaction between, for example, glucose and glycine, is the observance of a drop in the pH of the solution from an initial value of about 5.5 to about 4.5. This

SCHWARZ BIORESEARCH, INC.

initial increase in solution acidity is rapidly followed by the appearance of compounds within the solution which possess ultraviolet absorption maxima in the region of 285 to 296 m μ . Shortly thereafter, visible darkening of the solution occurs characterized by broad absorption over a range of several hundred millimicrons starting at about 408 m μ . As the solution becomes progressively darker, evidence of CO₂ evolution is noted along with the detection of strong characteristic aldehyde-like odors. The brown solution itself becomes increasingly viscous and ultimately sets to an insoluble hydrated gel.

Liquid diet solutions, by virtue of their composition and concentration, viz., one mole of total amino acids and two moles of glucose are excellent vehicles for studying the browning phenomenon. In terms of the molar concentration of the individual amino acids, the molar ratio of glucose to any given amino acid is between 20-40 times greater. Kinetically speaking, therefore, the concentration of glucose present within the media is essentially constant. If it is assumed that all the amino acids were of equal reactivity relative to reaction with the glucose in solution, then one could expect that the percent losses of all amino acids to be approximately equal. This, in fact however, is not the case. The basic amino acids, viz., histidine, lysine and arginine are decidedly more reactive than any of the other amino acids. So marked is this difference that it would appear that the basic amino acids are about twice as reactive as those amino acids in which the R group is a simple aliphatic substituent, ex., valine, leucine, alanine, etc. Thus, if one examines the data in Table 1 it may be seen, for example, that lysine having an amino group in the

SCHWARZ BIORESEARCH, INC.

€-position as well as one in the α -position disappears from solution just about twice as rapidly as glycine. The comparative data in diet #1 show losses of 61.5% lysine; 30.0% glycine while in diet #2 the values are 59.2% lysine and 29.8% glycine. Interestingly enough, diets #1 and #2 were liquid diet preparations of different compositions, formulated by different people in different parts of this country, stored in different types of containers, polyethylene versus glass, at ambient conditions which also differed and manufactured at time interval of two years from one another. Despite these differences, the same relationships are observed to hold internally within each diet solution.

Thus, whereas in lysine the basic reactive additional amino functionality is derived from the €-amino groups, in histidine this arises from the imidazole N, whereas in arginine, the basic amino nitrogen occurs at the 5-position, corresponding to the €-amino group in ornithine.

Columns 3 and 4 in Table 1 show those amino acids which are most liable to undergo C-N bond rupture and to liberate ammonia when subjected to either ultraviolet or ionizing radiation. This is approximately the same order in which the amino acids react with the aldehyde functional group of aldoses. If one, however, allows for the presence of two functional amino groups in the basic amino acids as well as the presence of R substituents in the amino acids which can either promote or retard the participation of the α -amino group in its reaction with glucose, it appears that the rate of condensation of amino acid with glucose is proportional to the total concentration of available amino groups in the reaction medium. The significance of

this fact will become evident in examination of the browning of protein with reducing sugars.

Table 2 summarizes the data of Lea and Hannon⁴. It clearly indicates that dry casein heated with glucose in moist air, selectively loses its basic amino acid components in addition to methionine. (In Table 1, it has previously been demonstrated that in a liquid diet solution, the same basic amino acids appeared to be twice as reactive as other simple aliphatic amino acids in reacting with glucose.) Their data tend to confirm the bifunctionality on the part of the amino groups of these amino acids in regard to reaction with glucose since even when these same amino acids are present in bound form in protein, they are still destroyed in the browning reaction.

The browning observed by Lea and Hannon in the dry state with casein along with its accompanying losses of specific basic amino acids was also found to occur in a solution of serum albumin heated with glucose at 53°C.⁵ Here too, amino acid destruction was confined to these same four acids to the extent indicated in Table 3. The essential points of these comparisons are the following:

1. That browning occurs both in the solid and liquid state.
2. That browning is not limited to amino acids but occurs equally well in proteins.
3. That at any given temperature the extent of amino acid loss is related to the number of exposed amino nitrogen or oxidizable groups in either protein or amino acid.

SCHWARZ BIORESEARCH, INC.

1. NUTRITIONAL CONSEQUENCE OF BROWNING

It was previously indicated in discussion of the stability of liquid diet preparations, that heating one such preparation for 12 days at 60° resulted in the death of all experimental animals when the browned diet was fed to weanling rats. The same formulation left to brown at room temperature for 4 months failed to produce any growth in test animals. The physical losses in amino acids that accompany these environmental exposures are recorded in Figure 1. The crossbars in this figure represent the National Research Council's recommended daily allowances of these respective amino acids.

Despite the drastic losses sustained by some of the essential amino acids in the heated diet, the observed lethality does not seem warranted by the magnitude of the losses sustained by the affected nutrients. Implicit in all of this is the possibility that one or more toxic products may have been formed during the browning process.

The distinct possibility that this may be the case is evident in the work of Adrian, Petit and Godon.⁶ These workers were able to cause a reduction in growth, a drop in the protein efficiency coefficient and decreased nitrogen balance in rats fed an adequate casein diet to which was added a solution of glucose and glycine that had previously been heated at 90°C for increasing periods of time. The observed toxicity of the reaction products of glucose and glycine to Phytophthora fragariae is consistent with this view.⁷ The possible nature of the toxic factor is considered in a later section of this report.

CHWARZ BIORESEARCH, INC.

However, part of the difficulty in characterizing "browning" has been the failure of previous workers to recognize the free radical nature of the browning reaction and particularly the role played by oxygen. Viewed in this light, many of the seemingly anomalous findings in prior browning studies are now comprehensible. This is particularly true in regard to the observed marked effect of temperature on browning.

It is very convenient to examine the rate of amino acid-reducing sugar interactions by measuring the increase in optical density at either 296m μ in the ultraviolet or at 408m μ in the visible region of the spectrum. The similarity in the shape of the curves at both wavelengths but absence of identity in their rates of formation, indicates that at each of these wavelengths, the curves represent the genesis of distinct intermediate compounds formed in the browning process, which may be related but are not identical. (Figures 2, 3, 4 and 5.)

Further, at the temperatures 50° and 60° the formation of compounds which absorb at 296m μ occurs at a faster rate than for those compounds absorbing at 408m μ . It is not inconceivable that the 296m μ absorbing material is the precursor of that absorbing at 408m μ . All curves show an initial induction period which increases in duration as the temperature is decreased. At both wavelengths, however, the dramatic increase in reaction rate with increasing temperature is apparent.

Therefore, more meaningful comparisons between the rates of reaction of two different amino acids with glucose can be expected at lower temperatures. Pursuing

SCHWARZ BIORESEARCH, INC.

this idea, a comparison of the rates of increase in optical density at 296m μ for the interaction of glucose with both lysine and glycine at a temperature of 50°C reveals that lysine is twice as reactive as glycine, a conclusion that had previously been arrived at on the basis of loss of these respective amino acids from solution during browning.

Finally, in passing it should be mentioned that the shape of all these curves is typical of reactions in which peroxide formation and decomposition is occurring.

2. ROLE OF OXYGEN IN BROWNING

Retrospective examination of the curves relating to the optical changes accompanying the interaction of glucose with amino acids reveals that oxygen involvement in the browning reaction was a distinct possibility. However, definite evidence as to the role played by oxygen came during an examination of the occurrence of browning in the solid state.

In one experiment, lollypops were prepared employing 20 parts of an amino acid hydrolysate and 80 parts of sucrose to which was added a red food coloring. The lollypops were cellophane-wrapped, and allowed to age at room temperature for several months. On reexamination, it was surprising to find that they had browned only at the surface. This observation was contrary to all expectations since the classical view of browning involves the simple condensation of an aldose sugar with an amino acid. If browning occurred at all, it could be expected to occur uniformly throughout the specimens. Here instead, however, browning was found to occur only at the surface and this effect was found in all specimens.

To further test this, tablets were prepared consisting of 20% pure amino acids in the presence of 80% glucose using different degrees of tablet compression. If oxygen diffusion were important to the reaction, the loosely compressed tablet could be expected to brown more rapidly than the densely compacted one. This result was confirmed by finding that the densely compacted tablets were only brown at the surface while the loosely pressed tablets were brown throughout. Next, compressed tablets consisting of amino acids and glucose were

SCHWARZ BIORESEARCH, INC.

prepared using various tablet lubricants, some of which contained saturated fatty acids; others an unsaturated fatty acid. Here the degree of tablet compression was uniform. Those tablets which contained amino acids, glucose and magnesium stearate remained white after three months at room temperature whereas those in which a linoleate ester had been used as the tablet lubricant, had browned. In these tablets, the extent of browning was related to the amount of unsaturated peroxidizable fatty acid present.

Having observed the relationship between oxygen diffusion and oxidation with the occurrence of browning in the solid state, it was decided to examine whether browning in solution also involved oxygen participation. To our knowledge, this relationship had never been reported. Since the browning reaction at moderate temperatures of solution is sufficiently slow as to extend over a considerable period of time, the diet solutions were placed in long ampuls, sealed at the top with parafilm, aged at both room temperature and 38° and the resulting changes recorded in time lapse movies. In the resulting film, it was seen that browning, as predicted, occurred in solution by proceeding from the air-liquid interface into the body of the liquid.

The role of oxygen diffusion in triggering the appearance of visual browning in solutions of amino acid-glucose was reconfirmed in diffusion studies run at ambient temperatures. Here, a liquid diet solution of 50% concentration was prepared containing 0.15% agar and 0.02% butyl paraben. The diet solutions were placed in long cylinders which were plugged with sterile cotton to keep out microorganisms and then overlaid with a thin polyethylene film to prevent

evaporation of the solution and yet allow the passage of oxygen into the solution. The presence of agar prevented the development of convection currents which might cause mixing within the solution. These solutions were left standing at ambient temperatures in the cylinders. Within several weeks, a dark zone appeared at the air-liquid interface which, with increasing time, increased in intensity and in magnitude. Here too, the penetration of browning into the interior of the diet solution from the surface was obviously proceeding in the wake of oxygen diffusion.

The picture that began to emerge from this study was that browning occurred as the result of peroxide attack upon the product resulting from the initial condensation of reducing sugar with amino acid. Further, it appeared that where peroxides were already extant in the amino acid-reducing sugar mixture, browning proceeded immediately. In those media where peroxides do not exist initially, the magnitude of the induction period observed prior to appearance of browning represents the time required for peroxide formation. The length of this induction period is markedly shortened with increasing temperature.

Figure 6 clearly shows the relationship between the number of peroxide molecules in the liquid diet solution and the degree of browning observed at three different temperatures at a given moment in time.

Specifically, Figure 6 represents liquid diet solutions maintained at 4°, 24° and 37° and to which 4mM/l of peracetic acid were added. To tubes of each of these solutions at all three temperatures, various

SCHWARZ BIORESEARCH, INC.

amounts of glutathione (reduced) were added and the optical densities of the resulting solution read at 408m μ . The resulting figure clearly indicates the stoicheometry involved in the chemical browning of the glucose-amino acid solutions.

The point of intersection of the tangents of the two slopes of the curve represents a stoicheometry such that 8 millimoles of reduced glutathione have decomposed 4 millimoles of peracetic acid. As one proceeds along the curve to the right of this point of intersection, the concentration of glutathione increases with no corresponding increase in optical density (browning). All values on the curve to the left of the point of interaction represent concentrations of peracetic acid in solution equal to the difference between the 4 millimoles of peracetic acid and one-half the value of the molar concentration of thiol on the X-axis.

The ability of thiols to inhibit browning (defined here as the increase in absorption 408m μ) in a solution of glucose and amino acid is clearly seen in Figure 7. With increasing concentration of thioglycerol the magnitude of the increased length of time of the induction period over that of the control of glucose and lysine alone at 60°, is progressively extended. In the presence of freely available oxygen, the thiol is able to inhibit browning only to the extent that it is able to destroy peroxides formed in solution. The parallel nature of the curves following the induction period indicates that once all of the thiols have been exhausted by chemical interaction with

SCHWARZ BIORESEARCH, INC.

peroxides, the rate of browning is governed solely by the rate at which peroxides formed within the given solution react with the amino acid-aldose condensate.

Extrapolation of the linear portion of this family of curves to the X axis yields a value which is a measure of the magnitude of protection afforded by any given concentration of inhibitor within the given system under study at the temperature in question. These points replotted in Figure 8 show that not much additional protection is to be attained beyond a 1% level of thioglycerol.

Figure 9 represents a linear plot obtained when a fixed quantity of peracetic acid was added to various diet solutions stored at 4°C and which also contained different levels of glutathione. The fact that these solutions had been previously stored for several weeks prior to the addition of the peroxide raises doubt as to the accuracy of the thiol value recorded on the X axis. However, this fact does not alter the linearity of this plot, only its slope is subject to change.

Having firmly established the role of both oxygen and peroxides within the framework of the browning reaction, it remained to be established at what stage oxygen entered into the browning mechanism. The observation of an induction period at temperatures of 50°C or lower in plots relating the magnitude of changes induced with time in systems containing both active aldehyde and amino functions indicates that the rate of peroxide formation within the media is limiting at least initially.

Restated, the presence of a measurable

SCHWARZ BIORESEARCH, INC

induction period indicates that under the stated conditions the rate at which, for example, glucose reacts with amino acid to form a peroxidizable substrate is faster than the rate at which oxygen is able to diffuse into the medium and form appreciable quantities of peroxide. However, as additional studies which will be presented in subsequent reports will further reveal, a steady state of peroxide formation and decomposition is attained during the browning reaction following the induction period. At this stage, peroxidation no longer is the limiting factor. Now the rate determining step becomes the rate at which amino acid is able to react with reducing sugar. The absence of an observed induction period in solution of amino acid-glucose heated to temperatures above 60°C confirms this fact. The slope of the plots of OD versus time (Figure 7) is a measure of the rate of glucose-lysine interaction. The identity of these slopes indicates that they are all measuring the same reaction.

3. REACTIONS OF MODEL COMPOUNDS

Part of the difficulties, it was felt, in attempting to trace the course of the browning reaction, arise from the fact that the carbohydrate involved is able to undergo a number of transformations which may not be related to the browning reaction at all. A case in point is the appearance within a mixture of glucose and amino acid undergoing browning of 5-hydroxymethyl furfural (5-HMF). Therefore, it was of decided interest to us to attempt to ascertain whether glucose per se was essential to the browning process or whether its function was simply to provide a water soluble aldehyde (5-HMF). To examine this possibility, the reaction of amino acid with α -pyrrolaldehyde (the N-containing analog of furfural) was studied. This compound, which itself obligingly absorbs at 293m μ , undergoes browning in the presence of amino acid forming a reaction product which absorbs at 426m μ .

The measurement of the increase in optical density at this wavelength with time permits a study of its rate of browning. The characteristic absorption spectrum of this compound both alone and subsequent to reaction with lysine are seen in Figure 10. The rate of browning of α -pyrrolaldehyde is depicted in Figure 11. where a plot of increase of OD at 426m μ with time is represented. The principal advantages of using α -pyrrolaldehyde in place of glucose are the thermodynamic stability of the pyrrol nucleus and the ability to determine the number of aldehyde groups participating in the reaction by a measurement of the nitrogen content of the product formed. Thus, on the basis of elemental analyses of the product, the requirement of an extensively conjugated system to account for its brown color

SCHWARZ BIORESEARCH, INC.

and the need to explain the role of oxygen in the browning process, the following mechanism is offered: (see Mechanism 1).

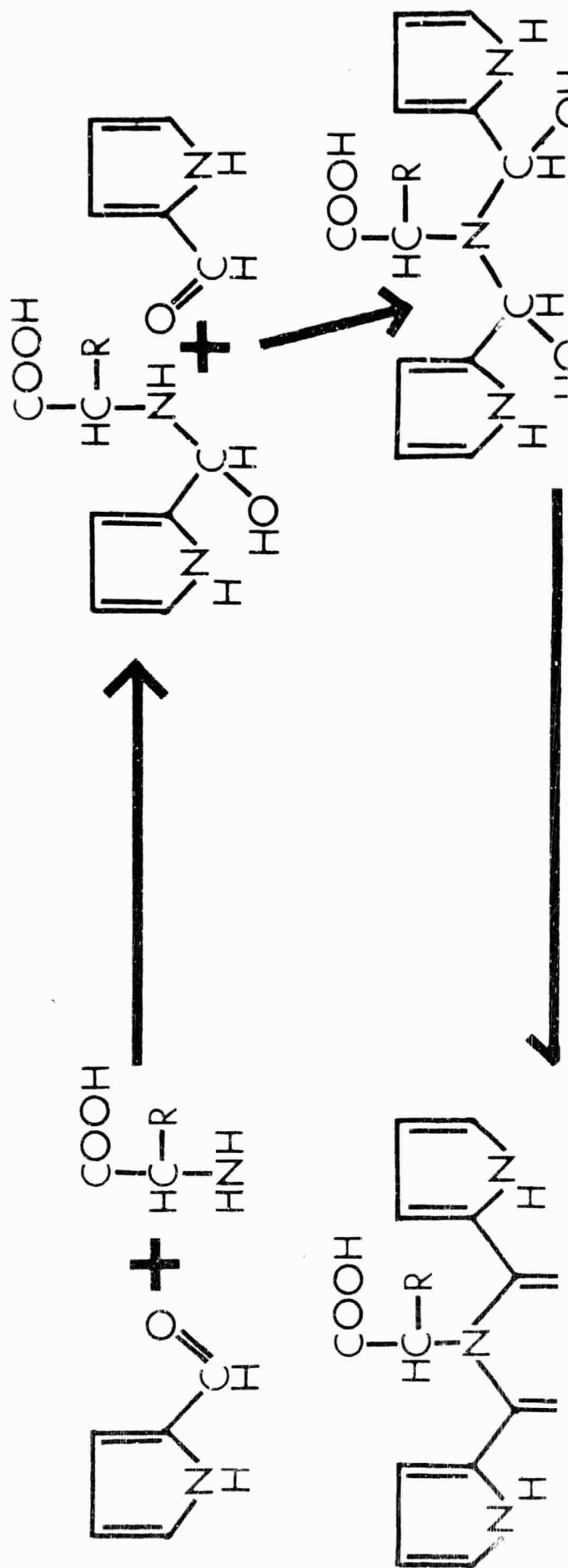
The essential features of this mechanism are first, a stoichiometry calling for two moles of aldehyde to react with one mole of amino acid to form an N,N, disubstituted amino acid. Two of these molecules are thought to be able by condensing and eliminating two moles of water, to form a carbon - carbon bond. If this reaction occurs in a head to head fashion as indicated in the above equation, the likelihood exists for the formation of a dihydropyrazine ring structure such as the type indicated in the outlined scheme. Whereas this explanation accounts for all of the known facts, the possibility also exists for the occurrence of a lateral coupling of a number of the N,N, disubstituted α -amino acids to form a substituted polyethylene azine type polymer.

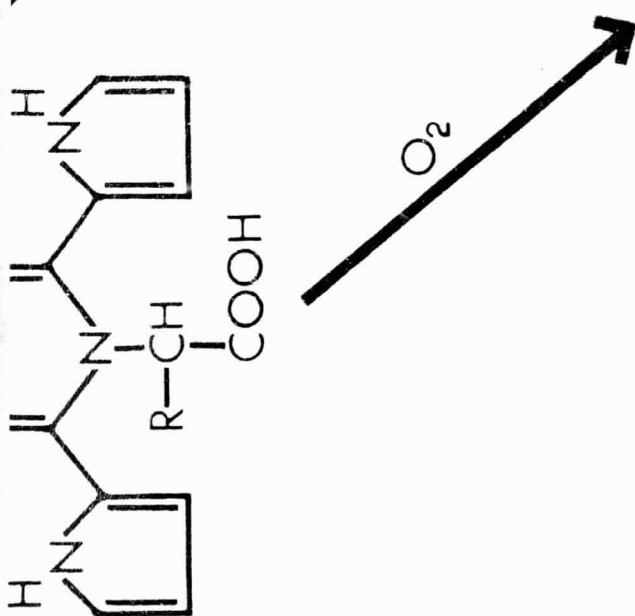
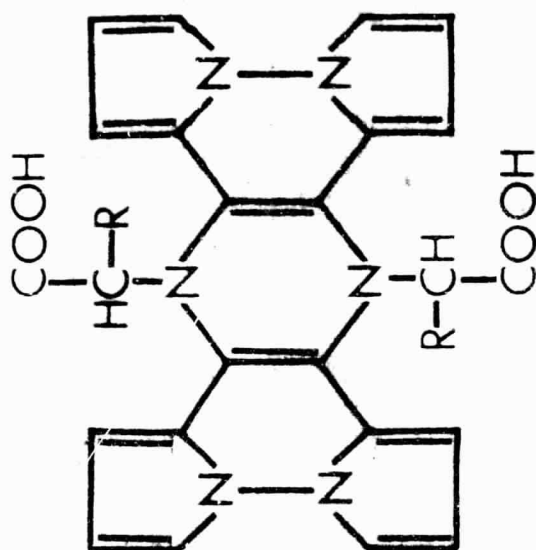
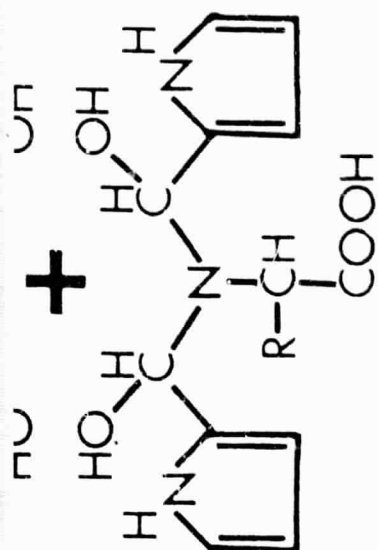
Adapting the mechanism proposed for the condensation of pyrrolaldehyde with amino acid to that of glucose and amino acid yields the scheme shown in Mechanism 2.

The significant modification introduced here is an additional step involving the peroxidation of the α -C-H bond of the amino acid. This latter peroxidized molecule is thought then to break down under the influence of thermal energy to liberate, via an α -keto acid, CO_2 and an aldehyde, with the simultaneous aromaticization of the dihydropyrazine ring to pyrazine. Here too, in addition to the option of forming pyrazine ring structures the possibility of forming polyethylene

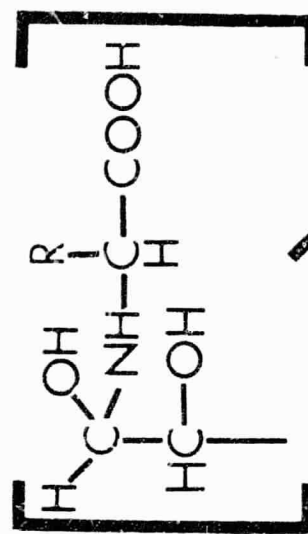
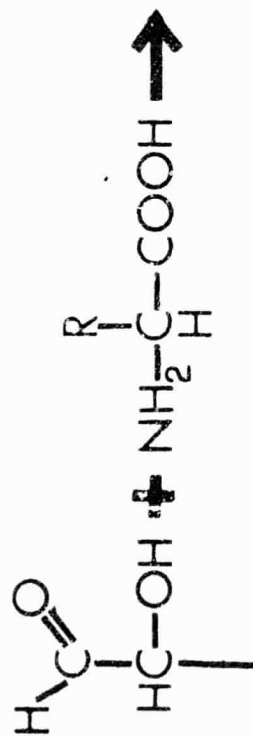
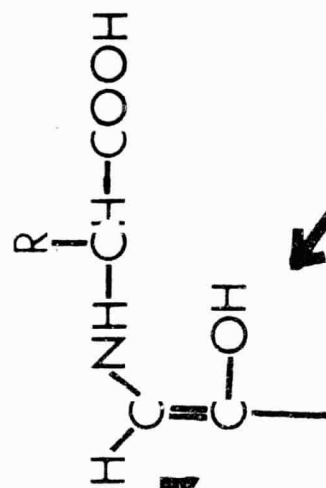
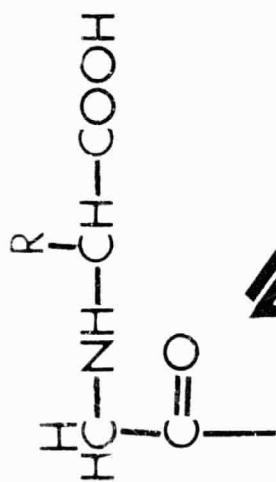
MECHANISM 1

AMINO ACID - PYRROLALDEHYDE BROWNING MECHANISM



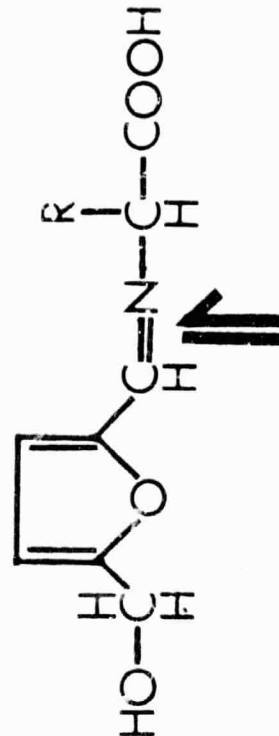
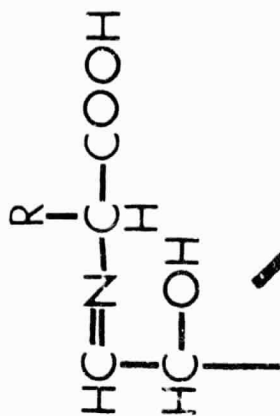


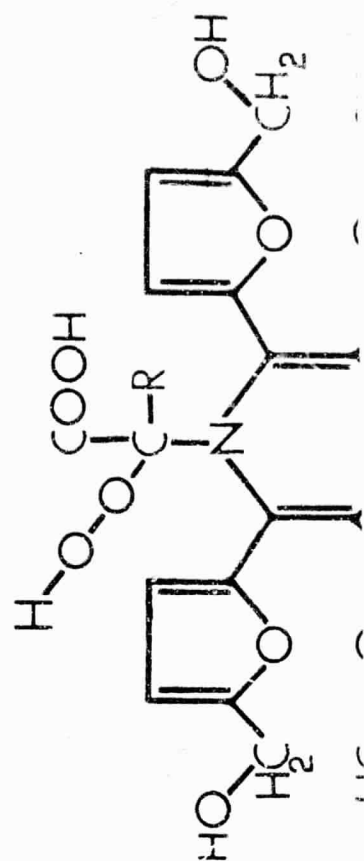
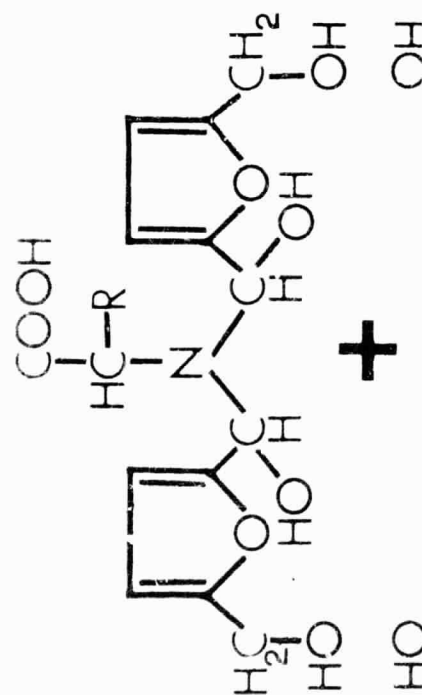
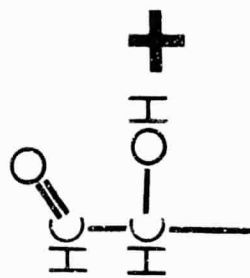
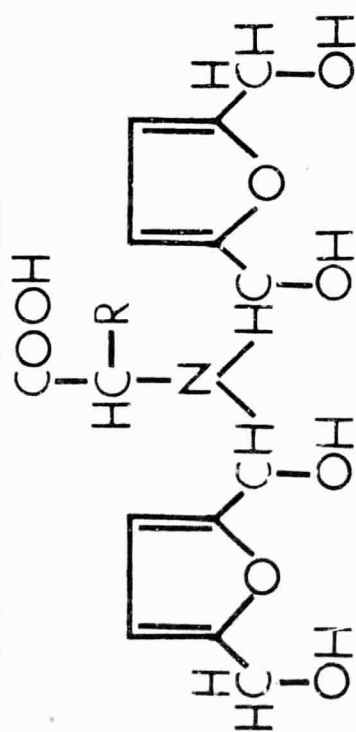
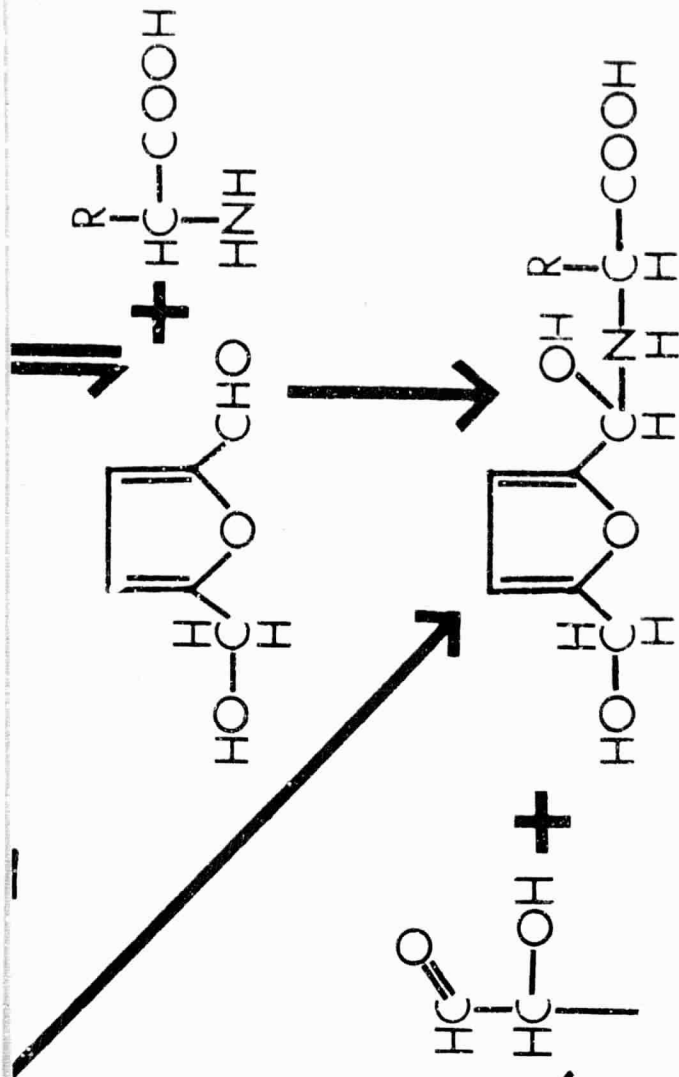
MECHANISM OF NON-ENZYMATIC BROWNING



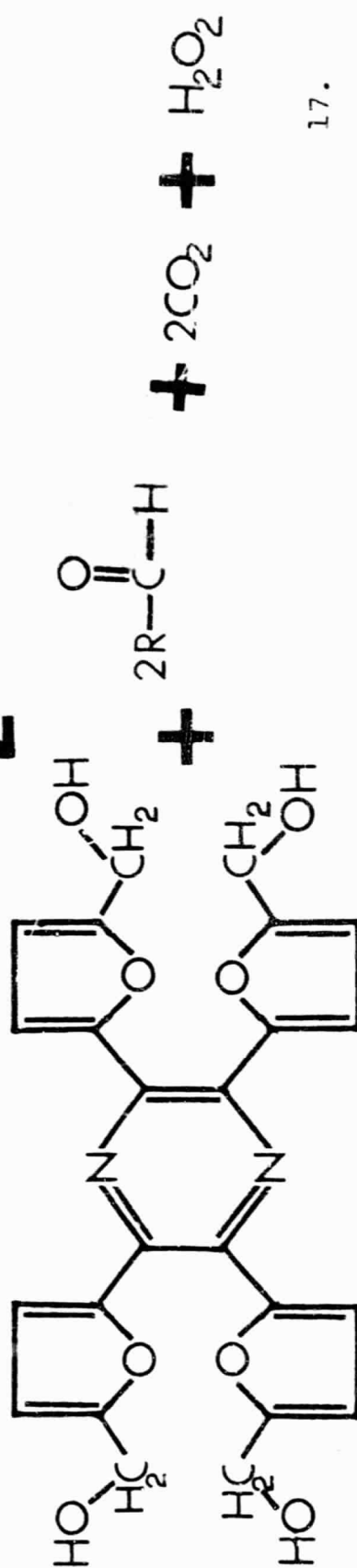
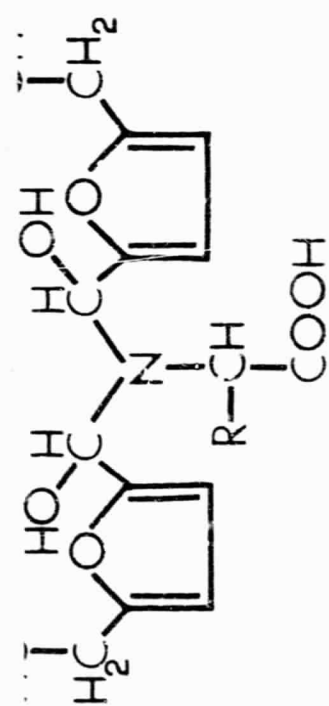
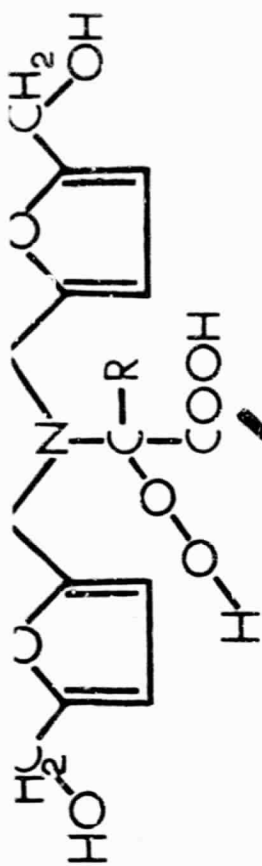
(-H₂O)

(-2H₂O)





2



SCHWARZ BIORESEARCH, INC

azine polymers, isoelectronic with pyrazine molecules, is very likely.

Without directly contravening the essential correctness of the pyrazine ring mechanism proposed it is felt that in actuality, two polymeric pathways leading to browning exist and may be the more important.*

These two types of browning reactions are thought to differ only with respect to whether the brown polyethylene azine polymer formed is mono or disubstituted, with substituents occurring at either carbon atom 1 or 2 or both of the chain backbone which itself is derived from the carbohydrate moiety. Although polymer formation by both pathways is most likely to be a simultaneous occurrence, the choice of the two routes is probably dependent upon the ratio of carbohydrate carbonyl groups to that of free amino groups within the reaction medium. Thus, a high ratio for example, of glucose to amino acid, would favor the formation of the disubstituted polymer while a lower ratio favors the monosubstituted polymer.

Superimposed on all of this is the fact that competitive with the formation of substituted polyethylene azines is the cyclization reaction leading to the synthesis of substituted pyrazines. The latter are only thought to form at fairly high temperatures by direct reaction of sugar and amino acid or from the cracking of polyethylene azine polymers.

There are several significant implications of the browning mechanisms proposed herein. The first of these is that browning is a free radical process, diffusion controlled at the lower temperatures where the rates of oxygen diffusion into the susceptible

*See following pages

SCHWARZ BIORESEARCH, INC.

medium and subsequent peroxidation are relatively slow. At higher temperatures or when preformed peroxides are present, the reaction begins immediately and is only related to the concentration of amino and carbonyl functions within the media. The liberation of hydrogen peroxide as a reaction product makes this reaction autocatalytic. The control of browning, therefore, is dependent upon the prevention of oxygen penetration into the medium as well as the destruction of extant peroxides. The favorable influence of low temperatures in retarding browning is related to the absence of sufficient energy to cause either peroxidation or the subsequent decomposition of peroxides.

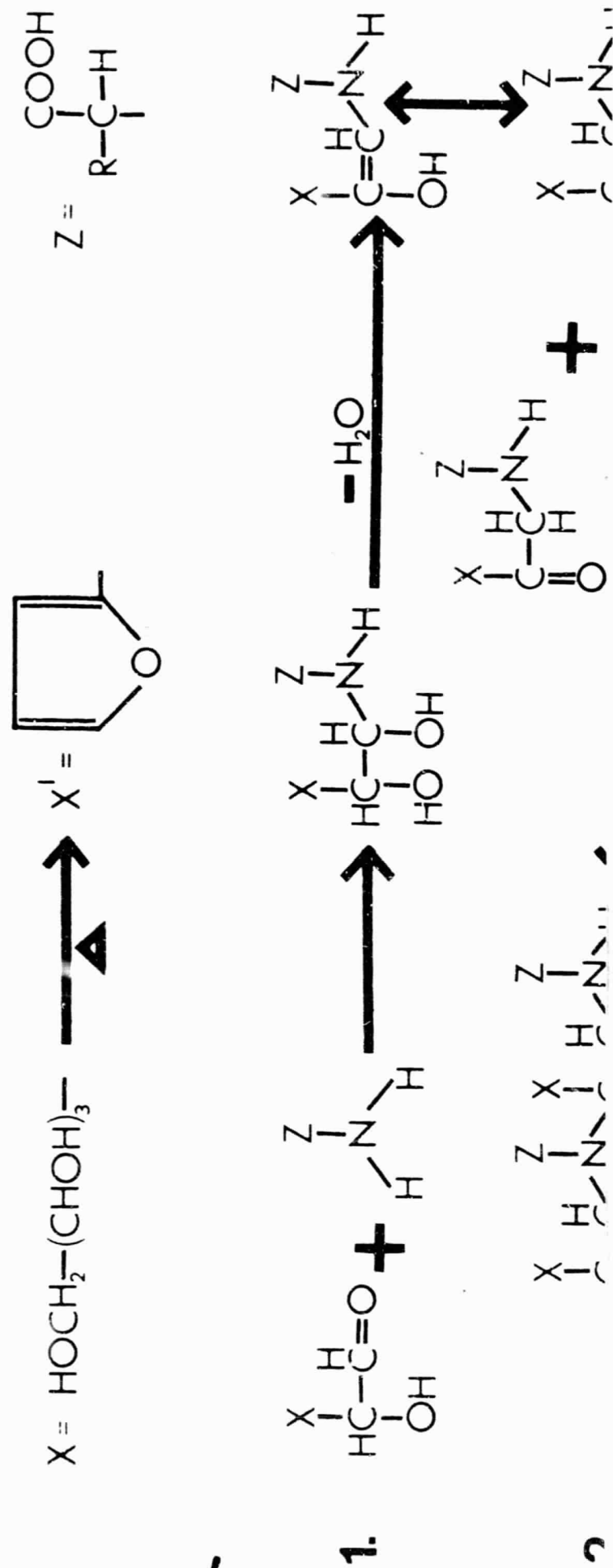
SCHWARZ BIORESEARCH, INC

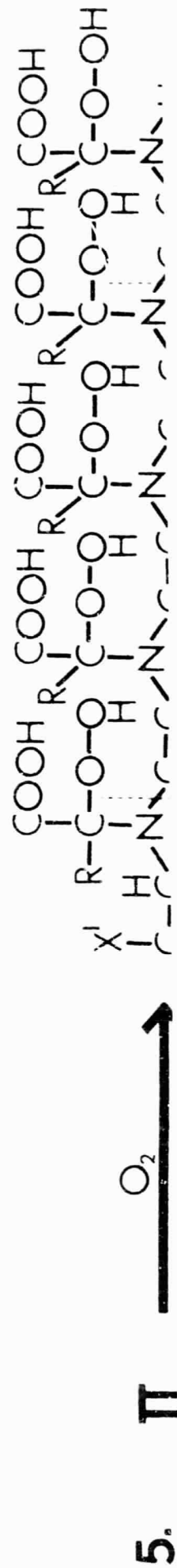
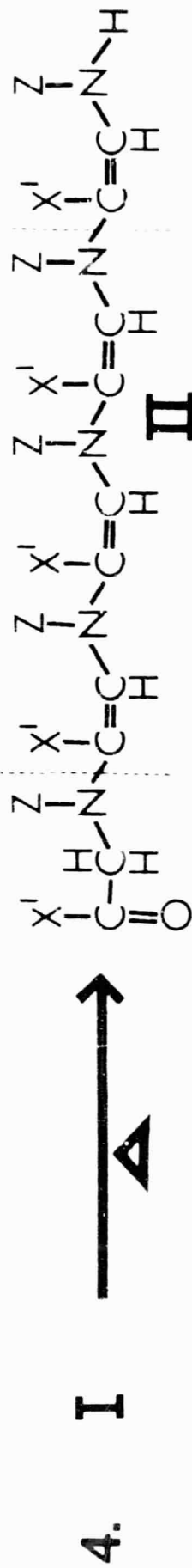
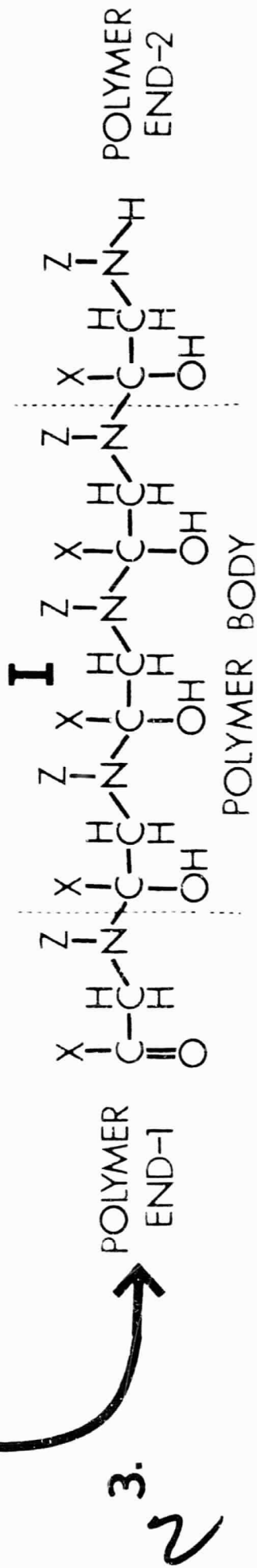
I. BROWNING MECHANISM VIA MONOSUBSTITUTED POLYETHYLENE
AZINE ROUTE

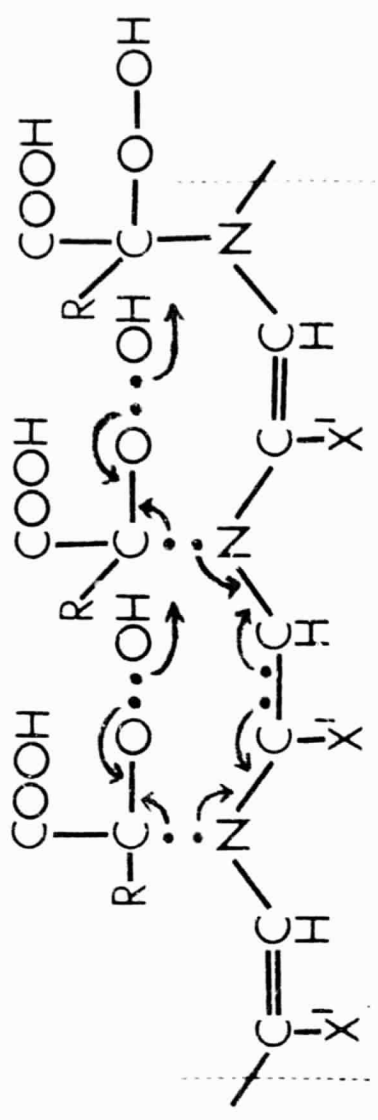
A. GLUCOSE-AMINO ACID SYSTEM

In this mechanism, the C_1 aldehyde group is thought to condense with the α -amino nitrogen to place a hydroxyl (OH) group at the C_1 position. Subsequent loss of this group in conjunction with a hydrogen atom from C_2 through the elimination of water, creates a double bond between C_1 and C_2 . Enolization of the hydroxyl group at C_2 forms the ketosamine or so-called Amadori rearrangement product. Self-condensation of several Amadori products leads to the polymers shown in equations (2) and (3). Dehydration within these polymers by removal of OH and H groups from carbon (2) and carbon (1) respectively generates a double bond. Oxygen attack at the site of the α -C-H bonds of the amino acid within these unsaturated polymers causes peroxidation. Thermal destruction of these peroxides leads to extensive conjugation of the unsaturated bonds along the polyethylene azine polymer backbone with the simultaneous liberation of CO_2 and aldehyde from the α -keto acid formed as well as an accompanying liberation of hydrogen peroxide. Whereas glucose is depicted here as the carbohydrate substrate, any carbohydrate moiety with a minimum of a $HOC-\overset{\overset{|}{H}}{\underset{\underset{H}{|}}{C}}=O$ group is capable by this mechanism of undergoing browning.

BROWNING MECHANISM VIA MONOSUBSTITUTED POLYETHYLENE AZINE ROUTE



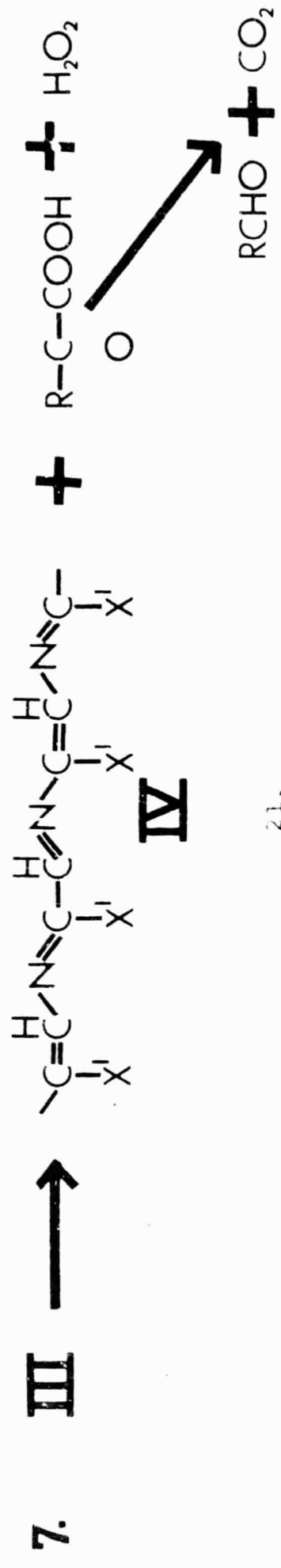




ENLARGED SEGMENT
OF III SHOWING
PEROXIDE DECOMPOSITION

6.

3



SCHWARZ BIORESEARCH, INC.

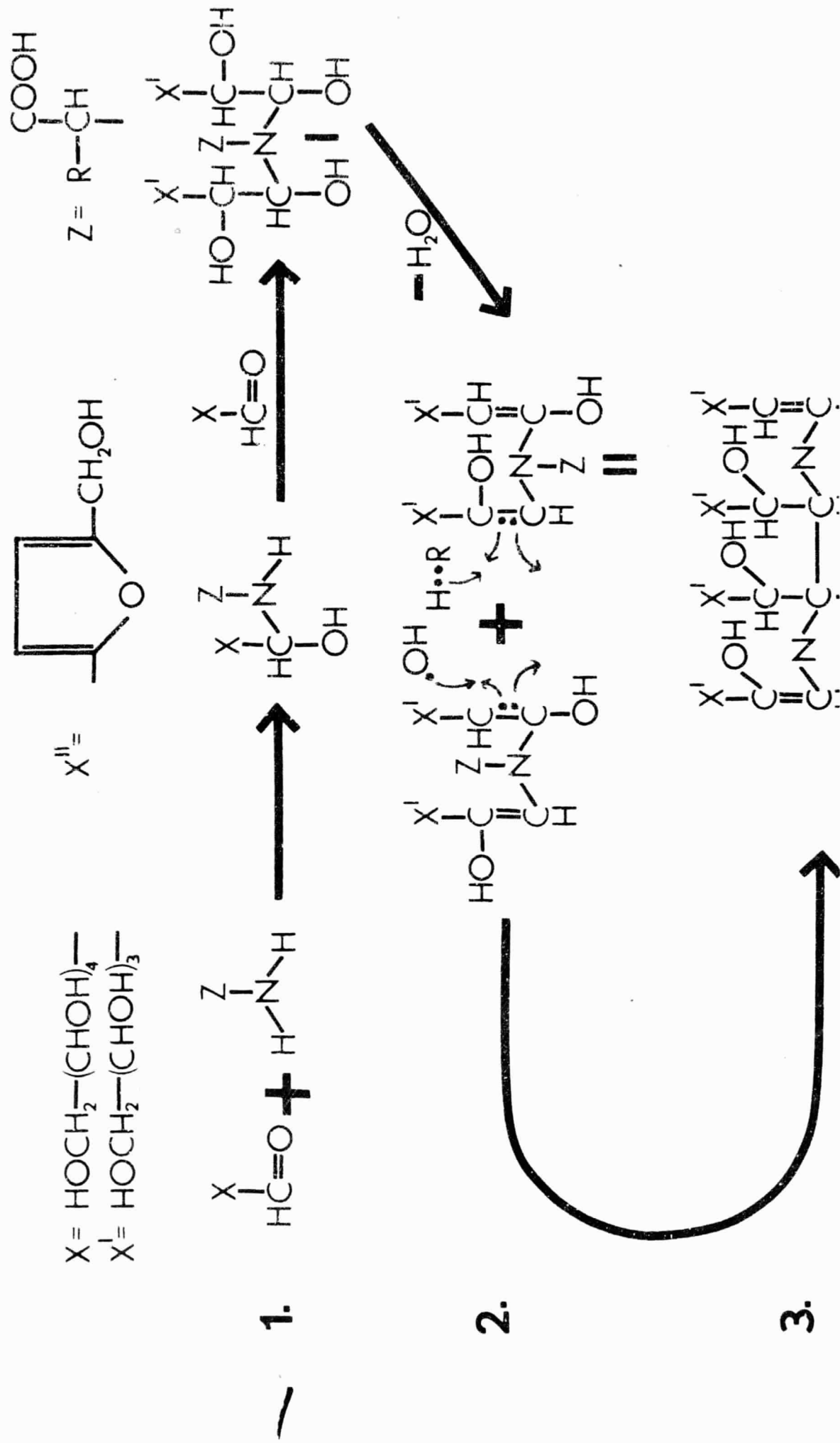
II. BROWNING MECHANISM VIA DISUBSTITUTED POLYETHYLENE AZINE ROUTE

A. GLUCOSE-AMINO ACID SYSTEM

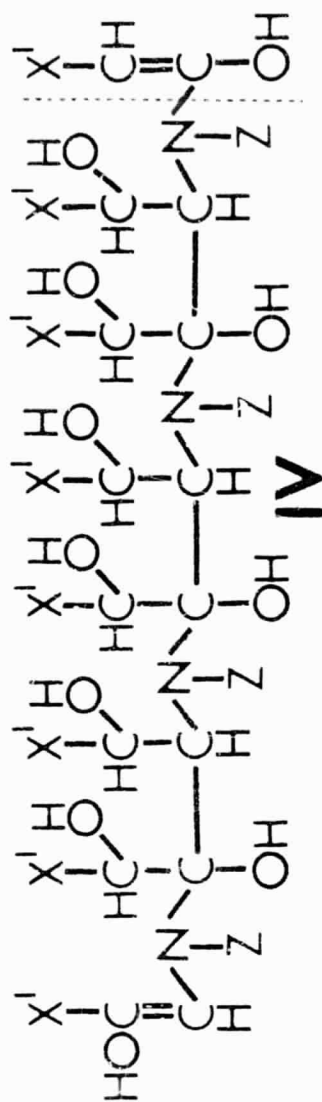
In this mechanism, the C_1 aldehyde group condenses with the α -amino nitrogen to place a hydroxyl (OH) group at the C_1 position, identical to the initial step in the monosubstituted polyethylene azine mechanism. However, this chemical entity now reacts with a second molecule of aldehyde to form (I) an N,N, disubstituted amino acid. Dehydration of this intermediate at the C_1 and C_2 positions in each of the former aldehyde moieties respectively produces the diunsaturated compound II. Successive condensation of the latter via a free radical initiated process yields (III) and (IV), higher polymeric units. Thermally induced dehydration of IV produces V, an unsaturated polymer believed responsible for ultraviolet absorption observed during browning. The peroxidation of V by O_2 at the site of the C-H bonds of the amino acid and subsequent thermal decomposition by a radical process of the peroxides thus formed, leads to formation of a disubstituted polyethylene azine polymer along with CO_2 , hydrogen peroxide and an aldehyde.

BROWNING MECHANISM VIA DISUBSTITUTED POLYETHYLENE AZINE ROUTE

SCHWABZ BIO RESEARCH, INC.



4.



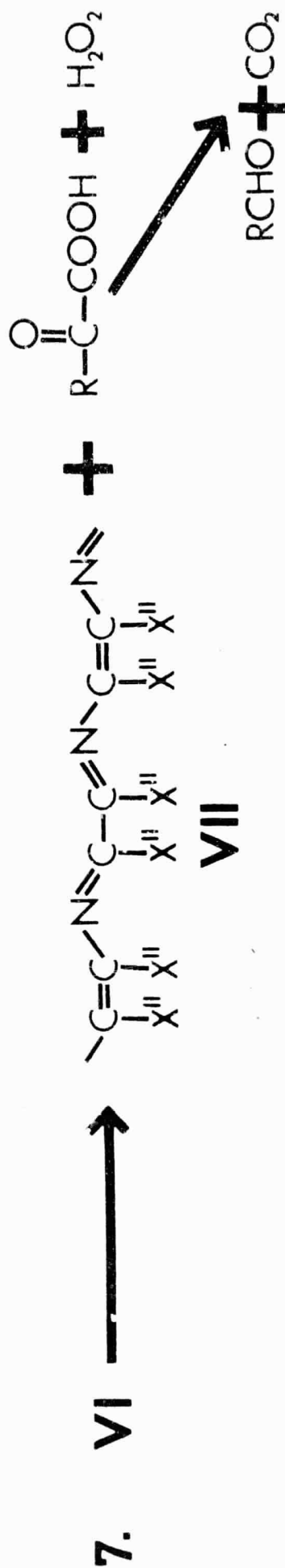
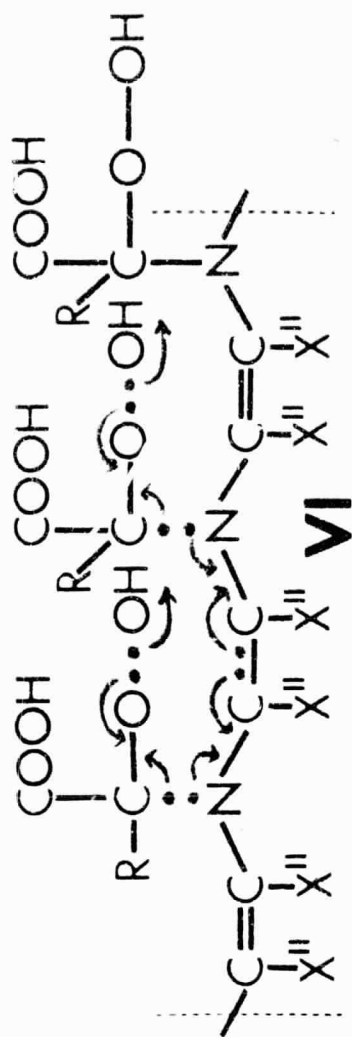


TABLE 1

COMPARISON BETWEEN EXTENT OF AMINO ACID - GLUCOSE REACTION
AND EASE OF AMINO ACID DEAMINATION

AMINO ACID	BROWNING INTERACTION		DEAMINATION DUE TO RADIATION	
	% Loss Liquid Diet #1 6 mos. -rm. temp.	% Loss Liquid Diet #2 12 mos. -rm. temp.	NH ₃ Liberation (mg/ml) (1) 5 At 1.45 x 10 ⁵ rep.	NH ₃ , % N of Total N UV Irradiation 2 hrs. (2)
HISTIDINE	69.5	85.3	11.8	---
ARGININE	---	62.4	7.5	---
LYSINE	61.5	59.2	3.7	20.9
METHIONINE	44.5	42.7	6.6	---
GLUTAMATE	---	43.5	---	---
ALANINE	28.0	---	7.2	10.4
GLYCINE	30.0	29.8	8.1	9.4
ISOLEUCINE	33.5	33.0	---	---
LEUCINE	31.5	29.9	---	---
VALINE	31.5	34.4	---	---
SERINE	16.0	24.8	---	---
THREONINE	24.5	16.9	---	---
PHENYLALANINE	18.0	17.0	---	7.3
ASPARTIC	24.5	17.4	---	---
PROLINE	6.9	0	---	---

(1) Kopicidova J., Kolousek A., Bubichy A., Liebster J., Nature 182, 1074 (1958)

(2) Greenstein J. and Winitz M. "Chemistry of the Amino Acids." p. 684

SCHWARZ BIORESEARCH, INC.

TABLE 2

DESTRUCTION OF AMINO ACID RESIDUES IN CASEIN ON
REACTION WITH GLUCOSE AT 37°C
(70% RELATIVE HUMIDITY)*

<u>AMINO ACID</u>	<u>5 DAY % LOSS</u>	<u>30 DAY % LOSS</u>
LYSINE	67.0	91.0
ARGININE	8.15	71.0
HISTIDINE	2.9	28.
METHIONINE	20.8	57.

*Data of Lea C H., and Hannan R.S., Biochem.
et Biophys. Acta, 5, 433 (1950)

SCHWARZ BIORESEARCH, INC.

TABLE 3

SIGNIFICANT LOSSES IN AMINO ACID CONTENT OF AN 8%
SOLUTION OF BOVINE SERUM ALBUMIN HEATED WITH 30%
GLUCOSE FOR 6 DAYS AT 53°C*

<u>AMINO ACID</u>	<u>% LOSS</u>
LYSINE	57
ARGININE	55
HISTIDINE	19
METHIONINE	12

*Data of Mohammad A., Frankel-Conrat H., and
Olcott H.S., Arch. Biochem. 24, 157 (1949).

FIGURE 1

EFFECT OF ENVIRONMENTAL EXPOSURE ON DIET AMINO ACID COMPOSITION



FIGURE 1

EFFECT OF TEMPERATURE ON GLUCOSE (2M)
GLYCINE (1M) INTERACTION



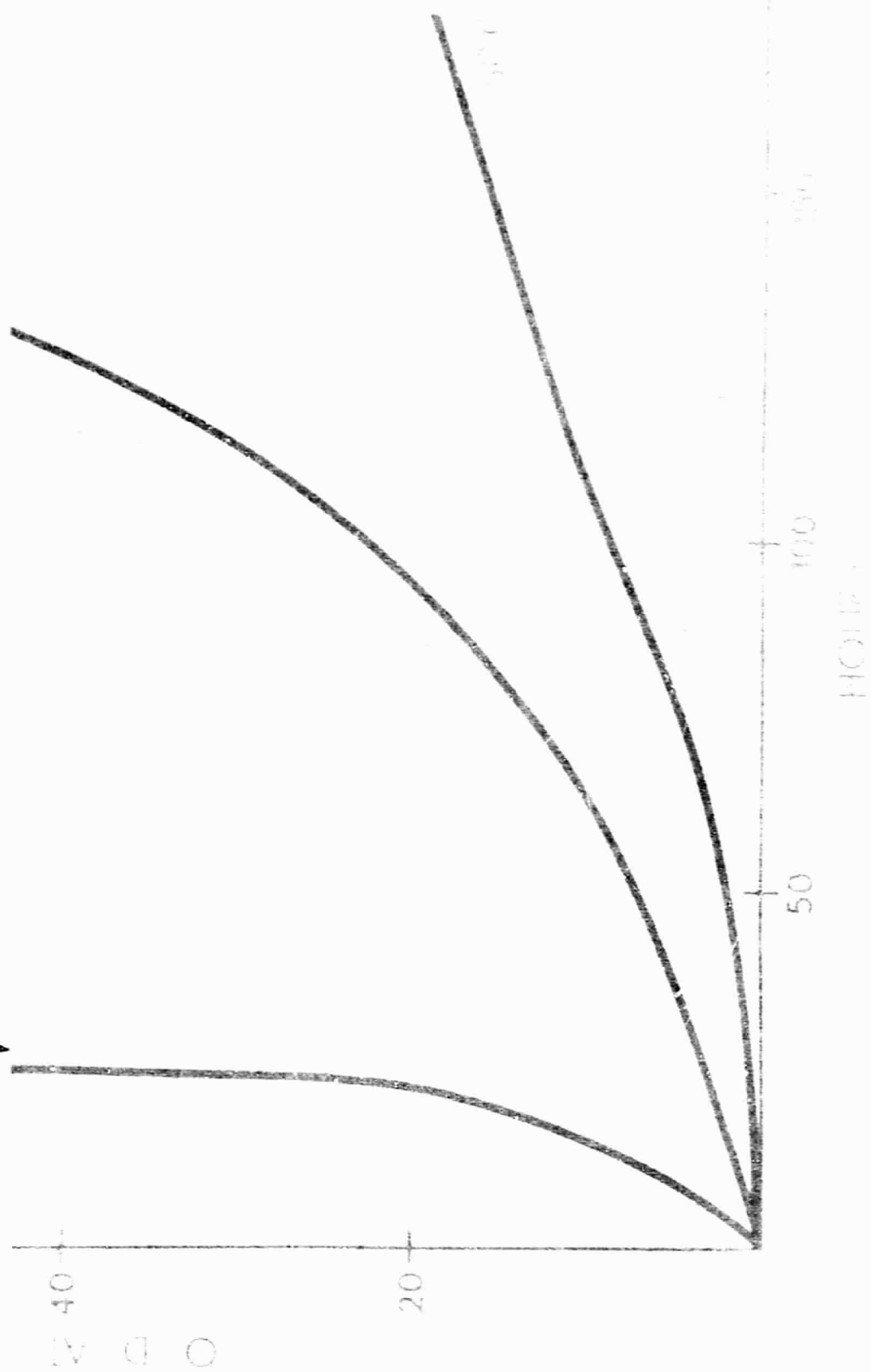
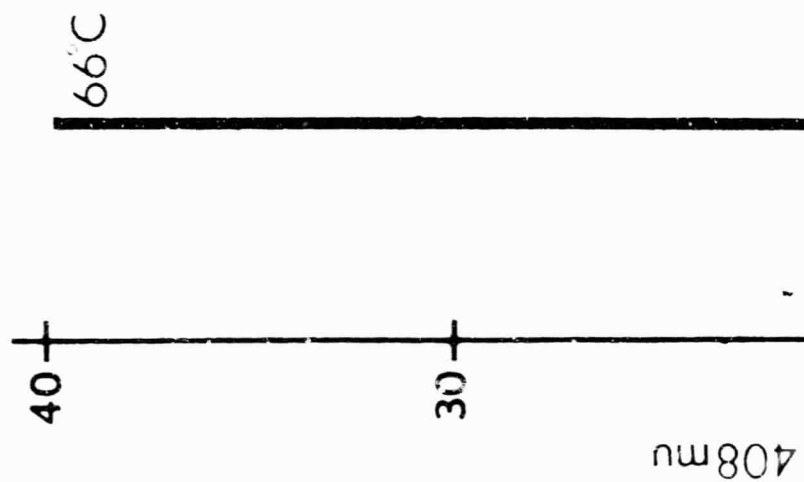
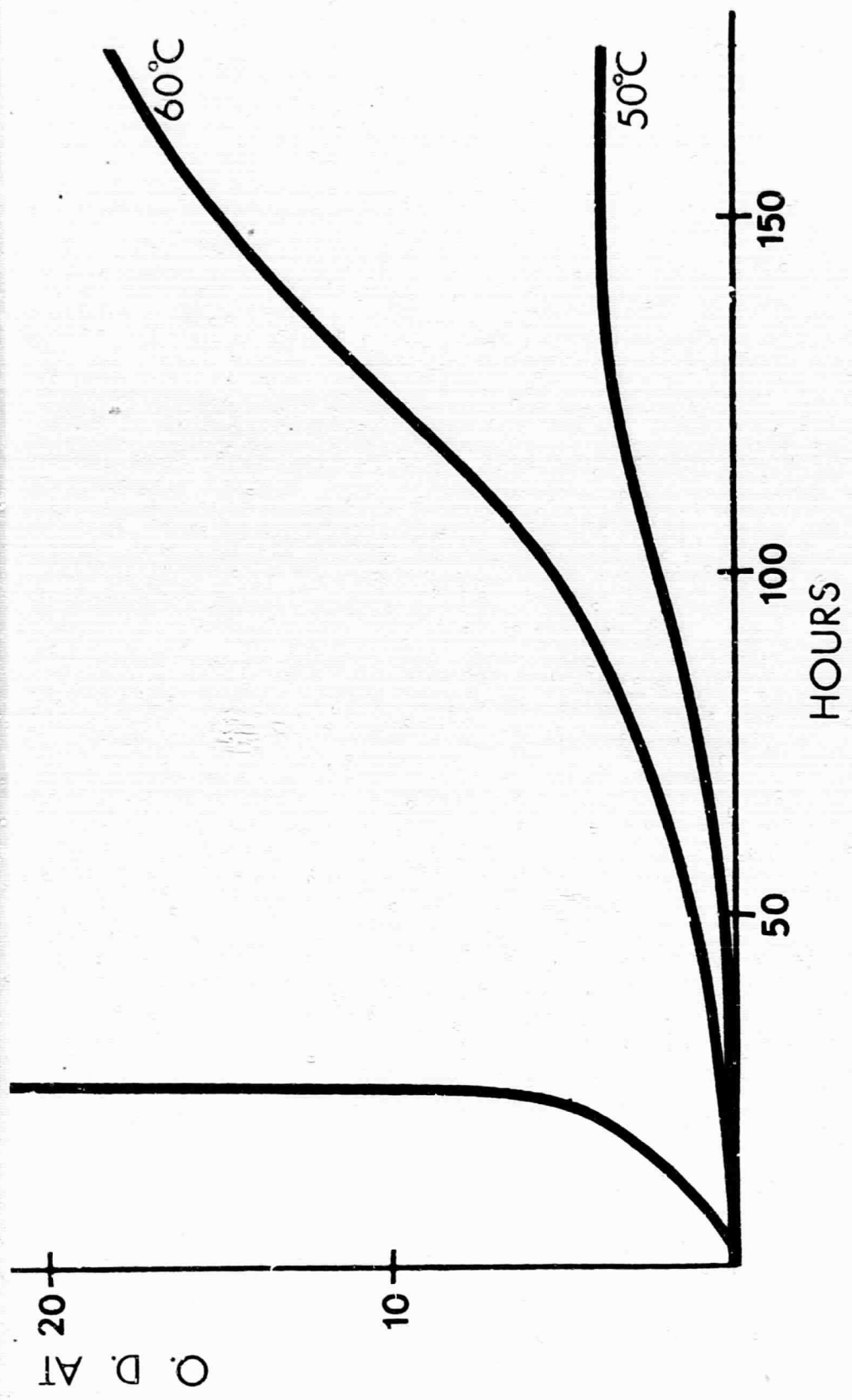
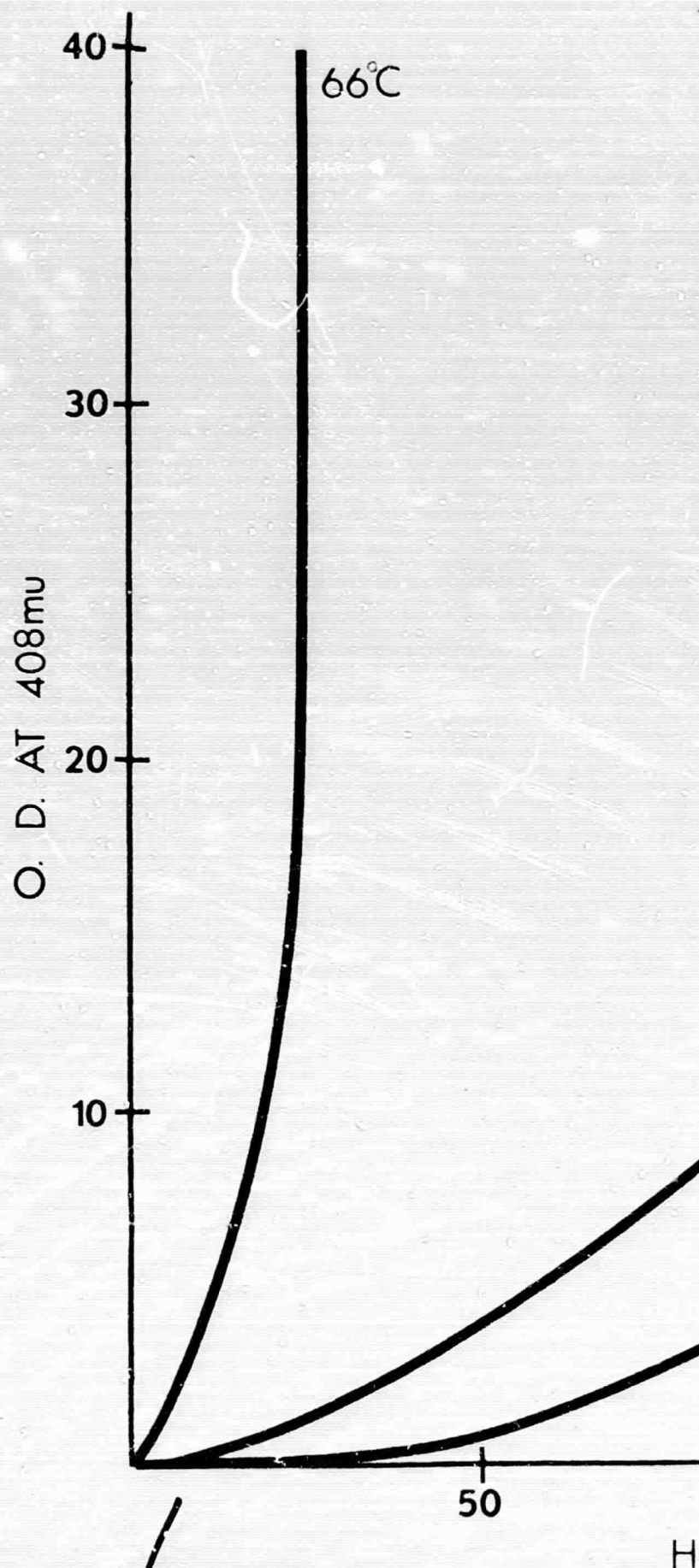


FIGURE 3

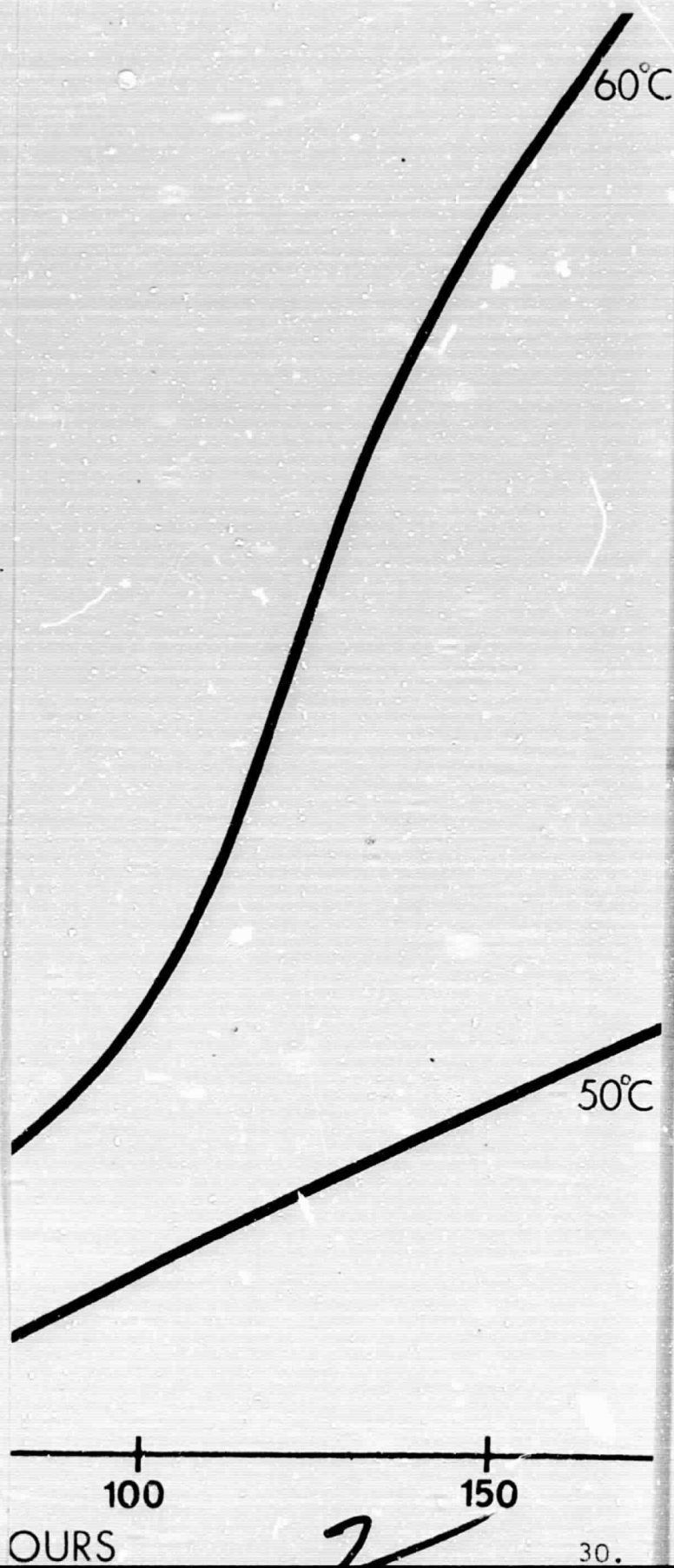
EFFECT OF TEMPERATURE ON GLUCOSE (2M) GLYCINE (1M) INTERACTION

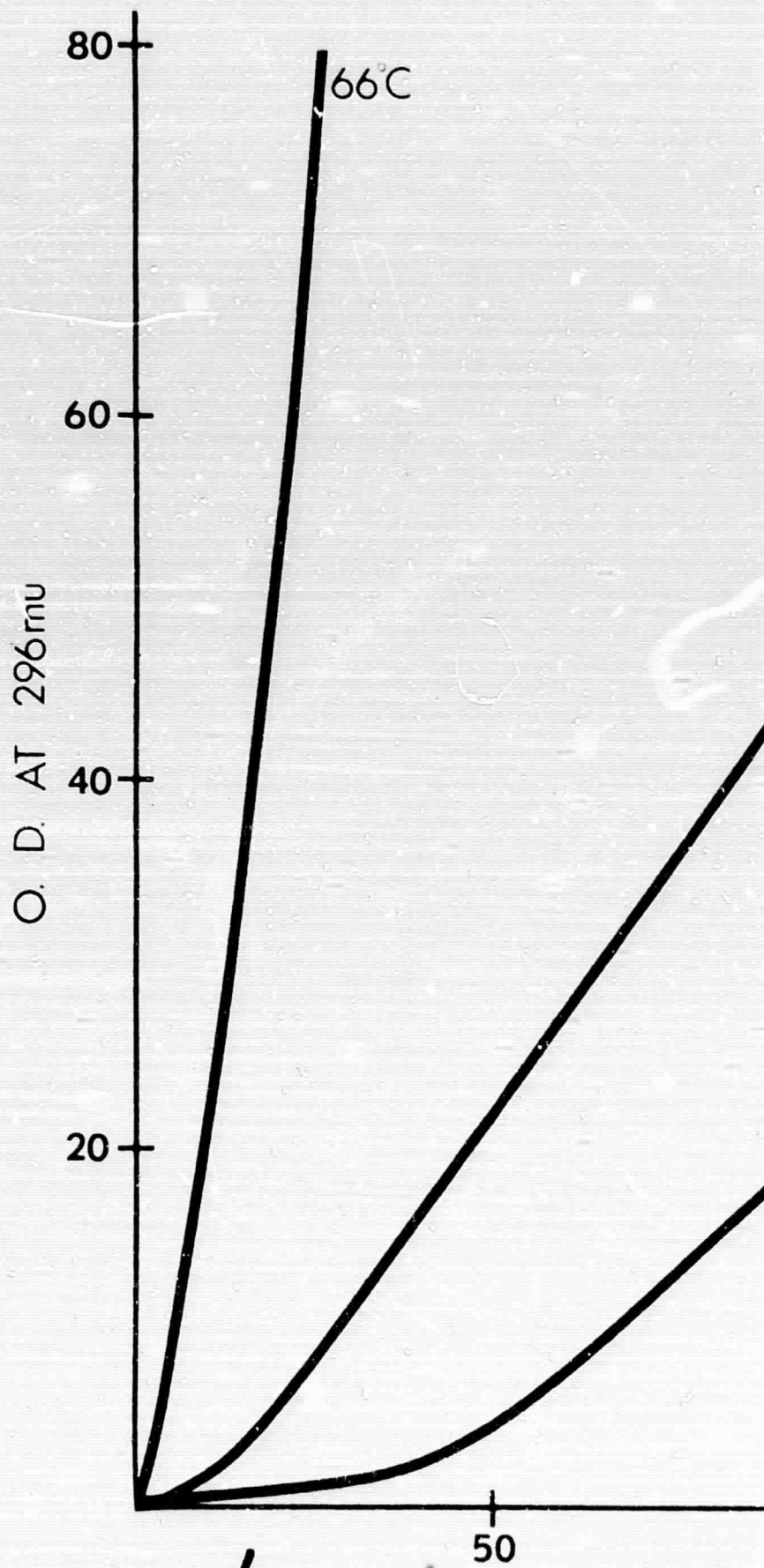




EFFECT OF TEMPERAT
LYSINE (1M)

URE ON GLUCOSE (2M) INTERACTION



EFFECT OF TEMPERATURE
LYSINE (1M) I

HOI

URE ON GLUCOSE (2M) INTERACTION

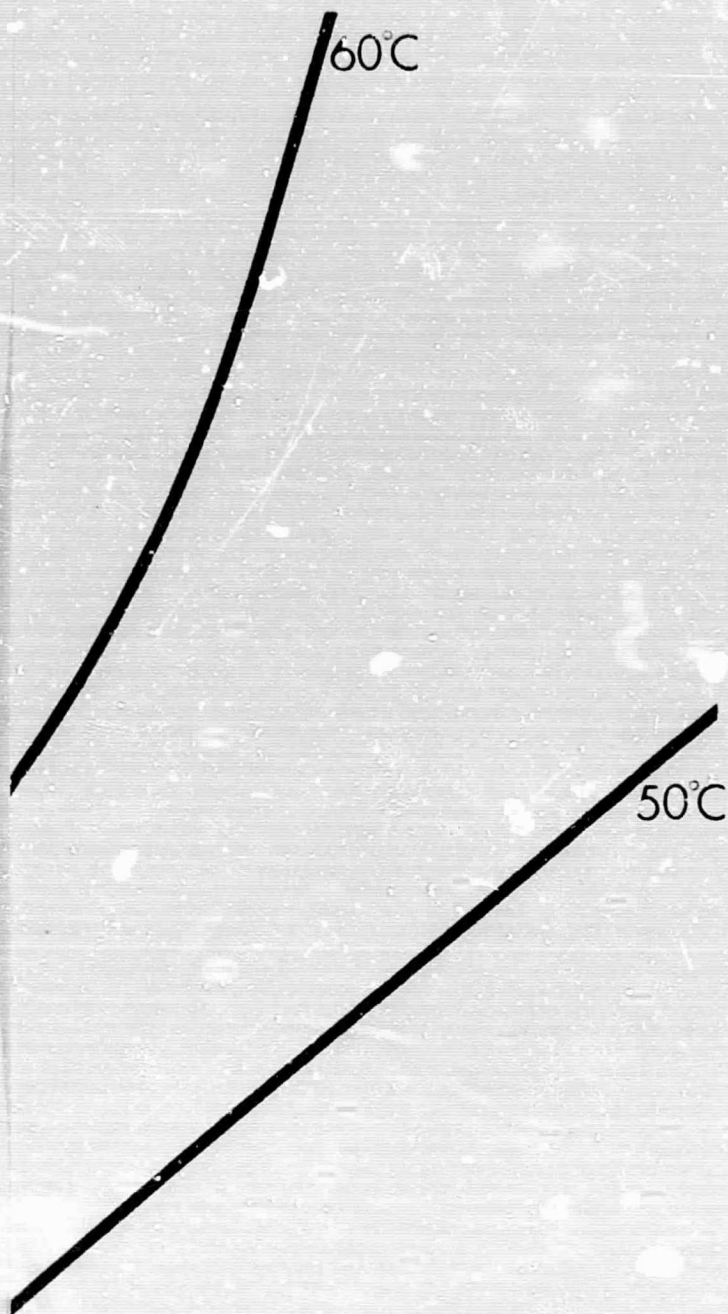
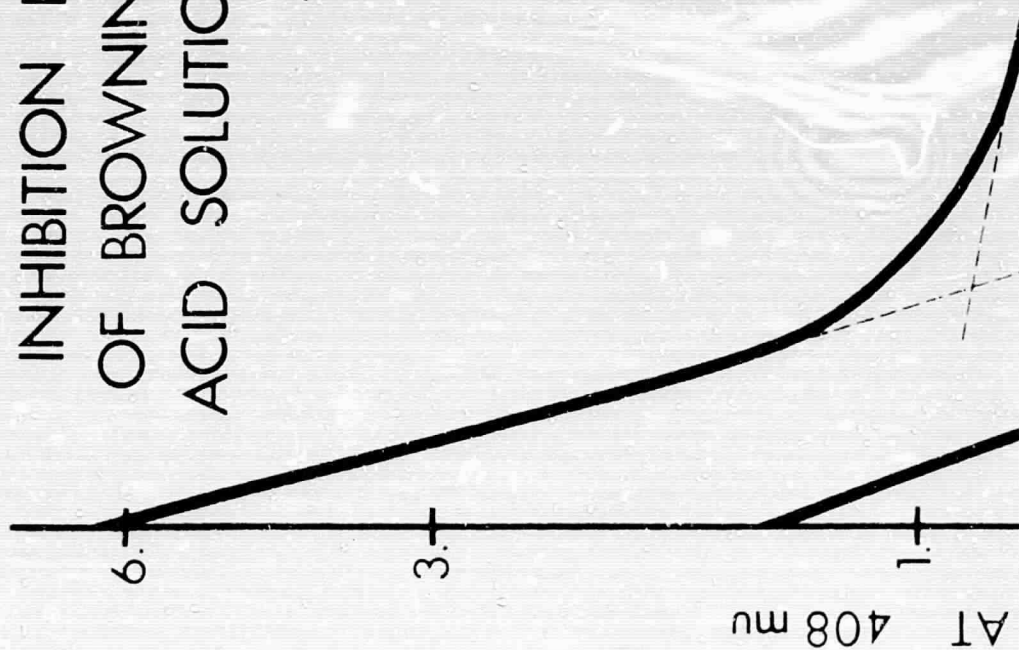
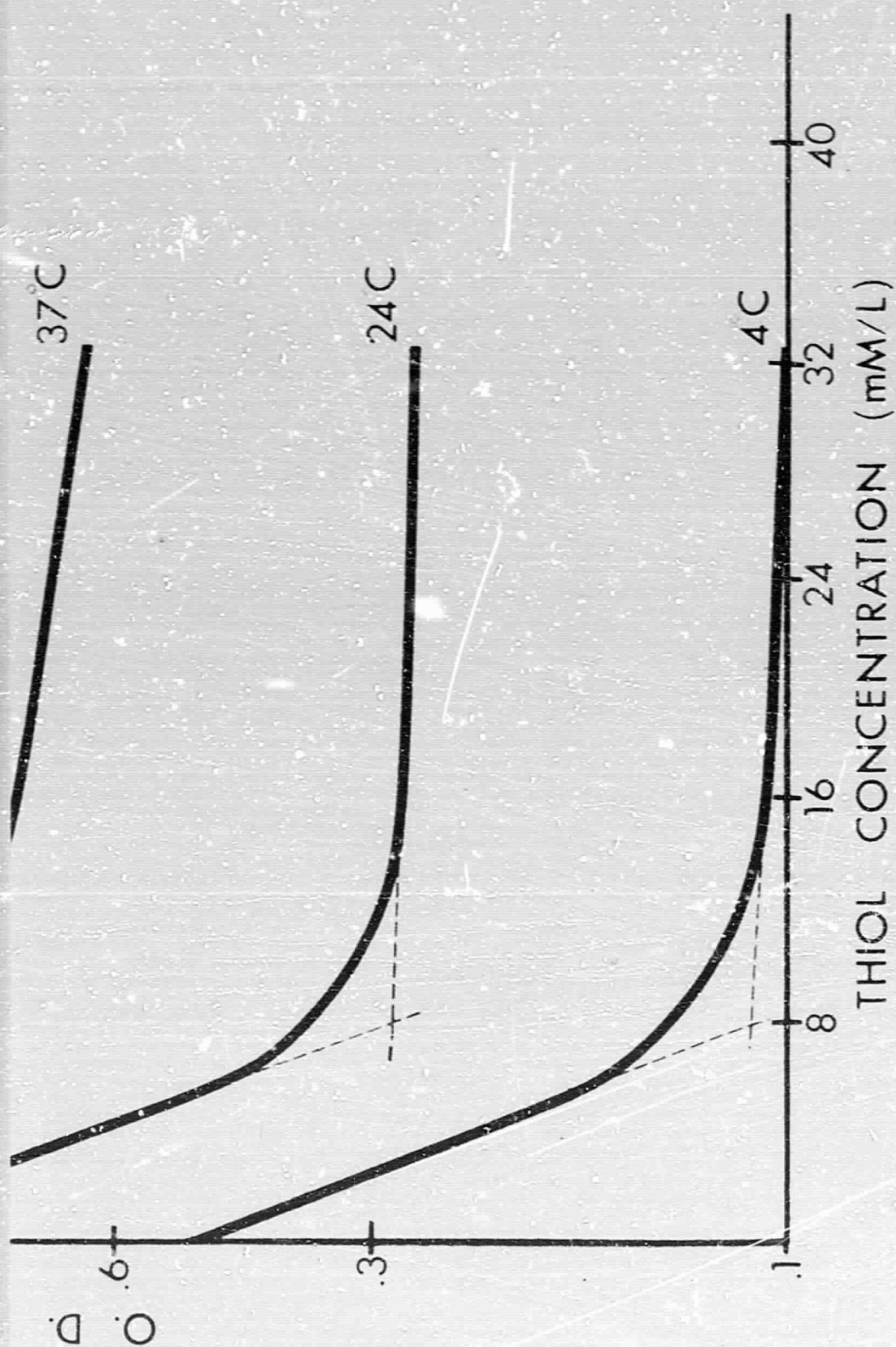


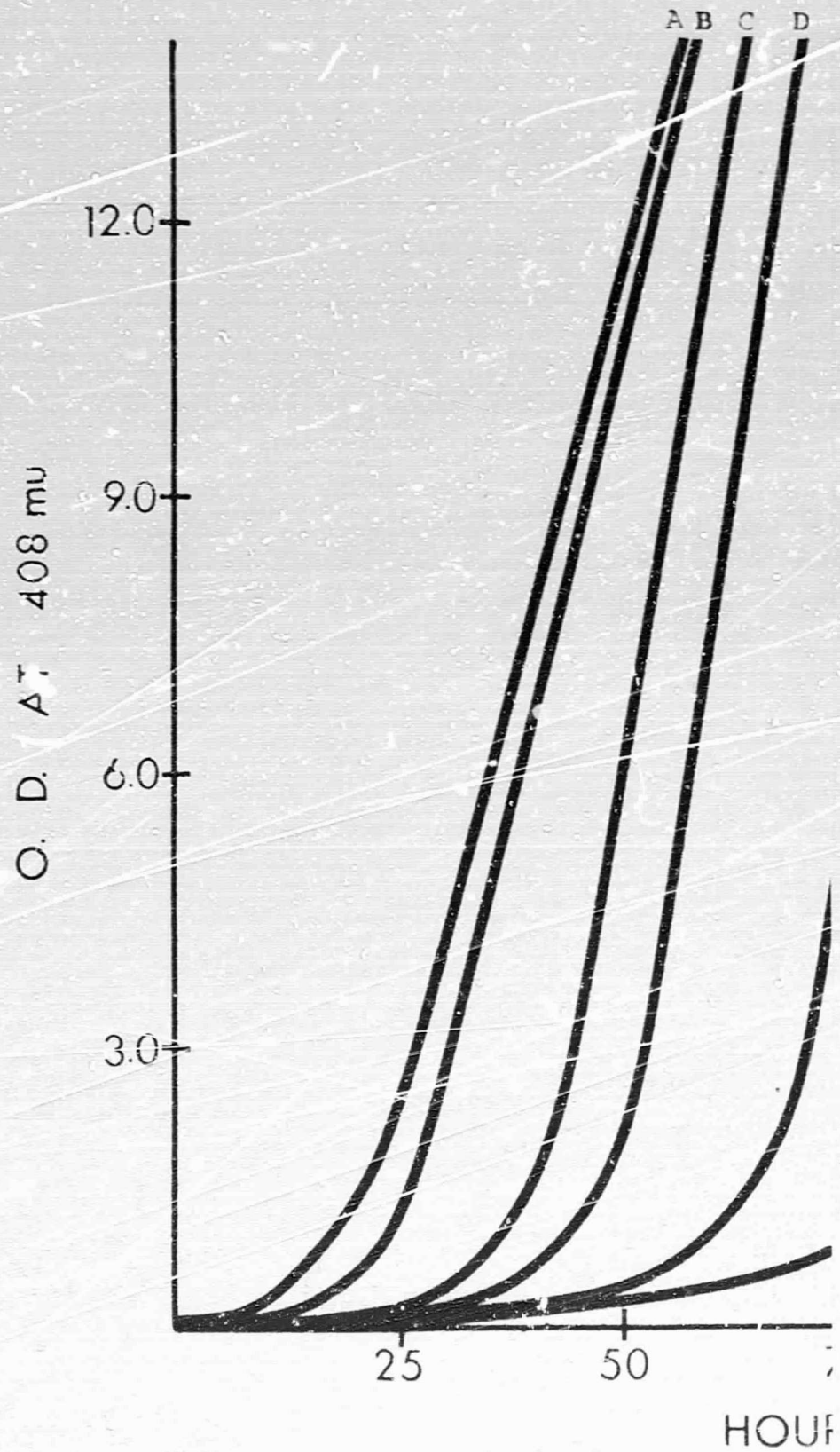
FIGURE 6

INHIBITION BY GLUTATHIONE (REDUCED)
OF BROWNING IN A GLUCOSE-AMINO
ACID SOLUTION CONTAINING PERACETIC
ACID (2mM/L)





THIOGLYCEROL INHIBITION (2M GLUCOSE - 1M LYSINE



OF BROWNING IN A
SOLUTION AT 60°C

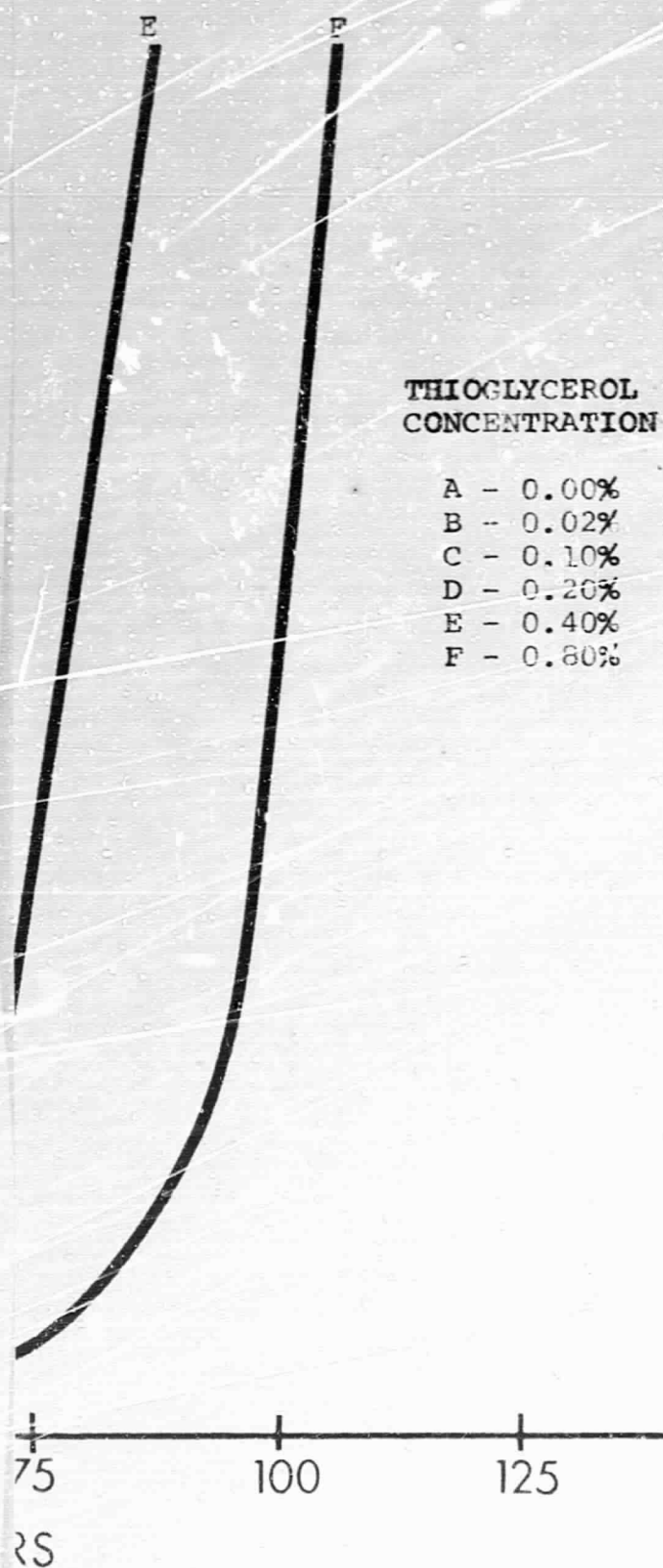
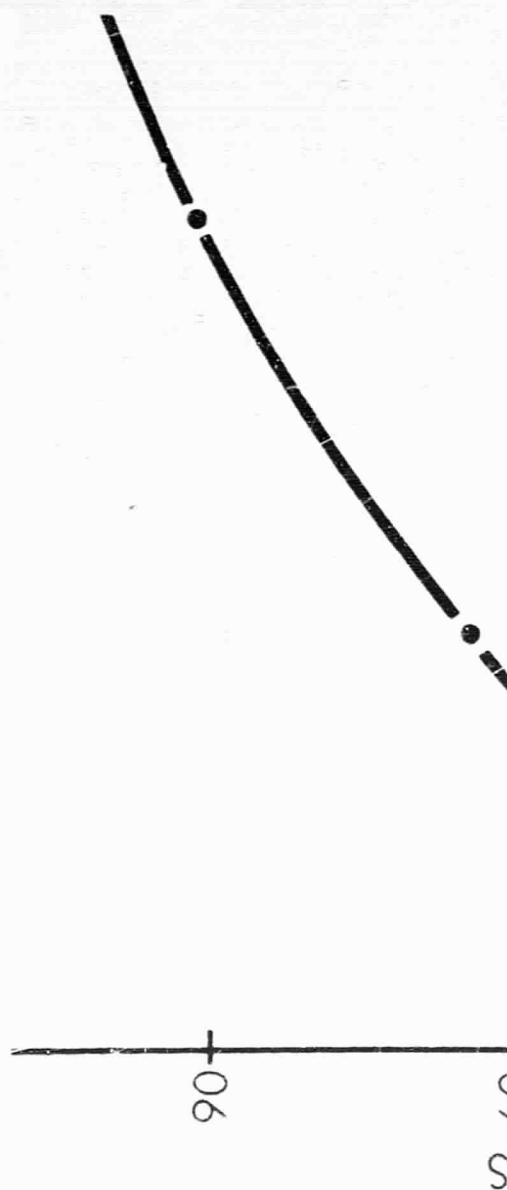


FIGURE 8

INHIBITION TIME OF BROWNING IN A GLUCOSE-
LYSINE SOLUTION AT 60°C WITH INCREASING
THIOGLYCEROL CONCENTRATION



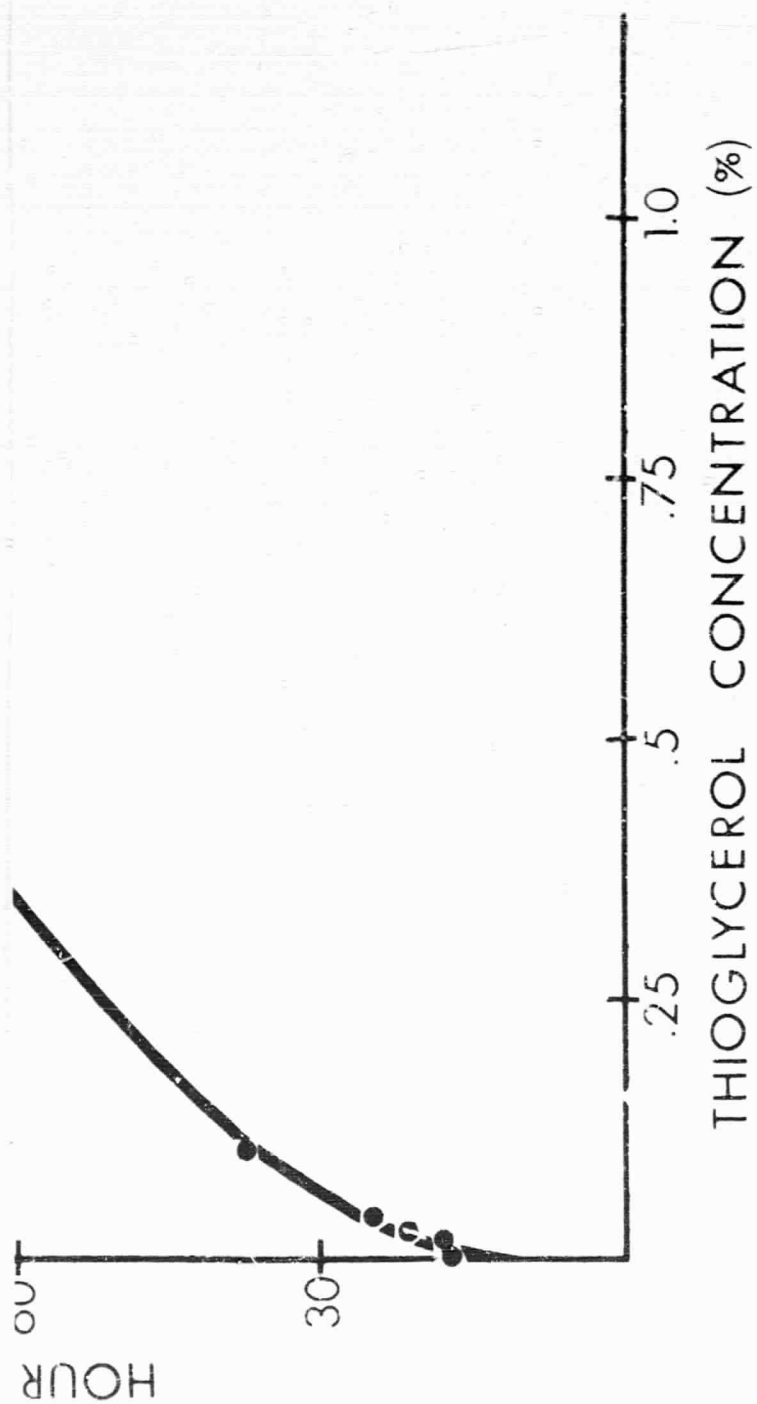
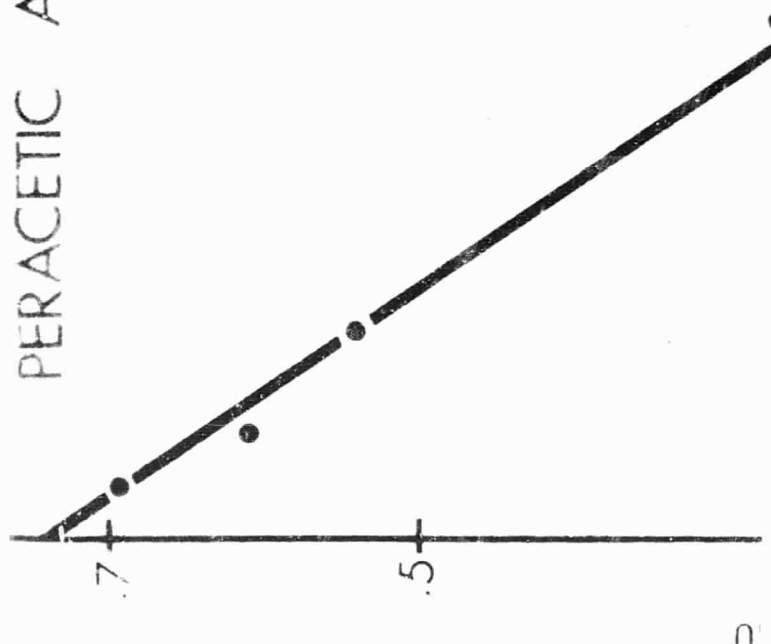


FIGURE 9

RATE OF BROWNING OF A GLUCOSE -- AMINO
ACID SOLUTION CONTAINING VARYING AMOUNTS
OF GLUTATHIONE (REDUCED) IN THE PRESENCE OF
PERACETIC ACID (0.002M AT 4°C)



2

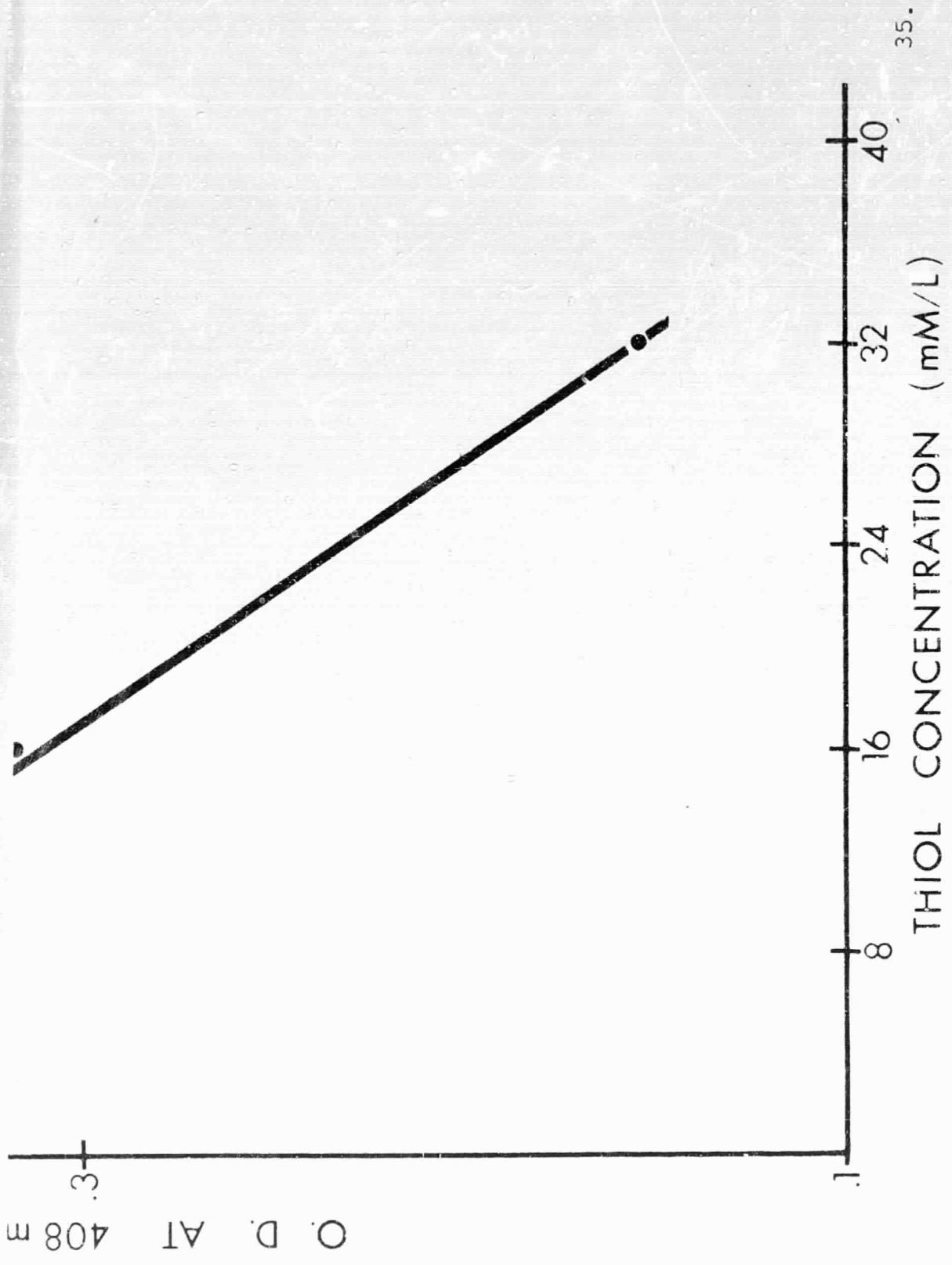
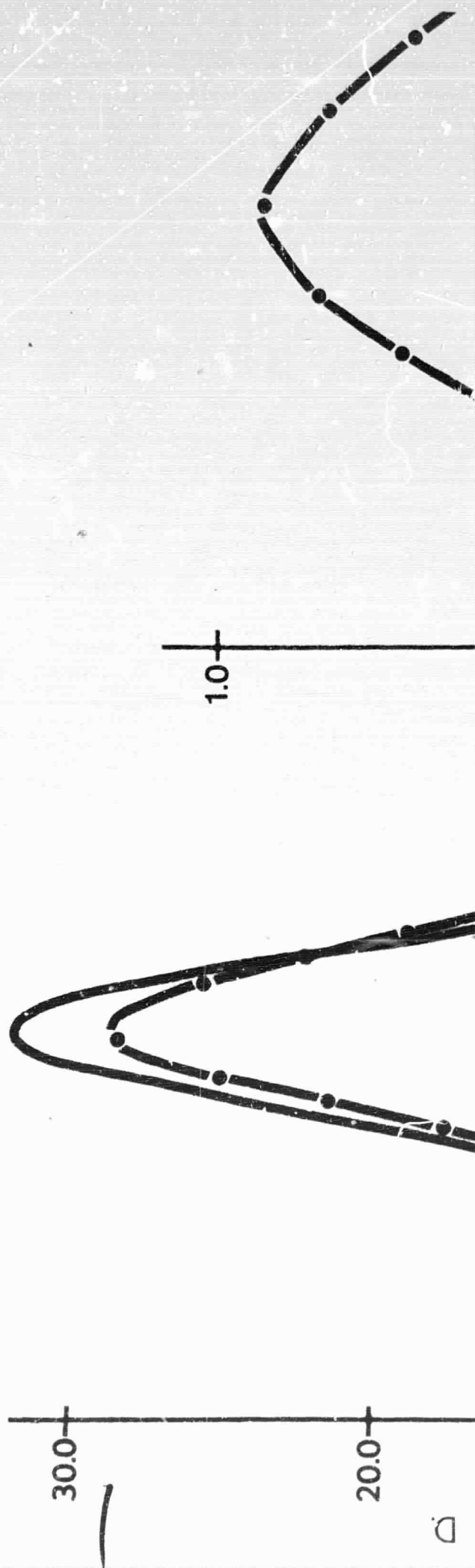


FIGURE 10

INTERACTION OF LYSINE (1M) WITH α -PYRROLALDEHYDE
(2M) IN A WATER - CELLOSOLVE SOLUTION AT 60°C



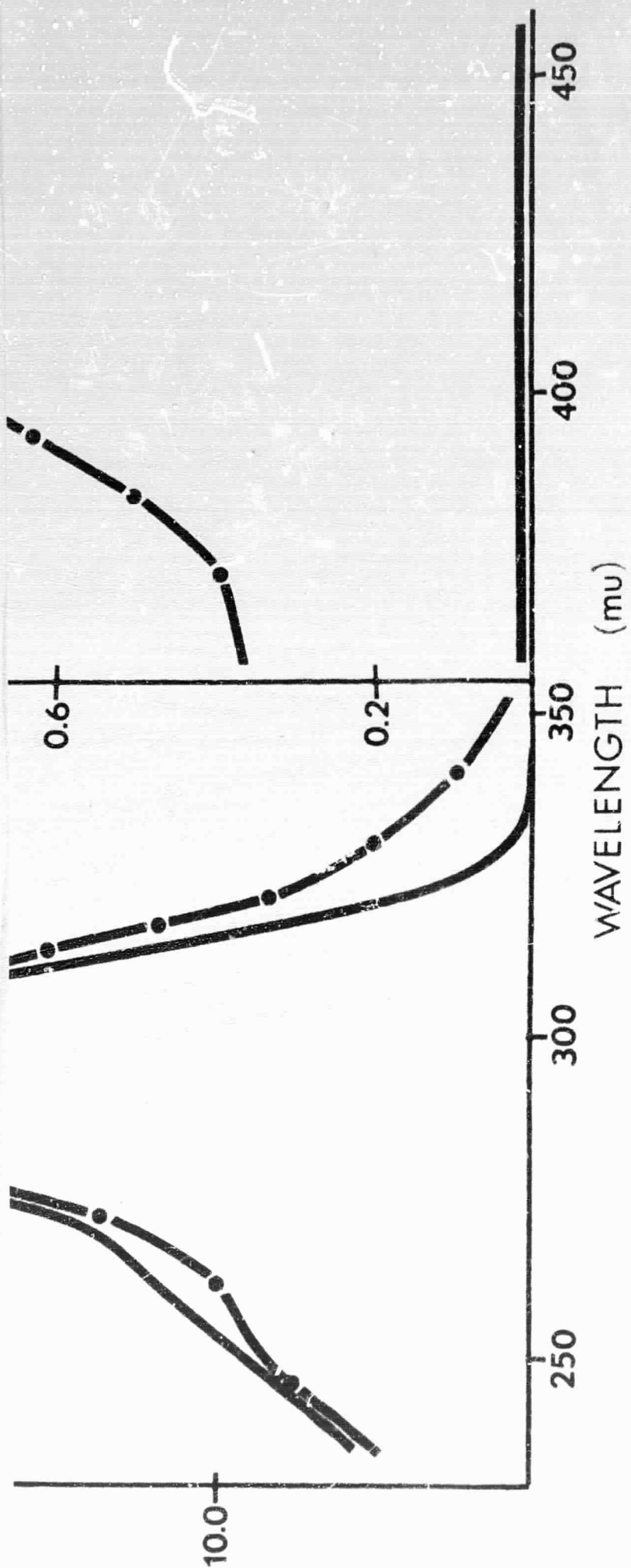
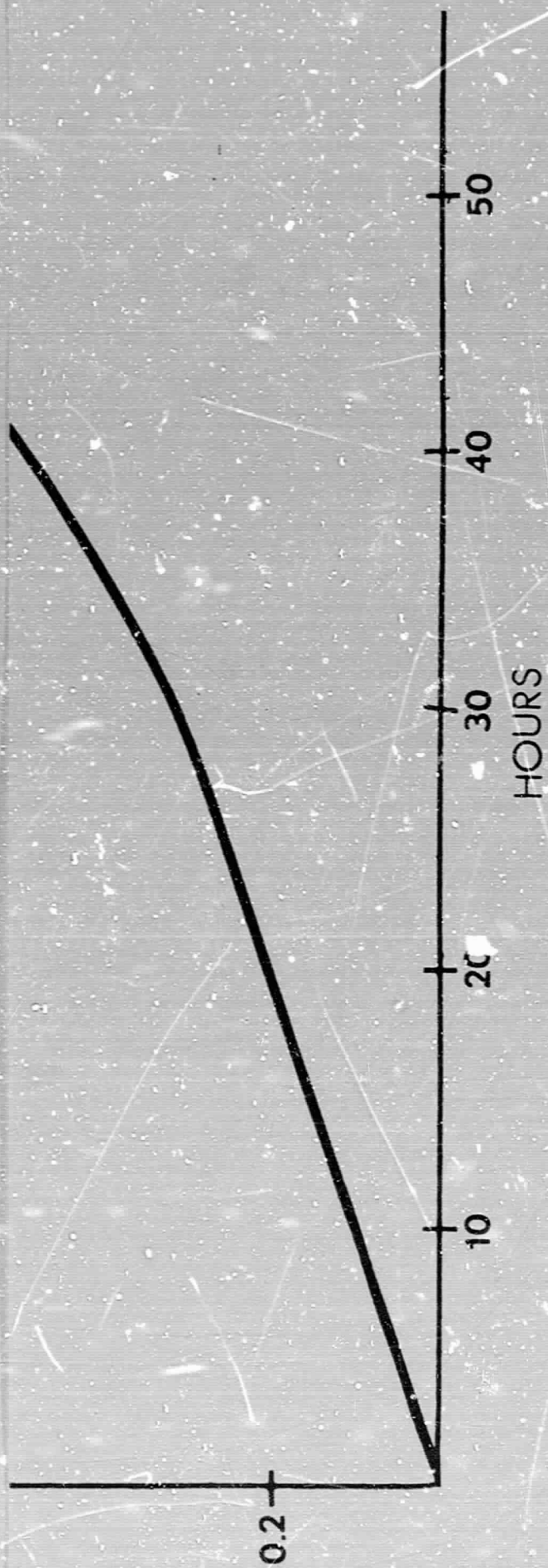


FIGURE 11

RATE OF BROWNING OF LYSINE (1M) - α PYRROLALDEHYDE
(2M) IN A WATER - CELLOSOLVE SOLUTION AT 60°C
MEASURED AT 426mu





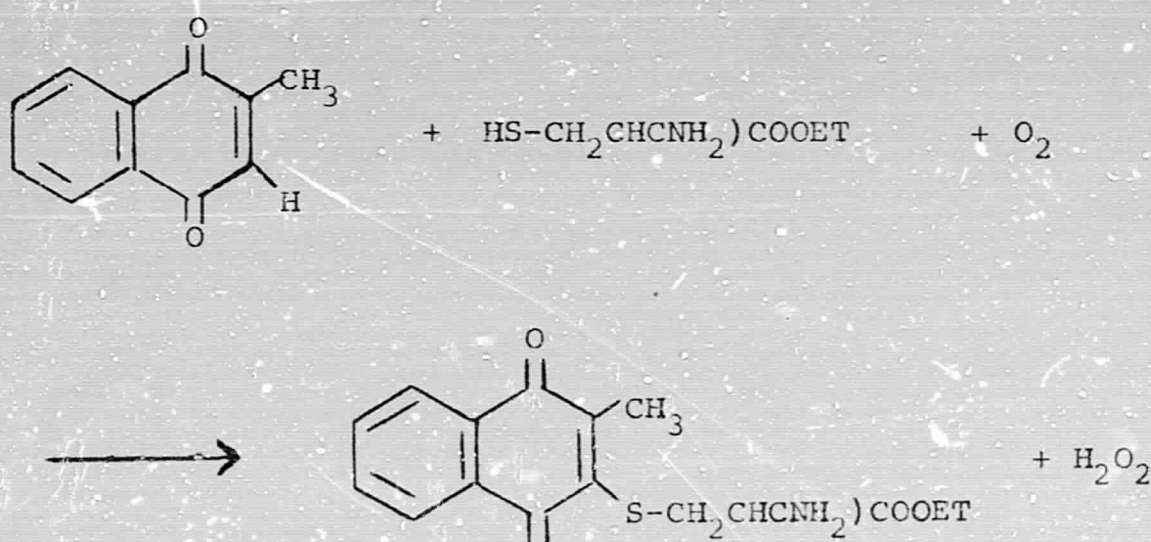
SCHWARZ BIORESEARCH, INC.

B. VITAMIN K--ETHYL CYSTEINATE INTERACTION

The presence in some liquid diet formulations of both ethyl cysteinate hydrochloride and vitamin K represents a chemical incompatibility which was only first recognized when it was observed that certain strains of rats (Fisher) unexpectedly developed a vitamin K deficiency. The remarkable feature here is that a vitamin K deficiency in rats normally is difficult to induce due to the vitamin K synthesizing capability of the intestinal microflora of the rat. The physiological evidence for this nutritional anomaly is presented in the nutritional sections that follow.

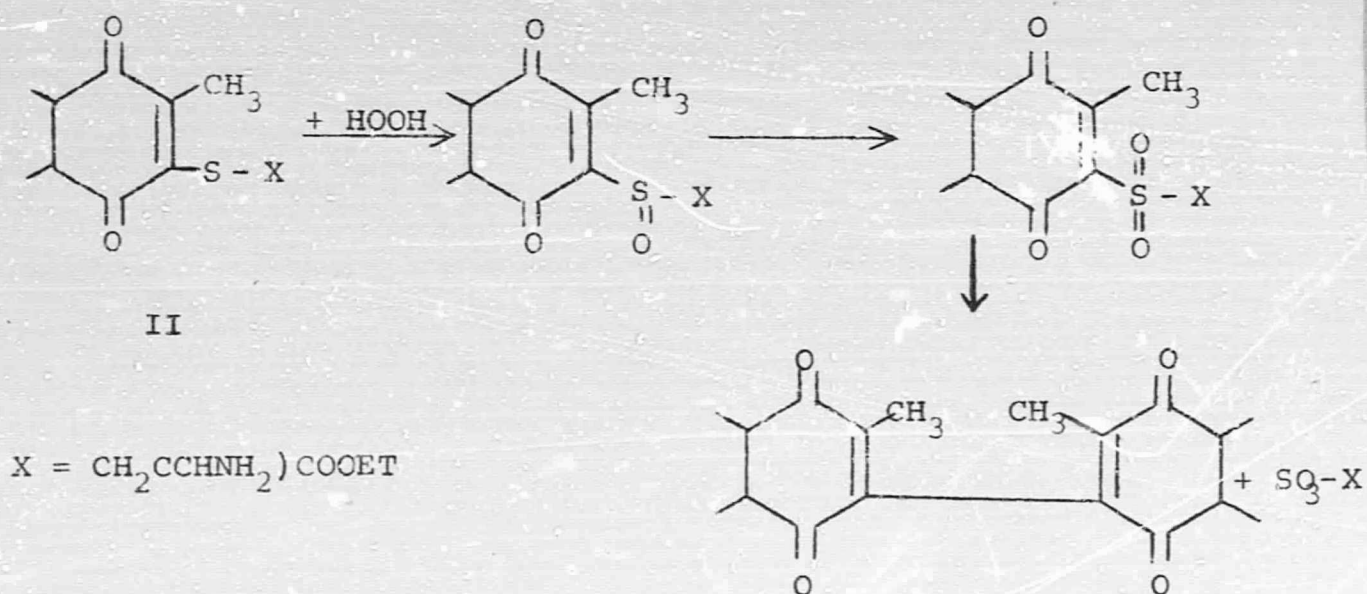
From the chemical standpoint, however, examination of the literature revealed that in earlier studies Nickerson⁸ had synthesized "thiodione", a vitamin K-thiol adduct produced by reaction of glutathione (reduced) with vitamin K. This reactivity was shown to be a property of the thiol function and that cysteine also reacts with vitamin K.

The reaction in question was pictured to be:



SCHWARZ BIORESEARCH, INC.

Whether Compound II on formation was capable of acting as an antagonist or antimetabolite which competed with vitamin K was unknown. It was also unknown whether this compound might interfere with vitamin K absorption within the intestine. Further, it was quite possible that compound II was not the compound responsible for these effects but rather some oxidation derivative was involved. The reason for suspecting the latter to be a distinct possibility was the observation that in the synthesis of compound II in our laboratory, a red compound was also produced. The addition of dilute hydrogen peroxide solution to II rapidly caused this red compound to form. The reaction involved is suspected to be:



The peroxides indicated in the reaction scheme are thought to be capable of arising either from peroxidized linoleate in the diet or peroxides formed during browning. The chemical and nutritional aspects of this problem are currently under investigation and will be reported on in our next report.

SCHWARZ BIORESEARCH, INC.

STABILITY STUDY, VITAMIN ASSAYS

INTRODUCTION:

A 57% (w/v) liquid diet was prepared and assayed periodically for a number of vitamins. The diet was subjected to storage under varying conditions of temperature, irradiation and time. The conditions chosen were those likely to be encountered during space flight or in ground simulator experiments.

Microbiological assay of several vitamins was undertaken as part of a stability study on liquid diets. The vitamins assayed were riboflavin, pyridoxine, niacin, pantothenate, folic acid, B₁₂, biotin, thiamine, vitamins A and E (di-alpha-tocopherol acetate).

Routinely, calculations of vitamin content are based on either turbidimetric readings or titrations with 0.1N NaOH. However, it was suggested⁹ that calculations for some of the B vitamins be based on pH readings. Preliminary experiments were done in this laboratory to compare the titration method with the pH method of vitamin assay. It was found that the pH method could be applied to five of the B vitamins under examination: riboflavin, niacin, pantothenate, folic acid and biotin. Of the two, the pH method is the preferred rapid assay method. Speed and ease of handling were greatly facilitated by the use of a single probe pH electrode which fit easily into the assay tubes.

Microbiological assays of vitamins are based on the principle that growth and/or acid production of the test organisms will be proportional, in a particular range, to the

SCHWARZ BIORESEARCH, INC.

concentration of a limiting vitamin. In practice, a medium is made up deficient in the vitamin which is to be assayed. Graded amounts of the particular vitamins are added to a series of tubes which contain this medium. This constitutes the assay standard. At the same time, several dilutions of the material being assayed are added to a second series of tubes containing the vitamin deficient medium. A standard curve is drawn from measurements on the first series of tubes. Measurements made from the second series of tubes are compared to values on the standard curve for determination of vitamin content.

METHODS:

Preparation of assay media, inocula and dilutions of vitamin standards were the same as described in "Difco Manual"¹⁰ pages 212-227. Pyridoxine was assayed gravimetrically, vitamin B₁₂ by electrometric titration to pH 7.0 and the other vitamins were assayed by pH measurement. The organisms used in the assays were:

1. Lactobacillus casei ATCC 7469 for riboflavin
2. Lactobacillus arabinosus ATCC 8014 for biotin, pantothenate and niacin.
3. Lactobacillus leichmanii ATCC 7830 for vitamin B₁₂.
4. Streptococcus lactis R ATCC 8043 for folic acid.
5. Neurospora sitophila ATCC 9276 for pyridoxine.

The vitamin concentration and diet dilutions used in the assays are shown in Table 4.

SCHWARZ BIO RESEARCH, INC

TABLE 4
VITAMIN CONCENTRATION AND DIET DILUTIONS USED IN ASSAYS

Vitamin	Vitamin concentration		Diet dilutions used in assay
	Calculated to be in diet µg/ml	Suggested assay range*	
Riboflavin	8.493	0.25-0.15 µg/tube	1/50, 1/100, 1/200
Pyridoxine-HCl	5.35	0.2-0.8	1/10, 1/20
Niacin	31.85	0.05-0.03	1/100, 1/200, 1/400
Calcium panto- thenate	42.47	0.02-0.12	1/400, 1/500, 1/1100
Folic acid	0.4247	0.002-0.006	1/75, 1/100, 1/150
B ₁₂ (0.1% tri- turate)	84.9	0.01-0.1 mg/tube	1/500, 1/1000, 1/2000
Biotin	0.2548	0.025-0.5 mg/tube	1/500, 1/1000, 1/5000
Vitamin A acetate	16.35 (i.u./ml)**	** Samples assayed by Wisconsin Alumni Research Foundation	
d, l -tocopherol acetate	28.5	Samples assayed by Wisconsin Alumni Research Foundation	
Vitamin D ₂	0.0035	Samples assayed by Wisconsin Alumni Research Foundation	

*Suggested in "Dilco Manual".

**Stock vitamin A assayed at 60% potency. Therefore, calculated value should be
9.8 i.u./ml.

SCHWARZ BIO RESEARCH, INC.

Diet samples were also sent to Wisconsin Alumni Research Foundation, Madison, Wisconsin for the assay of vitamin A, vitamin E and thiamine. A sample of the vitamin A used to prepare the diets was also sent to W.A.R.F. for analysis; as were certain samples for assay of vitamin B₆, B₁₂ and pantothenate. The methods used by W.A.R.F. are as follows:

Vitamin A:	Carr-Price Blue Color, Methods of Vitamin Assay, Ass'n. of Vitamin Chemists, Inc., Interscience Publishers, Inc., N.Y., 25 (1951)
Vitamin B ₁₂ :	U.S.P. XVI, 888 (1960)
Pyridoxine:	Atkins, Schultz, Williams and Frey, Ind. and Eng. Chem., Anal. Ed., 15, 141 (1943) (<u>S. carlsbergensis</u>)
Thiamine:	A.O.A.C., 655 (1960)
α -tocopherol Acetate:	Acta Chemica Scandinavica, 11, 34-43 (1957)
Calcium Pantothenate:	Nielands and Strong, Arch. of Bio. 19, 2 (1948)

Vitamin assays were performed on diet which had been subjected to the following storage conditions:

<u>Storage condition</u>	<u>Duration of exposure</u>
1. Initial control	0 weeks
2. 60°C dry heat	2 weeks
3. 5×10^5 and 5×10^6 roentgens	2 weeks
4. 5×10^5 and 5×10^6 roentgens in diet containing 0.16% ethyl cysteinate	2 weeks

SCHWARZ BIORESEARCH, INC.

<u>Storage condition</u>	<u>Duration of exposure</u>
5. Room temperature (in the dark	8 months
6. Room temperature (exposed to light)	4 months
7. Refrigeration temperature	8 months
8. Freezer temperature	8 months

RESULTS:

The Effect of Storage Temperature on Vitamin Stability

Diets stored at 60°C were assayed after 2 weeks storage. It was believed that the treatment was severe enough to exhibit its effects in this period of time. Therefore, this group can only be compared to the initial control or a group which had been assayed after 4 months. Using either comparison, the trends in the high temperature storage group are well-defined.

Using the data in Table 5 we can compare the group stored at 60°C with the two groups stored at reduced temperature. It can be seen that riboflavin, B₆ and niacin were relatively unaffected by the high temperature. Vitamin E and folic acid have a significantly reduced potency, but this is as much an effect of time as temperature (see Tables 5 and 9).

The vitamins most markedly affected by the high temperature were vitamin A, B₁₂, biotin and thiamine (Table 5).

The Effect of Long-Term Storage at Various Temperatures

Folic acid, thiamine and vitamin E are significantly reduced in potency after eight months even though stored at

SCHWARZ BIORESEARCH, INC.

reduced temperatures (Table 6). The remainder of the vitamins assayed are relatively stable for this period of time at low temperatures. Although stable at low temperatures, riboflavin and vitamin A are quite labile at room temperature over an eight month period. Folic acid, which was relatively unstable at low temperatures, is considerably more unstable at room temperature. Niacin and pantothenate were shown to be stable at low temperature and slightly less so at room temperature.

Vitamin B₁₂ exhibited no loss in potency after eight months storage at temperatures of 25°C or less. Oddly enough, vitamin B₆ appears to become more stable as the temperature is raised between 0° and 60°C.

The Effect of Light on Vitamin Stability

Diets exposed to sunlight and fluorescent light at room temperature exhibited nearly 100% loss of riboflavin after 4 months storage (Table 7). In the absence of light, the same diet showed a 25% decrease in riboflavin. Vitamin A acetate was shown to be quite sensitive to light. Vitamin E, niacin and folic acid exhibited a comparatively minor sensitivity to light. Pantothenate, thiamine and biotin showed little or no sensitivity to light. These diets turned dark brown after a few weeks storage, the darker color probably increased the stability of the light-sensitive vitamins.

The Effects of Irradiation in the Presence and Absence of Ethyl Cysteinate·HCl

Four groups of liquid diet were subjected to Co⁶⁰ irradiation at both 5×10^5 and 5×10^6 roentgens. One group of diet in each irradiation level contained 0.16% ethyl cysteinate·HCl; the other group contained no cysteinate.

SCHWARZ BIORESEARCH, INC.

With the exception of calcium pantothenate, all the vitamins were more sensitive to high level irradiation than to high temperature (Tables 8 and 5). For all practical purposes, vitamin A was completely destroyed by both high temperature and irradiation. The loss of vitamin E cannot be accurately assessed because a large part of its loss appears to be dependent upon time rather than treatment.

The presence of ethyl cysteinate reduced the radiation sensitivity of the B vitamins (Table 8). This effect was negligible, however, for thiamine but overwhelming with respect to B₁₂.

Ethyl cysteinate did not reduce the radiation sensitivity of the fat soluble vitamins tested.

The Effect of Time on Vitamin Stability

Diets stored at room temperature for eight months exhibited continual loss of activity of all vitamins but B₆ and B₁₂ (Table 9). Loss of niacin and pantothenate was moderate (10-15%) while thiamine, riboflavin, A, E, and folic acid exhibited a 40% or greater decrease in potency, folic acid being the highest with 80%. At lower temperatures, vitamin E exhibited the greatest loss in potency, i.e. 55-60% while loss of folic acid activity was second highest (35%) in both refrigerator and freezer storage (Tables 10 and 11).

B₁₂ is stable in diets stored at reduced temperatures. Pantothenate and niacin which were moderately labile at room temperature, are stable at reduced temperatures (Tables 10 and 11). As previously noted, B₆ appears to be less stable at reduced temperatures. The stability of vitamin A, thiamine, riboflavin and folic acid is greatly enhanced by lower temperatures (Tables 10 and 11).

SCHWARZ BIORESEARCH, INC.

CONCLUSIONS:

The greatest overall destruction of vitamins was caused by high level irradiation. The second most deleterious condition was high temperature. Ethyl cysteinate "protected" most of the B vitamins from the effects of irradiation. However, thiamine and the fat-soluble vitamins were not protected by this agent.

Riboflavin, pantothenate, folic acid and B₁₂ were relatively stable to low level irradiation, while riboflavin, B₆ and niacin were stable at high temperature.

Vitamin stability was not significantly enhanced by reducing the storage temperature from that of a refrigerator to that of a freezer. Stability was improved by storing the liquid diets in the cold as opposed to room temperature storage.

Eight months storage at room temperature resulted in a 25% or greater loss of vitamin activity for 5 of the 10 vitamins assayed, while storage at reduced temperature brought about corresponding losses in only 3 of the 10 vitamins (thiamine, folic acid and vitamin E). By increasing the diet levels of these three vitamins, a nutritionally adequate diet can be prepared to withstand long periods of storage at reduced temperature.

SCHWARZ BIO RESEARCH, INC.

TABLE 5
EFFECT OF STORAGE TEMPERATURE ON
VITAMIN STABILITY IN LIQUID DIET

Vitamin	Percent decrease in potency*		
	Freezer temp.	Refrigerator temp.	Room temp. 60°C**
Riboflavin	25	25	25 10
B ₆	--	0	0 0
Niacin	5	5	0 10
Folic acid	50	40	70 60
B ₁₂	--	0	5 25
Biotin	20	25	20 55
Thiamine	10	25	35 95
A	15	10	40 95
E	60	55	55 40
Pantothenate	0	0	0 40

*Compared to calculated potency, to nearest 5%.

**60°C samples assayed after 2 weeks storage, others assayed after 4 months.

SCHWARZ BIO RESEARCH, INC.

TABLE 6

THE EFFECT OF EIGHT MONTHS STORAGE AT VARIOUS
TEMPERATURES ON VITAMIN STABILITY IN LIQUID DIET

Vitamin	Percent decrease in potency*		
	Freezer temp.	Refrigerator temp.	Room temp.
Riboflavin	15	5	45
B ₆	15	5	0
Niacin	0	0	10
Folic acid	35	35	80
B ₁₂	0	0	0
Biotin	--	--	0
Thiamine	25	30	40
A	10	10	50
E	55	60	55
Pantothenate	0	0	15

*Compared to calculated potency, to nearest 5%.

SCHWARZ BIO RESEARCH, INC.

TABLE 7

EFFECT OF LIGHT ON VITAMIN STABILITY
IN LIQUID DIET

Vitamin	Percent decrease in potency*	
	Room temp. light	Room temp. dark
Riboflavin	100	25
B ₆	---	0
Niacin	15	0
Folic acid	80	70
B ₁₂	---	5
Biotin	25	20
Thiamine	35	35
A	70	40
E	70	55
Pantothenate	0	0

*Compared to calculated potency, to nearest 5%.
(This diet was stored four months.)

SCHWARZ BIO RESEARCH, INC.

TABLE 3

EFFECT OF IRRADIATION IN THE PRESENCE AND ABSENCE
OF ETHYL CYSTEINATE·HCl

Vitamin	Percent decrease in potency*			
	Low + cyst.**	Low***	High + cyst.****	High*****
Riboflavin	5	10	10	25
B ₆	10	35	40	30
Niacin	5	40	40	75
Folic acid	0	0	30	85
B ₁₂	0	0	0	95
Biotin	45	50	55	85
Thiamine	95	100	100	100
A	100	100	100	95
E	65	60	55	55
Pantothenate	0	0	0	0

*Compared to calculated potency, to nearest 5%. This
(This diet was stored two weeks.)

**5x10⁵ r in the presence of 0.16% ethyl cysteinate·HCl.
***5x10⁵ r.
****5x10⁶ r in the presence of 0.16% ethyl cysteinate·HCl.
*****5x10⁶ r.

SCHWARZ BIO RESEARCH, INC.

TABLE 9

THE EFFECT OF TIME ON VITAMIN STABILITY OF
LIQUID DIETS STORED AT ROOM TEMPERATURE

Vitamin	Percent decrease in potency*		
	0 months	4 months	8 months
Riboflavin	10	25	45
B ₆	0	0	0
Niacin	5	0	10
Folic acid	0	70	80
B ₁₂	0	5	0
Thiamine	0	35	40
A	0	40	50
E	10	55	55
Pantothenate	0	10	15

*Compared to calculated potency, to nearest 5%.

SCHWARZ BIORESEARCH, INC

TABLE 10

THE EFFECT OF TIME ON VITAMIN STABILITY OF
LIQUID DIETS STORED AT REFRIGERATOR TEMPERATURE

Vitamin	Percent decrease in potency*		
	0 months	4 months	8 months
Riboflavin	10	25	5
B ₆	0	0	5
Niacin	5	5	0
Folic acid	0	40	35
B ₁₂	0	0	0
Thiamine	0	25	30
A	0	10	10
E	10	55	60
Pantothenate	0	0	0

*Compared to calculated potency, to nearest 5%.

SCHWARZ BIORESEARCH, INC.

TABLE 11

THE EFFECT OF TIME ON VITAMIN STABILITY OF
LIQUID DIETS STORED AT FREEZER TEMPERATURE

<u>Vitamin</u>	<u>Percent decrease in potency*</u>		
	<u>0 months</u>	<u>4 months</u>	<u>8 months</u>
Riboflavin	10	25	15
B ₆	0	--	15
Niacin	5	5	0
Folic acid	0	50	35
B ₁₂	0	--	0
Thiamine	0	10	25
A	0	15	10
E	10	60	55
Pantothenate	0	0	0

*Compared to calculated potency, to nearest 5%.

SCHWARZ BIO RESEARCH, INC

MICROBIAL CONTROL IN LIQUID DEFINED DIETS

I. THE USE OF 2,3 EPOXYPROPANOL (GLYCIDOL) AS A STERILANT

INTRODUCTION

Chemically defined liquid diets are heat sensitive and must be cold sterilized. The liquid diets are routinely sterilized by filtration, but a means of chemical sterilization would make handling and packaging of diets much simpler.

It has been reported that 2,3-epoxypropanol (glycidol) can be used to sterilize aqueous compositions and that this compound is hydrolysed to glycerine in the process¹¹. This means of chemical sterilization seemed ideal for a liquid diet, as it would leave a biologically acceptable residue after exerting its microbiocidal effect. This agent was incorporated, at different levels, in a 50% (w/v) liquid diet and tested for its effectiveness as a sterilant.

MATERIALS AND METHODS

A series of screw-capped test tubes were made up to contain 23 ml of a sterile 50% diet, 1 ml of inoculum and 1 ml of a glycidol dilution or water. The inoculum was obtained from a 72 hour culture of airborne contaminants in Fluid Thioglycollate (Difco). The culture was diluted 1:1 with 0.85% sterile saline for use as the inoculum.

The tubes were incubated in the dark at room temperature for two weeks. One ml samples were removed from each tube immediately before the addition of glycidol, and

SCHWARZ BIORESEARCH, INC.

periodically thereafter. Serial dilutions were made from each sample and bacterial counts were obtained by routine plate counts methods. Bacterial counts listed in Table 12 as 0 organisms per ml were confirmed by subculturing duplicate 1 ml samples in Fluid Thioglycollate and Sabouraud Dextrose Broth (Difco) and incubating for 7 to 10 days at 37°C.

RESULTS

A glycidol concentration of 1% or higher sterilized the liquid diet in less than 3 days (Table 12). At a concentration of 0.1%, sterilization time was increased to 4 days, while nearly two weeks time was required for a 0.05% concentration of glycidol. It appears that the diet itself is somewhat bacteriostatic (Figure 12). The bacteriostatic property of liquid diets was tested in a later experiment.

CONCLUSIONS

Glycidol is an effective sterilant in liquid diets when used in concentration of 0.1% (u/v) or higher. It was noted, however, that a precipitate formed in diets containing glycidol. Preliminary evaluation of glycidol treated diets indicate that this agent is reacting with a portion of the diet. Effectiveness of glycidol against yeasts and molds was not tested because it was felt that reactions between glycidol and the diet components would contraindicate its use as a sterilant in this medium.

SCHWARZ BIORESEARCH, INC.

II. ATTEMPTS AT STERILIZING LIQUID DIETS

Glycidol (2,3-epoxypropanol) was added to liquid diets in order to test its antibacterial properties in this medium. It was observed, however, that glycidol caused a precipitation of some of the dietary ingredients of the liquid diet.

A larger amount of precipitate was formed when glycidol concentration was increased to 5% in diet-16. A sample of the supernatant liquid obtained from precipitated diet was submitted for amino acid analysis. The findings seem to indicate a greater than 50% loss of threonine, proline and phenylalanine.

III. THE USE OF METHYL AND PROPYL PARABENS AS LIQUID DIET PRESERVATIVES¹

INTRODUCTION

Methyl and propyl parabens have been used as preservatives in pharmaceutical and cosmetic products and were approved for use as food additives by the U.S. Food and Drug Administration in 1958. Liquid diets alone are somewhat bacteriostatic but will show signs of yeast growth within three days after inoculation when kept at room temperature. The addition of parabens is indicated for diets being fed to conventional animals, if the diet and container are to remain exposed to the environment for three or more days at a time. The F.D.A. has approved the use of methyl and propyl parabens up to 0.1% and the manufacturer recommends a combination of seven parts methyl to three parts propyl paraben.

¹Methyl and propyl esters of para hydroxybenzoic acid, Washine Chemical Corporation, Lodi, New Jersey.

SCHWARZ BIORESEARCH, INC.

TABLE 12

THE EFFECT OF GLYCIDOL ON AIRBORNE CONTAMINANTS
IN A LIQUID DEFINED DIET

Glycidol concentration (% v/v)	Number of organisms per ml						
	Time in days						
	0	1	3	4	7	11	14
0.0	4×10^5	6×10^4	2×10^4	8×10^3	1×10^3	2×10^2	1×10^2
0.05	1×10^6	---	6×10^3	2×10^3	8×10^1	$< 1 \times 10^2$	0
0.10	4×10^5	$< 1 \times 10^2$	$< 1 \times 10^1$	0	0	0	0
1.0	4×10^5	$< 1 \times 10^2$	0	0	0	0	0
5.0	5×10^5	$< 1 \times 10^2$	0	0	0	0	0

SCHWARZ BIORESEARCH, INC.

Two experiments were conducted. One illustrates the microbiostatic property of parabens in the presence of various levels of contamination in a liquid diet. The second illustrates the decreased efficiency of parabens in the presence of a fat mix. All our liquid diets contain the fat mix which is composed of fat soluble vitamins, ethyl linoleate and 60% polysorbate-80. Surfactants are known to interfere with the antimicrobial effectiveness of parabens. This leads us to believe that the polysorbate-80 fraction of the fat mix is responsible for the decreased efficiency of the parabens.

METHODS AND MATERIALS

The diets used in the following experiments were 30% (w/v) liquid, defined diets. When fat mix was present, 3 grams per liter of diet was used. When present, the parabens were used in a concentration of 0.5 gram methyl paraben + 0.15 gram propyl paraben per liter. The inoculum consisted of a mixed culture (bacteria and yeast) taken from a liquid diet which had been contaminated during administration to laboratory animals. To sterile 100 ml serum bottles, 50 ml of sterile diet 1 ml of inoculum was added. All diets were incubated at room temperature for the entire experimental period. One ml samples were removed periodically for enumeration of microorganisms by routine plate count methods.

RESULTS

Liquid diets with and without parabens were inoculated with various dilutions of a mixed culture to the levels indicated in Table . After 3 days growth, all the inoculated, paraben-free diets showed visible signs of fermentation.

SCHWARZ BIORESEARCH, INC

TABLE 14.

THE EFFECTIVENESS OF PARABENS IN THE PRESENCE
OF VARIOUS LEVELS OF MICROBIAL CONTAMINATION

Diet	Microbial count			
	Day			
	0	3	7	13
Microorganisms per ml of diet				
<u>With parabens</u>				
Uninoculated	0	0	0	0
Inoculated	1×10^1	4×10^1	3×10^3	2×10^5
Inoculated	1×10^2	2×10^2	4×10^4	3×10^6
Inoculated	1×10^3	2×10^3	2×10^6	3×10^7
Inoculated	1×10^4	4×10^3	8×10^6	3×10^7
<u>Without parabens</u>				
Uninoculated	0	0	0	0
Inoculated	1×10^1	1×10^6	1×10^6	---
Inoculated	1×10^2	1×10^6	1×10^6	---
Inoculated	1×10^3	1×10^6	1×10^6	---
Inoculated	1×10^4	1×10^6	1×10^6	---

SCHWARZ BIORESEARCH, INC.

The fermenting diets contained approximately 1×10^7 organisms per ml. Volume measurements and accurate counts were difficult to obtain because of the large amounts of dissolved gases in these samples. A sudden increase in the number of microorganisms occurs between the third and seventh day in diets containing parabens (Figure 13). This is true regardless of the initial level of contamination. This seems to indicate a qualitative rather than quantitative change in the microorganisms. The control curves in Figure 13 appear as straight lines. This is not the true shape of the curves but it serves to illustrate the time required for obtaining a large microbial population under the various conditions tested. It can be seen that parabens will retard the growth rate of microorganisms in a liquid diet, and its ability to do so is somewhat dependent upon the initial level of contamination (or inoculation).

Figure 14 clearly indicates that one or more factors in the fat mix will markedly reduce the antimicrobial effectiveness of the parabens. The steeper slope of the curve for the diet containing fat mix indicates a more rapid growth rate for microorganisms in this diet. After three weeks incubation at room temperature there is a hundred fold difference in the total number of microorganisms in the two diets.

CONCLUSIONS

Two experiments were conducted in which liquid diet was inoculated with a mixed culture of microorganisms. It was found that the number of microorganisms in a liquid diet containing parabens was dependent upon the initial level of contamination (Figure 13), and that the parabens were less effective in the presence of a fat mix (Figure 14).

SCHWARZ BIO RESEARCH, INC.

TABLE 13

THE EFFECT OF A FAT MIX (CONTAINING POLYSORBATE-80)
ON THE ANTIMICROBIAL EFFECTIVENESS OF PARABENS

Diet	Microbial count					
	Day					
	0	2	3	7	15	21
	Microorganisms per ml of diet					
With fat mix	7×10^2	9×10^3	1×10^4	2×10^7	5×10^7	8×10^7
Without fat mix	7×10^2	1×10^3	7×10^2	2×10^4	3×10^5	5×10^5

The responsible agent in the fat mix is believed to be polysorbate-80.

The results of this experiment indicate that parabens can retard microbial growth in a 30% (w/v) liquid diet, but not by a significant amount for long periods of time (e.g. more than two weeks). In addition, we have found that sterile liquid diets fed to rats for two days acquire a microbial content of approximately 1×10^5 organisms per ml. At this level of contamination, parabens will not greatly inhibit growth for more than a week.

Tests have not yet been conducted with parabens in a 50% (w/v) liquid diet. There are indications that the combined effect of parabens and the high osmotic pressure of a 50% diet should yield long term control of micro-organisms in diets being administered to laboratory animals.

STERILIZATION TECHNIQUES

Liquid diets were sterilized by filtration either through a microporous membrane or asbestos pads. For small volumes up to 50 liters, a type YY30, 293 mm filter holder¹ was used. A "Sanitype Filter" model ST² was employed for larger volumes, 50-300 liters.

In the YY30 filter holder we used the 293 mm, 0.22u (pore size) filter¹ along with a 257 mm type AP20 pre-filter¹. The complete unit was autoclaved for 35 minutes at 15 psi. and cooled to room temperature before use.

¹Millipore Filter Corporation, Bedford, Massachusetts

²F.R. Hormann and Company, Inc., Milldale, Connecticut

SCHWARZ BIORESEARCH, INC

The "Sanitype Filter" was set up with eight asbestos pads, Type D-10² and eight protective paper pads. The filter was sterilized by passing steam through the complete unit for 90 minutes.

The sterile filter units are aseptically attached to flexible plastic isolators which have been previously sterilized and contain previously sterilized bottles and bottling equipment. The isolators and their contents were sterilized with ethylene oxide. Ethylene oxide was introduced to a concentration of 200 mg per liter of isolator space with a minimum contact time of 18 hours. The system was then flushed with sterile air for a minimum of 16 hours before liquid diet was admitted to the system.

The liquid diet was sterilized by filtration under pressure using compressed nitrogen for the pressure. As the diet passed through the filter it flowed through plastic and glass tubing into the isolator where it could be sealed in bottles.

STERILITY CONTROLS

Spore inoculated samples were placed in the isolator each time it was sterilized. The samples were removed from the isolator and tested for viable spores by incubating for one week in Fluid Thioglycollate (Difco).

The spore inoculated samples³ consisted of the following: aluminum foil strips packaged in polyethylene-lined glassine envelopes, 500 cc cotton-plugged serum bottles, and rubber stoppers (for the above bottles) packaged in polyethylene-lined glassine envelopes. Each sample was

²F.R. Hormann and Company, Inc., Milldale, Connecticut

³Supplied by American Biological Control Laboratories, Inc.

SCHWARZ BIO RESEARCH, INC.

inoculated with 100,000 spores of an ethylene oxide resistant strain of Bacillus subtilis var. globigii, all samples were dried before use.

Representative samples of filter-sterilized diet were tested for sterility in accordance with procedures outlined in U.S.P. XVI.

The air supply to the isolator was checked for sterility by allowing the forced air to impinge upon the surface of two Nutrient Agar plates (Difco) for one hour. The plates were removed from the isolator, incubated for one week and checked for the presence of bacterial colonies.

The plastic isolator was checked for leaks by filling the isolator with sufficient freon and air to maintain a positive pressure. All outer surfaces, seams and gloves were then probed with a model TX-3⁴ halide leak detector.

⁴Bernzomatic Corporation, Rochester, New York

FIGURE 12

THE EFFECT OF GLYCIDOL CONCENTRATION ON RATE OF STERILIZATION AS
MEASURED IN A 50% (w/v) LIQUID DIET, INOCULATED WITH A
MIXED CULTURE OF AIRBORNE CONTAMINANTS

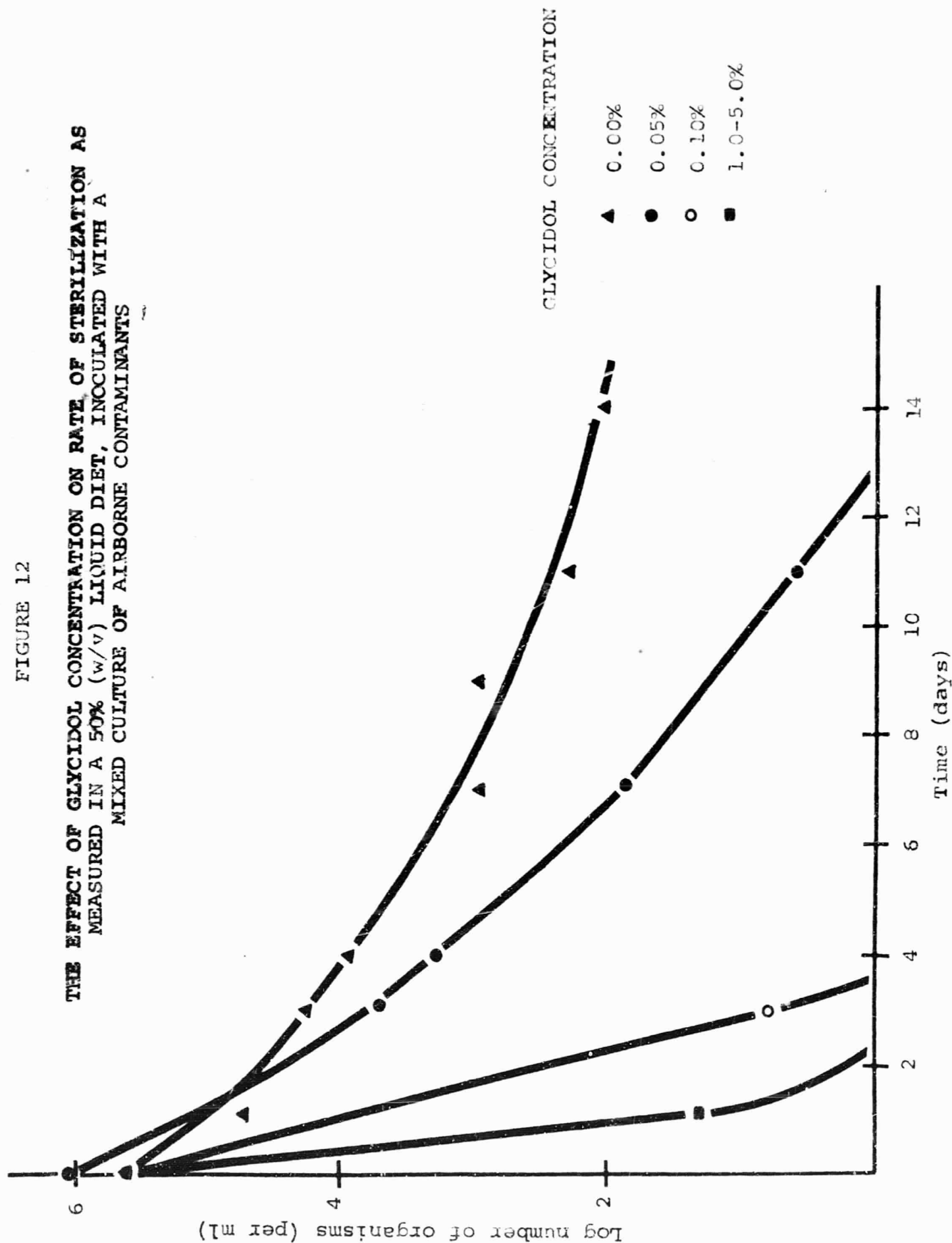


FIGURE 13

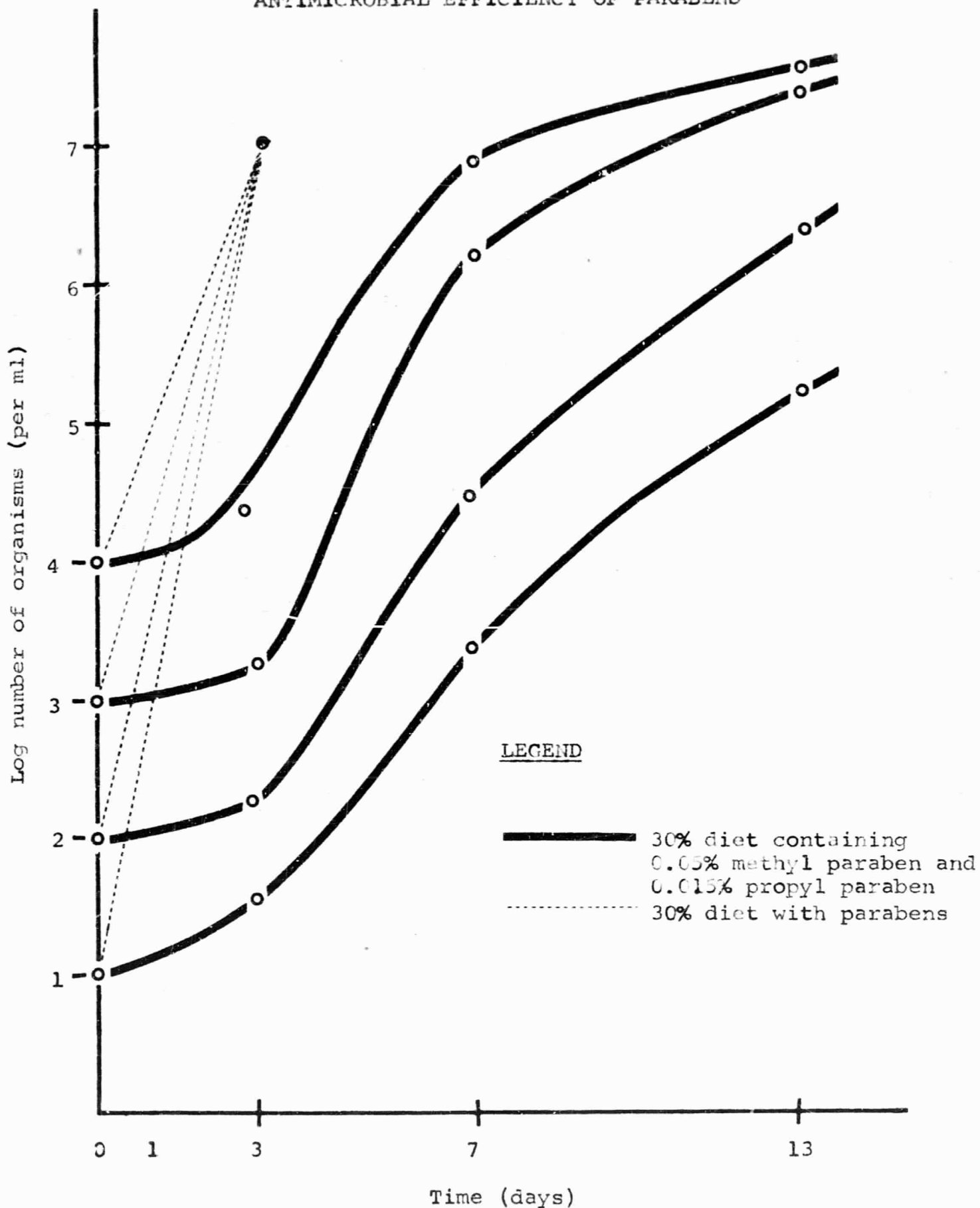
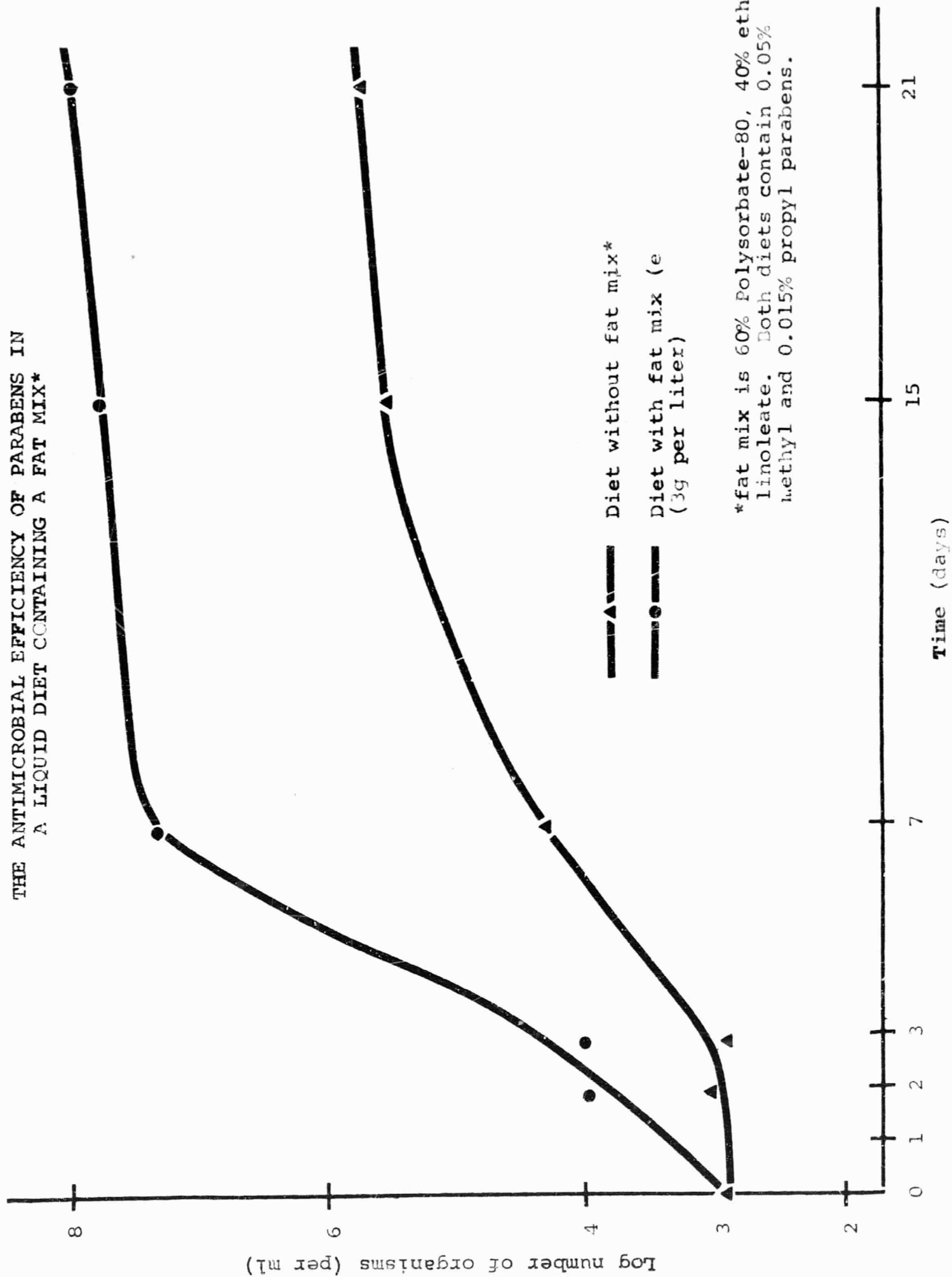
THE EFFECT OF INITIAL CONTAMINATION LEVEL ON
ANTIMICROBIAL EFFICIENCY OF PARABENS

FIGURE 14

THE ANTIMICROBIAL EFFICIENCY OF PARABENS IN
A LIQUID DIET CONTAINING A FAT MIX*



SCHWARZ BIORESEARCH, INC.

SUMMARY OF ANIMAL EXPERIMENTS

Animal experiments concerned with water metabolism, diet stability, physical form and amino acid composition of chemically defined liquid diets (Codelid diets) were conducted during the year.

The stability studies showed that conditions of storage markedly influence the stability of Codelid diets and that severity of environmental stress, browning, and nutrient loss were all directly related. Specifically, these studies showed that when refrigerated or frozen, a Codelid diet (diet-14) retains its nutritive value for at least 8 months. However, when the same diet was stored at room temperature for 4 months, regardless of whether it was sterile, kept in the dark or stored under nitrogen gas, nutrient losses occurred which caused growth retardation of rats consuming these diets. Growth retardation resulting from nutrient destruction also occurred when rats were fed diets which had been irradiated with 5×10^5 or 5×10^6 roentgens. Inclusion of ethyl cysteinate into these diets showed signs of radiation protection but the evidence was inconclusive. When the diet was kept at 60°C , marked destruction of all the amino acids occurred within 6 days and resulted in deficiencies of eight amino acids. Rats fed this diet suffered severe weight loss and eventually died. The inclusion of 0.5% glutathione into the diet prior to heating failed to prevent browning or nutrient losses.

A series of experiments were conducted to determine the adequacy of a water soluble, well defined, acid hydrolyzed protein as a replacement for the more expensive amino acids normally present in Codelid diets. It was found that excellent growth could be attained by rats fed diets containing this

SCHWARZ BIORESEARCH, INC

material at an equivalency of 9% protein (Nx6.25) and when supplemented with methionine, tryptophan, phenylalanine and lysine. The experiments indicated that a relatively inexpensive liquid diet could be developed with minimal loss of chemical definition and without loss of nutritional value.

Additional experiments concerning the amino acid moiety of Codelid diets showed that a diet (diet-17) incorporated into agar and containing asparagine, a high level of arginine and an amino acid pattern equivalent to 10% protein (Nx6.25), could produce growth of more than 5.5 gm/rat/day in CFE weanling rats. When this diet was fed as a liquid, without agar, growth was reduced to 4.8 gm/rat/day. The reduced growth was traceable to a lower diet intake of the liquid diet group compared to the group ingesting the diet in gel form.

In another experiment it was found that non-digestible bulk, in the form of agar or cellulose did not enhance diet utilization. However, as was the case for Codelid diet-17, providing the diet in gel form (agar) increased diet consumption which resulted in a corresponding increase in growth rate. It was suggested that with further dietary modifications agar may solve the age old problem of inadequate consumption of amino acid diets without loss of efficiency and that it may also afford an excellent means for incorporating high levels of fat into Codelid diets.

In two independent experiments, a hemorrhagic condition developed when Codelid diet-17 was fed to CDF strain rats. The condition was traced to vitamin K deficiency since intramuscular injection of menadione diphosphate prevented further hemorrhage. Inasmuch as menadione was present in the diet, it was hypothesized that chemical degradation may have caused interference with its biological utilization. It was also

SCHWARZ BIORESEARCH, INC.

suggested that the condition is strain specific since it was not observed in CFE rats. Studies are presently in progress to determine the factors responsible for this condition and to elucidate the nature of the strain difference in susceptibility.

Finally, studies in which a Codelid diet (diet-14) was supplied as the sole source of water and solid nutrients showed that a 30% (w/v) diet could best satisfy the water and nutrient requirements of the rat. The maximum tolerable concentration for satisfying these requirements was calculated to be 37% (w/v). At dietary concentrations in excess of 40% (w/v), the hypertonicity of the diet appeared too great for rats without access to drinking water. On the other hand, at dietary concentrations below 30% (w/v) the physical and physiological capacity of the rat was exceeded and it could not consume enough diet to satisfy its nutritive requirements. These findings suggested that a Codelid diet when provided as a sole source of water and solid nutrients, may be a practical approach to solving the nutritional, physiological and engineering problem associated with the space flight of humans and animals.

SCHWARZ BIORESEARCH, INC.

I. STABILITY STUDIES

In the previous sections of this report, the chemical nature of the losses sustained by a number of amino acids and vitamins in the diet following long term storage, was described along with an indication of the magnitude of such losses. Here, biological evaluation of these respective losses in terms of animal growth, is reported.

A. Pilot Experiment

In earlier experiments designed to determine an optimum liquid diet concentration for rats, diets were prepared at total nutrient concentrations of 10, 30, 50 and 70 percent (w/v).

The diet was initially prepared to contain 70% (w/v) solid nutrients and then appropriately diluted with distilled water to obtain the desired concentration. The composition of the 70% diet is shown in Table 15. All diets were sterilized by microfiltration through a millipore system and stored at 4°C. Bioassays were performed following storage for 3 and 6 months. The results are shown in Table 16. The data indicate that storage at 4°C had no adverse affect on the growth rate of animals fed the 70% (w/v) diet. The growth achieved after this diet was stored for either 3 or 6 months was slightly better than that of rats consuming freshly prepared diet at the same concentration. When diets containing 50% (w/v) or less solid nutrients were stored under the same conditions for 3 months, their growth promoting properties appeared to deteriorate. However, an insufficient supply of these dilute diets prevented additional bioassays.

It must be pointed out that growth in all groups was less than normally obtained with our liquid diets. This may be related to the inhibitory properties of ethyl cysteinate

TABLE 15

COMPOSITION OF BASAL DIET*

<u>Ingredient</u>	<u>gm/l</u>	<u>Ingredient</u>	<u>gm/l</u>
Sodium chloride	4.9	Arginine·HCl	1.59
Potassium hydroxide	4.31	Aspartate	13.58
Glucono-delta-lactone	18.76	Cysteinate·Ethyl	5.88
Magnesium oxide	0.511	Ester·HCl	
Ferrous ammonium sulfate	0.973	Glutamate, mono-	24.08
Mono-calcium fructose	70.0	sodium	
1,6-Diphosphate		Glycine	6.02
B-Vitamin pre-mix**	140.0	Histidine·HCl·H ₂ O	5.32
Trace Mineral pre-mix**	17.5 ml	Isoleucine	11.48
Fat pre-mix**	2.8	Leucine	15.54
Sodium hydroxide	4.06	Lysine·HCl	14.28
Methyl paraben	1.40	Methionine	5.46
Propyl paraben	0.42	Phenylalanine	9.66
Choline·HCl	2.10	Proline	7.42
Glucose	255.64	Serine	12.88
		Threonine	8.26
		Tryptophan	2.66
		Tyrosine·Ethyl	9.1
		Ester·HCl	
		Valine	12.32

*Values based on 70% (w/v) solid nutrients.

**For composition of B-vitamin, trace mineral and fat pre-mixes, see Table 11 of Annual Report 1965.

SCHWARZ BIORESEARCH, INC.

TABLE 16

A PRELIMINARY BIOASSAY ON THE STABILITY OF
LIQUID DIETS REFRIGERATED FOR 3 or 6 MONTHS*

Storage time	Diet concentration	Av. daily body weight gain	Av. daily diet consumption	Av. daily water consumption
	% solids	gm/rat/day	ml/rat/day	ml/rat/day
Initial**	70	2.0	16.6	16.8
	50	2.5	14.8	10.0
	30	3.2	45.0	8.9
	10	1.8	36.3	4.2
3 Months***	70	2.3	14.1	8.5
	50	1.5	14.0	7.2
	30	1.8	28.7	4.2
	10	0.2	50.9	1.6
6 Months****	70	2.4	13.4	18.1

*CFE male rats used in all experiments.

**Six rats allotted to each dietary treatment. Experimental period 28 days.

***Five rats allotted to each dietary treatment. Experimental period 14 days.

****Six rats allotted to the diet. Experimental period 27 days.

previously reported by this laboratory (Annual Report 1964). Perhaps also related to this inhibition was the reduced consumption observed in all groups, particularly the 30% (w/v) and 10% (w/v) groups, after the diet had been refrigerated for 3 months.

In view of the reduced growth and its possible relation to the inhibitory properties of ethyl cysteinate and in consideration of the preliminary nature of these experiments, a year long study was initiated to obtain definitive information on the stability of chemically defined liquid diets (Codelid diets). The design and progress of this experiment are reported below.

B. Long Term Stability Studies

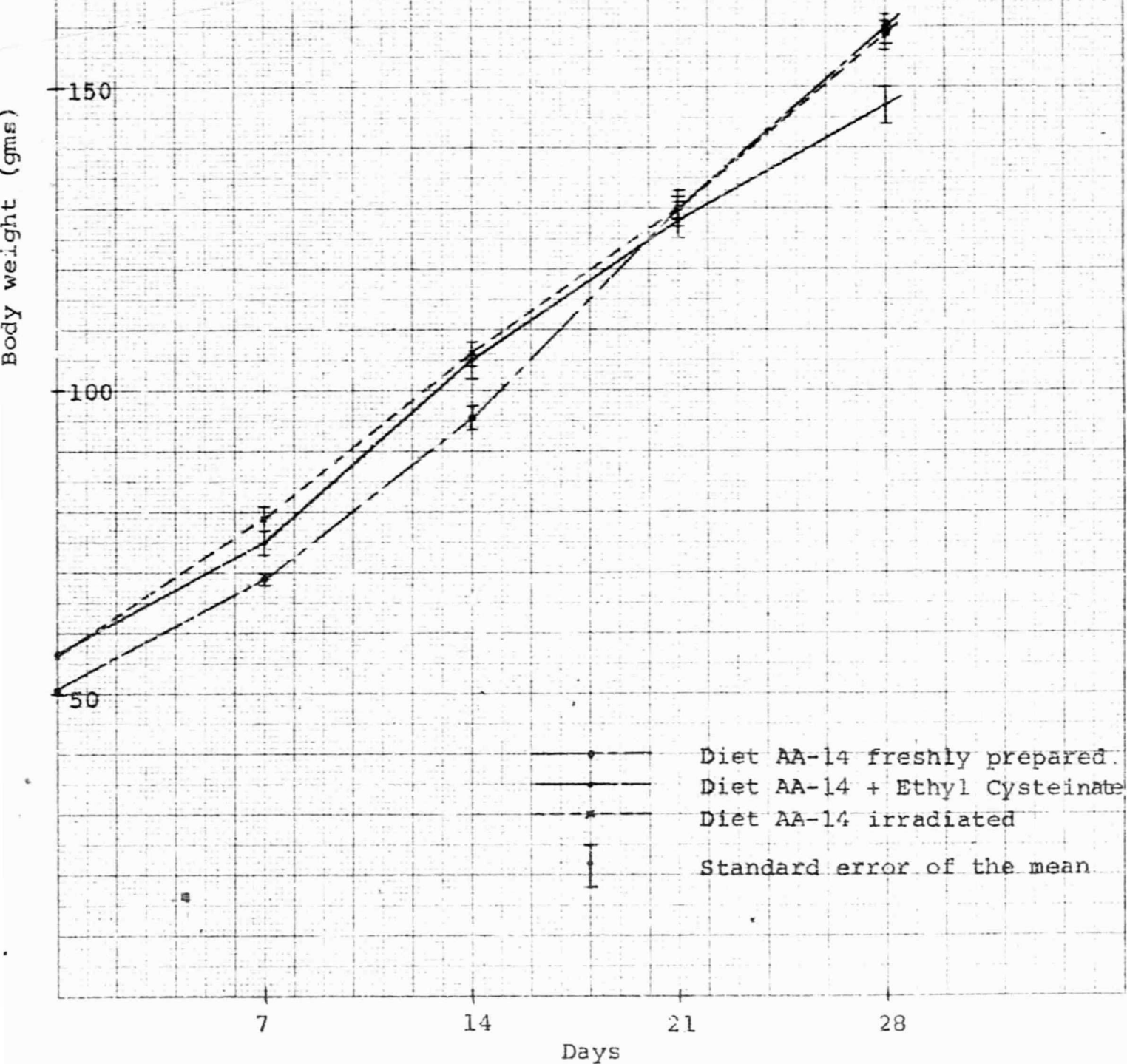
Long term stability studies in which diets are exposed to several invironmental conditions and then subjected to chemical and bioassay are still in progress. The first section of this report showed the chemical changes observed in diets exposed to a variety of environmental conditions. Corresponding bioassays are presented in this section.

The composition of the diet tested (Codelid diet-14) is shown in Tables 17 and 18. It should be noted that this diet was formulated to contain 57% solids (w/v). Although the diet was exposed to the various environmental conditions at this concentration, it was diluted to contain 50% solids (w/v) prior to bioassay.

Table 19 shows the growth rates, diet consumption and water consumption of rats fed the test diet after being

FIGURE 17

GROWTH OF RATS FED A FRESHLY PREPARED
CHEMICALLY DEFINED LIQUID DIET OR THE
SAME DIET IRRADIATED WITH 5×10^5 ROENTGENS



COMPOSITION OF TEST DIETS

FORMULATION AND NUMBER	14	16	17	18	15
<u>CARBOHYDRATES:</u>	<u>gm/l.</u>	<u>gm/l.</u>	<u>gm/l.</u>	<u>gm/l.</u>	<u>gm/l.</u>
Glucose	344.5	342.9	323.8	260.4	330.1
Glucono-delta-lactone	13.03	13.05	13.03	13.03	13.03
<u>MINERALS, Macro</u>					
Ferrous Ammonium Sulfate	0.70	-	0.70	0.70	0.70
Ferrous Gluconate	-	0.86	-	-	-
FDC (1)	25.00	25.25	25.00	25.00	25.00
Magnesium Oxide	0.38	0.37	0.38	0.38	0.38
Potassium Hydroxide	3.08	3.08	3.08	3.08	3.08
Sodium Bicarbonate	-	1.75	-	-	1.75
Sodium Chloride	3.50	2.40	3.50	3.50	3.50
Sodium Hydroxide	2.29	2.05	0.70	1.10	-
<u>MINERALS, Trace</u>	<u>mg/l.</u>	<u>mg/l.</u>	<u>mg/l.</u>	<u>mg/l.</u>	<u>mg/l.</u>
Ammonium molybdate·4H ₂ O	3.0	3.0	3.0	3.0	3.0
Cobalt Acetate·4H ₂ O	4.5	4.5	4.5	4.5	4.5
Cupric Acetate·4H ₂ O	7.5	7.5	7.5	7.5	7.5
Manganese Acetate·4H ₂ O	130.0	130.0	130.0	130.0	130.0
Potassium Iodide	15.0	15.0	15.0	15.0	15.0
Zinc Benzoate	11.0	11.0	11.0	11.0	11.0
<u>VITAMINS, Water Soluble</u>					
Ascorbic Acid	372.5	250.0	372.5	372.5	372.5
Biotin	0.22	0.15	0.22	0.22	0.22
B ₁₂ (1% Trituration)	74.5	50.0	74.5	74.5	74.5
Calcium Pantothenate	37.25	25.0	37.25	37.25	37.25
Calcium Chloride	1.30g	1.25g	1.30g	1.30g	1.30g
Folic Acid	0.37	0.25	0.37	0.37	0.37
Inositol	186.25	125.0	186.25	186.25	186.25
Niacin	27.94	18.75	27.94	27.94	27.94
Para-aminobenzoic acid	223.5	150.0	223.5	223.5	223.5
Pyridoxine·HCl	4.69	3.15	4.69	4.69	4.69
Riboflavin	7.45	3.75	7.45	7.45	7.45
Thiamine·HCl	3.73	2.5	3.73	3.73	3.73
<u>VITAMINS, Fat Soluble</u>					
Vitamin A Acetate 3000 IU/mg	5.0	5.0	5.0	5.0	5.0
Calciferol 40 IU/μg	3.5μg	3.5μg	3.5μg	3.5μg	3.5μg
Ethyl Linoleate	2.0g	2.0g	2.0g	2.0g	2.0g
Menadione	2.1	2.1	2.1	2.1	2.1
Polysorbate 80	3.0g	3.0g	3.0g	3.0g	3.0g
α-tocopherol acetate 1 IU/mg	25.0	25.0	25.0	25.0	25.0

(1) Monocalcium Fructose 1,6-Diphosphate

TABLE 18

COMPOSITION OF TEST AMINO ACID MIXTURES

FORMULATION AND NUMBER	14	16	17	18	15
COMPONENTS	gm/l.	gm/l.	gm/l.	gm/l.	gm/l.
<u>AMINO ACIDS:</u>					
L-Arginine·HCl	8.90	4.70	8.90	13.55	4.09
L-Histidine·HCl·H ₂ O	2.93	2.85	3.38	4.46	3.46
L-Isoleucine	6.45	4.40	5.50	8.20	4.30
L-Leucine	8.70	7.00	7.30	11.10	8.90
L-Lysine·HCl	8.00	6.50	11.80	18.00	11.30
L-Methionine	3.03	3.15	5.39	8.20	4.95
L-Phenylalanine	5.38	3.15	7.63	11.60	7.17
L-Threonine	4.70	4.40	5.00	5.30	3.58
L-Tryptophan	1.48	1.40	1.50	1.50	1.10
L-Valine	6.85	4.90	5.50	8.20	5.83
L-Alanine	-	3.20	2.30	3.50	3.16
L-Asparagine	-	-	3.95	6.00	-
L-Aspartate	7.63	6.75	2.30	3.50	5.02
L-Cysteine Ethyl Ester·HCl	-	0.55	2.43	3.70	-
L-Glutamate, Monosodium	13.38	25.60	27.95	44.49	24.95
Glycine	6.05	2.05	13.94	23.30	2.83
L-Proline	4.10	12.70	2.30	3.50	8.90
L-Serine	7.23	6.60	2.30	3.50	3.67
L-Tyrosine Ethyl Ester·HCl	5.08	8.40	3.14	3.78	0.65

TABLE 19

GROWTH RATE, DIET CONSUMPTION AND WATER CONSUMPTION
OF RATS FED A CHEMICALLY DEFINED LIQUID DIET
EXPOSED TO SEVERAL ENVIRONMENTAL CONDITIONS*

Environment**	Av. daily body weight gain gm/rat/day	Av. daily diet consumption ml/rat/day	Av. daily water consumption ml/rat/day
Control	3.9	26.6	12.6
Heat	(-) 1.6***	10.8***	7.9***
Low Irradiation	3.7	24.9	13.2
Low Irradiation + Ethyl Cysteinate	3.2	26.2	12.5
High Irradiation	1.6	21.2	12.7
High Irradiation + Ethyl Cysteinate	2.1	20.5	12.8
High Irradiation + Ethyl Cysteinate Non-sterile	2.2	21.7	12.9

*Experimental period 28 days. Twelve CFE male weanling rats of similar body weight were allotted to each dietary treatment. Growth curves are shown in Figures 15-18. Detailed diet consumption, water consumption, and growth data are presented in Table 1 in the Appendix.

**Environments: Control: freshly prepared diet AA-14.
Heat: diet AA-14 exposed to 60°C for 12 days.

Low Irradiation: diet AA-14 exposed to 5×10^5 Roentgen.

High Irradiation: diet AA-14 exposed to 5×10^6 Roentgen.

***All animals died during second week of experiment.

FIGURE 15

GROWTH OF RATS FED A FRESHLY PREPARED CHEMICALLY
DEFINED LIQUID DIET OR A STANDARD DIET
CONTAINING NATURAL FOOD INGREDIENTS

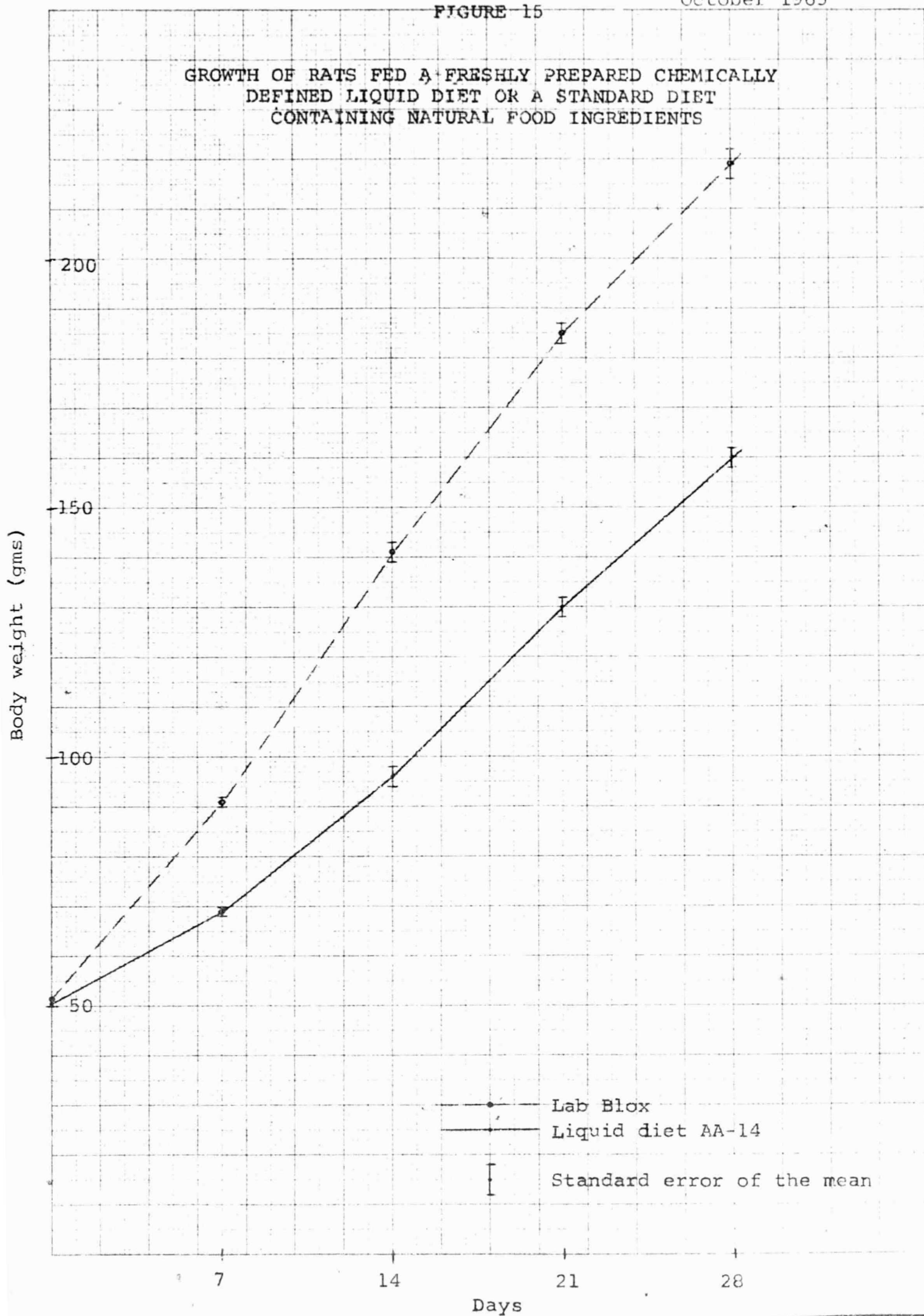


FIGURE 16

GROWTH OF RATS FED A FRESHLY PREPARED
CHEMICALLY DEFINED LIQUID DIET OR THE
SAME DIET KEPT AT 60°C FOR 12 DAYS

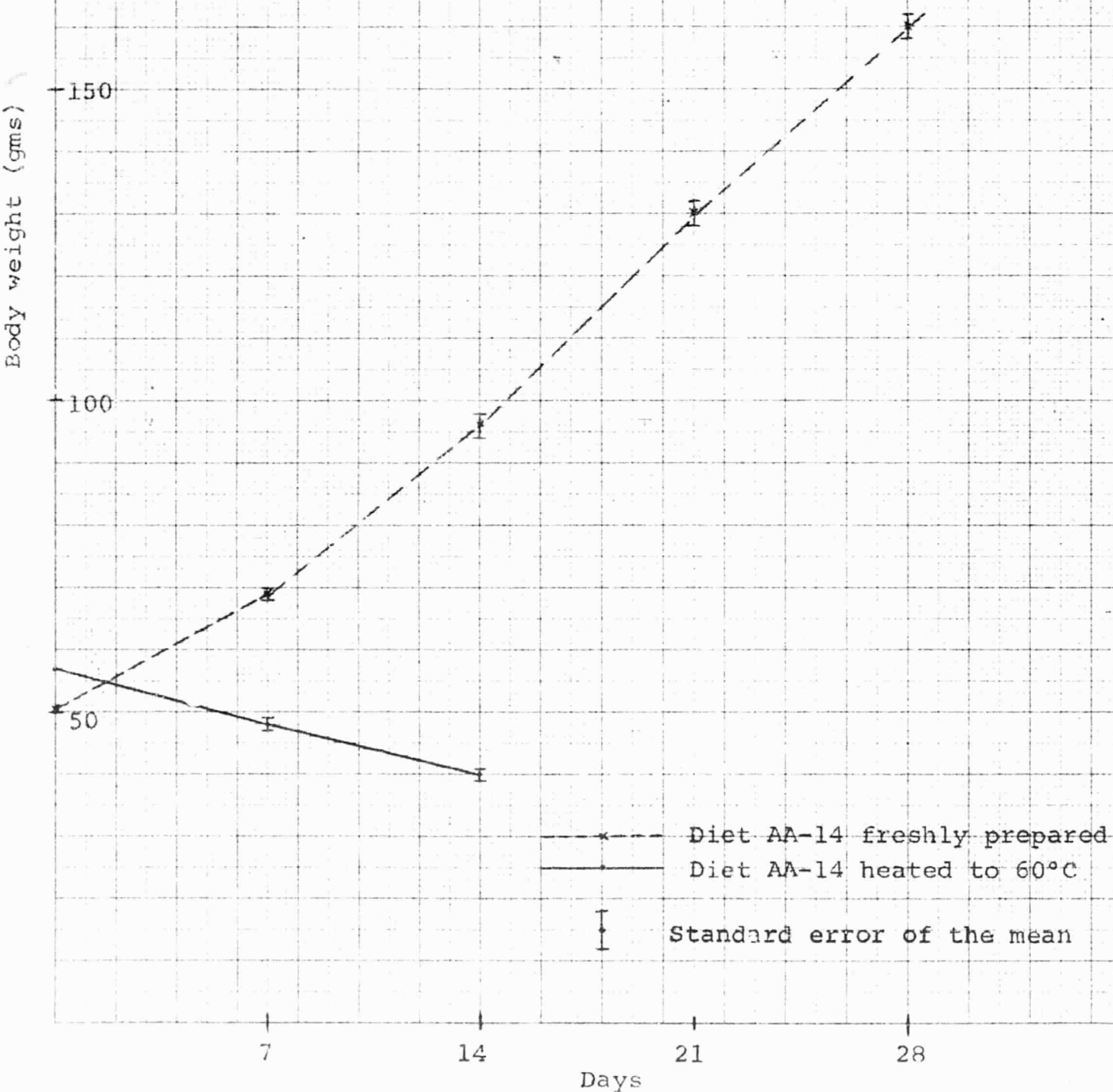


FIGURE 17

GROWTH OF RATS FED A FRESHLY PREPARED
CHEMICALLY DEFINED LIQUID DIET OR THE
SAME DIET IRRADIATED WITH 5×10^5 ROENTGENS

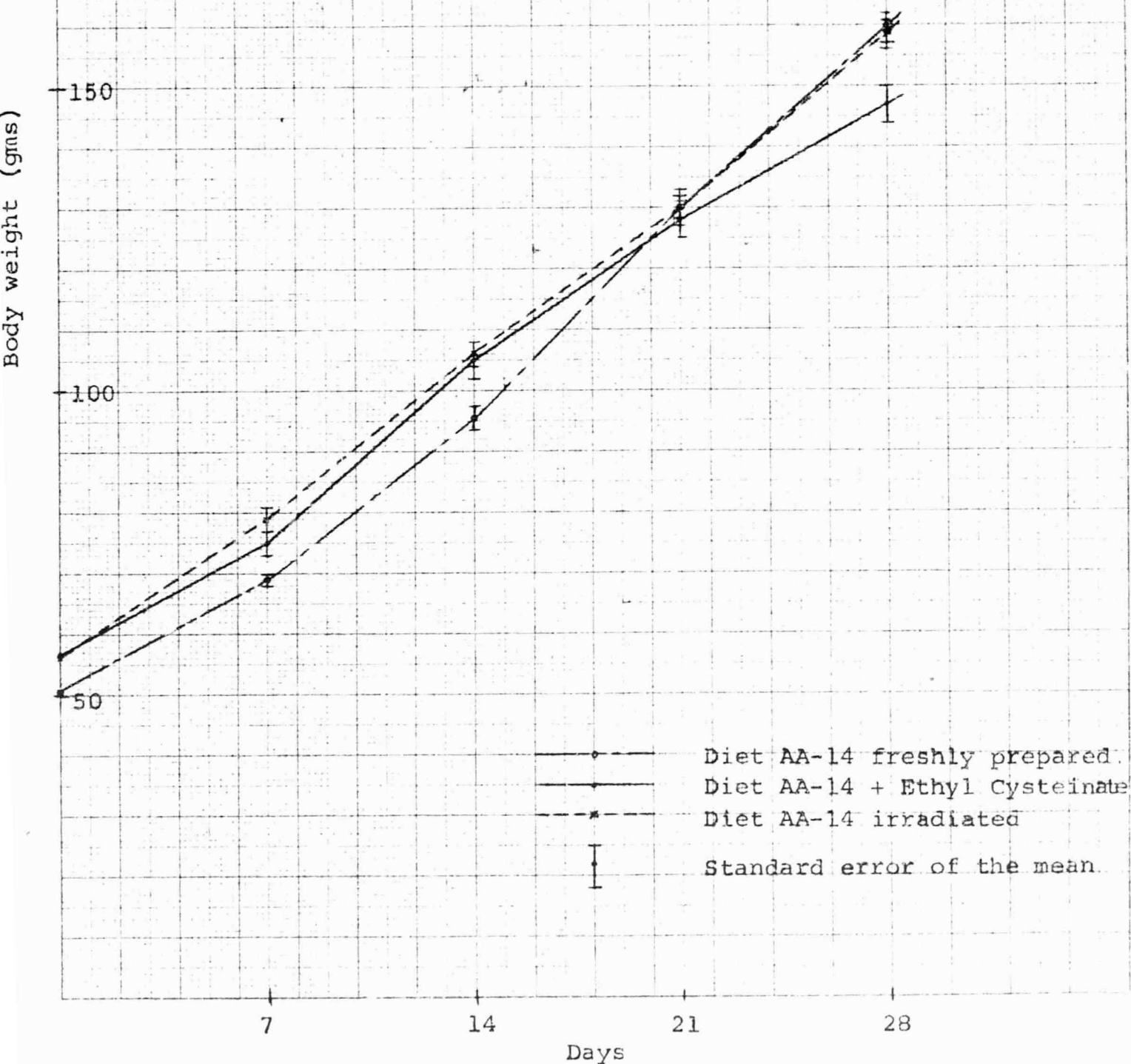
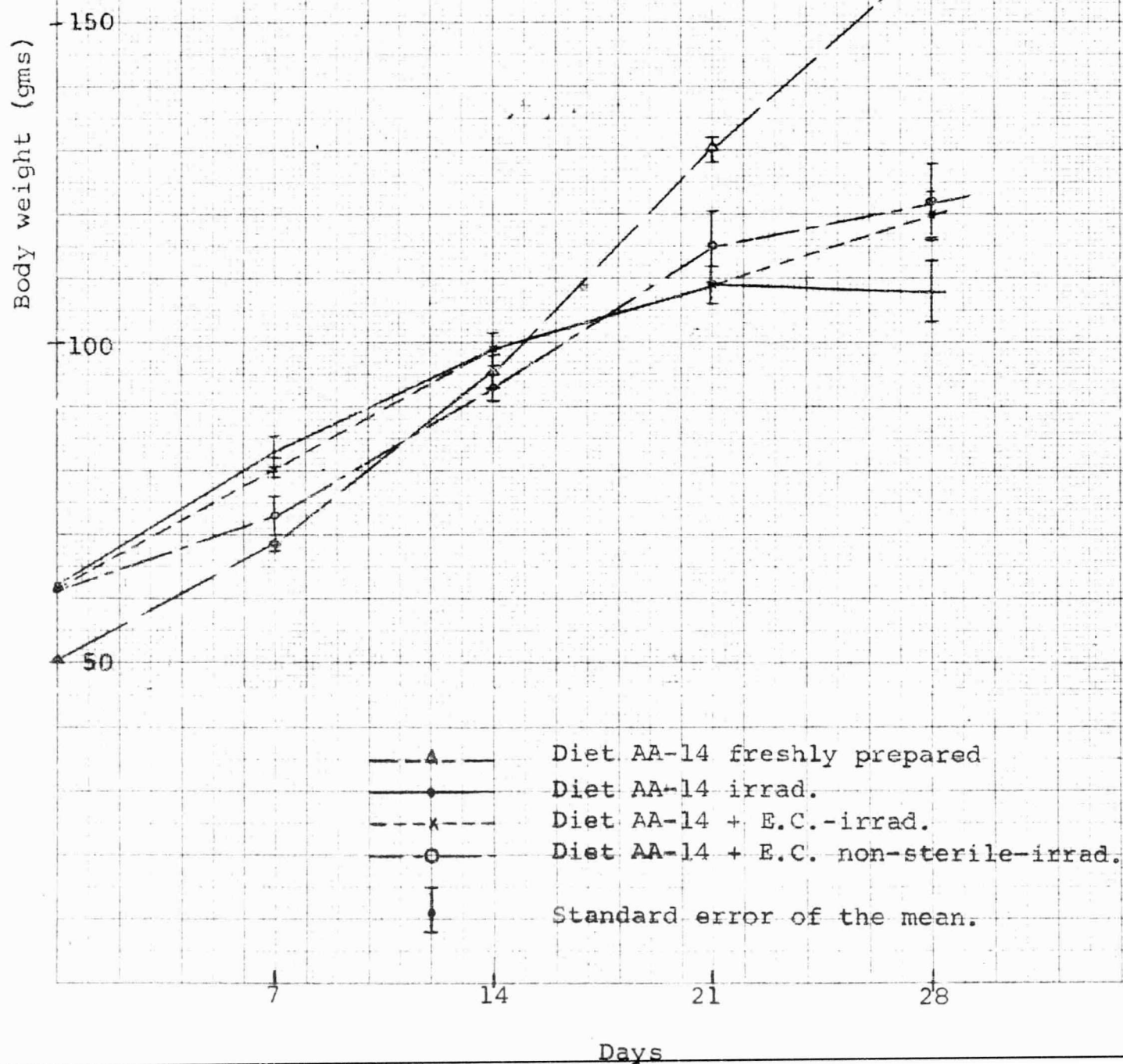


FIGURE 18

GROWTH OF RATS FED A FRESHLY PREPARED
CHEMICALLY DEFINED LIQUID DIET OR THE
SAME DIET IRRADIATED WITH 5×10^6 ROENTGENS



exposed to either heat (60°C for 12 days), low irradiation (5×10^5 Roentgens) or high irradiation (5×10^6 Roentgens). Corresponding growth curves are presented in Figures 15 through 18.

C. Heat

The biological response of animals fed the diet after it had been stored at 60°C for 12 days reflects the extensive chemical degradation cited earlier in this report. The marked body weight loss, reduced diet and water consumption and subsequent death of all animals suggests the formation of toxic products in addition to the destruction of essential nutrients.

Tables 20 and 21 and Figure 19 show the results of chemical and physical measurements performed on the diet before and after heat treatment. Marked destruction of all the amino acids occurred within 6 days after heating and resulted in dietary deficiencies of histidine, lysine, methionine, phenylalanine, valine, arginine, glycine, and glutamate. The magnitude of destruction and the wide range of amino acids destroyed indicates formation of antimetabolites and toxic products concurrently with the creation of the aforementioned deficiencies. The decreased dietary concentration of glucose suggests that one path of amino acid destruction is via the Maillard or "browning" reaction as discussed in section A.

Analyses of the diet for total nitrogen and phosphorus showed neither to be affected by heat. Of the vitamins measured (Table 21) only dietary concentrations of pyridoxine, folic acid, pantothenate and cyanocobalamin decreased. However, in no case was a deficiency created. Physical measure-

TABLE 20

AMINO ACID COMPOSITION* OF A CHEMICALLY DEFINED
LIQUID DIET AFTER EXPOSURE TO SEVERAL ENVIRONMENTAL CONDITIONS

Amino acid	Req.**	Control	Low Irradiation***		High Irradiation***		High Temperature-60°C	
			(-)E.C.	(+)E.C.	(-)E.C.	(+)E.C.	6 days	12 days
			gm/kg					
Histidine·HCl·H ₂ O	3.00	5.61	3.91	4.65	4.11	4.88	0.98	0.91
Isoleucine	5.00	12.49	11.81	12.02	12.23	12.19	5.61	5.79
Leucine	8.00	16.89	16.00	16.05	16.14	17.14	7.37	7.54
Lysine·HCl	9.00	16.53	14.82	14.77	14.17	15.35	2.40	2.23
Methionine	5.00	5.91	5.75	5.44	5.46	5.48	1.96	2.21
Phenylalanine	6.00	10.82	10.42	10.19	10.51	11.26	4.23	4.60
Threonine	5.00	9.67	9.02	8.35	9.16	9.72	6.26	8.14
Tryptophan	1.50				Not determined			
Valine	7.00	14.12	13.02	12.33	12.91	13.74	6.11	6.25
Arginine·HCl	5.00	17.72	17.37	16.81	15.44	16.86	2.53	2.12
Aspartate	2.30	15.35	14.67	13.77	14.86	15.61	7.68	7.96
Glycine	13.94	12.54	11.63	11.12	12.14	13.60	4.11	4.02
Monosodium glutamate	27.95	26.44	25.46	24.58	26.01	25.74	6.51	6.51
Proline	2.30	8.25	7.82	7.95	7.84	8.39	7.40	7.05
Serine	2.30	15.00	13.53	12.88	14.58	13.44	9.16	8.77
Alanine	2.30							
Cystine	2.30				Not determined			
Tyrosine	2.30				Not determined			
Asparagine	3.95							

*Determined chemically by column chromatography.

**Essential amino acid requirements listed in NRC publication 990. Values for non-essential amino acids obtained from G.S. Ranhotra and B. Connor Johnson, Proc.Soc.Expt'l.Biol.Med. 118:1197(1965).

5x10⁵ Roentgen.*5x10⁶ Roentgen.

TABLE 21
H

ASSAY OF A CHEMICALLY
EXPOSURE TO SEVERAL

Assay	Req.**	Control
Specific Gravity	---	1.1900
Osmotic Pressure mos/L (1:4 dil.)	---	835
pH	---	4.76
Glucose gm/kg	---	690.0
Nitrogen gm/kg	---	25.50
Phosphorus gr %	5.0	7.11
Vitamin A IU/gm	2.0	17.5
Vitamin E µg/gm	60	44.4
Thiamine µg/gm	1.25	7.2
Riboflavin µg/gm	2.50	11.8
Pyridoxine µg/gm	1.20	21.1
Biotin µg/gm	---	0.25
Niacin µg/gm	15.0	52.11
Folic Acid µg/gm	---	0.72

*Control - Freshly prepared diet; Low Irradiation - 5×10^5
Heat - 60°C for 12 days.

**Requirements listed in NRC publication 990.

***Ethyl cysteinate.

BLE 21

CONT'D

B

DEFINED LIQUID DIET AFTER
ENVIRONMENTAL CONDITIONS*

Low Irradiation		High Irradiation		Heat
(-)E.C.***	(+)E.C.	(-)E.C.	(+)E.C.	
1.1781	1.1789	1.1781	1.1789	1.1675
810	820	833	830	618
4.66	4.51	4.67	4.64	3.81
695.1	689.1	685.8	684.6	612.11
24.81	25.49	25.11	25.56	24.35
6.95	7.04	7.04	6.96	7.07
---	---	---	---	---
---	---	---	---	---
---	---	---	---	---
13.3	14.0	10.5	14.6	13.3
6.1	8.4	6.5	5.8	14.9
0.23	0.24	0.06	0.21	0.19
40.4	52.6	14.0	33.3	50.9
---	0.81	0.10	0.52	0.27

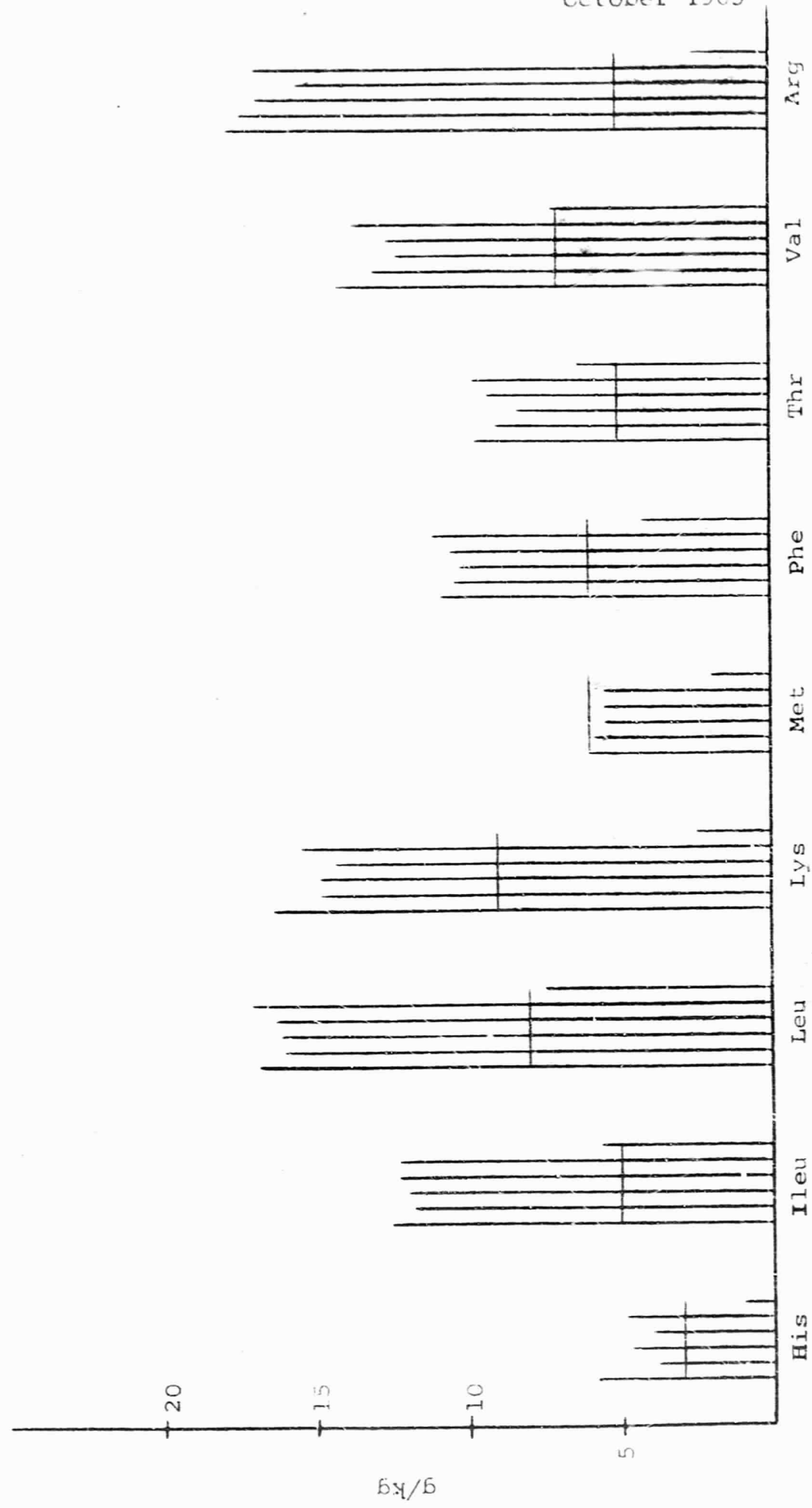
SCHWABZ BIORESEARCH, INC.

Contract NASW-517
Annual Report
October 1965

Roentgens; High Irradiation 5×10^6 Roentgens;

FIGURE 19

EFFECT OF ENVIRONMENTAL EXPOSURE ON DIET ESSENTIAL
AMINO ACID COMPOSITION



LEGEND:

Bar sequence (left to right): Control: Low Irradiation: High Irradiation + E.C.: High Irradiation + E.C.: High Temperature - 6 days. Shaded area represents NRC Requirements.

ments showed specific gravity, osmotic pressure and pH to decrease after the heat treatment.

D. Irradiation

Low Irradiation

Feeding animals diets which had been irradiated with 5×10^5 roentgen resulted in only a 5% reduction in growth rate. Apparently little loss of nutritive value occurred when the diet was exposed to low irradiation. This is shown by the comparison of nutrients in the diet before and after irradiation (Tables 20 and 21 and Figure 19). Of the nutrients measured only histidine, pyridoxine and niacin were partially destroyed. Nevertheless their residual concentration still exceeded the recommended requirement levels. While inclusion of ethyl cysteinate into the diet seemed to protect histidine and several of the vitamins from the effects of low irradiation, this was not reflected in the growth response of rats fed these diets. The inability to demonstrate a positive biological response to this radiation protection may be due to the adequacy of nutrients in the cysteine-free diet after irradiation (i.e. insufficient radiation dosage to cause deficiencies and produce by-products) and/or to a balance between the growth inhibitory properties of ethyl cysteinate (Annual Report 1964) and its radiation-protective properties in the cysteine containing diets.

High Irradiation

Feeding a diet which had been irradiated with 5×10^6 roentgens resulted in marked growth retardation. However,

SCHWARZ BIORESEARCH, INC.

in no instance was body weight loss observed and all animals survived. Food consumption was slightly reduced and water consumption was unaffected. Physical measurements of the diet revealed no difference from the control diets (Table 21). As with diets exposed to a lower dose of irradiation, histidine was partially destroyed. (Table 20 and Figure 19). In addition, minor destruction of arginine and lysine occurred. Of the vitamins and other nutrients measured (Table 21), pyridoxine, biotin, niacin, folic acid and cyanocobalamin showed evidence of marked destruction. However, due to the excess of these amino acids and vitamins in the diet prior to irradiation, no dietary deficiencies resulted. It, therefore, appears that the observed growth inhibition is probably due to the formation of antimetabolites and/or toxic end-products.

The inclusion of ethyl cysteinate in the diet partially protected the nutrients from high irradiation. This was shown in Tables 20 and 21 and is reflected in the diminished growth retardation of animals fed diets supplemented with ethyl cysteinate. It is of interest that despite these beneficial effects, diet consumption was the same in all groups fed the highly irradiated diets. This suggests that at the dietary concentration used in this study, the growth inhibitory properties of ethyl cysteinate (Annual Report 1964) may be secondary to its irradiation protection. However, it is not possible at this time to ascertain whether higher concentrations of ethyl cysteinate would provide additional protection against irradiation without concomitant growth inhibition.

SCHWARZ BIORESEARCH, INC.

It should be mentioned that one group of animals was fed an ethyl cysteinate containing diet which had purposely been contaminated by atmospheric air for 3 hours. Although contaminated prior to irradiation this diet was found to be sterile after exposure to 5×10^6 roentgens and produced the same growth response as observed in animals fed the same diet sterilized prior to irradiation. The importance of knowing the optimum level of ethyl cysteinate to be used in the diet for irradiation protection, therefore, becomes obvious if irradiation is to be considered as a method of sterilization.

E. Storage

Four Months

The data in Table 22 and Figure 20 show the growth rates diet consumption and water consumption of rats fed the diet after storage under several environmental conditions for four months. When weanling animals were fed the diet after being stored at 0-4°C or -6°C, they grew well and diet and water consumption was normal. However, when they were fed the diet after storage at room temperature, regardless of whether it was sterile, kept in the dark or stored under nitrogen gas, diet and water consumption was markedly reduced and marked growth retardation occurred.

These observations undoubtedly reflect the chemical alterations shown in Tables 23 and 24 and Figure 21. The amino acid analyses show that destruction (>15%) of all the amino acids occurred when the diet was stored at room

TABLE 22

GROWTH RATE, DIET CONSUMPTION AND WATER CONSUMPTION OF RATS
FED A CHEMICALLY DEFINED LIQUID DIET STORED UNDER
SEVERAL ENVIRONMENTAL CONDITIONS FOR FOUR MONTHS*

Storage conditions	Av. daily body weight gain gm/rat/day	Av. daily diet consumption ml/rat/day	Av. daily water consumption ml/rat/day
Control**	3.9	26.6	12.6
0-4°C	4.1	24.8	11.9
(-)6°C	4.1	25.6	11.2
24-26°C--dark	(0)	8.8	3.3
24-26°C--light	(-)	8.3	3.1
24-26°C--dark Non-sterile	(-)	8.1	2.7
24-26°C--dark Non-sterile-N ₂	(-)	8.5	3.6

*Experimental period 28 days. Twelve CFE male weanling rats of similar body weight were allotted to each dietary treatment. Growth curves are shown in Figure 20. Detailed diet consumption, water consumption and growth data are presented in Table 2 in the Appendix. Unless otherwise noted, all diets were stored as sterile solutions.

**Control: freshly prepared diet-14. Data obtained at zero time (see Table 14).

FIGURE 20

GROWTH OF RATS FED A CHEMICALLY DEFINED LIQUID DIET
STORED UNDER SEVERAL ENVIRONMENTAL CONDITIONS
FOR FOUR MONTHS

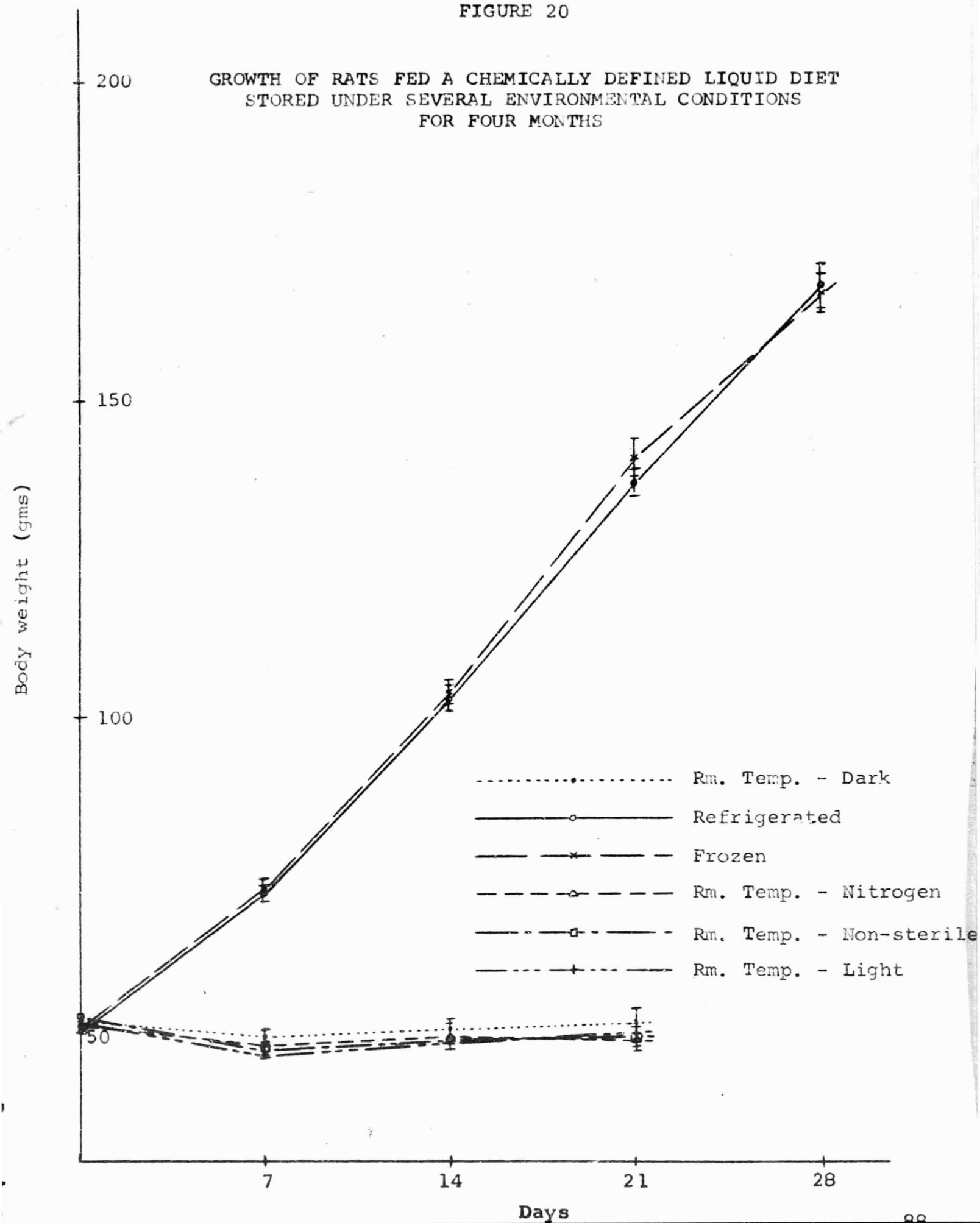


TABLE 24

ASSAY OF A CHEMICALLY DEFINED DIET
EXPOSURE TO SEVERAL ENVIRONMENTAL

Assay	Req.*	Control**	0-4°C
Specific Gravity	---	1.1900	1.1804
Osmotic Pressure mos/L (1:4 dil.)	---	835	829
pH	---	4.76	4.82
Glucose gm/kg	---	690.0	700.0
Nitrogen gm/kg	---	25.53	25.61
Phosphorus gm/kg	5.0	7.11	6.99
Vitamin A IU/gm	2.0	17.5	15.4
Vitamin E µg/gm	60	44.4	23.3
Thiamine µg/gm	1.25	7.2	5.5
Riboflavin µg/gm	2.50	11.8	11.4
Pyridoxine µg/gm	1.20	21.1	9.4
Niacin µg/gm	15.0	52.1	53.7
Folic Acid µg/gm	---	0.72	0.44

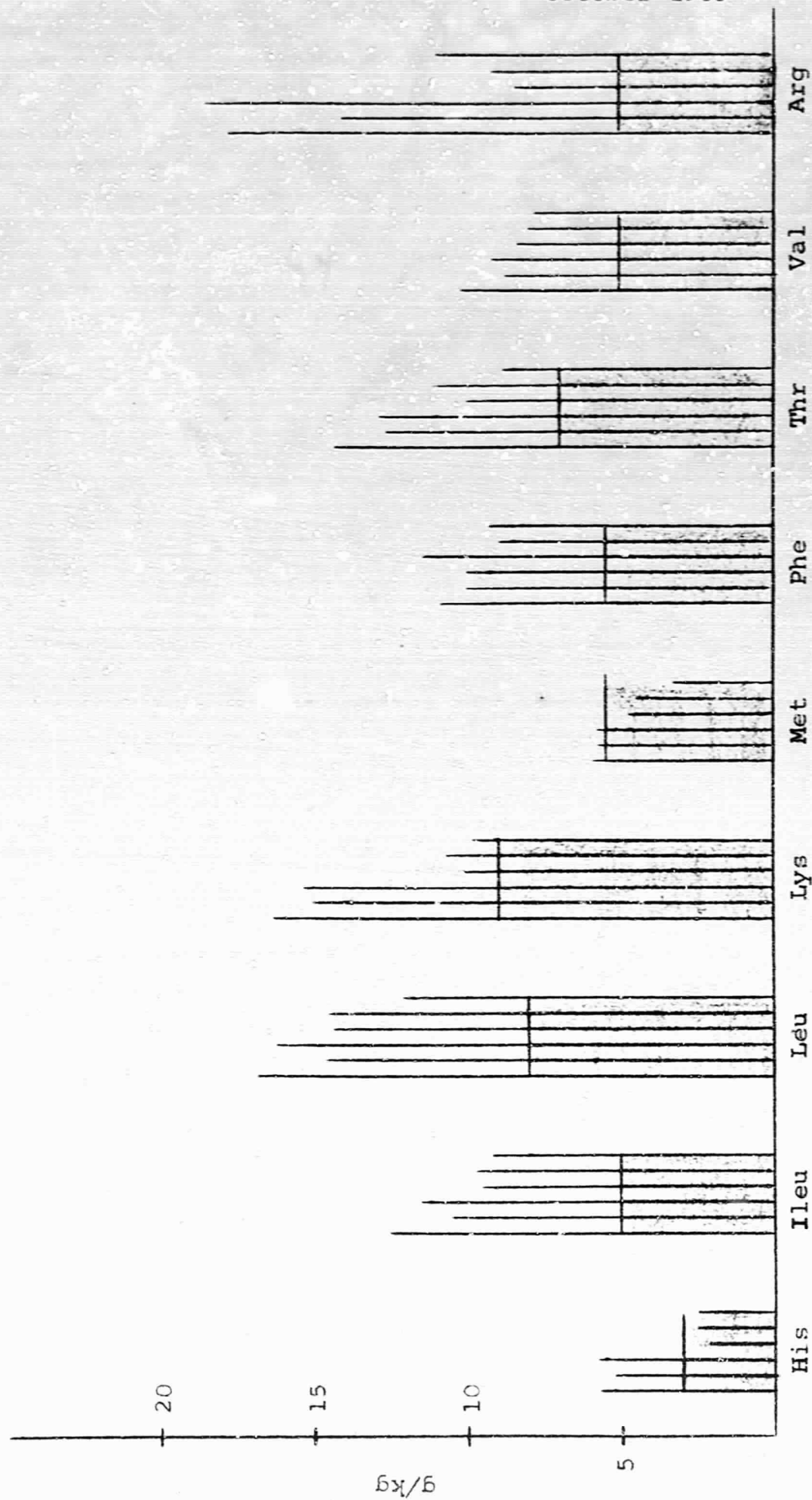
*Requirements listed in NRC publication 990.

**Freshly prepared diet (zero time).

LIQUID DIET AFTER
CONDITIONS FOR FOUR MONTHS

-6°C	Room Temperature 24-26°C			
	Dark	Nitrogen	Light	Non-Sterile
1.1803	1.1782	1.1797	1.1775	1.1939
814	791	791	788	876
4.87	4.67	4.67	4.65	4.57
684.9	695.7	688.5	695.0	686.9
25.61	25.61	25.78	25.61	---
7.07	6.88	6.89	6.89	6.89
14.9	10.6	10.2	5.2	3.4
20.2	22.6	22.6	15.0	11.7
6.4	4.7	5.1	4.7	5.4
11.4	11.4	11.4	0.4	11.4
---	9.5	---	---	---
52.6	64.9	49.1	48.4	52.6
0.35	0.23	0.35	0.14	0.23

FIGURE 21

EFFECT OF ENVIRONMENTAL EXPOSURE ON DIET ESSENTIAL
AMINO ACID COMPOSITIONLEGEND:

Bar sequence (left to right): Control; 4°C; -6°C; 24-26°C:- Dark; 24-26°C-Nitrogen:
24-26°C-Light.

SCHWARZ BIORESEARCH, INC.

temperature. This resulted in dietary deficiencies of the "essential" amino acids, histidine and methionine, and the "dispensable" amino acids, monosodium glutamate and glycine. When stored in the cold, destruction of monosodium glutamate and proline occurred at both 0-4°C and -6°C. This created a dietary deficiency of monosodium glutamate, which is ordinarily regarded as a "dispensable" amino acid. Partial destruction of arginine occurred only at 0-4°C.

Table 24 shows the results of the microbiological assays and chemical and physical measurements made on the diets. Osmotic pressure decreased in all diets kept at room temperature except the non-sterile diet where a slight increase was observed. Specific gravity and pH of these diets also showed slight changes. Dietary concentrations of glucose, nitrogen and phosphorus remained essentially unaltered. The vitamin assays of the room-temperature and cold-exposed diets showed losses of all vitamins measured except niacin. Riboflavin was stable under all conditions except when the diet was exposed to light. Light also enhanced the destruction of Vitamin A, Vitamin E and folic acid. Despite the partial destruction of the vitamins measured, only deficiencies of Vitamin E occurred and in the case of light exposure, only riboflavin was found to be deficient.

Eight Months

Table 25 and Figure 22 show the growth rates, diet consumption and water consumption of rats fed the diet after being stored at 0-4°C or -6°C for eight months.

TABLE 25

GROWTH RATE, DIET CONSUMPTION AND WATER CONSUMPTION
OF RATS FED A CHEMICALLY DEFINED LIQUID DIET
REFRIGERATED OR FROZEN FOR EIGHT MONTHS*

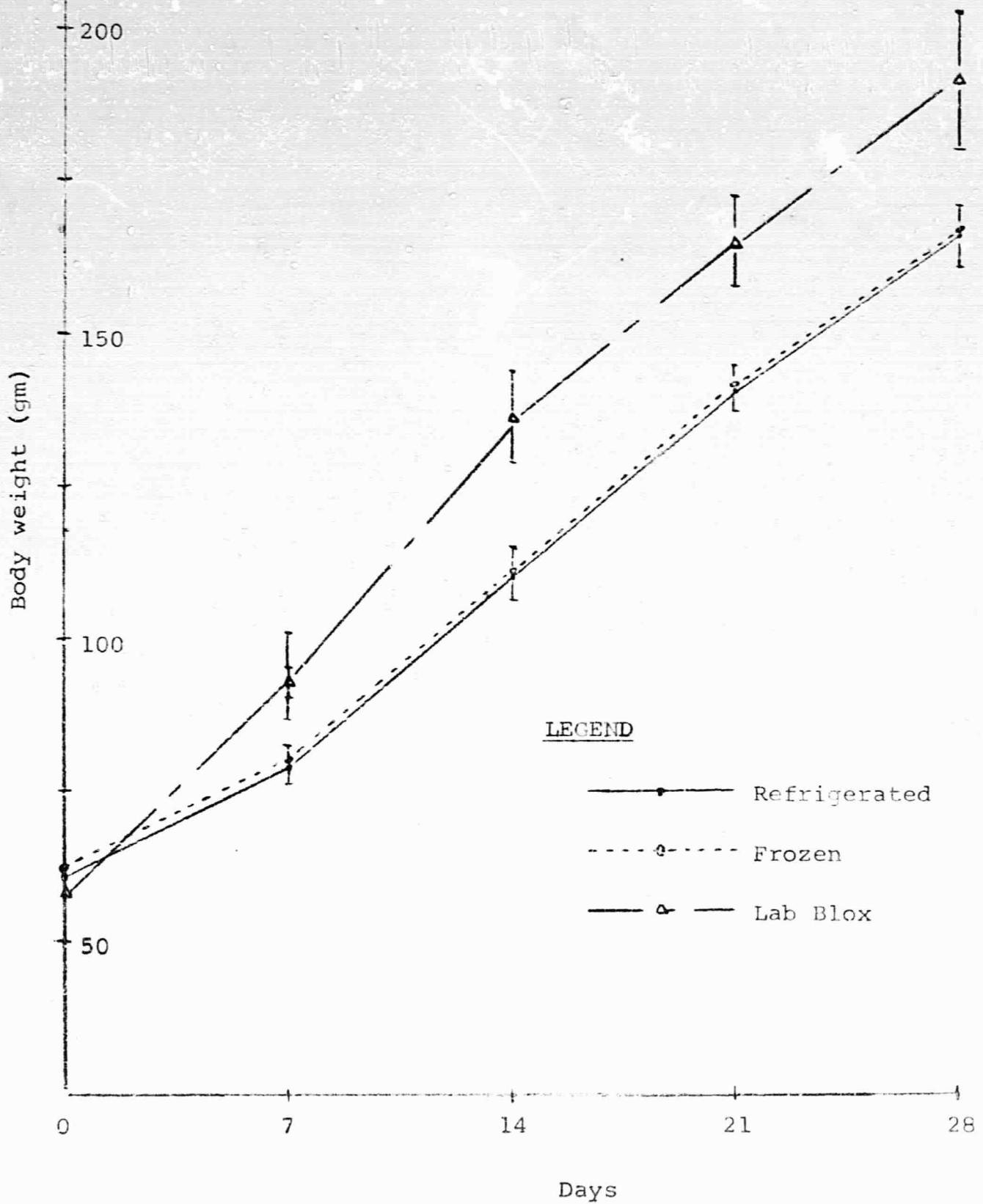
Storage condition	Av. daily body weight gain gm/rat/day	Av. daily diet consumption ml/rat/day	Av. daily water consumption ml/rat/day
Control**	3.9	26.6	12.6
0-4°C	3.8	25.5	9.4
(-)6°C	3.8	25.7	8.5

*Experimental period 28 days. Twelve CFE male weanling rats of similar body weight were allotted to each dietary treatment. Growth curves are shown in Figure 23. Detailed diet consumption, water consumption and growth data are presented in Table 3 in the Appendix. All diets were stored as sterile solutions.

**Control; freshly prepared diet-14. Data obtained at zero time (see Table 19).

FIGURE 22

GROWTH OF RATS FED CODELID DIET-14
REFRIGERATED OR FROZEN FOR EIGHT MONTHS



SCHWARZ BIO RESEARCH, INC

The growth rates of rats fed the frozen or refrigerated diet were equivalent to the rates obtained when freshly prepared diet was fed at the beginning of this study (zero time). Diet consumption was equivalent in all groups but water consumption was much less for animals receiving the two stored diets.

Tables 26 and 27 and Figure 23 show the results of chemical and microbiological assays of the stored diets compared with the freshly prepared diet and with NRC recommended requirements. Partial destruction of threonine, monosodium glutamate, and methionine occurred under both conditions of storage. Partial destruction of histidine also occurred when the diet was refrigerated. These changes resulted in slight deficiencies of methionine and monosodium glutamate. Glycine was also deficient but not as the result of destruction during storage.

Vitamin assays showed losses of all the vitamins measured except ribcflavin. However, only in the case of Vitamin E did a dietary deficiency occur. Glucose, nitrogen and phosphorus analyses showed very small losses after storage and specific gravity, osmotic pressure and pH remained virtually unchanged. Of the nutrient deficiencies determined, only a deficiency of methionine would be expected to inhibit growth. Glycine and glutamic acid are dietetically dispensable when the other dietary amino acids are adequate. Vitamin E is primarily required for reproduction. Apparently, in this study the loss of methionine was not critical and did not adversely affect growth.

SCHWARZ BIORESEARCH, INC.

TABLE 26

AMINO ACID COMPOSITION* OF A CHEMICALLY DEFINED
LIQUID DIET AFTER STORAGE AT 0-4°C OR (-)6°C
FOR EIGHT MONTHS

Amino acid	Req.**	Control	0-4°C	(-)6°C
	gm/kg			
Histidine·HCl·H ₂ O	3.00	5.61	4.50	5.29
Isoleucine	5.00	12.49	12.00	12.04
Leucine	8.00	16.89	15.79	15.65
Lysine·HCl	9.00	16.33	15.17	15.22
Methionine	6.00	5.91	5.03	5.13
Phenylalanine	6.00	10.82	10.81	10.63
Threonine	5.00	9.67	8.27	8.18
Tryptophan	1.50	----Not determined----		
Valine	7.00	14.12	13.02	13.33
Arginine·HCl	5.00	17.72	17.84	17.70
Aspartate	2.30	15.35	19.18	15.03
Glycine	13.94	12.54	12.25	12.21
Monosodium glutamate	27.95	26.44	20.99	20.74
Proline	2.30	8.25	8.15	8.16
Serine	2.30	15.00	14.65	14.10
Alanine	2.30			
Cystine	2.30	----Not determined----		
Tyrosine	2.30	----Not determined----		
Asparagine	3.95			

*Determined chemically by column chromatography.

**Essential amino acid requirements listed in NRC publication 990. Values for non-essential amino acids obtained from G.S. Ramlotra and B. Connor Johnson, Proc. Soc. Expt'l. Biol. Med. 118:1197(1965).

TABLE 2

ASSAY OF A CHEMICALLY DEFINED DIET
EXPOSURE TO SEVERAL ENVIRONMENTAL AGENTS

Assay	Req.*	Control**
Specific Gravity	---	1.1900
Osmotic Pressure mos/L (1:4 dil.)	---	835
pH	---	4.76
Glucose gm/kg	---	690.0
Nitrogen gm/kg	---	25.53
Phosphorus gm/kg	5.0	7.11
Vitamin A IU/gm	2.0	17.5
Vitamin E µg/gm	60	44.4
Thiamine µg/gm	1.25	7.2
Riboflavin µg/gm	2.5	11.8
Pyridoxine µg/gm	1.2	21.1
Niacin µg/gm	15.0	52.1
Folic Acid µg/gm	---	0.72

*Requirements listed in NRC publication 990.

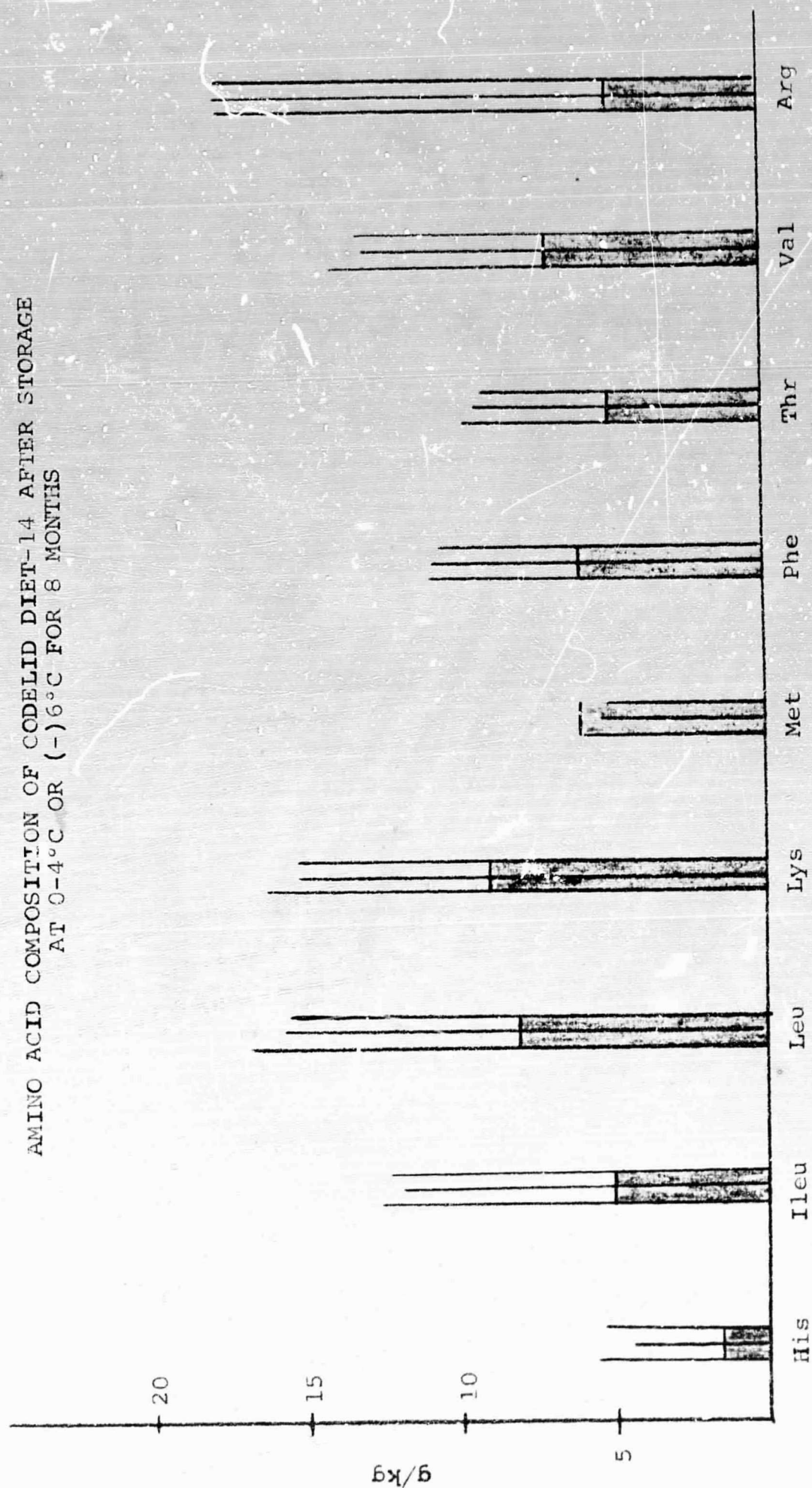
**Freshly prepared diet (zero time).

7

IED LIQUID DIET AFTER
CONDITIONS FOR EIGHT MONTHS

0-4°C	-6°C	<u>Room Temperature 24-26°C</u> Dark
1.1778	1.1739	1.1748
861	843	775
4.81	4.88	4.65
676.7	668.1	650.9
25.22	24.59	25.12
6.84	6.72	6.75
15.1	15.6	8.1
21.0	22.1	23.0
5.2	5.7	4.5
13.9	13.0	7.9
9.1	8.7	9.2
---	---	50.9
0.48	0.47	0.15

FIGURE 23

AMINO ACID COMPOSITION OF CODELID DIET-14 AFTER STORAGE
AT 0-4°C OR (-)6°C FOR 8 MONTHS**LEGEND:**

Bar sequence (left to right): Control; 0-4°C; (-)6°C. Shaded area represents NRC requirements.

SCHWARZ BIO RESEARCH, INC.

The results, therefore, indicate that Codelid diet-14 when refrigerated or frozen, retains its nutritive value for a period of eight months. Studies are still in progress to ascertain whether this diet remains stable for 12 months.

F. The Effect of Glutathione on Diet Stability

The results of the previous studies showed that conditions of storage markedly influence the stability of chemically defined liquid diets. The results also showed that severity of environmental stress, browning, and nutrient loss were all directly related. Based on these findings, it was suggested that browning was due to the reaction of certain dietary amino acids with the dietary reducing sugar glucose. The following study was conducted to determine whether a strong reducing substance such as glutathione can inhibit the glucose-amino acid interaction, prevent browning and thereby, preserve the nutritive value of diets exposed to a variety of environmental conditions.

The diet tested (Codelid diet-14) was the same as previously used at a concentration of 50% w/v (Tables 17 and 18). When called for, glutathione was added at a level of 0.3%. All diets were stored at the designated temperature for seven days prior to feeding. The experimental design and results are shown in Table 28 and Figures 24 through 27. Growth response and diet consumption were remarkably similar for rats consuming diets kept at 0-4°C, 20-24°C and 37°C. No significant ($P > 0.05$) increase in growth was attributable to glutathione. In all diets containing glutathione, browning did not appear as intense as in glutathione-free diets.

TABLE 28

GROWTH RATE, DIET CONSUMPTION AND WATER CONSUMPTION
OF RATS FED A CHEMICALLY DEFINED LIQUID DIET
EXPOSED TO SEVERAL ENVIRONMENTAL CONDITIONS
WITH AND WITHOUT GLUTATHIONE*

Storage condition**	Av. daily body weight gain gm/rat/day	Av. daily diet consumption ml/rat/day	Av. daily water consumption ml/rat/day
0-4°C	4.0	26.7	10.5
24-26°C	4.0	27.3	13.7
37°C	4.0	25.4	12.4
60°C***	-	-	-
0-4°C + GSH****	4.2	27.6	10.9
24-26°C + GSH	4.1	26.3	10.2
37°C + GSH	4.0	25.0	9.4
60°C + GSH***	-	-	-

*Experimental period 28 days. Twelve CFE male weanling rats allotted to each dietary treatment. Housed two per cage. Diet-14 fed to all groups. For details see Table 4 in the Appendix.

**The diet was stored at the designated temperature for seven days prior to feeding.

***Experiment terminated during third week due to death and severe loss of body weight.

****GSH - glutathione.

FIGURE 24

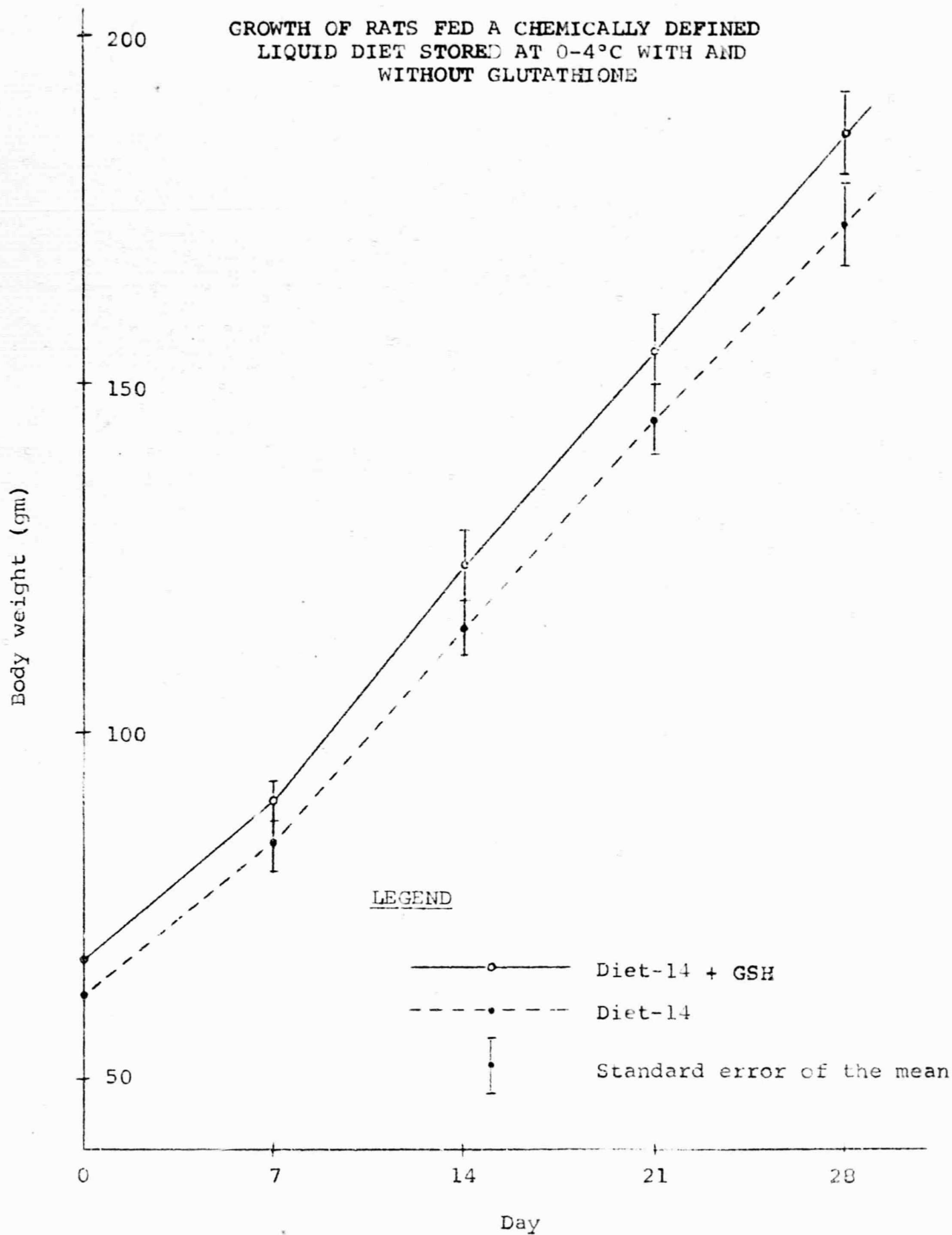


FIGURE 25

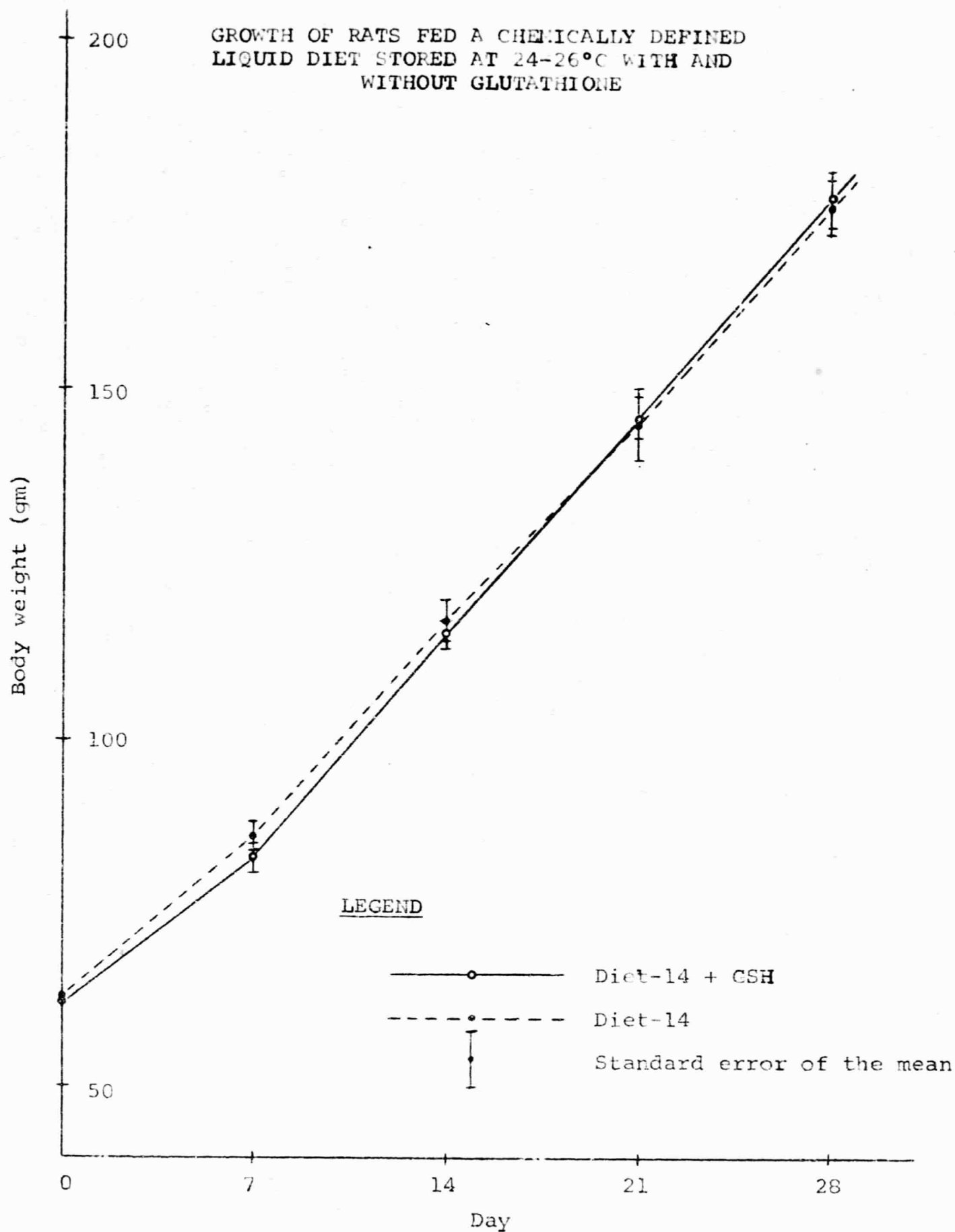


FIGURE 26

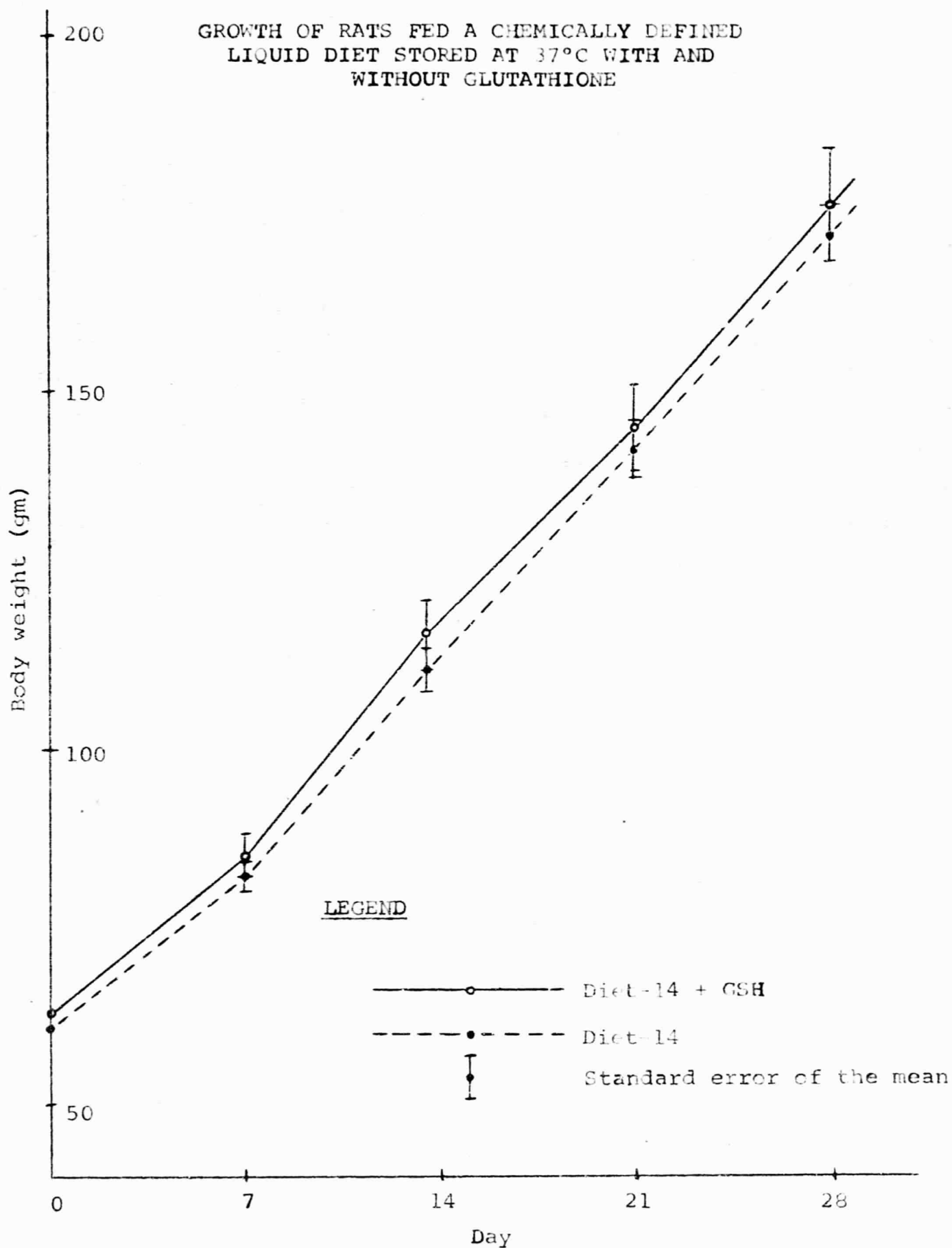
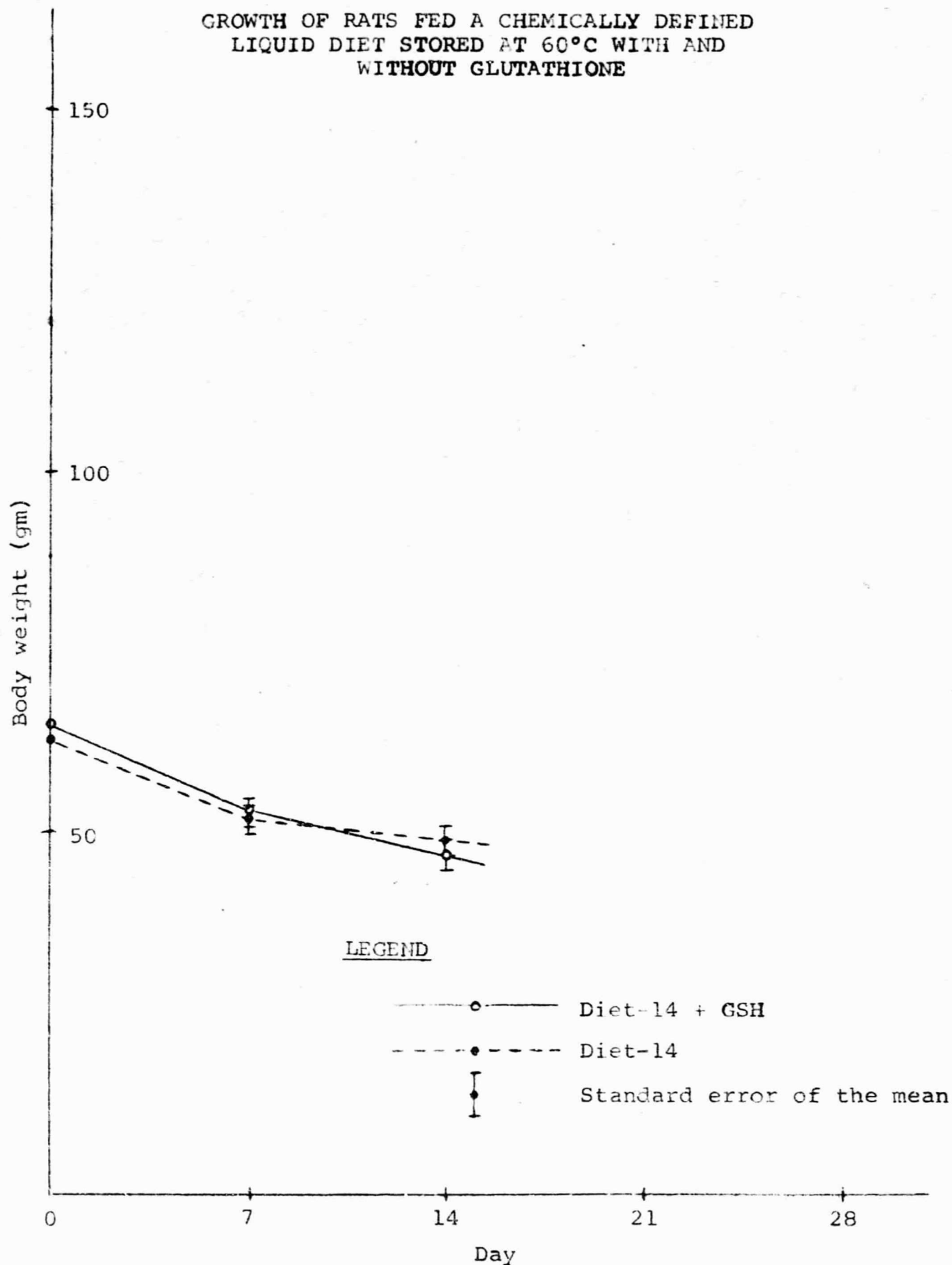


FIGURE 27

GROWTH OF RATS FED A CHEMICALLY DEFINED
LIQUID DIET STORED AT 60°C WITH AND
WITHOUT GLUTATHIONE



SCHWARZ BIORESEARCH, INC.

However, the degree of browning was not quantitatively determined due to the cloudiness of diets stored at room temperature or 37°C. Animals fed these diets either lost weight or died within 3 weeks.

The poor growth and eventual death of rats fed the diet after exposure to 60°C for 7 days regardless of the inclusion of 0.5% glutathione demonstrates the inadequacy of this compound in preserving the nutritive value of the diet under these drastic conditions.

SCHWARZ BIO RESEARCH, INC.

II. STUDIES ON THE AMINO ACID MOIETY OF CODELID DIETS*

A. Hydrolysate studies

This section describes a series of experiments in which a water soluble, well defined, acid hydrolyzed protein was tested as a replacement for the more expensive crystalline amino acids normally present in chemically defined diets. In these experiments, the relative quantities of the "protein-free" fraction of the diet with the exception of glucose was the same as shown for Codelid diet-14 in Table 17. After the inclusion of the appropriate quantities of hydrolysate and supplemental synthetic amino acids, each diet was brought to a final nutrient concentration of 50% (w/v) by adding glucose. The caloric concentration of each diet was calculated to be approximately 1.8 cal/ml.

In the first experiment (Table 29 and Figure 28), the hydrolysate was tested at dietary protein (Nx6.25) concentrations of 8.5, 10.6 and 12.8 percent. Tryptophan and arginine were added at each protein level and at all times represented 0.86 and 3.15% of the total dietary nitrogen.

The results show that 8.5% "protein" (Nx6.25) provided by an acid hydrolyzed protein, supplemented with arginine and tryptophan, can support the same rate of growth as an isonitrogenous diet containing a synthetic mixture of amino acids. The results also show that no benefit is derived from increasing the hydrolysate level to provide more than 98.5% protein. This is

*Codelid diets - Chemically defined liquid diets

SCHWARZ BIO RESEARCH, INC.

TABLE 29

THE GROWTH OF RATS FED GRADED LEVELS OF A PROTEIN
HYDROLYSATE SUPPLEMENTED WITH ARGININE AND TRYPTOPHAN*

Protein Source	Conc.** %	Av. daily body weight gain gm/rat/day	Av. daily diet consumption ml/rat/day	Av. daily water consumption ml/rat/day
Acid	8.5	3.4	24.6	14.9
Hydrolyzed	10.6	3.3	23.6	17.1
Protein	12.8	2.9	23.6	18.8
Amino acid mixture #14***	8.5	3.5	25.8	12.7

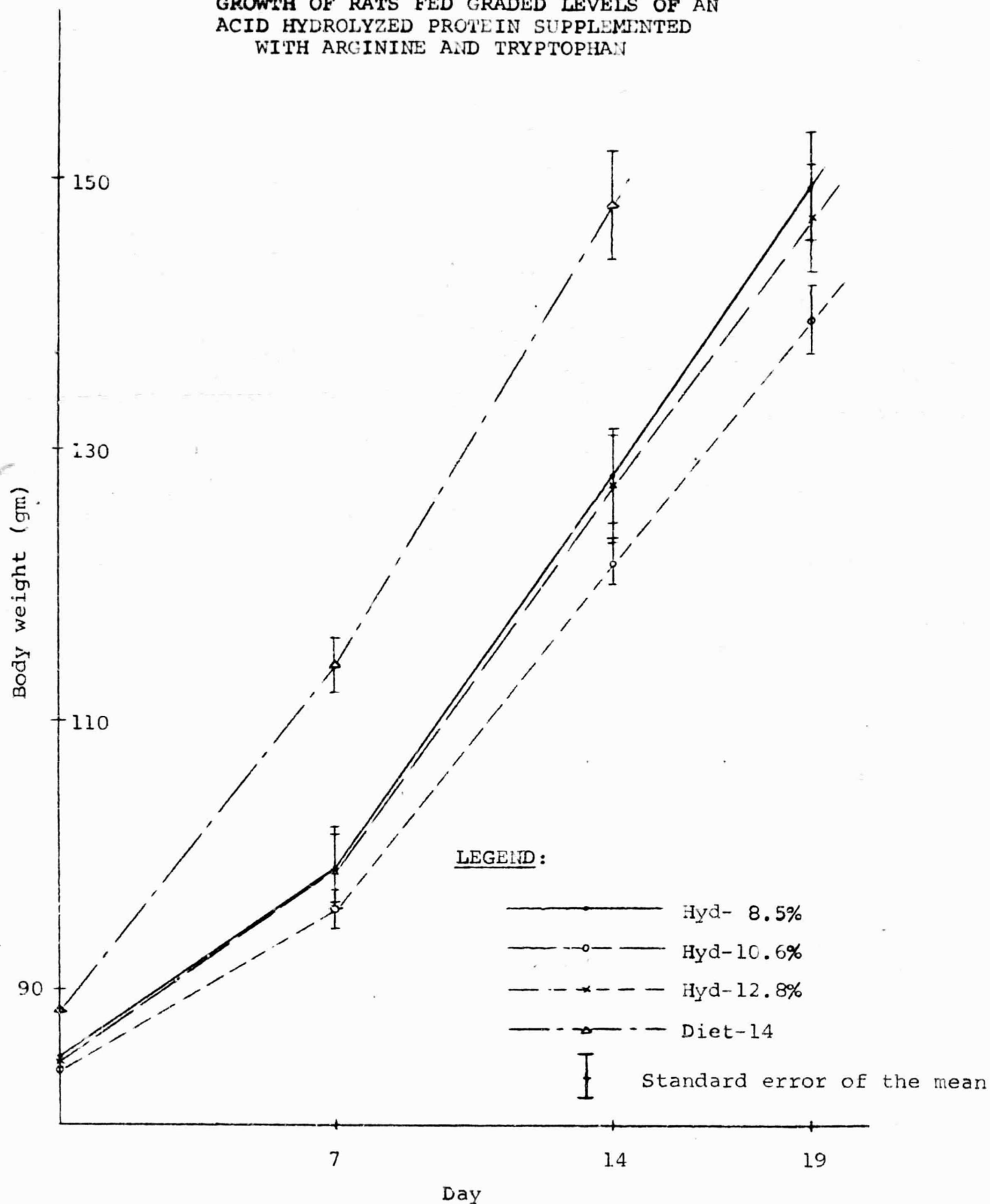
*Experimental period 19 days. Eight CFE male weanling rats allotted to each dietary treatment. For details, see Table 5 of the Appendix.

**N x 6.25.

***For composition, see Tables 17 and 18.

FIGURE 28

GROWTH OF RATS FED GRADED LEVELS OF AN
ACID HYDROLYZED PROTEIN SUPPLEMENTED
WITH ARGININE AND TRYPTOPHAN



SCHWARZ BIORESEARCH, INC.

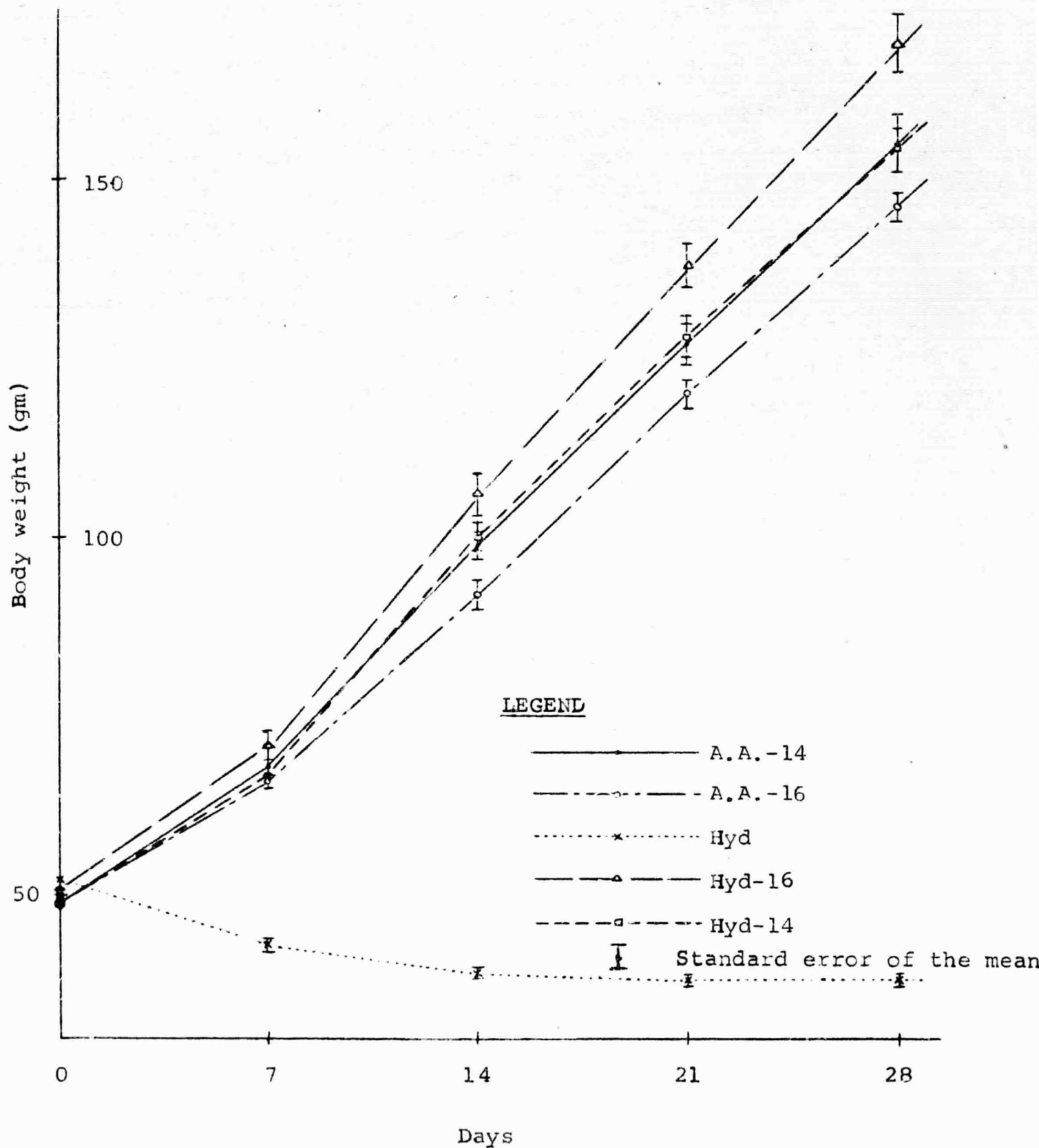
evidenced by the equivalent growth rate and diet consumption at the 8.5% and 10.6% protein levels and the slight growth retardation at the 12.8% level. Similar findings with synthetic amino acids have previously been reported by this laboratory (Annual Report, 1964). It should be noted, however, that the growth rates in both studies were not optimum for rats consuming a liquid diet.

In a second experiment, the growth of rats fed liquid diets containing either synthetic amino acid mixture-14 or -16 (Tables 17 and 18) was compared with the growth of rats receiving diets containing the protein hydrolysate supplemented to simulate each of these amino acid mixtures. While the amino acid composition of the supplemented hydrolysate was virtually equivalent to the synthetic mixtures, the diets were not isonitrogenous. This was due to the presence of nitrogenous constituents in the hydrolysate not normally found in the synthetic mixtures. The nitrogen concentrations of all diets is designated in Table

Figure 29 and Table 30 show the results of this experiment. Body weight loss and eventual death of half the rats occurred when the protein hydrolysate was fed without any amino acid supplementation. When the hydrolysate was supplemented to simulate mixture-14, growth was equivalent to those rats fed the synthetic amino acid mixture. When the hydrolysate was supplemented to simulate mixture-16, growth was faster than that observed in all other groups including those animals consuming synthetic amino acid mixture-16. However, in rechecking the diet formulations used in this study, it

FIGURE 29

GROWTH OF RATS FED CHEMICALLY DEFINED LIQUID DIETS
CONTAINING SYNTHETIC AMINO ACIDS OR AN
ACID HYDROLYZED PROTEIN SUPPLEMENTED WITH AMINO ACIDS



SCHWARZ BIO RESEARCH, INC.

TABLE 30

COMPARISON OF CHEMICALLY DEFINED LIQUID DIETS
CONTAINING SYNTHETIC AMINO ACID MIXTURES VS. A
PROTEIN HYDROLYSATE SUPPLEMENTED TO
SIMULATE THESE MIXTURES*

Dietary treatment**	Av. daily body weight gain gm/rat/day	Av. daily diet consumption ml/rat/day	Av. daily water consumption ml/rat/day
A.A.-14	3.8	24.1	10.6
A.A.-16	3.5	23.3	7.9
Hyd	(-)	7.6	3.9
Hyd-16	4.2	25.0	10.7
Hyd-14	3.6	24.6	11.9

*Experimental period 28 days. Ten CFE male weanling rats allotted to each dietary treatment. For detailed data, see Table 6 of the Appendix.

**For composition of amino acid mixtures 14 and 16, see Tables 17 and 18. Hyd-hydrolysate; Hyd-16-hydrolysate supplemented to simulate AA-16; Hyd-14-hydrolysate supplemented to simulate AA-14. Nitrogen concentration of diets: AA-14 1.28% N; AA-16 1.26% N; Hyd-14 1.4% N; Hyd-16 1.46% N.

SCHWARZ BIO RESEARCH, INC.

was found that synthetic amino acid mixture-16 contained one tenth the normal level of monosodium glutamate. Although this is a dietary dispensable amino acid, the reduced level markedly decreased the total nitrogen concentration from the theoretical level of 1.26% nitrogen to 1.06%. These alterations in composition, therefore, prevent a direct comparison of hydrolysate-16 with synthetic amino acid mixture-16. Nevertheless, results from other studies have shown the average growth rate of rats fed diet-16 to range from 3.8 gm/rat/day to 4.5 gm/rat/day. Since the rats used were of the same strain, age and sex as used in the present study, it would appear that hydrolysate-16 is at least equivalent to the synthetic amino acid mixture. Furthermore, based on the present findings, the adequacy of the hydrolysate is also shown by the equivalent growth of rats fed hydrolysate-14 and those fed synthetic mixture-14.

Using casein-amino acid diets, Rao and Johnson¹² determined the minimum essential amino acid requirements of the weanling rat (Table 31). These experimentally determined values approximate very closely, the National Research Council recommended allowances (Table 31). Comparison of the amino acid composition of our protein hydrolysate with the minimum requirements set forth by Rao and Johnson shows it to be deficient in methionine, tryptophan, phenylalanine, lysine, threonine and isoleucine (Table 31). A third experiment was, therefore, conducted to determine whether factorial supplementation of the hydrolysate with these amino acids would result in better growth of weanling rats.

SCHWARZ BIORESEARCH, INC.

TABLE 31

COMPARISON OF THE ESSENTIAL AMINO ACID COMPOSITION
OF AN ACID HYDROLYZED PROTEIN WITH RECOMMENDED REQUIREMENTS*

<u>Amino acid</u>	<u>NRC**</u> %	<u>Rao***</u> %	<u>Hyd****</u> %
Tryptophan	0.15	0.11	---
Histidine	0.30	0.25	0.25
Lysine	0.90	0.90	0.53
Leucine	0.80	0.70	0.85
Isoleucine	0.50	0.55	0.41
Phenylalanine	0.90	0.72	0.35
Methionine	0.60	0.50	0.25
Threonine	0.50	0.50	0.34
Valine	0.70	0.55	0.56

*Requirements expressed as percent of diet. For purposes of comparison the hydrolysate was calculated to be isonitrogenous with the recommended requirements of Rao and Johnson.

**Nutrient requirements of Laboratory Animals. National Research Council Publication 990.

***Rao and Johnson 1959. J. Nutr. 69:387.

****Amino acids determined by column chromatography.

SCHWARZ BIORESEARCH, INC.

As before, the amino acid-free portion of the diet was the same as used in all other experiments with the exception of glucose which was appropriately varied to keep all diets at a concentration of 50% (w/v). In contrast to the previous experiments, all diets with the exception of diet-16 were kept isonitrogenous at 1.6% nitrogen, equivalent to 10% protein (Nx6.25). The results and experimental design are shown in Table 32 and Figure 30. As in experiment #2 when the protein hydrolysate was patterned after diet-16, growth was very good. The slightly better growth observed in the present experiment (4.5 gm/rat/day), compared with the growth observed previously with diet-16 (Table 30) (4.2 gm/rat/day) may be due to the heavier starting weights of the former group of rats. Supplementation of the protein hydrolysate with methionine and tryptophan permitted only slow growth (2.9 gm/rat/day), whereas a marked increase was observed when phenylalanine was supplemented in addition to these two amino acids. The best growth (4.8 gm/rat/day) was obtained when the protein hydrolysate was supplemented with methionine, tryptophan, phenylalanine and lysine. Further supplementation with threonine and isoleucine did not enhance growth. In fact, the inclusion of isoleucine in the diet seemed to depress growth slightly suggesting the creation of an imbalance from the addition of this amino acid. These results again show that a properly supplemented protein hydrolysate can serve as an excellent source of amino acids in chemically defined water soluble diets. Furthermore, while the most limiting amino acids in our protein hydrolysate

TABLE 32

GROWTH, DIET CONSUMPTION AND WATER CONSUMPTION OF RATS FED A
CHEMICALLY DEFINED LIQUID DIET CONTAINING AN
ACID HYDROLYZED PROTEIN SUPPLEMENTED WITH AMINO ACIDS*

Group	Amino acid supplement	Av. daily body weight gain gm/rat/day	Av. daily diet consumption ml/rat/day	Av. daily water consumption ml/rat/day
I	Hyd***-16	4.5**	26.6**	10.1**
II	Hyd + 0.23% Met + 0.11% Try	2.9	24.1	12.3
III	As Group II + 0.35% Phe	4.3	25.9	12.7
IV	As Group III + 0.35% Lys	4.8	26.0	13.6
V	As Group IV + 0.14% Thr	4.6	25.9	15.3
VI	As Group V + 0.12% Ileu	4.4	25.6	13.5

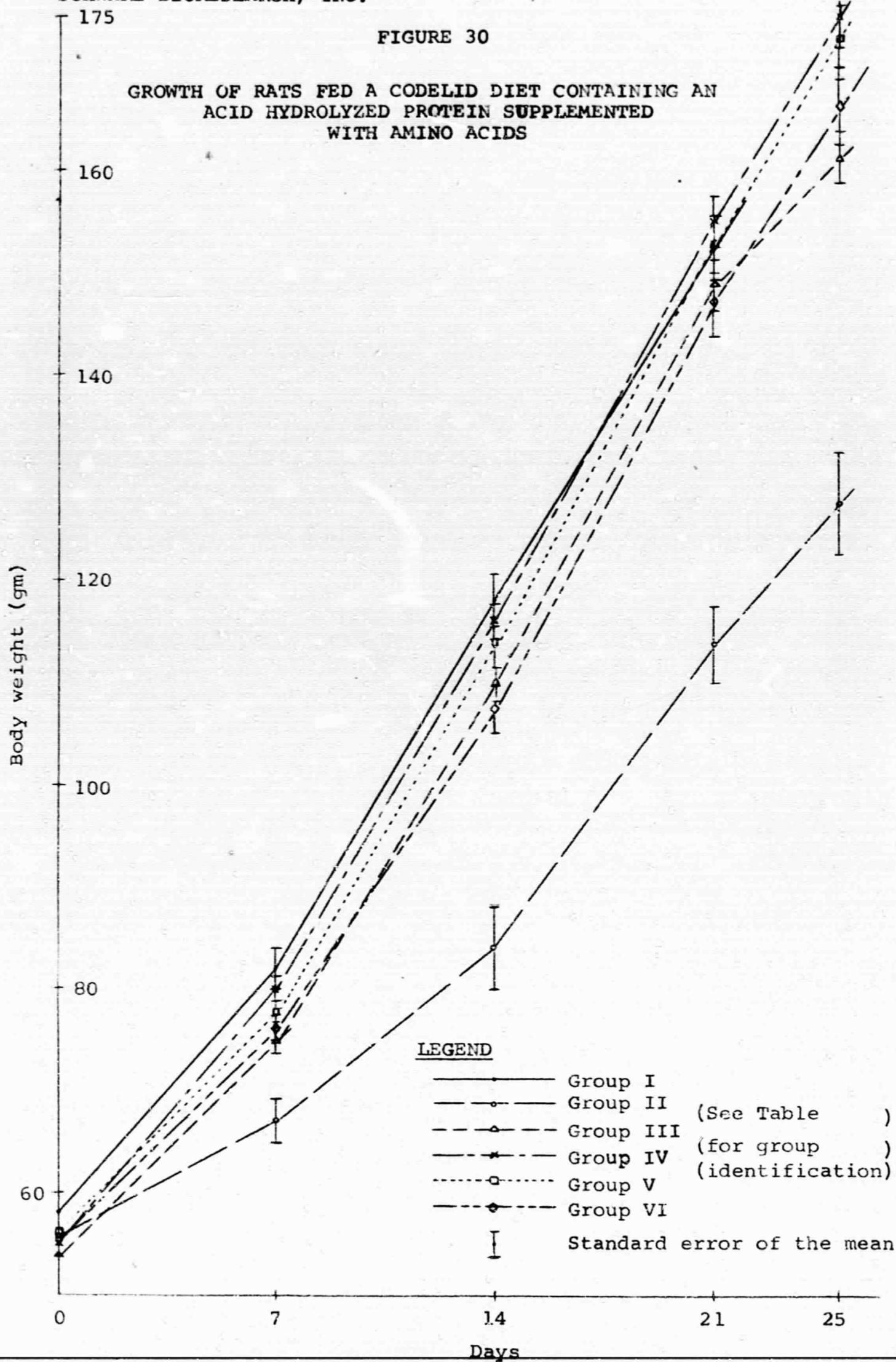
*Experimental period 25 days. Twelve CFE male weanling rats allotted to each treatment. Housed two per cage. For detailed data, see Table of the Appendix.

**Average values for 21 days.

***Hyd - acid hydrolyzed protein; Hyd-16 -- hydrolysate supplemented to simulate A.A. mixture 16.

FIGURE 30

GROWTH OF RATS FED A CODELID DIET CONTAINING AN
ACID HYDROLYZED PROTEIN SUPPLEMENTED
WITH AMINO ACIDS



SCHWARZ BIORESEARCH, INC.

were not biologically determined in these experiments, it is clear that such a hydrolysate need only be supplemented with very few essential amino acids to permit attainment of excellent growth.

B. Studies with agar and cellulose

Inadequate food intake has always been an important factor in obtaining normal growth of rats fed an amino acid diet. Recently, Rogers et al¹⁴ suggested that the reduction of food intake by rats fed such diets may be related to the osmotic effect of the amino acids. Using amino acid diets fed in an agar gel, on the assumption that this might reduce osmotic effects in the gastrointestinal tract, these workers observed weight gains in rats equivalent to or greater than those obtained with diets containing an equivalent quantity of casein supplemented with methionine. Their results also showed that the increased weight gain was due primarily to increased food intake. The experiment described below represents a preliminary study to determine whether the addition of non-digestible agar or ground cellulose to Codelid diets has any effect on diet consumption, diet utilization and growth rate of rats.

The experimental design and results are shown in Table 33. All diets were initially formulated to contain 50% solid nutrients patterned after diet-14 (Tables 17 and 18). Inasmuch as agar or cellulose were added to an already complete liquid diet all nutrients in the resulting diets were diluted by the quantity of

SCHWARZ BIORESEARCH, INC.

TABLE 33

THE EFFECT OF AGAR OR CELLULOSE SUPPLEMENTATION
OF LIQUID DIETS ON THE GROWTH RATE OF RATS*

Dietary treatment	Av. daily body weight gain gm/rat/day	Av. daily diet consumption gm/rat/day	Av. daily water consumption ml/rat/day
10% Cellulose**	3.2	31.1	11.6
20% Cellulose	2.1	34.4	8.8
20% Cellulose + 5% Corn oil	2.4	39.4	8.3
2.3% Agar	3.8	35.8	11.7
2.3% Agar + 5% Corn oil	4.1	36.1	13.4
Liquid diet--AA#14	3.8	23.8 ml	11.2

*Eight CFE male weanling rats allotted to each dietary treatment. Experimental period 28 days.

**Supplied as Solka Floc -- a product of Brown Company, Berlin, New Hampshire.

SCHWARZ BIORESEARCH, INC.

cellulose or agar + water* added.

The data in Table 33 show that a diet containing 2.3% agar and 5% corn oil promoted better growth than that attained with liquid diet. Supplementation of the liquid diet with 10% or 20% cellulose resulted in slight growth retardation. Inclusion of corn oil in either the cellulose or agar containing diets increased diet consumption and in the case of the agar diet, appeared responsible for the slight growth enhancement. Whether the effects of corn oil supplementation were due to improved palatability and increased caloric consumption or to the presence of additional essential and non-essential fatty acids remains to be determined.

Table 34 shows the estimated caloric concentration and caloric intake of the diets tested. These values were calculated using factors of 4, 9 and 4 calories per gram of carbohydrate, fat and amino acid respectively. While there appears to be no clear-cut relationship between caloric density of the diet and diet intake, the data show a parallelism between caloric intake and diet intake. However, as shown in Table 33 this parallelism was not completely reflected in growth rate. Although increased consumption of the agar diets resulted in increased growth, this did not hold true for rats consuming cellulose-containing diets.

*Agar was dissolved in a known quantity of hot distilled water and quickly added with vigorous stirring to the liquid diet. The diet containing the agar was allowed to cool with stirring until gel formation occurred.

SCHWARZ BIORESEARCH, INC.

TABLE 34

ESTIMATED ENERGY CONCENTRATION* AND ENERGY CONSUMPTION
OF CHEMICALLY DEFINED DIETS FED IN DIFFERENT PHYSICAL FORMS**

Dietary treatment	Calculated caloric concentration	Av. daily caloric consumption	Av. daily diet consumption
	Cal/gm	Cal/rat/day	gm/rat/day
10% Cellulose	1.58	49.1	31.1
20% Cellulose	1.45	49.9	34.4
20% Cellulose + 5% Corn oil	1.75	68.9	39.4
2.3% Agar	1.5	53.7	35.8
2.3% Agar + 5% Corn oil	1.84	66.4	36.1
Liquid diet--AA#14	1.74***	41.4	23.8ml

*Calculated on the basis of 4 Cal/gm carbohydrate; 9 Cal/gm fat;
4 Cal/gm amino acid.

**For growth data, see Table 33.

***Calories per ml.

SCHWARZ BIORESEARCH, INC.

Calculation of diet utilization (gm gain/cal consumed) shows that the cellulose diets were utilized least efficiently. The diet in liquid form was utilized most efficiently and again suggests that increased consumption would probably further enhance growth rate. Inclusion of agar into the diet increased diet and caloric intake without increasing growth. When corn oil was added to agar containing diets, growth increased although diet utilization was less than for rats consuming the liquid diet. Evidently, while agar seems to enhance diet consumption, it may also restrict absorption of certain nutrients. Consequently, liquid diets of higher nutrient concentration may be necessary if agar is to be included in the diet. This view is supported by the enhanced growth attributable to supplemental corn oil.

It may be concluded from this preliminary study, that at the concentrations studied, bulk, in the form of cellulose or agar does not enhance liquid diet utilization. On the other hand, while the mechanism is still unknown, providing liquid diets as a gel (agar) enhances diet consumption. It is possible, therefore, that with further modification, the use of agar may solve the problem of inadequate consumption of amino acid diets without loss of efficiency. Furthermore, the use of agar may also afford an excellent solution to the problem of homogeneous incorporation of high levels of fat into chemically defined liquid diets.

SCHWARZ BIO RESEARCH, INC.

C. New amino acid patterns

Experiments in our laboratory have shown that Codelid diets containing synthetic L-amino acids can support a moderate rate of growth in rats, but not a rate equivalent to that obtained with diets containing intact protein. Indeed, the literature indicates that maximal growth rate has never been obtained routinely. Recently, Rogers et al¹⁴ and Ranhotra and Johnson¹⁵ independently reported greater growth in rats fed L-amino acid diets than in those fed properly fortified casein diets. Rogers et al used an amino acid mixture equivalent to 15% protein and found that the inclusion of asparagine and agar were necessary to achieve maximal growth. Ranhotra and Johnson found that diets equivalent to 10% protein and containing asparagine and a high level of arginine were required for maximal growth. However, they were unable to demonstrate any need for agar.

The study described below represents a comparison of the Ranhotra and Johnson and Rogers et al amino acid mixtures in liquid and gel form and provided by L-amino acids or an appropriately supplemented acid hydrolyzed protein.

The diets tested are shown in Tables 17 and 18. The experimental design and results are shown in Table 35 and Figures 31 and 32. For purposes of identification, the Ranhotra and Johnson formulation is designated diet-17 and the Rogers et al formulation diet-18.

The data show good growth in all groups. The best growth rate was obtained with diet-17 in gel form with diet-18 as a liquid also producing a growth rate better

TABLE 35

GROWTH, DIET CONSUMPTION AND WATER CONSUMPTION OF RATS
FED LIQUID OR GEL FORMS OF CHEMICALLY DEFINED DIETS
CONTAINING DIFFERENT AMINO ACID MIXTURES*

Dietary treatment	Av. daily body weight gain gm/rat./day	Av. daily diet consumption ml/rat/day	Av. daily water consumption ml/rat/day
Diet-18	5.1	33.7	20.8
Diet-18-Hyd**	3.7	27.6	17.3
Diet-18-Gel***	4.6	31.4gm	19.6
Diet-17	4.8****	27.7	12.9
Diet-17-Hyd	4.5	27.7	13.3
Diet-17-Gel	5.6	35.0gm	14.6

*Experimental period 26 days. Twelve CFE male weanling rats allotted to each dietary treatment. For detailed data see Table 8 of the Appendix.

**Hyd - acid hydrolyzed protein

***Gel - agar.

****Average for 28 days.

FIGURE 31

GROWTH OF RATS FED CODELID DIET-17

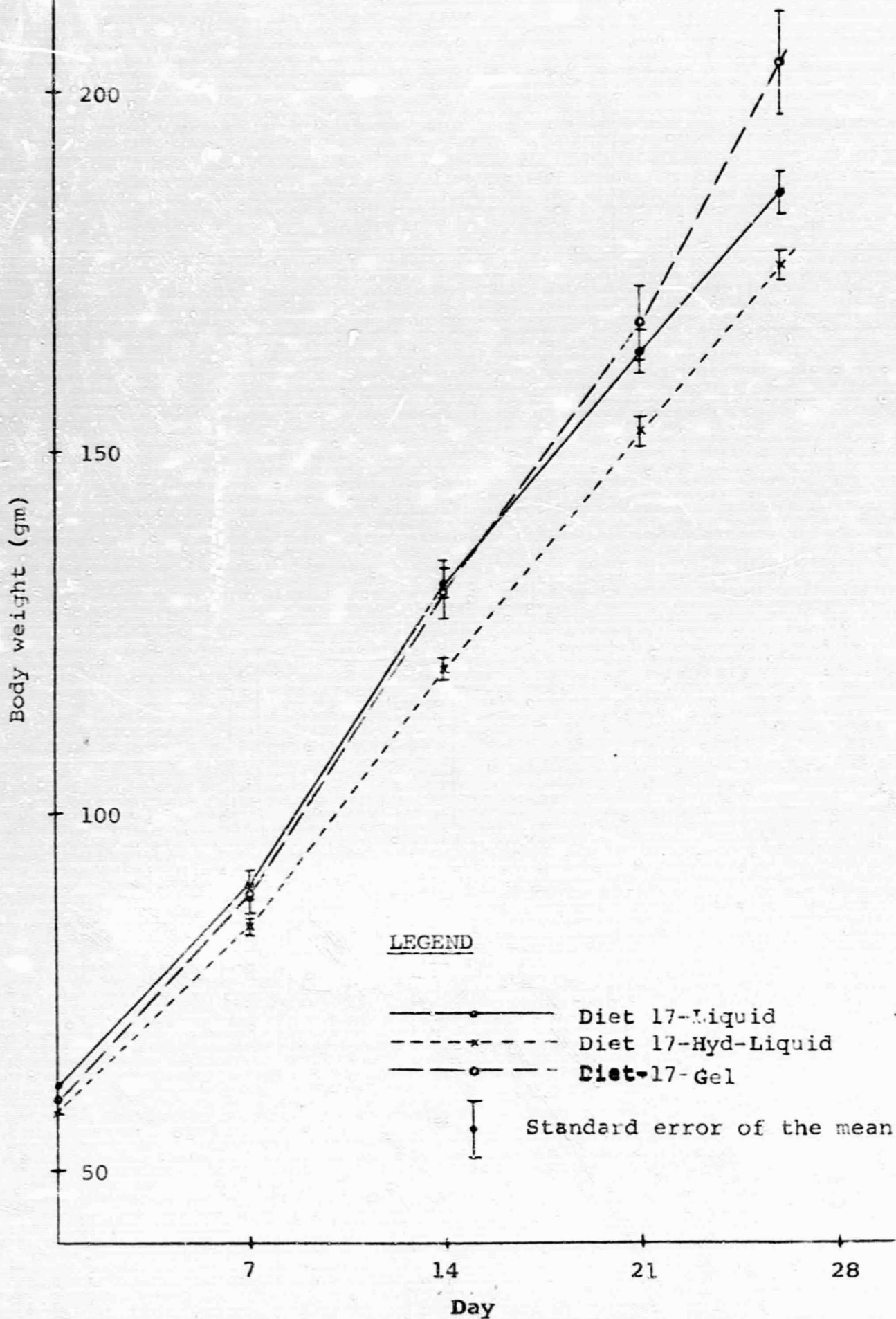
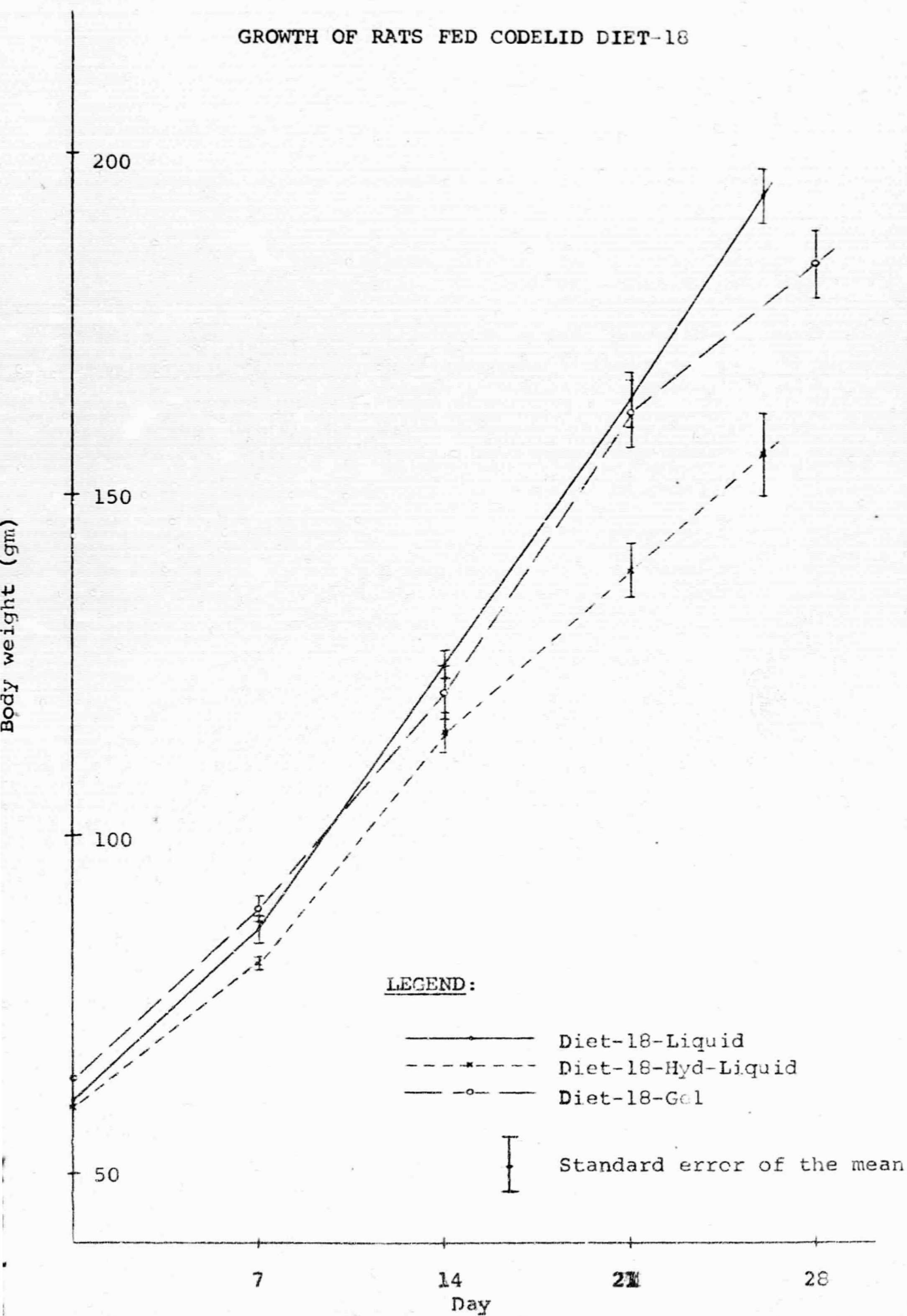


FIGURE 32



SCHWARZ BIO RESEARCH, INC.

than 5 gm/rat/day. No improvement resulted when diet-18 was incorporated into an agar gel. In fact, growth was reduced by about ten percent. Diet-18 provided as a supplemented acid hydrolysate produced the slowest growth of 3.7 gm/rat/day. Diet-17 in liquid form as the synthetic L-amino acid mixture or as the supplemented hydrolysate, were virtually equivalent in growth promoting properties. Diet consumption for all groups was greater than normally observed with CFE rats of the same age and sex, fed Codelid diets.

The inclusion of agar into diet-17 increased diet consumption which may have been responsible for the superior growth observed in this group. Incorporation of agar into diet-18 did not increase diet intake. However, it was observed that diet-17-gel was of a much softer texture than diet-18-gel. Whether this bears a relationship to diet consumption has not yet been ascertained. Water consumption by all groups was unusually high. This was particularly true for animals consuming diet-18 and is probably related to the very high amino acid concentration of this diet.

The results of this study are not consistent with the observations of Rogers et al¹⁴ or Ranhotra and Johnson¹⁵. Where Rogers et al improved growth by including agar in the diet, we observed no such response. On the other hand, we were able to enhance growth by including agar in diet-17 whereas Ranhotra and Johnson did not observe such an effect. While it is true that the diets used in this study were similar in amino acid composition, they differed greatly in their amino acid-free components. Although it seems doubtful that this

SCHWARZ BIORESEARCH, INC.

is responsible for the difference in results, such a possibility can not be ruled out.

The results of this study show that the amino acid mixtures tested can produce excellent growth in rats. Although the growth was still not equivalent to the normal rate of 6 gm/rat/day achieved by feeding a commercial lab chow (Lab Blox) such diets offer promise and slight modifications may further close the gap.

The different response to dietary incorporation of agar observed by Ranhotra and Johnson¹⁵, Rogers et al¹⁴ and this laboratory requires further investigation. Such factors as gel texture, osmotic pressure, conditions of formulation and feeding can all influence the results and should be carefully studied. Certainly the virtues of agar incorporation can not be properly evaluated until more extensive studies are performed.

D. Strain differences

It is well-recognized that there are species, age and strain differences in nutrient requirements. Since all the animal studies described in this report were conducted with CFE rats, it was decided to compare the adequacy of several Codelid diets with a different strain. Fisher animals were selected for this purpose and were housed and fed in the manner heretofore described. However, in contrast to the earlier system of selecting and distributing animals on the basis of body weight, all rats in the present study were selected from identified litters of 9-11 littermates. They were

SCHWARZ BIO RESEARCH, INC.

allocated to insure, as closely as possible, uniform distribution of littermates within each dietary treatment. The compositions of the diets studies are shown in Tables 17* and 18. The results are shown in Tables 36, 37 and 38.

The growth rates of rats fed diet-17 were significantly greater ($p < 0.01$) than all other groups through the third week of experiment. At this time, a hemorrhagic condition developed in the group fed diet-17 which resulted in the death of five animals. The syndrome was manifest by an enlarged eye "pop eye" condition in several rats, and external bleeding at the toes, nose, tail and penis. Death occurred in the heaviest animals. One rat died in the third week of experiment, two in the fourth week and two in the fifth week. All surviving rats were moribund and looked anemic. Most had signs of external hemorrhage and upon autopsy showed evidence of internal hemorrhage. Plasma prothrombin times of these animals were prolonged and concentrations of factors II, VII-X decreased. Platelet counts were normal as were the livers by gross and microscopic examination. Rats from the other groups showed no abnormalities and none died. Comparison of the growth rates of rats fed diet-16 versus diet-17 after the third week of experiment showed them to be equivalent ($p > 0.05$). Both diets promoted significantly ($p < 0.01$) better growth than the other diets despite the hemorrhagic condition in rats fed diet-17. Diet

*Diet-17 was modified so that the amino acid-free fraction of the diet was identical to diet-16.

SCHWARZ BIORESEARCH, INC.

TABLE 36

GROWTH OF MALE WEANLING FISHER RATS FED
CODELID DIETS DIFFERING IN AMINO ACID PATTERN

Dietary treatment		Body weight					
		Days					
		0	7	14	21	28	35
		gms					
Diet-14	$\bar{X}$	62.4	77.9	102.5	128.8	152.8	169.3
	SE	± 1.3	± 2.0	± 2.4	± 2.9	± 3.4	± 3.7
	BWG	--	2.2	3.5	3.8	3.4	2.3
Diet-15	$\bar{X}$	65.3	75.6	104.3	132.3	150.7	170.4
	SE	± 1.3	± 1.2	± 1.7	± 1.6	± 2.4	± 2.6
	BWG	--	1.5	4.1	4.0	2.6	2.7
Diet-16	$\bar{X}$	67.3	85.2	112.8	142.7	167.4	187.6
	SE	± 1.3	± 1.4	± 1.5	± 2.2	± 3.4	± 3.2
	BWG	--	2.6	3.9	4.3	3.5	3.0
Diet-17	$\bar{X}$	64.9	89.8	123.3	154.5**	176.2***	190.0****
	SE	± 2.0	± 2.6	± 3.9	± 3.1	± 4.7	± 5.9
	BWG	--	3.6	4.8	4.5	3.1	2.0
Diet-LB*****	$\bar{X}$	61.3	93.1	130.2	170.0	198.2	220.5
	SE	± 2.0	± 3.9	± 4.9	± 5.5	± 5.6	± 5.7
	BWG	--	4.5	5.3	5.7	4.0	3.2

*Experimental period 35 days. Twelve male weanling Fisher rats, 28 days of age, allotted to each dietary treatment. Housed two per raised wire bottom cage. All animals selected from identified litters of 9-11 littermates.

**Calculated on 11 animals.

***Calculated on 9 animals.

****Calculated on 7 animals.

*****Lab Blox - commercial laboratory chow manufactured by Wayne division of Allied Mills.

SCHWARZ BIORESEARCH, INC.

TABLE 37

DIET CONSUMPTION OF MALE WEANLING FISHER RATS FED
CODELID DIETS DIFFERING IN AMINO ACID PATTERN*

<u>Dietary treatment</u>	<u>Average daily diet consumption</u>				
	<u>Days</u>				
	<u>7</u>	<u>14</u>	<u>21</u>	<u>28</u>	<u>35</u>
	ml/rat/day				
Diet-14	18.0	23.9	27.8	31.1	31.3
Diet-15	16.3	18.8	24.9	29.6	29.5
Diet-16	19.8	26.3	32.3	32.3	34.9
Diet-17	18.8	24.8	26.9	29.8	28.8
Diet-LB	12.8gm	17.3gm	18.9gm	20.0gm	20.2gm

SCHWARZ BIORESEARCH, INC.

TABLE 38

WATER CONSUMPTION OF MALE WEANLING FISHER RATS FED
CODELID DIETS DIFFERING IN AMINO ACID PATTERN*

<u>Dietary treatment</u>	<u>Average daily water consumption</u>				
	<u>Days</u>				
	<u>7</u>	<u>14</u>	<u>21</u>	<u>28</u>	<u>35</u>
	ml/rat/day				
Diet-14	11.7	14.3	16.0	19.9	18.6
Diet-15	13.1	15.5	19.2	22.6	22.0
Diet-16	18.8	23.2	23.1	24.7	20.0
Diet-17	16.4	20.9	22.2	22.6	22.7
Diet-LB	19.1	23.1	27.0	29.0	29.7

SCHWARZ BIORESEARCH, INC.

consumption in all groups was normal.

Animals fed diet-15 had the lowest average daily consumption while those fed diet-16 had the highest intakes. Consumption of diets-14 and -17 were virtually equivalent. Water consumption in all groups was unusually high. Since rats consuming a commercial laboratory chow also consumed a large volume of water, this may be characteristic of the Fisher rat rather than directly attributable to the diets ingested.

The results of this experiment are different from those obtained with CFE rats in several respects. In all cases, Fisher animals grew at a slower rate than CFE's when fed diets of the same composition. As noted above, Fishers consumed more water than CFE animals although their diet consumption was slightly less. In terms of superiority for growth promotion, the diets were ranked $17 > 15 > 14 > 16$ for CFE rats whereas the ranking was almost completely reversed - $17 > 16 > 14 > 15$ - for Fisher rats. Finally, while diet-17 proved superior to the other Codelid diets tested for both strains, only in Fisher rats did it produce a hemorrhagic condition.

To be assured that an error was not made in diet preparation, another experiment was conducted in which Fisher rats were fed either diet-16 or -17. As before, all animals fed diet-16 were normal, whereas those fed diet-17 came down with the hemorrhagic condition within three weeks. The plasma prothrombin times were again prolonged and the manifestations were identical to those previously described. In attempting to determine the nutritional inadequacy, menadiol diphosphate was injected

SCHWARZ BIORESEARCH, INC.

intramuscularly and was found to prevent bleeding and to correct the prothrombin, factor II and factor VII-X deficiencies. Since menadione is present in diet-17, the therapeutic effect of menadiol diphosphate suggests destruction of vitamin K or chemical and/or biological interference with its utilization. Studies are presently in progress to determine the factors responsible for this condition and to elucidate the nature of the strain differences in susceptibility.

III. STUDIES ON THE WATER REQUIREMENTS OF RATS FED CODELID DIETS

Although water is an essential nutrient required in relatively large amounts, under ordinary conditions of animal care and maintenance, it is provided in liquid form apart from food. In a real sense, the presence of water in food is generally not as a nutrient per se but rather as a diluent of solid nutrients. Consequently, most studies concerned with thirst, water metabolism and water-food interrelationships have been conducted under conditions of separate feeding of food and water. Under these conditions, Adolph¹⁶, Strominger¹⁷ and many others have independently demonstrated quantitative relationships between food intake and water intake. In general, they have shown that restriction of water intake in normal rats depresses food intake and restriction of food intake reduces water intake. These findings have led to the hypothesis that a physiological mechanism exists which regulates food and water intake to maintain an approximate water:food ratio.

In contrast to the many studies in which food and water were fed separately relatively few concerning food-water interrelationships have been conducted when both are mixed

SCHWARZ BIORESEARCH, INC.

together and provided solely as a single formulation.

The classical studies of Adolph¹⁶ showed that when food and water are fed together as milk, the water intake is governed by caloric content even when this requires ingestion of the associated fluid. Using the same technique and a similar milk diet, Bruce and Kennedy¹⁸ found that central nervous control of food and water intake depends on different, though normally related features of the diet; food intake on the caloric content, and water intake on the demands imposed by the renal excretion.

The relationship of water to food intake assumes special importance in space travel. From the physiological standpoint, nutritional equilibrium, dehydration, renal clearance, urinary output and fecal waste all must be considered. Equally important are the physical problems of weight bulk, waste disposal, diet regeneration and food and water administration. The physical, chemical and nutritive properties of water soluble, chemically defined liquid diets (Codelid diets) offer a potential solution to many of these problems. In addition, they afford a unique opportunity for studying water-food interrelationships when the two constitute a single physical mixture. The following studies were, therefore, conducted to determine whether a water soluble, chemically defined diet can exclusively provide the water and nutrient requirements of the rat.

Tables 17 and 18 show the composition of the basal diet (Codelid diet-14) used in all studies to be reported in this section. The concentration of each nutrient is expressed on a weight:volume basis, the total concentration being 50%. The protein equivalency (Nx6.25) of this diet is 1.8 calories/ml. The amino acid pattern was modified

SCHWARZ BIO RESEARCH, INC.

after whole-egg protein. All basal diets and test diets were prepared with glass distilled water. Each test diet was derived from the basal mixture by diluting with an appropriate amount of distilled water to yield the designated dietary concentration. Such concentrations were varied in 10% increments from 10% to 50% solid nutrients. In all instances, glucose, nitrogen, phosphorus, osmotic pressure, specific gravity and pH determinations were performed on the basal diet. After dilution, phosphorus, specific gravity, osmotic pressure and pH measurements were made on each test diet.

In all experiments, the diet was administered in 100 ml capacity Richter tubes. These tubes were housed in specially designed units with sloped bottoms to permit quantitative collection of any dietary waste. The units were attached externally to the cage and a small circular opening permitted the animal to have access to the Richter tube, Figure 33. This procedure prevented the rat from wetting himself with diet, prevented dripping, and avoided the stickiness sometimes encountered in cages where liquid diets are used.

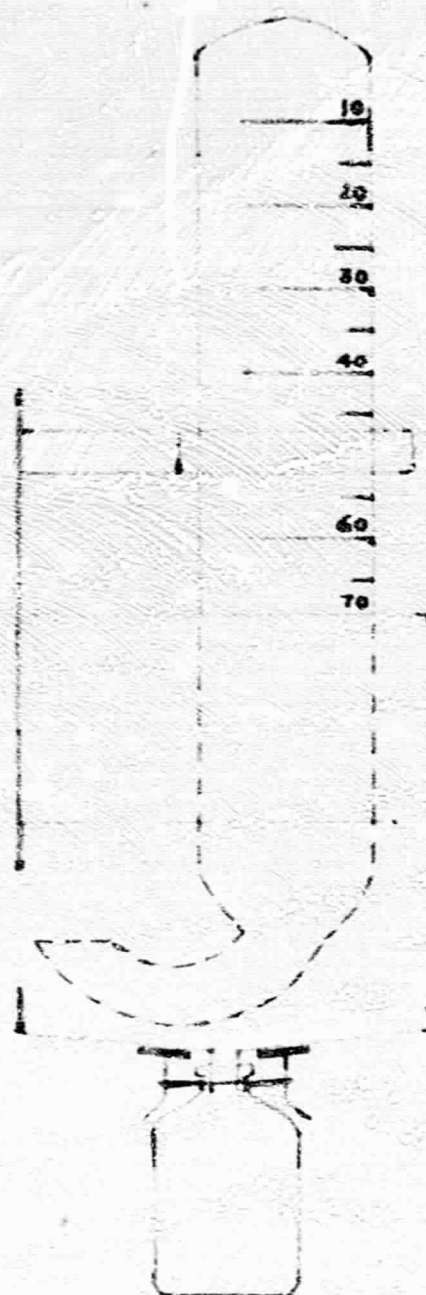
In cases where water was provided, it was administered in 200 ml capacity bottles with glass tubes extending into the cage. All ad libitum water was glass distilled prior to use.

In all but the first experiment, diet and water consumption were measured daily. In the first experiment, these factors were measured weekly. In all experiments, animals were weighed at weekly intervals after diet and water was removed from the cage for 1½ hours. Weighings were performed at the same time of day each week. CFE

SCHWARZ BIORESEARCH, INC.

FIGURE 33

RICHTER TUBE FEEDING APPARATUS



SCHWARZ BIORESEARCH, INC.

male, weanling rats were used in all experiments.

The growth curves of rats fed different concentrations of the previously described chemically defined diet with or without a separate supply of water is shown in Figures 34 and 35. The data was obtained over a 28 day experimental period using 10 CFE male weanling rats per dietary treatment. The animals were housed one per wire bottomed cage. Figure 34 shows the results obtained when water was allowed ad libitum. The initial lag period is characteristic of all our studies with water soluble, chemically defined diets and may reflect a period of adaptation. Thereafter, growth rates in all groups improved. There was no significant difference ($P > 0.01$) in growth between rats fed either of the three dietary concentrations.

Figure 35 shows the growth response of rats fed the same diets but not allowed a separate supply of water. Again, the characteristic initial lag period is observed, with growth rates improving during the last three weeks of the experiment. When compared with animals fed corresponding diets with ad libitum water (Figure 34), only those consuming the 30% diet promoted equivalent growth, the 40% and 50% dietary concentrations producing slower growth rates.

The average daily body weight gain over the entire 28 day period is shown in Table 39. All growth rates were compared to the control group. This was the group which received the 50% diet with ad libitum water. As previously indicated, there was no significant difference ($P > 0.01$) in growth between rats fed either of the three dietary concentrations when ad libitum water was provided. However,

FIGURE 34

GROWTH OF RATS FED DIFFERENT CONCENTRATIONS
OF A CHEMICALLY DEFINED LIQUID DIET
WITH AD LIBITUM WATER

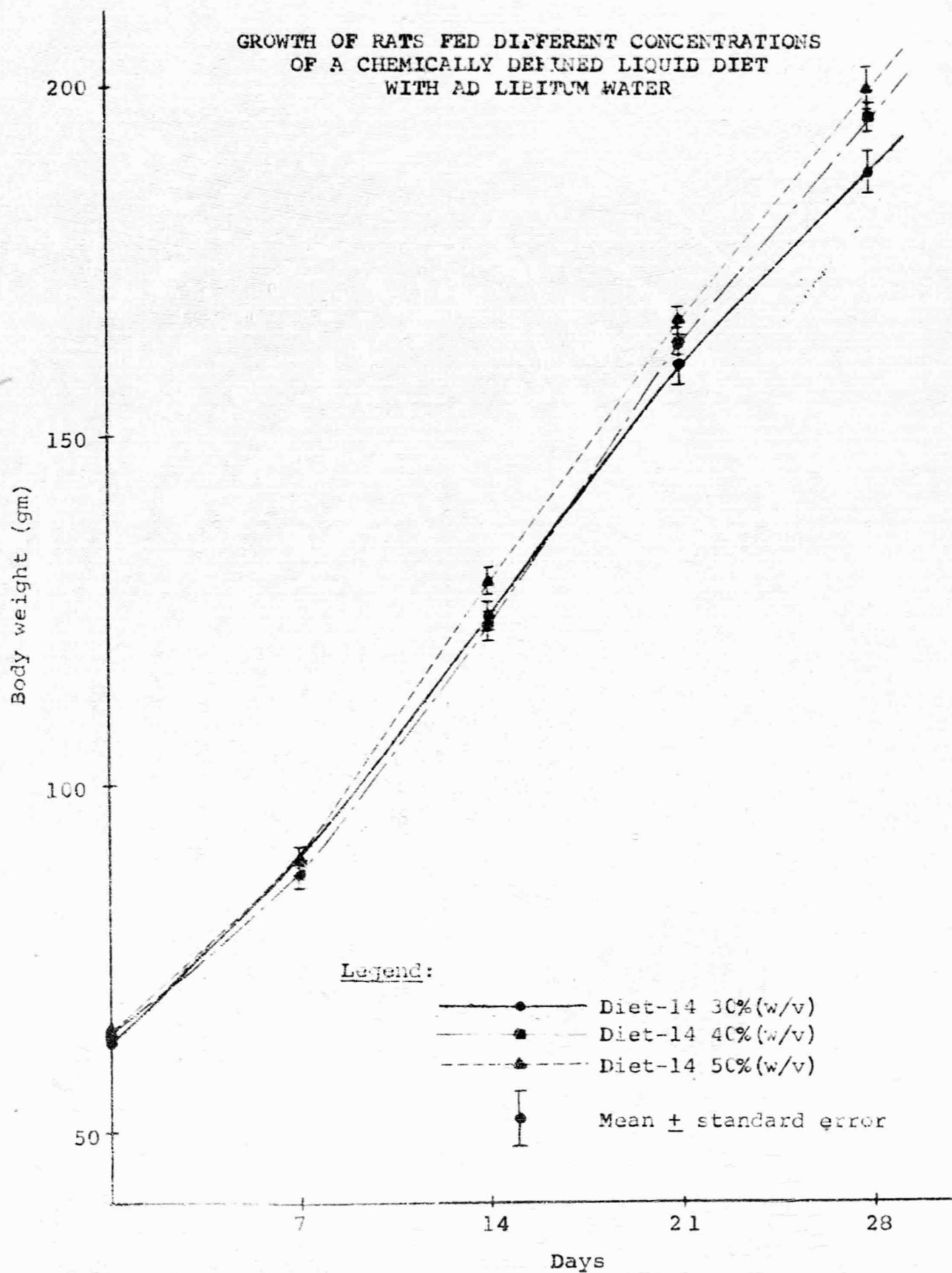


FIGURE 35

GROWTH OF RATS FED DIFFERENT CONCENTRATIONS
OF A CHEMICALLY DEFINED LIQUID DIET
WITHOUT AD LIBITUM WATER

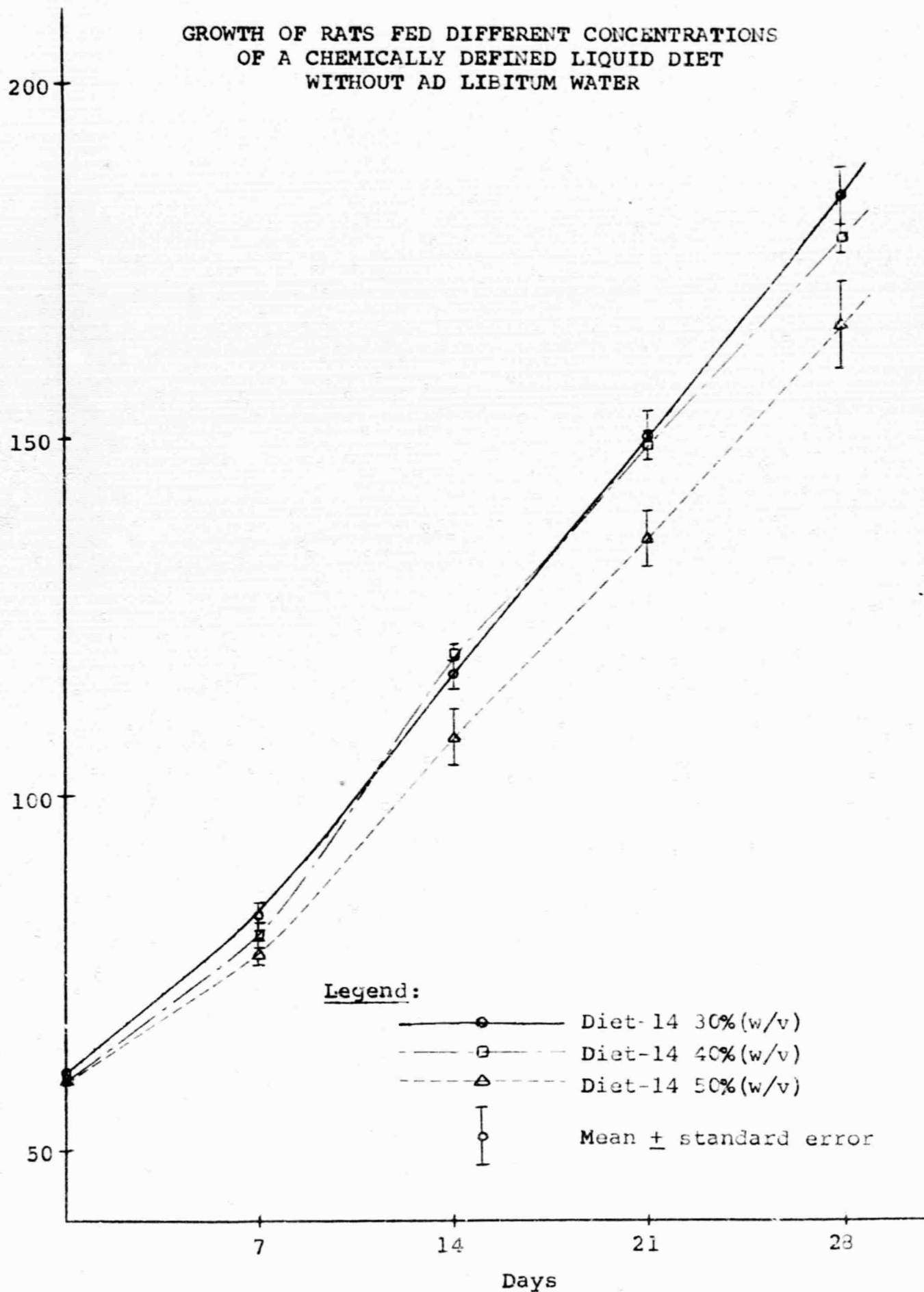


TABLE 39

THE EFFECT OF AD LIBITUM WATER AND LIQUID DIET
CONCENTRATION ON THE GROWTH RATE OF RATS*

Dietary treatment % solids***	Av. daily body weight gain g/rat/day	p**
50	3.7±0.21****	<0.001
40	4.2±0.11	<0.01
30	4.3±0.14	>0.01
50 + H ₂ O	4.8±0.10	
40 + H ₂ O	4.7±0.08	>0.01
30 + H ₂ O	4.4±0.08	>0.01

*Experimental period 28 days. Ten CFE male weanling rats allotted to each dietary treatment. Housed one per wire bottomed cage. For details see Tables 9 and 10 in the Appendix.

**P value for t-test of significance.

***Percentage solid nutrients in diet expressed on a weight:volume basis.

****Mean value ± standard error.

SCHWARZ BIO RESEARCH, INC.

when the diets were the sole source of solid nutrients and water, only the 30% diet promoted growth which was not significantly less ($P > 0.01$) than the control group.

Table 40 shows the diet and calculated energy intake of the rats on this experiment. The caloric intake was calculated from the generally accepted values of 4, 9 and 4 calories per gram of carbohydrate, fat and protein respectively. As previously observed, diet intake regardless of access to ad libitum water, increased with decreasing diet concentration. More significant is the fact that at all but the 50% level without access to drinking water, the caloric consumption was virtually the same.

Thus, just as the rat alters its intake of high roughage or high fat diets to satisfy its energy needs, so it alters its intake of a water soluble chemically defined diet. It is not surprising, therefore, that the NRC recommended energy requirements¹³ for male rats of the age, weight and sex employed in these experiments is around 54-65 calories of metabolizable energy per day -- very close to the caloric intakes observed in this experiment.

The essentially equivalent growth observed in all groups but the 40% and 50% without ad libitum water can be attributed to equivalent caloric intakes and adequate water consumption. The slower growth of the 50% group without ad libitum water is undoubtedly due to its inadequate intake which is probably related to the hypertonicity of the diet. The slower growth of the group consuming the 40% diet without drinking water also seems related to diet hypertonicity although in this case, it did not cause inadequate caloric consumption.

TABLE 40

THE EFFECT OF AD LIBITUM WATER AND LIQUID DIET
CONCENTRATION ON DIET AND ENERGY INTAKE OF RATS*

Dietary treatment	Av. daily diet consumption	Av. daily caloric intake	p**
% solids***	ml/rat/day	cal/rat/day	
50	24.2±0.9	43.6	<0.001
40	38.3±0.7	55.6	>0.05
30	51.4±0.9	55.5	>0.05
50 + H ₂ O	32.1±1.4	57.8	
40 + H ₂ O	37.5±0.6	54.0	>0.05
30 + H ₂ O	51.5±1.2****	55.5	>0.05

*Data tabulated for first 24 days of a 28 day experimental period. For details see Tables 9 and 10 in the Appendix.

**P value for t-test of significance.

***Percentage solid nutrients in diet expressed on a weight:volume basis.

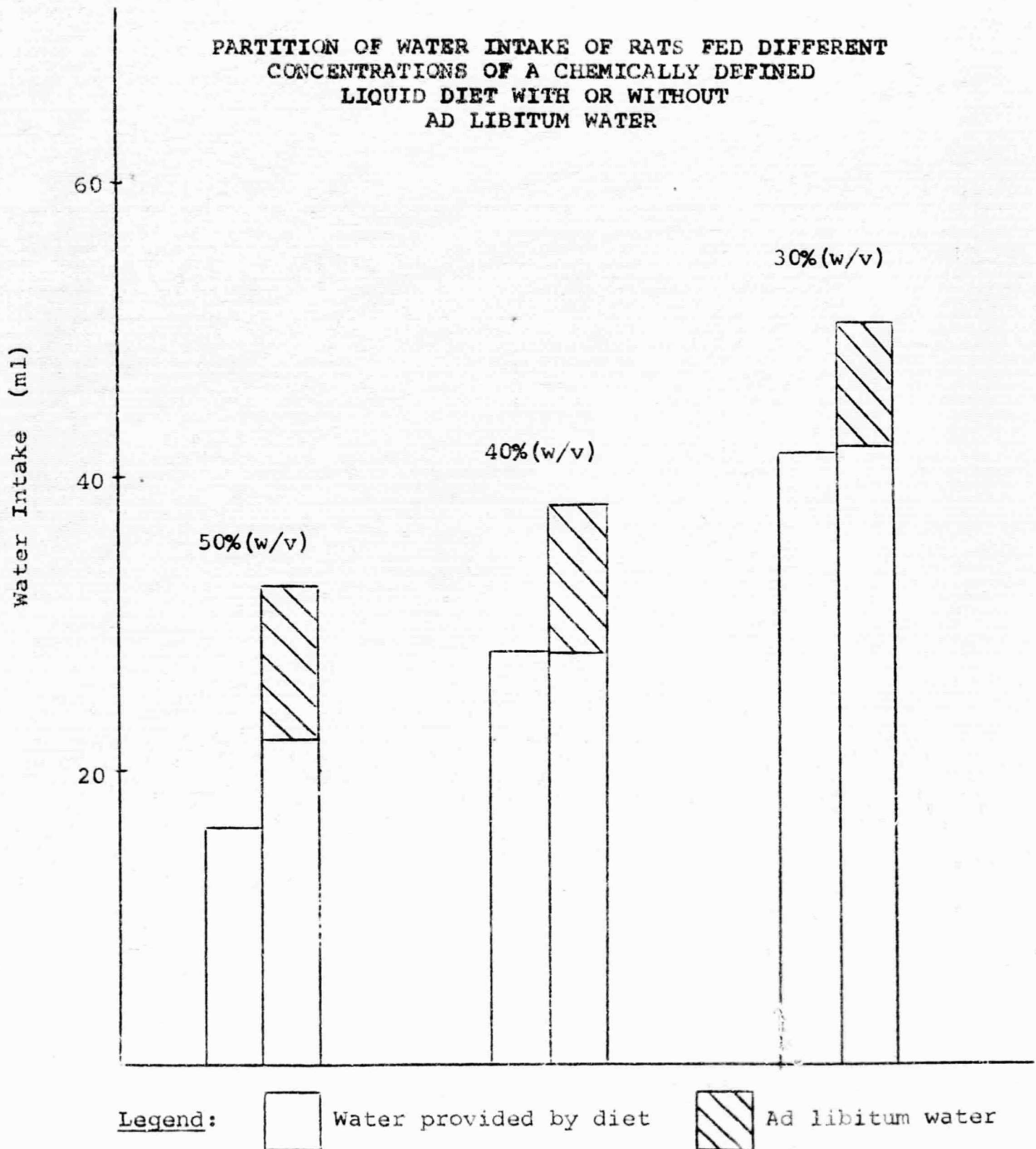
****Mean value for 10 rats ± standard error.

SCHWARZ BIORESEARCH, INC.

Figure 36 and Table 41 show the daily water consumption of rats on this experiment. The white bar shows the water provided by the diet, the lined bar shows the water provided by ad libitum water. As expected, total daily water intake increased with decreasing dietary concentration. This was the result of the interdependence of water and caloric intake, so that total water intake increased as the rats attempted to fulfill their energy requirements. Ad libitum water intake was virtually equivalent at the 40% and 50% dietary concentrations but decreased at the 30% level.

From this data, one might calculate the maximum dietary concentration which will simultaneously satisfy the water and solid nutrient requirements of the growing rat fed a chemically defined diet of this type. It will be remembered from Table 39 that rats consuming the 40% diet without ad libitum water did not grow as well as the control group (50% + ad libitum water) despite virtually equivalent caloric and solid nutrient intakes. Only after ad libitum water was provided was growth rate of the 40% group equivalent to the control (50% + ad libitum water). Since the solid nutrient intake was virtually the same in each of these groups -- approximately 16 grams -- (control and both 40% groups) it would appear that the minimal water requirement compatible with normal diet utilization is represented by the control group. This value corresponds to 33 ml/rat/day. The total volume of a diet combining 33 ml of water and 16 grams of solids into a single source would be 43 ml. This is equivalent to a 37% diet on a weight:volume basis and would represent the maximum dietary concentration capable of simultaneously satisfying the water and solid nutrient requirement of the growing rat. In view of these findings, it is interesting ~~that~~ the amount of ad libitum water consumed

FIGURE 36



At all dietary concentrations bar on right represents diet being consumed with ad libitum water.

TABLE 41

PARTITION OF WATER INTAKE OF RATS FED DIFFERENT
CONCENTRATIONS OF A CHEMICALLY DEFINED
LIQUID DIET WITH OR WITHOUT
AD LIBITUM WATER*

Dietary treatment	Average daily water consumption**		
	Diet	Ad libitum water	Total
% solids***	ml/rat/day	ml/rat/day	ml/rat/day
50	16.2	----	16.2****
40	28.3	----	28.3****
30	41.5	----	41.5
50 + H ₂ O	21.9	10.6	32.5
40 + H ₂ O	27.9	10.3	38.2
30 + H ₂ O	41.9	8.5	50.4

*Data tabulated for first 24 days of a 28 day experimental period. For details see Tables 9 and 10 in the Appendix.

**Mean values for 10 rats.

***Percentage solid nutrients in diet expressed on a weight:volume basis.

****Significant decrease ($P < 0.05$) compared with animals receiving 50% (w/v) diet plus ad libitum water.

SCHWARZ BIO RESEARCH, INC.

by the 40% and 30% groups was not necessary to satisfy their minimal water requirements. It would appear, therefore, that under these conditions, factors other than liquid diet consumption may be regulating *ad libitum* water intake.

Throughout this experiment, urine and feces were collected for purposes of estimating the over-all water balance of the animals. Table 42 shows some of the data obtained during these collections. As would be expected, urine volume increased with increasing diet intake. The urinary osmotic pressure and specific gravity reflected diet concentration and accessibility to supplemental water. It is noteworthy that in all cases where *ad libitum* water was permitted, the osmotic pressure was below 1000 milliosmoles per liter and the specific gravity was below 1.03. The only diet permitting urinary O.P.'s and S.G.'s falling within this range when no *ad libitum* water was available was the 30% diet. If these levels are critical in terms of kidney function then the higher levels observed in the urine of animals consuming the 50% and 40% diets without *ad libitum* water may reflect poor diet utilization and, consequently, could explain the poorer growth of these two groups.

Figure 37 illustrates this point. The dotted lines in all cases represent diets fed without *ad libitum* water -- the solid lines represent those groups where supplemental water was accessible. The hypertonicity of the 50% diet is reflected in the extremely high urinary osmolarity throughout the experimental period when animals were fed this diet without *ad libitum* water. The group consuming the 40% diet without *ad libitum* water on the other hand had a marked increase in urinary osmotic pressure only after the 18th day of the experiment. This sudden increase corresponds

TABLE 42

VOLUME, OSMOTIC PRESSURE AND SPECIFIC GRAVITY
OF URINE FROM RATS FED DIFFERENT CONCENTRATIONS
OF A CHEMICALLY DEFINED LIQUID DIET WITH AND
WITHOUT AD LIBITUM WATER*

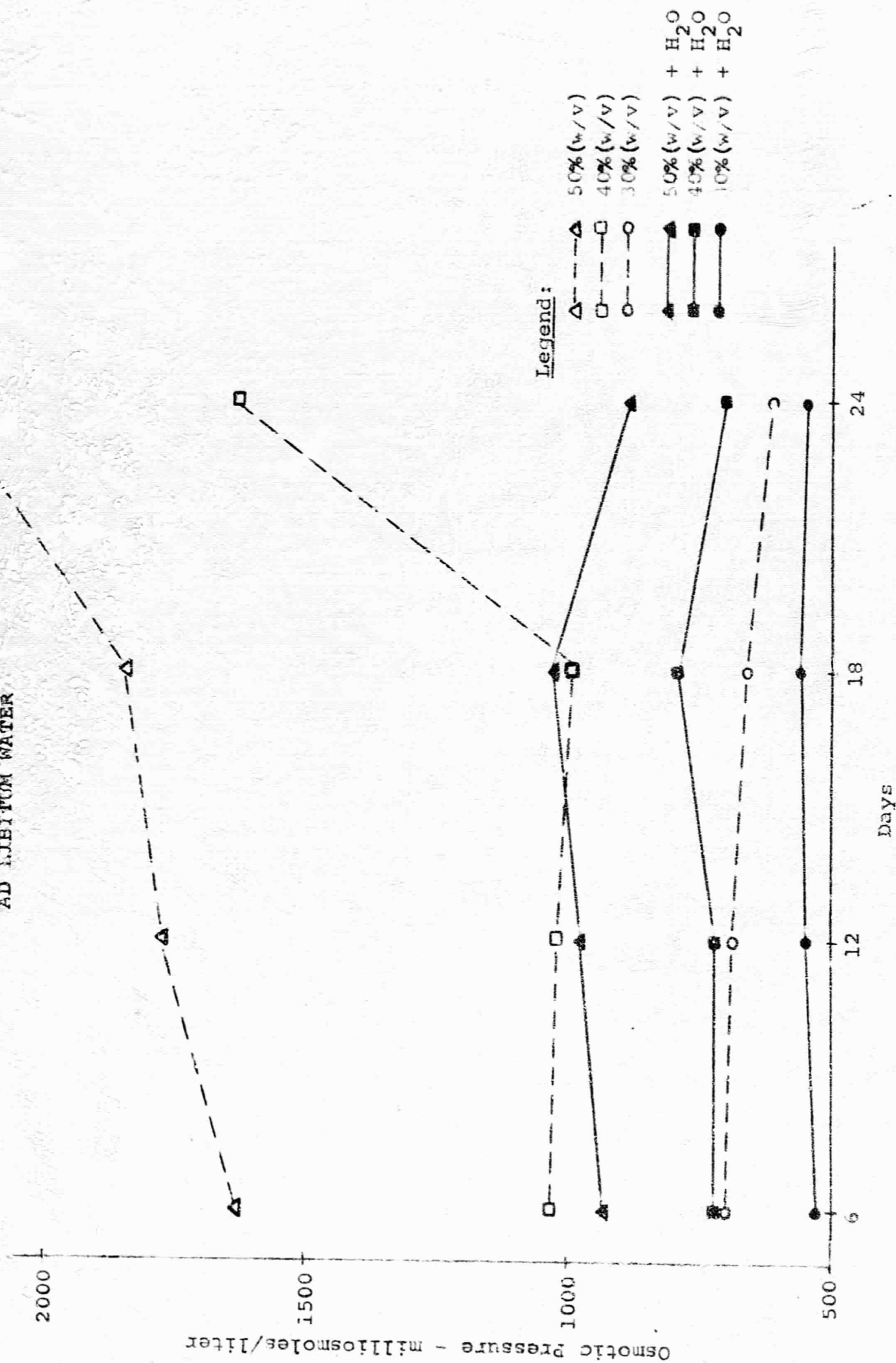
Dietary treatment % solids**	Av. daily urine volume ml/rat/day	Osmotic pressure mOsm/l	Specific gravity
50	7.0	1881	1.0564
40	14.8	1186	1.0322
30	22.9	665	1.0213
50 + H ₂ O	18.6	954	1.0295
40 + H ₂ O	22.1	737	1.0214
30 + H ₂ O	34.3	542	1.0177

*Data tabulated for first 24 days of a 28 day experiment.
Ten rats allotted to each dietary treatment.

**Percentage solid nutrients in diet expressed on a weight:
volume basis.

FIGURE 22

OSMOTIC PRESSURE OF URINE FROM RATS FED DIFFERENT
CONCENTRATIONS OF A CHEMICALLY DEFINED
LIQUID DIET WITH AND WITHOUT
AD LIBITUM WATER



SCHWARZ BIORESEARCH, INC.

to the time at which the growth rate of this group slackened and may be associated with an observed drop in diet consumption.

Figure 38 shows the calculated osmotic pressure of fluids present in the G.I. tract when rats consumed the various test diets. These calculations were based on the assumption shown to be valid for man, that 10% of body weight is intestinal fluid. As was the case for urinary osmotic pressures, the O.P.'s in the G.I. tract of rats consuming the 40% or 50% diets without ad libitum water was higher than for the other groups. Based on the previous data, it would appear that an intestinal osmolarity of around 1800-1900 milliosmoles in the G.I. tract is critical and that higher levels may result in poor diet utilization. Again, the borderline value of the 40% diet is illustrated by its close proximity to this critical level.

The data suggests that diet osmolarity affects diet utilization in different ways and at different times depending on the level of osmolarity. In the case of a hypertonic 50% diet, when ad libitum water is not provided, a chronic effect is observed which appears to directly influence food intake. On the other hand, when a 40% diet is fed without ad libitum water, the osmolarity does not seem to be as critical and its effect is not immediately apparent. Undoubtedly, depending upon degree, dietary osmolarity may exert its influence via such mechanisms as appetite control, alteration of intestinal flora, renal clearance, intestinal absorption and cellular utilization.

We currently are initiating studies on the influence of diet osmolarity on several of these factors.

Table 43 shows the growth, diet and energy consumption

FIGURE 38

CALCULATED OSMOTIC PRESSURE IN THE GI TRACT OF RATS FED DIFFERENT
CONCENTRATIONS OF A CHEMICALLY DEFINED LIQUID DIET
WITH AND WITHOUT AD LIBITUM WATER

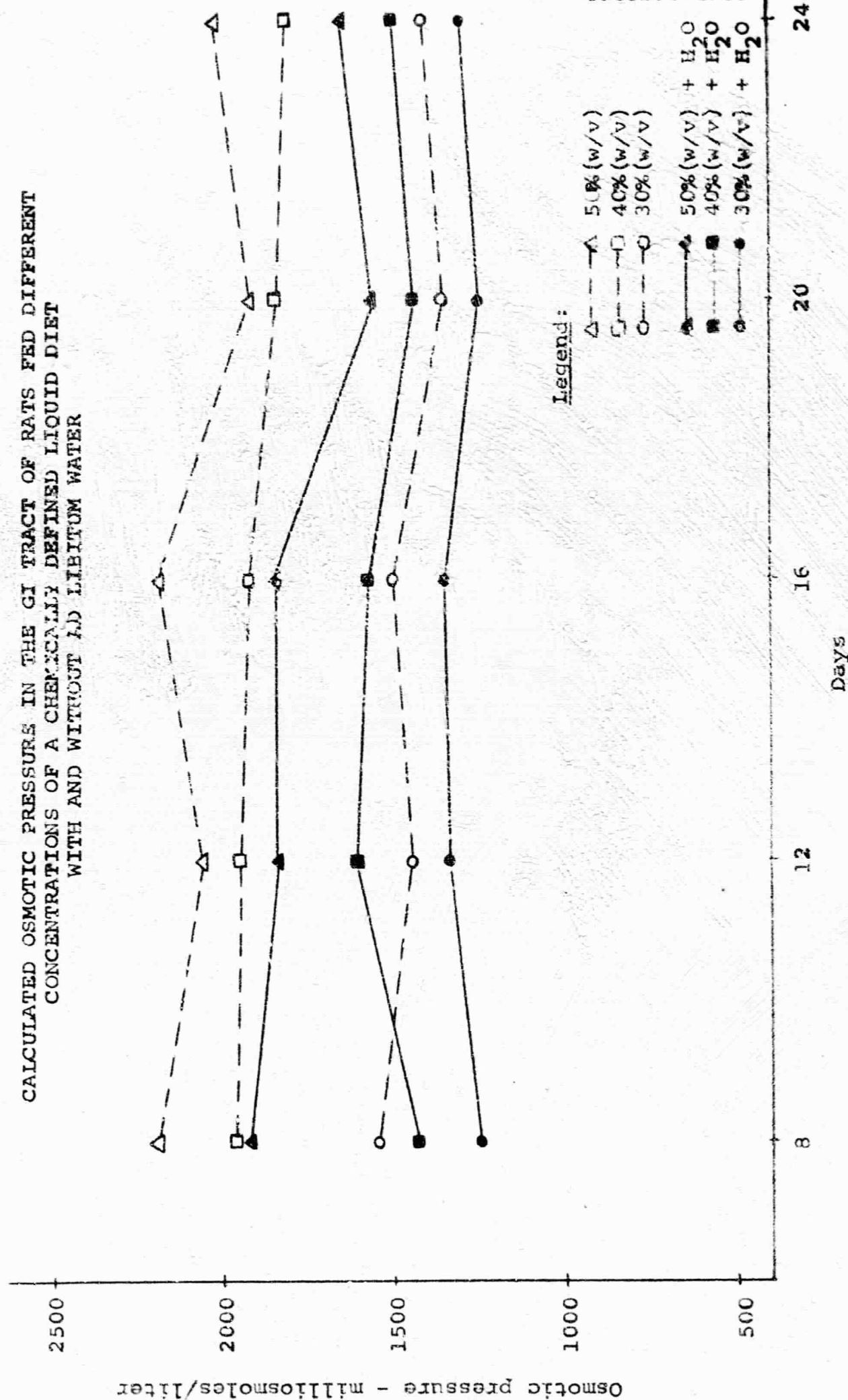


TABLE 43

THE EFFECT OF AD LIBITUM WATER AND
LIQUID DIET CONCENTRATION ON DIET
AND ENERGY INTAKE OF RATS*

Dietary treatment	Av. daily body weight gain	Av. daily diet consumption	Av. daily caloric intake
% solids**	gm/rat/day	ml/rat/day	cal/rat/day
20	3.7 $\pm$ 0.3***	91.8	66.1
10	2.4 $\pm$ 0.1	131.8	47.4
20 + H ₂ O	3.8 $\pm$ 0.2	90.2	64.9
10 + H ₂ O	2.4 $\pm$ 0.1	138.8	50.0
50 + H ₂ O (Control)	4.8 $\pm$ 0.1	32.1	57.8

*Experimental period 28 days. CFE male weanling rats housed one per wire bottomed cage. Values for each 20% group obtained from 7 animals. Values for each 10% group obtained from 10 animals.

**Percentage solid nutrients in diet expressed on a weight: volume basis.

***Mean value $\pm$ standard error.

SCHWARZ BIORESEARCH, INC.

of rats fed 10% and 20% diets compared with the control group from the previous experiments. Except for concentration, the composition of the diet was the same in both experiments. It can be seen that the dilute diets could not produce growth equivalent to the control group. The data also show that the availability of ad libitum water had no influence on diet utilization at these extremely dilute concentrations. Thus, growth rate and diet consumption of corresponding diets was equivalent regardless of ad libitum water intake. It is interesting that the caloric intake of the groups consuming 20% diets was greater than the control group--and yet the animals did not grow as well. This is undoubtedly due to several factors, not the least of which is physical activity--and the need for the animal to continually consume diet. In this case, diet consumption of the 20% groups was almost three times that of the control. It is not unreasonable, therefore, that as the result of consuming and excreting large volumes of fluid, the energy requirement of the rats increase beyond the normally accepted values. Certainly this is the case for animals consuming the 10% diet. Here the situation was further complicated by the physical inability of the animal to handle large volumes of diet. Consequently, while there was a marked increase in diet consumption, it was still inadequate in terms of normal caloric requirements and markedly inadequate in terms of the increased activity associated with the consumption of large volumes of diet.

The results of these studies show chemically defined water soluble diet is particularly well-suited for studying water:nutrient interrelationships and for identifying the role played by the various physiological mechanisms controlling these relationships. When supplied as a sole source

SCHWARZ BIORESEARCH, INC.

of water and solid nutrients, such a diet can satisfy the total nutritive requirements of the growing rat. The dietary concentration that can best do this was found to be 30% on a weight:volume basis. The maximal concentration at which such a diet can simultaneously satisfy water and solid nutrient requirements was calculated to be 37%.

The results suggest that several mechanisms are operative in regulating diet intake and diet utilization when rats are fed a chemically defined liquid diet as the sole source of water and solid nutrients. These mechanisms are related to caloric density of the diet, diet osmolarity and the physical capacity of the rat.

It was observed that depending on dietary concentration, animals consumed diets to fulfill their energy requirements. However, at dietary concentrations in excess of 40% (w/v), the hypertonicity of the diet appeared too great for rats without access to drinking water. On the other hand, at dietary concentrations below 30% (w/v) the physical capacity of the rat was exceeded and it could not consume enough diet to satisfy its nutritive requirements.

These findings indicate that a chemically defined water soluble diet, when provided as a sole source of water and nutrients, may be a practical approach to solving the nutritional, physiological and engineering problems associated with human space flight.

A. D₂O studies

The previous study showed that rats fed 40% or 30% (w/v) diets consumed more ad libitum water than was found necessary to satisfy their minimal water requirements. The data suggested that factors other than liquid diet consumption might be regulating

ad libitum water intake and that there could be a difference in the metabolism and/or tissue distribution of drinking water vs. dietary water. The present study represents a preliminary experiment to develop a technique for determining by use of D_2O , the distribution of drinking water and dietary water in tissues of rats consuming a Codelid diet.

Twelve rats, housed in separate metabolism cages, were divided into two groups. The rats in group I were fed 40% (w/v) liquid diet-14 and were given free access to water containing 20% (v/v) D_2O . The rats in group II were fed 40% (w/v) liquid diet-14 formulated in 27% (v/v) D_2O and 73% (v/v) water. These rats had free access to distilled water containing no D_2O . Urinary D_2O concentration of each rat was determined periodically. When the D_2O concentration in the urine stabilized, or seemed to decrease, each rat was sacrificed and the liver, kidney, heart, leg muscle and back muscle were excised and promptly frozen for subsequent D_2O analysis. In all cases except the liver, the appropriate organs from each group of animals were pooled. Livers were assayed individually.

1. Procedure for urinary D_2O determination

All urine samples were collected under toluene at three day intervals except for the initial two day collection. After recording the volume excreted, the urine was refluxed through an air condenser for approximately 1-1½ hours and subsequently distilled under vacuum. A few crystals of oven dried (105°C) $KMnO_4$ were then added to the distillate which was again distilled and used for D_2O determination.

SCHWARZ BIORESEARCH, INC.

2. Procedure for tissue D₂O determination

The liver from each animal was treated individually while the leg muscles, kidneys, hearts, and back muscles were each pooled before treatment. The samples were homogenized by hand with a ground glass tissue grinder. The homogenate was transferred to a 25 ml boiling flask and distilled under a vacuum into a dry ice acetone bath. Several crystals of oven dried (105°C) KMnO₄ were then added and the sample was re-distilled. This sample was then used in the Falling Drop Apparatus for D₂O determination.

In order to determine the extent of D₂O incorporation into tissue protein or fat, the tissue residue was lyophilized for 24 hours at 25°C. It was then pulverized with a mortar and pestle, re-lyophilized for another 24 hours at 60°C and finally combusted on a Coleman C-H Analyzer in a stream of pure scrubbed oxygen. Instead of absorbing the D₂O and water formed in the conventional manner, the absorption tubes were immersed in a dry ice bath to trap the water of combustion. For better cold conduction, the absorption tubes were packed with 1/8" glass helices. (Four combustions of a 25-35 mg sample were required to produce enough sample for the Falling Drop Apparatus.) The absorption tube was removed from the analyzer after the fourth sample had been combusted and the contents emptied into a 25 ml boiling flask. This sample was distilled under a vacuum into a micro boiling flask. After warming to 25°C, this sample was run on the Falling Drop Apparatus.

3. Procedure for D₂O assay

D₂O was assayed by the Falling Drop technique for determining relative density. This method allows a substance of unknown density to fall through an immiscible solvent of known density. Since the fall distance drop size and solvents are the same, the density of the unknown is a function of the fall time.

In the present study, a drop of unknown was administered with a pipette controlled by a screw top pipette controller. The apparatus was filled with water and immersed in a bath to control temperature. The tubes corked at both ends were filled with a designated solvent or mixture of solvents and pre-adjusted to the desired density. The solvents used included: 0-fluorotoluene used in the range of 4-20% D₂O and a mixture consisting of 69.5% m-xylene and 30.5% bromobenzene for the 0-5% D₂O range. All samples were read against standard curves which were prepared weekly. All values are expressed as a percent of either urinary water, tissue water or water of combustion.

The results are shown in Tables 44 through 47. Urinary D₂O concentration (Table 44) reached a maximum between the fifth and fourteenth days of experiment. Most animals ingesting D₂O with their drinking water reached a plateau in urinary D₂O concentration by the fifth day, whereas those ingesting D₂O with their diet reached a plateau around the eleventh day. The plateau in urinary D₂O concentration was taken as an indication of D₂O equilibration within the body and the animals were, therefore, sacrificed on the fourteenth day of

SCHWARZ BIO RESEARCH, INC.

TABLE 44

D₂O CONCENTRATION OF THE URINE FROM RATS FED DIETARY
D₂O OR D₂O IN THEIR DRINKING WATER*

Treatment	Animal #	D ₂ O concentration					
		2 days	5 days	8 days	11 days	14 days	
		%	%	%	%	%	%
D ₂ O in drinking water	1A	3.30	7.32	6.55	6.45	4.75	
	1B	3.52	7.00	6.32	4.15	5.70	
	1C	3.67	3.72	3.85	3.18	1.61	
	1D	<3.00	3.49	3.68	4.00	2.83	
	1E	<3.00	<3.00	----	2.45	1.73	
	1F	<3.00	<3.00	<3.00	1.35	1.93	
D ₂ O in diet	2A	4.35	9.98	11.50	12.60	12.61	
	2B	3.67	9.44	13.22	13.30	12.87	
	2C	4.22	9.73	12.50	15.45	13.90	
	2D	4.48	9.80	12.72	15.40	15.53	
	2E	4.82	11.08	14.30	15.30	15.04	
	2F	4.27	10.21	14.68	14.55	14.51	

*All animals were sacrificed on the fourteenth day since all showed signs of having reached a plateau in D₂O excretion by this time.

TABLE 45

D₂O INTAKE OF RATS FED DIETARY D₂O OR D₂O
IN THEIR DRINKING WATER

Treatment	Animal #	Diet consumption		Water consumption		D ₂ O as percent of total fluid intake
		Total diet ml	D ₂ O ml	Total water ml	D ₂ O ml	
D ₂ O in drinking water	1A	667		324	65	6.6
	1B	626		305	61	6.6
	1C	668		142	28	3.5
	1D	640		206	41	4.9
	1E	635		120	24	3.2
	1F	732		145	29	3.3
D ₂ O in diet	2A	624	125	252		14.3
	2B	633	127	275		13.5
	2C	628	126	192		15.4
	2D	634	127	96		17.4
	2E	660	132	68		18.1
	2F	664	137	167		16.1

SCHWARZ BIO RESEARCH, INC.

TABLE 46

DISTRIBUTION OF D₂O IN THE ORGANS AND TISSUES FROM RATS
FED DIETARY D₂O OR D₂O IN THEIR DRINKING WATER*

Treatment	D ₂ O in drinking water		D ₂ O in diet	
	Homogenate %	Combusted tissue %	Homogenate %	Combusted tissue %
Liver	2.82**	1.74**	11.12	2.34
Kidney	3.25	2.22	9.20	2.65
Heart	3.08	2.07	12.10	2.78
Leg muscle	3.95	1.85	12.15	1.73
Back muscle	2.75	0.17	12.00	3.62

*Values represent the mean values for 6 rats in each group.

**Mean value for 5 rats.

TABLE 47

EFFECT OF FEEDING D₂O VIA DIET OR DRINKING WATER
ON GROWTH AND ORGAN SIZE OF RATS*

Measurement	D ₂ O via drinking water	D ₂ O via diet
Body weight gain	gm/rat/day	
Liver weight	gm	
Kidney weight	gm	
Heart weight	gm	
	3.0	2.2
	10.4	8.2
	2.2	1.9
	1.2	1.0

*Mean values for 6 rats per group.

SCHWARZ BIO RESEARCH, INC.

experiment in order to determine D_2O distribution in the preselected tissues. The results in Table 46 show a uniform distribution of D_2O in the tissues measured regardless of the source of D_2O (i.e. drinking water vs. diet). The concentration in the tissues was a function of the amount of D_2O ingested so that rats fed D_2O with their drinking water had an average D_2O concentration of around 3% of tissue water whereas those consuming larger quantities (Table 45) of D_2O via their diets had an average concentration around 11% of tissue water.

With the exception of back muscle, the concentration of D_2O in the water of combustion was similar for the respective tissues regardless of whether the D_2O was ingested via the diet or the drinking water. Since the ratio of D_2O concentrate in the tissue water of rats fed D_2O via diet versus those fed D_2O via drinking water was approximately 4:1, one might expect the same ratio in the water from the combusted tissues. However, this was not the case, since the ratio approached 1:1. This suggests that there might be a D_2O tolerance level in living tissue which curtails incorporation of this material into tissue protein or fat. Such a tolerance level may be related to inhibition of active transport of D_2O into tissue protein and fat or to the fact that a C-D bond requires much more energy for stretching or rupture than does a C-H bond. In the latter case, once deuterium becomes bound to a carbon atom, further reactions at this site may be considerably retarded if not completely blocked.

Table 47 shows the weight of the organs and growth rate of the two groups of animals. Rats consuming D_2O

SCHWARZ BIO RESEARCH, INC.

via their diet and consequently ingesting a relatively large quantity of this material grew less and had smaller organs. It was also noted upon autopsy that these rats had intestinal abnormalities manifested by a swollen, puffed-up appearance and marked discoloration. It is possible, therefore, that in addition to the aforementioned metabolic effects such factors as alteration in normal absorption, adverse changes in osmotic pressure or interference with the solvent function of water, may occur when the animal consumes relatively large quantities of D_2O .

The results of this study show that D_2O can be used as a tool for elucidating the interrelationships of dietary water and drinking water in experimental rats. The data indicate that there is probably no difference in the metabolic pathways or tissue distribution of these two sources of D_2O and that the concentration of D_2O incorporated into tissue water is related to the amount ingested. Additional data on combusted tissue suggested that there is a D_2O tolerance level in living tissue. Several hypothesis related to this tolerance level were advanced and must still be tested.

APPENDIX

TABLE 1

BODY WEIGHT, DIET CONSUMPTION, AND
FED LIQUID DIETS EXPOSED TO

Environment**	Body weight				
	Day				
	0	7	14	21	
	gm/rat				
Control	50.6±1.2***	69.0±0.8	95.8±1.9	129.5±1.6	1
Heat	56.9±0.9	47.9±1.0	39.9±0.8	----	
Low Irradiation	56.4±1.5	78.6±2.0	105.9±2.2	130.3±3.0	1
Low Irradiation + Ethyl Cysteinate	56.9±1.6	75.3±2.2	104.8±2.8	128.3±3.1	1
High Irradiation	62.3±1.0	82.8±2.3	99.1±2.6	108.7±3.0	1
High Irradiation + Ethyl Cysteinate	61.4±1.2	80.4±1.6	98.8±2.9	109.3±3.1	1
High Irradiation + Ethyl Cysteinate Non-sterile	61.3±1.1	72.8±3.1	92.5±2.1	114.8±5.6	1

*Experimental period 28 days. Twelve CFE male weanling rats allotted summarized data, see Figures 15 through 18 and Table 19.

**Control - Freshly prepared diet; Heat - 60°C for 12 days; Low I

***Mean value ± standard error.

WATER CONSUMPTION OF RATS
ENVIRONMENTAL STRESS*

28	Diet consumption				Water consumption			
	Day				Day			
	7	14	21	28	7	14	21	28
	ml/rat/day				ml/rat/day			
59.5±1.8	15.7	25.0	29.3	36.6	7.3	12.1	13.9	17.7
-----	10.8	11.6	-----	-----	7.9	12.3	-----	-----
58.5±3.3	22.1	22.1	27.7	32.1	8.2	12.6	14.0	15.2
46.8±3.1	22.5	19.9	27.6	27.1	9.7	12.3	14.7	14.9
08.0±4.8	20.9	20.1	21.1	22.7	9.8	11.9	13.9	15.3
19.6±3.7	19.6	18.4	19.3	20.8	10.4	15.7	16.6	16.1
21.8±5.8	21.4	20.4	21.8	22.9	12.5	14.5	12.6	14.7

ted to each dietary treatment. Diet-14 fed to all groups. For
rradiation - 5×10^5 roentgens; High Irradiation - 5×10^6 roentgens.

2

APPEN
TABLE

BODY WEIGHT, DIET CONSUMPTION
FED LIQUID DIETS EXPOSED

Environment**	Body weight			
	Day			
	0	7	14	21
	gm/rat			
Refrigerated	50.5±1.0****	72.5±1.0	103.1±1.6	136.5±
Frozen	51.3±0.9	72.8±1.1	103.5±1.7	141.1±
Room Temperature -- Dark	52.2±1.1	49.6±1.2	50.8±1.5	52.3±
Room Temperature -- Light	51.8±0.8	47.3±1.2	48.9±1.1	49.7±
Room Temperature -- Nitrogen	51.5±0.7	48.8±0.9	49.7±1.1	48.8±
Room Temperature -- Non-sterile	52.5±1.1	47.5±1.3	49.2±1.4	49.3±

*Experimental period 28 days. Twelve CFE male weanling rats summarized data, see Figure 20 and Table 22.

**Diets stored 4 months. Room temperature - 24°-26°C; Refrige

***Data not available.

****Mean value ± standard error.

DIX

2

AND WATER CONSUMPTION OF RATS
TO ENVIRONMENTAL STRESS*

		Diet consumption				Water consumption			
		Day				Day			
	28	7	14	21***	28	7	14	21	28
		ml/rat/day				ml/rat/day			
-2.3	167.8 $\pm$ 3.5	15.9	20.7		26.8	8.4	11.5	13.6	14.4
-2.6	167.1 $\pm$ 2.7	19.0	20.5		26.6	8.8	9.5	12.2	14.4
-1.7		12.8	8.7			3.8	3.3	2.7	
$\pm$ 1.0		11.7	8.7			3.8	3.3	2.4	
$\pm$ 1.1		12.6	8.4			4.0	3.2	3.6	
$\pm$ 1.6		12.7	8.2			2.5	2.2	2.5	

lotted to each dietary treatment. Diet-14 fed to all groups. For
rated 0°-4°C; Frozen -6°C.

2

APPENDIX

TABLE 3

BODY WEIGHT, DIET CONSUMPTION AND
FED LIQUID DIETS REFRIGERATED

Environment**	Body weight			
	Day			
	0	7	14	21
	gm/rat			
Refrigerated	60.8±1.1***	78.2±2.0	110.4±2.8	141.0±2.8
Frozen	61.4±1.0	78.9±1.3	111.6±1.8	142.4±2.8
Lab Blox	57.4±1.0	92.8±5.7	135.6±5.9	165.4±7.8

*Experimental period 28 days. Twelve CFE male weanling rats al
For summarized data see Figure 23 and Table 25.

**Refrigerated 0°-4°C; Frozen -6°C.

***Mean value ± standard error.

****Expressed in grams/rat/day.

WATER CONSUMPTION OF RATS
OR FROZEN FOR 8 MONTHS*

	Diet consumption				Water consumption			
	Day				Day			
28	7	14	21	28	7	14	21	28
	ml/rat/day				ml/rat/day			
165.5± 3.0	17.0	26.1	28.1	30.6	8.7	10.9	10.8	7.2
166.5± 3.8	18.0	25.4	29.0	30.5	7.9	8.3	8.3	9.5
192.4±10.1	14.8**	19.1	19.3	20.5	17.4	19.6	17.5	26.0

lotted to each dietary treatment. Diet-14 fed to all groups.

APPE

TABL

BODY WEIGHT, DIET CONSUMPTION AND WATER CONSUMPTION
EXPOSED TO SEVERAL ENVIRONMENTAL CONDITIONS

Storage conditions	Body weight			
	Day			
	0	7	14	21
	gm/rat			
0-4°C	62.3±2.8**	83.8±3.2	114.5±4.1	144.7±5
24-26°C	63.2±1.4	85.8±1.8	116.8±2.5	145.3±4
37°C	61.3±1.9	82.0±2.1	111.3±3.3	141.7±3
60°C***	63.4±1.8	52.1±1.5	49.1±2.4	---
0-4°C + GSH****	67.3±2.3	89.6±3.2	123.7±4.6	155.3±5
24-26°C + GSH	61.9±1.3	83.3±1.8	114.6±2.4	146.0±3
37°C + GSH	62.7±2.2	84.5±2.9	116.3±4.5	144.5±6
60°C + GSH***	64.8±1.9	53.3±1.6	47.4±1.5	---

*Experimental period 28 days. Twelve CFE male weanling rat
For summarized data, see Table 28 and Figures 24-27. Diet-

**Mean value ± standard error.

***Experiment terminated during third week due to excessive d

****GSH - glutathione.

DIX

4

ION OF RATS FED A CHEMICALLY DEFINED LIQUID DIET DITIONS WITH AND WITHOUT GLUTATHIONE*

		Diet consumption				Water consumption			
		Day				Day			
	28	7	14	21	28	7	14	21	28
		ml/rat/day				ml/rat/day			
.3	172.9 $\pm$ 5.7	15.4	29.2	29.7	32.5	7.0	8.6	12.8	13.6
.7	176.0 $\pm$ 4.1	18.7	26.2	31.1	33.1	9.0	10.3	17.5	18.1
.8	172.1 $\pm$ 3.9	17.5	24.5	28.1	31.5	7.5	10.2	15.9	16.0
	---	9.7	9.3	--	--	5.8	4.5	--	--
.1	186.1 $\pm$ 6.3	18.3	26.8	31.5	33.6	7.9	9.2	14.0	12.7
.3	177.2 $\pm$ 4.2	18.2	25.4	28.9	32.6	7.1	7.8	12.1	13.9
.3	176.0 $\pm$ 7.5	17.3	24.3	27.8	30.4	6.4	8.7	11.0	11.6
	---	10.0	8.5	--	--	6.5	5.7	--	--

s allotted to each dietary treatment. Housed two per cage.
14 fed to all groups.

eath and severe loss of body weight.

APPENDIX

TABLE 5

BODY WEIGHT, DIET CONSUMPTION AND WATER
GRADED LEVELS OF A PROTEIN HYDROLYSATE SUPPLEMENT

Protein		Body weight			
Source	Conc. **	Day			
		0	7	14	
	%	gm/rat			
Acid	8.5	85.0 <u>±</u> 2.6***	99.0 <u>±</u> 2.6	128.0 <u>±</u> 3.4	149
Hydrolyzed	10.6	84.5 <u>±</u> 2.2	98.9 <u>±</u> 3.1	127.4 <u>±</u> 3.9	146
Protein	12.8	83.8 <u>±</u> 1.0	96.3 <u>±</u> 1.4	121.5 <u>±</u> 1.6	139
Amino Acid					
Mixture #14****		88.6 <u>±</u> 1.3	114.0 <u>±</u> 2.7	147.8 <u>±</u> 4.2	155

*Experimental period 19 days. Eight CFE male weanling rats alle data, see Figure 28 and Table 29.

N x 6.25.

***Mean value $\pm$ standard error.

****For composition, see column 2, Table 12, Annual Report, 1965.

APPENDIX

TABLE 6

BODY WEIGHT, DIET CONSUMPTION AND WATER CONSUMPTION
CONTAINING SYNTHETIC AMINO ACIDS OR AN ACID HYDROLYSATE

Dietary treatment**	Body weight				
	Day				
	0	7	14	21	28
	gm/rat				
A.A.#14	48.9±2.0***	68.4±1.2	98.7±2.3	127.4±3.0	159.7±3.5
A.A.#16	48.9±1.2	65.7±1.1	91.7±2.1	119.7±2.4	146.7±2.4
Hyd	52.8±1.2	43.0±0.7	38.6±0.8	37.5±0.8	38.6±0.8
Hyd-16	51.3±1.0	70.8±1.9	105.9±2.9	138.4±3.5	168.4±3.5
Hyd-14	48.5±1.0	67.4±1.4	100.2±1.7	128.3±2.6	158.3±2.6

*Experimental period 28 days. Ten CFE male weanling rats allotted to each treatment group (Figure 29 and Table 30).

**For composition of amino acid mixtures 14 and 16, see Table 12 of Figure 29 and Table 30. Hyd-14-hydrolysate supplemented to simulate AA16;

***Mean value ± standard error.

CONSUMPTION OF RATS FED
FED WITH ARGININE AND TRYPTOPHAN*

	Diet consumption			Water consumption		
	Day			Day		
	7	14	19	7	14	19
	ml/rat/day			ml/rat/day		
.3+4.2	19.0	25.5	31.2	10.4	16.5	19.1
.9+4.1	18.2	24.7	30.9	10.0	18.4	25.3
.4+2.5	17.9	25.0	29.5	12.4	19.5	26.5
.9+6.3	22.1	25.7	31.1	8.3	13.0	18.3

tted to each dietary treatment. For summarized

2

CONSUMPTION OF RATS FED LIQUID DIETS
 VIZED PROTEIN SUPPLEMENTED WITH AMINO ACIDS*

	Diet consumption				Water consumption			
	Day				Day			
28	7	14	21	28	7	14	21	28
	ml/rat/day				ml/rat/day			
5.3 $\pm$ 3.9	16.6	23.5	26.4	29.7	7.3	10.1	11.2	13.9
5.0 $\pm$ 2.4	12.9	26.2	25.9	28.1	5.4	9.0	8.5	8.8
3.4 $\pm$ 1.7	7.2	8.0	4.9	10.2	3.7	4.5	3.1	4.2
3.8 $\pm$ 3.7	15.9	23.9	28.0	32.0	6.3	9.1	12.5	14.8
4.7 $\pm$ 3.4	15.2	26.4	26.4	30.2	7.2	10.8	12.1	17.3

to each dietary treatment. For summarized data, see

Annual Report, 1965; Hyd-hydrolysate; Hyd- 16-hydrolysate
 o simulate AA14.

2

APPE

TABL

BODY WEIGHT, DIET CONSUMPTION AND WATER CO
LIQUID DIET CONTAINING AN ACID HYDROLYZE

Group	Amino acid supplement	Body weight			
		Day			
		0	7	14	
		gm/rat			
I	Hyd***-16	58.3±1.0**	82.0±2.1	118.3±2.4	15
II	Hyd + 0.23% Met + 0.11% Try	55.6±0.7	67.2±2.2	83.9±3.8	11
III	As Group II + 0.35% Phe	54.0±0.9	75.6±1.0	110.3±1.5	14
IV	As Group III + 0.35% Lys	54.9±1.0	80.3±1.1	116.4±2.0	15
V	As Group IV + 0.14% Thr	56.1±0.5	78.4±0.8	114.4±2.2	15
VI	As Group V + 0.12% Ileu	55.5±0.8	76.2±1.0	107.6±2.1	14

*Experimental period 25 days. Twelve CFE male weanling rats at
data see Table 32 and Figure 30.

**Mean ± standard error.

***Hyd - acid hydrolyzed protein. Hyd-16 - hydrolysate suppleme

NDIX

E 7

NSUMPTION OF RATS FED A CHEMICALLY DEFINED
D PROTEIN SUPPLEMENTED WITH AMINO ACIDS*

		Diet consumption				Water consumption			
		Day				Day			
21	25	7	14	21	25	7	14	21	25
		ml/rat/day				ml/rat/day			
2.5+3.3	---	19.4	26.7	33.7	--	7.2	9.8	13.3	--
3.8+3.6	127.8+5.1	14.5	22.5	28.4	30.9	7.6	11.9	13.7	15.9
9.1+2.6	161.0+2.6	17.9	25.1	30.0	30.6	8.1	10.3	14.0	18.3
4.8+2.7	175.3+2.8	18.8	24.0	32.0	29.2	8.6	12.5	13.7	19.4
3.2+2.6	171.9+3.2	17.4	25.1	33.4	27.5	8.8	16.5	18.5	17.4
7.1+3.0	166.2+3.9	18.0	26.2	32.5	25.6	8.1	11.5	15.9	18.5

lotted to each treatment. Housed two per cage. For summarized

nted to simulate A.A. mixture 16.

2

APPEN

TABLE

BODY WEIGHT, DIET CONSUMPTION AND WATER
FORMS OF CHEMICALLY DEFINED DIETS CONTA

Dietary treatment	Body weight				
	Day				
	0	7	14	21	
	gram				
Diet 18	61.4±1.0**	86.3±2.0	124.8±2.3	164.3±4.1	194.
Diet 18 - Hyd***	59.8±1.0	81.4±1.2	114.5±2.6	139.0±4.2	155.
Diet 17	62.3±1.2	89.9±1.7	132.2±2.4	164.0±3.0	186.
Diet 17 - Hyd***	58.0±0.8	84.1±1.2	119.7±1.4	152.9±1.8	175.
Diet 17 - Gel	59.7±2.2	88.8±2.6	130.9±4.1	168.4±4.9	204.
Diet 18 - Gel	64.1±1.1	89.3±2.2	121.1±4.1	162.2±4.8	183.

*Experimental period 26 days. Twelve CFE male weanling rats all
Table 35 and Figures 31 and 32.

**Mean value ± standard error.

***Acid hydrolyzed protein.

****Value at 28 days.

DIX

8

CONSUMPTION OF RATS FED LIQUID OR GEL
CONTAINING DIFFERENT AMINO ACID MIXTURES*

	Diet consumption				Water consumption			
	Day				Day			
	7	14	21	26	7	14	21	26
	ml/rat/day				ml/rat/day			
26								
0+4.3	21.1	31.7	38.5	43.6	11.4	19.9	21.6	30.2
7+5.9	19.0	25.4	27.9	38.0	11.1	17.8	18.0	22.3
0+2.7	20.1	29.1	29.1	32.3	10.8	11.0	15.7	14.2
5+2.1	19.2	26.6	31.4	33.7	9.6	13.7	16.0	13.8
2+6.8****	26.2	35.5	38.2	40.1****	10.1	14.5	15.6	18.0****
8+5.1	25.5	32.9	32.4	34.6	11.2	15.8	24.6	26.7

otted to each dietary treatment. For summarized data, see

APPENDIX

TABLE 9

BODY WEIGHT AND DIET CONSUMPTION OF RATS FED A CHEM
AT THREE DIFFERENT CONCENTRATIONS* WITHOUT

Diet concentration % solids (w/v)	Body weight			
	Day			
	0	7	14	21
			grams	
30	60.5±1.0***	82.7±1.9	116.5±2.5	150.9±4.2
40	59.9±1.0	80.8±2.2	120.3±1.9	148.9±2.3
50	60.1±0.7	77.6±2.5	108.3±4.4	136.2±4.4

*Solid nutrient concentration expressed on a weight/volume basis.

**Experimental period 28 days. Ten CFE male weanling rats allotted to groups. For summarized data see Tables 39-41 and Figure 35.

***Mean value ± standard error.

ICALLY DEFINED LIQUID DIET
AD-LIBITUM WATER**

	Diet consumption			
	Day			
	8	16	24	28
28	ml/rat/day			
183.7 $\pm$ 4.1	44 $\pm$ 0.8	52 $\pm$ 1.7	58 $\pm$ 1.2	66 $\pm$ 2.2
178.9 $\pm$ 3.1	28 $\pm$ 0.8	42 $\pm$ 1.3	46 $\pm$ 1.0	45 $\pm$ 2.0
165.7 $\pm$ 5.9	17 $\pm$ 1.7	27 $\pm$ 0.9	28 $\pm$ 1.3	34 $\pm$ 1.7

o each dietary treatment. Diet-14 fed to all

2

SCHWARZ BIORESEARCH, INC.

APPENDIX

TABLE 10

BODY WEIGHT, DIET CONSUMPTION AND WATER CONSUMPTION
LIQUID DIET AT THREE DIFFERENT CONCENTRATIONS

Diet concentration % solids (w/v)	Body weight					
	Day					
	0	7	14	21	28	8
	grams					
30	63±0.9***	89±1.6	124±1.6	160±2.9	187±2.4	35.8±0
40	64±1.0	87±1.9	123±2.2	163±2.3	195±2.2	26.2±0
50	65±0.8	89±0.8	129±1.5	166±2.5	199±3.0	25.8±0

*Solid nutrient concentration expressed on a weight/volume basis.

**Experimental period 28 days. Ten CFE male weanling rats allotted to summarized data see Tables 39-41 and Figure 34.

***Mean value ± standard error.

ION OF RATS FED A CHEMICALLY DEFINED
IONS* WITH AD-LIBITUM WATER**

	Diet consumption			Water consumption			
	Day			Day			
	16	24	28	8	16	24	28
	ml/rat/day			ml/rat/day			
6	57.2±0.4	61.7±0.8	62.2±2.2	6.4±0.3	7.7±0.4	11.3±0.6	13.8±1.2
1	40.1±0.3	45.9±0.4	47.1±1.3	9.5±0.3	8.8±0.4	12.6±0.7	12.9±1.1
2	33.3±0.2	37.1±0.4	42.6±1.2	10.2±0.3	9.1±0.3	12.4±0.3	13.7±0.9

each dietary treatment. Diet-14 fed to all groups. For

2

SCHWARZ BIORESEARCH, INC.

REFERENCES

1. Annual Report, 1964 submitted under contract NASw-517 by Schwarz BioResearch, Inc.
2. Greenstein, J. P., Birnbaum, S. M., Winitz, M. and Otey, M. C. Arch. Biochem. and Biophys., 72, 400 (1957).
3. Maillard, L. C., Compt. rend., 154, 66 (1912).
4. Lea, C. H. and Hannan, R. S. Biochem. et Biophys. Acta, 5, 433 (1950).
5. Mohammad, A., Frankel-Conrat, H. and Olcott, H. S. Arch. Biochem., 24, 157 (1949).
6. Adrian, J., Petit, L. and Godon, B. Compt. rend., 255, 391 (1962).
7. McKeen, W. E. Science, 123, 509 (1956).
8. Nickerson, W. J., Falcone, G. and Strauss, G. Biochemistry, 2, 537 (1963).
9. "Research Today", Eli Lilly & Company, Indianapolis, Indiana, 1946.
10. "Difco Manual", 9th Ed., Difco Laboratories, Inc., Detroit, Michigan, 1953.
11. Rifkin, C. and Marcus, D. U.S. Patent 3,143,464, August 1964.
12. Rama Rao, P. B., Metta, V. C., Johnson, B. C. J. Nutr. 69, 387 (1959).
13. National Research Council publication 990, 1962.
14. Rogers, O. R., and Harper, A. E. J. Nutr., 87, 267 (1965).
15. Ranhotra, G. S. and Johnson, B. C. Proc. Soc. Exp. Biol. Med., 118, 1197 (1965).
16. Adolph, E. F. Am. J. Physiol., 151, 110 (1947).
17. Strominger, J. L. Yale J. Biol. Med. 19, 279 (1947).
18. Bruce, H.M. and Kennedy, G. C. Proc. Roy. Soc. London, Series B, 138, 528 (1951).

SCHWARZ BIORESEARCH, INC.

COST STATEMENT

I. Statement of Total Cost:

	<u>Cumulative Costs Incurred</u>	<u>Estimated Compl. Costs</u>
Direct Labor	\$ 124,480.44	\$ 16,500.00
Direct Materials	100,901.05	13,500.00
Other Direct Charges	22,080.13	4,000.00
Overhead @ 100% Direct Labor	124,480.44	16,500.00
Total Direct Costs	371,942.06	50,500.00
G&A @ 15% Total Direct Costs	55,791.33	7,575.00
Total	427,733.39	58,075.00
Gov't. Owned Equipment	27,348.86	1,357.00
Fixed Fee	28,624.67	3,727.00
Total Billed thru 9/30/65	<u>\$ 483,706.92</u>	<u>\$ 63,159.00</u>

II. Expenditure Rate:

<u>Period</u>	<u># of Mos.</u>	(in thousands)	
		<u>Total Billing</u>	<u>Average Monthly Billing</u>
10/4/62 - 12/31/62	3	\$ 14.1	\$ 4.7
1/1/63 - 2/28/63	2	37.1	18.5
3/1/63 - 5/31/63	3	65.3	21.8
6/1/63 - 10/4/63	4	47.7	11.9
10/4/63 - 3/31/64	6	92.9	15.5
4/1/64 - 9/30/64	5	72.6	14.5
10/1/64 - 9/30/65	<u>12</u>	<u>154.0</u>	<u>12.8</u>
	<u>35</u>	<u>\$483.7</u>	<u>\$13.8</u>