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SCHWARZ BIORESEARCH, INC.

SUPERIOR DIET

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ABSTRACT

1. Experiments were conducted to determine the stability of chemically defined liquid diets under several environmental conditions and to determine their effect on the intestinal flora of rats.

2. Nutritionally complete liquid diets containing free L-amino acids, glucose, minerals, vitamins and ethyl linoleate were found to be stable during long term storage (one year) only if refrigerated or frozen.

3. Deterioration via the Maillard reaction was found to be a function of temperature and is related to the presence of substances in the diet which can initiate free radical formation through peroxidation reactions. This results in a loss of available essential amino acids accompanied by formation of toxic adducts. These reactions can be delayed by additives which inhibit free radical formation and can be avoided by segregating the amino acid components from the sugar components of the diet.

4. For long term storage without refrigeration, the amino acid and carbohydrate components of liquid diets should be stored separately and mixed just before feeding if they are to be mixed at all.

5. Based on radiochemical studies with model systems of C^{14} glucose and C^{14} glycine, a new mechanism of non-enzymatic browning (Maillard reaction) is proposed. This mechanism involves an induction period in which equimolar amounts of amino acid and aldose react to form an enolamine or ketosamine followed by free radical attack of the amine to cause oxidative polymerization.

6. Vitamin K deficiency is produced in Charles River Fischer rats fed liquid diets which contain menadione and high levels of ethyl cysteinate·HCl. Under the same conditions, vitamin K deficiency does not occur in the CFE strain, or in A. R. Schmidt Fischer rats. Equimolar substitution of menadiol sodium di-phosphate for menadione or removal of ethyl cysteinate·HCl from the diet prevents the deficiency syndrome.

7. Differences exist in the intestinal flora of different rat strains and in rats of the same strain obtained from different sources. These differences do not seem to be responsible for the unique susceptibility of Charles River Fischer rats to vitamin K deficiency.

8. Under conditions where coprophagy is not prevented, the intestinal microflora of rats is not significantly modified by feeding chemically defined liquid diets.

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INTRODUCTION

During the last four years, evidence has been gathered in our laboratories[1] and those of others[2] demonstrating the nutritional efficacy of chemically defined liquid diets in man and laboratory animals. Experiments in our laboratories showed that these diets promote good growth in weanling rats and maintain nitrogen equilibrium in adult animals[1]. Studies with human subjects showed that they can be maintained in a healthy condition for almost a half year when fed chemically defined diets as their sole source of nutrition[2].

In the aforementioned experiments, the liquid diets studied were always freshly prepared. In order to determine their suitability for prolonged space flight where it may not be possible to prepare fresh diets, we also studied their stability when stored under a variety of environmental conditions[5]. We found that refrigerated diets retained their nutritional value for 8 months, whereas marked losses occurred when the diets were irradiated (Co_{60} $5x10^6$ Rad), stored at room temperature (24-26°C) for 1 month, or stored at 60°C for 12 days.

The studies presented in this report were concerned with the nature of the chemical interactions occurring in liquid diets under various environmental conditions and the relation of these reactions to the nutritional value of the diets. Chemical experiments included studies of: the mechanism of non-enzymatic browning; the role of ethyl linoleate and peroxides in diet stability; and the stabilizing effect of anti-browning agents. Animal experiments included: the nutritional evaluation of diets after refrigeration or freezing

for one year; an attempt to identify and isolate the factors responsible for the toxicity and growth retardation resulting from aging and heating (accelerated aging); and the biological evaluation of diets containing thiols as anti-browning agents. In addition, an intensive study was made of the factors responsible for a hemorrhagic syndrome resulting from the ingestion of certain liquid diets.

Finally, primarily as the result of a report that chemically defined liquid diets reduce the microbial population of the human intestine[3], but also related to our studies of the hemorrhagic syndrome, we studied the influence of these diets on the intestinal flora of several rat strains. The results of these studies are also presented in this report.

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NATURE OF CHEMICAL INTERACTIONS OCCURRING WITHIN LIQUID DIET SOLUTIONS

1. Investigations into the Mechanism of Non-enzymatic Browning.

The present interest in non-enzymatic browning developed in the course of efforts designed to assess the potential for use in space flight of chemical diet formulations of the type described by Greenstein and co-workers[4]. In an attempt to investigate the role played by water in the Maillard reaction, both amino acids and sugars were physically solubilized in a highly concentrated aqueous medium in the form of solid lollypops. As we have previously described in an earlier report[5], over a period of several months at ambient temperatures, browning was observed to initiate and be most intense at the air-solid interface. With time it proceeded to progress gradually into the interior of the medium. This observation was unanticipated. According to the classical view of the interaction of an aldose with an amino acid, if browning occurs at all, it could be expected to occur uniformly throughout the specimen.

In another experiment, a mixture of amino acids was compressed together into tablet form with glucose. In this case, browning was most extensive in those tablets in which an unsaturated peroxidizable fatty acid (sorbital linoleate) had been employed as tablet lubricant. Furthermore, the extent of browning was proportional to the quantity of peroxidizable tablet lubricant added. The results of these experiments suggested that browning is a diffusion controlled process in which oxygen diffuses to some readily

peroxidizible substrate formed within the reaction medium. In the first experiment, water served as the diffusion medium. In the latter experiment where the role of water was minimized, the presence of peroxidizable fatty acids permitted oxygen diffusion.

At this point, an effort was undertaken to establish whether as in the case of the lollypops, non-enzymatic browning in solution also occurs via a diffusion controlled process in which the ability of oxygen to penetrate into the medium is a determining factor. We postulated that the reason the diffusion controlled nature of the Maillard reaction was not heretofore observed, was that its occurrence was obscured by thermal convection and diffusion currents generated in the solution. To eliminate these factors, a liquid diet solution was immobilized in 0.15% agar. The solution in turn, was placed into a tall cylinder, stoppered with a cotton plug, and allowed to stand at ambient temperature for several weeks. Within this period, 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.

In order to assess the part played by peroxides in non-enzymatic browning in aqueous media, a fixed quantity of acetic acid peroxide was added to chemical diet solutions maintained at several temperatures. However, prior to the addition of the peroxide, a thiol compound, specifically, reduced glutathione, was added to the solutions in increasing increments to insure that any preexisting peroxides present in solution were destroyed.

A number of important conclusions concerning the browning process were revealed in this experiment:

(1) Browning is a free radical reaction wherein the rate of browning is proportional to the concentration of peroxides present, and derivatively, the concentration of available peroxy free radicals.

(2) As the thiol content of the solution is increased, a greater quantity of peroxides are destroyed with a resulting reduction in the degree of browning. When a stoichiometry of two (2) moles of thiol to one (1) mole of peroxide is attained, browning ceases.

The effect of the addition of increasing quantities of thiol to a solution of glucose and glycine heated at 60°C over an extended period was also examined. As the thiol content increases, the duration of the induction period, i.e. the time lag before the onset of visible browning also increases. The interpretation to be drawn here is that no browning occurs as long as the thiol content of the solution exceeds the concentration of peroxides (free radicals) present in solution. However, once all of the thiol has been consumed, peroxides attack the reaction product present in solution whereupon browning starts. The identity of this peroxidizible substrate is thought to be the enolamine tautomer of 1-deoxy-1-N-carboxymethylamino-D-fructose, i.e., the enolic form 1-deoxy-1-N-carboxymethylamino-D-erythro-hexos-1-ene.

Examination of the effect of temperature on the rate of browning of an amino acid-aldose solution reveals that the induction period is markedly shortened with increasing temperature and the rate of browning is accelerated. It is obvious, especially at the higher temperatures, that

the rate of oxygen diffusion is not a limiting factor here and that this reduction in the induction period and acceleration of browning must correspond to the formation of increasing quantities of both peroxide and oxidizable substrate.

Figure 1 provides additional evidence in support of the point that some reducing substance is formed in the amino acid-aldose reaction medium prior to the onset of browning. Here we see a simultaneous measurement of both optical density and redox potential changes which occur in a solution of glucose and glycine heated at 60°C. After a brief period of quiescence one notes a sudden increase in reduction potential indicating the formation of a reducing compound. The formation of this compound occurs well in advance of visible browning.

Browning Reaction - Stoichiometry and Mechanism

In order to attach a reasonable degree of confidence to any proposed chemical mechanism, it is necessary to satisfy any existing doubts relative to the actual participation in the reaction of the proposed reaction intermediates. This is particularly true in the case of non-enzymatic browning where many reaction intermediates resulting from fragmentation of both amino acid and aldose have been postulated and where specific products have been formed in model systems with solvents other than water[6].

As a technique to examine both the question of product fragmentation and reactant stoicheometry, simultaneous radiochemical experiments were run in the model system consisting of glycine and glucose in water. To specifically determine whether fragmentation actually did

occur and to establish the extent to which either the aldose or amino acid moieties were involved, randomly labeled glycine and glucose were employed. In addition, to determine whether decarboxylation of the amino acid played any significant part under the reaction conditions employed, an additional parallel run was made using glycine-1-C^{14}.

During the course of the reaction run at 60°C in an aqueous solution composed of (2) molar glucose and (1) molar glycine, the radioactivity of the products as well as reactants were determined quantitatively by scintillation counting following their chromatographic separation from aliquot samples removed from the reaction medium at various times. Correlation of losses sustained by both glucose and glycine in the course of the reaction clearly indicates that a stoicheomtery of one mole of glucose to one mole of glycine is involved in the formation of an initial reaction product, which we subsequently identified to be the enolamine tautomer of the ketosamine. The fact that the product of the reaction using glycine-1-C^{14} retains its radioactive label, and does so in the same molar specific activity as the product of the reaction using randomly labeled glycine-C^{14}, indicates that the formation of this product does not involve decarboxylation.

The equal molecular participation of both glucose and glycine in the formation of the ketosamine suggested the obvious kinetics, i.e. of a second order rate reaction. Insight into the kinetics of non-enzymatic browning process was gained through the use of C^{14} labeled glycine and glucose in a series of parallel determinations. The reactions were run at 60°C and terminated on the 25th day at which

time the browned contents of the reaction vessel had completely gelled. During the interim, however, at specified intervals, aliquots were withdrawn from the reaction and chromatographed on paper. Following this, the paper chromatogram was radioautographed to permit the location of the reaction intermediate and end products formed in the course of the reaction. To simplify the method of identification, only one of the reactants, i.e. either glucose or glycine were radiolabeled. The chemical identities of the chromatography spots were established by color generated in unlabeled controls in comparable positions when ninhydrin and aniline formate reagents were employed.

The replication of the 17 day reaction chromatogram is seen in Figure 2. N-butanol-water-acetic acid 2:1:1 was the primary solvent system employed with all chromatograms. A second solvent system, ethanol-tertiary butanol-formate-water 12:4:1:3 was used periodically to double check for the presence of new spots. Several salient features are immediately evident. The first is the observed absence of multiple spots, clearly indicating that neither fragmentation of glucose nor glycine occurred during the browning reaction[7]. Second, the absence of radioactive spots other than that of the single reaction product (ketosamine) located between the polymer which is retained at the origin and either that of glucose or glycine, further demonstrates that families of intermediate brown polymers possessing differential solubilities in the solvent system employed, viz., (n-butanol-water-acetic acid) (2:1:1) are not present. The excision from the paper chromatograms of those radioactive areas corresponding to darkened regions on the radioautograph permitted the determination of the corresponding radioactivity and hence the molar concentration of both reactants and products.

From a knowledge of the initial molar concentration of reactants and the subsequent percentages of the total radioactivity found to be distributed between reactants and products, the changes in the molar concentrations of the reactants with time were established. These data are recorded in Table 1. Substitution of these data in the second order rate equation yielded a value of 0.0017 moles^{-1} day^{-1} as the value of the specific rate constant for the rate at which ketosamine is generated under these reaction conditions (Tables 2 and 3).

Kinetic Measurements - Experimental Techniques

Four, ten (10) ml volumetric flasks containing 10 milliliters of a solution which was 2 molar in glucose and 1 molar in glycine were covered with Parafilm and placed in a constant temperature bath maintained at 60°C. Flask number (1) was run as a control. To flask number (2), 500 microcuries of glycine-1-C^{14} were added such that the glycine present had a specific activity of 50 μc/millimole. To flask number (3) 150 microcuries of randomly labeled glycine-C^{14} were added such that the glycine present possessed a specific activity of 15 μc/millimole. To flask number (4) 300 μc of randomly labeled glucose-C^{14} was added to bring glucose to a specific activity of 15 μc/millimole. At the specified time intervals of 0, 3, 5, 7, 10, 14, 17 and 20 days, both 2λ and 4λ samples were withdrawn from each of the four respective flasks and applied to Whatman #40 filter paper of dimensions 15½" x 5½". These sheets were subjected to ascending chromatography at room temperature for a period of 16 hours. The solvent system used was n-butanol-glacial acetic acid-water 2:1:1. The chromatograms were dried and radioautographed by exposure to Kodak

medical X-ray film for a period of 48 hours and then developed and fixed. The identity of the optically dense areas on the negative was established by spray applications to paper chromatogram control #1 of 0.25% ninhydrin in acetone and 2% aniline formate in acetone reagents. Those areas of the paper corresponding to the location of the various compounds present were individually excised, cut into thin shreds, placed in 22mm width scintillation vials together with 10 ml of scintillation fluid and counted for one minute in a Packard Tri-Curb Liquid Scintillation Spectrometer Series 314E.

The rate of browning usually measured in a plot of $OD_{408m\mu}$ versus time is in actuality then not the initial rate at which ketosamine is formed but rather the rate at which the ketosamine oxidatively couples to form brown polymer. This fact becomes apparent when a comparison is made of a plot of polymer formation determined radiochemically as a function of time with that of a plot of optical density at 408mμ with time (Figure 4).

The similar shape of these two curves indicates the identity of the reaction in question. Similarly, the parallel rate of development of reducing compound in the reaction medium with that of ketosamine formation determined radiochemically also indicates that both measurements involve determination of the formation of the same compound. The parallel nature of both sets of curves indicates quite lucidly that the mechanism of non-enzymatic browning involves an initial formation of colorless ketosamine which builds up in solution prior to the visual appearance of brown polymer.

Viewed in retrospect, it is now possible to

rationally interpret some of the unique characteristics of the Maillard reaction, viz., the role of temperature, the observation of an induction period, as well as our finding relative to the effect of peroxides in accelerating browning and of thiols in inhibiting it. Non-enzymatic browning, therefore, for the sake of convenience, may be viewed as occurring in three distinct phases:

Phase I--This phase corresponds to the "induction period" in which the solution remains yellow and where the sole physical evidence of change are the drop in pH of the medium, the development of enhanced reducing capacity and loss of nutritional value on bioassay in rats. From the chemical standpoint, this phase represents the reaction of amino groups with aldose to form enolamine or alternatively, ketosamine with an accompanying initial peroxide formation.

Phase II--Here, the solution begins to darken and fluorescent compounds posessing ultraviolet absorption maxima in the region of 289mμ and 330mμ appear. The compound possessing 330mμ absorption is also found to be ninhydrin positive.

From the standpoint of the chemistry involved, the peroxides which have along with ketosamine built up in solution, now begin to break down autocatalytically into free radicals. The latter now attack the ketosamine causing its oxidative polymerization to brown polymer as well as 1,2-ketose-imine formation (Schiff base). The latter compound is believed to be the one responsible for the 330mμ absorption. This phase of the reaction

is dependent upon the concentration of both free oxygen and peroxides present in the medium. The introduction of thiols to this medium in the early part of Phase II inhibits the appearance of visible browning by destroying both free radicals and peroxides present but may allow the continued formation of ketosamine.

Phase III--This represents the phase of the reaction in which solid brown polymer is formed quickly by autocatalysis accompanied by the entire reaction medium setting to a crosslinked gel. From the chemical standpoint, it represents a rapid decomposition of peroxides with an accompanying liberation of free radicals which, in turn, attack the ketosamine present causing it to polymerize.

Proposed Mechanism of Non-enzymatic Browning (Maillard Reaction)

In actuality, non-enzymatic browning (Maillard Reaction) is not simply the reaction of amino acids with an aldose, but rather a process consisting of a number of consecutive reactions of which only the first involves the reaction of amino acid with aldose. From the mechanistic standpoint, the non-enzymatic browning process is thought to be initiated by an ionic condensation reaction which is then followed by a series of free radical reactions leading ultimately to the formation of brown polymer. Three successive mechanistic steps are believed to be involved in non-enzymatic browning.

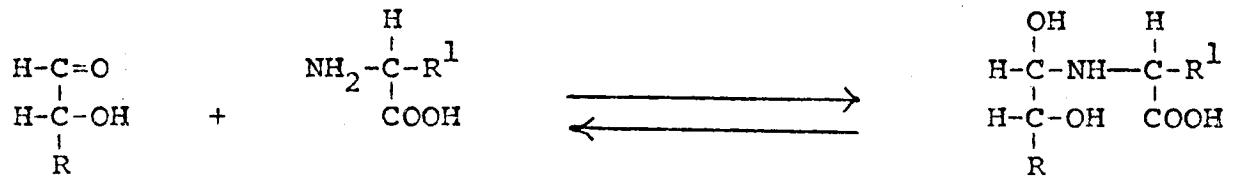
The first step requires the addition of the amino groups of the amino acid to the carbonyl function of the aldose to form intermediate (I), which is believed to have a transient existence. In the second step, intermediate (I) is thought to dehydrate by loss of the elements of water from the respective groups on carbon atoms (1) and (2) of the aldose to form the stable isolatable enolamine depicted as (II). This compound by either of two possible tautomeric shifts can exist in the form of the ketosamine (II_A) as well as in the form of the substituted amino acid (II_B). The specific enol-keto tautomerism involved in the formation of the ketosamine (II_A) can be regarded as a step in the Amadori Rearrangement.

Additional reactions ascribable to (II) are: peroxidation at the C-3 position, oxidation to a 1,2 ketosimine, and free radical initiated vinyl polymerization..

It is the latter reaction, however, which is believed to be responsible for both visible browning and polymer formation. In the third step of the non-enzymatic browning process, breakdown of peroxides liberates free radicals which attack the double bond of the enolamine (II) to initiate a vinyl type polymerization process. The polymer (IV) thus formed by successive dehydration process, is postulated to produce a polymer whose backbone consists of alternating single and double carbon-carbon bonds (V_A). Structures of the type of (V_A) could account for both fluorescence and brown color of the polymer formed. In addition, the possible dehydration or cyclization of the side chain R, to a furan ring would further contribute to the overall brown color of the polymer. In the specific case of the polymer formed by reaction of glycine with

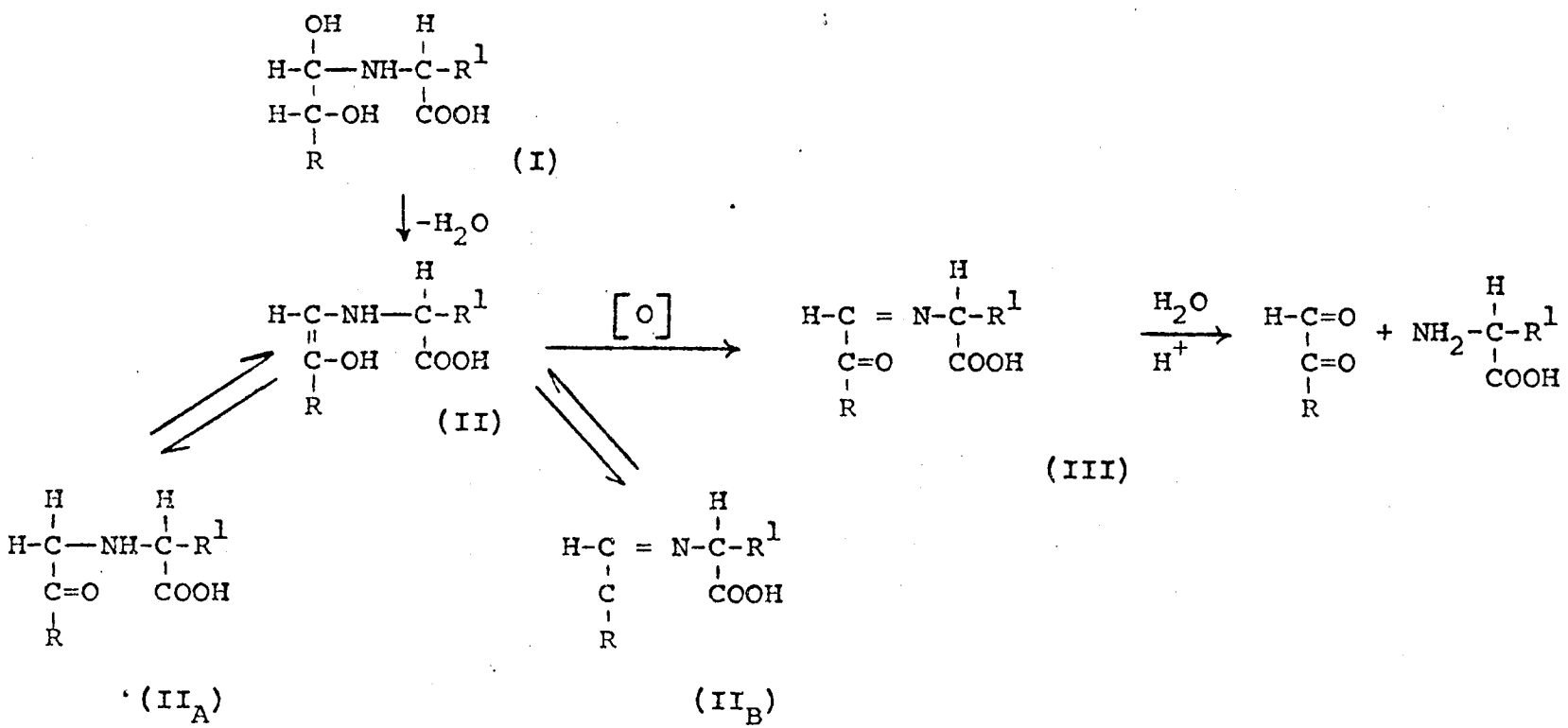
PROPOSED MECHANISM OF NON-ENZYMATIC BROWNING (MAILLARD REACTION)

1.



R = aldose moiety : R^1 = amino acid substituted (I)

2.



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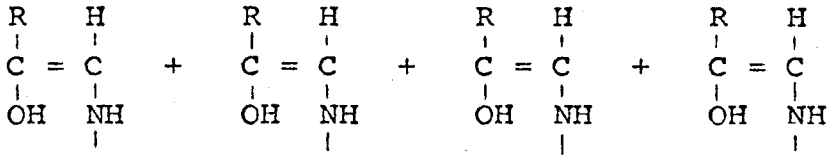
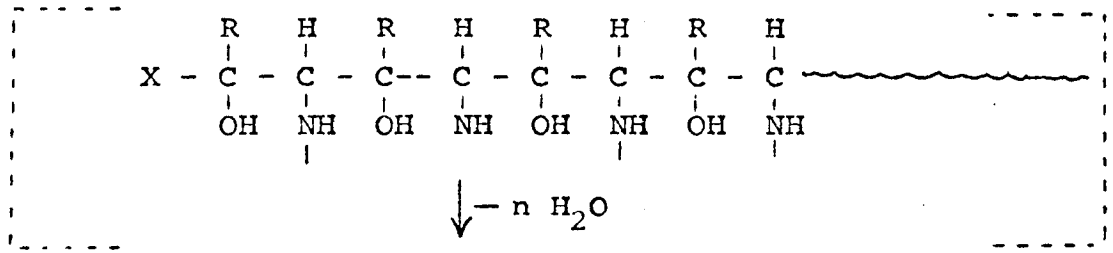
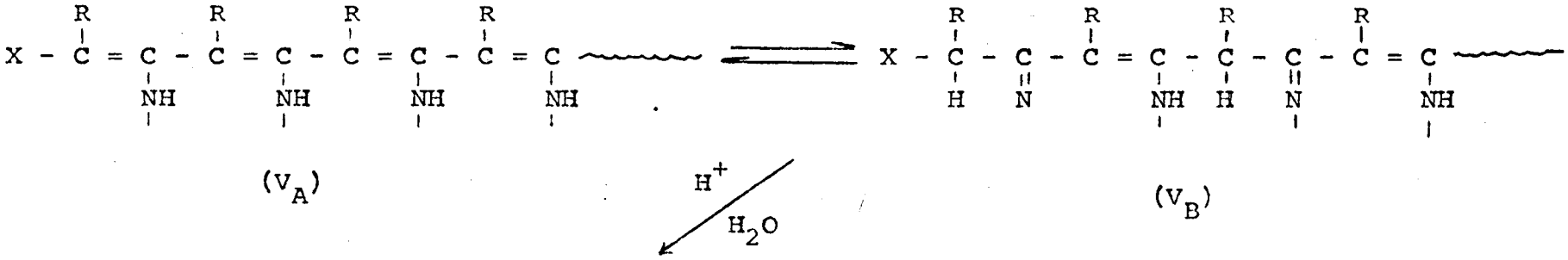
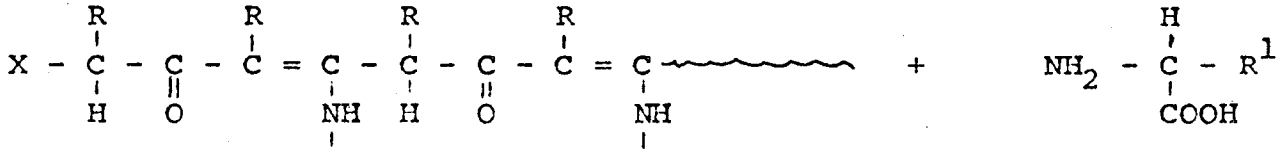
PROPOSED MECHANISM OF NON-ENZYMATIC BROWNING (MAILLARD REACTION)

3. 



(IV)

4. 

5. 

(VI)

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glucose, the polymer predicted to form would be one having structures (V_B) and VI and in which R is a furan ring.

In view of the importance attached to the C-1 and C-2 positions of the aldose in the Maillard reaction[8] relative to other carbon atoms, the reaction of glycine with both ribose and glucose as well as with their deoxy-counterparts was also studied (see Table 4).

The finding of twice as many carbon atoms relative to nitrogen in brown polymer formed from the reaction of deoxyaldoses with glycine as those present in polymers produced from the same reaction in which aldoses are used, clearly suggests that in the case of deoxyaldoses, an acyloin condensation of two or more deoxyaldose moieties to produce a hydroxy-ketone intermediate occurs very early in the reaction. This new acyloin is then believed to react with amino acids to form brown polymeric substances in the manner analogous to that proposed for glucose.

Amino acid chromatograms of liquid diet solutions which have undergone browning as the consequence of extended storage or accelerated "aging" (heating) invariably reveal the presence of more ninhydrin positive materials than were found to exist initially. Fortuitously from the analytical standpoint, seven of the major ninhydrin positive contributors appear in the first 4 hours of a 22 hour elution cycle, well in advance of the normal diet components. By the process of selective withdrawal of amino acids from the diet solution, it was established that the peak designated #5 (elution time 2 hours and 40 minutes to 3 hours) corresponds to a reaction product formed from glucose and glycine. This same peak is found to occur in simple solutions of glycine and glucose which have undergone non-enzymatic browning. By means of a cationic

exchange resin Dowex-50, (pyridine form, 200-400 mesh) the two ninhydrin positive materials could be separated from the glucose and recovered by eluting the column with 0.2N pyridine formate buffer solution (pH 2.9). Alternatively, the components of the entire mixture could be separated using either ascending or descending chromatography employing the solvent system (n-butanol-glacial acetic acid-water 2:1:1). This technique was employed to study the kinetics of non-enzymatic browning using radiolabeled glycine and glucose. The oxidized form of the ketosamine would be expected to possess ultraviolet absorptivity, ninhydrin positive reactivity and to liberate amino acid on hydrolysis.

2. Ethyl Linoleate - Peroxidation

The rate at which non-enzymatic browning occurs in the liquid diet was previously shown[5] to be dependent upon the concentration of peroxide molecules present in the medium. Also, the ability of diet-17 to induce a vitamin K deficiency in CDF rats was found to be related to either the age of the linoleate-fat soluble vitamin mixture employed or to the degree to which a freshly prepared linoleate-fat soluble mixture had been aerated. Therefore, the ease of formation of peroxides and their respective levels in the diet are important parameters to be considered in evaluating the nutritional adequacy of preparations of this type.

Early attempts to determine directly the extent to which the ethyl linoleate present in the diet had undergone peroxidation were unsuccessful. This inability to assess the degree to which this unsaturated fatty acid ester had reacted with oxygen was related to the small quantity of this material (0.2%) present relative to other lipid soluble materials, ex: Tween 80. Efforts to extract the fat soluble components of the diet with solvents and to ascertain its peroxide content by reaction with thiobarbituric acid were frustrated by the fact that the surfactant, Tween 80, also produced a red color with this reagent. Thus, in order to gain insight into the fate of the unsaturated linoleate in the diet, it was necessary to resort to model systems.

Ethyl linoleate was subjected to aeration at different temperatures. The extent of resulting peroxidation was estimated by loss of peak area using gas-liquid chromatography and by the intensity of the thiobarbituric color

developed at 530 millimicrons.

Thiobarbituric Acid Method

This method is based on the reaction between thiobarbituric acid and malonaldehyde liberated on oxidation of unsaturated fatty acids[9].

Procedure

1. Reagent:

   Dissolve 0.67 gm of thiobarbituric acid (TBA) in a sufficient volume of water to make a total volume of 100 ml. Add glacial acetic acid to bring the total to 200 ml.

2. Method:

   To 3.0 grams of ethyl linoleate previously aerated, 10 ml of carbon tetrachloride is added. Now, 10 ml of the TBA reagent is added and the tubes containing the solutions shaken for 4 minutes at the rate of 120-125 oscillation per minute. The aqueous phase is now withdrawn, heated in boiling water bath for one hour and then read at 530 mμ. The TBA number is calculated as the number of milligrams of malonaldehyde liberated per 1,000 grams of fatty acids.

A plot of the data in Table 8 yields an S shaped curve which reveals the autocatalytic nature of this process once a temperature of 30°C is exceeded. Attempts to arrest this process by the addition of 0.1% of butylated hydroxyanesole were unsuccessful.

The presence of peroxidized fatty acid or the potential for peroxidation of the unsaturated fatty acids present in the diet probably constitutes the greatest single source of diet instability. The primary sources of unsaturation in the diet are derived from the oleate moiety of the Tween 80 and the ethyl linoleate present.

The reason why peroxides are so detrimental to the chemical integrity of these diets is their ability to initiate free radical processes. One such process is the addition of ethyl cysteinate to menadione leading to the destruction of the latter's ability to function physiologically as a synthetic vitamin K. Another free radical initiated process is, as we have shown in this report, the generation of brown pigments by oxidative coupling of ketosamine units formed by reaction of glucose with amino acid.

Prevention of the onset of visible non-enzymatic browning was achieved by the addition of thiols or of compounds capable of liberating thiols. This finding was reported earlier by us[5]. The addition of thiols to a diet solution, while effective in sweeping peroxides from the medium, causes the destruction of the menadione present by formation of an addition product of the type described. The solution to the dual problems of peroxide removal and retention of vitamin K activity was found to be the substitution of menadiol phosphate, the water soluble reduced form of menadione which possesses vitamin K activity but which will not react with thiol compounds.

Ethyl Cysteinate-Menadione Adduct Formation

The accelerated onset of the hemorrhagic syndrome, associated with a vitamin K deficiency in those rats of the

CDF strain receiving liquid diet formulation #17, a formulation characterized by high levels of ethyl cysteinate (2.5 gram/liter) clearly pointed to the possibility of menadione-ethyl cysteinate interaction leading to vitamin K destruction. Nickerson et al.[10] had earlier shown that reduced glutathione reacted with menadione to form "thiodione" the name given to the resulting product. Under conditions simulating those employed in diet preparations, menadione and ethyl cysteinate were permitted to react and the reaction product was isolated.

Preparation:

Twenty-one grams of 2-methyl napthoquinone (0.14 moles) were dissolved in 1,470 ml of 95% ethanol (3A). In 490 ml of deionized water, 25.8 gm of ethyl cysteinate hydrochloride (0.14 moles) were dissolved. The cysteine solution was added to the menadione solution accompanied by agitation of solution. The mixture began to darken and evidence of gas liberation was observed. Following two days of refrigerated storage, the precipitate formed was washed with 95% ethanol. Unlike menadione, the reaction product was found to be insoluble in cold toluene. Consequently, 12 grams of crude product were dissolved in 300 ml of hot toluene. The filtered toluene solution which was now red in color was chilled in a refrigerator. The yellow crystals formed (7.5 gm) were washed with cold toluene and then dried over P_2O_5. On solution in ethanol, the yellow solution rapidly turns red. On long standing, red colored crystals appear in the solution. The latter was thought to be some oxidation product of the reaction.

Elemental analyses of the yellow product formed

in the reaction of menadione with ethyl cysteinate·HCl showed:

%C - 64.04; %H - 4.93; %N - 3.19;
%S - 10.87. Mp. 239-240°C.

The reaction product was bioassayed in male, weanling rats to determine whether it possessed vitamin K properties or whether it exhibited properties antagonistic to that of vitamin K. These results are reported in the animal experimental section.

3. Diet Stability - Evaluation of Long Term Potential

Despite the judicious combination of menadiol and a thiol compound to increase the shelf life of the liquid diet in terms of retention of optical clarity and vitamin K activity, the gain so achieved is but a temporary respite. As our earlier report indicates[5], once all of the thiol compound has been consumed in reaction with the peroxides present, the formation of newly generated peroxides (free radicals) causes visible non-enzymatic browning to resume. In addition, even in the absence of linoleate or Tween 80, peroxides are found to be generated in reaction of amino acid and aldose in the presence of oxygen.

More germane to the issue of diet stability and nutritional adequacy is the question of the nutritional significance of the physical appearance of browning within the diet solution. Restated in chemical terms, the issue becomes whether the chemical reactions occurring prior to the visible appearance of browning are the ones in which irreversible nutritional losses occur and/or in which toxic intermediates are generated. The failure of brown polymer (formed in the reaction of glycine and glucose) when added to a freshly prepared diet solution to cause a reduction in the normal rate of growth of rats indicates the innocuousness of this material. Significantly enough, however, the inability of reduced glutathione, or homocysteine thiolactone to prevent the nutritional deterioration of liquid diets in which they were contained, while successfully inhibiting the appearance of visible browning (Figure 3), clearly indicates that nutritional changes

have occurred prior to browning. Thus, it would seem that the intermediate colorless ketosamines formed from amino acid-glucose interaction may be the products responsible for the observed toxicity during browning. Petit et al.[11] have found similar evidence in their study of the reaction products of glucose and glycine.

In comparison with conventional foods, therefore, liquid diets are, by their chemical nature, inherently more prone to nutritional deterioration. This instability is the result of the fact that liquid diets contain a maximum number of functionally reactive aldehyde and amino groups compared to conventional foods in which, for example, the carbohydrate source is starch or sucrose and in which protein is the precursor of the amino acids. In these latter polymeric materials, the free reactive functional groups are limited to chain terminals and pendant side chains. The retention of nutritional adequacy and the small amino acid losses in liquid diet solutions which have been refrigerated (0-4°C) or frozen (-6°C) for periods exceeding one year, clearly indicates that under these conditions interaction of amino acid with glucose is inappreciable. At higher temperatures, however, the sole assurance that liquid diets will deliver their full nutritive potential is prompt usage shortly after preparation.

TABLE 1

KINETICS OF REACTION OF GLUCOSE (2M) WITH GLYCINE (1M) AT 60°C IN WATER USING:

50μc Glycine-1-C^{14}/ml, run (2)
15μc Glycine-C^{14}/ml, run (3)
30μc Glucose-C^{14}/ml, run (4)

Day	% Glycine		Glycine (moles)		% Glucose	Glucose (moles)
	(2)	(3)	(2)	(3)	(4)	(4)
0	100	100	1.0	1.0	100	2.00
3	94.0	92.3	0.940	0.923	94.0	1.880
5	89.2	87.6	0.892	0.876	94.2	1.884
7	86.7	86.6	0.867	0.866	91.9	1.838
10	80.9	79.7	0.809	0.797	90.8	1.816
14	73.3	68.9	0.733	0.689	82.9	1.658
17	67.5	66.5	0.675	0.665	77.7	1.554
20	64.3	49.6	0.643	0.496	75.5	1.510

TABLE 2

KINETICS OF REACTION OF (2M) GLUCOSE WITH (1M) GLYCINE AT 60°C IN WATER USING:

50μc Glycine-1-C^{14}/ml, run (2)
15μc Glycine-C^{14}/ml, run (3)
30μc Glucose-C^{14}/ml, run (4)

IN TERMS OF PRODUCTS FORMED

Day	Run #	Ketosamine %	Ketosamine moles/l	Polymer %	Polymer moles/l
0	2	0	0	0	0
	3				
	4				
3	2	6.0	0.06	0	0
	3	7.7	0.077	0	0
	4	6.0	0.120	0	0
5	2	10.4	0.104	0.4	0.004
	3	11.7	0.117	0.7	0.007
	4	5.1	0.102	0.7	0.014
7	2	12.5	0.125	0.8	0.008
	3	12.5	0.125	0.8	0.008
	4	7.3	0.146	0.8	0.016
10	2	17.3	0.173	1.7	0.017
	3	17.8	0.178	2.5	0.025
	4	7.5	0.150	1.7	0.034
14	2	22.3	0.223	4.3	0.086
	3	23.8	0.228	7.3	0.146
	4	12.1	0.242	5.0	0.100
17	2	25.8	0.258	6.7	0.134
	3	26.8	0.268	6.7	0.134
	4	13.4	0.268	8.8	0.176
20	2	26.8	0.268	8.9	0.178
	3	30.6	0.306	19.9	0.398
	4	12.2	0.244	12.4	0.248

TABLE 3

CALCULATION OF 2nd ORDER RATE CONSTANT FOR REACTION
2M GLUCOSE -- 1M GLYCINE IN SOLUTION AT 60°C

$$\left(\frac{1}{2.3} \times \frac{1}{t} \times \frac{1}{(a-b)} \quad \log \frac{b}{a} \frac{(a-x)}{(b-x)} = k\right)$$

a = glucose - 2.0 moles/l, b = glycine - 1.0 moles/l

t (days)	$\log \left(1/2 \frac{(a-x)}{(b-x)}\right)$	2.3 (k) (moles^{-1} day^{-1})	k (moles^{-1} day^{-1}
0	0	0	0
3	log 1.010 = 0.00432	0.00144	0.00063
5	log 1.069 = 0.02898	0.00580	0.00252
7	log 1.060 = 0.02531	0.00361	0.00157
10	log 1.132 = 0.0534	0.00539	0.00234
14	log 1.162 = 0.0653	0.00465	0.00202
17	log 1.160 = 0.0645	0.00380	0.00165
20	log 1.172 = 0.0690	0.00345	0.00150
		0.02784	
	mean	0.0039	mean 0.00170

TABLE 4

ELEMENTAL COMPOSITION OF BROWN POLYMERS FORMED IN THE REACTION IN AQUEOUS SOLUTION OF 2M ALDOSE WITH 1M GLYCINE AT 60°C

Polymer Composition	Empirical Formula	Elemental Analysis*			
		%C	%H	%N	%O
Ribose-Glycine	$C_{10.6}H_{12.5}N_1O_{4.3}$	56.96	5.62	6.78	31.14
Deoxyribose-Glycine	$C_{20.8}H_{21.4}N_1O_{3.9}$	63.88	5.53	3.59	16.00
Glucose-Glycine	$C_{12.2}H_{13.2}N_1O_{3.2}$	65.06	5.90	6.27	22.77
Deoxyglucose-Glycine	$C_{24.5}H_{31.5}N_1O_{10}$	59.26	6.31	2.74	31.69

*Schwarzkopf Laboratories, Maspeth, N. Y.

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TABLE 5

ETHYL LINOLEATE AERATED AT ROOM TEMPERATURE AT RATE OF 3 LITERS PER MINUTE

TBA Number	Time in Hours
1.25	00
2.20	1.5
3.70	3
5.90	6
6.40	9

TABLE 6

ETHYL LINOLEATE AERATED AT ROOM TEMPERATURE AT RATE OF 3 LITERS PER MINUTE--- OXIDATION DETERMINED VIA TBA METHOD

OD at 530 mμ	Time in Hours
0.823	0
0.958	2
1.120	7
1.210	24
1.95	31
3.432	97

TABLE 7

ETHYL LINOLEATE -- EXTENT OF OXIDATION AT AMBIENT TEMPERATURES AERATED AT RATE OF 0.5 LITERS PER MINUTE

Ethyl Linoleate (4 gm)	Additive (0.05 gm)	OD at 530 mμ
(+)	0	0.608
(+)	H_2O	0.724
(+)	Ammonium molybdate	0.718
(+)	Cobalt acetate	2.000
(+)	Copper acetate	2.300

TABLE 8

EFFECT OF TEMPERATURE ON EXTENT OF OXIDATION OF ETHYL LINOLEATE (3 gm) FOLLOWING 30 MINUTES EXPOSURE IN 50 ML BEAKER AS DETERMINED BY GLC

Temperature °C	Extent of Peroxidation (%)
8	17.5
21	19
37	48
55	95
69	95

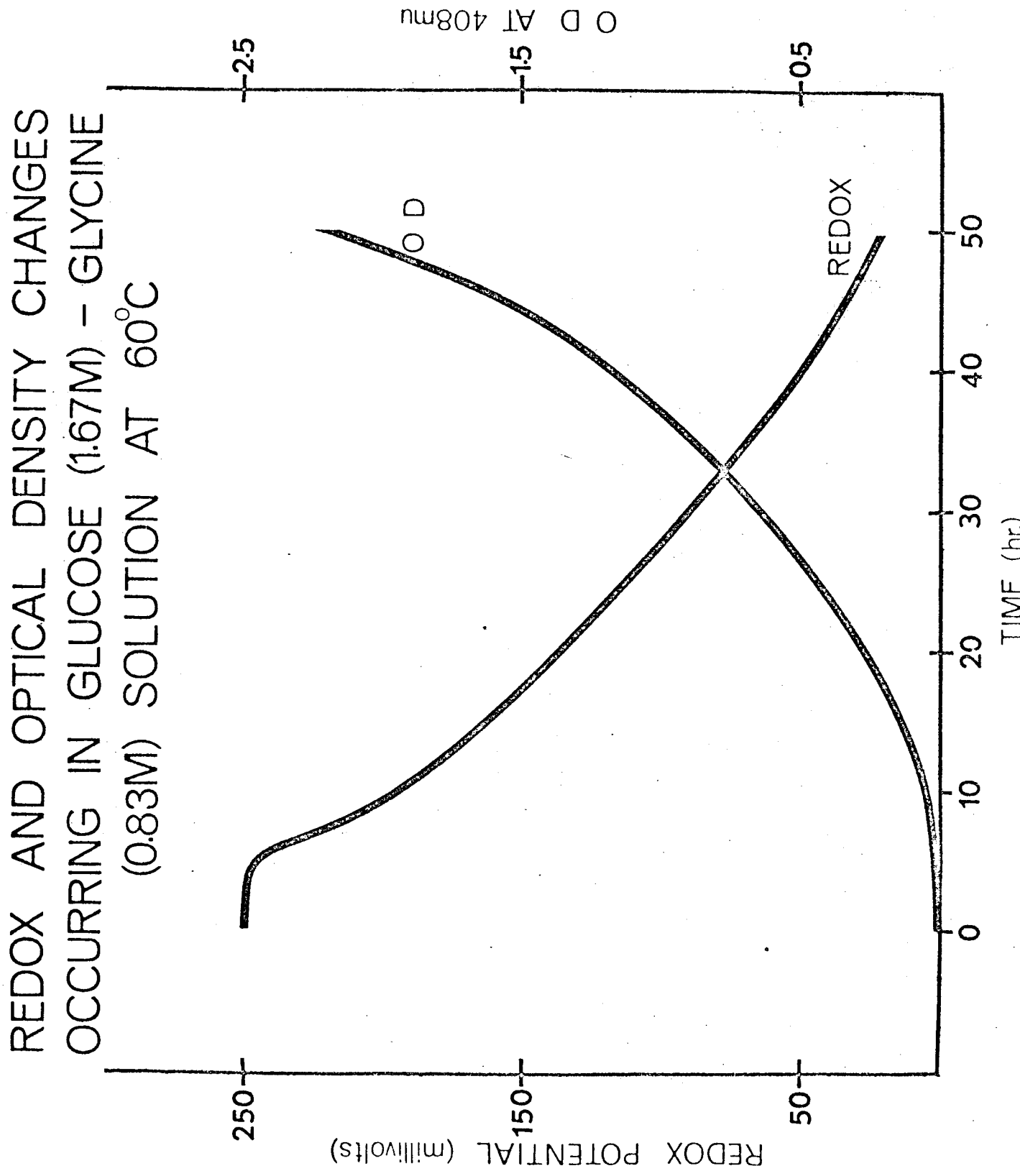
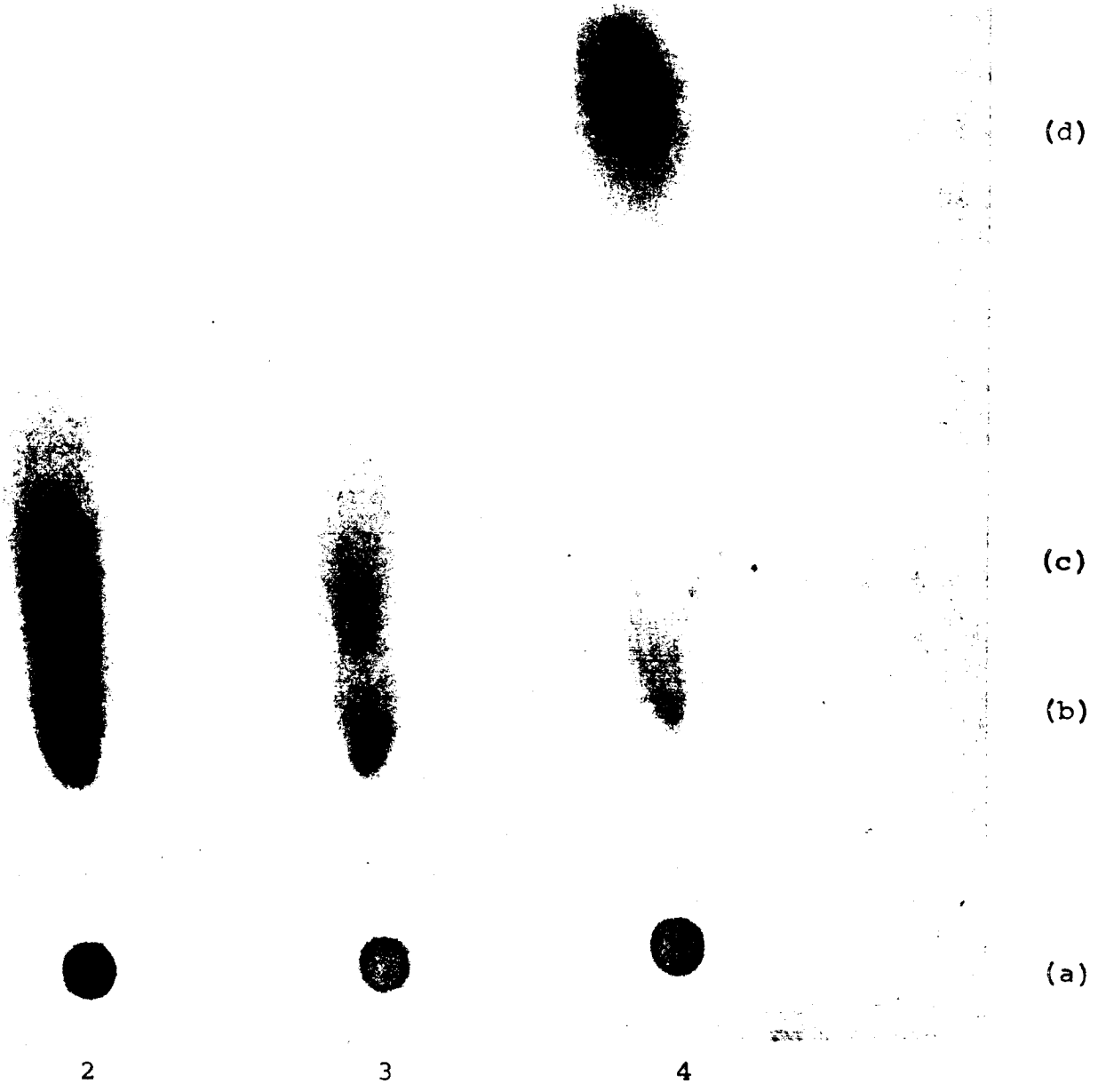

REDOX AND OPTICAL DENSITY CHANGES
OCCURRING IN GLUCOSE (1.67M) - GLYCINE
(0.83M) SOLUTION AT 60°C
O D AT 408mu
2.5
1.5
0.5
O D
REDOX
250
150
50
REDOX POTENTIAL (millivolts)
0
10
20
30
40
50
TIME (hr.)

FIG. 1

RADIOAUTOGRAPH OF CHROMATOGRAPHED REACTION PRODUCTS FORMED BY GLYCINE (1M)-GLUCOSE (2M) IN SOLUTION AT 60°C. AFTER 17 DAYS

2 = glycine-1-C^{14} specific activity 50 μc/mmole
3 = glycine-C^{14} specific activity 15 μc/mmole
4 = glucose-C^{14} specific activity 15 μc/mmole



(a) = fluorescent brown polymer
(b) = product
(c) = glycine
(d) = glucose

FIG. 2

FIG. 3

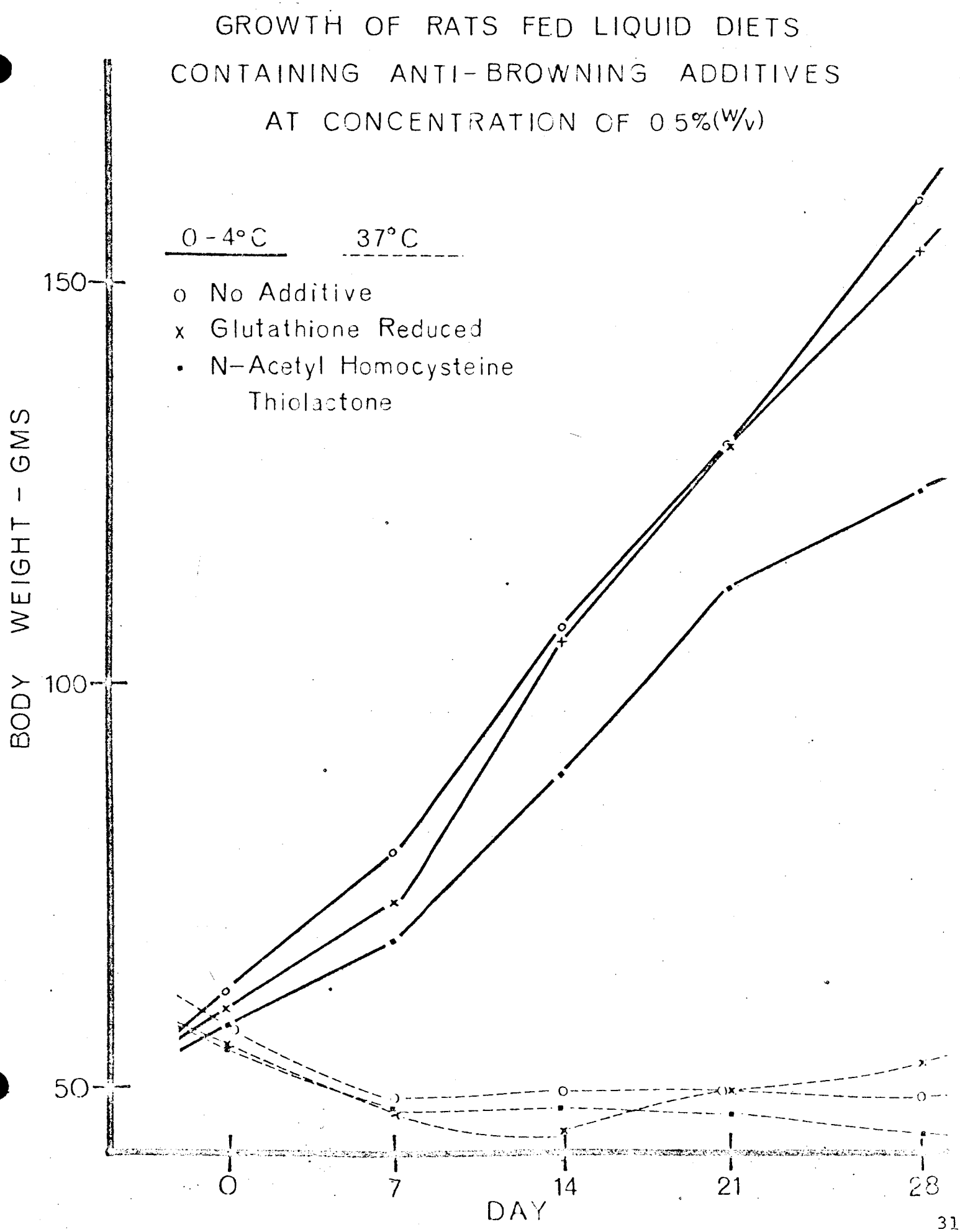
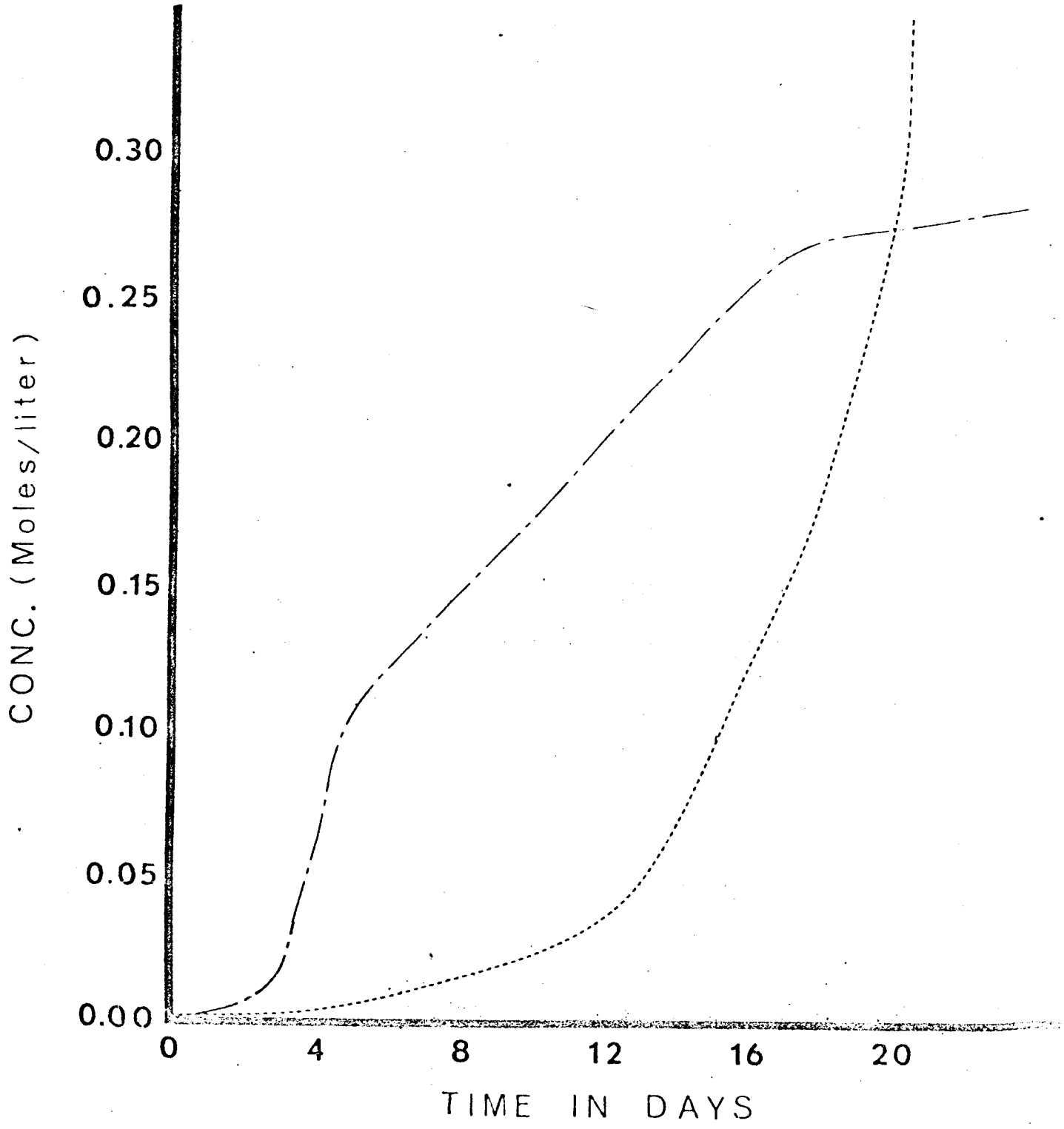

GROWTH OF RATS FED LIQUID DIETS
CONTAINING ANTI-BROWNING ADDITIVES
AT CONCENTRATION OF 0.5%(W/V)
0-4°C
37°C
o No Additive
x Glutathione Reduced
• N-Acetyl Homocysteine Thiolactone
BODY WEIGHT - GMS
150
100
50
0
7
14
21
28
DAY

FIG. 4

REACTION OF 2M GLUCOSE– 1M GLYCINE IN AQUEOUS SOLUTION AT 60° C



Ketoseamine ——·——·——; Polymer ·····

ANIMAL EXPERIMENTS

Introduction

The animal experiments conducted during this contract year were primarily concerned with the relationship of the nutritional value of liquid diets to their chemical stability. Three major areas were studied:

1) Long term stability studies in which liquid diets were refrigerated or frozen for one year and then chemically and biologically assayed.

2) Short term stability studies where diets were subjected to heat stress followed by attempts to identify and isolate the factors responsible for the resulting toxicity and growth retardation.

3) Blood coagulation studies where the dietary constituents responsible for inducing hemorrhage in Fischer strain rats were identified and the mechanism of action elucidated.

STABILITY STUDIES

1. Long Term Stability Studies

In our last Annual Report[5], stability studies were described in which Codelid* diet-14 was stored under several environmental conditions for eight months and then chemically and biologically assayed. The results showed that when refrigerated or frozen for eight months, the diets retained their nutritional value as measured by growth response. Only minor nutrient losses, as the result of chemical degradation, occurred under these conditions. The data

*Codelid is an acronym used to designate completely defined liquid diets.

described below were obtained with diets refrigerated or frozen for 12 months and represent the completion of the one year stability program heretofore outlined[5]. The diet, (Codelid diet-14) (Tables 9 and 10), storage conditions, and all procedures were the same as those employed in our previous experiments[5].

Table 11 and Figure 5 compare the response of rats fed the frozen or refrigerated diets with the response of those fed an identical diet at zero time, freshly prepared diet-14, or Lab Blox. The growth of all groups fed the Codelid diets was less than for those consuming Lab Blox. Rats fed the refrigerated or frozen diets exhibited equivalent growth rates which were slightly better than the growth rates of the control rats or those consuming the freshly prepared diet. Diet and water consumption of all Codelid diet groups were essentially the same. Diet utilization (gm gain/ml diet consumed) was equivalent for rats consuming fresh diet-14 and the two stored diets. The lower diet utilization and slower growth of the control group was probably due to the particular rat shipment and the longer experimental period (28 vs. 24 days) rather than to a dietary effect.

Table 12 and Figure 6 show the results of amino acid analyses of the two stored refrigerated diets compared with the control diet and with NRC recommended requirements[12]. Partial destruction of valine, methionine, arginine, lysine, histidine, and monosodium glutamate occurred. The greatest breakdown was in the case of histidine where only 74.4% of its original concentration was found. The other degraded amino acids were present at about 85% of their original concentration. Of the amino acids measured, only degradation of methionine resulted in a slight dietary deficiency. This

deficiency, however, did not appear to adversely affect growth rate.

Amino acid breakdown was much less in the frozen diet. Here, only slight losses in serine and methionine were observed. All the other amino acids measured appeared to be stable.

These data conclusively show that when refrigerated or frozen, Codelid diet-14 retains its nutritive value, as measured by growth of rats, for a period of at least one year. The results also indicate that freezing retards amino acid loss more than refrigeration. In both instances, however, amino acid losses are very small.

2. Heated Diets and Growth Inhibition

Our previous studies showed a marked loss in the nutritional value of Codelid diet-14 when it was stored at room temperature or at 60°C[5]. Rats fed the diet after it had been stored at room temperature for 4 months decreased their diet and water consumption and lost body weight. Rats fed the diet after storage at 60°C for 12 days exhibited a similar pattern and died within three weeks. In all cases, growth retardation and death were accompanied by the "browning" of the diets. It was hypothesized that the adverse effects could be the consequence of amino acid deficiencies, formation of hydroxylated amino acid antagonists and/or the formation of toxic brown polymers. The present study was conducted to determine the nutritional effect of dietary amino acid losses resulting from heat treatment and to ascertain the toxicity of the brown polymer.

The experimental design and results are shown in Table 13. Diets were formulated to contain an amino acid pattern simulating Codelid diet-14 after exposure to 60°C for

12 days (Table 14). The chromatographic results of our earlier studies were used to establish this pattern[5]. The composition of the basal diet and the amino acid pattern (Codelid diet-14) prior to heat treatment are shown in Tables 9 and 10. The polymer tested in this experiment was the product of a glycine-glucose interaction.

The data in Table 13 shows that the loss of amino acids due to heating creates deficiencies and imbalances which are reflected in dramatic growth retardation (Groups IV, V and VI). This was particularly evident when tryptophan was removed from the diet Group IV. Addition of the brown polymer (glucose-glycine condensate) to the complete diet or to the diet deficient in amino acids did not adversely effect growth rate (Groups II, III and V). Diet and water consumption were normal for all groups receiving the complete amino acid diets regardless of the presence of the polymer. Marked reduction in diet and water consumption occurred in the groups ingesting the amino acid deficient diets.

These findings indicate that depending upon the severity of the heat treatment, the growth retardation and toxicity heretofore observed in rats consuming Codelid diets may be due to independent factors. In the present study, growth depression as well as reduced diet and water intake resulted from feeding an amino acid mixture simulating a mixture kept at 60°C for 12 days. These results were not complicated by the presence of hydroxy amino acids, brown polymer or other addition products in the diet. This type of situation could well occur under less severe environmental stress as, for example, limited storage at room temperature. On the other hand, at elevated temperatures, the formation

of toxic products in addition to amino acid deficiencies may occur. In such cases, as in our earlier studies[5], the deficiencies could add to, or could be masked by, the effects of the toxic factors.

The lack of toxicity of the glycine-glucose condensate used in this study suggests that the polymer or pigment which renders the diet brown under conditions of "browning", is not toxic. If this also holds true for other amino acid-glucose condensates the toxicity observed in our earlier studies could have been due to the presence of hydroxylated amino acids as well as other antagonists such as linoleate hydro-peroxides.

Finally, the body weight loss observed when tryptophan was omitted from the diet warrants comment. Since this amino acid is required primarily for maintenance[13] its absence is most critical when growth becomes secondary to maintenance. Such a situation occurs when rats ingest diets deficient in amino acids required for growth and, consequently, the absence of tryptophan in Group VI prevented the maintenance of body weight.

Although the results of the previous experiment suggest that the growth retardation observed in rats fed heat treated diets is due primarily to amino acid deficiencies resulting from amino acid destruction, they do not show whether the products of destruction also cause growth inhibition or toxicity. The following experiments were, therefore, set up to test these possibilities and to determine whether such products, if formed, are related to specific amino acids. The experimental designs and growth data are shown in Tables 15 through 21.

The data presented in Tables 15 through 19 were obtained from experiments in which amino acids were factorially excluded from the diet prior to heating and then added in the amounts called for in Codelid diet-14 after the 6 day 60°C heat treatment. The selected amino acids excluded from the diets were those found to be most labile when the complete diet was heated. These included histidine, arginine, lysine monosodium glutamate (MSG) and glycine. Since tryptophan was not chromatographically assayed, it was also excluded from the diets in order to determine whether it contributes to the growth inhibitory properties of heated diets.

The data show that, with the exception of tryptophan, the exclusion of the aforementioned amino acids from the diet prior to heating and their addition afterwards, reversed the body weight loss observed when rats were fed heated diets. In these instances, however, the growth rate never reached the level attained with a non-heated diet (Table 15).

The factorial elimination of glycine, histidine, arginine and MSG prior to heating resulted in a progressive increase in growth rate (Table 15). This trend did not hold, however, when lysine was excluded from a diet already void in these amino acids (Table 16).

The exclusion of carbohydrates from the diet prior to heating and their addition afterwards also resulted in diets which reversed the body weight loss one observes when rats are fed diets which are complete before heating. In fact, when monocalcium fructose 1,6-diphosphate (FDC), glucose and potassium and magnesium gluconates were absent from an otherwise complete diet during heating, the diets produced growth almost equivalent to that attained with the non-heated control diet (Table 16).

In all cases, consumption of heat treated diets was less than for the non-heated control diet. However, there was a trend towards increased diet consumption and improved utilization (grams gain/ml diet consumed) when amino acids or carbohydrates were excluded from the diet prior to heating. Arginine and lysine were exceptions to these trends. In the former case, diet utilization improved but consumption did not increase and in the latter case, consumption and utilization were markedly reduced.

The data in Table 17 shows the amino acid composition of the diets fed to the rats. The values are expressed as a percentage of the amino acids normally present in fresh Codelid diet-14 (Table 10). The diets represent heated diets to which the excluded amino acids or carbohydrates were added after heating. Amino acid destruction (Table 17) and deficiencies (Table 18) decreased with the factorial exclusion of the amino acids and carbohydrates prior to heating. Although several specific ninhydrin positive compounds disappeared with the exclusion of specific amino acids, there was no general trend towards a decrease in the number or concentration of new compounds (Table 19). The exclusion of carbohydrates on the other hand, resulted in diminished amino acid destruction and in the disappearance of most new ninhydrin positive compounds. When glucose was excluded before heating, only methionine was destroyed enough to result in a deficiency. However, when in addition to glucose, FDC and the gluconates were absent from the diet during heating, no essential amino acid deficiencies resulted.

As indicated in our earlier experiments, the data suggest that the growth inhibitory properties of heated diets are due primarily to the amino acid deficiencies created. In

the present study, growth inhibition was reduced by excluding amino acids from the diet prior to heating and adding them afterwards. However, this procedure reduced, but did not completely eliminate the essential amino acid deficiencies resulting from heating. Consequently, while growth improved, it never equalled that attained with a non-heated diet. Only when all of the carbohydrates were excluded from the diet was growth virtually equivalent to the control group (rats fed non-heated diet) and only then were all of the essential amino acids present in adequate amounts.

One cannot rule out the possibility that other factors also contribute to the growth inhibitory properties of heated diets. Such factors could include deficiencies due to destruction of components of the non-amino acid moiety of the diet with possible formation of toxic products; amino acid imbalances resulting from drastically altered dietary amino acid patterns; and formation of toxic products resulting from amino acid destruction. The data obtained in these experiments do not permit a clear-cut differentiation between these factors. Almost all ninhydrin positive compounds detectable by chromatography disappeared simultaneously with the disappearance of essential amino acid deficiencies. Similarly, when the essential amino acids were present in adequate amounts, total amino acid destruction was small and disruption of the normal amino acid pattern minimal, thus making it difficult to differentiate between imbalance, deficiency and toxicity as growth inhibitory factors.

Finally, the excellent growth observed when all the carbohydrates were excluded from the diet suggests that the non-amino acid moiety does not independently contribute to the observed growth retardation. Although the gluconates

were excluded as the potassium and magnesium salts and the fructose as the calcium salt, the improved growth response was probably due to decreased interaction between reducing sugars and amino acids rather than to the elimination of calcium, potassium or magnesium.

The last experiment in this series was designed to determine whether the growth retardation of rats fed heated diets was due to amino acid deficiencies or to the formation of toxic products resulting from amino acid destruction. Female, CFE weanling rats were used in this study. The experimental design and results are shown in Tables 20 and 21. All amino acid-glucose mixtures were added to a complete, freshly prepared diet. The amino acids of each supplement were provided in the same proportions and amounts as in Codelid diet-14. Glucose was added to each amino acid mixture in the ratio of 2 moles glucose/mole amino acid. The amino acid-glucose mixtures were added to the complete diet directly or heated 6 days at 60°C and then added to the complete diet.

The results show that in the presence of adequate amounts of the essential amino acids, the addition of amino acid-glucose solutions did not severely depress growth rate. When a non-heated or heated glycine-glucose mixture (Table 20, Group 2,3) was supplemented in the amounts stipulated above, normal growth was observed. Only when the dietary level was increased threefold was slight growth depression observed (Group 4). The addition of an arginine, histidine, lysine and glucose solution to the diet did not depress growth rate. However, when this solution was heated prior to supplementation, growth retardation resulted. In contrast, good growth was observed when a heated mixture of arginine, histidine, lysine, glucose and glycine was added to the diet (Group 3)

whereas addition of the non-heated solution caused growth inhibition.

With the exception of the control group (Group 1), diet consumption was virtually equivalent in all groups. Consequently, diet utilization decreased in those cases where growth inhibition occurred. Water consumption was fairly uniform in all groups with the exception of those fed diets containing supplemental arginine, lysine, histidine and glycine where intake increased slightly.

The failure to produce growth depression through supplementation of a glycine-glucose reaction mixture at 1x and 2x, the level of glycine normally present in the diet is in accordance with our earlier observations (Tables 13 and 15). The slight growth depression resulting from a threefold increase in supplementation of this reaction mixture suggested that the end products of the glycine-glucose interaction are non-toxic or are not present in sufficiently high amounts to be harmful. If the products are toxic, the latter explanation may account for the slight growth depression observed when diet is supplemented with a glycine-glucose solution heated for 23 days rather than for the standard 6 day period. Thus, the formation of toxic products after a 6 day heat phase may be too small to be effective whereas after 23 days, it may be enough to inhibit growth.

Adrian et al.[11] produced growth inhibition in rats fed a casein diet supplemented with a previously heated glycine-glucose solution. However, their conditions of heating (90°C for 6 hours) were different from ours and may have resulted in qualitative and quantitative differences in the products formed. Chromatographic and infra-red analyses suggest that under our conditions, the product of glycine-glucose interaction is either a ketosamine or its enolamine tautomer.

Adrian et al.[11] did not identify the end products of their reaction mixture. Nevertheless, it is clear that the severity and duration of heating can alter the end product formation at least to the extent of inducing differences in biological response.

The growth depression observed when a heated solution of arginine, histidine, lysine and glucose is added to the diet is undoubtedly due to the presence of new products in the reaction mixture. The possibility that the depressed growth was due to an imbalance of amino acids is ruled out by the good growth of rats fed a diet containing an unheated mixture of the same amino acids. When glycine was added to an unheated mixture of arginine, histidine and lysine (Group 8) growth inhibition resulted. However, when this mixture was heated, growth was essentially equivalent to that of the control group (Group 1).

In view of the growth depression observed when rats were fed a diet supplemented with a heated amino acid mixture containing arginine, histidine, lysine and glucose (Group 6), it was surprising that a heated mixture containing glycine in addition to these ingredients (Group 9) did not also depress growth. Apparently the newly formed ninhydrin positive compounds were not toxic at the levels present in the diet (Table 21). Since the diet fed to Group 6 contained the same amino acid mixture minus glycine, one might expect a similar chromatogram except for the absence of peaks directly attributable to glycine. If this is the case, the growth inhibition observed in this group would be due to something other than the presence of newly formed ninhydrin positive compounds. This would also seem to be true for Group 8 where the concentrations and number of new ninhydrin compounds were less

than for the diet containing the heated amino acid mixture (Group 9) but where slower growth was observed. Clearly, further experimentation is necessary to elucidate the factors responsible for these unusual findings.

3. The Effect of Anti-Browning Agents

In our last Annual Report[5], evidence was presented to show that the browning of heated diets is an oxidation process which can be inhibited by such thiol containing compounds as reduced glutathione, N-acetyl homocysteine thiolactone (HAR) and sodium-S-cysteine sulfonate (Bunte Salt). The effect of these browning inhibitors on the toxicity of Codelid diet-14 was studied in the experiment described below.

Freshly prepared diets were supplemented with 0.5% (w/v) additive and then kept at 0°-4°C or 37°C for 45 days, or at 60°C for 6 days. They were then chemically assayed and fed to rats for three weeks.

The data in Table 22 and Figure 3 show that the additives did not prevent the growth inhibition resulting from feeding heated diets. Without exception, body weight loss occurred when rats were fed diets kept at 37°C for 45 days. Diets heated at 60°C for 6 days caused body weight loss and death regardless of the presence of additives. When refrigerated diets were fed, growth rate was slower than usually obtained with Codelid diet-14 (Table 11). The presence of 0.5% homocysteine thiolactone in the refrigerated diet depressed growth slightly whereas the inclusion of Bunte Salt improved growth rate.

Consumption of the refrigerated diets was fairly uniform between groups although somewhat lower than usually observed (Table 11). When heated diets were fed, consumption was markedly reduced particularly if the diets had been

heated to 60°C. Water consumption also decreased when the rats ingested the heat treated diets.

Tables 23, 24 and 25 show the chemical data obtained in these studies. Amino acid losses occurred in all instances at 37°C with very little protection provided by the thiol additives. Glutathione appeared to be the most protective additive at 37°C but at 60°C it was ineffective.

Methionine deficiency occurred in all diets kept at 37°C. Histidine deficiency resulted when no additives were in the diet and threonine deficiency occurred in diets kept at 37°C and containing HAR or glutathione. Interestingly, in the absence of any additives and at 60°C, threonine was not destroyed.

The number of new ninhydrin positive compounds formed from amino acid destruction was not inhibited by the presence of antibrowning agents (Table 25). The amount of each new compound formed was effected by these agents. When glutathione was present in diets kept at 37°C, 2 ninhydrin positive compounds decreased in quantity, 6 increased, one new peak was formed, and one disappeared. Under the same conditions but with Bunte salt in the diet, 5 ninhydrin positive peaks increased, 3 decreased, 1 was not altered and 4 new peaks appeared on the chromatogram. The HAR appeared to be the most effective in reducing the quantity of each new peak formed. In this instance, at 37°C 9 peaks decreased, none increased and 4 new peaks were formed. The identification of the ninhydrin positive peaks appearing in all cases and their elution pattern is shown in Figure 7.

The biological data obtained in this study suggest that amino acid deficiencies as well as toxic factors are responsible for the body weight loss and death of rats fed

the heated diets. This is in contrast to our earlier findings (Table 15 and 16) where few deaths occurred and where we attributed the growth retardation primarily to amino acid deficiencies. Comparison of the chemical data obtained in these different experiments show that the amino acid deficiencies in the 60°C (GSH) containing diet were more severe than previously observed in a diet of the same composition heated to 60°C for 6 days in the absence of GSH (Table 24 vs. 18). Furthermore, while the number and distribution of new ninhydrin positive compounds were similar in both instances, the amounts formed in the GSH containing diet were considerably larger (Table 19 vs. 25). Similarly, although the amino acid deficiencies were not as severe in the 37°C diets, the formation of new ninhydrin positive compounds was greater than heretofore observed in similar diets heated to 60°C and may have been responsible for the observed toxicity in the present study. At this time, it is difficult to explain why diets kept at 37°C for 45 days have higher concentrations of newly formed ninhydrin positive material than similar diets kept at 60°C for 6 days. One possibility may be that the newly formed compounds are rapidly destroyed at the elevated temperature by organic peroxides or other intermediates which act catalytically.

The results of this study show that at the levels employed, GSH, HAR and Bunte salt do not prevent amino destruction or the formation of new ninhydrin positive compounds in diets kept at 37°C for 45 days or at 60°C for 6 days. Although ineffective at 60°C, they do inhibit browning at 37°C (see chemical section). However, since the present findings show growth retardation and toxicity to occur in all diets kept at 37°C, it is unlikely that browning *per se* contributes to this biological response.

TABLE 9

COMPOSITION OF TEST DIETS

Formulation and Number	14	16	17
Carbohydrates:	gm/L	gm/L	gm/L
Glucose	344.5	342.9	323.8
Glucono-delta-lactone	13.03	13.05	13.03
Minerals, Macro			
Ferrous Ammonium Sulfate	0.70	-	0.70
Ferrous Gluconate	-	0.86	-
FDC(1)	25.00	25.25	25.00
Magnesium Oxide	0.38	0.37	0.38
Potassium Hydroxide	3.08	3.08	3.08
Sodium Bicarbonate	-	1.75	-
Sodium Chloride	3.50	2.40	3.50
Sodium Hydroxide	2.29	2.05	0.70
Minerals, Trace	mg/L	mg/L	mg/L
Ammonium molybdate·$4H_2O$	3.0	3.0	3.0
Cobalt Acetate·$4H_2O$	4.5	4.5	4.5
Cupric Acetate·$4H_2O$	7.5	7.5	7.5
Manganese Acetate·$4H_2O$	130.0	130.0	130.0
Potassium Iodide	15.0	15.0	15.0
Zinc Benzoate	11.0	11.0	11.0
Vitamins, Water Soluble			
Ascorbic Acid	372.5	250.0	372.5
Biotin	0.22	0.15	0.22
B_{12} (1% trituration)	74.5	50.0	74.5
Calcium Pantothenate	37.25	25.0	37.25
Calcium Chloride	1.30g	1.25g	1.30g
Folic Acid	0.37	0.25	0.37
Inositol	186.25	125.0	186.25
Niacin	27.94	18.75	27.94
Para-aminobenzoic Acid	223.5	150.0	223.5
Pyridoxine·HCl	4.69	3.15	4.69
Riboflavin	7.45	3.75	7.45
Thiamine·HCl	3.73	2.5	3.73
Vitamins, Fat Soluble			
Vitamin A Acetate 3000 IU/mg	5.0	5.0	5.0
Calciferol 40 IU/µg	3.5µg	3.5µg	3.5µg
Ethyl Linoleate	2.0g	2.0g	2.0g
Menadione	2.1	2.1	2.1
Polysorbate 80	3.0g	3.0g	3.0g
α-tocopherol acetate 1 IU/mg	25.0	25.0	25.0

(1) Monocalcium fructose 1,6-diphosphate

TABLE 10

COMPOSITION OF TEST AMINO ACID MIXTURES

Formulation and Number	14	16	17
Components	gm/L	gm/L	gm/L
Amino Acids:			
L-Arginine·HCl	8.90	4.70	8.90
L-Histidine·HCl·H_2O	2.93	2.85	3.38
L-Isoleucine	6.45	4.40	5.50
L-Leucine	8.70	7.00	7.30
L-Lysine·HCl	8.00	6.50	11.80
L-Methionine	3.03	3.15	5.39
L-Phenylalanine	5.38	3.15	7.63
L-Threonine	4.70	4.40	5.00
L-Tryptophan	1.48	1.40	1.50
L Valine	6.85	4.90	5.50
L-Alanine	-	3.20	2.30
L-Asparagine	-	-	3.95
L-Aspartate	7.63	6.75	2.30
L-Cysteine Ethyl Ester·HCl	-	0.55	2.43
L-Glutamate, Monosodium	13.38	25.60	27.95
Glycine	6.05	2.05	13.94
L-Proline	4.10	12.70	2.30
L-Serine	7.23	6.60	2.30
L-Tyrosine Ethyl Ester·HCl	5.08	8.40	3.14

TABLE 11

GROWTH RATE, DIET CONSUMPTION AND WATER CONSUMPTION OF RATS FED CODELID DIET 14 AFTER STORAGE AT 0-4°C OR (-)6°C FOR 12 MONTHS*

Storage Conditions	Av. daily body weight gain	Av. daily diet consumption	Av. daily water consumption
	gm/rat/day	ml/rat/day	ml/rat/day
Control**	3.9	26.6	12.6
Fresh diet	4.2	23.3	13.2
Refrigerated (0-4°C)	4.5	25.5	13.2
Frozen (-6°C)	4.5	24.5	10.7
Lab Blox	6.3	18.7	24.7

*Experimental period 24 days. Eight CFE male weanling rats from identified litters of 9-11 littermates allotted to each dietary treatment. Housed 2 per cage. Four rats allotted to the Lab Blox diet. For details see Table 1 of the Appendix.

**Control: freshly prepared diet at zero time. Data obtained from 12 CFE male weanlings kept on experiment 28 days. For details see Appendix, Table 1 , Annual Report 1965.

Figure 5

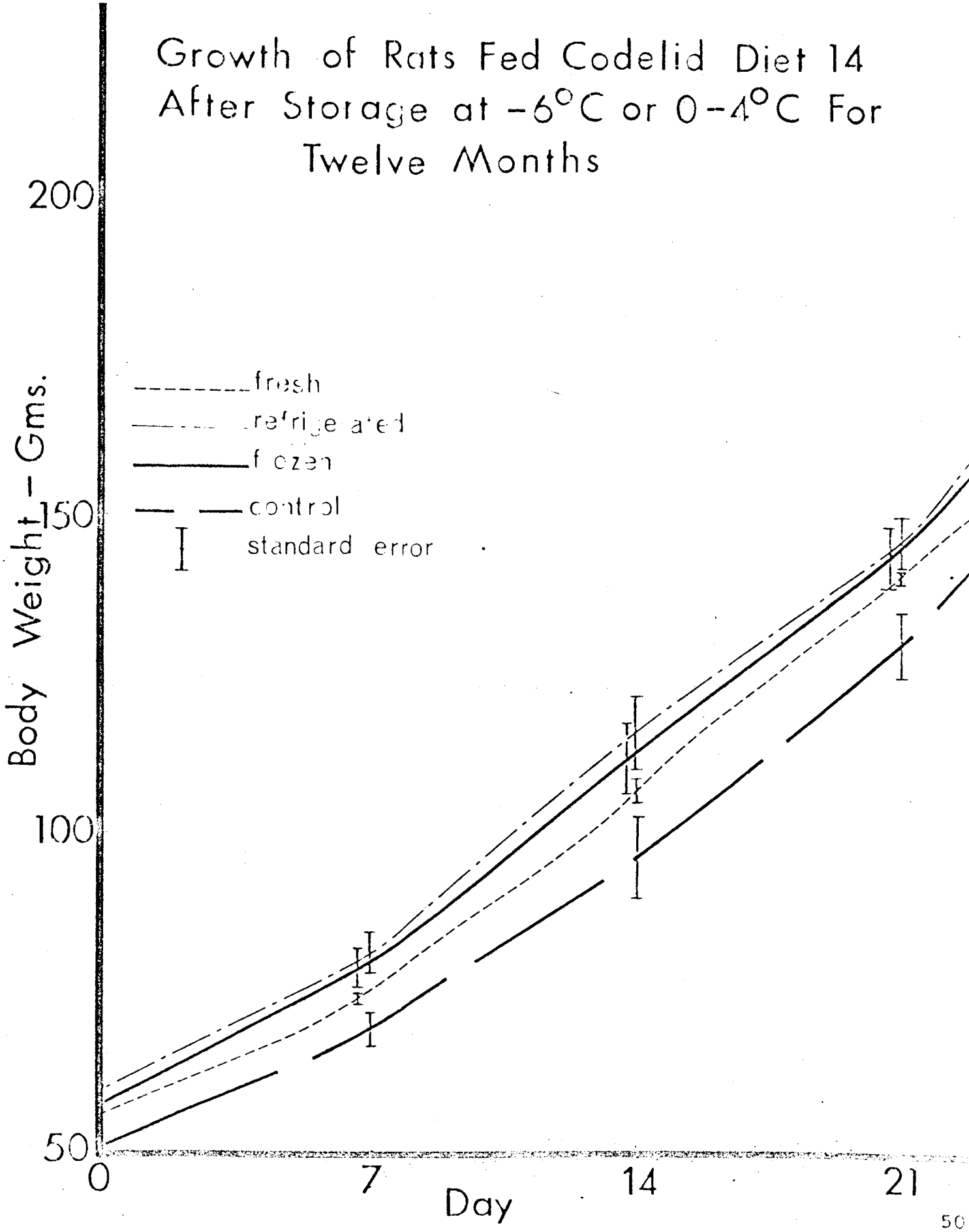

Growth of Rats Fed Codelid Diet 14 After Storage at -6°C or 0-4°C For Twelve Months
fresh
refrigerated
frozen
control
standard error
Body Weight - Gms.
200
150
100
50
0
7
14
21
Day

TABLE 12

AMINO ACID COMPOSITION OF A CHEMICALLY DEFINED LIQUID DIET AFTER STORAGE AT 0-4°C OR (-)6°C FOR 13 MONTHS*

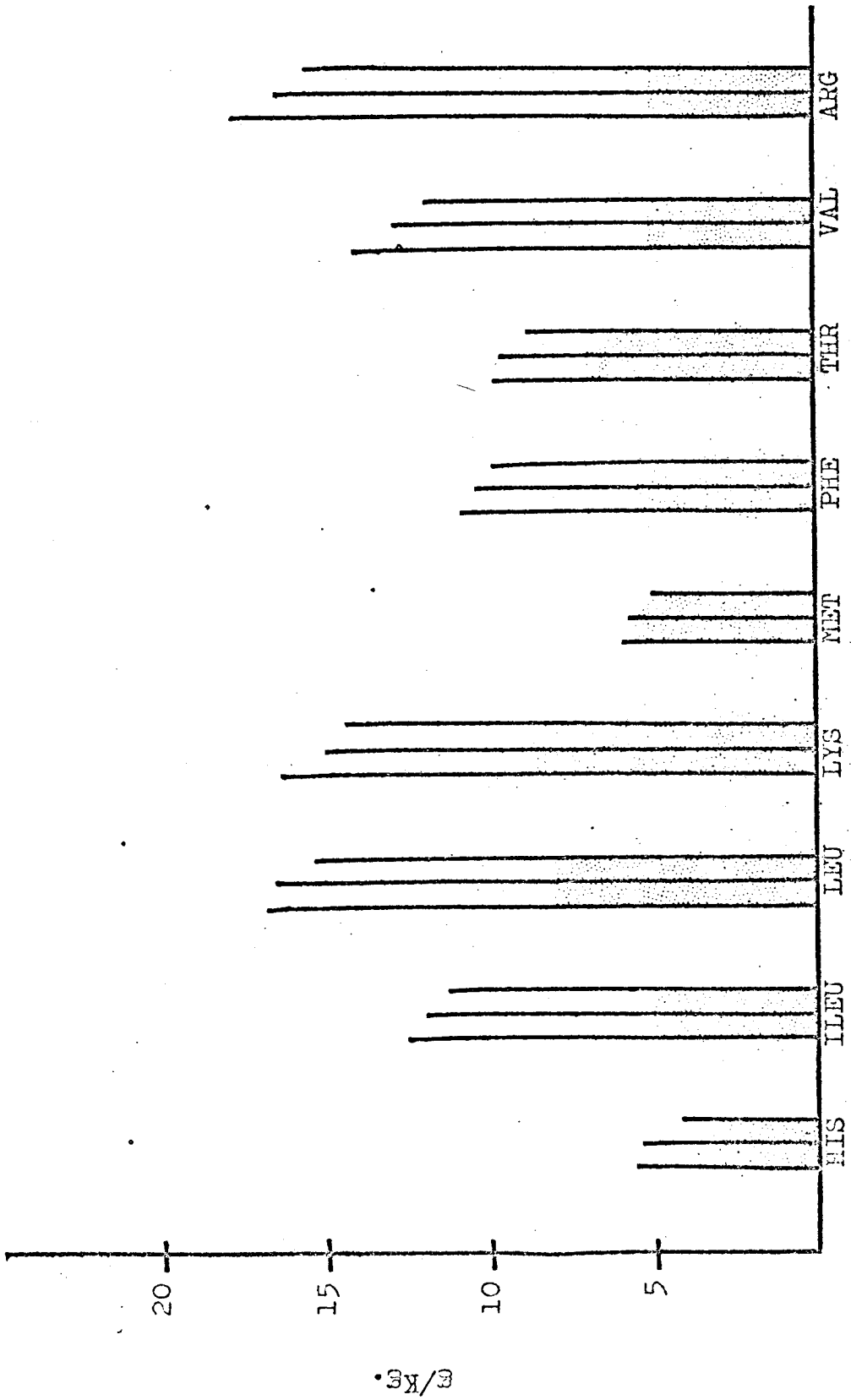
Amino Acid	Req**	Control	0-4°C	(-)6°C
		gm/kg		
Histidine·HCl·H_2O	3.00	5.61	4.17	5.35
Isoleucine	5.00	12.49	11.32	11.98
Leucine	8.00	16.89	15.26	16.51
Lysine·HCl	9.00	16.33	14.35	15.0
Methionine	6.00	5.91	5.0	5.72
Phenylalanine	6.00	10.82	9.84	10.35
Threonine	5.00	9.67	8.75	9.63
Tryptophan	1.50	-------	Not determined	-------
Valine	7.00	14.12	11.82	12.75
Arginine·HCl	5.00	17.72	15.56	16.65
Aspartate	2.30	15.35	14.46	14.0
Glycine	13.94	12.54	11.70	11.63
Monosodium glutamate	27.95	26.44	22.86	23.70
Proline	2.30	8.25	9.23	9.28
Serine	2.30	15.00	13.60	13.42

*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).

Figure 6

EFFECT OF REFRIGERATING OR FREEZING A CODELID DIET FOR 13 MONTHS ON ESSENTIAL AMINO ACID STABILITY



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

TABLE 13

GROWTH OF RATS FED DIETS DESIGNED TO SIMULATE CODELID DIET 14 AFTER ITS EXPOSURE TO HEAT*

Group	Dietary Treatment	Av. daily body weight gain	Av. daily diet consumption	Av. daily water consumption
		gm/rat/day	ml/rat/day	ml/rat/day
I	Diet 14	4.2	23.3	13.2
II	Diet 14+Polymer**-1gm/L	3.9	22.3	11.2
III	Diet 14+Polymer-5gm/L	4.4	22.7	13.1
IV	Diet 14+A.A. as after 60°C ***	0.2	16.4	7.9
V	As Gp. IV+Polymer-1gm/L	0.3	12.8	6.3
VI	As Gp. IV minus Trypt.	(-)0.4	9.7	6.5
VII	Lab Blox	6.3	18.7gm	24.7

*Experimental period 24 days except for Gps. II and III where experimental period was 21 days. Ten CFE male weanling rats from identified litters of 9-11 littermates allotted to each dietary treatment. Housed 2 per cage. Four rats allotted to the Lab Blox diet. For details see Table 2 of the Appendix.

**Polymer: product of glucose-glycine interaction.

***For composition of A.A. pattern see Table 23 .

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TABLE 14

COMPOSITION OF AMINO ACID MIXTURE SIMULATING A MIXTURE KEPT AT 60°C FOR 12 DAYS*

Amino Acid	Concentration	Percent of Theoretical**	Percent of Requirements***
	gm/liter	%	%
L-Arginine·HCl	1.06	11.9	42.4
L-Histidine·HCl·H_2C	0.45	15.4	30.3
L-Isoleucine	2.89	44.8	115.8
L-Leucine	3.77	43.3	94.2
L-Lysine·HCl	1.11	13.9	24.8
L-Methionine	1.11	36.6	36.8
L-Phenylalanine	2.30	42.8	76.7
L-Threonine	4.07	86.6	162.8
L-Tryptophan	****	----	----
L-Valine	3.12	45.5	89.3
L-Aspartate	3.98	52.2	
L-Glutamate, Monosodium	3.25	24.3	
Glycine	2.01	33.2	
L-Proline	3.53	86.1	
L-Serine	4.39	60.7	
L-Tyrosine, Ethyl Ester·HCl	****		

*Determined chemically by column chromatography.

**Percentage of amino acids normally present in fresh AA mix-14 (see Table 10).

***Percentage of amino acid requirements listed in NRC publication 990. Calculated on a dry matter basis.

****Tryptophan and Ethyl Tyrosinate·HCl not determined chromatographically.

5.0gm/L Tyrosine ethyl ester added to diets IV, V and VI (Table 13).

1.5gm/L Tryptophan added to diets IV and V (Table 13).

TABLE 15

THE EFFECT OF AMINO ACID EXCLUSION ON THE TOXICITY OF HEAT TREATED CHEMICALLY DEFINED LIQUID DIETS*

Group	Dietary treatment**	Body weight Initial g	Body weight Final g	Av. daily body weight gain g/rat/day	Av. daily diet consumption ml/rat/day	Av. daily water consumption ml/rat/day
1	Diet-14 fresh	66.4±2.0***	150.3±4.4	4.0	23.5	13.3
2	Diet-14 - 60°C - 6 days	71.3±3.6	55.5±1.9+	---	9.9	10.6
3	As Gp 2 minus Trypt. and Glyc.	69.9±3.3	81.9±3.4	0.6	13.0	10.0
4	As Gp 3 minus Hist.	67.9±3.0	95.0±4.8	1.3	16.3	15.2
5	As Gp 4 minus Arg.	64.6±2.9	95.8±5.3‡	1.5	16.2	13.6
6	As Gp 5 minus Lys. and MSG	65.6±2.5	116.7±5.9	2.4	17.1	11.3
7	As Gp 2 minus Glucose	70.6±2.6	122.1±2.7	2.5	18.6	10.9
8	Diet-14 + Glyc-Glucose rkn mix	69.1±2.3	152.4±5.1	4.0	20.8	13.2

*Experimental period 21 days. Seven CFE male weanling rats per treatment. For details, see Appendix Table 3.

**Those amino acids excluded from the diet prior to heating were added in appropriate amounts after the 6 day heat phase.

***Mean value ± standard error.

+Represents average of 4 rats; Three rats died during third week of experiment.

‡Represents average of 6 rats; One rat died during third week of experiment.

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TABLE 16

THE EFFECT OF AMINO ACID AND CARBOHYDRATE EXCLUSION ON THE TOXICITY OF HEAT TREATED CHEMICALLY DEFINED LIQUID DIETS*

Group	Dietary treatment**	Body weight		Av. daily body weight gain	Av. daily diet consumption	Av. daily water consumption
		Initial	Final			
		g	g	g/rat/day	ml/rat/day	ml/rat/day
1	Diet-14 fresh	59.7±1.4***	141.1±4.3	3.9	29.9	11.4
2	Diet-14 - 60°C - 6 days	61.3±1.6	50.7±3.5	---	8.7	7.0
3	As Gp 2 minus Trypt.	62.3±1.2	44.3±2.2+	---	11.6	13.4
4	As Gp 3 minus Arg. Hist. Lys. Glyc.	59.6±2.9	67.7±2.3	0.4	11.7	10.6
5	As Gp 2 minus Glucose, Gluconates and FDC	61.4±2.6	133.3±4.3	3.4	22.8	12.8
6	Diet-14 + Glyc.-Glucose rkn mix-prolonged heat	61.3±2.0	134.7±2.3	3.5	16.9	11.9

*Experimental period 21 days. Seven CFE male weanling rats per treatment. Housed 1 or 2 per wire bottomed cage. For details, see Table 4 of the Appendix.

**Those amino acids and carbohydrates excluded from the diet prior to heating were added in appropriate amounts after the 6 day heat phase.

***Mean value ± standard error.

+Represents average of 5 rats; two rats died during the third week of experiment.

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TABLE 17

AMINO ACID COMPOSITION[+] OF DIETS HEATED IN THE ABSENCE OF SELECTED CARBOHYDRATES AND AMINO ACIDS

Variable[‡]	None	Try.	Try.Gly.	Try.Gly. His.	Try.Gly. His.Arg.	Try.Gly. His.Arg. Lys.	Try.Gly. His.Arg. Lys.MSG	Glucose	Glucose Glucon. FDC
				Percent of Theoretical*					
Arg.	11.9	17.3	30.5	30.9	90.9	>100	90.5	---	---
His.	15.4	34.2	21.2	>100	84.4	96.4	92.5	83.3	>100
Ileu.	44.8	45.6	54.9	64.2	54.1	46.5	72.7	78.3	83.3
Leu.	43.3	45.4	72.0	68.1	55.3	45.8	74.6	83.7	88.5
Lys.	13.9	20.4	22.9	28.0	25.8	>100	83.0	71.9	76.2
Met.	36.6	49.5	57.8	56.8	45.7	44.2	65.0	74.9	97.7
Phe.	42.8	48.7	55.5	59.3	51.7	44.3	73.7	95.6	97.5
Thr.	86.6	89.2	79.6	99.4	75.9	99.6	89.2	78.3	97.2
Val.	45.5	54.3	63.4	61.9	49.8	49.9	70.2	78.8	94.5
Asp.	52.2	63.2	80.0	80.0	63.9	59.5	81.5	81.0	>100
Glu.	24.3	28.3	40.4	48.1	46.9	30.9	89.3	61.4	---
Gly.	33.2	40.5	---	>100	80.4	89.3	96.9	69.6	97.9
Pro.	86.1	89.5	>100	>100	73.8	82.0	94.4	>100	---
Ser.	60.7	67.4	>100	79.7	60.6	64.0	73.9	---	97.0

[+]Determined chemically by column chromatography.

[‡]The amino acid or carbohydrate excluded from the diet prior to heating and added to complete the diet after the 6 day 60°C heat phase.

*Percentage of amino acids normally present in fresh diet-14 (see Table 10).

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TABLE 18

ESSENTIAL AMINO ACID COMPOSITION+ OF DIETS HEATED IN THE ABSENCE OF SELECTED CARBOHYDRATES AND AMINO ACIDS

Variable‡	None	Try.	Try.Gly.	Try.Gly. His.	Try.Gly. His.Arg.	Try.Gly. His.Arg. Lys.	Try.Gly. His.Arg. Lys.MSG	Glucose	Glucose Glucon. FDC
				Percent of Requirement*					
Arg.	42.4	61.6	>100	>100	>100	>100	>100	--	--
His.	30.3	67.3	41.7	>100	>100	>100	>100	>100	>100
Ileu.	>100	>100	>100	>100	>100	>100	72.7	>100	>100
Leu.	94.2	98.8	>100	>100	>100	99.6	>100	>100	>100
Lys.	24.8	36.4	40.9	50.0	46.0	>100	>100	>100	>100
Met.	36.8	49.8	58.1	57.1	45.9	44.4	65.4	75.3	98.2
Phe.	76.7	87.3	99.5	>100	92.7	79.4	>100	>100	>100
Thr.	>100	>100	>100	>100	>100	>100	>100	>100	>100
Val.	89.3	>100	>100	>100	97.7	97.9	>100	>100	>100

+Determined chemically by column chromatography.

‡The amino acid or carbohydrate excluded from the diet prior to heating and added to complete the diet after the 6 day 60°C heat phase.

*Percentage of amino acid requirements listed in NRC publication 990. Calculated on a dry matter basis.

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TABLE 19

ESTIMATED CONCENTRATION OF NINHYDRIN POSITIVE COMPOUNDS[+] PRESENT IN DIETS HEATED AT 60°C FOR 6 DAYS

Diet Variable[‡]	Peak Number													
	1	2	3	5	6	7	10	11	15	16	17	18	19	20
	Estimated Concentration* millimoles/L													
None	3.2	6.2	10.4	8.1	10.5	4.9	6.3		4.3	3.7	35.0	2.0		
Try.	9.2	12.4	20.9	19.2	21.8	9.2	2.9	12.5	8.6	10.6	11.3	5.9	10.5	2.6
Try.Gly.	2.0	8.1	12.4		10.9	3.9			4.7	8.6	6.7	7.6	1.9	1.0
Try.Gly. His.	10.5	14.6	19.4		22.9	8.8	3.3	12.4	7.5	13.0	11.8	11.6	12.9	3.7
Try.Gly. His.Arg.	6.7	12.5	17.9	3.4	17.0	8.2	7.0	11.7		12.2	10.1		6.9	1.5
Try.Gly. His.Arg. Lys.	9.9	13.7	24.0	25.3		10.1	6.9	10.9					10.2	9.8
Try.Gly. His.Arg. Lys.MSG	5.2	11.4	15.7	1.7	16.6	7.6	5.2	6.9		26.9				
Glucose										1.0			12.9	4.5
Glucose-Glucon.-FDC													9.8	

[+]Determined chemically by chromatography; for identification see Figure 7.

[‡]The amino acid or carbohydrate was excluded from the diet prior to heating and added to complete the diet afterwards.

*Estimated by calculation: O.D. unknown peak $\times \frac{1}{\frac{\text{O.D. peak No. X}}{\text{O.D. A.A. of known conc.}}}$

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TABLE 20

GROWTH, DIET CONSUMPTION AND WATER CONSUMPTION OF RATS FED CODELID DIET-14 SUPPLEMENTED WITH HEATED AND NON-HEATED AMINO ACID MIXTURES+

Group	Supplement‡	Body weight Initial	Body weight Final	Av. daily body weight gain	Av. daily diet consumption	Av. daily water consumption
		g	g	g/rat/day	ml/rat/day	ml/rat/day
1	None	77.8±3.0*	136.5±2.7	2.9	24.1	12.3
2	Glyc.-Glucose mix	70.1±3.0	124.4±4.1	2.7	20.7	11.4
3	As Gp 2 - heated	74.6±2.8	131.6±2.4	2.9	20.3	11.1
4	As Gp 3 - 2x	76.9±2.5	131.1±2.8	2.7	20.5	11.6
5	As Gp 3 - 3x	70.9±2.4	121.3±3.4**	2.5	19.3	12.8
6	Arg. Hist. Lys. Glucose mix	77.1±1.8	136.3±2.5	3.0	20.6	16.6
7	As Gp 6 - heated	80.6±2.9	131.1±5.2	2.5	20.3	14.7
8	Arg. Hist. Lys. Glyc. Glucose mix	78.1±2.9	124.1±5.8	2.3	20.5	12.0
9	As Gp 8 - heated	78.3±2.9	134.6±2.5	2.8	20.8	13.4

+Experimental period 20 days. Eight CFE female weanling rats allotted to each test diet. Housed 2 per wire bottomed cage. For details, see Appendix Table 5.

‡Amino acid supplements were heated for 6 days at 60°C.

*Mean value ± standard error.

**Mean value ± standard error for 7 rats; one rat died during third week of experiment.

TABLE 21

ESTIMATED CONCENTRATION OF NINHYDRIN POSITIVE COMPOUNDS[+] IN DIETS SUPPLEMENTED WITH HEATED OR NON HEATED AMINO ACID MIXTURES

Group	Amino Acid Mixture[‡]	Peak Number						
		5	6	16	17	18	19	20
		Estimated Concentration* millimoles/L						
2	Glycine-Glucose			0.5				4.3
8	Arg.Hist.Lys.Glyc.-Glucose			1.6			10.0	6.8
9	Arg.Hist.Lys.Glyc.-Glucose-Heated	22.5	0.6	14.6	13.4	8.8	15.2	6.2

[+]Determined chemically by chromatography. For identification, see Figure 7.

[‡]All heated amino acid mixtures were kept at 60°C for 6 days and then added to Codelid diet-14.

*Estimated by calculation: O.D. unknown peak $\times \frac{1}{\frac{\text{O.D. peak No. X}}{\text{O.D. A.A. of known conc.}}}$

TABLE 22

EFFECT OF VARIOUS BROWNING INHIBITORS ON THE TOXICITY OF CHEMICALLY DEFINED LIQUID DIETS KEPT AT THREE DIFFERENT TEMPERATURES*

Temperature	Additive	Body weight Initial	Body weight Final	Av. daily body weight gain	Av. daily diet consumption	Av. daily water consumption
		g	g	g/rat/day	ml/rat/day	ml/rat/day
0-4°C 45 days	None	62.2±3.3**	129.8±3.2	3.2	22.8	11.4
	GSH	61.2±3.9	131.2±6.7	3.3	21.9	12.1
	Bunte salt	61.7±3.4	139.2±6.4	3.7	22.2	11.1
	HAR	57.7±2.2	112.3±3.5	2.6	20.4	6.8
37°C 45 days	None	56.5±1.8	49.3±2.3	---	11.9	6.4
	GSH	53.7±3.4	46.0±2.9	---	13.4	6.0
	Bunte salt	56.2±2.6	47.3±2.0	---	11.1	7.2
	HAR	54.4±2.9	47.5±2.2***	---	14.8	5.6
60°C 6 days	None	58.0±2.3	41.4±2.5****	---	9.9	8.6
	GSH	58.0±2.3	49.2±2.1+	---	7.7	8.8
	Bunte salt	55.2±1.8	42.2±5.4‡	---	9.4	8.1
	HAR	60.5±3.1	49.8±3.0‡	---	6.0	7.4

*Experimental period 21 days. Five or six CFE male weanling rats per treatment. GSH - Glutathione; HAR - Homocysteine thiolactone. For detailed data see Table 6 of the Appendix.

**Mean value ± standard error.

***Mean value of 5 rats - 1 death during second week of experiment.

****Mean value of 5 rats at end of first week of experiment - 1 death during first week, 3 deaths during second week and 1 death during third week.

+Mean value of 5 rats at end of first week of experiment - 1 death during first week, 3 deaths during second week and 2 deaths during third week.

‡Mean value of 6 rats at end of second week of experiment - 5 deaths during third week.

‡Mean value of 6 rats at end of first week of experiment - 3 deaths during second week and 3 deaths during third week.

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TABLE 23

EFFECT OF BROWNING INHIBITORS ON THE AMINO ACID COMPOSITION* OF HEATED CODELID DIET-14

Temperature	37°C - 45 Days				60°C - 6 days
Inhibitor**	None	GSH	Bunte Salt	HAR	GSH
	% of Theoretical+				
Arginine	72.5	89.1	60.2	61.9	10.2
Histidine	41.6	60.4	50.5	68.9	23.2
Isoleucine	81.1	77.7	71.9	80.8	32.1
Leucine	82.9	94.0	82.1	82.8	32.8
Lysine	68.8	89.3	56.0	66.6	15.5
Methionine	63.7	74.6	66.7	72.6	28.4
Phenylalanine	73.1	84.7	76.9	84.5	32.0
Threonine	99.6	44.9	82.6	45.3	>100
Valine	77.4	66.7	74.3	77.1	81.0
Aspartate	>100	44.6	83.0	83.1	70.8
Glutamate	80.9	58.0	60.1	65.1	37.8
Glycine	98.5	88.8	76.7	82.1	--
Proline	--	>100	95.1	95.1	>100
Serine	>100	68.2	87.0	76.1	73.9

*Determined chemically by column chromatography.

**Added at the level of 0.5% w/v. GSH - Glutathione, reduced; Bunte Salt - Sodium-S-Cysteine Sulfonate; HAR - N-Acetyl homocysteine thiolactone.

+Percentage of amino acids normally present in fresh diet-14 (see Table 10).

TABLE 24

EFFECT OF BROWNING INHIBITORS ON THE AMINO ACID COMPOSITION* OF HEATED CODELID DIET-14

Temperature	37°C - 45 days				60°C - 6 days
Inhibitor**	None	GSH	Bunte Salt	HAR	GSH
	% of Requirement+				
Arginine	>100	>100	>100	>100	36.4
Histidine	81.9	>100	99.4	>100	45.7
Isoleucine	>100	>100	>100	>100	82.9
Leucine	>100	>100	>100	>100	71.3
Lysine	>100	>100	99.9	>100	27.7
Methionine	64.1	75.0	67.1	73.0	28.5
Phenylalanine	>100	>100	>100	>100	57.4
Threonine	>100	84.4	>100	85.2	>100
Valine	>100	>100	>100	>100	>100

*Determined chemically by column chromatography.

**Added at the level of 0.5% w/v. GSH - glutathione reduced; Bunte Salt - Sodium-S-cysteine sulfonate; HAR - N-Acetyl homocysteine thiolactone.

+Percentage of amino acid requirements listed in NRC publication 990. Calculated on a dry matter basis.

TABLE 25

ESTIMATED CONCENTRATION OF NINHYDRIN POSITIVE COMPOUNDS+ PRESENT IN DIETS HEATED TO 37°C OR 60°C IN THE PRESENCE OF BROWNING INHIBITORS

Test Condition	Inhibitor‡	Peak Number														
		1	2	3	4	5	6	7	10	11	15	16	17	18	19	20
		Estimated Concentration* millimoles/L														
0-4°C	None														3.8	7.5
37°C 45 Days	None	5.4	12.6	16.4		13.5	6.3	5.3				10.5	10.6	12.9		
	GSH	7.8	18.8	20.1		17.9	9.2					11.6	7.9	10.3	2.7	
	Bunte Salt	5.1	14.1	16.4	2.4	13.3	19.5	8.8		9.6	2.9	19.3	0.4	15.1	15.8	
	HAR	2.9	7.3	8.2	1.9	8.2	10.9	4.2		3.6	1.5	9.6	4.6	7.1	11.5	
60°C	None		see Table													
6 Days	GSH	10.9		25.9	2.1	31.2	27.1	13.3			11.3	20.6		10.4	9.1	

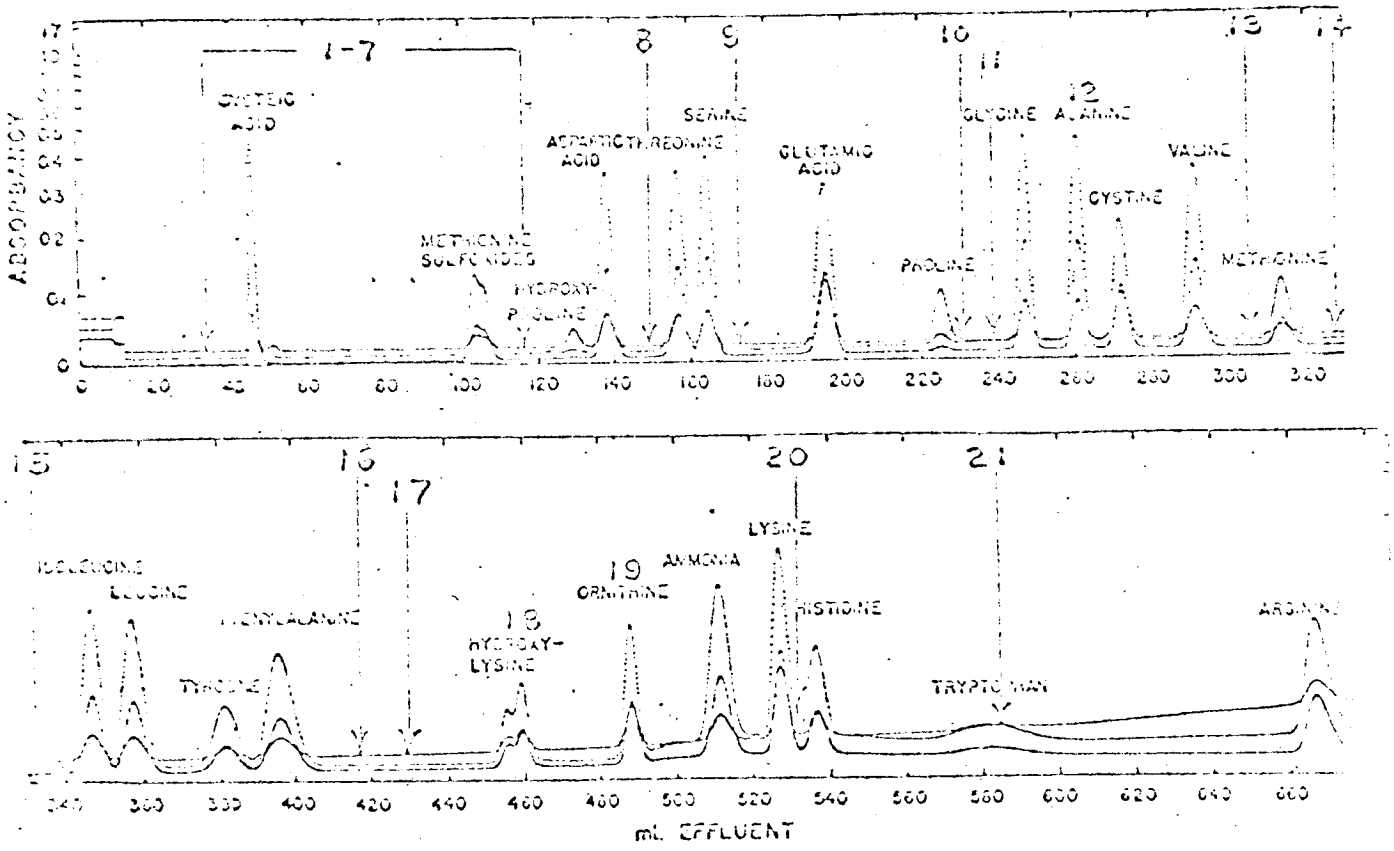
+Determined chemically by chromatography. For identification, see Figure 7.

‡All inhibitors added at level of 0.5% (w/v); GSH - glutathione, reduced; HAR - N-acetyl homocysteine thiolactone; Bunte Salt - sodium-S-cysteine sulfonate.

*Estimated by calculation: O.D. unknown peak x $\frac{1}{\frac{\text{O.D. Peak No. X}}{\text{O.D. A.A. of known conc.}}}$

FIGURE 7

TYPICAL AMINO ACID CHROMATOGRAM WITH ELUTION PATTERN OF NEWLY FORMED NINHYDRIN POSITIVE COMPOUNDS*



*For identification of newly formed peaks, see Legend on page 67.

LEGEND FOR FIGURE 7

Peak No.	Tentative Identification
1	------
2	Threo-β-hydroxyaspartic acid
3	------
4	------
5	Glucosaminic acid or similar 8C chain
6	Urethro-β-hydroxyaspartic acid or hydroxy glutamic acid
7	Hydroxy glutamic acid
8	Asparagine or methionine sulfone
9	Glutamine or homoserine
10	Citrulline
11	------
12	Alanine
13	------
14	------
15	γ-amino butyric acid
16	------
17	------
18	D-allo-hydroxylysine
19	Ornithine
20	G-hydroxy tryptophan
21	Tryptophan

BLOOD COAGULATION STUDIES

Introduction:

In previous experiments designed to elucidate strain differences in the utilization of completely defined liquid diets (Codelid diets) we reported an unexpected hemorrhagic condition in male, weanling, CDF Fischer rats ingesting Codelid diet-17[5]. The condition was observed after rats had consumed the diet for two weeks and was manifested by internal and external bleeding and prolonged prothrombin times. Intramuscular injection of menadiol diphosphate prevented hemorrhage and restored prothrombin times to normal. Since menadione was present in the diet, the therapeutic effect of the phosphate analogue suggested destruction and/or biological interference with menadione utilization.

The experiments described below were designed to determine the factors responsible for the hemorrhagic condition and to elucidate the nature of the strain differences in susceptibility between CFE and Fischer rats.

Methods:

Unless otherwise noted, male, CFE or Fischer, weanling rats of 9-11 littermates were used in all studies. They were housed 2 per wire bottomed cage in a temperature-controlled room (72-76°) and were allotted to insure, as closely as possible, uniform distribution of littermates within each dietary treatment. Diet and water were provided ad libitum. All liquid diets were fed via Richter tubes. Residual diet was measured and discarded daily and clean Richter tubes were then replenished with refrigerated diet.

Fresh water was provided on alternate days. The compositions of the diets are shown in Tables 9 and 10. In the initial studies, Guaiac tests to detect internal hemorrhage were conducted daily on the feces under each cage. When the Guaiac tests were distinctly positive or if the animals showed signs of anemia, external bleeding or appeared moribund, they were sacrificed with chloroform and bled by heart puncture. The blood samples were then centrifuged and plasma prothrombin times were immediately determined by the method of Quick[14]. Whenever prolonged prothrombin times were observed, Hicks-Pitney thromboplastin generation time tests were performed to detect deficiencies in the Stage I phase of the blood clotting mechanism[14]. After blood samples had been withdrawn, the kidneys and liver were removed and weighed. Body weights were determined at weekly intervals or immediately prior to death after diet and water were removed for 1-2 hours.

1. The role of ethyl cysteinate·HCl

In our earlier studies, only Codelid diet-17 produced hemorrhages in CDF rats[5]. This diet differs from the other diets tested (Codelid diets-14, 15 and 16) primarily in amino acid concentration and pattern (Tables 9 and 10). One of the major differences is the much higher concentration of ethyl cysteinate·HCl in diet-17 compared with the other diets. This variance is of particular interest since cysteine is known to react with menadione to form a quinone-thioether[15]. The following experiment was conducted to determine whether ethyl cysteinate·HCl is related to the onset of hemorrhage in CDF rats.

The experimental design and growth data are shown in Table 26. Although the duration of the experiment was 9

weeks, the results were tabulated for only 4 weeks since animals were subsequently sacrificed at different time intervals. Detailed body weight, diet consumption and water consumption data for the full 9 week period are presented in Table 7 of the Appendix.

The data in Table 26 shows the growth of all groups consuming diet-17 to be significantly ($p < 0.01$) greater than that attained by rats consuming diet-16. A similar difference in growth rate between rats fed diet-16 and 17 was also observed in our earlier experiments[5]. The growth rate of rats receiving diet-17 was 85% of that of rats consuming a commercial laboratory chow (Lab Blox)*. In all cases, growth was less than observed in CFE male rats of similar age and consuming similar diets[5]. Diet consumption was also less than normally observed with CFE animals. The level of diet consumption corresponded to the rate of growth. Water consumption was unusually high for all groups.

Unlike earlier experiments where abnormal prothrombin times were observed within 2-3 weeks, prothrombin deficiencies in the present experiment were not consistently observed until the 8th and 9th week of feeding (Table 27). At this time, all rats exhibiting prolonged prothrombin times also exhibited abnormal thromboplastin generation times, indicating a deficiency in both the Stage I and Stage II phases of the blood clotting mechanism.

Examination and autopsy of the animals sacrificed during the 8th and 9th weeks showed rats consuming diet-17 with 1.22 or 2.43 gm/liter ethyl cysteinate·HCl to have a

*Wayne Lab Blox - A product of Allied Mills, Inc., Chicago, Ill.

ruffled emaciated appearance and bleached eyes. Autopsy revealed the livers of the animals to be pale yellow and smaller than the livers excised from rats fed the other diets (Appendix Tables 8, 9 and 10). In several animals fed Codelid diet-17 with the high levels of ethyl cysteinate·HCl, the gastrointestinal tract was abnormally yellow. Animals from these groups also showed an unusual amount of fat deposition in the peritoneal cavity, particularly around the small intestine and kidneys. Kidney size (Appendix Tables 8 and 10) was slightly smaller in rats consuming the higher levels of ethyl cysteinate·HCl and appeared pale in those animals exhibiting pale livers.

It is clear from these results that Codelid diet-17, when supplemented with high levels of ethyl cysteinate·HCl, causes a hemorrhagic condition in male, weanling CDF rats. The limited occurrence of hemorrhage in littermates receiving diet-16 with an equivalent level of ethyl cysteinate·HCl suggests that additional factors present in diet-17 may be responsible for, or enhance, the onset of this syndrome. In the present experiment, prothrombin deficiency was not observed until the 9th week of feeding. In our earlier studies, the deficiency appeared within 3 weeks. This delayed response was probably due to slower destruction or inactivation of menadione within the diet rather than to such biological factors as increased resistance to vitamin K deficiency or inhibition of some metabolic pathway leading to hypoprothrombenemia. Delayed destruction of menadione could come about by the slow formation of reaction products within the diet which independently, or together with ethyl cysteinate, may regulate the rate of menadione destruction during the course of an experiment.

2. The role of menadione-thioether

Our earlier studies showed that the hemorrhagic syndrome in male weanling CDF rats consuming Codelid diet-17 is related to vitamin K deficiency[5]. The previously described experiment demonstrated the involvement of ethyl cysteinate·HCl in producing this condition. Since menadione can react with cysteine·HCl to form menadione-thioether (2-methyl, 3-cysteinyl, 1,4 naphthoquinone)[15] it was postulated that this adduct may behave as an antimetabolite of vitamin K.

The experiment described below was conducted to determine whether the inclusion of chemically synthesized menadione-thioether induces hemorrhage in rats.

Due to the unavailability of CDF rats, the experiment was conducted with CFE male, weanlings. The design and results are shown in Tables 28 and 29. Growth was slightly less than for rats consuming diet-16 without menadione-thioether (Table 30). However, the magnitude of the differences was small and the greater growth in the latter group was probably attributable to their heavier starting weight (Appendix Table 14). The exclusion of vitamin K from the diet did not affect weight gain or diet consumption. Water consumption was uniform between groups and was in the range commonly observed with CFE rats fed Codelid diets.

Table 29 shows the effect of menadione-thioether on the prothrombin times of these animals. Without exception, prothrombin times were normal in all groups, regardless of the level of thioether included in the diet or the presence of menadione. All animals appeared healthy. Autopsies revealed no sign of internal hemorrhage or gross morphological abnormalities. The livers and kidneys from these rats had no

lesions, and were of normal size and color (Appendix Tables 12 and 13).

The results show that over a 9 week experimental period, male CFE weanling rats fed Codelid diet-16 with menadione-thioether, in the presence or absence of dietary menadione, grow normally and show no signs of hemorrhage or hypoprothrombenemia. The lack of any observable anti-vitamin K activity by the thioether may be related to the rat strain used in this study. Similar resistance to vitamin K deficiency was evident in our earlier studies[5]. Here, a hemorrhagic condition was not observed in CFE rats fed a diet known to induce bleeding and prolonged prothrombin times in CDF rats of the same age and sex. The apparent strain difference in susceptibility to vitamin K deficiency, therefore, prevents any conclusions at this point concerning the anti-vitamin K activity of menadione-thioether.

3. Strain differences in susceptibility to vitamin K deficiency

Strain differences in susceptibility to vitamin K deficiency have previously been reported in the literature[16]. Such differences are related to the prevention or practice of coprophagy[17] and may also be related to the nature of the diet used to induce the deficiency[16]. The previous studies and those presented in our earlier report[5] indicated a strain difference in susceptibility to vitamin K deficiency in rats fed Codelid diets. The following experiments were conducted to elucidate this difference between CFE and CDF rats.

The data in Tables 30 and 31 show the results of feeding male, weanling CFE rats Codelid diets with or without

menadione. Microbiological data obtained during these experiments are presented in another section of this report (pp 118-202). Growth rates, diet consumption and water consumption were normal in all groups whether or not menadione was in the diet. Growth of rats consuming diet-16 and 17 were virtually the same, but significantly less ($p < 0.01$) than those ingesting Lab Blox. Prothrombin times were not affected by excluding menadione from the diet (Table 31). Livers and kidneys were normal in size and appearance (Appendix Tables 15 and 16). The small difference in organ weights reflect the age of the animal at autopsy and were not due to dietary treatment.

Tables 32 and 33 show the results of feeding Codelid diet-16 with or without menadione to CDF, male weanling rats. Growth of rats fed the menadione-free diet was slightly but not significantly ($p > 0.01$) less than those fed the complete diet. Growth of rats consuming Lab Blox was significantly ($p > 0.01$) greater than for the two groups consuming liquid diet. Diet consumption was normal for all groups and was not altered by excluding menadione from the diet. Water consumption was higher than usually observed with CDF rats fed liquid diets but was not influenced by the absence of menadione from the diet. Water consumption of the Lab Blox group was normal.

The prothrombin data shown in Table 33 clearly demonstrates a marked susceptibility of the male, weanling CDF rat to vitamin K deficiency. Nine out of twelve rats ingesting Codelid diet-16 without menadione exhibited prolonged prothrombin times. Only one abnormal prothrombin time was observed in rats fed the same diet but containing menadione. Although the livers and kidneys from rats fed the Codelid diets were smaller than those from rats fed Lab Blox (Appendix Tables 18 and 19) this was simply a reflection of the larger

body weight of the latter group of rats. The livers from rats fed the menadione-free diet were slightly smaller than from those fed a complete diet, but again, this was due to their smaller body size. Kidney size was virtually the same for both liquid diet groups. The kidneys and particularly the livers from rats fed the menadione-free diet were lighter in color compared to those from rats fed the diet containing menadione or Lab Blox.

These findings explain why we have never observed a hemorrhagic syndrome in CFE rats fed Codelid diet-17. It is evident that there is a strain difference in susceptibility to induced vitamin K deficiency when rats are fed Codelid diets. Male, weanling CFE rats seem resistant to this deficiency whereas CDF rats of the same age and sex are susceptible. Our microbiological data (see pp 118 to 202) also show quantitative differences in the aerobic vitamin K synthesizing organisms between these strains. Whether these microbiological differences are related to susceptibility to dietary vitamin K deficiency must still be determined.

Finally, in agreement with our earlier findings and regardless of strain, growth rates, diet consumption, and water consumption, liver size and kidney size were essentially unaffected by excluding menadione from the diet. The only signs of vitamin K deficiency in the present study were the prolonged prothrombin times and abnormal thromboplastin-generation times in CDF rats. In contrast, our previous studies showed external and internal signs of anemia and hemorrhage in addition to prothrombin inadequacy. This supports our earlier view that several dietary factors regulate, via chemical interaction, both the severity and rate of onset of the hemorrhagic syndrome.

4. Rat origin and vitamin K deficiency

In view of the importance of the intestinal microflora in the synthesis of vitamin K[16] it was thought that the strain differences observed in the previous study could be related to differences in the intestinal flora of CFE and Fischer rats. It was also suggested that Codelid diet-17 may induce vitamin K deficiency by altering the intestinal flora of the animal. The following experiment was conducted to test these hypotheses.

Microbiological assays were performed on cecal and fecal specimens from CFE and Fischer rats fed either Lab Blox, Codelid diet-16 or Codelid diet-17. Since Fischer rats were not available from our normal supplier, Charles River Breeding Laboratories, Wilmington, Massachusetts, they were obtained from the A. R. Schmidt Co., Madison, Wisconsin. The microbiological sampling procedures, techniques and results are presented elsewhere in this report (pp 118-202).

The growth data in Table 34 show that CFE rats grow at a significantly ($p < 0.01$) faster rate than ARS Fischer rats regardless of the diet being fed. In both strains, diet-17 produced greater growth than diet-15 but in all cases, the growth rate was significantly ($p < 0.01$) lower than attained with Lab Blox. Diet consumption was greater for CFE rats but utilization (gm gain/ml diet consumed) was equivalent in both strains and for both diets. Water consumption was similar for both strains when liquid diets were fed but was unusually low for Fischer rats ingesting Lab Blox.

The data in Table 35 again show that diet-17 does not produce prolonged prothrombin times in CFE rats. As expected, normal prothrombin times were also found in all rats

of both strains ingesting diet-16 or Lab Blox. However, contrary to our earlier findings, diet-17 did not produce hypoprothrombenemia in Fischer rats.

Since the diets used in this study were of the same composition as diets heretofore shown to induce a hemorrhagic condition in CDF Fischer rats, it was suggested that A. R. Schmidt (ARS) Fischer rats may be more resistant to vitamin K-deficiency than Fischer rats originating from Charles River Breeding Laboratories. An experiment was, therefore, conducted to determine the susceptibility of ARS rats to vitamin K deficiency when fed menadione-free Codelid diets.

The results in Table 36 show that the absence of menadione from the diets of ARS rats did not effect growth rate, diet consumption or water consumption. As previously observed with Fischer rats (Table 34), those fed Codelid diet-17 grew at a significantly ($p < 0.01$) greater rate than those fed Codelid diet-16 even though menadione was excluded from the diet.

The prothrombin data in Table 37 show that unlike CDF Fischer rats, Fischer rats of A. R. Schmidt origin do not come down with vitamin K deficiency when fed menadione-free diets. This explains why Codelid diet-17 did not induce a hemorrhagic condition in the ARS Fischer rats used in the previous experiment. This finding is particularly significant since it points up the importance of considering the derivation as well as the strain of experimental animals employed in any study.

The strain and source differences in susceptibility to vitamin K deficiency could be attributed to a number of factors acting independently or in combination. Such factors could include the genetic make-up of the experimental animal,

the environment in which it was raised, the food it was fed prior to any test period and its microbial population. Our present work has been confined to comparing the population of the intestinal flora of rats susceptible and resistant to vitamin K deficiency. The results (see pp 118 to 202) show that while the intestinal microflora are related to K-deficiency susceptibility, other factors also play a significant role.

5. The role of other dietary factors

Our previous results showed that diets of the same composition but formulated at different times, caused hemorrhagic syndromes which differed in severity and rate of onset. We also found that while ethyl cysteinate·HCl is the primary agent responsible for the vitamin K deficiency and hemorrhagic condition which occurs when male, CDF, Fischer rats are fed Codelid diet-17, it was ineffective when provided at the same concentration in diet-16 (Table 27). These findings suggest that other dietary factors may influence the hemorrhagic syndrome and the destruction or inactivation of vitamin K either directly or indirectly through their action on ethyl cysteinate·HCl. The following experiments were conducted to test this hypothesis. Male, CDF, Fischer rats were used in all studies.

The first experiment of this series was designed to determine the effect of menadione-thioether and an oxygenated fat mix on prothrombin times and the onset of hemorrhage. The composition of the fat mix was the same as used in all studies and is shown in Table 9. It was prepared and frozen 4 weeks before the start of the experiment. A portion

of the mixture was oxygenated by bringing it to room temperature and bubbling oxygen through the solution for 18 hours. Menadione thioether was synthesized by the method previously described[15]. It was added to the diet in the same quantities as in our experiments with CFE rats (Table 28).

The results in Table 38 show that growth rate, diet consumption and diet utilization were not effected by adding menadione-thioether or an oxygenated fat mix to the diet. Water consumption was high in all groups, but was in the range usually observed with CDF rats of similar age. When ethyl cysteinate, menadione-thioether or an oxygenated fat mix were the only diet variables, water consumption was less than for the control group. However, when in addition to an oxygenated fat mix ethyl cysteinate or menadione-thioether was also added to the diet, water intake increased.

The hemorrhagic syndrome as measured by deaths due to bleeding, abnormal prothrombin times and rate of onset, was moderate in all groups (Table 39). The incidence and severity of abnormal prothrombin times was greatest when the diets contained 2.42 g ethyl cysteinate·HCl/liter. The addition of oxygenated fat mix to the diet influenced the hemorrhagic syndrome but the effect was not pronounced. At both levels of ethyl cysteinate·HCl it increased the incidence and elevated the range of abnormal prothrombin times. When the diet contained 0.55 g ethyl cysteinate/liter, 4 out of 10 rats were abnormal when oxygenated fat mix was present, whereas only 1 out of 10 was abnormal when the standard fat mix was used (control group). At higher concentrations of ethyl cysteinate·HCl (2.42 g/L) 8 out of 10 rats were abnormal compared to 6 out of 10 when the standard fat mix was

used. In addition, the range of prothrombin times was higher when the oxygenated fat mix was included in the diet.

When menadione-thioether was added to the diet, 5 out of 10 rats had abnormal prothrombin and thromboplastin generation times. However, the number of abnormalities was the same regardless of the presence of the oxygenated fat mix although in the latter instance, the range of prothrombin times was elevated.

The hypoprothrombenemia produced by adding 2.42 g/L ethyl cysteinate to diet-16 is in disagreement with our earlier findings. This is of particular interest since it suggests that other than the high concentration of ethyl cysteinate·HCl, the dietary factors responsible for the condition are not peculiar to diet-17. Furthermore, the variations observed indicate that the factor(s) involved in producing the condition are probably intermediates or end-products of interactions occurring within the diet whose rate of formation and concentration can not be readily controlled. Consequently, their occurrence may differ in diets of the same composition but formulated at different times and may also depend on the duration and conditions of storage during the course of an experiment. In this connection, it is noteworthy that the fat mixture used in this study was frozen for 4 weeks prior to use. If organic peroxides were formed during this time, and if they contribute to the hemorrhagic syndrome, they could account for the hypoprothrombenemia produced when 2.42 g/L ethyl cysteinate·HCl was added to diet-16 in the present experiment contrasted to our earlier findings. Similarly, if organic peroxides function in this

capacity, their presence in the fat mix prior to oxygenation could mask any response attributable to this treatment. They could also be responsible for, or contribute to the effect ascribed to menadione-thioether.

Although the treatment differences observed in the previous experiment were small and the number of animals tested limited, the evidence supports the view that the state of oxidation of the fat mix influences the hemorrhagic syndrome induced by diet-17. Since ethyl linoleate is a major component of the fat mix and in view of its susceptibility to auto-oxidation, the next experiment was conducted to determine its role in producing the syndrome.

The results and experimental design are shown in Tables 40 and 41. It is clear that the removal of ethyl linoleate from the diet decreased growth rate and diet utilization although the effect was not as pronounced when ethyl cysteinate·HCl was also absent from the diet. When ethyl cysteinate·HCl was removed, diet utilization decreased as the result of a relatively large increase in diet consumption compared to the small increase in growth rate. The increased growth, however, was probably sufficient to partially compensate for the growth depression due to the removal of ethyl linoleate and may account for the response observed when the two components were simultaneously absent from the diet. Water consumption was not influenced by dietary treatment.

The data in Table 41 show that under the dietary conditions studied, ethyl cysteinate·HCl is required to produce the hemorrhagic syndrome. In no instance were deaths or prolonged prothrombin times observed when diet-17 was completely free of ethyl cysteinate. On the other hand, 14 of the 15 rats tested were abnormal when it was present in diet-17

at its normal concentration (2.42 g/L). Ethyl linoleate did not influence the incidence of abnormalities. However, the condition appeared to be more severe when it was present in the diet (i.e. 6 vs. 1 hemorrhagic death within the first 6 weeks).

The results of the previous two experiments suggest that while the fat pre-mix and ethyl linoleate may influence the severity of the hemorrhagic condition, their contribution is small and ineffective in the absence of ethyl cysteinate·HCl. The results also indicate that it may be the oxidative state of these components and not the components per se which is responsible for their action. In view of these findings and the fact that the fat mix (which also contains ethyl linoleate) constitutes only 0.5% (w/v) of the diet, it was thought that the oxidative state of the complete diet, not just that of the fat mix, may in some manner regulate the action of ethyl cysteinate·HCl in causing the syndrome. The following experiment represents an attempt to test this by determining the influence of antioxidants on the onset and severity of the hemorrhagic condition. The antioxidants selected for investigation were water-soluble ascorbic acid (vitamin C) and fat-soluble α-tocopherol acetate (vitamin E). They were added to the diet at twice their ordinary levels (Table 9).

The experimental design and results are shown in Tables 42 and 43. The rats used in this experiment were older (51 days of age) than used in our earlier experiments (30-35 days of age). Growth was depressed by doubling the ascorbic acid and α-tocopherol acetate levels in the diet. However, the depression appeared to be related to the rapid onset and severity of the hemorrhagic condition in these two groups rather than to direct inhibition. Diet and water consumption

were not effected by dietary treatment. The higher intakes than observed in previous experiments (Tables 26, 32, 38 and 40) were probably due to the age and heavier starting weights of the animals.

The data in Table 43 once again shows that Codelid diet-17 produces a hemorrhagic syndrome in male, CDF rats. Without exception, prothrombin times and thromboplastin generation times were prolonged. The rapid onset and severity of the condition when the antioxidant level in the diet was increased was unexpected. When the dietary concentration of vitamin C and vitamin E were doubled both in the presence and absence of ethyl linoleate, over 50% of the rats tested died from hemorrhage and all the survivors had prolonged prothrombin times. It is clear from these data that either directly or in their capacity as antioxidants, ascorbic acid and/or α-tocopherol acetate play an important role in producing the hemmorhagic syndrome. However, the function of each, individually, and their mechanism of action, is not presently known.

The next experiment was designed to determine the individual contribution of α-tocopherol acetate and ascorbic acid to the onset of the hemorrhagic syndrome. Since others have shown that high levels and sometimes even normal dietary levels of vitamin A can cause hypoprothrombenemia[18, 19], the influence of this vitamin on the onset of the hemorrhagic condition under our experimental conditions was also studied. It was thought that ascorbic acid and α-tocopherol acetate could exert their action by protecting vitamin A which would then be available to function in its hypoprothrombenemic capacity.

The experimental design and results are shown in Tables 44 and 45. In general, growth rate was not appreciably influenced by dietary treatment. A small improvement occurred

in all cases where vitamin A was removed from the diet (Groups IV, V and VIII). Slight growth retardation was observed when excess ascorbic acid was added (Group II) but this effect disappeared when excess vitamin E was also present or when vitamin A was absent from the diet. Diet and water consumption were fairly uniform between all diet groups. Although the prothrombin data in Table 45 are presented for the full 7 week experimental period, the values for Groups I, III, VI, VII and VIII should be considered for the first 6 weeks only. The reason for this is that these groups were fed freshly prepared diets at the beginning of the 7th week of experiment and since the extent of dietary interaction may be governed by the duration of storage, the final outcome could be markedly altered.

In no instance was the incidence or extent of the hemorrhagic syndrome as severe as previously observed although as before, the onset was fairly rapid (Table 43). The independent supplementation of α-tocopherol acetate and ascorbic acid did not seem to influence the extent of hypoprothrombenemia in the presence or absence of dietary vitamin A (Groups I, II, IV and V). Similarly, the removal of vitamin A from the diet (Group III) did not alter the incidence or severity of hypoprothrombenemia. When the two antioxidants were added simultaneously to a vitamin A-free diet, only 1 rat was abnormal (Group VI). This is in contrast to our earlier findings where a severe hemorrhagic condition was induced by the addition of the two antioxidants to a diet containing vitamin A (Table 43). In accordance with our earlier findings, ethyl cysteinate was found to be the most important factor governing the onset of the hemorrhagic syndrome. When ethyl cysteinate was absent from the diet, regardless of whether the diet contained vitamin A, the simultaneous supplementation

of ascorbate and α-tocopherol acetate failed to produce any abnormalities (Groups VII and VIII).

Due to the moderate hypoprothrombenemia observed in the present study, and in view of the limited number of rats tested, it is difficult to ascribe a role to either vitamin E or vitamin C in the hemorrhage syndrome. For these same reasons, little can be said about the role of vitamin A. The results suggest, however, that this vitamin is not required in Codelid diet-17 to produce the syndrome (Group III). This was also found to be the case when α-tocopherol acetate or ascorbic acid were independently added to the diet. However, the failure to produce hypoprothrombenemia in a vitamin A-free diet when the two antioxidants were supplemented simultaneously, contrasted to our earlier findings (Table 43), suggests that in such a situation, their action may be mediated through vitamin A. It is clear that further experimentation is necessary to elucidate the relative contribution of vitamin A, E and C to the hemorrhagenicity of Codelid diet-17.

Our previous results showed that the hemorrhagic syndrome produced by diet-17 is due to vitamin K deficiency[5] which probably results from the interaction of ethyl cysteinate·HCl with menadione. Others have shown that the -SH group of cysteine reacts with menadione at the 3 position of the quinone nucleus to yield a quinone-thioether (2-methyl, 3 cysteinyl, 1,4 naphthoquinone)[15]. The following study was conducted to determine the specificity of menadione and ethyl cysteinate·HCl for producing the hemorrhagic syndrome. In addition, we studied the influence of hydrogen peroxide on the onset of the condition. It was suggested that the improtance of the oxidative state of the diet may be due to organic peroxides generated during the course of an experiment

which could directly or indirectly influence menadione inactivation or could oxidize menadione-thioether to form a more effective antimetabolite, i.e. sulfoxide, sulfone or a dimer of menadione.

The experimental design and results are shown in Tables 46 and 47. To test the specificity of the menadione molecule, an equimolar amount of Synkavite*, (menadiol sodium diphosphate) was added to the diet. This compound was selected because the 3-position is no longer an active site for ethyl cysteinate·HCl addition due to the reduced state of the quinone nucleus. The specificity of ethyl cysteinate·HCl was tested by replacing it with the diethylester. The configuration of this molecule is such that free -SH groups are no longer available for attachment at the 3 position of the menadione molecule.

The results show that replacing menadione with Synkavite did not effect growth rate, diet consumption or water consumption. Diethylcysteinate caused a slight reduction in growth but diet and water intake were essentially unaltered. Diet utilization was virtually the same in each of these groups. The addition of peroxides to the diet caused growth depression and decreased diet utilization. Diet and water consumption were also reduced.

The data in Table 46 clearly show that replacing menadione with Synkavite and ethyl cysteinate·HCl with diethyl cysteinate prevents the hypoprothrombenemia observed when these ingredients are normally present in the diet. Seven out of eight rats had prolonged prothrombin and thromboplastin generation times when ethyl cysteinate·HCl and menadione were present in the diet. On the other hand, no abnormalities occurred when they were replaced by diethyl

*Synkavite - Trademark for menadiol sodium diphosphate, a product of Roche Laboratories, Nutley, N. J.

cysteinate·HCl or Synkavite respectively.

The addition of hydrogen peroxide to the diet at a level of 8 g/L did not increase the incidence or severity of hypoprothrombenemia when ethyl cysteinate·HCl was in the diet and was completely ineffective in its absence. Only one abnormality was found when the H_2O_2 concentration in the diet was increased to 16 g/L. The addition of 8 g/L H_2O_2 to a diet containing menadione-thioether did not cause any prolonged prothrombin times.

Although the results clearly demonstrate the specificity of menadione and ethyl cysteinate·HCl for producing the hemorrhagic syndrome, they do not show any effect from hydrogen peroxide. However, the concentrations of peroxides studied may have been considerably higher than the concentration normally generated in the diet and consequently, may have been inhibitory rather than catalytic with respect to menadione inactivation. An example of such inhibition could be the rapid oxidation of ethyl cysteinate·HCl due to the presence of high concentration of free peroxides in the diet. This view is suggested by the reduced occurrence of hypoprothrombenemia when the H_2O_2 concentration of the diet was increased from 8 g/L to 16 g/L. That the concentration of peroxides may have been too high is also suggested by the initial bleaching and subsequent browning of the diets several days after their addition. This type of color change was not heretofore observed in diet-17.

The failure to produce any abnormalities by adding peroxide to a diet containing menadione-thioether may also be due to the high concentration of peroxide employed. If

the supply of peroxide were exhausted because of its rapid interaction with other dietary ingredients, then the evidence would indicate that at the concentration studied, menadione-thioether is not an antimetabolite of menadione. On the other hand, if the peroxide reacted with the thioether to form sulfoxides, sulfones or a dimer of menadione, the data would indicate that these adducts are also ineffective as antimetabolites. The results of this study and our earlier studies strongly suggest that, while menadione and ethyl cysteinate react to cause a hemorrhagic syndrome, the condition is due to vitamin K deficiency per se and not to antimetabolite formation.

The idea that oxidation of ethyl cysteinate renders it ineffective for reaction with menadione was tested in the next experiment. A 50% solution of ethyl cysteinate·HCl was oxygenated for 18 hours at room temperature and then incorporated into diet-17. The results of feeding this diet to male, CDF rats are shown in Tables 48 and 49.

In accordance with our earlier findings, growth of rats fed diet-17 was slightly greater than those fed diet-16. The oxygenation of ethyl cysteinate had no effect on growth rate. Diet and water consumption was essentially equivalent for all groups.

Hypoprothrombenemia and hemorrhage occurred in virtually all rats fed diet-17. Replacing ethyl cysteinate with the oyxgenated solution did not reduce the occurrence of abnormalities. Four of the animals in this group died from bleeding and the 4 surviving animals were all found to be hypoprothrombenemic. Three out of ten rats were abnormal when diet-16 was fed.

In view of these findings, the oxygenated solution of ethyl cysteinate·HCl was chemically assayed for -SH groups. It was found that only a 10% loss of -SH occurred as the result of oxygenation so that enough ethyl cysteinate·HCl was still available to inactivate menadione and, thereby, cause the observed hemorrhagic syndrome.

It is of interest that 3 abnormalities were observed in the group ingesting diet-16. This diet contains only 0.55 g/L ethyl cysteinate·HCl and does not usually produce hemorrhagic symptoms. The prolonged prothrombin times observed in the present experiment suggests that other dietary factors were present in sufficient quantity to render this small concentration of ethyl cysteinate·HCl effective, if only to a limited extent. This was previously shown to be the case when an oxygenated fat mix was added to diet-16 containing the same amount (0.55 g/L) of ethyl cysteinate·HCl (Table 39). It would appear, therefore that ethyl cysteinate·HCl must be completely absent from the diet to avoid all risk of producing hypoprothrombenemia.

The last experiment in this series was conducted to determine the effect of dietary menadione concentration on the onset of hemorrhage in CDF rats fed Codelid diet-17. Since the molar concentration of ethyl cysteinate·HCl present in diet-17 is 10^3 X that of menadione, it was hypothesized that if antimetabolites are formed from their interaction, the addition of more menadione to the diet would lead to the formation of additional antimetabolite and a severe hemorrhagic condition would result. On the other hand, if this were not the case, then increasing the menadione level in the diet would tend to prevent the onset of vitamin K deficiency. The experimental design and results are shown in Tables 50 and 51.

The data show that growth rate is not significantly altered by dietary menadione concentration although slight

retardation was observed at the highest concentration. Diet consumption and water consumption were also uneffected by dietary treatment.

The prothrombin data in Table 51 show only a very small occurrence (4 out of 14 rats) of hypoprothrombenemia in the group receiving 10^{-5} moles/L of menadione. Only 2 abnormalities were found in the remaining three groups receiving higher levels of menadione. Since hemorrhage-producing diet-17 normally contains 10^{-5} moles/L menadione, we expected a greater incidence of hypoprothrombenemia than observed. The unusually mild response in this experiment makes it difficult to draw any definite conclusions but the almost complete absence of hypoprothrombenemia in the higher concentration groups suggests that increasing the menadione concentration in the diet prevents the onset of vitamin K deficiency and does not lead to the formation, if any, of effective amounts of antimetabolite.

TABLE 26

GROWTH RATE, DIET CONSUMPTION AND WATER CONSUMPTION OF RATS FED CODELID DIET 16 OR 17 WITH GRADED LEVELS OF ETHYL CYSTEINATE*

Test Diet	E.C. Level**	Av. daily body weight gain	Av. daily diet consumption	Av. daily water consumption
	gm/L	gm/rat/day	ml/rat/day	ml/rat/day
Diet 16	0.55	3.3	21.7	17.4
Diet 16	2.43	3.4	19.6	15.2
Diet 17	--	4.3	24.9	18.5
Diet 17	1.22	4.0	22.5	19.0
Diet 17	2.43	4.1	22.6	18.5
Lab Blox	--	5.0	15.5gm	25.2

*Experimental period 27 days. Twelve CDF, male weanling rats from identified litters of 9-11 littermates allotted to each dietary treatment. Housed 2 per cage. For detailed data obtained over the entire 9 week experimental period, see Table 7 of the Appendix.

**Ethyl cysteinate·HCl.

TABLE 27

PROTHROMBIN TIMES OF RATS FED CODELID DIET 16 OR 17 WITH GRADED LEVELS OF ETHYL CYSTEINATE·HCl*

Diet	16		17			Lab Blox
E.C. level gm/L	0.55	2.43	0	1.22	2.43	---
Weeks on Exp't.	Prothrombin Time Seconds					
4	19.8	15.8	11.2	10.5	120(+)**	10.9
	11.3	10.9	9.8	11.7	12.5	
	10.7					
5	11.6	20.0**	28.1**	11.2	10.6	
		18.4	9.8		11.0	
7	13.2	11.2	11.4		11.9	
		11.5			11.3	
8	11.2	11.7	10.4	17.8**	120(+)**	10.3
	11.7	11.2	11.8	11.9	14.7**	11.2
9	13.4	13.4	11.8	68.1**	115.0**	15.3
	12.9	12.7	10.8	15.6**	21.1**	13.7
	11.8	11.4	10.7	18.2**	25.5**	12.9
	11.1	11.1	11.1	19.6**	23.2**	13.7
	10.4		10.7	15.4**	60(+)**	
				47.4**		
				34.1**		
				120(+)**		

*CDF male weanling rats from identified litters of 9-11 littermates allotted to each dietary treatment. Housed 2 per cage.

**Prolonged thromboplastin generation time.

TABLE 28

THE EFFECT OF MENADIONE THIOETHER ON THE GROWTH RATE, DIET CONSUMPTION AND WATER CONSUMPTION OF CFE RATS FED CODELID DIET 16*

Test Diet	Menadione Thioether	Av. daily body weight gain	Av. daily diet consumption	Av. daily water consumption
	mg/L	gm/rat/day	ml/rat/day	ml/rat/day
Diet 16	5	4.2	29.1	14.8
Diet 16	15	4.1	28.1	13.2
Diet 16 (-)Vit.K	5	4.0	29.6	13.1

*Experimental period 28 days. Ten CFE male rats 24 days of age from identified litters of 9-11 littermates allotted to each dietary treatment. Housed 2 per cage. For detailed data obtained over 7 weeks see Table 11 of the Appendix. For data on CFE male rats of similar age consuming Lab Blox or Codelid diet-16 without menadione-thioether see Table 30.

TABLE 29

THE EFFECT OF MENADIONE THIOETHER ON THE PROTHROMBIN TIMES OF CFE RATS FED CODELID DIET 16*

	Prothrombin Times		
Test Diet	16	16	16(-)Vit.K
Menadione Thioether Conc. mg/L	5	15	5
Weeks on Exp't		seconds	
7	10.2	10.6	10.4
	10.8	9.7	10.2
	10.7	10.2	9.7
8	10.5	10.3	10.3
	10.2	10.3	10.7
	10.1	10.2	10.7
9	11.2	11.7	10.7
	12.2	10.7	10.7
	12.7	10.6	11.2
			12.8

*CFE male rats 24 days of age from identified litters of 9-11 littermates allotted to each dietary treatment. For data on CFE male rats of similar age consuming Lab Blox see Table 31.

TABLE 30

GROWTH RATE, DIET CONSUMPTION AND WATER CONSUMPTION OF CFE RATS FED CODELID DIET 16 OR 17 WITH OR WITHOUT MENADIONE*

Test Diet	Menadione Level	Av. daily body weight gain	Av. daily diet consumption	Av. daily water consumption
	mg/L	gm/rat/day	ml/rat/day	ml/rat/day
Diet 16	2.0	4.4	25.0	8.8
Diet 16	0.0	4.6	25.8	12.6
Diet 17	2.0	4.4	25.7	12.3
Diet 17	0.0	4.6	25.5	10.9
Lab Blox	---	6.3	18.9gm	25.2

*Experimental period 28 days. Twelve CFE rats 25 days of age from identified litters of 9-11 littermates allotted to each treatment. Housed 2 per cage. For detailed data obtained over the entire 5 week experimental period, see Table 14 of the Appendix.

TABLE 31

PROTHROMBIN TIMES OF CFE RATS FED CODELID DIET 16 OR 17 WITH OR WITHOUT MENADIONE*

	Prothrombin Time				
Test Diet	16	16	17	17	Lab Blox**
Menadione Level mg/L	2.0	0.0	2.0	0.0	
			seconds		
	10.9***	10.9	11.7	11.3	10.3
	10.7***	10.7	11.3	11.7	9.9
	10.9	11.2	10.8	10.4	10.8
	12.8	11.2	10.8	10.9	10.8
	11.4	12.7	11.8	11.1	10.2
	12.5	11.7	12.2	11.2	9.7
	19.6	10.9	11.8	11.2	12.5
	16.6	11.9	12.0	11.5	11.2
	10.7		12.6	13.1	10.7
	11.2		13.6	12.2	11.1
				10.2	10.2
					10.8

*All samples were taken during the 5th week of experiment unless otherwise noted. Rats were 25 days of age at start of experiment.

**All samples from this group taken between the 7th and 9th week of experiment.

***Samples taken during 4th week of experiment.

TABLE 32

GROWTH RATE, DIET CONSUMPTION AND WATER CONSUMPTION OF CDF RATS FED CODELID DIET 16 WITH OR WITHOUT MENADIONE*

Test Diet	Menadione Level	Av. daily body weight gain	Av. daily diet consumption	Av. daily water consumption
	mg/L	gm/rat/day	ml/rat/day	ml/rat/day
Diet 16	2.0	3.3	23.9	24.0
Diet 16	0.0	3.1	22.1	23.0
Lab Blox	---	4.7	16.6	27.7

*Experimental period 28 days. CDF male rats 30 days of age from identified litters of 9-11 littermates allotted to each dietary treatment. Housed 2 per cage. Twelve rats allotted to each liquid diet group. Ten rats allotted to the Lab Blox group. For detailed data obtained over the entire 35 day experimental period see Table 17 of the Appendix.

TABLE 37

PROTHROMBIN TIMES OF FISCHER* RATS FED VITAMIN K-FREE CODELID DIETS**

	Test diet		
	Diet-16	Diet-16 minus Menadione	Diet-17 minus Menadione
Weeks on exp't.	Prothrombin Time seconds		
6	20.8+	10.3	10.3
	11.1	11.8	13.8+
8	12.8	10.0	10.8
	11.3	11.6	10.8
	11.0	9.6	10.3
	10.3	10.1	10.5
9	12.3	11.3	11.0
	10.8	11.3	11.1
	11.3	10.8	11.1
	11.3		10.8

*Source of Fischer rats -- A.R. Schmidt Inc., Madison, Wisconsin.

**Twelve male Fischer rats 25 days of age from identified litters started on each test diet. Housed 2 per wire bottomed cage.

+Prolonged thromboplastin generation time.

TABLE 35

PROTHROMBIN TIMES OF CFE AND FISCHER RATS
FED CODELID DIETS*

Rat strain	CFE**			Fischer**		
Test diet	16	17	LB+	16	17	LB+
Weeks on exp't.	Prothrombin Time - Seconds					
6	10.7	11.0	10.7	10.7	11.1	10.8
	11.4	11.2	11.4	10.8	11.0	11.1
	11.3	11.3	9.8	10.8	46.3‡	11.2
	11.8	10.8	11.2	11.4	11.2	
	11.8		10.1	10.2	11.7	
7	10.2	9.8	10.2	10.2	10.7	10.5
	10.8	10.5	10.8	9.8	10.8	10.7
	10.2	11.6	10.6	10.8	10.8	11.3
	11.3		11.8	11.3	11.8	12.0
8	10.3	10.3	9.8	10.3	10.8	10.4
	10.2	10.3	9.8	10.8	10.7	10.3
	11.7	11.8	10.3	11.5	11.3	11.3
	10.3	11.6	10.3	10.1	11.8	10.8
	10.3	10.8	9.8	10.8	12.0	10.3
9	11.3	10.1	10.8	10.3	10.3	10.8
	9.8	10.8	12.9	11.8	10.8	10.8
	10.1	10.8	10.3		13.5	10.6
	13.3	11.8	10.8		11.7	10.8

*Eighteen rats 27-30 days of age from identified litters of 9-11 littermates allotted to each test diet. Housed 2 per wire bottomed cage.

**Source of CFE rats - Carworth Inc., New City, N. Y.
Source of Fischer rats - A. R. Schmidt, Inc., Madison, Wisc.

+LB - Lab Blox product of Wayne Division of Allied Mills.

‡Prolonged thromboplastin generation time.

TABLE 36

GROWTH RATE, DIET CONSUMPTION AND WATER CONSUMPTION OF FISCHER* RATS FED VITAMIN K-FREE DIETS**

Test diet	Menadione level mg/L	Av. daily body weight gain	Av. daily diet consumption	Av. daily water consumption
Diet-16	2.0	2.9	22.1	13.6
Diet-16	0.0	2.8	21.5	13.1
Diet-17	0.0	3.6	25.5	13.3

*Source of Fischer rats - A. R. Schmidt, Inc., Madison, Wis.

**Experimental period 28 days. Twelve male rats, 25 days of a age from identified litters started on each test diet. Housed 2 per wire bottomed cage. For detailed data, see Appendix Table 33.

TABLE 34

GROWTH RATE, DIET CONSUMPTION AND WATER CONSUMPTION OF CFE AND FISCHER RATS FED CODELID DIET-16 OR -17+

Strain	Diet	Av. daily body weight gain g/rat/day	Av. daily diet consumption ml/rat/day	Av. daily water consumption ml/rat/day
CFE	16	4.0	27.1	14.2
Fischer	16	3.0	18.9	16.4
CFE	17	4.4	30.3	15.3
Fischer	17	3.6	23.9	15.3
CFE	Lab Blox	5.9	17.9g	21.2
Fischer	Lab Blox	4.4	12.9g	16.2

+Experimental period 28 days. Eighteen CFE or Fischer (A. R. Schmidt) male rats, 26-27 days of age from identified litters of 9-11 littermates allotted to each dietary treatment. Housed 2 per wire bottomed cage. For detailed data obtained over 42 days see Table 20 of the Appendix.

TABLE 33

PROTHROMBIN TIMES OF CDF RAT FED CODELID DIET 16 WITH OR WITHOUT MENADIONE*

	Prothrombin Time		
Test Diet	Diet 16	Diet 16	Lab Blox
Menadione Level mg/L	2.0	0.0	
		seconds	
	13.2	52.3**	10.8
	10.2	22.3**	10.2
	10.2	10.2	10.2
	10.2	32.8**	10.1
	9.8	10.2	10.1
	19.4**	28.8**	10.3
	11.4	120(+)**	10.2
	11.2	120(+)**	9.8
	10.3	35.1**	10.3
	11.7	10.2	9.2
	12.0	15.7**	
		65.1**	

*CDF male rats 30 days of age from identified litters of 9-11 littermates allotted to each dietary treatment. Housed 2 per cage. All samples taken after 5th week of experiment.

**Prolonged thromboplastin generation time.

TABLE 38

THE EFFECT OF MENADIONE THIOETHER, OXYGENATED FAT MIX AND ETHYL CYSTEINATE ON GROWTH, DIET CONSUMPTION AND WATER CONSUMPTION OF FISCHER RATS FED CODELID DIET-16*

Diet supplement**	Av. daily body weight gain g/rat/day	Av. daily diet consumption ml/rat/day	Av. daily water consumption ml/rat/day
None	3.0	27.8	17.9
E.C.	2.9	27.1	14.7
Thioether	3.1	27.3	14.7
Oxygenated fat mix	3.0	28.3	14.3
Oxygenated fat mix + E.C.	3.1	27.7	18.1
Oxygenated fat mix + thioether	3.1	26.9	18.3

*Data obtained over first 28 days of a 63 day experimental period. Ten CDF male rats, 30 days of age from identified litters of 9-11 littermates, allotted to each dietary treatment. Housed 2 per cage. For detailed data, see Table 21 of the Appendix.

**E.C. - Ethyl Cysteinate·HCl - 2.42 gm/L; Thioether - Menadione thioether - 5.5 mg/L; Oxidized fat mix - 5.03gm to replace normal fat mix.

TABLE 39

THE EFFECT OF MENADIONE THIOETHER, OXYGENATED FAT MIX AND ETHYL CYSTEINATE CONCENTRATION ON THE PROTHROMBIN TIMES OF FISHER RATS FED CODELID DIET-16*

Dietary treatment		Prothrombin time					
Ethyl Cysteinate·HCl	gm/L	0.55	2.42	0.55	0.55	2.42	0.55
Menadione-thioether	mg/L	--	--	5.5	--	--	5.5
Fat mix	gm/L	5.03	5.03	5.03	--	--	--
Oxygenated fat mix	gm/L	--	--	--	5.03	5.03	5.03
Weeks on experiment		seconds					
4		10.2	25.3**	9.7	10.2	88.3**	10.1
		9.9	16.8**	9.7	10.6	49.6**	10.7
5		11.6	13.3	14.8	11.2	10.2	11.7
		11.5	10.1	9.8	10.7	54.0**	11.4
		D***				DH	
6		18.7**	10.7	21.6**	17.3**	58.7**	16.7**
		10.7	10.5	17.6**	12.7	11.4	13.7
8			59.4**				84.9**
			DH				
9		13.7	28.0**	12.1	14.8**	120+**	51.8**
		11.8	48.0**	16.8**	13.4	120+**	17.8**
		D		15.2**	17.7**	26.9**	16.8**
				18.7**	23.9**		

*CDF male rats 30 days of age from identified litters of 9-11 littermates allotted to each dietary treatment. Housed 2 per cage.

**Prolonged thromboplastin generation time.

***D - Death due to undetermined cause; DH - Death due to hemorrhage.

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TABLE 40

THE EFFECT OF ETHYL LINOLEATE AND ETHYL CYSTEINATE·HCL ON THE GROWTH RATE, DIET CONSUMPTION AND WATER CONSUMPTION OF CDF RATS FED CODELID DIET-17+

Test diet‡	Av. daily body weight gain g/rat/day	Av. daily diet consumption ml/rat/day	Av. daily water consumption ml/rat/day
Diet-17	3.4	21.7	18.5
Diet-17 minus E.L.	2.8*	21.1*	21.3*
Diet-17 minus E.C.	3.5	24.7	20.3
Diet-17 minus (E.L. + E.C.)	3.2	24.9	20.0

+Experimental period 28 days. Fifteen CDF rats 33 days of age from identified litters of 9-11 littermates allotted to each dietary treatment. Housed 1 or 2 per wire bottomed cage. Unless otherwise noted, values represent 14 rats. For detailed data obtained over the entire 6 week experimental period, see Table 24 of the Appendix.

‡E.L. - Ethyl Linoleate; E.C. - Ethyl Cysteinate·HCl.

*Mean value for 13 rats.

TABLE 41

THE EFFECT OF ETHYL LINOLEATE AND ETHYL CYSTEINATE ON THE HEMORRHAGIC SYNDROME IN RATS FED CODELID DIET-17+

Ethyl cysteinate·HCl gm/L	2.42	2.42	0.0	0.0
Ethyl linoleate gm/L	2.0	0.0	2.0	0.0
Weeks on experiment	Prothrombin time seconds			
1		DH‡		
3	DH			
4		D		
6	DH, DH, D, DH, DH			
7	DH	18.8*	9.8	10.3
	55.5*	14.3*	11.3	10.8
	120(+)*	43.3*	9.8	11.8
	120(+)*	41.6*	12.3	11.8
	120(+)*	80.4*	11.8	11.5
	19.8*	63.0*	11.0	10.8
8	120(+)*	19.8*	11.3	10.8
	12.6	22.8*	10.3	10.3
	18.8*	19.3*	11.5	10.8
		10.4		
		67.6*		
		17.0*		
		26.8		
9			10.3	10.8
			9.9	11.6
			10.8	10.1
			10.3	10.3
			10.0	9.8
				9.9

+Fifteen CDF male rats 33 days of age from identified litters of 9-11 littermates allotted to each dietary treatment. Housed 1 or 2 per wire bottomed cage.

‡DH - death due to hemorrhage; D - death due to undetermined cause.

*Prolonged thromboplastin generation time.

TABLE 42

THE EFFECT OF DIETARY ANTIOXIDANTS ON THE GROWTH RATE, DIET CONSUMPTION AND WATER CONSUMPTION OF CDF RATS FED CODELID DIET-17+

Test diet‡	Av. daily body weight gain	Av. daily diet consumption	Av. daily water consumption
	g/rat/day	ml/rat/day	ml/rat/day
Diet-17	3.7	32.3	24.2
Diet-17 + Antioxidants	2.3	29.6	28.0
Diet-17 + Antioxidants minus E.L.	3.1	32.1	28.3

+Experimental period 28 days. Ten CDF male rats, 51 days of age from identified litters of 9-11 littermates allotted to each dietary treatment. Housed 2 per wire bottomed cage. For detailed data obtained over the entire 7 week experimental period see Table 26 of the Appendix.

‡Antioxidants - Ascorbic acid and α-tocopherol acetate; E.L.- Ethyl Linoleate.

TABLE 43

THE EFFECT OF DIETARY ANTIOXIDANTS ON THE ONSET OF HEMORRHAGE AND PROTHROMBIN TIMES OF CDF RATS FED CODELID DIET-17*

Dietary treatment	17	Prothrombin times 17 + Anti-oxidants	17-EL** + antioxidants
Weeks on experiment		seconds	
3		DH+	18.8‡
		DH	DH
		DH	DH
			DH
5		DH	DH
		DH	
6		DH	DH
		D	DH
7	17.8‡	120(+)‡	72.1‡
	21.1‡	26.7‡	34.7‡
	21.8‡	120(+)‡	17.6‡
	21.0‡	DH	
	23.3‡		
	24.1‡		
	32.3‡		
	30.6‡		
	18.0‡		
	19.8‡		

*Ten CDF male rats, 51 days of age, from identified litters, allotted to each dietary treatment. Housed 2 per wire bottomed cage.

**EL - Ethyl linoleate.

+DH - Death due to hemorrhage; D - Death due to unknown cause.

‡Prolonged thromboplastin generation time.

TABLE 44

EFFECT OF ANTIOXIDANTS, VITAMIN A AND ETHYL CYSTEINATE·HCL ON THE GROWTH RATE, DIET CONSUMPTION AND WATER CONSUMPTION OF CDF RATS FED CODELID DIET-17+

Group	Diet variable‡	Av. daily body weight gain g/rat/day	Av. daily diet consumption ml/rat/day	Av. daily water consumption ml/rat/day
I	Excess Vit. E (50mg/L)	3.4	31.7	12.1
II	Excess Vit. C (500mg/L)	3.0	31.2	12.6
III	Vit. A-free	3.4	30.1	12.9
IV	As Gp I minus Vit. A	3.6	30.8	14.1
V	As Gp II minus Vit. A	3.3	30.3	13.9
VI	As Gp III + Excess Vit. E + Excess Vit. C	3.4	31.6	12.9
VII	E.C.-free + Excess Vit. E + Excess Vit. C	3.3	29.3	12.8
VIII	As Gp VII minus Vit. A	3.7	30.9	14.5

+Eight CDF male rats 40-42 days of age from identified litters of 9-11 littermates started on each test diet. Housed 2 per wire bottomed cage. For detailed data, see Table 28 of the Appendix.

‡Diet-17 normally contains: Vit. E (1 IU/mg)-25mg/L; Vit C-250mg/L; Vit. A (1,760 IU/mg) 5mg/L and E.C. (Ethyl Cysteinate·HCl) 2.43g/L.

TABLE 45

THE EFFECT OF DIETARY ANTIOXIDANTS, VITAMIN A AND ETHYL CYSTEINATE ON THE PROTHROMBIN TIMES OF CDF RATS FED CODELID DIET-17*

Diet Variable**	Group							
	I	II	III	IV	V	VI	VII	VIII
Vit. A free			X	X	X	X		X
Excess Vit. C		X			X	X	X	X
Excess Vit. E	X			X		X	X	X
E.C.-free							X	X
Weeks on exp't.	Prothrombin Times seconds							
3	33.7+	DH***	26.5+	48.3+	31.6+	12.6	13.5	14.6
	32.0+	35.3+	25.5+	DH	45.6+			
4		D		DH				
5	14.9+	9.8	22.6+	12.6	DH	17.6+	11.3	11.0
					44.0+			
6	12.2	9.8	9.3	9.8	16.1	11.5	9.5	10.0
	13.9						10.8	10.8
							10.3	9.8
7	15.3	12.6	10.8	10.0	12.8	13.6	11.1	9.5
	14.8	15.0	11.3	11.5	9.5	11.8	11.1	10.6
	11.8	10.9	10.0	10.3	9.3	11.8	10.5	10.6
			10.3			10.8		

*Eight CDF male rats 40-42 days of age from identified litters of 9-11 littermates started on each test diet. Freshly prepared diet was fed to Groups I, II, IV and V at beginning of 7th week.

**Excess Vit. C - 500 mg/L; Excess Vit. E - 50 mg/L; Vit. A - 1,760 IU/L; E.C. (ethyl cysteinate·HCl) 2.42 g/L.

***DH - death due to hemorrhage; D - death due to undetermined cause.

+Prolonged thromboplastin generation time.

TABLE 46

THE EFFECT OF SYNKAVITE[+], DIETHYL CYSTINATE·HCL, HYDROGEN PEROXIDE AND MENADIONE-THIOETHER ON THE GROWTH RATE, DIET CONSUMPTION AND WATER CONSUMPTION OF CDF RATS FED CODELID DIET-17[‡]

Test diet	Av. daily body weight gain	Av. daily diet consumption	Av. daily water consumption
	g/rat/day	ml/rat/day	ml/rat/day
Diet-17	4.0	25.2	19.5
Diet-17 w Synkavite (6.5mg/L)	4.0	24.9	20.7
Diet-17 w Diethylcystinate·HCl (2.43g/L)	3.6	23.1	20.1
Diet-17 + H_2O_2 (8g/L)	3.4	23.2	18.7
Diet-17 minus E.C.* + H_2O (8g/L)	2.3	21.8	16.9
Diet-17 + H_2O_2 (16g/L)	2.8	21.4	18.9
Diet-17 + H_2O_2 (8g/L) + Menadione-thioether (5mg/L)	2.2	18.8	17.3

[+]Synkavite - menadiol sodium diphosphate, Roche Laboratories, Nutley, New Jersey.

[‡]Experimental period 28 days. Eight CDF male rats 36 days of age started on each test diet. Housed 2 per wire bottomed cage. For detailed data obtained over 63 days see Table 29 of the Appendix.

*Ethyl cysteinate·HCl.

TABLE 47

THE EFFECT OF SYNKAVITE†, DIETHYL CYSTINATE·HCL AND HYDROGEN PEROXIDE ON THE PROTHROMBIN TIMES OF CDF RATS FED CODELID DIET-17‡

Diet Variable								
Menadione	mg/L	2.1	--	2.1	2.1	2.1	2.1	2.1
Synkavite‡	mg/L	--	6.5	--	--	--	--	--
Ethyl Cysteinate	g/L	2.43	2.43	--	2.43	0.0	2.43	2.43
Diethylcystinate·HCl	g/L	--	--	2.43	--	--	--	--
H_2O_2	g/L	--	--	--	8	8	16	8
Menadione-thioether	mg/L	--	--	--	--	--	--	5.5
Weeks on Experiment		Prothrombin Time seconds						
5		38.3*	9.9	9.6	21.6*	11.3	10.5	12.0
		35.1*	9.8	10.2	24.6*	11.3	10.8	11.3
6		20.8*	11.2	10.3	10.3	10.3	19.8*	10.3
		11.1	11.3	10.8	11.3	10.8	10.8	11.3
8		35.0*	11.3	11.3	12.7	11.5	10.9	10.7
		34.8*						
9		38.3*	10.3	9.8	33.9*	9.3	10.5	10.6
		20.6*	12.1	10.3	10.3	10.3	9.8	10.6
			10.2	11.0	18.6*	9.6	10.8	9.8

†Synkavite - Menadiol sodium diphosphate, Roche Laboratories, Nutley, New Jersey

‡Eight CDF male rats 36 days of age were started on each diet. Housed 2 per wire bottomed cage.

*Prolonged thromboplastin generation time.

TABLE 48

GROWTH RATE, DIET CONSUMPTION AND WATER CONSUMPTION OF RATS FED CODELID DIETS WITH OR WITHOUT OXYGENATED ETHYL CYSTEINATE·HCL*

Test diet	Av. daily body weight gain g/rat/day	Av. daily diet consumption ml/rat/day	Av. daily water consumption ml/rat/day
Diet-16	2.9	24.5	17.3
Diet-17	3.3	24.9	17.5
Diet-17-oxid. E.C.	3.2	24.5	17.2

*Experimental period 42 days. Ten CDF male rats 30 days of age from identified litters of 9-11 littermates allotted to each dietary treatment. Housed 2 per wire bottomed cage. For detailed data see Table 30 of the Appendix.

TABLE 49

THE EFFECT OF OXYGENATING ETHYL CYSTEINATE·HCl ON THE PROTHROMBIN TIMES OF CDF RATS*

Test diet	Prothrombin time		
	Diet-16	Diet-17	Diet-17-Oxid. E.C.
Weeks on exp't.	seconds		
7	61.8**	83.9**	120(+)**
	31.7**	31.4**	52**
		54.2**	DH
8	DH+	DH	
9	10.9	18.1**	120(+)**
	11.3	120(+)**	70.4**
		DH	12.9
		DH	DH
			DH
			DH
10	10.8	56.7**	66.6**
	13.1	120(+)	
	11.3		
	11.5		
	12.1		

*Ten CDF male rats 30 days of age from identified litters of 9-11 littermates allotted to each dietary treatment. Housed 2 per wire bottomed cage.

**Prolonged thromboplastin generation time.

+DH - Death due to hemorrhage.

TABLE 50

THE EFFECT OF MENADIONE CONCENTRATION ON THE GROWTH RATE, DIET CONSUMPTION AND WATER CONSUMPTION OF CDF RATS FED CODELID DIET-17*

Menadione Concentration moles/liter	Av. daily body weight gain g/rat/day	Av. daily diet consumption ml/rat/day	Av. daily water consumption ml/rat/day
$1x10^{-5}$	3.4+	26.0	14.7
$1x10^{-4}$	3.7	25.8	13.6
$1x10^{-3}$	3.6	26.0	15.8
$1x10^{-1}$	3.0	25.9	14.4

*Fourteen CDF male littermates from identified litters, 36 days of age, allotted to each dietary treatment. Housed 2 per wire bottomed cage.

+Average value for 13 rats.

TABLE 51

THE EFFECT OF MENADIONE CONCENTRATION ON THE PROTHROMBIN TIMES OF CDF RATS FED CODELID DIET-17*

Weeks on Exp't.	Molar concentration of dietary menadione			
	$1x10^{-5}$	$1x10^{-4}$	$1x10^{-3}$	$1x10^{-1}$
	Prothrombin Times seconds			
5	12.8	13.6	11.6	11.8
	15.6+	9.8	10.8	11.1
	9.5	11.1	10.3	10.3
	10.2	10.5		11.3
6	11.8	11.8	>60+	12.1
	10.8	14.7	10.6	11.5
7	13.3	11.3	11.0	12.0
	13.1	15.3	10.8	9.4
8	20.3+	10.6	11.0	10.0
	19.4+	12.0	10.6	10.6
9	12.6	10.8	10.8	10.5
	16.1+	10.0	10.8	10.3
	10.1	10.8	10.8	12.9+
		12.0	10.3	11.0

*Fourteen CDF male littermates from identified litters, 36 days of age allotted to each dietary treatment. Housed 2 per wire bottomed cage.

+Prolonged thromboplastin generation time.

STUDIES OF THE RELATIONSHIP OF NUTRITIONAL INPUT TO THE MICROFLORAL POPULATION OF RATS

Introduction

A gross change in an animal's diet can be expected to affect the numbers and types of microflora in the animal's intestinal tract. The effect of diets on intestinal microflora have been reported by several workers. A few examples are Nath et al[20] and Porter and Rettger[21] studies on rats, Gall et al on mice[22] and more recently, Gall et al on humans[23] and chimpanzees[24].

In the present study, we fed several chemically defined liquid diets and one natural chow diet to CDF and CFE rats. The CDF rats were obtained from two suppliers, the CFE from a third. The effects of the various diets were measured in terms of bacterial counts on cecal contents of the rats. Bacterial counts were compared between different diet groups and among different rat strains on the same diet.

Statistically significant differences in bacterial counts arose from variation in both diet, and rat strain.

The CDF rats supplied by Charles River Breeding Laboratories developed a hemorrhagic condition when fed diet-17 with menadione and diet-16 without menadione. This condition could not be related in this study to the presence or absence of specific microflora, but may be related to a high ratio of anaerobes to aerobes in the Charles River CDF rats fed diet-17. Cecal contents of CDF Charles River rats were administered orally to germ-free CFE rats 11 times during an 8 week period.

The microflora of the CDF rats were found to establish themselves in the intestinal tract of the CFE animals. However, the hemorrhagic condition did not develop. The ratio of anaerobes to aerobes was found to be higher in the Charles River rats, but owing to the smaller number of germ-free A. R. Schmidt rats, a statistically significant difference was not evident.

Materials and Methods

Mature, male rats (4 to 10 week post-weaning) were used in this study. Feeding and maintenance of these rats is described in the previous section of this report (Animal Experiments). Cecal contents of these rats were examined during the fourth to ninth week after initiation of dietary treatment. Three types of rats were used; CFE rats purchased from Carworth, Inc.[1], Fischer 344 rats (CDF-CR) purchased from Charles River Breeding Laboratories, Inc.[2], and Fischer 344 rats (CDF-ARS) purchased from A. R. Schmidt Co., Inc.[3].

The experimental groups consisted of the three types of rats on each of four diets; diet-16[4], diet-16 without menadione, diet-17[4] and Lab Blox[5]. The compositions of the liquid diets tested are shown in Tables 9 and 10.

Bacterial counts were made initially on freshly extruded fecal samples in the earlier part of this study (Semi-Annual Report). These results were subsequently averaged along with later counts made on the cecal contents of rats receiving the same diets as there were no differences in cecal and fecal counts.

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1. Carworth, Inc., New City, N.Y.
2. Charles River Breeding Laboratories, Inc., Wilmington, Mass.
3. A. R. Schmidt Co., Inc., Madison, Wisconsin
4. Schwarz BioResearch, Inc.
5. Wayne Lab Blox, Allied Mills, Inc., Chicago, Illinois

The p values obtained from this t test are listed in tables 61 to119. Tables 61 to 87 compare the effects of various diets on bacterial counts while tables 88 to 119 compare the effects of rat strains on bacterial counts.

Tables 52 to 60 were constructed to show the degree of variation in bacterial counts within the rat groups.

Results

1. The distribution of bacterial counts within experimental groups.

Bacterial counts obtained from the cecal contents of the rats tested and the distribution of these counts are presented in tables 52 to 60.

It can be seen in table 52 that the number of E. coli present in cecal specimens varies widely within each group. For example, for CFE rats the range is as great as 0 to 10^9 E. coli per gram (wet weight) of cecal contents. The range of differences for any one type of rat receiving a particular diet is from 4 to 9 log units between the highest and lowest E. coli counts for that group. However, the mean number of E. coli isolated from all the groups has a much narrower range; 3.2×10^4 to 4.6×10^6 E. coli per gram (wet weight) of cecal contents.

If the samples yielding no E. coli are omitted from table 52, the remaining figures show a 6-7 log unit distribution of counts for CFE rats, while the CDF rats maintain a 3 to 5 log unit distribution.

The distribution observed was not caused by a

KF streptococcal agar - isolation identification and enumeration of enterococci. Trypticase soy agar with 5% defibrinated Sheep's blood - isolation and enumeration of total aerobes. Phenylethyl alcohol agar - isolation and enumeration of aerobes in the presence of large numbers of Proteus.

All media were incubated at 37°C. EMB and Staphylococcus medium No. 110 were incubated for 24 hours, anaerobic cultures were incubated for 72 hours, all other cultures were incubated for 48 hours before colony counts were made. Anaerobic cultures were incubated in Brewer jars[6] using Gaspaks[6] to obtain anaerobic conditions. LBS and KF Streptococcal agar cultures were incubated in candle jars.

Statistical Methods

Colony counts of bacteria isolated from the various dietary groups were compared statistically using Student's t-test of significance. The colony counts for individual rats were corrected for dilution, averaged and converted to logarithm form. The logarithm of the colony counts for individual rats was used in the statistical formula:

$$t = \sqrt{\frac{(m_1 m_2)\ (m_1+m_2-2)}{(m_1+m_2)\ (\Sigma x_1^2+\Sigma x_2^2)}} \cdot (\bar{x}_1-\bar{x}_2)$$

where m = number of samples, $\bar{x}$ = the mean log number of bacteria, $\Sigma x^2 = \Sigma(x-\bar{x})^2$, x = the individual sample log number of bacteria, subscripts 1 and 2 are for the two groups being compared. The formula was obtained from Snedecor[29] for comparing two groups of different sizes.

6. Baltimore Biological Laboratories, Baltimore, Md.

Cecal and/or fecal specimens were collected from each rat, maintaining a minimum exposure of the specimens to air. Cecal specimens were obtained by removing the cecum of chloroform anesthetized rats and extruding approximately 0.5 g of cecal contents into 4.5 ml of dilution fluid. The dilution fluid used was Gall's medium containing cysteine and bicarbonate[23].

These specimens were weighed in tared tubes of dilution fluid immediately after collection. They were then dispersed in the dilution fluid by trituration with a glass rod. Serial dilutions were made in tenfold increments from 10^{-1} to 10^{-7}. Two dilutions from the series were plated in duplicate for each of six plating media. Plate counts were made by surface inoculations of the media with 0.1 ml of the dilution. The inoculum was spread with a glass rod "hockey stick" using a revolving dish turntable.

The plating media and method of organism identification employed were based upon the procedures of Zubrzycki and Spaulding[25] and Graber, O'Neal and Rabin[26]. Brucella agar with 5% blood suggested for the isolation of Bacteroides[27] was used for isolation and enumeration of total anaerobic flora. Zubrzycki and Spaulding used a pour plate method for isolation and identification of enterococci. We, however, used a surface inoculation which is the preferred method according to Rogers and Sarles[28]. The media employed and their uses were as follows:

Levine Eosine methylene Blue Agar - isolation enumeration and identification of E. coli, Aerobacter and Proteus. Staphylococcus medium No. 110 - isolation and enumeration of Staphylococcus and Bacillus. LBS medium isolation and enumeration of Lactobacillus.

unidirectional change in bacterial counts, as these mature rats have already gone through the progressive changes involved in establishing their flora. Work conducted in the earlier part of this study indicates that the number of E. coli in fecal samples taken from a single rat will fluctuate from day to day, the variation being similar to that observed for the entire group. The degree of variation in bacterial counts cannot be attributed to changes in the food or water content of the cecum, as evidenced by the failure to observe a comparable variation in counts of Staphylococcus (table 55), total aerobes (table 57), total anaerobes (table 58), Lactobacillus (table 59) and enterococcus (table 60) which were obtained from the same specimens as those used for enumeration of E. coli.

Despite the wide variation within the groups, statistically significant differences among these groups are demonstrable, (tables 61, 70, 79, 88, 97, 102 and 111).

The distribution of samples based upon counts of Aerobacter (table 53) and Proteus (table 54) does not vary as widely as that for E. coli. However, we find that many of the samples contained no Aerobacter and many contained no Proteus.

An outstanding feature of the distribution tables for Aerobacter and Proteus is the large number of samples from which these organisms were not isolated. The presence of these two organisms appears to be dependent upon the source of rats. Aerobacter is present in 7 to 26% of the CFE rats, 6-38% of the

CDF-ARS rats and 56-85% of the CDF-CR rats. Proteus was isolated from 0 to 5% of the CFE rats, 0 to 11% of the CDF-ARS rats and 55 to 63% of the CDF-CR rats. As may be expected, the numbers of Aerobacter and Proteus present in CDF-CR rats are significantly higher than in CFE and CDF-ARS rats, tables

Staphylococcus counts ranged between 10^3 and 10^6 per gram (wet weight) of cecal contents in nearly 99% of all rats samples (table 55). For rats receiving a given diet, Staphylococcus counts were significantly higher ($p < 0.05$) in CDF-CR rats, tables 91, 100 and 105. The only exception was the comparison of CDF-CR rats and CFE rats when both receive Lab Blox (table 114). The CDF-CR counts in this comparison were not significantly higher ($p > 0.100$).

The distribution data for Bacillus counts (table 56) reveal a marked absence of this organism in most samples with the exception of those samples obtained from animals consuming Lab Blox. Examination of the diets revealed the presence of Bacillus in Lab Blox, in numbers approximately equal to that found in the same weight of feces or cecal contents. Very few or no Bacillus was isolated from the liquid diets. Rats which had been fed Lab Blox and were subsequently switched to one of the liquid diets generally had no isolatable Bacillus in their cecal contents and feces. Thus, it appears that the isolation of Bacillus from the cecal contents of these rats is primarily an indication that the organism is present in the diet.

The total aerobic flora isolated from the various groups of rats was found to be in the range of 10^7 to 10^9 bacteria per gram (wet weight) of cecal contents (table 57).

The distribution of cecal samples according to the number of total anaerobes isolated is within a comparatively narrow range of bacterial counts (table 58). The mean number of total anaerobes per gram (wet weight) of cecal contents for all experimental groups ranges from 1.2×10^9 to 4.0×10^9. Although there is little difference among the various groups, there are some statistically significant differences (tables

Levels of Lactobacillus in cecal contents are shown in table 59. Distribution of counts and mean number of Lactobacilli were fairly consistent within each rat strain tested. There were significant differences, in the mean number of Lactobacilli, among the various rat groups consuming diet-17 and Lab Blox (tables 109 and 118).

Enterococci were isolated from more than 98% of the cecal samples. The mean number of enterococci ranged from 3.0×10^5 to 2.8×10^6 per gram of cecal contents (table 60), and 95% of the samples contained enterococci at a level of 10^5-10^7 per gram.

2. The effect of diet on bacterial counts.

Tables 61 to 69 are arranged to show the effect of diet on mean number of bacteria isolated from CDF-ARS rats. Each table lists the results of statistical analyses for a single bacterial type.

These data for CDF-CR rats are contained in tables 70 to 78 and for CFE rats in tables 79 to 87. Rats consuming Lab Blox consistently had lower levels of E. coli. CDF-ARS rats fed diets 16 and diet-16 without menadione (16-K) had significantly more E. coli than the groups consuming either diet-17 or Lab Blox (table 61). This trend is reversed in the CFE rats (table 79). In this group, diet-17 produced higher E. coli counts than either diet-16 or Lab Blox.

Aerobacter counts for CDF-ARS rats were higher with diets 16 and 16-K than Lab Blox and higher with 16-K than diet-17 (table 62). Diets 16 and 16-K also produced higher counts than Lab Blox in CDF-CR rats (table 71). Aerobacter counts for CFE rats were higher with diet-16 than diet-17 (table 80). The differences between 16-K and 17, and diet-16 and Lab Blox are not quite at the significant level ($p > .05 < .10$) in CFE rats.

There are virtually no significant differences in Proteus counts caused by variation in diet (tables 63, 72 and 81). Table 81 reveals significantly more isolatable Proteus from CFE rats consuming diet-16 than from those consuming diet-17. However, the mean number of Proteus is 4.5×10^0 in the diet-16 group and 0 in the diet-17 group. This appears to be a very small difference in spite of what the statistical formula has shown.

Staphylococcus counts for both CDF-ARS and CDF-CR rats were significantly lower in the Lab Blox group than in the groups consuming diets 16,

16-K or 17 (tables 64 and 73). The CFE rats, however, have the highest Staphylococcus counts in the Lab Blox group (table 82), but the counts are significantly higher than diet-17 only.

Rats consuming Lab Blox had significantly higher Bacillus counts than the other dietary groups (tables 65, 74 and 83). This was true for all rat strains, CFE, CDF-ARS and CDF-CR. In addition, CDF-CR rats consuming diet-16 had a higher level of Bacillus than those consuming diets 16-K or diet-17.

There was no significant difference in counts of the total aerobes isolated from among the CDF-ARS dietary groups (table 66). The CDF-CR and the CFE rats had higher total aerobe counts in the Lab Blox group (tables 75 and 84) and by comparison, diet-16 counts were higher than diet-17 in the CDF-CR rats (table 75).

Tables 76 and 85 show that the total anaerobe counts for CFE rats and CDF-CR rats did not vary with diet. In CDF-ARS rats the counts were significantly higher in the group which consumed diet-17 than in the groups which consumed either diet-16 or Lab Blox (table 67).

CDF-CR rats consuming Lab Blox had higher Lactobacillus counts than the diet-17 group (table 77). Lactobacillus counts did not vary with diet in either the CFE or CDF-ARS groups (tables 68 and 86).

There were no significant differences in enterococcus counts of CFE rats (table 87). The Lab Blox

group of CDF-ARS rats had lower counts than the other dietary groups (table 69), while in CDF-CR rats only the diet 17 group was significantly higher than the Lab Blox group (table 78).

3. The effect of rat type on bacterial counts.

Three types of rats were used in this study. One was a CFE rat purchased from Carworth, Inc. The other two were Fischer 344 rats (CDF), purchased from Charles River Breeding Laboratories. (CDF-CR) and A. R Schmidt Co., Inc. (CDF-ARS). Statistically significant differences in bacterial counts were noted among the three types of rats consuming the same diet.

Statistical comparisons of the mean number of bacteria isolated from the three types of rat are presented in tables 88 to 119. Comparisons for rats consuming diet-16 are presented in tables 88 to 90, diet 16-K in tables 97 to 101, diet-17 in tables 102 to 110 and Lab Blox in tables 111 to 119.

Each type of rat consuming diet-16 was significantly different from the other types in terms of E. coli counts (table 88). The CDF-CR rats had the highest counts and CFE the lowest. When the three types of rats were fed Lab Blox, there were no significant differences in E. coli counts (table 111). The CDF-CR rats had significantly higher E. coli counts than the other rats when all were consuming diet 16-K (table 97). Both CDF-CR and CFE rats had higher E. coli counts than CDF-ARS rats when diet-17 was consumed (table 102).

Regardless of diet, the CDF-CR rats always had significantly higher counts of Aerobacter than CFE rats (tables 89, 98, 103 and 112). CDF-ARS rats had higher Aerobacter counts than CFE rats consuming diets 16-K or 17 (tables 98 and 103). There was no significant difference when the same groups were fed diet-16 or Lab Blox (tables 89 and 112). Aerobacter counts for CDF-CR rats are significantly higher than for CDF-ARS rats on all diets but 16-K (tables 89, 98, 103 and 112.

The number of Proteus isolated was always significantly higher in CDF-CR rats than in the CDF-ARS or CFE rats (tables 90, 99, 104 and 113). The CDF-ARS and CFE rats did not differ from each other in numbers of Proteus isolated from their cecal contents.

CDF-CR rats, regardless of diet, had higher counts of Staphylococcus than CDF-ARS rats (tables 91, 100, 105 and 114). Except for the Lab Blox groups (table 114), CDF-CR rats had significantly higher Staphylococcus counts than CFE rats. CDF-ARS rats had higher counts than CFE rats consuming diet-16, 16-K or 17. However, when these rats were fed Lab Blox, the CFE rats had significantly higher Staphylococcus counts than the CDF-ARS rats.

When the rats were fed Lab Blox, the CDF-CR group was found to have the highest Bacillus counts (table 115). There were virtually no Bacillus isolated from the groups consuming liquid diets (tables 92, 101 and 106).

Cecal contents of rats consuming diet-16 yielded

the same number of total aerobes (table 93) for each type of rat. When the rats received diet-17, the number of total aerobes isolated was greater in CFE rats than CDF-CR rats (table 107) and somewhat greater in CDF-ARS than CDF-CR rats ($p > .050$, $< .100$). Both the CFE and CDF-CR rats consuming Lab Blox have a higher count of total aerobes than the CDF-ARS rats (table 116). Counts of total anaerobes were the same for all rats consuming diet-17 (table 108). Counts were higher in CFE than CDF-ARS rats consuming diet-16 (table 94) or Lab Blox (table 117), while CDF-CR counts were higher than CDF-ARS on the Lab Blox diet only (table 117).

Lactobacillus counts did not vary with the type of rat when the rats were consuming diet-16 (table 95). The CFE rats had higher Lactobacillus counts than the others consuming diet-17 (table 109). When a Lab Blox diet was fed to the rats, Lactobacillus counts were higher in the CDF-CR and CDF-ARS rats (table 118).

The only significant difference in enterococcus counts is seen in the groups consuming diet-16 (table 96). Here the CDF-ARS rats have higher counts than CFE rats.

Discussion and Conclusions

A comparison of bacterial counts on cecal contents of three types of rats receiving liquid diets or a solid diet has been made. The results of this comparison reveal significant differences in bacterial levels among the types of rats and among the different dietary groups within each type of rat.

Nine genera or groups of bacteria were enumerated. Total anaerobes, total aerobes, Lactobacillus, enterococcus and E. coli were chosen as representatives of the predominant groups of intestinal flora. E. coli, Aerobacter, Staphylococcus aureus, Proteus, Bacillus cereus and Bacillus subtilis have been described by Almquist[30] as vitamin K-synthesizing bacteria and so these were included in the groups of bacteria to be enumerated.

Only the CDF-CR rats developed a hemorrhagic condition associated with a vitamin-K deficiency. The deficiency arose from ingestion of diet-17 and diet 16-K. The CDF-CR rats generally had higher cecal levels of the vitamin-K synthesizing bacteria than the other rats. One would expect from the data that CDF-CR rats would be less susceptible to a K deficiency because of their levels of K-synthesizing flora. However, Nightingale[31] has indicated that a vitamin K-deficiency can develop in rats with a normal level of K-synthesizing flora if a substance such as dihydroxystearic acid is included in the diet. This fatty acid does not inhibit the bacteria but apparently prevents them from synthesizing vitamin K. The nature of the diet-caused hemorrhagic condition and the possible mechanism of vitamin K inactivation are discussed in other sections of this report.

In an attempt to delineate the role of the intestinal flora in the hemorrhagic syndrome two possibilities were considered, one being a deficiency in K-synthesizing bacteria, the other an abundance of K-requiring bacteria in the intestine. It has been shown that the rats which developed the hemorrhagic syndrome had an abundance of

K-synthesizing organisms in their cecum. The cecal contents were not assayed for the presence of vitamin K, thus it is possible that these organisms may not have been producing the vitamin in the cecum of CDF-CR rats receiving diet-17 or diet 16-K.

Five male weanling gnotobiotic CFE rats were rendered normal by intestinal transplantation with the intestinal flora of CDF-CR rats. After nine weeks of receiving diet-17, the CDF-CR rats were shown to have developed prolonged prothrombin times, while the CFE rats which now carry the CDF-CR intestinal flora, exhibited normal prothrombin times. This indicates that either the vitamin K requiring organisms are present in insufficient numbers to successfully compete with the CFE rat for the vitamin, or that the organism is present in CDF-CR rats but failed to establish in the CFE. A third possibility is that a metabolic difference between the two rat strains exists. Comparative plate counts on cecal and/or fecal specimens of both groups of rats show a very similar flora but with a rather high E. coli level in the conventionalized CFE rats. Thus, it has not been established whether or not competition, between the host CDF-CR rat and its flora, for vitamin K has induced the hemorrhagic syndrome by increasing the overall vitamin K requirement.

Gross qualitative or quantitative changes in the intestinal microflora were not observed when rats were switched from a Lab Blox diet to a chemically defined liquid diet. Winitz et al[32], reported having used chemically defined liquid diets in studies relating to the regulation of human intestinal flora with chemical

diets. Human fecal samples were found to be nearly devoid of intestinal bacteria after the subjects had consumed liquid diets for a few weeks. The most dramatic response was obtained with diets containing glucose. The loss of bacteria was slower when other carbohydrates were substituted for glucose. The liquid diets used in the present study contained glucose and were very similar to diets used in the Winitz study. The vast difference between the results of the present study with rats and the Winitz study on humans, warrants at least a brief conjecture as to the discrepancy in results. Rats practice coprophagy and, therefore, will reinoculate themselves and at the same time, supply bulk material (feces) thought necessary to support the growth of bacteria in the intestinal tract. It was noted in the human study that addition of bulk to the diet retarded the diminution of fecal flora. In addition, the well-developed cecum of the rat may serve as a reservoir of bacterial growth. Thus, it seems quite possible that humans and rats may exhibit markedly different responses to a chemically defined liquid diet.

The effects of chemically defined liquid diets on the rats' intestinal flora appear to be relatively moderate, thought quantitative in terms of certain groups of bacteria.

CDF-CR and CDF-ARS rats when consuming liquid diets usually had higher counts of E. coli, Aerobacter, Proteus, Staphylococcus and enterococcus than the same rats consuming Lab Blox. CFE rats consuming liquid diets had higher counts of E. coli and enterococcus than the same rats consuming Lab Blox.

There was also a general trend for CDF-CR rats to have higher counts of E. coli, Aerobacter, Proteus, Staphylococcus, and Bacillus than the other rats, regardless of diet. This strain was grossly different from the others in presence and numbers of Proteus and Aerobacter isolated from cecal contents.

The CFE rats generally had the greatest numbers of total aerobes, total anaerobes and Lactobacillus.

The three types of rat had nearly equal counts of enterococcus, however, the CDF-ARS had a significantly higher count than the CFE rats on diet-16 only.

The cecal flora of the various rats appears to be rather stable. There is more variation in counts of the gram negative bacilli than in the other groups and some organism not reported here, such as Pseudomonas, Corynebacterium and Streptococcus viridans were occasionally isolated in moderate numbers. The microfloral population profile appears to be somewhat more dependent upon the strain or source of rat than the diet being consumed.

TABLE 52

DISTRIBUTION OF CECAL SAMPLES ACCORDING TO NUMBER OF *E. COLI* PER GRAM (WET WEIGHT) OF SAMPLE

Rat Strain	Diet	Mean Number of *E. coli*	Sample Frequency: Number of *E. coli*										
			0	10^1	10^2	10^3	10^4	10^5	10^6	10^7	10^8	10^9	10^{10}
CFE	16	9.3×10^4	3		1	2	14	4	5	5	1		
	17	2.4×10^6				2	5	5	5	2	6	1	
	Lab Blox	4.5×10^4	5		1	7	5	7	10	4			
CDF-CR*	16	4.6×10^6						6	14	9	2		
	17	9.3×10^5					2	12	14	2			
	Lab Blox	2.3×10^5	4			1	8	15	8	6			
CDF-ARS**	16	1.3×10^6						10	4	1	1		
	17	2.2×10^5	1				2	9	6				
	Lab Blox	3.2×10^4	1		1	1	6	5	2				

*CDF Fischer rats supplied by Charles River Labs., Inc.

**CDF Fischer rats supplied by A. R. Schmidt, Co., Inc.

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TABLE 53

DISTRIBUTION OF CECAL SAMPLES ACCORDING TO NUMBER OF AEROBACTER PER GRAM (WET WEIGHT) OF SAMPLE

Rat Strain	Diet	Mean Number of Aerobacter	Sample Frequency Number of Aerobacter										
			0	10^1	10^2	10^3	10^4	10^5	10^6	10^7	10^8	10^9	10^{10}
CFE	16	1.0×10^1	25		1	6	1		1				
	17	1.7×10^0	25		1	1							
	Lab Blox	2.2×10^0	25			4							
CDF-CR*	16	1.8×10^4	5			3	10	14	2				
	17	2.5×10^3	8			2	7	1	1				
	Lab Blox	2.0×10^2	15			13	5	1					
CDF-ARS**	16	3.3×10^1	10		1	2	2	1					
	17	1.9×10^1	13				4	1					
	Lab Blox	1.7×10^0	16			1							

*CDF Fischer rats supplied by Charles River Labs., Inc.

**CDF Fischer rats supplied by A. R. Schmidt, Co., Inc.

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TABLE 54

DISTRIBUTION OF CECAL SAMPLES ACCORDING TO NUMBER OF *PROTEUS* PER GRAM (WET WEIGHT) OF SAMPLE

Rat Strain	Diet	Mean Number of *Proteus*	Sample Frequency: Number of *Proteus*										
			0	10^1	10^2	10^3	10^4	10^5	10^6	10^7	10^8	10^9	10^{10}
CFE	16	4.5×10^0	29			1	4						
	17	0	27										
	Lab Blox	1.4×10^0	37		1	1							
CDF-CR*	16	2.0×10^3	11			2	4	4	6	1			
	17	1.8×10^3	6			1	2	4	3				
	Lab Blox	5.4×10^2	15				10	7	1				
CDF-ARS**	16	0	15										
	17	4.4×10^0	16					2					
	Lab Blox	0	15										

*CDF Fischer rats supplied by Charles River Labs., Inc.

**CDF Fischer rats supplied by A. R. Schmidt, Co., Inc.

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TABLE 55

DISTRIBUTION OF CECAL SAMPLES ACCORDING TO NUMBER OF *STAPHYLOCOCCUS* PER GRAM (WET WEIGHT) OF SAMPLE

Rat Strain	Diet	Mean Number of *Staphylococcus*	Sample Frequency · Number of *Staphylococcus*										
			0	10^1	10^2	10^3	10^4	10^5	10^6	10^7	10^8	10^9	10^{10}
CFE	16	5.0×10^4			1	4	19	9	2				
	17	2.8×10^4			1	6	19	7	1				
	Lab Blox	8.5×10^4				2	21	16	1				
CDF-CR*	16	8.9×10^5					1	15	19				
	17	6.3×10^5					2	13	8				
	Lab Blox	1.3×10^5					14	27	2				
CDF-ARS**	16	2.2×10^5					2	13		1			
	17	1.5×10^5					9	9	1				
	Lab Blox	3.3×10^4				3	12	2					

*CDF Fischer rats supplied by Charles River Labs., Inc.

**CDF Fischer rats supplied by A. R. Schmidt, Co., Inc.

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TABLE 56

DISTRIBUTION OF CECAL SAMPLES ACCORDING TO NUMBER OF *BACILLUS* PER GRAM (WET WEIGHT) OF SAMPLE

Rat Strain	Diet	Mean Number of *Bacillus*	Sample Frequency: Number of *Bacillus*										
			0	10^1	10^2	10^3	10^4	10^5	10^6	10^7	10^8	10^9	10^{10}
CFE	16	1.5×10^0	33		1	1							
	17	1.5×10^0	33		1	1							
	Lab Blox	3.2×10^3	1	1	3	35							
CDF-CR*	16	2.5×10^1	22		1	3	6	1					
	17	0	23										
	Lab Blox	7.9×10^3	1		5	27	7						
CDF-ARS**	16	0	16										
	17	1.5×10^0	19			1							
	Lab Blox	1.8×10^3	1		3	12	1						

*CDF Fischer rats supplied by Charles River Breeding Labs., Inc.

**CDF Fischer rats supplied by A. R. Schmidt, Co., Inc.

TABLE 57

DISTRIBUTION OF CECAL SAMPLES ACCORDING TO NUMBER OF TOTAL AEROBES PER GRAM (WET WEIGHT) OF SAMPLE

Rat Strain	Diet	Mean Number of Total Aerobes	Sample Frequency: Number of Total Aerobes										
			0	10^1	10^2	10^3	10^4	10^5	10^6	10^7	10^8	10^9	10^{10}
CFE	16	1.7×10^8								7	10	1	
	17	2.6×10^8								3	12	3	
	Lab Blox	9.8×10^8									10	6	
CDF-CR*	16	2.2×10^8								1	4	2	
	17	6.0×10^7								8	5	1	
	Lab Blox	2.0×10^9									2	10	
CDF-ARS**	16	2.0×10^8								2	12	1	
	17	1.4×10^8								7	10	2	
	Lab Blox	2.5×10^8								3	12		

*CDF Fischer rats supplied by Charles River Breeding Labs., Inc.

**CDF Fischer rats supplied by A. R. Schmidt, Co., Inc.

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TABLE 58

DISTRIBUTION OF CECAL SAMPLES ACCORDING TO NUMBER OF TOTAL ANAEROBES PER GRAM (WET WEIGHT) OF SAMPLE

Rat Strain	Diet	Mean Number of Total Anaerobes	Sample Frequency: Number of Total Anaerobes										
			0	10^1	10^2	10^3	10^4	10^5	10^6	10^7	10^8	10^9	10^{10}
CFE	16	3.5×10^9									3	13	3
	17	4.0×10^9									2	13	3
	Lab Blox	3.3×10^9										13	2
CDF-CR*	16	3.3×10^9										5	
	17	3.2×10^9									1	8	1
	Lab Blox	3.6×10^9									1	11	
CDF ARS**	16	1.4×10^9									5	11	
	17	3.8×10^9								1	2	13	3
	Lab Blox	1.2×10^9									4	9	

*CDF Fischer rats supplied by Charles River Breeding Labs., Inc.

**CDF Fischer rats supplied by A. R. Schmidt, Co., Inc.

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TABLE 59

DISTRIBUTION OF CECAL SAMPLES ACCORDING TO NUMBER OF LACTOBACILLUS PER GRAM (WET WEIGHT) OF SAMPLE

Rat Strain	Diet	Mean Number of Lactobacillus	Sample Frequency: Number of Lactobacillus										
			0	10^1	10^2	10^3	10^4	10^5	10^6	10^7	10^8	10^9	10^{10}
CFE	16	2.2×10^8							1	6	4	7	
	17	4.2×10^8								5	5	7	1
	Lab Blox	4.2×10^8									10	2	
CDF-CR*	16	1.1×10^8								1	1		
	17	3.1×10^7							3	4	4		
	Lab Blox	4.6×10^8								1	8	3	
CDF-ARS**	16	7.4×10^7							1	6	8		
	17	7.1×10^7							1	10	4	2	
	Lab Blox	1.2×10^8								4	9	1	

*CDF Fischer rats supplied by Charles River Breeding Labs., Inc.

**CDF Fischer rats supplied by A. R. Schmidt, Co., Inc.

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TABLE 60

DISTRIBUTION OF CECAL SAMPLES ACCORDING TO NUMBER OF ENTEROCOCCUS PER GRAM (WET WEIGHT) OF SAMPLE

Rat Strain	Diet	Mean Number of Enterococcus	Sample Frequency Number of Enterococcus										
			0	10^1	10^2	10^3	10^4	10^5	10^6	10^7	10^8	10^9	10^{10}
CFE	16	6.2×10^5					3	8	8				
	17	1.1×10^6				1		7	8	3			
	Lab Blox	3.8×10^5	1				1	5	8				
CDF-CR*	16	9.3×10^5						4	1	1			
	17	2.8×10^6						1	11	1			
	Lab Blox	4.1×10^5	1					6	4	1			
CDF-ARS**	16	2.5×10^6						3	13				
	17	1.5×10^6						4	15	1			
	Lab Blox	3.0×10^5						16	1				

*CDF Fischer rats supplied by Charles River Breeding Labs., Inc.

**CDF Fischer rats supplied by A. R. Schmidt, Co., Inc.

TABLE 61

STATISTICAL COMPARISON OF NUMBERS OF E. COLI ISOLATED FROM CDF-ARS RATS FED VARIOUS DIETS

Diet	No. of Animals	Mean No. of E. coli	Probability
16	16	1.3×10^6	
16-K	12	4.4×10^5	$p > .100$
16	16	1.3×10^6	
17	18	2.2×10^5	$p > .050$
16	16	1.3×10^6	
Lab Blox	16	3.2×10^4	$p < .005$
16-K	12	4.4×10^5	
17	18	2.2×10^5	$p > .400$
16-K	12	4.4×10^5	
Lab Blox	16	3.2×10^4	$p < .025$
17	18	2.2×10^5	
Lab Blox	16	3.2×10^4	$p > .100$

TABLE 62

STATISTICAL COMPARISON OF NUMBERS OF AEROBACTER ISOLATED FROM CDF-ARS RATS FED VARIOUS DIETS

Diet	No. of Animals	Mean No. of Aerobacter	Probability
16	16	3.3×10^1	p >.050
16-K	12	1.2×10^3	
16	16	3.3×10^1	p >.500
17	18	1.9×10^1	
16	16	3.3×10^1	p <.050
Lab Blox	17	1.7×10^0	
16-K	12	1.2×10^3	p <.050
17	18	1.9×10^1	
16-K	12	1.2×10^3	p <.001
Lab Blox	17	1.7×10^0	
17	18	1.9×10^1	p >.050
Lab Blox	17	1.7×10^0	

TABLE 63

STATISTICAL COMPARISON OF NUMBERS OF *PROTEUS* ISOLATED FROM CDF-ARS RATS FED VARIOUS DIETS

Diet	No. of Animals	Mean No. of *Proteus*	Probability
16	15	0	
16-K	12	0	$p > .500$
16	15	0	
17	18	4.4×10^0	$p > .100$
16	15	0	
Lab Blox	15	0	$p > .500$
16-K	12	0	
17	18	4.4×10^0	$p > .200$
16-K	12	0	
Lab Blox	15	0	$p > .500$
17	18	4.4×10^0	
Lab Blox	15	0	$p > .100$

TABLE 64

STATISTICAL COMPARISON OF NUMBERS OF STAPHYLOCOCCUS ISOLATED FROM CDF-ARS RATS FED VARIOUS DIETS

Diet	No. of Animals	Mean No. of Staphylococcus	Probability
16	16	2.2×10^5	p >.200
16-K	12	1.4×10^5	
16	16	2.2×10^5	p >.200
17	19	1.5×10^5	
16	16	2.2×10^5	p <.001
Lab Blox	17	3.3×10^4	
16-K	12	1.4×10^5	p >.500
17	19	1.5×10^5	
16-K	12	1.4×10^5	p <.001
Lab Blox	17	3.3×10^4	
17	19	1.5×10^5	p <.005
Lab Blox	17	3.3×10^4	

TABLE 65

STATISTICAL COMPARISON OF NUMBERS OF *BACILLUS* ISOLATED FROM CDF-ARS RATS FED VARIOUS DIETS

Diet	No. of Animals	Mean No. of *Bacillus*	Probability
16	16	0	p >.500
16-K	12	0	
16	16	0	p >.200
17	20	1.5×10^0	
16	16	0	p <.001
Lab Blox	17	1.8×10^3	
16-K	12	0	p >.400
17	20	1.5×10^0	
16-K	12	0	p <.001
Lab Blox	17	1.8×10^3	
17	20	1.5×10^0	p <.001
Lab Blox	17	1.8×10^3	

TABLE 66

STATISTICAL COMPARISON OF NUMBERS OF TOTAL AEROBES ISOLATED FROM CDF-ARS RATS FED VARIOUS DIETS

Diet	No. of Animals	Mean No. of Total Aerobes	Probability
16	15	2.0×10^8	
17	19	1.4×10^8	$p > .200$
16	15	2.0×10^8	
Lab Blox	15	2.5×10^8	$p > .500$
17	19	1.4×10^8	
Lab Blox	15	2.5×10^8	$p > .200$

TABLE 67

STATISTICAL COMPARISON OF NUMBERS OF TOTAL ANAEROBES ISOLATED FROM CDF-ARS RATS FED VARIOUS DIETS

Diet	No. of Animals	Mean No. of Total Anaerobes	Probability
16	16	1.4×10^9	
17	19	3.8×10^9	$p < .050$
16	16	1.4×10^9	
Lab Blox	13	1.2×10^9	$p > .500$
17	19	3.8×10^9	
Lab Blox	13	1.2×10^9	$p < .025$

TABLE 68

STATISTICAL COMPARISON OF NUMBERS OF LACTOBACILLUS ISOLATED FROM CDF-ARS RATS FED VARIOUS DIETS

Diet	No. of Animals	Mean No. of Lactobacillus	Probability
16	15	7.4×10^7	p >.500
17	17	7.1×10^7	
16	15	7.4×10^7	p >.200
Lab Blox	14	1.2×10^8	
17	17	7.1×10^7	p >.200
Lab Blox	14	1.2×10^8	

TABLE 69

STATISTICAL COMPARISON OF NUMBERS OF ENTEROCOCCUS ISOLATED FROM CDF-ARS RATS FED VARIOUS DIETS

Diet	No. of Animals	Mean No. of Enterococcus	Probability
16	16	2.5×10^6	
17	20	1.5×10^6	$p > .100$
16	16	2.5×10^6	
Lab Blox	17	3.0×10^5	$p < .001$
17	20	1.5×10^6	
Lab Blox	17	3.0×10^5	$p < .001$

TABLE 70

STATISTICAL COMPARISON OF NUMBERS OF *E. COLI* ISOLATED FROM CDF-CR RATS FED VARIOUS DIETS

Diet	No. of Animals	Mean No. of *E. coli*	Probability
16	31	4.6×10^6	
16-K	19	7.2×10^6	$p > .200$
16	31	4.6×10^6	
17	30	9.3×10^5	$p < .001$
16	31	4.6×10^6	
Lab Blox	53	2.3×10^5	$p < .001$
16-K	19	7.2×10^6	
17	30	9.3×10^5	$p < .001$
16-K	19	7.2×10^6	
Lab Blox	53	2.3×10^5	$p < .001$
17	30	9.3×10^5	
Lab Blox	53	2.3×10^5	$p > .050$

TABLE 71

STATISTICAL COMPARISON OF NUMBERS OF *AEROBACTER* ISOLATED FROM CDF-CR RATS FED VARIOUS DIETS

Diet	No. of Animals	Mean No. of *Aerobacter*	Probability
16	34	1.8×10^4	
16-K	19	4.9×10^4	$p > .400$
16	34	1.8×10^4	
17	27	2.5×10^3	$p > .100$
16	34	1.8×10^4	
Lab Blox	37	2.0×10^2	$p < .001$
16-K	19	4.9×10^4	
17	27	2.5×10^3	$p > .050$
16-K	19	4.9×10^4	
Lab Blox	37	2.0×10^2	$p < .001$
17	27	2.5×10^3	
Lab Blox	37	2.0×10^2	$p > .050$

TABLE 72

STATISTICAL COMPARISON OF NUMBERS OF *PROTEUS* ISOLATED FROM CDF-CR RATS FED VARIOUS DIETS

Diet	No. of Animals	Mean No. of *Proteus*	Probability
16	28	2.0×10^3	
16-K	19	2.1×10^2	$p > .200$
16	28	2.0×10^3	
17	16	1.8×10^3	$p > .500$
16	28	2.0×10^3	
Lab Blox	33	5.4×10^2	$p > .400$
16-K	19	2.1×10^2	
17	16	1.8×10^3	$p > .200$
16-K	19	2.1×10^2	
Lab Blox	33	5.4×10^2	$p > .500$
17	16	1.8×10^3	
Lab Blox	33	5.4×10^2	$p > .500$

TABLE 73

STATISTICAL COMPARISON OF NUMBERS OF *STAPHYLOCOCCUS* ISOLATED FROM CDF-CR RATS FED VARIOUS DIETS

Diet	No. of Animals	Mean No. of *Staphylococcus*	Probability
16	35	8.9×10^5	
16-K	19	9.8×10^5	$p > .500$
16	35	8.9×10^5	
17	32	6.3×10^5	$p > .200$
16	35	8.9×10^5	
Lab Blox	55	1.3×10^5	$p < .001$
16-K	19	9.8×10^5	
17	32	6.3×10^5	$p > .200$
16-K	19	9.8×10^5	
Lab Blox	55	1.3×10^5	$p < .001$
17	32	6.3×10^5	
Lab Blox	55	1.3×10^5	$p < .001$

TABLE 74

STATISTICAL COMPARISON OF NUMBERS OF *BACILLUS* ISOLATED FROM CDF-CR RATS FED VARIOUS DIETS

Diet	No. of Animals	Mean No. of *Bacillus*	Probability
16	33	2.5×10^1	
16-K	19	0	$p < .010$
16	33	2.5×10^1	
17	32	0	$p < .001$
16	33	2.5×10^1	
Lab Blox	49	7.9×10^3	$p < .001$
16-K	19	0	
17	32	0	$p > .500$
16-K	19	0	
Lab Blox	49	7.9×10^3	$p < .001$
17	32	0	
Lab Blox	49	7.9×10^3	$p < .001$

TABLE 75

STATISTICAL COMPARISON OF NUMBERS OF TOTAL AEROBES ISOLATED FROM CDF-CR RATS FED VARIOUS DIETS

Diet	No. of Animals	Mean No. of Total Aerobes	Probability
16	7	2.2×10^8	$p < .025$
17	13	6.0×10^7	
16	7	2.2×10^8	$p < .001$
Lab Blox	12	2.0×10^9	
17	13	6.0×10^7	$p < .001$
Lab Blox	12	2.0×10^9	

TABLE 76

STATISTICAL COMPARISON OF NUMBERS OF TOTAL ANAEROBES ISOLATED FROM CDF-CR RATS FED VARIOUS DIETS

Diet	No. of Animals	Mean No. of Total Anaerobes	Probability
16	5	3.3×10^9	
17	10	3.2×10^9	$p > .500$
16	5	3.3×10^9	
Lab Elox	12	3.6×10^9	$p > .500$
17	10	3.2×10^9	
Lab Blox	12	3.6×10^9	$p > .500$

TABLE 77

STATISTICAL COMPARISON OF NUMBERS OF LACTOBACILLUS ISOLATED FROM CDF-CR RATS FED VARIOUS DIETS

Diet	No. of Animals	Mean No. of Lactobacillus	Probability
16	2	1.1×10^8	p >.200
17	11	3.1×10^7	
16	2	1.1×10^8	p >.050
Lab Blox	12	4.6×10^8	
17	11	3.1×10^7	p <.001
Lab Blox	12	4.6×10^8	

TABLE 78

STATISTICAL COMPARISON OF NUMBERS OF ENTEROCOCCUS ISOLATED FROM CDF-CR RATS FED VARIOUS DIETS

Diet	No. of Animals	Mean No. of Enterococcus	Probability
16	6	9.3×10^5	$p > .050$
17	13	2.8×10^6	
16	6	9.3×10^5	$p > .400$
Lab Blox	12	4.1×10^5	
17	13	2.8×10^6	$p < .010$
Lab Blox	12	4.1×10^5	

TABLE 79

STATISTICAL COMPARISON OF NUMBERS OF *E. COLI* ISOLATED FROM CFE RATS FED VARIOUS DIETS

Diet	No. of Animals	Mean No. of *E. coli*	Probability
16	35	9.3×10^4	
16-K	16	5.6×10^5	$p > .100$
16	35	9.3×10^4	
17	26	2.4×10^6	$p < .010$
16	35	9.3×10^4	
Lab Blox	39	4.5×10^5	$p > .500$
16-K	16	5.6×10^5	
17	26	2.4×10^6	$p > .200$
16-K	16	5.6×10^5	
Lab Blox	39	4.5×10^4	$p > .050$
17	26	2.4×10^6	
Lab Blox	39	4.5×10^4	$p < .005$

TABLE 80

STATISTICAL COMPARISON OF NUMBERS OF AEROBACTER ISOLATED FROM CFE RATS FED VARIOUS DIETS

Diet	No. of Animals	Mean No. of Aerobacter	Probability
16	34	1.0×10^1	
16-K	16	8.1×10^0	$p > .500$
16	34	1.0×10^1	
17	27	1.7×10^0	$p < .050$
16	34	1.0×10^1	
Lab Blox	39	2.2×10^0	$p > .050$
16-K	16	8.1×10^0	
17	27	1.7×10^0	$p > .050$
16-K	16	8.1×10^0	
Lab Blox	39	2.2×10^0	$p > .100$
17	27	1.7×10^0	
Lab Blox	39	2.2×10^0	$p > .500$

TABLE 81

STATISTICAL COMPARISON OF NUMBERS OF *PROTEUS* ISOLATED FROM CFE RATS FED VARIOUS DIETS

Diet	No. of Animals	Mean No. of *Proteus*	Probability
16	34	4.5×10^0	p >.100
16-K	16	0	
16	34	4.5×10^0	p <.050
17	27	0	
16	34	4.5×10^0	p >.050
Lab Blox	39	1.4×10^0	
16-K	16	0	p >.500
17	27	0	
16-K	16	0	p >.200
Lab Blox	39	1.4×10^0	
17	27	0	p >.200
Lab Blox	39	1.4×10^0	

TABLE 82

STATISTICAL COMPARISON OF NUMBERS OF *STAPHYLOCOCCUS* ISOLATED FROM CFE RATS FED VARIOUS DIETS

Diet	No. of Animals	Mean No. of *Staphylococcus*	Probability
16	35	5.0×10^4	
16-K	16	4.7×10^4	$p > .500$
16	35	5.0×10^4	
17	35	2.8×10^4	$p > .100$
16	35	5.0×10^4	
Lab Blox	40	8.5×10^4	$p > .100$
16-K	16	4.7×10^4	
17	35	2.8×10^4	$p > .200$
16-K	16	4.7×10^4	
Lab Blox	40	8.5×10^4	$p > .100$
17	35	2.8×10^4	
Lab Blox	40	8.5×10^4	$p < .001$

TABLE 63

STATISTICAL COMPARISON OF NUMBERS OF *BACILLUS* ISOLATED FROM CFE RATS FED VARIOUS DIETS

Diet	No. of Animals	Mean No. of *Bacillus*	Probability
16	35	1.5×10^0	
16-K	16	0	$p > .200$
16	35	1.5×10^0	
17	35	1.5×10^0	$p > .500$
16	35	1.5×10^0	
Lab Blox	40	3.2×10^3	$p < .001$
16-K	16	0	
17	35	1.5×10^0	$p > .200$
16-K	16	0	
Lab Blox	40	3.2×10^3	$p < .001$
17	35	1.5×10^0	
Lab Blox	40	3.2×10^3	$p < .001$

TABLE 84

STATISTICAL COMPARISON OF NUMBERS OF TOTAL AEROBES ISOLATED FROM CFE RATS FED VARIOUS DIETS

Diet	No. of Animals	Mean No. of Total Aerobes	Probability
16	18	1.7×10^8	p >.200
17	18	2.6×10^8	
16	18	1.7×10^8	p <.001
Lab Blox	16	9.8×10^8	
17	18	2.6×10^8	p <.010
Lab Blox	16	9.8×10^8	

TABLE 85

STATISTICAL COMPARISON OF NUMBERS OF TOTAL ANAEROBES ISOLATED FROM CFE RATS FED VARIOUS DIETS

Diet	No. of Animals	Mean No. of Total Anaerobes	Probability
16	19	3.5×10^9	
17	18	4.0×10^9	$p > .500$
16	19	3.5×10^9	
Lab Blox	15	3.3×10^9	$p > .500$
17	18	4.0×10^9	
Lab Blox	15	3.3×10^9	$p > .500$

TABLE 86

STATISTICAL COMPARISON OF NUMBERS OF LACTOBACILLUS ISOLATED FROM CFE RATS FED VARIOUS DIETS

Diet	No. of Animals	Mean No. of Lactobacillus	Probability
16	18	2.2×10^8	p >.400
17	18	4.2×10^8	
16	18	2.2×10^8	p >.400
Lab Blox	12	4.2×10^8	
17	18	4.2×10^8	p >.500
Lab Blox	12	4.2×10^8	

TABLE 87

STATISTICAL COMPARISON OF NUMBERS OF ENTEROCOCCUS ISOLATED FROM CFE RATS FED VARIOUS DIETS

Diet	No. of Animals	Mean No. of Enterococcus	Probability
16	19	6.2×10^5	p >.200
17	19	1.1×10^6	
16	19	6.2×10^5	p >.500
Lab Blox	15	3.8×10^5	
17	19	1.1×10^6	p >.200
Lab Blox	15	3.8×10^5	

TABLE 88

STATISTICAL COMPARISON OF NUMBERS OF *E. COLI* ISOLATED FROM VARIOUS RATS FED DIET 16

Rat Strain	No. of Animals	Mean No. of *E. coli*	Probability
CFE	35	9.3×10^4	
CDF-CR	31	4.6×10^6	$p < .001$
CFE	35	9.3×10^4	
CDF-ARS	16	1.3×10^6	$p < .050$
CDF-CR	31	4.6×10^6	
CDF-ARS	16	1.3×10^6	$p < .050$

TABLE 89

STATISTICAL COMPARISON OF NUMBERS OF AEROBACTER ISOLATED FROM VARIOUS RATS FED DIET 16

Rat Strain	No. of Animals	Mean No. of Aerobacter	Probability
CFE	34	1.0×10^1	
CDF-CR	34	1.8×10^4	$p < .001$
CFE	34	1.0×10^1	
CDF-ARS	16	3.3×10^1	$p > .200$
CDF-CR	34	1.8×10^4	
CDF-ARS	16	3.3×10^1	$p < .001$

TABLE 90

STATISTICAL COMPARISON OF NUMBERS OF *PROTEUS* ISOLATED FROM VARIOUS RATS FED DIET 16

Rat Strain	No. of Animals	Mean No. of *Proteus*	Probability
CFE	34	4.5×10^0	
CR	28	2.0×10^3	$p < .001$
CFE	34	4.5×10^0	
ARS	15	0	$p > .100$
CR	28	2.0×10^3	
ARS	15	0	$p < .001$

TABLE 91

STATISTICAL COMPARISON OF NUMBERS OF STAPHYLOCOCCUS ISOLATED FROM VARIOUS RATS FED DIET 16

Rat Strain	No. of Animals	Mean No. of Staphylococcus	Probability
CFE	35	5.0×10^4	
CR	35	8.9×10^5	$p < .001$
CFE	35	5.0×10^4	
ARS	16	2.2×10^5	$p < .010$
CR	35	8.9×10^5	
ARS	16	2.2×10^5	$p < .001$

TABLE 92

STATISTICAL COMPARISON OF NUMBERS OF *BACILLUS* ISOLATED FROM VARIOUS RATS FED DIET 16

Rat Strain	No. of Animals	Mean No. of *Bacillus*	Probability
CFE	35	1.5×10^0	$p < .005$
CR	33	2.5×10^1	
CFE	35	1.5×10^0	$p > .200$
ARS	16	0	
CR	33	2.5×10^1	$p < .025$
ARS	16	0	

TABLE 93

STATISTICAL COMPARISON OF NUMBERS OF TOTAL AEROBES ISOLATED FROM VARIOUS RATS FED DIET 16

Rat Strain	No. of Animals	Mean No. of Total Aerobes	Probability
CFE	18	1.7×10^8	
CR	7	2.2×10^8	$p > .500$
CFE	18	1.7×10^8	
ARS	15	2.0×10^8	$p > .500$
CR	7	2.2×10^8	
ARS	15	2.0×10^8	$p > .500$

TABLE 94

STATISTICAL COMPARISON OF NUMBERS OF TOTAL ANAEROBES ISOLATED FROM VARIOUS RATS FED DIET 16

Rat Strain	No. of Animals	Mean No. of Total Anaerobes	Probability
CFE	19	3.5×10^9	$p > .500$
CR	5	3.3×10^9	
CFE	19	3.5×10^9	$p < .050$
ARS	16	1.4×10^9	
CR	5	3.3×10^9	$p > .100$
ARS	6	1.4×10^9	

TABLE 95

STATISTICAL COMPARISON OF NUMBERS OF LACTOBACILLUS ISOLATED FROM VARIOUS RATS FED DIET 16

Rat Strain	No. of Animals	Mean No. of Lactobacillus	Probability
CFE	18	2.2×10^8	p >.500
CR	2	1.1×10^8	
CFE	18	2.2×10^8	p >.100
ARS	15	7.4×10^8	
CR	2	1.1×10^8	p >.500
ARS	15	7.4×10^8	

TABLE 96

STATISTICAL COMPARISON OF NUMBERS OF ENTEROCOCCUS ISOLATED FROM VARIOUS RATS FED DIET 16

Rat Strain	No. of Animals	Mean No. of Enterococcus	Probability
CFE	19	6.2×10^5	$p > .500$
CR	6	9.3×10^5	
CFE	19	6.2×10^5	$p < .005$
ARS	16	2.5×10^6	
CR	6	9.3×10^5	$p > .050$
ARS	16	2.5×10^6	

TABLE 97

STATISTICAL COMPARISON OF NUMBERS OF E. COLI ISOLATED FROM VARIOUS RATS FED DIET 16-K

Rat Strain	No. of Animals	Mean No. of E. coli	Probability
CFE	16	5.6×10^5	p <.010
CR	19	7.2×10^6	
CFE	16	5.6×10^5	p >.500
ARS	12	4.4×10^5	
CR	19	7.2×10^6	p <.001
ARS	12	4.4×10^5	

TABLE 98

STATISTICAL COMPARISON OF NUMBERS OF *AEROBACTER* ISOLATED FROM VARIOUS RATS FED DIET 16-K

Rat Strain	No. of Animals	Mean No. of *Aerobacter*	Probability
CFE	16	8.1×10^0	$p < .010$
CR	19	4.9×10^4	
CFE	16	8.1×10^0	$p < .010$
ARS	12	1.2×10^3	
CR	19	4.9×10^4	$p > .050$
ARS	12	1.2×10^3	

TABLE 99

STATISTICAL COMPARISON OF NUMBERS OF PROTEUS ISOLATED FROM VARIOUS RATS FED DIET 16-K

Rat Strain	No. of Animals	Mean No. of Proteus	Probability
CFE	16	0	p <.005
CR	19	2.1×10^2	
CFE	16	0	p >.500
ARS	12	0	
CR	19	2.1×10^2	p <.010
ARS	12	0	

TABLE 100

STATISTICAL COMPARISON OF NUMBERS OF STAPHYLOCOCCUS ISOLATED FROM VARIOUS RATS FED DIET 16-K

Rat Strain	No. of Animals	Mean No. of Staphylococcus	Probability
CFE	16	4.7×10^4	$p < .001$
CR	19	9.8×10^5	
CFE	16	4.7×10^4	$p < .050$
ARS	12	1.4×10^5	
CR	19	9.8×10^5	$p < .001$
ARS	12	1.4×10^5	

TABLE 101

STATISTICAL COMPARISON OF NUMBERS OF *BACILLUS* ISOLATED FROM VARIOUS RATS FED DIET 16-K

Rat Strain	No. of Animals	Mean No. of *Bacillus*	Probability
CFE	16	0	p >.500
CR	19	0	
CFE	16	0	p >.500
ARS	12	0	
CR	19	0	p >.500
ARS	12	0	

TABLE 102

STATISTICAL COMPARISON OF NUMBERS OF E. COLI ISOLATED FROM VARIOUS RATS FED DIET 17

Rat Strain	No. of Animals	Mean No. of E. coli	Probability
CFE	26	2.4×10^6	
CR	30	9.3×10^5	$p > .200$
CFE	26	2.4×10^6	
ARS	18	2.2×10^5	$p < .050$
CR	30	9.3×10^5	
ARS	18	2.2×10^5	$p < .050$

TABLE 103

STATISTICAL COMPARISON OF NUMBERS OF *AEROBACTER* ISOLATED FROM VARIOUS RATS FED DIET 17

Rat Strain	No. of Animals	Mean No. of *Aerobacter*	Probability
CFE	27	1.7×10^0	
CR	27	2.4×10^3	$p < .001$
CFE	27	1.7×10^0	
ARS	18	2.0×10^1	$p < .025$
CR	27	2.4×10^3	
ARS	18	2.0×10^1	$p < .005$

TABLE 104

STATISTICAL COMPARISON OF NUMBERS OF *PROTEUS* ISOLATED FROM VARIOUS RATS FED DIET 17

Rat Strain	No. of Animals	Mean No. of *Proteus*	Probability
CFE	27	0	
CR	16	1.8×10^3	$p < .001$
CFE	27	0	
ARS	18	4.4×10^0	$p > .050$
CR	16	1.8×10^3	
ARS	18	4.4×10^0	$p < .005$

TABLE 105

STATISTICAL COMPARISON OF NUMBERS OF STAPHYLOCOCCUS ISOLATED FROM VARIOUS RATS FED DIET 17

Rat Strain	No. of Animals	Mean No. of Staphylococcus	Probability
CFE	35	2.8×10^4	
CR	32	6.3×10^5	$p < .001$
CFE	35	2.8×10^4	
ARS	19	1.5×10^5	$p < .001$
CR	32	6.3×10^5	
ARS	19	1.5×10^5	$p < .001$

TABLE 106

STATISTICAL COMPARISON OF NUMBERS OF *BACILLUS* ISOLATED FROM VARIOUS RATS FED DIET 17

Rat Strain	No. of Animals	Mean No. of *Bacillus*	Probability
CFE	35	1.5×10^0	
CR	32	0	$p > .100$
CFE	35	1.5×10^0	
ARS	20	1.5×10^0	$p > .500$
CR	32	0	
ARS	20	1.5×10^0	$p > .200$

TABLE 107

STATISTICAL COMPARISON OF NUMBERS OF TOTAL AEROBES ISOLATED FROM VARIOUS RATS FED DIET 17

Rat Strain	No. of Animals	Mean No. of Total Aerobes	Probability
CFE	18	2.6×10^8	
CR	13	6.0×10^7	$p < .025$
CFE	18	2.6×10^8	
ARS	19	1.4×10^8	$p > .200$
CR	13	6.0×10^7	
ARS	19	1.4×10^8	$p > .050$

TABLE 108

STATISTICAL COMPARISON OF NUMBERS OF TOTAL ANAEROBES ISOLATED FROM VARIOUS RATS FED DIET 17

Rat Strain	No. of Animals	Mean No. of Anaerobes	Probability
CFE	18	4.0×10^9	
CR	10	3.2×10^9	$p > .500$
CFE	18	4.0×10^9	
ARS	19	3.8×10^9	$p > .500$
CR	10	3.2×10^9	
ARS	19	3.8×10^9	$p > .500$

TABLE 109

STATISTICAL COMPARISON OF NUMBERS OF LACTOBACILLUS ISOLATED FROM VARIOUS RATS FED DIET 17

Rat Strain	No. of Animals	Mean No. of Lactobacillus	Probability
CFE	18	4.2×10^8	
CR	11	3.1×10^7	$p < .005$
CFE	18	4.2×10^8	
ARS	17	7.1×10^7	$p < .025$
CR	11	3.1×10^7	
ARS	17	7.1×10^7	$p > .200$

TABLE 110

STATISTICAL COMPARISON OF NUMBERS OF ENTEROCOCCUS ISOLATED FROM VARIOUS RATS FED DIET 17

Rat Strain	No. of Animals	Mean No. of Enterococcus	Probability
CFE	19	1.1×10^6	
CR	13	2.8×10^6	$p > .200$
CFE	19	1.1×10^6	
ARS	20	1.5×10^6	$p > .500$
CR	13	2.8×10^6	
ARS	20	1.5×10^6	$p > .050$

TABLE 111

STATISTICAL COMPARISON OF NUMBERS OF *E. COLI* ISOLATED FROM VARIOUS RATS FED LAB BLOX

Rat Strain	No. of Animals	Mean No. of *E. coli*	Probability
CFE	39	4.5×10^4	p >.100
CR	53	2.3×10^5	
CFE	39	4.5×10^4	p >.500
ARS	16	3.2×10^4	
CR	53	2.3×10^5	p >.050
ARS	16	3.2×10^4	

TABLE 112

STATISTICAL COMPARISON OF NUMBERS OF AEROBACTER ISOLATED FROM VARIOUS RATS FED LAB BLOX

Rat Strain	No. of Animals	Mean No. of Aerobacter	Probability
CFE	39	2.2×10^0	$p < .001$
CR	37	2.0×10^2	
CFE	39	2.2×10^0	$p > .500$
ARS	17	1.7×10^0	
CR	37	2.0×10^2	$p < .001$
ARS	17	1.7×10^0	

TABLE 113

STATISTICAL COMPARISON OF NUMBERS OF *PROTEUS* ISOLATED FROM VARIOUS RATS FED LAB BLOX

Rat Strain	No. of Animals	Mean No. of *Proteus*	Probability
CFE	39	1.4×10^0	$p < .001$
CR	33	5.4×10^2	
CFE	39	1.4×10^0	$p > .200$
ARS	15	0	
CR	33	5.4×10^2	$p < .001$
ARS	15	0	

TABLE 114

STATISTICAL COMPARISON OF NUMBERS OF *STAPHYLOCOCCUS* ISOLATED FROM VARIOUS RATS FED LAB BLOX

Rat Strain	No. of Animals	Mean No. of *Staphylococcus*	Probability
CFE	40	8.5×10^4	
CR	55	1.3×10^5	$p > .100$
CFE	40	8.5×10^4	
ARS	17	3.3×10^4	$p < .025$
CR	55	1.3×10^5	
ARS	17	3.3×10^4	$p < .001$

TABLE 115

STATISTICAL COMPARISON OF NUMBERS OF BACILLUS ISOLATED FROM VARIOUS RATS FED LAB BLOX

Rat Strain	No. of Animals	Mean No. of Bacillus	Probability
CFE	40	3.2×10^3	
CR	49	7.9×10^3	$p < .025$
CFE	40	3.2×10^3	
ARS	17	1.8×10^3	$p > .200$
CR	49	7.9×10^3	
ARS	17	1.8×10^3	$p < .010$

TABLE 116

STATISTICAL COMPARISON OF NUMBERS OF TOTAL AEROBES ISOLATED FROM VARIOUS RATS FED LAB BLOX

Rat Strain	No. of Animals	Mean No. of Total Aerobes	Probability
CFE	16	9.8×10^8	
CR	12	2.0×10^9	$p > .050$
CFE	16	9.8×10^8	
ARS	15	2.5×10^8	$p < .005$
CR	12	2.0×10^9	
ARS	15	2.5×10^8	$p < .001$

TABLE 117

STATISTICAL COMPARISON OF NUMBERS OF TOTAL ANAEROBES ISOLATED FROM VARIOUS RATS FED LAB BLOX

Rat Strain	No. of Animals	Mean No. of Total Anaerobes	Probability
CFE	15	3.3×10^9	
CR	12	3.6×10^9	$p > .500$
CFE	15	3.3×10^9	
ARS	13	1.2×10^9	$p < .005$
CR	12	3.6×10^9	
ARS	13	1.2×10^9	$p < .005$

TABLE 118

STATISTICAL COMPARISON OF NUMBERS OF *LACTOBACILLUS* ISOLATED FROM VARIOUS RATS FED LAB BLOX

Rat Strain	No. of Animals	Mean No. of *Lactobacillus*	Probability
CFE	12	4.2×10^8	
CR	12	4.6×10^8	$p > .500$
CFE	12	4.2×10^8	
ARS	14	1.2×10^8	$p < .005$
CR	12	4.6×10^8	
ARS	14	1.2×10^8	$p < .005$

TABLE 119

STATISTICAL COMPARISON OF NUMBERS OF ENTEROCOCCUS ISOLATED FROM VARIOUS RATS FED LAB BLOX

Rat Strain	No. of Animals	Mean No. of Enterococcus	Probability
CFE	15	3.8×10^5	
CR	12	4.1×10^5	$p > .500$
CFE	15	3.8×10^5	
ARS	17	3.0×10^5	$p > .500$
CR	12	4.1×10^5	
ARS	17	3.0×10^5	$p > .500$

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SCHWARZ BIORESEARCH, INC.

COST STATEMENT

Statement of Total Cost:

	Cumulative Costs Incurred
Direct Labor	$ 178,573.35
Direct Materials	121,610.07
Other Direct Charges	24,358.80
Overhead @ 100% Direct Labor	178,573.35
Total Direct Costs	$ 503,115.57
G&A @ 15% Total Direct Costs	75,467.33
Total	$ 578,582.90
Gov't. Owned Equipment	29,916.10
Fixed Fee	38,087.00
Total Billed thru 9/30/66	$ 646,586.00

Expenditure Rate:

Period	# of Mos.	(in thousands) Total Billing	Average Monthly Billing
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	12	154.0	12.8
10/1/65 - 9/30/66	12	162.9	13.6
	47	$646.6	$ 13.8

APPENDIX

TABLE 1

BODY WEIGHT, DIET CONSUMPTION AND WATER CONSUMPTION OF RATS FED CODELID DIET-14 AFTER STORAGE AT 0-4°C OR (-)6°C FOR 12 MONTHS*

Storage condition	Body weight, Day 0	Day 7	Day 14	Day 21	Day 24
	g/rat				
Fresh diet	55.8±1.8	74.7±1.7	107.4±2.7	141.1±2.9	155.9±3.0
Refrigerated (0-4°C)	60.4±2.0	81.1±2.7	115.9±4.4	146.0±4.8	168.8±3.4
Frozen (-6°C)	57.3±1.7	77.8±3.1	113.3±4.2	145.0±4.5	165.9±5.0
Lab Blox	56.8±3.6	99.0±6.4	150.5±7.1	182.5±11.8	208.5±10.9
	Diet consumption, ml/rat/day				
Fresh diet		16.0	22.9	29.2	27.4
Refrigerated (0-4°C)		19.1	27.0	28.6	29.7
Frozen (-6°C)		18.3	25.7	27.9	28.2
Lab Blox		12.4g	19.7g	20.9g	25.6g
	Water consumption, ml/rat/day				
Fresh diet		6.1	14.1	17.5	17.4
Refrigerated (0-4°C)		6.5	11.9	18.9	18.8
Frozen (-6°C)		6.3	11.2	15.2	9.2
Lab Blox		15.0	33.9	29.9	15.1

*Eight CFE male weanling rats from identified litters of 9-11 littermates allotted to each dietary treatment. Housed 2 per wire bottomed cage. Four rats allotted to the Lab Blox diet. For summarized data see Table 11 of the Text.

APPENDIX

TABLE 2

BODY WEIGHT, DIET CONSUMPTION AND WATER CONSUMPTION OF RATS FED A DIET DESIGNED TO SIMULATE CODELID DIET-14 AFTER HEAT DESTRUCTION*

Group**	Body weight, Day 0	Day 7	Day 14	Day 21	Day 24
	g/rat				
I	55.8±1.8	74.7±1.7	107.4±8.4	141.1±2.9	155.9±3.0
II	54.8±0.8	70.8±1.6	101.2±2.6	136.1±3.6	---
III	58.9±2.0	78.0±2.9	114.5±3.9	150.3±3.3	---
IV	56.1±1.5	55.8±1.4	57.8±1.7	59.2±4.3	61.7±1.6
V	58.8±2.0	58.5±1.8	61.2±2.3	62.6±2.0	65.6±1.9
VI	58.1±1.3	52.6±1.2	48.7±1.3	46.0±1.2	49.2±1.5
VII	56.8±3.6	99.0±6.4	150.5±7.1	182.5±11.8	208.5±10.9
	Diet consumption, ml/rat/day				
I		16.0	22.9	29.2	27.4
II		15.3	23.3	28.3	--
III		15.6	22.5	29.9	--
IV		8.3	14.2	24.6	21.4
V		4.4	15.9	18.6	11.5
VI		5.5	11.6	9.7	14.9
VII		12.4g	19.7g	20.9g	25.6g
	Water consumption, ml/rat/day				
I		6.1	14.1	17.5	17.4
II		5.4	12.4	15.7	--
III		6.8	14.9	17.7	--
IV		4.8	9.3	10.5	5.5
V		3.4	7.2	9.3	3.6
VI		4.5	7.7	8.7	2.9
VII		15.0	33.9	29.9	15.1

*Ten CFE male weanling rats from identified litters of 9-11 littermates allotted to each dietary treatment. Housed 2 per wire bottomed cage. Four rats allotted to the Lab Blox diet. For summarized data see Table 13 of the text.

**Gp I-Diet 14; Gp II-Diet14+Polymer-1g/L; Gp III-Diet14+Polymer-5g/L; Gp IV-Diet 14+A.A. after 60°C; Gp V-As Gp IV+Polymer-1g/L; Gp VI-As Gp IV minus Trypt.; Gp VII-Lab Blox.

APPENDIX

TABLE 3

BODY WEIGHT, DIET CONSUMPTION AND WATER CONSUMPTION OF RATS FED HEAT TREATED CODELID DIET-14 FROM WHICH SELECTED AMINO ACIDS AND GLUCOSE WERE REMOVED PRIOR TO HEATING+

Group‡	Body weight			
	Day			
	0	7	14	21
	g/rat			
I	66.4±2.0	84.4±2.4	119.7±4.2	150.3±4.6 (7)
II	71.3±3.6	57.7±2.8	54.6±3.0	55.5±1.9 (4)
III	69.9±3.3	65.9±2.8	78.4±2.9	81.9±3.4 (7)
IV	67.9±3.0	68.1±3.8	85.4±3.5	95.0±4.8 (7)
V	64.6±2.9	68.4±3.1	88.3±3.7	95.8±5.3 (6)
VI	65.6±2.5	77.0±3.0	101.1±3.2	116.7±5.9 (7)
VII	70.6±2.6	76.1±2.4	100.6±2.8	122.1±2.7 (7)
VIII	69.1±2.3	85.7±3.6	119.3±4.6	152.4±5.1 (7)
IX	58.4±1.6	109.0±1.8	158.0±5.9	205.0±5.2 (5)

(continued)

APPENDIX

TABLE 3 (continued)

Group‡	Diet consumption			Water consumption		
	Day			Day		
	7	14	21	7	14	21
	ml/rat/day			ml/rat/day		
I	20.0	22.8	27.8	8.5	10.7	14.8
II	12.4	8.1	9.3	7.1	7.7	16.9
III	11.6	15.3	12.1	7.8	7.2	15.1
IV	12.9	16.3	19.6	10.0	14.5	21.1
V	12.9	16.0	19.6	9.2	11.7	19.9
VI	15.0	18.2	18.0	7.1	9.4	17.4
VII	14.0	16.8	24.9	9.5	10.0	13.3
VIII	16.7	21.6	24.2	10.1	11.5	17.9
IX	15.5g	20.3g	21.0g	13.3	22.0	24.5

†Seven CFE male weanling littermates started on each test diet. Housed 1 or 2 per wire bottomed cage. For summarized data see Table 15 of the text.

‡Gp I - Diet-14 fresh; Gp II - Diet-14 - 60°C - 6 days; Gp III as II minus Trypt. and Glyc.; Gp IV as III minus Hist.; Gp V - as IV minus Arg.; Gp VI - as V minus Lys. and MSG; Gp VII as II minus glucose; Gp VIII - diet-14 + Glyc-glucose rkn mix; Gp IX - Lab Blox. Those ingredients excluded from the diet prior to heating were added in appropriate amounts to complete the diet after the 6 day heat phase.

*Mean value ± standard error for 7 rats unless otherwise noted. Numbers in brackets represent survivors. All deaths occurred during third week of experiment.

**Mean value ± standard error for 5 rats.

APPENDIX

TABLE 4

BODY WEIGHT, DIET CONSUMPTION AND WATER CONSUMPTION OF RATS FED HEAT TREATED CODELID DIET-14 FROM WHICH SELECTED AMINO ACIDS AND CARBOHYDRATES WERE REMOVED PRIOR TO HEATING+

Group‡	Body weight, Day 0	Body weight, Day 7	Body weight, Day 14	Body weight, Day 21
	g/rat			
I	59.7±1.4*	83.3±1.5	117.4±2.9	141.1±4.3
II	61.3±1.6	53.9±1.8	51.0±2.9	50.7±3.5
III	62.3±1.2	58.7±1.8	59.1±1.8	44.3±2.2**
IV	61.4±2.6	80.0±3.1	107.6±3.2	133.3±4.3
V	61.3±2.0	83.3±2.3	110.7±2.5	134.7±2.3
VI	59.7±1.8	103.3±3.7	144.6±3.6	179.4±3.4
		Diet consumption		
		ml/rat/day		
I		17.6	42.4	29.9
II		12.2	5.4	8.5
III		11.2	12.7	10.8
IV		18.0	22.9	27.4
V		12.0	17.8	21.0
VI		16.6g	18.7g	19.6g
		Water consumption		
		ml/rat/day		
I		7.9	14.4	12.0
II		5.7	7.2	8.2
III		9.4	14.9	15.9
IV		6.7	14.6	17.2
V		7.6	11.3	16.9
VI		12.6	24.7	30.9

*Seven CFE male weanling rats started on each test diet. Housed 1 or 2 per wire bottomed cage. For summarized data, see Table 16 of the text.

‡Gp I - Diet-14 fresh; Gp II - Diet-14 - 60°C - 6 days; Gp III - as II minus trypt.; Gp IV - as II minus carbohydrate; Gp V - as Gp I + Glyc-glucose rkn mix (prolonged heat); Gp VI - Lab Blox. Those ingredients excluded from the diet prior to heating were added in appropriate amounts to complete the diet after the 6 day heat phase.

*Mean ± standard error for 7 rats unless otherwise noted.
**Mean ± standard error for 5 rats.

APPENDIX

TABLE 5

BODY WEIGHT, DIET CONSUMPTION AND WATER CONSUMPTION OF RATS FED CODELID DIET 14 SUPPLEMENTED WITH HEATED AND NON-HEATED AMINO ACID MIXTURES+

Group	Supplement‡	Body weight			
		Day			
		0	7	14	20
		g/rat			
1	None	77.8±3.0*	107.1±2.2	123.1±2.1	136.5±2.7
2	Glyc.Glucose mix	70.1±3.0	94.6±3.2	111.3±3.4	124.4±4.1
3	As Gp 2 heated	74.6±2.8	103.0±3.5	112.0±3.0	131.6±2.4
4	As Gp 3 2X	76.9±2.5	103.9±2.3	117.5±2.5	131.1±2.8
5	As Gp 3 3X	70.9±2.4	93.3±3.0	108.5±2.9	121.3±3.4**
6	Arg.Hist.Lys.-Glucose mix	77.1±1.8	100.5±1.6	121.3±2.2	136.3±2.5
7	As Gp 6 heated	80.6±2.9	104.4±3.4	117.0±4.7	131.1±5.2
8	Arg.Hist.Lys.Gly.Glucose mix	78.1±2.9	97.6±3.9	113.0±4.9	124.1±5.8
9	As Gp 8 heated	78.3±2.9	102.5±2.2	119.5±2.3	134.6±2.5
10	Lab Blox	75.0±3.4	123.5±3.1	142.6±3.5	160.4±3.9

(continued)

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APPENDIX

TABLE 5 (continued)

Group	Diet consumption			Water consumption		
	Day			Day		
	7	14	20	7	14	20
	ml/rat/day			ml/rat/day		
1	20.7	24.6	27.0	7.4	11.9	17.5
2	18.4	21.7	22.0	6.9	10.3	17.1
3	18.6	16.8	25.6	7.5	10.4	15.3
4	18.2	20.5	22.7	8.5	12.3	14.1
5	15.6	19.6	22.6	8.1	13.2	17.1
6	18.4	19.8	23.7	9.0	19.2	21.5
7	17.5	20.7	22.7	11.3	12.8	20.1
8	20.0	19.8	21.8	8.7	11.0	16.4
9	18.2	19.2	25.0	8.5	12.6	19.0
10	18.9g	18.2g	21.8g	16.8	23.1	22.9

[+]Eight CFE female weanling rats allotted to each test diet. Housed 2 per wire bottomed cage. For summarized data, see Table 20 of the text.

‡Amino acid supplements were heated for 6 days at 60°C.

*Mean value ± standard error for 8 rats unless otherwise noted.

**Mean value ± standard error for 7 rats; one rattdied during third week of experiment.

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APPENDIX

TABLE 6

BODY WEIGHT, DIET CONSUMPTION AND WATER CONSUMPTION OF RATS FED CODELID DIET-14 KEPT AT THREE DIFFERENT TEMPERATURES AND CONTAINING THREE DIFFERENT "ANTI-BROWNING" AGENTS+

Group‡	Body weight			
	Day			
	0	7	14	21
	g/rat			
I	62.2±3.3*	79.0±3.9	107.2±5.3	129.8±6.2 (6)
II	612±3.9	73.2±3.6	105.2±5.8	131.2±6.7 (6)
III	61.7±3.4	72.8±3.3	108.7±4.2	139.2±6.4 (6)
IV	57.7±2.2	67.8±2.2	88.8±2.0	112.3±3.5 (6)
V	56.5±1.8	48.5±1.6	49.3±2.3	49.3 (4)
VI	53.7±3.4	47.7±2.9	46.0±2.9	50.3 (4)
VII	56.2±2.6	47.7±2.4	47.3±2.0	44.8 (4)
VIII	54.4±2.9	47.7±2.2	47.5±2.2	46.3 (4)
IX	58.0±2.3**	41.4±2.5	41.0 (2)	40.0 (1)
X	58.0±2.3**	49.2±2.1	40.0 (2)	-- (0)
XI	55.2±1.8	45.7±1.3	42.2±5.4	-- (0)
XII	60.5±3.1	49.8±3.0	47.3 (3)	-- (0)
XIII	65.0±3.1**	100.2±5.0	152.2±7.4	194.6±5.3 (5)

(continued)

APPENDIX

TABLE 6 (continued)

Group[+]	Diet consumption			Water consumption		
	Day			Day		
	7	14	21	7	14	21
	ml/rat/day			ml/rat/day		
I	12.4	23.4	32.7	8.7	11.4	14.1
II	12.4	21.5	32.0	9.0	12.0	15.2
III	13.9	20.4	32.3	8.0	11.0	14.2
IV	13.6	18.1	29.6	5.7	7.1	7.7
V	8.3	13.0	14.4	7.0	6.2	6.1
VI	9.2	14.4	16.6	5.1	6.4	6.5
VII	9.0	11.0	13.3	6.1	7.5	8.1
VIII	11.5	14.9	18.2	4.9	8.3	3.6
IX	8.0	11.9	--	5.8	11.3	--
X	8.0	7.4	--	6.8	10.7	--
XI	7.1	11.6	--	7.7	8.5	--
XII	2.9	9.1	--	7.1	7.6	--
XIII	14.6	23.3	21.2	16.1	30.1	33.9

[+]Five or six CFE male weanling rats started on each test diet. Housed 1 or 2 per wire bottomed cage. For summarized data see Table 22 of the text.

[‡]Temperature: 0-4°C (45 days) Gps I-IV; 37°C (45 days) Gps V-VIII; 60°C (6 days) Gps IX-XII. Additive: None- Gps I, V, IX; Glutathione - Gps II, VI, X; Bunte Salt- Gps III, VII, XI; Homocysteine thiolactone - Gps IV, VIII, XII; Lab Blox - Gp XIII.

*Mean ± standard error for 6 rats unless otherwise noted.

**Mean value ± standard error for 5 rats. Numbers in brackets represent survivors.

APPENDIX

TABLE 7

BODY WEIGHT, DIET CONSUMPTION AND WATER CONSUMPTION OF RATS FED CODELID DIET 16 OR 17 WITH GRADED LEVELS OF ETHYL CYSTEINATE·HCL*

Group	Test diet	E.C. level+ g/L	Body weight Day 0	Day 6	Day 13	Day 20	Day 27
			g/rat				
I	Diet 16	0.55	43.1±2.4‡	59.3±3.0	84.4±3.8	111.0±4.7	136.6±5.4
II	Diet 16	2.43	44.9±2.2	60.5±2.5	84.8±3.3	111.6±4.2	138.0±5.0
III	Diet 17	--	44.4±1.6	66.6±2.2	98.1±2.9	128.6±3.5	159.5±3.7
IV	Diet 17	1.22	43.6±1.7	66.3±2.6	96.2±3.3	125.3±4.0	151.5±4.3
V	Diet 17	2.43	43.8±2.2	65.8±3.0	95.7±3.4	126.7±4.4	155.9±5.2
VI	Lab Blox	--	46.0±2.0	73.6±3.5	110.9±4.8	146.9±5.5	181.0±5.9

(continued)

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APPENDIX

TABLE 7 (continued)

Group	Test diet	E.C. level+ g/L	Body weight Day 35 g/rat	Day 42 g/rat	Day 49 g/rat	Day 56 g/rat
I	Diet 16	0.55	161.8±6.4	185.9±11.3	200.7±7.0	217.0±7.3
II	Diet 16	2.43	159.2±6.3	187.6± 6.8	207.0±6.5	226.5±6.6
III	Diet 17	--	184.3±3.5	209.3± 3.4	224.7±4.8	240.3±4.7
IV	Diet 17	1.22	172.6±4.8	195.0± 5.3	222.4±5.7	233.3±4.6
V	Diet 17	2.43	177.2±5.6	207.2± 5.8	222.6±8.0	234.4±9.2
VI	Lab Blox	--	212.4±6.9	231.4± 7.3	241.3±7.4	266.6±6.5

(continued)

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APPENDIX

TABLE 7 (continued)

Group	Test diet	E.C. level+ g/L	Diet consumption Day 6	13	20	27	35	42
			ml/rat/day					
I	Diet 16	0.55	14.0	19.3	25.3	28.1	32.1	32.4
II	Diet 16	2.43	12.9	19.9	24.7	20.9	29.1	29.9
III	Diet 17	--	15.0	20.5	27.9	36.1	35.2	41.0
IV	Diet 17	1.22	14.5	21.5	25.7	28.2	33.4	30.8
V	Diet 17	2.43	14.3	20.8	27.4	27.9	34.5	33.1
VI	Lab Blox	--	11.0g	14.5g	16.8g	19.6g	20.2g	19.7g
			Water consumption ml/rat/day					
I	Diet 16	0.55	13.8	16.8	18.6	20.3	16.7	16.8
II	Diet 16	2.43	13.3	14.1	16.4	17.0	16.3	15.8
III	Diet 17	--	12.8	16.5	21.7	23.0	21.9	27.7
IV	Diet 17	1.22	13.6	16.7	22.1	23.4	22.3	22.5
V	Diet 17	2.43	12.7	16.3	20.6	24.5	23.3	20.4
VI	Lab Blox	--	17.5	23.7	27.5	32.2	34.9	29.6

*Twelve CDF male weanling rats from identified litters of 9-11 littermates allotted to each treatment. Housed 2 per wire bottomed cage. For summarized data, see Table 26 of the text.

+Ethyl Cysteinate·HCl.

‡Mean value ± standard error. All values calculated for 12 animals except as follows: Gp I--day 35-9, 42-8, 49 and 56-7. Gp II--day 35 and 42-10, day 49 and 56-6. Gp III--day 20 and 27-11, 35-10, 42, 49 and 56-7. Gp IV--day 35-11, 42, 49, and 56-10. Gp V--day 35-11, 42-9, 49 and 56-7. Gp VI--day 35, 42, 49 and 56-9.

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APPENDIX

TABLE 8

AVERAGE SIZE OF LIVERS AND KIDNEYS EXCISED FROM RATS FED CODELID DIET 16 OR 17 WITH GRADED LEVELS OF ETHYL CYSTEINATE*

Group	Test Diet	E.C. Level	Liver		Kidneys	
		gm/L	gm	% of body weight	gm	% of body weight
I	Diet 16	0.55	12.3	5.23	2.1	0.91
II	Diet 16	2.43	12.9	5.63	2.2	0.98
III	Diet 17	--	13.4	5.31	2.4	0.94
IV	Diet 17	1.22	10.7	4.69	2.1	0.93
V	Diet 17	2.43	10.7	4.56	2.0	0.87
VI	Lab Blox	--	12.7	4.43	2.4	0.84

*Organs removed from CDF male rats 12 weeks of age (week 9 of experiment). Values represent the average for: Group I - 5 rats; Group II - 3 rats; Group III - 5 rats; Group IV - 7 rats; Group V - 4 rats and Group VI - 3 rats. For individual data obtained over the entire 9 week experimental period, see Tables 6 and 7.

APPENDIX

TABLE 9

SIZE OF LIVERS EXCISED FROM RATS FED CODELID DIET 16 OR 17
WITH GRADED LEVELS OF ETHYL CYSTEINATE*

	Liver Size											
Diet	16		17			Lab Blox	16		17			Lab Blox
E.C. level gm/L	0.55	2.43	0	1.22	2.43	--	0.55	2.43	0	1.22	2.43	--
Weeks on Exp't.	grams						% of body weight					
7	9.6	8.2	9.3		9.2		4.59	4.58	4.10		4.13	
		9.8			11.3			4.69			5.00	
8	10.4	12.2	12.5	10.7	13.9	11.8	4.73	5.17	4.92	4.26	5.31	5.00
	9.5	11.5	11.1	9.1	6.4	13.0	5.28	4.91	4.66	4.14	3.48	4.98
9	11.6	13.1	13.4	11.9	8.0	15.0	5.11	5.90	5.30	5.24	3.36	4.09
	12.3	12.4	13.7	11.1	14.0	10.8	5.17	5.85	5.46	5.11	5.26	3.91
	13.4	13.2	14.7	12.3	11.6	12.3	5.40	5.16	5.44	5.44	5.35	5.30
	11.7		12.1	10.7	9.0		4.89		5.28	4.53	4.27	
	12.4		12.9	10.4			5.56		5.08	4.33		
				11.3						5.04		
				7.2						3.14		
$\bar{x}$	11.4	11.5	12.5	10.5	10.4	12.6	5.09	5.18	5.03	4.58	4.56	4.66

*CDF male weanling rats from identified litters of 9-11 littermates.

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TABLE 10

SIZE OF KIDNEYS EXCISED FROM RATS FED CODELID DIET 16 OR 17 WITH GRADED LEVELS OF ETHYL CYSTEINATE*

	Kidney Size											
Diet	16		17			Lab Blox	16		17			Lab Blox
E.C. level qm/L	0.55	2.43	0	1.22	2.43	--	0.55	2.43	0	1.22	2.43	--
Weeks on Exp't.	grams						% of body weight					
7	2.4	1.7	2.0		1.7		1.15	0.95	0.88		0.76	
		2.0			2.1			0.96	0.7		0.93	
8	2.4	2.4	2.4	1.9	2.7	2.2	1.09	1.02	0.94	0.76	1.03	0.93
	1.9	2.2	2.3	1.8	1.6	2.4	1.06	0.94	0.97	0.82	0.87	0.92
9	2.0	2.2	2.4	2.2	2.1	2.3	0.88	0.99	0.95	0.97	0.88	0.91
	2.0	2.3	2.6	2.1	2.2	2.4	0.84	1.08	1.04	0.97	0.82	0.79
	2.3	2.2	2.7	2.3	2.1	2.5	0.93	0.86	1.00	1.02	0.97	0.81
	2.2		2.0	2.0	1.7		0.92		0.87	0.85	0.81	
	2.2		2.1	2.2			0.99		0.83	0.92		
				2.3						1.03		
				1.7						0.74		
$\bar{X}$	2.2	2.1	2.3	2.1	2.0	2.4	0.98	0.97	0.94	0.90	0.88	0.87

*CDF male weanling rats from identified litters of 9-11 littermates.

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APPENDIX

TABLE 11

BODY WEIGHTS OF CFE RATS FED GRADED LEVELS OF MENADIONE THIOETHER WITH CODELID DIET 16+

Test Diet	Menadione Thioether	Body weight							
		Day							
		0	7	14	21	28	35	42	49
	mg/L	g/rat							
Diet 16	5	51.9±2.0‡	74.6±3.1	105.7±4.4	138.9±5.7	168.9±7.4	180.6±8.6	203.7±10.0	213.1±9.4
Diet 16	15	51.5±1.8	74.5±1.8	107.5±2.7	139.0±3.3	166.2±4.5	181.7±6.7	206.5±7.3	220.0±7.9
Diet 16 (-)Vit. K	5	51.0±2.3	72.7±3.0	104.6±3.2	130.1±3.2	162.5±2.7	183.3±5.8	192.6±6.5	214.3±5.5

(continued)

APPENDIX

TABLE 11 (continued)

Test Diet	Menadione Thioether mg/L	Diet consumption Day 7	14	21	28	35	42	49
		ml/rat/day						
Diet 16	5	22.2	28.0	31.4	34.9	39.6	37.4	43.9
Diet 16	15	21.9	27.0	30.0	33.4	34.6	35.6	40.8
Diet 16 (-)Vit. K	5	23.5	29.1	30.7	35.0	37.2	37.1	39.4
		Water consumption ml/rat/day						
Diet 16	5	12.2	14.5	18.7	13.7	18.3	22.1	21.0
Diet 16	15	11.1	15.4	14.3	11.8	17.7	22.4	20.2
Diet 16 (-)Vit. K	5	11.0	15.4	15.4	10.5	15.6	25.5	20.5

+Ten CFE male rats 24 days of age from identified litters of 9-11 littermates allotted to each dietary treatment. Housed 2 per wire bottomed cage. For summarized data see Table 28 of the text.

++Mean value ± standard error. Calculated for 10 rats, days 0-35; 7 rats, day 42 and 4 rats, day 49.

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APPENDIX

TABLE 12

SIZE OF LIVERS FROM CFE RATS FED MENADIONE-THIOETHER WITH CODELID DIET 16*

	Liver Size					
Test Diet	16	16	16 (-)Vit.K	16	16	16 (-)Vit.K
Menadione Thioether Conc. mg/L	5	15	5	5	15	5
Weeks on Exp't	grams			% of body weight		
	8.7	11.2	10.3	3.88	4.60	4.31
7	7.0	8.8	8.2	3.91	3.80	4.29
	10.6	12.7	10.4	4.09	5.38	4.66
	9.0	9.8	8.3	4.37	4.41	4.0
8	9.6	11.1	10.4	4.05	4.10	3.87
	11.8	8.4	10.8	4.04	3.93	4.54
	10.9	9.8	11.0	5.34	3.75	4.62
	11.6	13.2	11.8	4.60	4.58	4.90
9	12.0	13.2	13.3	4.72	5.95	5.47
			12.6			4.83
$\bar{X}$	10.1	10.9	10.7	4.33	4.5	4.55

*CFE male rats 24 days of age from identified litters of 9-11 littermates allotted to each dietary treatment. For data on CFE male rats of similar age consuming Lab Blox see Table 18 of the Appendix.

APPENDIX

TABLE 13

SIZE OF KIDNEYS FROM CFE RATS FED MENADIONE THIOETHER WITH CODELID DIET 16*

	Kidney Size					
Test Diet	16	16	16 (-)Vit.K	16	16	16 (-)Vit.K
Menadione Thioether Conc. mg/L	5	15	5	5	15	5
Weeks on Exp't	grams			% of body weight		
	2.1	2.3	2.2	0.94	0.95	0.92
7	1.7	2.2	1.9	0.95	0.95	0.99
	2.4	2.6	2.5	0.93	1.10	1.12
	2.0	2.6	1.9	0.97	1.17	0.92
8	2.2	2.4	2.5	0.93	0.89	0.93
	2.3	2.0	2.1	0.79	0.93	0.88
	1.8	1.6	2.5	0.88	0.61	1.05
9	2.4	2.8	2.5	0.95	0.97	1.04
	2.1	2.4	2.4	0.83	1.08	0.99
			2.9			1.11
$\bar{X}$	2.1	2.3	2.3	0.91	0.96	0.99

*CFE male rats 24 days of age from identified litters of 9-11 littermates allotted to each dietary treatment. For data on CFE male rats of similar age consuming Lab Blox see Table 19 of the Appendix.

APPENDIX

TABLE 14

BODY WEIGHT OF CFE RATS FED CODELID DIET
16 OR 17 WITH OR WITHOUT VITAMIN K*

Dietary Treatment	Body weight, Day 0	Day 7	Day 14	Day 21	Day 28	Day 35
	g/rat					
Diet-16	64.5±2.5**	92.5±3.7	128.3±4.8	162.1±6.1	187.1±9.5***	207.8±21.7‡
Diet-16 (-)Vit.K	67.7±2.3	96.8±3.2	134.4±3.2	169.3±3.7	196.5±4.3	235.2± 5.7+
Diet-17	63.4±2.0	99.8±2.8	135.2±4.2	164.2±6.4	186.3±8.8	209.2±12.0+
Diet-17 (-)Vit.K	64.7±2.0	98.7±2.6	135.6±2.8	163.8±4.4	193.8±4.9	228.0± 8.3+
Lab Blox	64.7±2.3	110.9±3.4	161.8±4.4	192.3±5.4	240.5±5.0	246.0± 5.2

(continued)

APPENDIX

TABLE 14 (continued)

Dietary Treatment	Diet consumption ml/rat/day				
	7	14	21	28	35
Diet-16	17.3	25.6	27.6	29.5	28.2
Diet-16 (-)Vit.K	18.5	26.2	26.5	31.9	32.6
Diet-17	18.8	26.1	28.5	29.5	34.2
Diet-17 (-)Vit.K	17.3	26.3	29.1	29.4	33.7
Lab Blox	13.7gm	20.5gm	20.8gm	20.7gm	21.0gm
	Water consumption ml/rat/day				
Diet-16	7.9	8.6	9.2	9.6	12.3
Diet-16 (-)Vit.K	10.9	13.3	13.5	12.8	16.3
Diet-17	11.3	13.3	11.0	13.7	17.9
Diet-17 (-)Vit.K	11.5	11.9	11.0	9.2	13.0
Lab Blox	21.0	27.8	27.0	24.8	28.4

*Twelve CFE rats 25 days of age from identified litter of 9-11 littermates allotted to each treatment. Housed 2 per cage. For summarized data see Table 30 of the text.

**Mean value ± standard error for 12 rats unless otherwise noted.

***Calculated for 10 rats.

+Calculated for 6 rats.

‡Calculated for 4 rats.

APPENDIX

TABLE 15

SIZE OF LIVERS EXCISED FROM CFE RATS FED CODELID DIET 16 OR 17 WITH OR WITHOUT MENADIONE*

	Liver Size							
Test Diet	16		17		16		17	
Menadione Level mg/L	2.0	0.0	2.0	0.0	2.0	0.0	2.0	0.0
Wks. on Exp't	grams				% of body weight			
4	7.1				4.13			
	8.5				5.31			
5	10.6	8.3	10.8	8.6	4.69	3.49	4.66	4.08
	9.3	10.3	8.2	9.4	4.87	4.64	5.58	4.80
	9.0	7.9	10.3	9.2	4.09	4.18	4.36	4.44
	11.2	8.6	11.3	9.4	5.02	4.08	4.67	4.10
	9.2	9.1	11.4	10.6	4.74	4.10	5.73	4.36
	12.8	7.8	11.6	9.9	5.20	4.02	5.18	4.63
	11.0	10.3	10.4	12.4	4.83	4.17	4.56	4.61
	7.0	11.6	8.8	12.3	4.07	4.85	5.83	5.10
		12.8	15.0	15.0		5.52	6.02	6.38
		13.0	12.9	12.3		5.22	6.17	5.87
		14.8	15.9	13.9		5.64	6.05	6.21
		18.0	13.9	15.8		6.41	6.62	6.35
$\bar{X}$	9.6	11.0	11.7	11.6	4.7	4.7	5.5	5.1

*CFE male rats 24 days of age from identified litters of 9-11 littermates allotted to each dietary treatment.

APPENDIX

TABLE 16

SIZE OF KIDNEYS EXCISED FROM CFE RATS FED CODELID DIET 16 OR 17 WITH OR WITHOUT MENADIONE*

	Kidney Size							
Test Diet	16		17		16		17	
Menadione Level mg/L	2.0	0.0	2.0	0.0	2.0	0.0	2.0	0.0
Wks. on Exp't	grams				% of body weight			
4	2.0				1.16			
	2.2				1.38			
5	2.1	2.5	2.2	2.3	0.93	1.05	0.95	1.09
	2.2	2.2	1.5	2.0	1.15	0.99	1.02	1.02
	2.6	2.0	2.4	2.2	1.18	1.06	1.02	1.06
	2.2	2.3	2.4	2.2	0.99	1.09	0.99	0.96
	2.1	2.1	2.2	2.0	1.08	0.95	1.11	0.82
	2.5	2.0	2.3	2.6	1.02	1.03	1.03	1.21
	2.1	2.6	2.4	2.3	0.92	1.05	1.05	0.86
	1.9	2.4	1.5	2.4	1.10	1.00	0.99	1.00
		2.3	2.3	2.3		0.99	0.99	0.98
		2.8	2.8	2.3		1.12	1.12	1.10
		2.8	2.8	2.3		1.07	1.07	1.03
		3.2	3.2	2.6		1.14	1.14	1.04
$\bar{X}$	2.2	2.4	2.3	2.3	1.09	1.04	1.04	1.01

*CFE male rats 24 days of age from identified litters of 9-11 littermates allotted to each dietary treatment.

APPENDIX

TABLE 17

BODY WEIGHT OF CDF RATS FED CODELID DIET 16 WITH OR WITHOUT VITAMIN K*

Dietary Treatment	Body Weight					
	Day					
	0	7	14	21	28	35
	gm/rat					
Diet 16	48.8±1.4**	68.6±1.7	92.8±2.1	118.3±2.3	141.8±2.2	170.8±3.5***
Diet 16 (-)Vit.K	49.0±1.1	65.3±1.3	86.6±1.6	111.1±2.3	134.3±2.7	161.8±3.7***
Lab Blox	50.4±1.1	83.0±1.5	118.5±2.3	151.9±2.8	181.8±3.4	206.8±3.9***

(continued)

APPENDIX

TABLE 17 (continued)

Dietary Treatment	Diet consumption				
	7	14	21	28	35
	ml/rat/day				
Diet-16	18.0	22.2	27.1	28.1	33.8
Diet-16 (-)Vit.K	17.2	22.5	25.7	22.9	32.6
Lab Blox	12.0	16.0	18.2	20.3	20.9
	Water consumption				
	ml/rat/day				
Diet-16	15.9	23.7	26.1	30.4	18.6
Diet-16 (-)Vit.K	14.8	22.8	24.1	30.3	17.9
Lab Blox	18.6	27.8	28.3	36.2	33.7

*Twelve CDF male rats 30 days of age from identified litters of 9-11 littermates allotted to each dietary treatment. Ten rats allotted to the Lab Blox group. For summarized data, see Table 32 of the text.

**Mean value $\pm$ standard error. Calculated for 12 rats.

***Calculated for 6 rats.

APPENDIX

TABLE 18

SIZE OF LIVERS EXCISED FROM CDF RATS FED CODELID DIET 16 WITH OR WITHOUT MENADIONE*

	Liver Size					
Test Diet	16		Lab Blox	16		Lab Blox
Menadione Level mg/L	2.0	0.0		2.0	0.0	
Weeks on Exp't	grams			% of body weight		
	6.4	6.4	8.9	4.13	4.05	4.49
	7.9	7.9	11.0	4.57	5.90	5.24
	7.0	6.5	11.6	4.43	4.45	5.25
	8.0	6.6	10.7	5.03	4.37	5.19
5	7.2	6.6	11.8	4.39	4.82	5.59
	7.4	5.9	10.4	4.59	3.99	5.31
	7.1	6.3	12.2	4.10	4.12	5.02
	8.5	5.4	9.7	4.64	3.94	4.64
	7.4	7.2	11.4	4.07	6.99	5.28
	9.3	7.8	10.5	5.11	4.97	4.69
	8.2	8.9		4.80	5.20	
	9.4	7.3		5.19	4.45	
$\bar{X}$	7.8	6.9	10.7	4.59	4.77	5.07

*CDF male rats 30 days of age from identified litters of 9-11 littermates allotted to each dietary treatment.

APPENDIX

TABLE 19

SIZE OF KIDNEYS EXCISED FROM CDF RATS FED CODELID DIET 16 WITH OR WITHOUT MENADIONE*

	Kidney Size					
Test Diet	16	Lab Blox		16	Lab Blox	
Menadione Level mg/L	2.0	0.0		2.0	0.0	
Weeks on Exp't	grams			% of body weight		
5	1.4	1.5	1.6	0.90	0.95	0.81
	1.7	1.4	1.9	0.98	1.04	0.90
	1.7	1.4	2.1	1.08	0.96	0.95
	1.6	1.5	1.8	1.01	0.99	0.87
	1.5	1.4	2.0	0.91	1.02	0.95
	1.5	1.3	1.8	0.93	0.88	0.92
	1.5	1.3	2.1	0.87	0.85	0.86
	1.8	1.3	1.8	0.98	0.95	0.86
	1.7	1.6	1.9	0.93	0.98	0.88
	1.8	1.5	1.9	0.99	0.96	0.85
	1.7	1.7		0.99	0.99	
	1.8	1.6		0.99	0.98	
$\bar{X}$	1.6	1.5	1.9	0.96	0.96	0.89

*CDF male rats 30 days of age from identified litter of 9-11 littermates allotted to each dietary treatment.

APPENDIX

TABLE 20

BODY WEIGHT, DIET CONSUMPTION AND WATER CONSUMPTION OF CFE AND CDF RATS FED CODELID DIET-16 OR -17+

Group‡	Body weight						
	Day						
	0	7	13	21	28	35	42
	g/rat						
I	71.4±1.2*	96.0±1.6	122.8±2.0	155.6±2.8	183.0±3.6	207.5±4.1	221.6±6.2
II	35.3±1.1	53.0±1.0	69.4±1.5	97.0±1.9	120.4±10.7	149.5±3.0	170.8±4.4
III	73.2±1.6	103.2±4.4	133.2±2.8	168.4±2.6	194.7±3.9	220.3±6.7	239.6±7.8
IV	33.8±0.9	56.9±1.2	78.5±1.4	109.1±1.9	134.2±3.3	164.1±3.6	185.5±4.0
V	73.1±1.8	103.4±3.4	152.6±3.5	200.1±3.3	236.1±3.6	256.7±4.6	292.2±3.7
VI	34.1±0.8	65.1±2.4	88.4±1.4	125.6±1.8	158.6±2.3	186.7±3.1	211.1±3.5

(continued)

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TABLE 20 (continued)

Group‡	Diet consumption					
	Day					
	7	13	21	28	35	42
	ml/rat/day					
I	16.6	25.6	29.6	36.5	38.5	35.3
II	12.0	14.5	23.0	26.1	28.7	33.0
III	19.1	26.6	28.3	47.3	42.2	38.8
IV	12.5	16.3	22.3	44.5	36.5	47.7
V	12.9g	20.3g	18.0g	20.6g	20.9g	22.8g
VI	7.6g	14.2g	13.7g	16.4g	18.9g	20.2g
Group‡	Water consumption					
	ml/rat/day					
I	4.7	14.2	18.3	19.4	20.8	23.7
II	5.1	16.0	24.0	20.3	21.4	27.9
III	4.8	14.8	20.9	20.7	24.0	25.2
IV	4.8	14.9	21.4	19.9	24.5	30.5
V	6.9	20.3	27.1	30.4	37.6	39.4
VI	5.4	13.5	21.6	24.3	30.7	33.5

†Eighteen CFE and 18 Fischer (A. R. Schmidt) male rats 26-27 days of age, all from identified litters of 9-11 littermates allotted to each dietary treatment. Housed 2 per wire bottomed cage. For summarized data see Table 34 of the text.

‡Strain: Gp I, III and V represent CFE rats; Gp II, IV and VI represent Fischer rats.

Diet: Gp I and II, diet-16; Gp III and IV, diet-17; Gp V and VI, Lab-Blox.

*Mean ± standard error for 18 rats unless otherwise noted. Due to death or sacrifice: Gp I--13 rats day 35, 10 day 42; Gp II--16 rats day 7 through 28, 11 day 35, 8 day 42; Gp III--15 rats day 28, 13 day 35 and 10 day 42; Gp IV--20 rats day 0 through day 21; 16 day 28, 14 day 35 and 11 day 42; Gp V and VI 13 rats day 35 and 10 day 42.

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TABLE 21

BODY WEIGHT, DIET CONSUMPTION AND WATER CONSUMPTION OF RATS FED CODELID DIET-16 SUPPLEMENTED WITH MENADIONE-THIOETHER, OXYGENATED FAT MIX AND ETHYL CYSTEINATE*

Diet supplement**	Body weight, Day 0	Day 7	Day 14	Day 21	Day 28
	g/rat				
None	59.6±2.8†	75.0±2.8	94.7±4.0	125.7±4.9	143.3±6.1
E.C.	56.7±2.4	74.8±2.9	95.1±4.5	119.8±5.9	138.0±5.7
Thioether	56.3±2.5	74.6±3.0	96.6±3.3	125.8±3.9	143.8±4.9
Oxid. fat mix	58.4±1.9	73.8±2.5	97.4±3.3	123.5±4.1	142.4±4.6
Oxid. fat mix + E.C.	54.7±2.4	73.5±2.6	96.3±3.4	122.0±3.5	140.2±4.5‡
Oxid. fat mix + thioether	58.1±3.1	75.7±3.0	97.7±3.6	122.6±5.2	143.7±5.3

	Diet consumption, Day 7	Day 14	Day 21	Day 28
	ml/rat/day			
None	23.5	25.2	29.6	32.8
E.C.	21.6	25.0	28.1	33.8
Thioether	22.0	25.7	28.6	32.9
Oxid. fat mix	23.0	27.7	28.7	33.6
Oxid. fat mix + E.C.	21.8	25.7	28.8	34.3
Oxid. fat mix + thioether	22.1	26.9	28.7	30.2

	Water consumption, Day 7	Day 14	Day 21	Day 28
	ml/rat/day			
None	13.2	18.0	24.5	16.0
E.C.	10.0	13.4	19.2	16.0
Thioether	11.2	13.5	17.4	16.6
Oxid. fat mix	9.4	12.9	20.3	14.4
Oxid. fat mix + E.C.	13.1	15.4	25.9	17.9
Oxid. fat mix + Thioether	10.7	16.2	25.3	21.1

*Experimental period 28 days. Ten CDF males 30 days of age allotted to each dietary treatment. Housed 2 per wire bottomed cage. For summarized data, see Table 38 of the text.

**E.C. - Ethyl cysteinate 2.42 gm/L; Thioether - Menadione thioether 5.5 mg/L; Oxidized fat mix 5.03 gm to replace normal fat mix.

†Mean value ± standard error.

‡Calculated for 9 animals.

APPENDIX

TABLE 22

SIZE OF LIVERS EXCISED FROM RATS FED CODELID DIETS CONTAINING MENADIONE-THIOETHER, OXYGENATED FAT MIX AND ETHYL CYSTEINATE·HCL+

Diet Variable												
Ethyl Cysteinate·HCl g/L	0.55	2.42	0.55	0.55	2.42	0.55	0.55	2.42	0.55	0.55	2.42	0.55
Menadione-thioether mg/L	--	--	5.5	--	--	5.5	--	--	5.5	--	--	5.5
Fat mix g/L	5.03	5.03	5.03	--	--	--	5.03	5.03	5.03	--	--	--
Oxygenated fat mix g/L	--	--	--	5.03	5.03	5.03	--	--	--	5.03	5.03	5.03
Weeks on Experiment	Liver Size											
	grams						% of body weight					
4	7.1	6.7	7.2	5.4	4.6	6.0	4.49	4.75	4.39	4.35	4.22	4.17
	6.1	5.8	8.1	6.4	6.7	5.7	4.18	5.09	5.19	4.85	4.65	4.71
5	7.4	7.2	6.3	6.0	7.8	6.2	4.30	4.59	4.17	3.89	4.43	4.49
	9.0	9.6	6.9	7.7	5.5	8.4	5.17	4.66	4.26	4.38	3.48	4.72
6	7.0	7.7	7.3	8.4	5.5	8.5	4.07	4.48	3.80	3.89	3.35	4.01
	9.0	6.3	6.1	7.6	7.7	9.0	4.36	4.29	4.04	3.88	3.89	4.15
8		7.0				8.7		3.76				3.72
9	11.8	11.7	9.8	12.1	6.7	11.0	5.04	4.64	4.21	5.43	3.49	4.74
	11.4	9.7	10.7	10.0	5.8	10.7	5.33	3.79	4.69	4.27	2.89	4.63
			6.9	9.3	8.1	9.6			3.22	4.03	3.93	4.42
			10.5	10.1					4.30	4.65		

+Ten CDF male rats from identified litters of 9-11 littermates allotted to each dietary treatment. Housed 2 per wire bottomed cage. For summarized data see Tables 38 and 39 of text.

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TABLE 23

SIZE OF KIDNEYS EXCISED FROM RATS FED CODELID DIETS CONTAINING MENADIONE-THIOETHER, OXYGENATED FAT MIX AND ETHYL CYSTEINATE·HCL+

Diet Variable													
Ethyl Cysteinate·HCl g/L	0.55	2.42	0.55	0.55	2.42	0.55	0.55	2.42	0.55	0.55	2.42	0.55
Menadione-thioether mg/L	--	--	5.5	--	--	5.5	--	--	5.5	--	--	5.5
Fat mix g/L	5.03	5.03	5.03	--	--	--	5.03	5.03	5.03	--	--	--
Oxygenated fat mix g/L	--	--	--	5.03	5.03	5.03	--	--	--	5.03	5.03	5.03
Weeks on Experiment	Kidney Size											
	grams						% of body weight					
4	1.6	1.4	1.8	1.2	1.1	1.3	1.01	0.99	1.10	0.97	1.01	0.90
	1.3	1.2	1.8	1.3	1.6	1.1	0.89	1.05	1.15	0.98	1.11	0.91
5	1.6	1.5	1.4	1.2	1.6	1.4	0.93	0.96	0.93	0.78	0.91	1.01
	1.6	1.7	1.6	1.6	1.5	1.6	0.92	0.83	0.99	0.91	0.95	0.90
6	1.4	1.6	1.7	1.7	1.4	1.7	0.81	0.93	0.89	0.79	0.85	0.80
	1.8	1.3	1.2	1.7	1.7	1.8	0.87	0.88	0.79	0.87	0.86	0.83
8		1.9				1.8		1.02				0.77
9	2.1	2.2	2.0	2.2	1.4	2.1	0.90	0.87	0.86	0.99	0.73	0.91
	2.1	1.9	2.1	2.0	1.6	2.0	0.98	0.74	0.92	0.85	0.80	0.87
			1.6	1.8	1.6	2.0			0.75	0.78	0.78	0.92
			2.1	1.9					0.86	0.88		

+Ten CDF male rats from identified litters of 9-11 littermates allotted to each dietary treatment. Housed 1 or 2 per wire bottomed cage. For summarized data see Tables 38 and 39 of the text.

APPENDIX

TABLE 24

BODY WEIGHT, DIET CONSUMPTION AND WATER CONSUMPTION OF CDF RATS FED CODELID DIET-17 WITH OR WITHOUT ETHYL LINOLEATE AND ETHYL CYSTEINATE·HCL+

Group‡	Body weight, Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
	g/rat‡						
I	52.1±2.5	71.3±2.4	97.9±2.5	123.0±3.3	145.9±4.2	165.9±3.7	178.1±3.9*
II	49.3±2.7	64.3±3.1	87.6±3.7	108.7±4.0	129.0±4.3**	147.5±5.2	164.0±5.8
III	48.0±2.5	65.4±3.2	91.8±3.2	118.3±4.0	145.3±4.9	168.5±5.1	195.5±5.9
IV	49.1±1.5	67.6±3.0	90.9±3.4	116.2±3.5	138.5±4.5	156.7±4.6	174.7±4.9
	Diet consumption, ml/rat/day						
I		12.7	20.6	24.1	29.4	30.8	30.2*
II		11.0	22.1	22.7	28.6**	28.7	33.4
III		14.0	23.6	28.4	32.6	34.9	35.8
IV		15.0	20.8	26.8	36.8	32.6	31.5
	Water consumption, ml/rat/day						
I		8.2	17.4	22.2	26.2	19.9	20.2*
II		9.6	20.9	28.1	26.6**	22.4	16.1
III		9.6	19.2	25.6	26.6	21.0	15.7
IV		9.3	19.0	24.7	26.8	13.8	--

+Fifteen CDF rats 33 days of age from identified litters of 9-11 littermates allotted to each dietary treatment. Housed 1 or 2 per wire bottomed cage. Unless otherwise noted values represent 14 rats. For summarized data, see Table 40 of the text.

‡Gp I--Diet-17; Gp II--Diet-17 minus E.L.; Gp III--Diet-17 minus E.C.; Gp IV--Diet-17 minus (E.L. + E.C.).

‡Mean ± standard error for 14 rats unless otherwise noted.

*Mean value for 9 rats.

**Mean value for 13 rats.

APPENDIX

TABLE 25

SIZE OF LIVERS EXCISED FROM RATS FED CODELID DIET-17
WITH AND WITHOUT ETHYL CYSTEINATE·HCL AND ETHYL LINOLEATE*

	Liver size							
Test diet**	17	17-EL	17-EC	17-(EL + EC)	17	17-EL	17-EC	17-(EL + EC)
Weeks on Exp't.	grams				% of body weight			
6	8.6	10.0	6.9	7.8	4.9	5.2	4.5	4.0
	5.9	8.3	7.7	7.7	3.7	5.4	4.1	4.3
	7.5	7.5	9.2	6.4	4.4	4.5	4.0	3.8
	8.8	7.3	9.5	8.4	4.5	4.9	4.2	4.1
	9.6	8.4	7.9	8.2	5.0	4.7	4.0	4.5
		5.5	8.4	5.2		3.8	4.2	3.8
7	7.8	7.7	8.0	7.9	4.0	4.1	3.9	4.1
	8.4	9.4	8.4	9.1	3.7	4.3	3.6	4.8
	8.6	11.1	6.8	8.5	4.7	5.2	3.5	4.6
		8.8				5.2		
		7.5				4.1		
		11.0				5.1		
		9.3				4.8		
8			13.0	9.4			5.1	4.9
			11.4	10.3			4.6	4.6
			11.3	10.3			4.8	4.8
			10.6	10.4			4.2	4.7
			12.1	11.4			4.9	4.7
			11.7	11.3			4.8	4.9
$\bar{x}$	8.2	8.6	9.5	8.8	4.4	4.7	4.3	4.4

*Fifteen CDF male rats 33 days of age were started on each dietary treatment. Housed 1 or 2 per wire bottomed cage.

**E.L. - Ethyl Linoleate; E.C. - Ethyl Cysteinate·HCl.

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TABLE 25A

SIZE OF KIDNEYS EXCISED FROM RATS FED CODELID DIET-17
WITH AND WITHOUT ETHYL CYSTEINATE·HCL AND ETHYL LINOLEATE*

	Kidney size							
Test diet**	17	17-EL	17-EC	17-(EL + EC)	17	17-EL	17-EC	17-(EL + EC)
Weeks on Exp't.	grams				% of body weight			
6	1.9	1.7	1.4	1.7	1.09	0.88	0.92	0.86
	1.5	1.6	1.6	1.7	0.94	1.04	0.95	0.96
	1.7	1.6	1.9	1.5	0.98	0.96	0.82	0.89
	1.8	1.5	1.7	1.6	0.91	1.01	0.75	0.78
	1.7	1.3	1.8	1.7	0.89	0.73	0.91	0.92
		1.4	1.9	1.3		0.97	0.94	0.95
7	1.6	1.6	1.7	1.7	0.83	0.86	0.84	0.89
	2.0	2.1	2.0	1.7	0.87	1.00	0.85	0.90
	1.7	2.1	1.4	2.0	0.93	0.98	0.73	1.07
		1.6				0.95		
		1.7				0.94		
		2.1				0.97		
		1.7				0.89		
8			2.3	1.7			0.91	0.88
			2.2	2.0			0.88	0.89
			2.1	2.0			0.89	0.93
			2.2	2.1			0.87	0.94
			2.2	2.2			0.88	0.91
			2.2	2.1			0.90	0.90
$\bar{x}$	1.7	1.7	1.9	1.8	0.93	0.94	0.86	0.91

*Fifteen CDF male rats 33 days of age were started on each dietary treatment. Housed 1 or 2 per wire bottomed cage.

**EL - Ethyl Linoleate; E.C. - Ethyl Cysteinate·HCl.

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TABLE 26

BODY WEIGHT, DIET CONSUMPTION AND WATER CONSUMPTION OF CDF RATS FED CODELID DIET-17 WITH SUPPLEMENTARY ANTIOXIDANTS+

Group‡	Body weight, Day 0	Day 7	Day 14	Day 21	Day 28	Day 35
	g/rat					
I	117.3±3.0*	142.8±3.7	171.7±4.2	198.1±4.2	221.8±4.1	242.3±4.8
II	113.7±3.2	138.7±3.3	162.2±4.8	175.6±6.1**	177.8±9.7**	181.6±12.8**
III	111.4±3.2	135.0±4.1	161.7±4.6	181.3±5.4***	197.9±8.9***	209.5±12.1***
	Diet consumption					
	ml/rat/day					
I		26.1	31.1	33.4	38.4	33.3
II		25.5	30.0	29.6**	33.4**	25.4**
III		26.7	31.0	31.7***	38.8***	44.3***
	Water consumption					
	ml/rat/day					
I		15.4	20.0	29.7	31.6	20.3
II		17.2	21.2	32.3**	41.2**	29.1**
III		14.0	20.2	32.1***	46.7***	25.7***

+Ten CDF male rats 51 days of age from identified litters of 9-11 littermates allotted to each dietary treatment. Housed 2 per wire bottommed cage. For summarized data, see Table 42 of the text.

‡Gp I--Diet-17; Gp II--Diet-17 + Ascorbic Acid and α-tocopherol acetate; Gp III--Diet-17 + Ascorbic Acid + α-tocopherol acetate minus ethyl linoleate.

*Mean ± standard error for ten rats unless otherwise noted.

**Mean value for 9 rats - 21st day; 8 rats 28th day and 7 rats 35th day.

***Mean value for 9 rats - 21st day; 7 rats 28th day and 6 rats 35th day.

APPENDIX

TABLE 27

SIZE OF KIDNEYS AND LIVERS EXCISED FROM CDF RATS FED CODELID DIET-17 WITH OR WITHOUT SUPPLEMENTARY ANTIOXIDANTS*

Test diet**		Kidney size		Liver size	
		grams	% of body weight	grams	% of body weight
17		12.6	4.4	2.2	0.77
		12.5	4.3	2.4	0.82
		10.8	3.8	2.3	0.80
		11.2	4.3	2.2	0.85
		10.8	4.2	1.7	0.66
		12.1	4.2	2.2	0.76
		12.0	4.4	2.0	0.73
		11.8	4.4	1.8	0.67
		12.7	4.9	2.2	0.84
		12.2	4.5	2.2	0.80
	$\bar{x}$	11.9	4.3	2.1	0.77
17 + Antioxidants		7.9	3.6	1.9	0.87
		11.2	4.8	2.1	0.90
		12.2	4.3	2.2	0.78
	$\bar{x}$	10.4	4.2	2.1	0.85
17 + Antioxidants minus E.L.		6.8	3.7	1.6	0.87
		10.5	4.2	2.1	0.84
		11.7	5.1	2.1	0.92
	$\bar{x}$	9.7	4.3	1.9	0.88

*CDF male rats were 51 days of age at start of experiment. Specimens were obtained only when the rats were sacrificed for blood samplings (seventh week of experiment). For details see Table 26 of the Appendix.

**Antioxidants - Ascorbic acid and α-tocopherol acetate; E.L. - ethyl linoleate.

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TABLE 28

BODY WEIGHT, DIET CONSUMPTION AND WATER CONSUMPTION OF CDF RATS FED CODELID DIET-17 CONTAINING DIFFERENT LEVELS OF VITAMIN A, VITAMIN E, ASCORBIC ACID AND ETHYL CYSTEINATE·HCL+

Group‡	Body weight				
	Day				
	0	7	14	21	28
	g/rat				
I	89.9±6.1*	112.8±5.2	140.4±5.8	173.4±5.2	184.2±9.2**
II	83.4±5.8	106.4±8.2	134.5±8.2	158.9±6.4	168.0±8.9**
III	84.4±4.7	112.7±4.6	135.6±5.8	159.1±6.3	179.5±9.5**
IV	87.3±3.1	115.3±2.9	143.3±3.6	169.5±4.5	187.7±4.7***
V	86.5±3.3	111.3±4.3	136.5±5.4	162.1±6.1	177.5±8.4**
VI	89.1±4.3	117.8±4.4	142.6±5.5	164.4±5.9	184.6±5.5***
VII	78.5±5.0	98.8±4.6	124.6±5.3	146.4±3.9	169.9±4.1***
VIII	81.2±2.6	110.1±3.3	140.6±3.7	164.6±3.7	184.0±6.3***

(continued)

APPENDIX

TABLE 28 (continued)

Group‡	Diet consumption				Water consumption			
	Day				Day			
	7	14	21	28	7	14	21	28
	ml/rat/day				ml/rat/day			
I	19.1	38.9	36.5	32.4	6.2	15.8	12.9	13.5
II	22.6	33.6	33.6	34.9	7.8	13.7	14.7	14.1
III	22.7	33.0	32.7	32.0	6.0	17.0	15.8	13.1
IV	22.4	33.2	35.4	32.2	7.2	17.0	17.3	14.9
V	21.3	33.5	34.9	31.4	6.8	18.9	16.2	13.5
VI	23.0	31.7	35.4	36.1	7.3	14.2	14.6	15.5
VII	20.8	29.5	34.4	32.5	7.5	15.5	12.8	15.4
VIII	20.6	34.7	34.4	33.7	6.8	17.5	19.0	14.8

†Eight CDF male rats 40-42 days of age from identified litters of 9-11 littermates started on each test diet. For summarized data, see Table 44 of the text.

‡Gp I - 50mg/L Vit. E; Gp II - 500mg/L Vit. C; Gp III - Vit. A-free; Gp IV as I minus Vit. A; Gp V as II minus Vit. A; Gp VI - as III with 50mg Vit. E and 500mg/L Vit. C; Gp VII - Vit. E 50mg/L; Vit. C - 500mg/L minus Ethyl Cysteinate·HCl; Gp VIII - as VII minus Vit. A.

*Mean value ± standard error for 8 rats unless otherwise noted.

**Mean value ± standard error for 6 rats.

***Mean value ± standard error for 7 rats.

APPENDIX

TABLE 29

BODY WEIGHT, DIET CONSUMPTION AND WATER CONSUMPTION OF CDF RATS FED CODELID DIET-17 CONTAINING VARIOUS ADDITIVES RELATED TO BLOOD COAGULATION+

Group‡	Body weight							
	Day							
	0	7	14	21	28	35	42	49
	g/rat							
I	71.4±4.9*	101.1±5.1	132.1±5.4	167.3±5.2	183.8±4.8	196.2±6.9	221.8**	244.3***
II	70.3±1.9	96.4±5.2	129.9±4.4	160.0±4.4	181.3±3.7	196.8±6.9	214.0±7.5	245.3
III	61.3±1.7	85.5±2.7	113.9±3.5	143.9±5.0	161.4±5.1	184.0±8.0	199.3±8.1	205.5
IV	60.8±3.1	82.8±4.7	108.1±6.0	135.6±5.8	155.5±6.6	173.7±5.8	186.3±5.4	208.8
V	68.0±3.6	83.1±3.5	105.8±4.6	124.1±7.3	131.1±9.9	151.3±15.4	170.8**	167.0
VI	61.1±3.6	78.6±4.0	101.0±5.2	123.8±6.1	137.6±7.2	131.5±10.2	127.7±12.4	136.8
VII	52.6±2.6	68.4±2.4	87.3±3.1	104.8±3.7	112.9±5.7	114.5±10.4	115.7±14.7	143.5

(continued)

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TABLE 29 (continued)

Group‡	Diet consumption						
	Day						
	7	14	21	28	35	42	49
	ml/rat/day						
I	20.1	24.7	29.0	26.8	29.1	30.0**	44.7***
II	20.5	24.9	25.7	28.6	27.0	27.3	28.6
III	18.7	20.5	28.4	24.7	32.2	31.0	27.9
IV	18.0	21.7	25.3	27.6	28.6	25.3	31.1
V	18.0	21.2	23.7	24.2	24.0	24.5**	21.2
VI	16.2	19.8	24.0	25.5	21.1	18.0	17.6
VII	16.9	18.3	18.3	21.7	16.3	18.8	22.1
Group‡	Water consumption						
	ml/rat/day						
I	16.3	18.9	23.2	19.4	15.1	16.0**	19.0***
II	16.3	21.3	24.8	20.4	19.9	18.6	18.8
III	15.3	18.4	25.9	20.8	19.6	19.3	15.0
IV	13.3	16.9	24.4	20.2	17.1	16.0	16.0
V	12.7	15.9	22.3	16.8	15.6	17.3**	15.4
VI	16.4	17.4	24.0	17.6	13.9	14.4	14.6
VII	13.4	16.3	22.3	17.1	14.4	17.2	19.0

†Eight CDF rats 36 days of age started on each test diet. Housed 2 per wire bottomed cage. For summarized data, see Table 46 of the text.

‡Gp I--Diet-17; Gp II--Diet-17 with menadiol sodium diphosphate; Gp III--Diet-17 w Diethylcystinate·HCl; Gp IV--Diet-17 w H_2O_2; Gp V--Diet-17 minus E.C. w H_2O_2; Gp VI--Diet-17 w 2x H_2O_2; Gp VII--Diet-17 w H_2O_2 and menadione-thioether.

*Mean value ± standard error for 8 rats, 0-28 days and for 6 rats 35-42 days unless otherwise noted. Data on the 49th day represents mean value of 4 animals per group.

**Mean value of 5 rats.

***Mean value of 3 rats.

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TABLE 30

BODY WEIGHT, DIET CONSUMPTION AND WATER CONSUMPTION OF RATS FED CODELID DIETS WITH OR WITHOUT OXIDIZED ETHYL CYSTEINATE·HCL*

Test diet	Body weight, Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
				g/rat			
Diet-16	39.3±1.4**	53.1±1.2	71.2±0.9	96.7±1.7	120.9±1.8	146.9±2.2	161.5±1.9
Diet-17	37.8±1.8	58.5±1.8	81.0±2.2	105.8±2.8	133.7±2.8	158.3±5.1	177.2±5.0
Diet-17 w oxid.E.C.	40.8±1.5	60.6±1.4	81.9±1.6	108.8±3.9	135.3±4.2	157.9±4.0	173.6±5.0

	Diet consumption, Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
			ml/rat/day			
Diet-16	16.2	19.3	23.3	26.7	29.9	31.7
Diet-17	15.3	21.2	26.5	28.5	28.8	29.5
Diet-17 w oxid.E.C.	14.4	18.9	25.4	27.4	28.7	32.2

	Water consumption, Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
			ml/rat/day			
Diet-16	5.8	17.5	20.1	16.3	20.1	24.2
Diet-17	6.5	19.2	20.1	18.1	19.9	21.1
Diet-17 w oxid.E.C.	5.7	17.9	21.7	19.8	19.4	18.5

*Ten CDF male rats 30 days of age from identified litters of 9-11 littermates allotted to each dietary treatment. Housed 2 per wire bottomed cage. For summarized data, see Table 48 of the text.

**Mean ± standard error for ten rats.

APPENDIX

TABLE 31

SIZE OF LIVERS EXCISED FROM RATS FED CODELID DIETS WITH OR WITHOUT OXIDIZED ETHYL CYSTEINATE·HCL*

Test diet	Liver Size					
	16	17	17-Oxid. E.C.	16	17	17-Oxid. E.C.
Weeks on exp't.	grams			% of body weight		
7	7.0	8.7	6.5	4.24	4.26	3.87
	5.4	10.2	8.9	3.80	5.34	4.61
9	6.2	8.2	7.0	3.16	3.83	3.35
	7.7	7.9	5.9	3.95	3.45	2.73
			5.7			3.39
10	9.5	7.7	6.0	4.17	3.17	3.19
	7.8	6.1		3.64	3.72	
	7.7			3.62		
	7.4			3.38		
	8.2			3.87		
$\bar{X}$	7.32	8.13	6.67	3.76	3.96	3.52

*Ten CDF male rats 30 days of age were started on each dietary treatment. Housed 2 per wire bottomed cage.

APPENDIX

TABLE 32

SIZE OF KIDNEYS EXCISED FROM RATS FED CODELID DIETS WITH OR WITHOUT OXIDIZED ETHYL CYSTEINATE·HCL*

Test diet	Kidney Size					
	16	17	17-Oxid. E.C.	16	17	17-Oxid. E.C.
Weeks on exp't.	grams			% of body weight		
7	1.6	2.1	1.9	0.97	1.03	1.12
	1.6	2.0	2.1	1.13	1.05	1.09
9	1.4	1.7	1.6	0.71	0.79	0.77
	1.9	1.7	1.6	0.97	0.74	0.74
			1.6			0.95
10	2.1	1.8	1.5	0.92	0.74	0.80
	2.0	1.7		0.93	1.04	
	1.6			0.75		
	2.1			0.96		
	2.0			0.94		
$\bar{X}$	1.81	1.83	1.72	0.92	0.90	0.91

*Ten CDF male rats 30 days of age were started on each dietary treatment. Housed 2 per wire bottomed cage.

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APPENDIX

TABLE 33

BODY WEIGHT, DIET CONSUMPTION AND WATER CONSUMPTION
OF FISCHER RATS FED VITAMIN K-FREE DIETS+

	Body weight							
	Day							
Test Diet	0	7	14	21	28	35	42	49
	g/rat							
Diet-16	37.3±0.7‡	49.9±1.1	72.1±1.4	95.9±1.7	119.3±1.9	138.1±1.7	161.9±2.9*	179.1±3.6**
Diet-16, K-free	35.5±1.2	50.7±1.5	68.8±1.5	95.5±2.0	115.0±4.3	134.8±3.5	145.9±3.6*	165.8±4.6**
Diet-17, K-free	35.3±0.9	58.7±1.3	85.3±1.7	110.5±2.5	134.8±2.7	154.8±3.9	164.8±4.6*	179.9±2.0**

(continued)

APPENDIX

TABLE 33 (continued)

Test Diet	Diet consumption*** Day 7	14	21	28	35	42	49
	ml/rat/day						
Diet-16	12.7	21.9	24.8	29.0	30.7	31.4	33.2
Diet-16, K-free	13.2	20.3	24.1	28.4	31.6	25.3	32.9
Diet-17, K-free	15.0	22.8	28.9	35.0	34.3	32.8	30.7
	Water consumption						
	ml/rat/day						
Diet-16	8.1	14.1	14.8	17.5	18.2	12.6	10.6
Diet-16, K-free	7.2	12.6	15.6	17.0	13.0	12.9	14.1
Diet-17, K-free	7.6	12.8	15.0	17.9	17.0	13.2	15.1

+Twelve male Fischer rats (A. R. Schmidt Inc., Madison, Wisconsin) 25 days of age from identified litters started on each test diet. Housed 2 per wire bottomed cage. For summarized data see Table 36 of the text.

‡Mean ± standard error for 12 rats unless otherwise noted.

*Mean ± standard error for 10 rats.

**Mean ± standard error for 8 rats.

***Spillage was not measured in this experiment.

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