CATARACT FORMATION IN YOUNG RATS AS A CONSEQUENCE OF MATERNAL DIETS CONTAINING EXCESS PHENYLALANINE AND LOW IN TRYPTOPHAN AND/OR VITAMIN E

by

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Chapter I

INTRODUCTION

Cataracts have been reported to be the single largest cause of blindness in the world (1). Each year the vision of well over a million people is affected by cataracts (2). It is the most frequent and important lesion found in the lens.

A cataract or lens opacity is a region in the lens which causes the scattering of light. As a result the light which passes through the lens produces a distorted image or, if severe enough, no image at all.

The orgin of the term stems from the Latin word "cataracta" which in turn can be traced back to the Greek word "kataraktes." Both words mean waterfall. Cataracts are not a new phenomenon. The ancient code of Hammurabi, 1800 B.C., mentions the penalties of unsuccessful cataract removal. The code states that "if a physician opened a man's eye with a bronze needle and the patient lost his eye, the physician lost his hands" (3). Other historic references can be found in Egyptian papyrus dating back to 1300, 1500 and 1600 B.C.

In spite of man's early knowledge of cataracts today there still is no cure. A partial remedy does exist which

involves removal of the lens and substitution by a spectacle lens.

In the majority of cases the etiolgies of both congenital and senile cataracts are obscure. Nutrition has often been put forth as a possible factor; however, until recently there has been little evidence to support this hypothesis. The relationship which has recently been discovered is the critical need for vitamin E in concert with tryptophan during the gestation and lactation period of rats (4).

Based on this evidence the following project was undertaken. The objective of the study was to gain a better understanding of the interaction of vitamin E and tryptophan in cataract formation. It is hoped that this information may in some way lead to a better understanding of human cataract formation and a possible method of prevention.

Chapter II

REVIEW OF LITERATURE

A. Structure of the Eye

The eye is the organ associated with the sense of sight. It consists of three coats: protective, vascular, and neuro-ephithelial, the inner cavity consists of the aqueous humor, lens, and vitreous body (Figure 1). The protective coat is made up of the transparent cornea and sclera; the vascular coat is composed of the choroid, ciliary body, and iris; and the retina constitutes the neuro-epithelial layer.

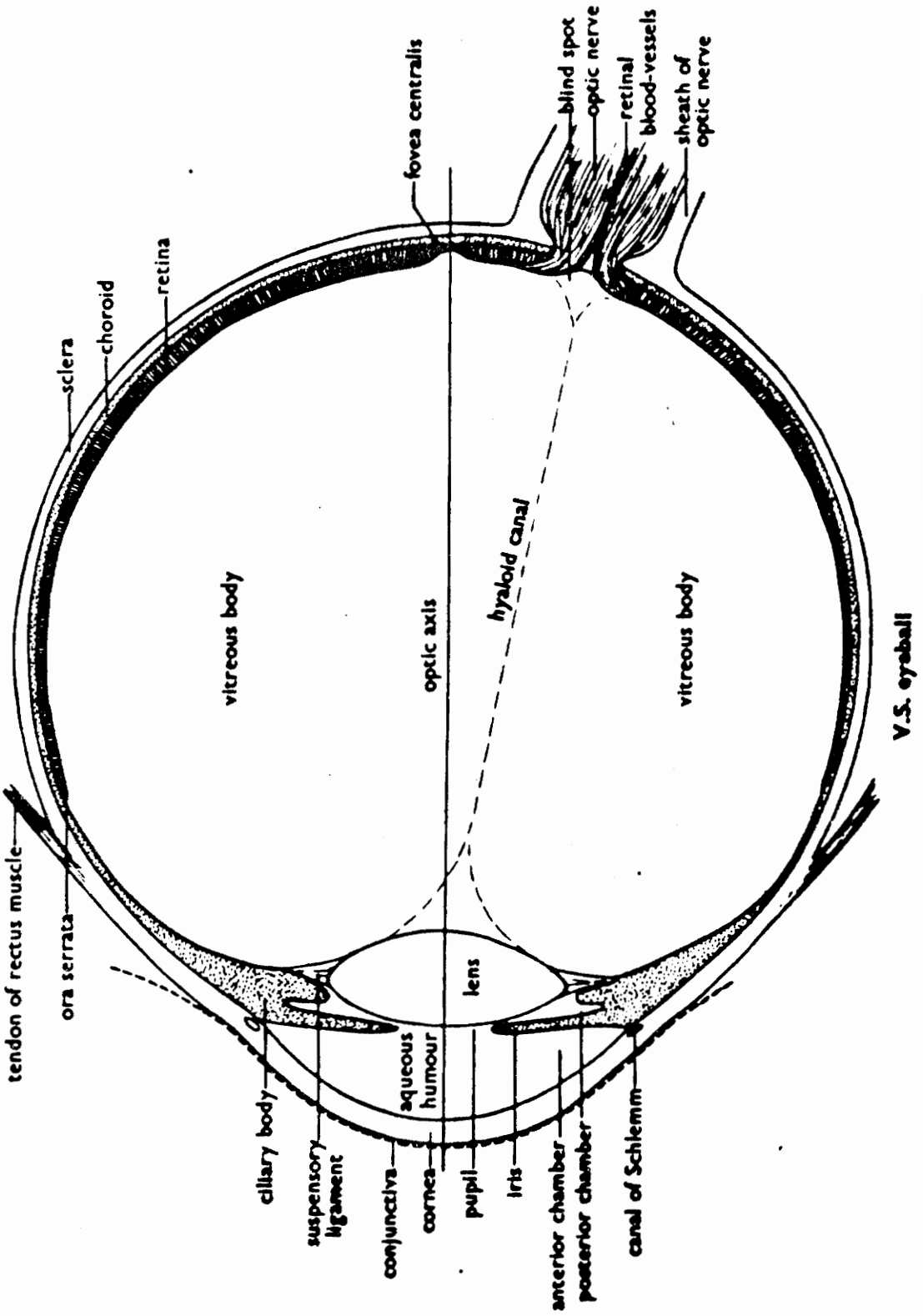
B. Lens Structure

The lens can be divided into three structural regions: (a) the capsule, an elastic membrane which surrounds the exterior surface of the lens; (b) the epithelium, a single layer of cells which extends under the anterior portion of the capsule to the equator, (it is from this single layer that the lens fiber cells are produced); and (c) the lens which consists of fibers and interstitial material. (Figure 2). The lens itself is derived from ectodermal tissue. The lens is composed of 35 percent solid matter, most of which is protein, and 65 percent water. Lens fiber cells contain a

Figure 1.

The Human Eye

Diagrammatic showing a longtudinal cross section of the human eye.


tendon of rectus muscle
ora serrata
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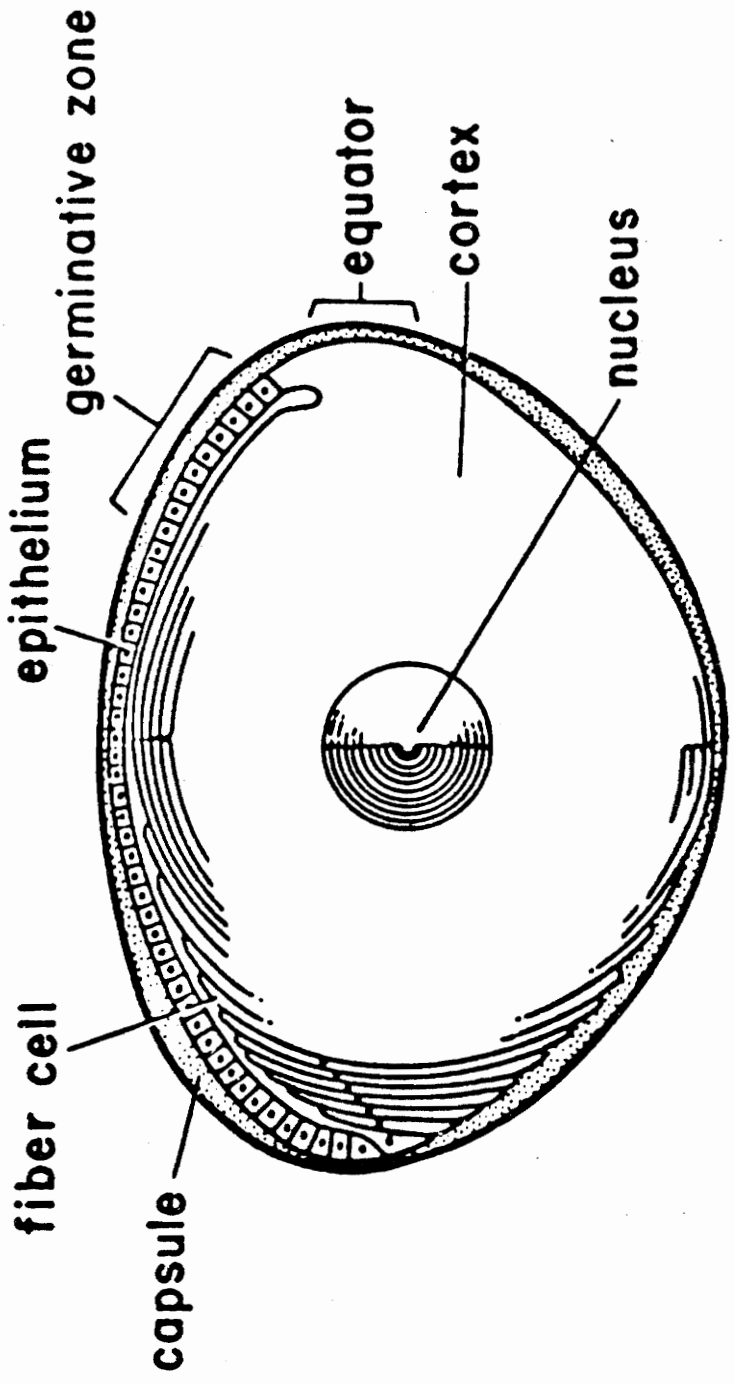
choroid
retina
ciliary body
suspensory ligament
conjunctiva
aqueous humour
cornea
pupil
lens
iris
anterior chamber
posterior chamber
canal of Schlemm
vitreous body
optic axis
hyaloid canal
vitreous body
fovea centralis
blind spot
optic nerve
retinal blood-vessels
sheath of optic nerve
V.S. eyeball

Figure 2

The Human Lens

Front view of the lens showing the three structural regions.


germinative zone
equator
cortex
nucleus
epithelium
fiber cell
capsule

variety of different proteins or crystallins. These crystallins are subdivided into three main groups of soluble structural proteins: alpha (α); beta (β); and gamma (γ) crystallins.

The α-cyrstallins have the largest molecular size of the soluble proteins (400,000-900,000, depending on the species) while β- and γ-cyrstallins are smaller (50,000-250,000, and 20,000, respectively). There is also an insoluble lens protein, called the albuminoid.

The lens can also be subdivided into an outer cortical and central nuclear region. The dividing point between these regions is not clear-cut. But the nuclear space is composed of the embryonic protein of the oldest fiber cells which have been compressed by the growth of new fiber cells.

C. Classification of Cataracts

Cataracts can be classified into two types: senile and congenital or developmental. The most common are senile cataracts which occur most frequently in elderly individuals but are not inevitable accompaniment of old age. There are two main types of senile cataracts: cortical and nuclear. Both may be seen in the same lens.

In cortical cataracts the cataractous regions occur in the outer layers of the lens. This opacity arises from a disturbance in the arrangement of lens fibers (2). The

fibers become swollen or distorted causing gaps which may fill with water or debris.

Nuclear cataracts are caused by protein changes in the area of the lens (2). The position of this type of cataract will interfere with vision. Cortical cataracts may not seriously interfere with vision.

Senile cataracts are more common in some parts of the world than in others. Rates of occurrence in the sub-continent of India are particularly high in comparison to those in the United States or Europe. In India nearly 7 percent of the population suffers from cataract and nearly 1.5 percent of the population is blind due to cataract (5).

Maternal or early infantile malnutrition in one of the suspected causes of congenital or developmental cataract formation. This type of cataract is of primary importance in this study. Other causes of congenital cataracts include virual, genetic and toxic compounds.

D. Vitamin E and the Incidence of Congenital Cataract Formation

The appearance of cataracts as the result of low tocopherol intake by weanling or older rats has not been well documented. Most studies relating to tocopherol deficiencies during pregnancy have employed prolonged dietary restriction, and have resulted in resorption of the fetus rather than development of congenital defects.

The relationship of the eye disorder retrolental fibroplasia has been reported by Owens and Owens (6) in a study of eye disorders in premature infants. They noted that vitamin E was the only fat soluble vitamin which was not added to the infant formula, and the low fat diet recommended for premature infants contained very little vitamin E.

Cheng et al. (7) also observed congenital defects of the eye as the result of low tocopherol in the diet. They fed weanling female Holtzman rats 18-20 percent casein diets deficient in tocopherol throughout growth, from weanling to breeding age. At different times during pregnancy they were administered 2 mg DL-a-tocopherol acetate by gavage. When the tocopherol supplement was not given until the twelfth day of gestation resorption of the fetus occurred. However, when the supplement was provided on the tenth day of gestation, the young were successfully delivered but multiple congenital abnormalities were observed. Eye abnormalities were not the most common congenital defect. Only one or two cases of eye inflammation were detected among 112 fetuses. This data suggests that cataracts or associated congenital eye defects are incidental but not predominant when the maternal diet is limited only in tocopherol.

Callison and Orent-Keiles (8) found that the off-spring of rats fed vitamin E deficient diets had smaller eyes than normal. The eye lids sometimes failed to open, and an opaque membrane was observed behind the pupil.

Reports by Ferguson et al. (1, 10) describe a cloudiness in the central portion of the lens in the offspring of turkeys fed an all vegetable protein diet deficient in vitamin E. The diet consisted of soybean oil meal, ground yellow corn and ground milo with supplements of minerals and selected vitamins. This lens disorder was associated with high embryonic mortality. Supplementation with α-tocopherol acetate did, however, prevent the eye abnormalities and restore normal hatchability. These results have also been confirmed by Jensen and McGinnis (11).

E. Placental Transfer of Vitamin E

Results from studies concerning placental transfer of vitamin E are conflicting. Early work by Gordon et al. (12) indicates that supplementing the mother with vitamin E does not have an effect on the levels of vitamin E in the premature infant.

Oski and Barnes (13) in a double blind study, gave high doses of vitamin E to every other mother for several months before delivery. Some infants were born with higher plasma vitamin E levels, but statistical significance was not found.

Bieri (14) has shown that the plasma lipid status of premature infants is very low, and becomes progressively lower with a greater degree of prematurity. Since α-tocopherol is transported via a lipoprotein carrier, increased vitamin E levels in the infant may not be possible due to the low plasma lipid levels. Horwitt et al. (15) has shown very little correlation between the blood tocopherol levels of the mother and the child at birth. Johnson et al. (16) similarily showed very little relationship between serum tocopherol levels of the mother and child. Supplementing the mother did not ensure vitamin E transport across the placenta when serum tocopherol concentrations were measured. It is possible that vitamin E may have been deposited in other tissues of the fetus. In this case the blood levels would not indicate the true vitamin E status. As previously mentioned, a decrease in serum lipoprotein levels may reduce the capacity of premature infants to carry tocopherol. On the other hand Minkowski et al. (17) have shown that plasma vitamin E levels of the infant at birth could be influenced by injecting tocopherol into the mother prior to delivery. In addition, Lenonard et al. (18) have reported that there was a direct relationship between the plasma levels of vitamin E in the infant at birth and that of the mother. Mino and Nishino (19) studied the correlation between maternal and cord blood of infants at delivery. Among the

mothers supplemented with vitamin E acetate prior to delivery, the mean serum vitamin E level in the mothers blood and the cord blood was significantly higher in the vitamin E supplemented group than in the control groups. However, the difference in the mean serum vitamin E levels between the two groups was less in the cord blood than in the maternal blood. These investigators concluded that a moderate correlation exists between the cord blood and the maternal blood.

F. Tryptophan Deficiency and Congenital Cataract Formation

There have been numerous reports of the effects of diets low in tryptophan on the generation of cataracts during postnatal development.

Curtis et al. (20) in their account of tryptophan deficiency in rats mention a "white opaqueness of the eye and loss of the characteristic colors of the eye." Their results were confirmed by Totter et al. (21).

Tryptophan deficiencies have also resulted in cataracts in other species including pigs (Cartwright et al. (22)) and guinea pigs (Von Sallmann et al. (23)). Frequently in these studies acid hydrolzed protein, which was tryptophan free, or combinations of tryptophan low proteins were used. However the combinations of these tests resulted in a relatively poor appearance and debilitation of the

experimental animals, and there were considerable differences between the intensity and rate of cataract development among the various studies.

Buschke (24) reports that there are two types of tryptophan deficiency cataract, the acute and chronic. Acute consists of feathery opacities in the posterior cortex. The chronic type consists of fine dots in the cortex. The nucleus remains clear.

Schaeffer and Murray (25) studied whether tryptophan per se plays a role in lens metabolism or whether tryptophan deficiency results in interference of protein synthesis. Rats were fed a diet deficient in tryptophan but later supplemented with the limiting amino acid. In order for protein synthesis to take place all the essential amino acids must be available simultaneously. Despite the large amounts of tryptophan ingested the rats developed cataracts. This indicated that tryptophan must be available for protein synthesis to prevent cataract formation. No differences were found in the tryptophan content of the lens from normal and animals on the tryptophan deficient diet.

Pike (26) was the first to study the interaction of tryptophan and niacin deficiencies during the gestation period in producing congenital cataracts in the fetuses. The diet used consisted of acid-hydrolyzed casein, sucrose, cornstarch, brewers yeast, salt, crisco, and cod liver oil

supplemented with l-cysteine and nicotinic acid. L-tryptophan was added at either a 0.025% or 0.2% levels. This diet was fed to Spraque-Dawley rats for 21 days during gestation. At 22 days the progeny were killed and the eyes of the progeny examined histologically for cataracts. Seven of the eleven young selected at random from females fed the lower level of tryptophan had cataracts. All of the lens from the offspring of females receiving the 0.2% tryptophan diet had normal lens. The only source of vitamin E was cod liver oil. Vitamin E content of cod liver oil depends on the source and age of the oil. It is possible Pike may have been working with a dual deficiency of Vitamin E and tryptophan.

Chaves et al. (27) reported the occurrence of unilateral and bilateral cataract in about 30 percent of the progeny of female rats fed throughout gestation and lactation on a 10 percent protein diet in which cowpeas and cashew nuts served as the sole source of protein (low tryptophan diet). When the vegetable protein was replaced by casein no eye abnormalities were observed. Elevation of the protein level of the vegetable protein diet to 21 percent without changing the ratio of cowpeas to cashews (3:1) also prevented cataracts.

Shortly thereafter, Bunce et al. (28) studied the cause of this congenital defect by supplementation of the

cataractogenic diet described above with purified nutrients. Although several amino acids were present in less than recommended levels, only tryptophan and methionine were added to the supplmented diet, since these were the most limiting amino acids. The results showed that DL-tocopherol (40 mg.%) was as effective in the prevention of cataracts as a combined supplement of 0.5g methionine, 0.5g tryptophan and 10 mg% niacin. Cataracts were never observed in the mated dams nor in weanling rats maintained on the standard lab chow diet for 10 weeks. Twelve young rats with cataracts were placed on a standard lab chow diet immediately after weaning. Reexamination of these animals after three weeks revealed no change in appearance of the eyes; after six weeks only three had opacities of the original intensity and the other nine had either clear or cloudy lenses.

The most recent work by Bunce and Hess (4) has shown a more conclusive relationship existing between vitamin E and tryptophan. The relative effect upon lens transparency of a diet low in both tryptophan and vitamin E was compared to diets low in either nutrient alone. The results showed that at weanling stage 33 percent of the 126 progeny had either unilateral or bilateral lens opacities. This occurred when the maternal diet during gestation and lactation provided only 75 mg L-tryptophan and 0.1 mg DL-tocopherol acetate per 100 g diet.

No opacities were found in 179 progeny when tryptophan alone was restricted. Only 6 percent (7 rats of 111 total) showed an incidence of cataracts when vitamin E was the sole limiting nutrient. Seventy-five percent of the weanling rats from normally nourished mothers developed extensive opaque regions within ten weeks when tryptophan was limited to 0.05 g per 100 g diet whether tocopherol was present or not.

G. Effect of Nitrogen Intake on Tryptophan Requirements for the Pregnant Rat

Although tryptophan requirements for growth in young rats has been studied, very little has been reported on the effects of varying levels of nitrogen intake on the tryptophan requirements for pregnancy. Lojkin's (29) reports indicate that for pregnant rats fed a niacin-free diet containing 3 percent nitrogen the minimal tryptophan intake required ranged between .093% and .136%. These levels were required for normal growth and viability of the fetuses. Pike's (26) work indicates that an intake of 0.2% tryptophan was needed by the pregnant rat fed a niacin-free 14.7% casein hydrolysate diet. This was the level required for normal weight gain and protection of the fetus from congential cataract.

In Lojkin's (30) more recent study an attempt was made to study the effect of varying levels of amino acids and nonspecific nitrogenous substances on the tryptophan requirement for pregnancy in the rat. Normally imbalanced diets produce reduced growth and reduced food intake. Lojkin, however, found that the nitrogen imbalances created either by decreasing the percentage of dietary tryptophan or by increasing the percentage of dietary nitrogen did not affect the food intake of pregnant rats fed these diets. But more importantly, Lojkin's work showed that an increase in dietary nitrogen subsequently causes an increase in the requirements for tryptophan in the pregnant rat.

H. Deficiencies of Protein and Amino Acids in Congenital Cataract Formation

Lens damage resulting from amino acid deficiencies, other than tryptophan, have not been as thoroughly investigated.

Sydenstricker *et al.* (31) reported that in rats deficiencies of protein or each of the essential amino acids except arginine resulted in some lens damage. The most marked changes occurred in rats fed diets deficient in histidine or phenylalanine.

Bagchi (32) found that rats fed a low protein diet exhibited a lowering of the glutathione and protein bound

sulfhydryl groups in the lens. He was able to produce cataracts by injecting animals on the diet with methionine sulfoximine. This process was reversed by dosing with methionine or cystine. This indicates that low sulfur amino acid content in the diet was responsible for the lens changes. Methionine sulfoximine is a methionine anti-metabolite.

Few reports can be found on feeding an abundance of protein. Mitchell et al. (33) have shown that an abundance of protein in the diet decreases the incidence and rate of galactose cataract development in rats.

I. P-chlorophenylalanine (PCP) Induced Cataract.

P-chlorophenylalanine (PCP) is an inhibitor of tryptophan hydroxylase (1.13.1.13) and phenylalanine hydroxylase (1.14.3.1) (Figure 3). PCP has been used in conjunction with phenylalanine loading in order to produce an animal model for phenylketonuria (PKU). In addition, PCP has been shown to produce cataracts in young rats, either when given as a long term dietary supplement or when injected subcutaneously over a period of weeks.

Brown et al. (34) administered PCP to newborn rats for twenty days. Weaned pups were then placed on a diet containing PCP and phenylalanine. The progeny developed

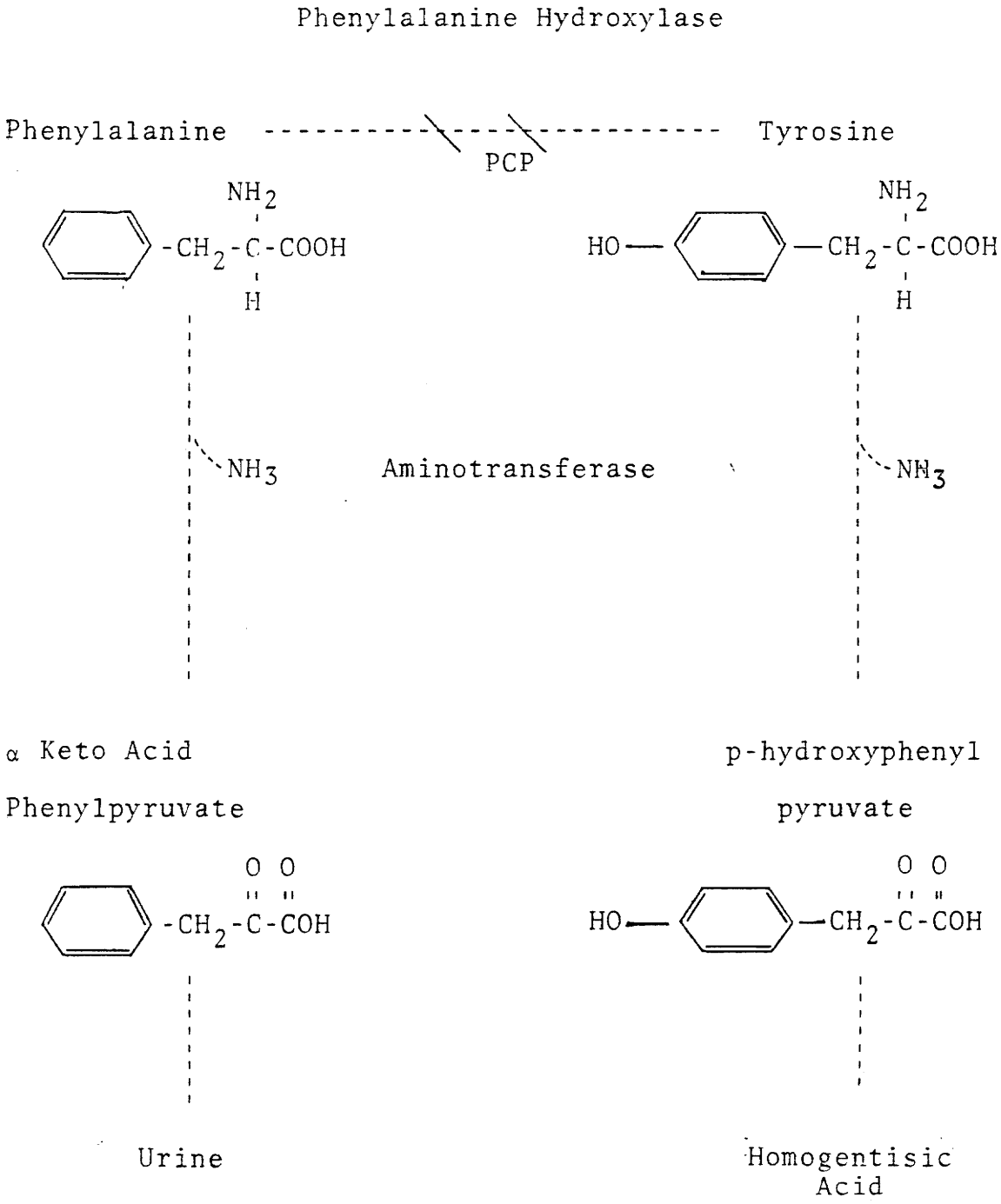


Figure 3. Metabolic Pathway of Phenylalanine

cataracts within fourteen days post weaning. Liver phenylalanine hydroxylase activity was inhibited while serum and tissue phenylalanine concentrations rose. Controls fed a regular diet supplemented with 5% phenylalanine alone showed no sign of cataract. PCP does not interfere with protein synthesis but alters aromatic amino acid transport (35). This mechanism could reduce the availability of L-phenylalanine to the lens where it is important for maintenance of clarity.

J. Role of Vitamin E in Heme Biosynthesis

Nair's laboratory (36, 37) has shown that vitamin E is involved in heme biosynthesis. This may have serious consequences in the female rat and her offspring, deprived of vitamin E. There may be a decreased capacity in the female to carry out catabolic reactions for phenylalanine and tyrosine, since these catabolic pathways also involve heme containing microsomal mixed function oxidases. The combined effect of vitamin E deprivation and increased amino acid levels could lead to reduced capacity of these enzyme systems. It may also cause an elevation in maternal and fetal phenylalanine levels and or their keto acid analogues. The credibility of this hypothesis depends upon the accuracy of Nair's claim. Several labs have attempted to duplicate Nair's work but all attempts have been unsuccessful.

K. Experimental Rationale

In view of the literature cited above the experimental basis for this project was formulated. The diet formulation used by Bunce and Hess (4) were used as a basis for this project. Using this as a starting point several interesting experiments suggested prospects for an experimental design. The first was the use of PCP injections and excess phenylalanine diets fed to females produce cataracts in their progeny. Because PCP blocks the normal metabolic pathway for phenylalanine metabolism an alternative pathway must be taken. The alternative pathway which it follows is the production of its α-keto acid by-products, one of which is phenylpyruvate. The second idea concerns Nair's hypothesis that vitamin E is involved in heme synthesis. Because the mixed function oxidases responsible for phenylalanine metabolism are heme related enzymes it seems entirely possible that a deficiency of vitamin E could restrict the production of mixed function oxidases and thus cause phenylalanie to be shunted into other alternative pathways. This would result in a similar build up of keto acids as in the administration of PCP and could result in cataract formation in the progeny of females fed a diet containing excess phenylalanine and a vitamin E restriction. Therefore it is expected that a phenylalanine imbalance imposed on the diet

formulation described by Bunce and Hess might exacerbate the incidence of cataract which they reported.

To examine this possibility urinary phenylpyruvate would be measured in the pregnant female to determine if keto acid build up is occurring as the result of a vitamin E restriction.

Whether vitamin E is ever involved in cataract formation depends on the view one takes on placental transfer of this fat soluble vitamin. As noted above the views on placental transfer of this vitamin are conflicting. However, since a high incidence of cataract was observed with a simultaneous deficiency of vitamin E and tryptophan and no cataracts were observed when the diets were supplemented with these two nutrients, there is definitely something which is transferred to the fetus. But what is transferred? Is it vitamin E, tryptophan or a metabolite of a tryptophan and vitamin E interaction.

The question involved here is whether the fetus is ever exposed to a tryptophan deficiency. It might be possible that the female is able to breakdown her own body protein stores in order to supply the fetus with adequate tryptophan. If this is the case the fetus would never be exposed to a tryptophan deficiency.

Note that we have been talking about placental transfer of nutrients and not what takes place postpartum. The time

period for cataract formation is therefore another question involved. The cataract may be formed in the fetal stage or postpartum. If the cataract is nuclear then it would probably have formed in the fetal stage. Nuclear cataracts are formed from the very earliest cells in the lens. The period for formation of other types of cataracts would be more difficult to determine.

The other question involved is whether the cataracts, if produced, resulted from a phenylalanine imbalance or a nitrogen imbalance. One of the key parameters to examine here are the by-products of nitrogen metabolism found in the urine of the pregnant females.

The pregnant female and developing fetus serve as an excellent model for this examination. Other data of interest will be food consumption patterns for the females during gestation. The efficiency of these diets to produce adequate weight gains during gestation and sufficient breast milk to support weight gains in their progeny. Lens analysis will be considered to determine if variants between groups, as well as amino acid analysis on the soluble lens homogenates and blood from weanling pups. Phenylalanine hydroxylase activity in the livers of weanling rats will also be considered, and finally, one of the most significant factors, the incidence of cataracts between groups.

All these factors will be considered in order to enhance our knowledge about the relationship which appears to exist between vitamin E and tryptophan in cataract formation.

Chapter III

EXPERIMENTAL PROCEDURES AND METHODS

This project was composed of three experiments. Within each experiment are several subdivisions: animal care and mating procedure; diet and feeding procedure; collection and analysis of urine; collection and analysis of lens; collection and processing of plasma.

Sprague-Dawley rats were used in each of the three experiments. Eight to nine-week old females were ordered from Flow Laboratories in Dublin, Virginia. Their weights varied between 175-200 g. Both males and females were adjusted to their assigned diets for one week. Males were also adjusted to the diet because rats are coprophagous. This measure insured that the feces were limiting in vitamin E and or tryptophan.

The basal diets for experiments I and II and experiment III are shown in Tables 1 and 2 respectively. Varying combinations and levels of vitamin E and tryptophan were added to these diets. Table 3 shows the combinations of diets used in the three experiments. All diets were stored between -11 to -8°C when not in use.

After a one week adjustment period male and female rats were housed in a one-to-one pairing for up to 14 days in

Table 1

Composition of Basal Diet I for Experiments I and II

g/10 kg

L-Amino Acids[a]

Histidine	54
Lysine	155
Leucine	80
Isoleucine	50
Phenylalanine	560
Methionine	30
Cysteine	30
Tryosine	30
Threonine	50
Valine	70
Arginine	75
Glutamic Acid	90
Aspartic Acid	90
Proline	90
Alanine	103
Glycine	90
Serine	90
Tryptophan	7.75
Total	1744.5

Gelatin	600
Glucose	5200
Cornstarch	806
Vitamin Mix[b]	250
(Vitamin E Omitted)	
Alphacel[c]	500
Rh Salt Mix[c]	400
Corn Oil	1000
(tocopherol stripped)	
Vitamin E[c]	.01ml
(α-tocopherol acetate)	
(1g/10 ml ether)	

[a]Obtained from Ajinomoto, New York, N.Y.

[b]Obtained from Icn Pharmacuticals, Inc., Cleveland, Ohio. Provides per loog diet (in g) ascorbic acid, 4.5; (in Iu); retinyl acetate, 1980; cholecalciferol, 220; (in mg); inosetol choline chloride, 165; menadione, 5; p-amino-benzoic acid, 4; niacin, 9.9; riboflavin, 2.3; pyridoxine-HcL, 2.2; thiamine-HcL, 2.2; calcuim-pantothenate, 6.6; (in ug) biotin, 44; folic acid, 198; and B12, 3.

[c]Obtained from Icn Pharmacuticals, Inc., Cleveland, Ohio.

Table 2

Composition of Basal Diet II for Experiement III

g/10 kg

L-Amino Acids[a]

Histidine	108
Lysine	310
Leucine	160
Isoleucine	100
Phenylalanine	120
Methionine	60
Cysteine	60
Tyrosine	60
Threonine	100
Valine	140
Arginine	150
Glutamic Acid	180
Aspartic Acid	180
Proline	180
Alanine	206
Glycine	180
Serine	180
Tryptophan	7.5
Total	2481.5

Glucose	5800
Cornstarch	806
Vitamin Mix[b]	250
(Vitamin E Omitted)	
Alphacel[c]	500
Rh Salt Mix[c]	400
Corn Oil	1000
(tocopherol stripped)	
Vitamin E[c]	0
(α-tocopherol acetate)	

[a]Obtained from Ajinomoto, New York, N.Y.

[b]Obtained from Icn Pharmacuticals, Inc., Cleveland, Ohio. Provides per 1oog diet; (in g) ascorbic acid, 4.5; (in Iu), retinyl acetate, 1980; cholecalciferol, 220; (in mg), inositol choline chloride, 165; menadione, 5; p-aminobenzoic acid, 11; niacin, 9.9; riboflavin, 2.2; pyridoxine-HCL, 2.2; thiamine-HCL, 2.2; calicum pantothinate, 6.6; (in ug) biotin, 44; folic acid, 198; and B-12, 3.

[c]Obtained from Icn Pharmacuticals, Inc.,Cleveland, Ohio.

Table 3

Nutrient Supplements for Experiments I, II, and III

g/10 kg

	Treatments		
Experiment I[1]	A -E -TRP	B +E -TRP	C +E +TRP
tryptophan[3]	---	---	42.50
vitamin E[4]	---	4.0	4.10

Experiment II[1]	A -E -TRP	B +E -TRP	C +E +TRP	D -E +TRP
tryptophan[3]	---	---	42.50	42.50
vitamin E[4]	---	4.0	4.0	---

	24% amino acid mixture				12% amino acid mixture	
Experiment III[2]	A_{24} -E -TRP	B_{24} +E -TRP	C_{24} +E +TRP	D_{24} +E +TRP	A_{12} -E -TRP	C_{12} +E +TRP
tryptophan[3]	---	---	42.50	42.50	---	42.50
vitamin E[4]	---	4.0	4.0	---	---	4.0

[1]Basal Diet I

[2]Basal Diet II

[3]Tryptophan added to basal diet at expense of alanine.

[4]Added in ether solution (1g/10 ml ether).

stainless steel wire mesh cages. Food and water was provided ad libitum. Males and females of the same age were used in experiment I. In experiments II and III some males from the previous studies were used. Papers underneath the cages were changed daily to facilitate the detection of copulation plugs. The plugs are easily distinguishable from food and fecal material due to their hardness and light yellowish appearance. The mating period ended when copulation plugs were observed underneath the cages. The data was recorded as the first day of gestation. Females were then separated from the males, weighed and transferred to opaque polypropylene cages approximately 48 x 28 x 15 cm. Each cage was provided with shredded softwood bedding, food, water and enclosed in a bonnet type cage filter. The bonnet filtered out dust and provided the females with a less distracting environment.

Animal rooms were maintained on an automatic 12 hour light-dark cycle. Room temperature varied between 25-28°C in experiments I and II. Experiment III was performed in an air conditioned room where the temperature was maintained between 21-25°C.

At the beginning of the 7th day of gestation the females were weighed and placed in metabolic cages. Forty-eight hour urine collections were made. One ml of 2N HCL

2N H_2SO_4 was placed into the receiving flasks to prevent bacterial growth. In order to minimize urine contamination the metabolic cages were washed daily and the urine was filtered before analysis. If analyses could not be performed on the collection day it was frozen for later analysis.

Urine in experiments I and II were analyzed for phenylpyruvate (α-keto acid of phenylalanine) and creatinine.

The method for phenylpyruvate analysis was described by Coburn et al. (38). One ml of filtered urine was diluted with distilled water (1:4). Following addition of 4g NaCl and acidification with concentrated HCl, phenylpyruvate was extracted twice with 5 ml ethyl acetate in a screwtop centrifuge tube. Samples were mixed by a mechanical shaker for fifteen minutes. The ethyl acetate layers were transferred to 3 ml of phosphate buffer (.2M, ph. 8.1). These were mechanically shaken for five minutes centrifuged for five minutes at 1200 x G. One ml of the phosphate phase was pipetted into each of two test tubes containing 2 ml distilled water and 2 ml of borate buffer (1.0M, ph. 8.1) respectively. The samples were mixed well. After one hour the absorbance for each pair of test tubes was read at 300nm on a Beckman DB Spectophotometer. Simultaneously a blank and a standard were processed.

Urine creatinine was also measured by the picrate method (39). Fifty ul of urine and 10 ml water were placed in a 50 ml volumetric flask. Ten ml of alkaline picrate was added. The solution was diluted to 50 ml mixed well and after 15 minutes the absorbance was measured at 495 nm on an Eppendorf Spectophotometer. A distilled water blank was used to determine zero concentration. Fresh alkaline picrate was made for each analysis since the solution tends to darken with age. A standard curve was prepared using commercial creatinine. Concentration of keto acid was expressed as mg of keto acid per 48 hours per mg creatinine per 48 hours.

In experiment III urine was analyzed for total nitrogen, urea, and creatinine. Urine collections were made as described previously. Samples were frozen for later analysis. The samples were run on a Technicon Autoanalyzer II. Total nitrogen and creatinine were measured on the same analyzer. A separate Technicon Autoanalyzer II BUN (blood urea nitrogen) was set up for urea analysis.

At the end of the fourteenth day of gestation the females were removed from the metabolic cages, weighed and placed in their respective plastic cages. Bedding in these cages changed each week when the females were weighed. In experiments I and II the females were weighed before and

after giving birth. These weights were not taken in experiment III in hopes that pup survival would increase. Pups were weighed and counted at birth in experiments I and II. For the same reason as given above these weights were not taken in experiment III. Dead pups were removed from the cage. Weight records of the pups were kept on a weekly basis. The first week after birth is the most critical. If the female cares for her progeny during this period chances for survival are usually increased.

In each experiment the females were removed from their progeny between 21 or 30 days after birth. After a 12 hour fast the pups were sacrificed in experiments I and II. In experiment III the females were returned, after a 12 hour fast, to their progeny for 2 hours then removed. This was done to ensure that the pups had consumed only breast milk and not the solid diet. Five to six hours were allowed to elapse before the pups were killed. Reports by McLaughlin and Illaman (40) and Stockland et al. (41) state that the most significant relationship of plasma amino acid levels to the adequancy of intake were obtained during this span of time.

Rats were sacrificed by decapitation. Blood draining from the neck was collected into heparinized tubes and placed on ice. The blood samples were centrifuged at 14,000

x G. Plasma was removed and frozen for later amino acid analysis.

The eyes were removed and the lens extracted. The lens was examined before and after extraction for the presence of cataracts. The lenses were combined within groups in a tared 5 ml beaker and placed on ice. Reweighing the beaker as soon as possible was critical in order to determine an accurate wet weight on the lenses. The lenses were then homogenized in a 4 ml Potter Elvehjem homogenizer. The homogenizer was rinsed 2 or 3 times with distilled water. The total volume was kept between 2-3 ml. Samples were centrigued at 24,000 x G for twenty minutes and the soluble protein removed, leaving a pellet of insoluble protein. The insoluble protein was suspended in 1 to 2 mls of distilled water and the volume recorded.

The separation of the water soluble lens proteins into four fractions (alpha-, beta(heavy)-, beta (light)-. and gamma crystallins) was done using a Sephadex G-200 column. Initially a column 7.5 mm x 90 cm was used but due to poor resolution it was exchanged for a larger column 15 mm x 90 cm. The gel was swollen for five hours at 100°C in the buffer. It was poured into the column and two void volumes of buffer were pumped through before the samples were applied. The sample was eluted with a buffer containing

0.65M Tris-hydrochloric acid (ph. 7.6), 0.05M sodium chloride, and 0.001 M methylenediamine tetraacetic acid (EDTA). Flow rate of the column ranged from .1-.15ml/ minute. Fractions were collected very 15 minutes. The absorbance was measured at 280nm on a Beckman DB Spectophotometer. Tris buffer was used as a blank. Each group of lenses was run in duplicate, when there was enough sample. When the column was not in use, 0.02% sodium azide buffer was pumped through the column in order to prevent any bacterial growth. Total protein was determined on the soluble lens homogenate using the Lowry method (42).

Amino acid analysis was performed on both plasma and soluble lens homogenates. Plasma amino acid analysis was performed in experiments I and II and the amino acid composition of soluble lens homogenates was performed in experiment III. Both lens homogenate and plasma were prepared for amino acid analysis by similar methods. One ml of homogenate or plasma was pipetted into a test tube and 0.25 ml of 20% sulfosalicylic acid solution added. The mixture was shaken vigorously and cooled in an ice bath for 30 minutes. At the end of this period the sample was centrifuged at 14,000 x G for 15 minutes. The supernatant was filtered through a funnel with a tight plug of glass wool in the stem. Samples were charged with N_2 and frozen for later

analysis. The samples were run on a Beckman Model 120B Amino Acid Analyzer with an Autolab System AA integrator.

Livers from the progeny were removed and analyzed for phenylalanine hydroxylase enzyme. This analysis was conducted by M. Pallansch (43) using the method of Kaufman (44) as modified by Bublitz (45) and Gillam et al. (46).

Statistical Analysis

Comparisons between means were analyzed using the Statistical Analysis System (SAS) (47), and an IBM/360 computer. When significant differences were found, Duncan's Multiple Range Test was used to determine the location of those differences.

Chapter IV

RESULTS

Experiment I

A. Physical Appearance of Females During Gestation and Lactation

No noticeable differences were observed in the physical appearance of the females in each experimental group during gestation or lactation. One of the most commonly occurring observations, however, was an eye condition, diagnosed as conjunctivitis which affected some of the animals. The eye condition generally occurred several days before parturition. A medicated salve was applied to the infected area in an attempt to eliminate this problem. But, in most cases this treatment was not successful. Later analysis revealed that the bleeding-like secretions at the nose and eye lids probably resulted from dehydration. As a result of the pigmentation food consumption patterns decreased with subsequent decreases in body weights. In a few cases this resulted in a severe debilitation of the female. When this occurred the female usually died prior to or during parturition. Those that survived usually gave birth to dead pups or refused to care for them. In all

cases that survived the pigmentation disappeared or was noticeably improved within a week after parturition.

Loss of hair was also observed in some dams while nursing. This occurred most often in the double deficient group (diet A) or where the litter sizes were larger than normal (average size was 8 pups).

B. Food Consumption Records for Females During Gestation

Three diets (Table 3) were used in this experiment; diet A, limited in both vitamin E and tryptophan; diet B, supplemented with vitamin E but limiting in tryptophan; and diet C, with supplements of vitamin E and tryptophan. All three diets contained 5% phenylalanine.

The mean total food consumption (±SD) during gestation were not significantly different ($P > 0,05$) between groups (Table 4). Similarly the feed efficiency ratios between groups were not significantly different ($P > 0.05$).

C. Body Weights and Weight Gain Records for Females Through Gestation

Mean body weights (±SD) for females during the gestation period (Table 5) continued to increase for each group during this period. Following parturition, mean body weights returned to levels slightly below, for groups A and B, and

Table 4

Experiment I

Mean Food Consumption Records for Females Through Gestation

(Expressed in g of diet consumed/24 hours)

	Experimental Treatments[1]		
Day of Gestation	A -E -TRP	B +E -TRP	C +E +TRP
1-7	19±5	18±4	17±4
7-14	19±5	19±3	17±4
14-21	21±8	19±8	17±8
Mean Total Food[1,2] Consumption during Gestation	410±91[a]	381±36[a]	347±52[a]
Feed Efficiency Ratio[1,2]	.10±05[a]	.14±.04[a]	.19±.05[a]

[1]Means ± SD

[2]Means with different letters are signficiantly different, $P < 0.05$.

Table 5

Experiment I

Mean Body Weight Records For Females
(in g)

	Experimental Treatments[1]		
	A^{-E}_{-TRP}	B^{+E}_{-TRP}	C^{+E}_{+TRP}
Day of Gestation			
1	212±11	220±17	201±14
7	229±9	240±17	229±16
14	244±16	260±16	251±16
21	260±16	275±18	269±16
Days Postpartum			
1	201±18	199±16	220±56

[1]Mean ± SD

above, for group C, those weights recorded on the first day of gestation. The mean weight gains (±SD) for females during gestation (Table 6) declined in all groups during the 14-21st days of gestation.

The mean total weight gains during gestation were significantly different ($P < 0.05$). Mean total weight gains for group C were significantly larger than either groups A or B. Mean total weight gains for groups A and B, however, were not significantly different ($P > 0.05$).

D. Reproductive Data

Fertility, survival at parturition and postnatal viability records are shown in Table 7. For all groups the fertilization was very successful. The number of females that died at parturition was greatest for group B (42%). Group A showed a slightly lower survival percentage (33%) and group C had the lowest percentage of pup survival (25%).

E. Weight Records for Pups

The mean body weights (±SD) for pups within each group from the 1st through the 14th day postpartum (Table 7) were quite similar. Analysis of the mean weight gains between groups during this period did not indicate any significant differences ($P > 0.05$).

Table 6

Experiment I

Mean Weight Gains For Females During Gestation (in g)

	Experimental Treatments[1]		
	A^{-E}_{-TRP}	B^{+E}_{-TRP}	C^{+E}_{+TRP}
Periods during Gestation (days)			
1-7	15±6	21±4	27±5
7-14	17±8	21±8	22±5
14-21	16±5	12±8	20±9
Mean Total Body Weight Gain During[1,2] Gestation	44±14[a]	55±14[a]	69±7[b]

[1]Mean ± SD

[2]Means with different letters are significantly different, $P < 0,05$.

Table 7

Experiment I

Reproduction Data

Fertility, Survival at Parturition and Postnatal Viability

Diet	Females Preg/ Females Mated		Females dying at parturition females mated		Pups surviving at weaning/ pups delivered	
	(no.)	(%)	(no.)	(%)	(no.)	(%)
A^{-E}_{-TRP}	11/12	92	2/11	18	23/69	33
B^{+E}_{-TRP}	10/10	100	1/10	10	47/112	42
C^{+E}_{+TRP}	10/10	100	3/10	30	22/87	25

Table 8

Experiment I

Mean Body Weight Records For Pups
(in g)

	Experimental Treatments[1]		
	A -E -TRP	B +E -TRP	C +E -TRP
Days Postpartum			
1	6±1	5±1	5±1
7	11±1	11±2	11±1
14	22±5	20±3	21±2
Mean Total Weight Gain[1,2]	16±6[a]	13±2[a]	16±7[a]

[1]Mean ± SD

[2]Means with different letters are significantly different, $P < 0.05$.

F. Incidence of Cataract in Weanling Rats

Table 9 shows the number of pups with cataracts and the number of litters within each group with cataracts.

No cataracts were observed in pups from Group C. However the incidence of cataract for groups A and B was 65% and 74% respectively (Table 9).

In group A eleven pups died or were killed postpartum. Four pups were killed because they were too weak to survive. The lids were removed and the lenses examined for cataract. The examination revealed 3 pups with bilateral or unilateral cataract. Seven other pups from a different litter in group A died during the night and were discovered the next morning. A postmorteum examination revealed bilateral cataracts in each of the fourteen lenses. Postmorteum changes may have been responsible for these observations.

Exposing the lens prior to the period for normal lid opening can cause a cataract called a spontaneous cataract. Including these figures in group A increases the incidence of cataract to the same level as that recorded for group B.

G. G-200 Separation Patterns on Soluble Lens Homogenates from Weanling Rats

The protein lens pattern for group A (Figure 5) was obtained using a G-200 column and fresh soluble lens

Table 9

Experiment I

Incidence of Cataract at Weaning

Diet	No. of Cataracts/ No. of Pups		No. of Litters with Cataracts	
	(no.)	(%)	(no.)	(%)
A^{-E}_{-TRP}	15/23	65	3/3	100
	**25/34	** 74	**5/5	**100
B^{+E}_{-TRP}	35/47	74	5/5	100
C^{+E}_{+TRP}	0/22	0	0/3	0

**Including postmortem pups.

homogenate, a rerun of this group (Figure 6) showed a different protein lens pattern. A possible explanation for this is that the soluble lens protein was frozen and thawed a number of times between runs. Freezing and unthawing appears to affect the soluble protein cyrstallins.

Patterns for group B (Figure 7) and group C (Figure 8) were run with fresh soluble lens homogenate. Comparisons between patterns for each group did not reveal any consistent relationships which would identify one group (cataractous) of lenses from another group (noncataractous). All patterns show four peaks of varying heights and shapes. For each pattern the gamma peak is consistantly larger than any of the other peaks with the exception of group A in which the alpha peak is larger in height and more distinctly separated than either of the Beta fractions.

H. Plasma Amino Acid Analysis

Plasma amino acid patterns from weanling rats (Table 10) were obtained after a 12 hour fasting period. The amino acid patterns showed a great deal of variability between and within each group.

Figure 4

G-200 Separation Pattern of the Soluble Lens Homogenate from Weanling Rates in Group A^{-E}_{-TRP}

The curve is a separation pattern of soluble lens homogenate from weanling rats in diet group A. The flow rate was 6-9 ml/hour and each fraction contained 1 ml. A total of 5.1 mg protein was applied to the colunn. All lenses (16) were cataractous.

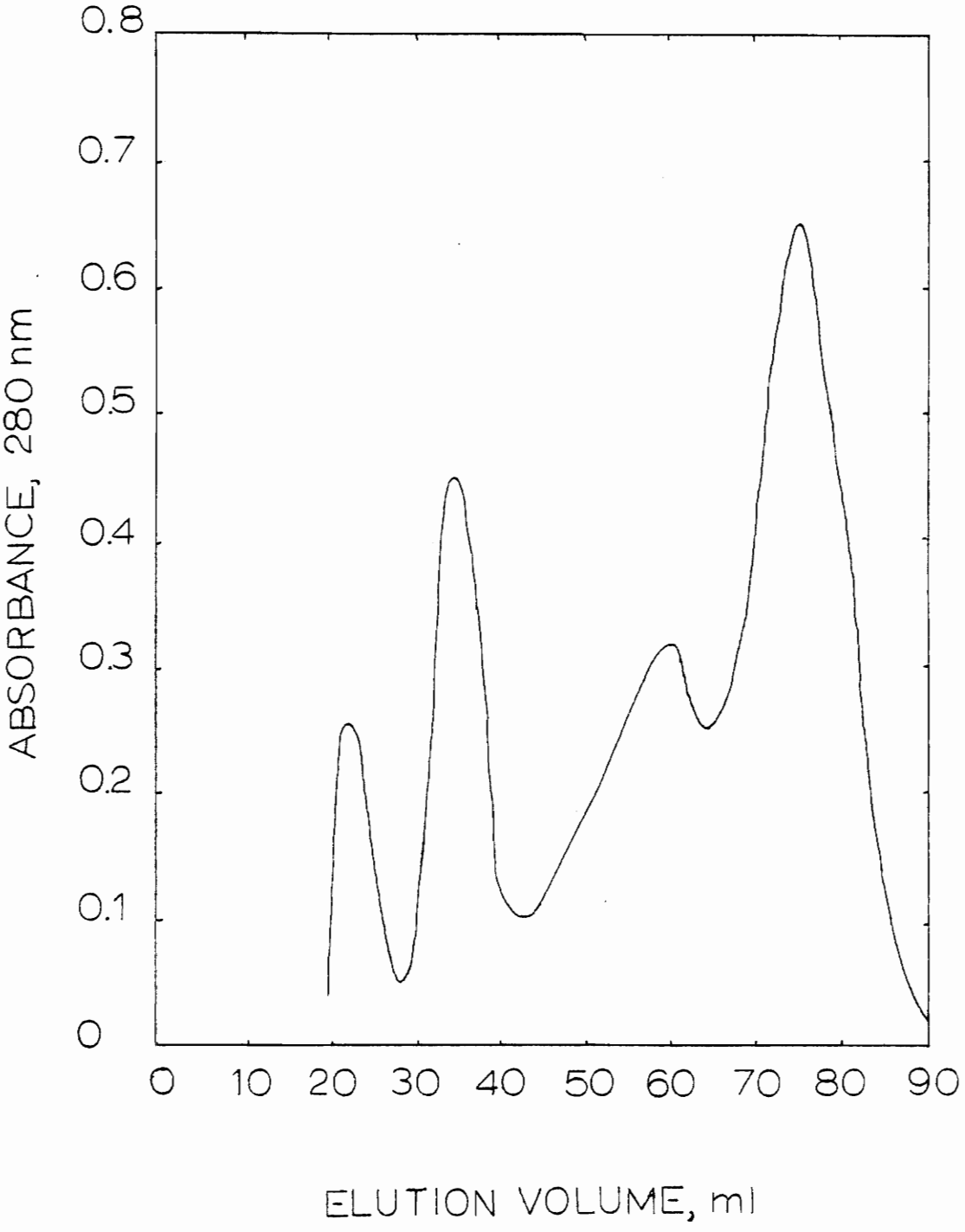

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
ABSORBANCE, 280 nm
0
10
20
30
40
50
60
70
80
90
ELUTION VOLUME, ml

Figure 5

Rerun of G-200 Separation Pattern of the Soluble Lens Homogenate from Weanling Rats in Group A^{-E}_{-TRP}

The curve is a rerun of the soluble lens homogenate from weanling rats in diet group A after the homogenate had been frozen and unthawed. The flow rate was 6-9 ml/hour and each fraction contained 1 ml. A total of 5.1 mg protein was applied to the column. All lenses (16) were cataractous.

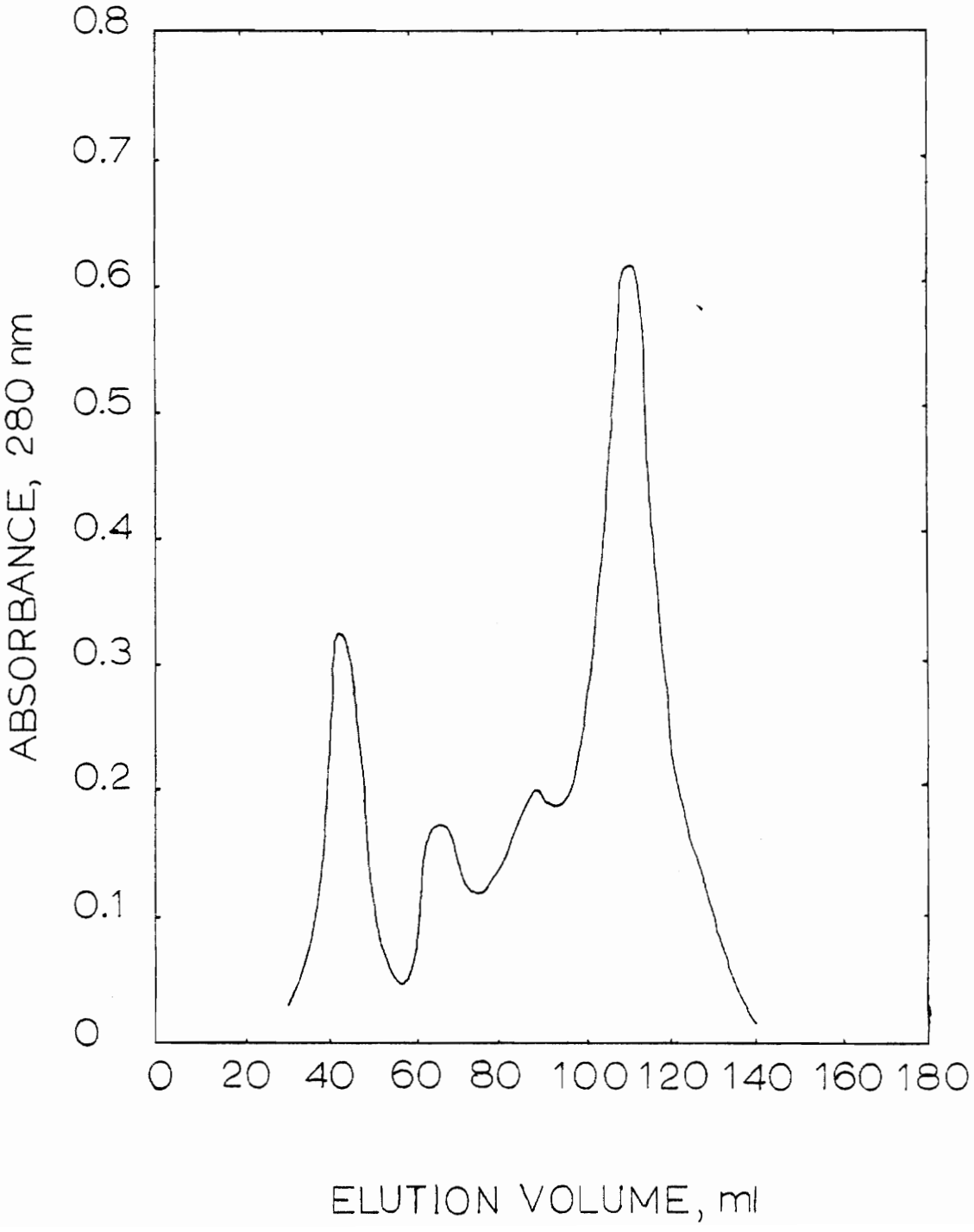

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
ABSORBANCE, 280 nm
0 20 40 60 80 100 120 140 160 180
ELUTION VOLUME, ml

Figure 6

G-200 Separation Pattern of the Soluble Lens Homogenate from Weanling Rats in Group B^{+E}_{-TRP}

The curve is a separation pattern of the lens homogenate of weanling rats in group B. The flow rate was 6-9 ml/hour and each fraction contained 1 ml. A total of 9.9 mg protein was applied to the column. All lenses (20) were cataractous.

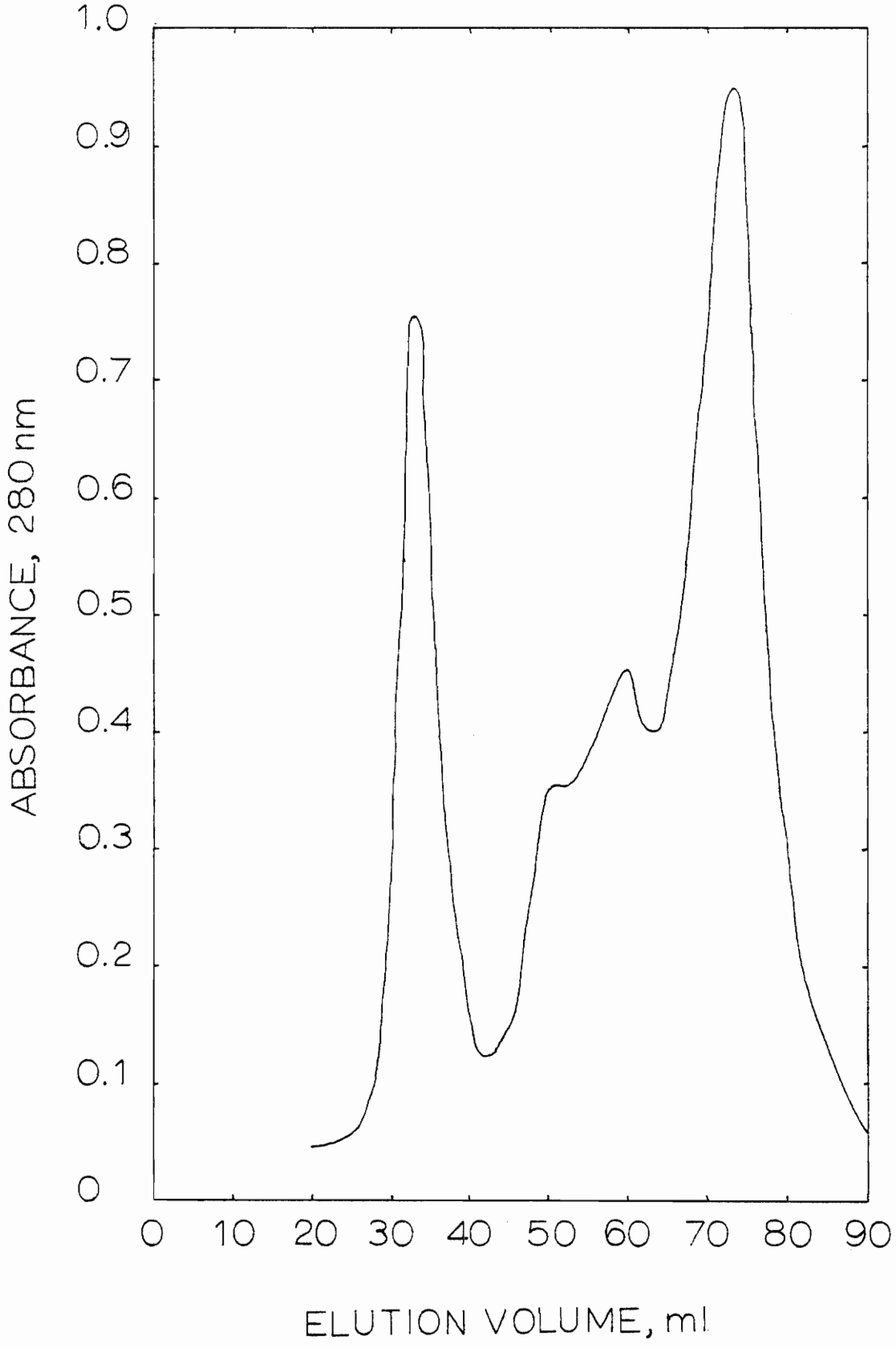

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
ABSORBANCE, 280 nm
0
10
20
30
40
50
60
70
80
90
ELUTION VOLUME, ml

Figure 7

G-200 Separation Pattern of the Soluble Lens Homogenate from Weanling Rats in Group C^{+E}_{+TRP}

The curve is a separation pattern of soluble lens homogenate from weanling rats in diet group C. The flow rate was 6-9 ml/hour and each fraction contained 1 ml. A total of 15.1 mg protein was applied to the column. No cataracts in lenses (24) from group C.

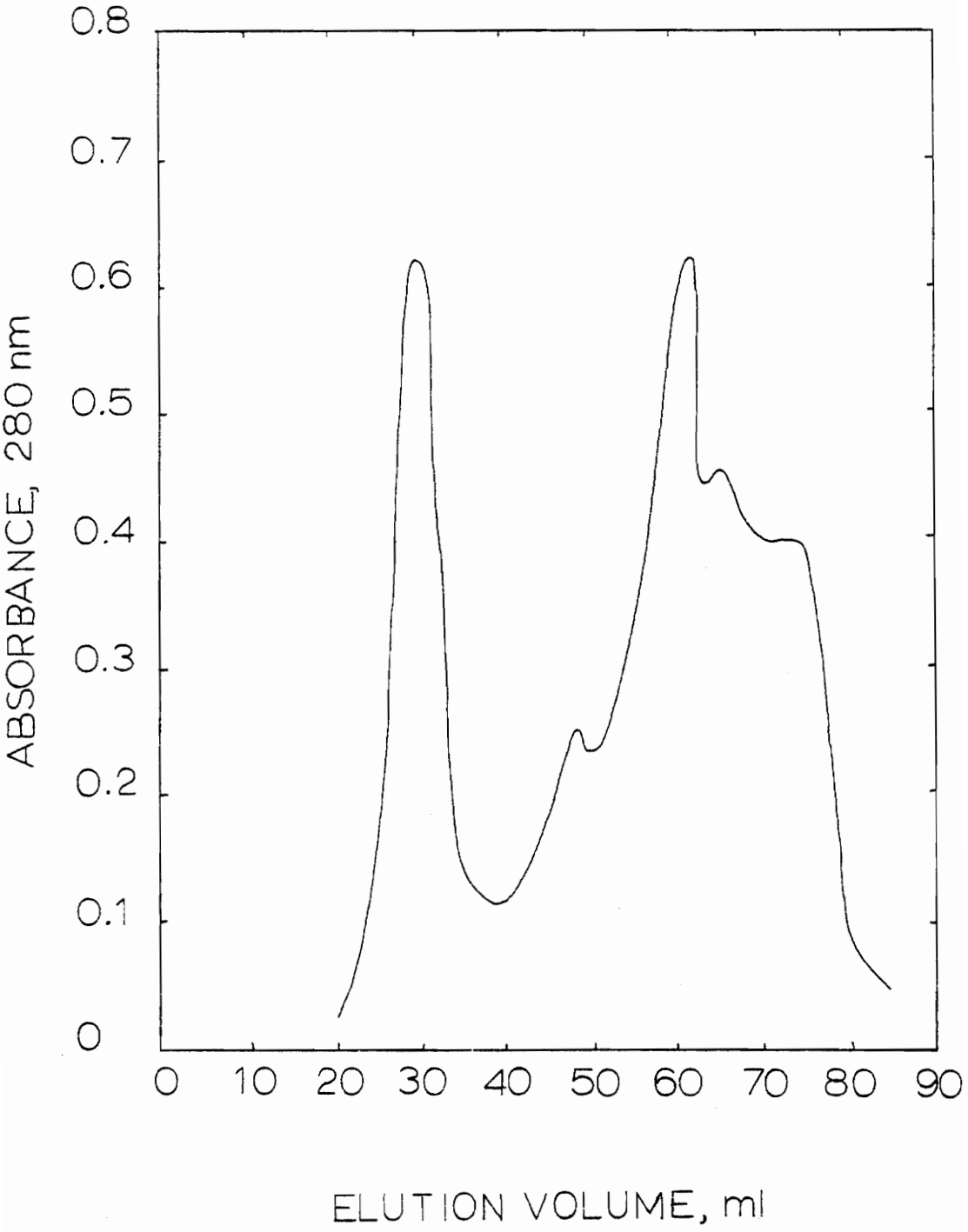

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
ABSORBANCE, 280 nm
0
10
20
30
40
50
60
70
80
90
ELUTION VOLUME, ml

Table 10

Experiment I

Amino Acid Patterns on Plasma From Fasting Weanling Rats
(concentration nM/ml)

Amino Acid	Experimental Treatments				
	A-E 1-TRP	A-E 2-TRP	B+E -TRP	C +E 1+TRP	C +E 2+TRP
Tryptophan	25	17	----	----	----
Lyscine					
Histidine	131	135	102	27	57
Arginine					
Aspartic Acid	54	104	46	50	49
Threonine					
Serine					
Glutamic Acid	184	184	200	183	202
Proline					
Glycine					
Alanine					
Cysteine	16	21	33	----	----
Valine	136	121	116	68	99
Methionine					
Isoleucine	64	56	70	61	64
Leucine	96	70	88	48	71
Tyrosine	95	77	43	73	42
Phenylalanine	112	82	52	39	47

Blank Space = Concentration greater than standard curve values.

---- = Undectable.

I. Keto Acid (phenylpyruvate) Analysis of Urine

The man urinary phenylpyruvate concentrations (±SD) were obtained from females during the 7-14 days of gestation (Table 11). Concentrations of phenylpyruvate in groups B and C showed an apparent increase between the 7-12th days of gestation but showed a slight decrease during the 13-14th days of gestation. Group A, however, does not show an increase during the 9-12th days but shows an increase in concentrations excreted during the 13-14th days of gestation. The mean phenylpyruvate excretion for the 7-14th days of gestation were not significantly different ($P > 0.05$) between groups.

Experiment II

A. Introduction

Based on the results obtained from experiment I this experiment was designed to test the reproducibility of the results of experiment I and in addition test a new diet. This new diet (D) was the direct opposite of diet B in that it contained limited amounts of vitamin E and was supplemented with tryptophan.

Table 11

Experiment I

Mean Urinary Keto Acid (Phenylpyruvate) Excretion of Pregnant Female Rats[1]

(µg Keto Acid/µg Creatinine/48 Hours)

	Experimental treatments[1]		
	A -E -TRP	B +E -TRP	C +E +TRP
Days of Gestation			
7-8	8±2	7±3	7±2
9-10	14±12	15±7	16±6
11-12	14±2	18±3	19±9
13-14	16±3	17±6	16 9
Mean[2] Phenylpyruvate Excretion	13±3[a]	14±16[a]	15±16[a]

[1]Mean ± SD

[2]Means with different letters are significantly different, $P < 0.05$.

B. Physical Appearance of Females During Gestation and Lactation

Similar physical observations were noted in this experiment as in experiment I. The bleeding-like secretions and hair losses were still observed just prior to parturition and in lactation respectively.

C. Food Consumption Records for Females During Gestation

The mean food consumption records (±SD) from the first day of gestation through parturition (Table 12). In general indicate that food consumption patterns between groups were quite similar. During the period of 14-21 days of gestation food consumption patterns decreased for groups A, C and D while group B showed an increase. The mean total food consumption (±SD) patterns during gestation were not significantly different ($P > 0.05$) between groups. The mean food efficiency ratios (±SD) were not significantly different ($P > 0.05$) for each group.

D. Body Weights and Weight Gain Records for Females During Gestation

Mean body weights (±SD) in each group (Table 13) increased steadily throughout gestation. Following parturition

Table 12

Experiment II

Mean Food Consumption Records For Females Through Gestation
(expressed in g of diet consumed/24 hours)

	Experimental Treatments[1]			
	A-E -TRP	B+E -TRP	C+E +TRP	D-E +TRP
Days of Gestation				
1-7	22±5	21±3	19±6	20±4
7-14	20±4	21±4	21±5	20±5
14-21	19±8	27±4	19±8	15±8
Mean total food consumption during gestation[1,2]	408±48[a]	452±30[a]	416±88[a]	387±74[a]
Feed Efficiency Ratio[1,2]	.18±.04[a]	.20±.02[a]	.16±.0[a]	.16±.02[a]

[1]Mean ± SD

[2]Means with different letters are significantly different, $P < 0.05$.

Table 13

Experiment II

Mean Body Weight Records For Females
(in g)

	Experimental Treatments[1]			
	A^{-E}_{-TRP}	B^{+E}_{-TRP}	C^{+E}_{+TRP}	D^{-E}_{+TRP}
Day of Gestation				
1	230±7	238±17	220±20	225±18
7	255±10	256±18	244±20	248±21
14	274±5	279±19	277±28	276±22
21	304±21	332±26	288±40	290±36
Days Postpartum				
1	233±18	251±32	213±33	222±25

[1]Mean ± SD

mean body weights returned to levels slightly above, for groups A and B, and slightly below, for groups C and D, those weights recorded on the first day of gestation.

Mean weight gains (±SD) increased up to the 14th day of gestation for all groups with the exception of group A which showed a decline. During the period of the 14-21st day of gestation mean weight gains increased for groups A and B but declined in groups C and D from previous gains (Table 14).

The mean total weight gain (±SD) between groups during gestation were significantly different ($P > 0.05$). Mean total weight gains for groups B were significantly larger than either groups A, C or D. Mean weight gains for groups A, C, and D were not significantly different ($P > 0.05$).

E. Reproductive Data

Fertility, survival at parturition, and postnatal viability records for experiment II are shown in Table 15. Fertility was more successful for groups A and B as compared to groups C and D.

The number of females that died at parturition was greatest for group D (42%) and lowest for group B (0%). Group C however showed a higher percentage (27%) of dead females than group A (10%).

Table 14

Experiment II

Mean Weight Gains For Females During Gestation (in g)

	Experimental Treatments[1]			
	A $^{-E}_{-TRP}$	B $^{+E}_{-TRP}$	C $^{+E}_{+TRP}$	D $^{-E}_{+TRP}$
Periods during Gestation (Days)				
1-7	25±8	18±5	24±8	23±4
7-14	19±9	22±8	33±3	28±5
14-21	30±20	51±9	11±12	14±12
Total body weight gain during gestation	74±24[a]	93±12[a]	68±26[a]	65±23[a]

[1]Mean ± SD

[2]Means with different letters are significantly different, $P < 0.05$.

Table 15

Experiment II

Reproduction Data

Fertility, Survival at Parturition and Postnatal Viability

Diet	Females preg/ females mated		Females dying at partiurition/ females mated		Pups surviving at weaning/ pups delivered	
	(no.)	(%)	(no.)	(%)	(no.)	(%)
A$^{-E}_{-TRP}$	10/10	100	1/10	10	17/90	19
B$^{+E}_{-TRP}$	11/11	100	0/11	0	61/129	47
C$^{+E}_{+TRP}$	11/14	78	3/11	27	10/127	7
D$^{-E}_{+TRP}$	12/14	86	5/12	42	2/106	2

The percentage of pups surviving as compared to the total number delivered was greatest for group B (47%), and lowest for group D (2%). Groups A and C had 10% and 27% survival rates respectively.

F. Weight Records for Pups

Mean weight gains for pups were lowest for group D during the first week postpartum. During the second week postpartum group B showed lower weight gains than the other groups. Mean weight gains during the third week were greater for all groups over the previous weeks gains (Table 16).

The mean total weight gain between groups were not significantly different ($P > 0.05$) for groups A and B or groups A and C. Group D had significantly larger weight gains than the other groups, however, the number of pups in this group was limited ($n = 2$).

G. Incidence of Cataract in Weanling Rats

No cataracts were observed in the lenses of pups from group C. Each of the other groups showed a high incidence of cataract, with Group A showing the larger incidence (Table 17). Each of the litters in groups A and D showed

Table 16

Experiment II

Mean Body Weight Records for Pups
(in g)

	Experimental Treatments[1]			
	A^{-E}_{-TRP}	B^{+E}_{-TRP}	C^{+E}_{+TRP}	D^{-E}_{+TRP}
Days Postpartum				
1	5±1	6±1	6±1	4±1
7	11±3	10±1	12±1	4±0
14	18±6	15±2	21±1	25±0
21	31±8	23±3	44±3	83±5
Mean total weight gain[1,2]	26±8[a,b]	17±2[b]	38±3[a]	79±1[c]

[1]Mean ± SD

[2]Means with different letters are significantly different, $P < 0.05$.

Table 17

Experiment II

Incidence of Cataract at Weaning

Diet	No. of Cataracts/ No. of Pups		No. of Litters with Cataracts	
	(no.)	(%)	(no.)	(%)
A^{-E}_{-TRP}	10/17	59	2/2	100
B^{+E}_{-TRP}	29/61	48	6/7	85
C^{+E}_{+TRP}	0/10	0	0/2	0
D^{-E}_{+TRP}	1/2	50	1/1	100

cataracts. However, one whole litter in group B did not show any signs of cataracts.

H. G-200 Separation Patterns on Soluble Lens Homogenates from Weanling Rats

Protein lens patterns obtained using a G-200 Sephadex column and soluble lens homogenate are shown for groups A (Figure 9), group B (Figure 10), group C (Figure 11), and group D (Figure 12).

Comparisons of these patterns reveals that the gamma (γ) peak is consistently larger than the other peaks, with the exception of group B where the alpha (α) and gamma (γ) peaks are the same height. The alpha (α) fraction in each group has a consistently larger peak height than either of the beta (β) fractions. However, in groups A and C the beta (heavy) fraction is quite distinct and the lens patterns are similar.

I. Plasma Amino Acid Analysis

Plasma amino acid patterns from weanling rats were obtained after a 12 hour fasting period (Table 18). Comparisons of these patterns showed the same variability as seen in experiment I.

Figure 8

G-200 Separation Pattern of the Soluble Lens Homogenate from Weanling Rats in Group A^{-E}_{-TRP}

The curve is a separation pattern of soluble lens homogenate from weanling rats in diet group A. The flow rate was 6-9 ml/hour and and each fraction contained 2 ml. A total of 9.3 mg protein was applied to the column. All lenses (16) were cataractous.

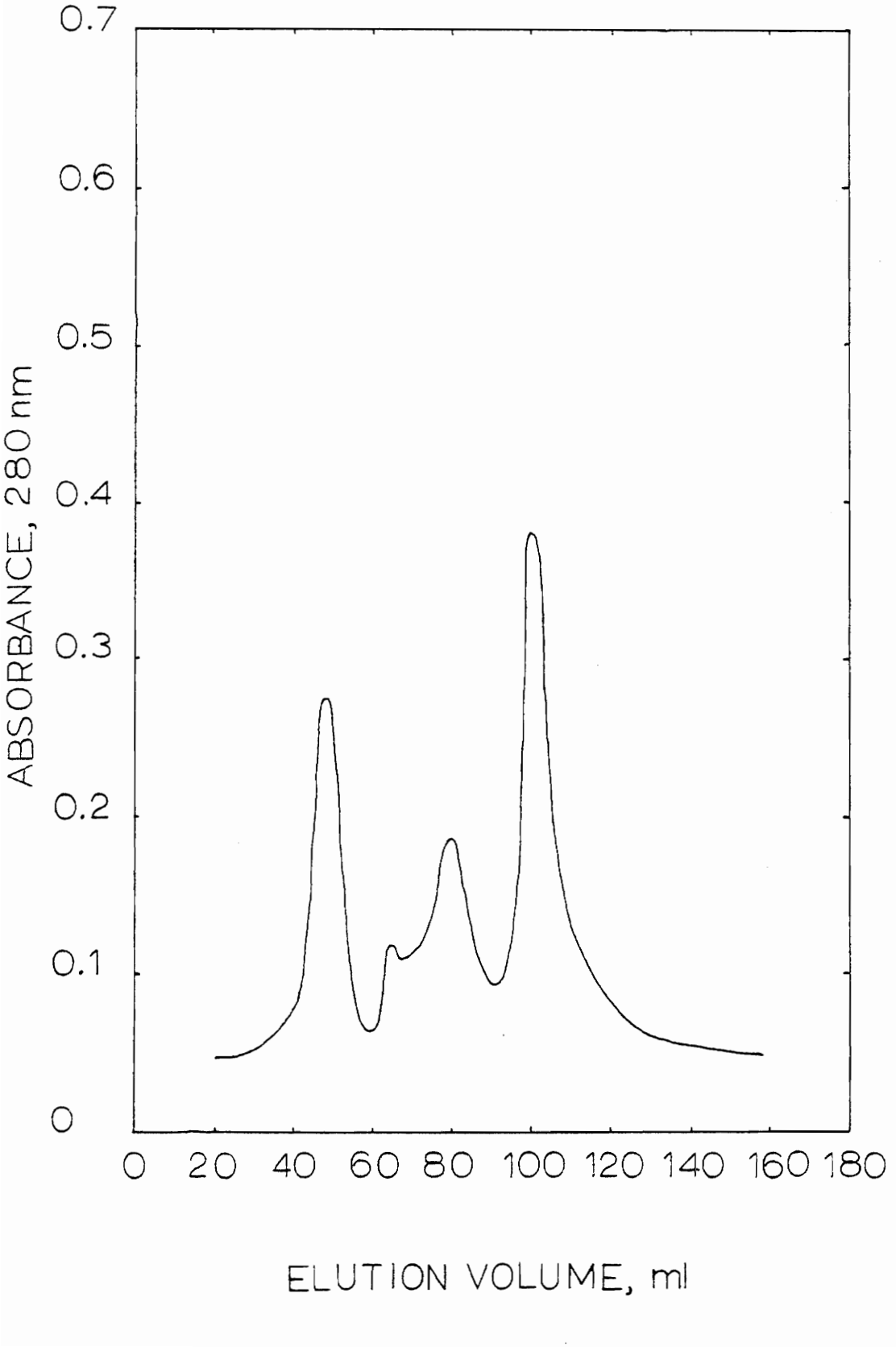

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
ABSORBANCE, 280 nm
0 20 40 60 80 100 120 140 160 180
ELUTION VOLUME, ml

Figure 9

G-200 Separation Pattern of the Soluble Lens Homogenate From Weanling Rats in Group B^{+E}_{-TRP}

The curve is a separtion pattern of soluble lens homogenate from weanling rats in diet group B. The flow rate was 6-9 ml/hour and each fraction contained 2 ml. A total of 8.4 mg protein was applied to the column. All lenses (17) were cataractous.

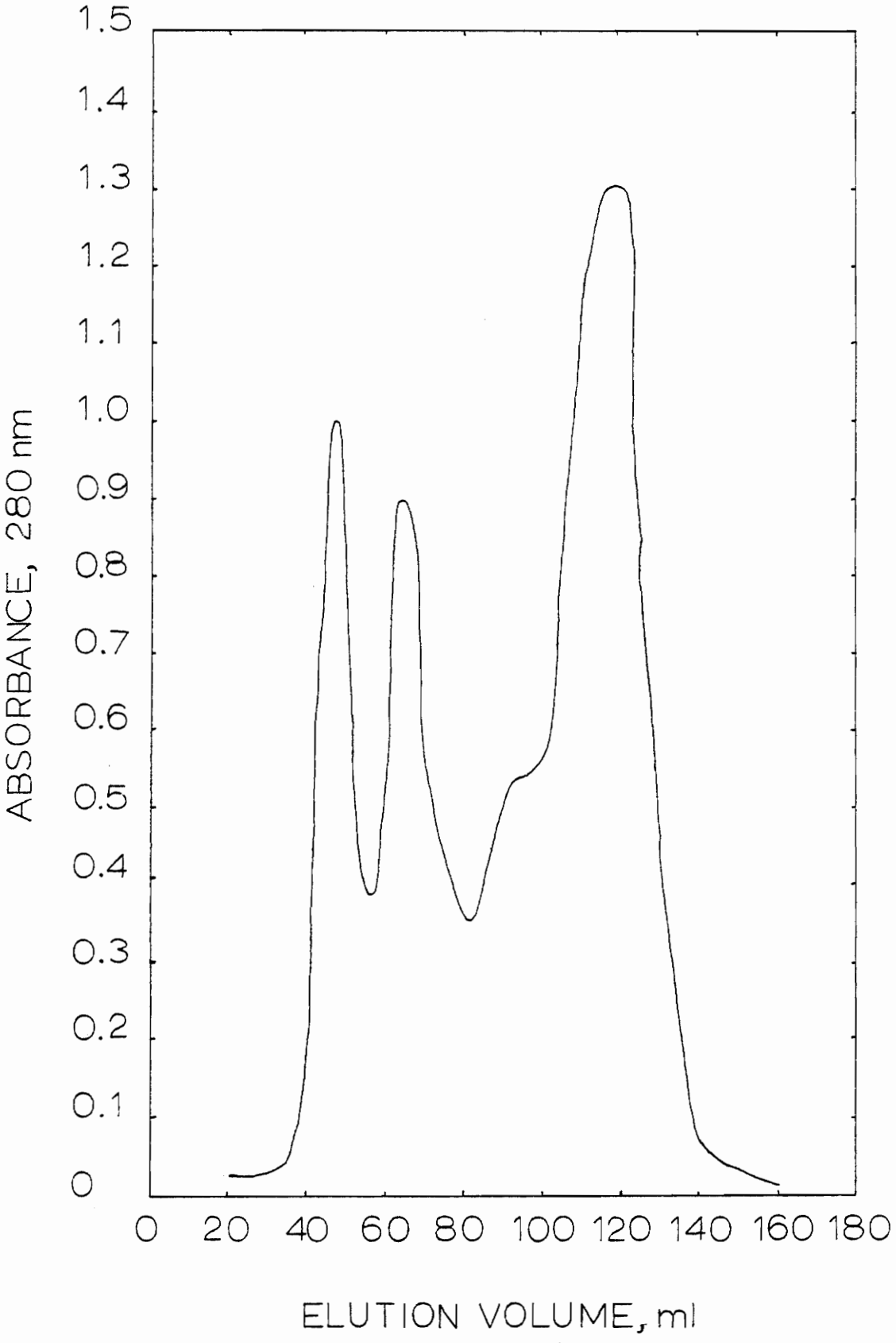

1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
ABSORBANCE, 280 nm
0
20
40
60
80
100
120
140
160
180
ELUTION VOLUME, ml

Figure 10.

G-200 Separation Pattern of the Soluble Lens Homogenate from Weanling Rats in Group C^{+E}_{+TRP}

The curve is a separation pattern of soluble lens homogenate from weanling rats in diet group C. The flow rate was 6-9 ml/hour and each fraction contained 2 ml. A total of 8.3 mg protein was applied to the column. No cataracts in lenses (10) from group C.

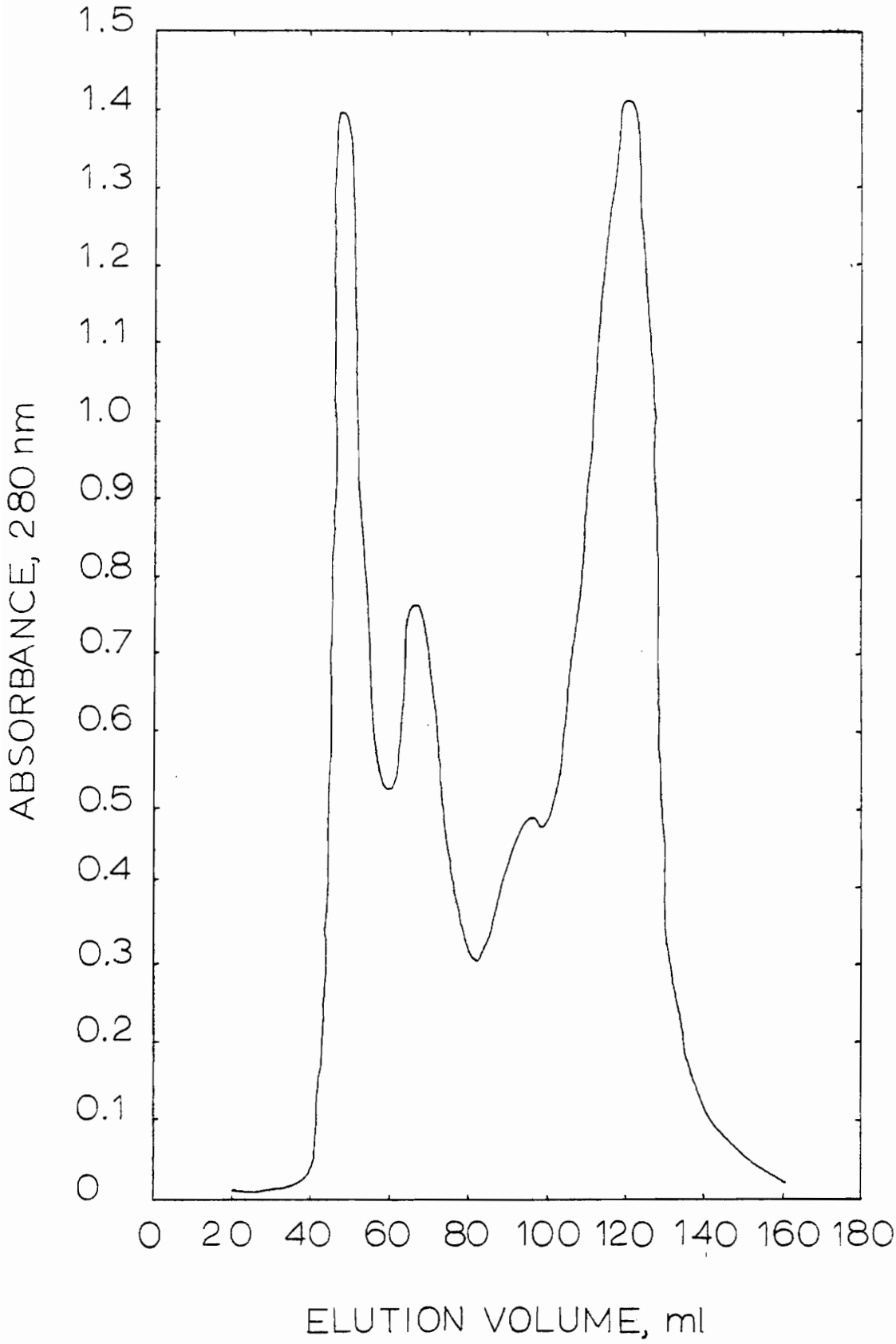

ABSORBANCE, 280 nm
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0 20 40 60 80 100 120 140 160 180
ELUTION VOLUME, ml

Figure 11

G-200 Separation Pattern of the Soluble Lens Homogenate from Weanling Rats in Group D^{-E}_{+TRP}

The curve is a separation pattern of soluble lens homogenate from weanling rats in diet group D. The flow rate was 6-9 ml/hour and each fraction contained 2 ml. A total of 5.8 mg protein was applied to the column. Lenses were mixed, cataractous (2) and no cataractous (2).

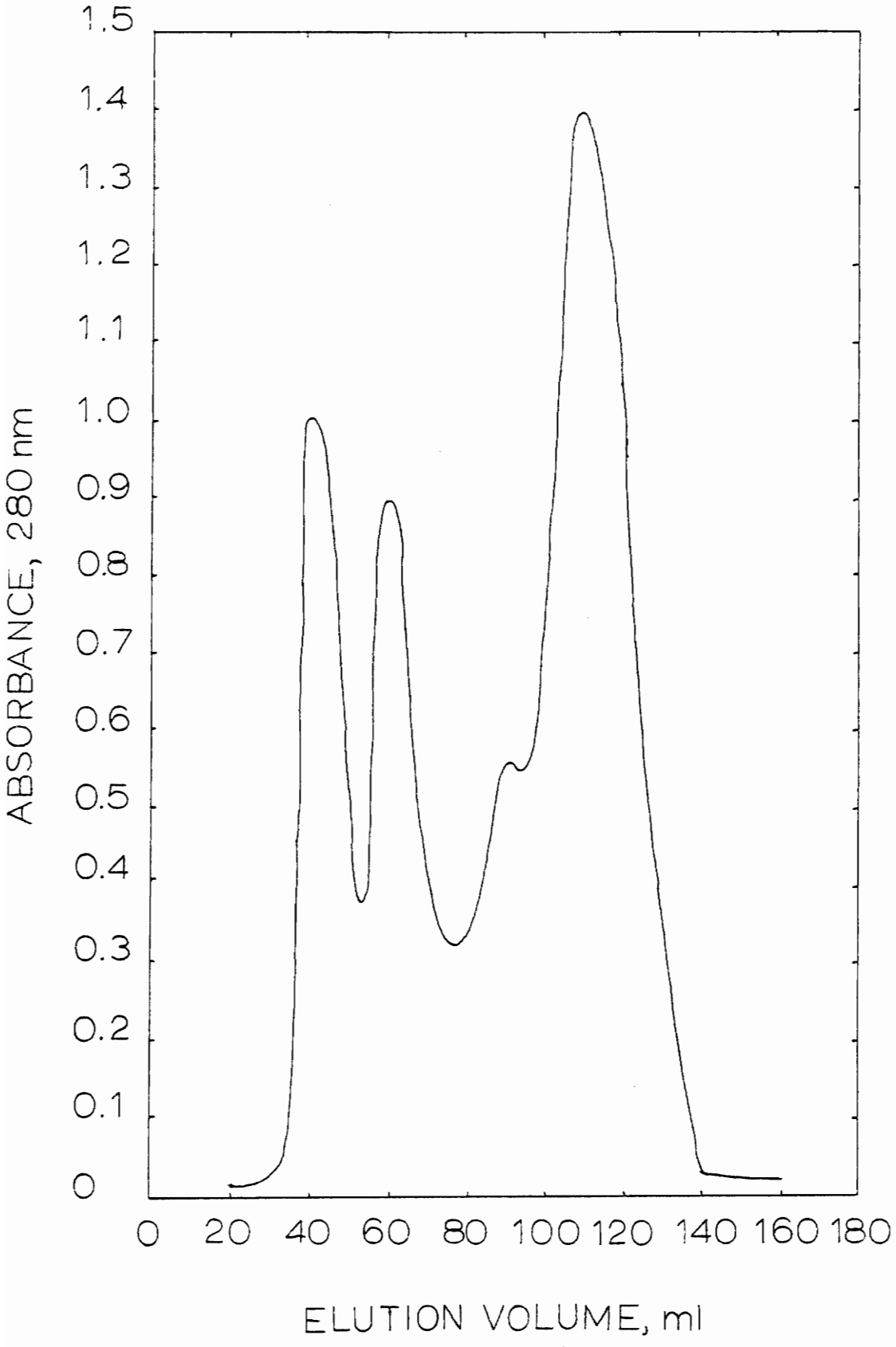

ABSORBANCE, 280 nm
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0 20 40 60 80 100 120 140 160 180
ELUTION VOLUME, ml

Table 18

Experiment II

Amino Acid Patterns on Plasma From

Fasting Weanling Rats
(concentration nM/ml)

Amino Acid	Experimental Treatments			
	A^{-E}_{-TRP}	B^{+E}_{-TRP}	C^{+E}_{+TRP}	D^{-E}_{+TRP}
Tryptophan	----	25	----	----
Lysine				
Histidine	98	117	68	69
Arginine				
Aspartic Acid	104	57	81	97
Threonine				
Serine				
Glutamic Acid	253	143	209	228
Proline				
Glycine				
Alanine				
Cysteine	----	23	----	----
Valine	88	104	62	54
Methionine				
Isoleucine	71	49	49	46
Leucine	76	63	47	56
Tyrosine	47	41	38	53
Phenylalanine	36	34	29	30

Blank Space = Concentration greater than standard curve values.

---- = Undectable.

J. Keto Acid (phenylpyruvate) Analysis of Urine

Based on the increase in keto acid (phenylpyruvate) concentrations for each group in experiment I, during the 7-14th days of gestation the sampling period was extended to include the 16th day of gestation.

The mean phenylpyruvate excretion (±SD) patterns for female rats within each group from the 7-16th days of gestation (Table 19) revealed no consistent patterns between or within each group. The mean phenylpyruvate excretion for the 7-16th days of gestation were not significantly different ($P > 0.05$) between groups.

Experiment III

A. Introduction

Experiment III was designed to test whether nitrogen or phenylalanine imbalances, imposed in experiments I and II, were responsible for cataract formation. One of the primary purposes of this experiment was to examine the metabolic pathways for nitrogen during gestation and determine some of the relationships between tryptophan and vitamin E. This was accomplished by examining the metabolic nitrogen by-products in urinary excretions, such as urea, total nitrogen content and creatinine.

Table 19

Experiment II

Mean Urinary Keto Acid (Phenylpyruvate) Excretion of Pregnant Rats
(μg Keto Acid/μg Creatinine/48 Hours)

	Experimental Treatments[1]			
	A -E -TRP	B +E -TRP	C +E +TRP	D -E +TRP
Days of Gestation				
7-8	20±2	22±9	27±18	19±4
9-10	21±7	15±3	18±5	17±9
11-12	18±5	19±14	32±23	18±7
13-14	15±7	17±5	22±5	23±8
15-16	25±20	15±10	21±4	18±7
Mean[1,2] phenylpryuvate excretion	20±3[a]	17±2[a]	24±5[a]	18±0[a]

[1]Mean ± SD

[2]Means with different letters were significantly different, $P < 0.05$.

B. Physical Appearance of Females During Gestation and Lactation

As described in experiments I and II the only physical changes which were also observed in this experiment were the bleeding-like secretions and hair losses before parturition and during lactation, respectively. These characteristics similarly affected each group but seemed to be more prominent in those groups deficient in both vitamin E and tryptophan.

During the course of the experiment bilateral cataracts were observed in one of the females from group A_{12}. This occurred at a period just prior to parturition. After parturition the dam failed to care for her progeny and they died. At this time the dam was placed on a standard rat chow diet. An examination of this female one week later revealed a noticeable improvement in the bilateral cataracts. The cataracts were still visible but not as distinct.

C. Food Comsumption Records for Females through Gestation

Six diets (Table 3) were used in this experiment: four diets A_{24}, B_{24}, C_{24}, and D_{24} contained a 24% purified amino acid mixture, and two diets designated A_{12} and C_{12} contained a 12% purified amino acid mixture. The

concentration of tryptophan varied for each group as in the previous experiments. Vitamin E, however, was not added to those diets deficient in this vitamin. Other exceptions were that a phenylalanine imbalance was not imposed nor was a gelatin supplement added to any of the diets.

The mean food consumption (±SD) (Table 20) generally increased from the 1st to the 21st day of gestation. During the 14-21st days of gestation, however, mean food consumption declined for groups A_{24}, C_{24} and C_{12}. A comparison of the mean total food consumption (±SD) during gestation did not show significant differences ($P > 0.05$) between groups.

Analysis of the mean feed efficiency ratios between groups, for the first two weeks of gestation, showed that the feed efficiency ratio for group B was significantly larger than the other groups. Feed efficiency ratios for the other groups were not significantly different ($P > 0.05$) from one another.

D. Body Weights and Weight Gain Records for Females During Gestation

A record of mean body weights (±SD) were obtained for females during the first two weeks of gestation.

Table 20

Experiment III

Mean Food Consumption Records For Females Through Gestation
(expressed in g of diet consumed/24 hours)

	Experimental Treatments[1]					
	24% Amino Acid				12% Amino Acid	
	$A_{24\text{-TRP}}^{-E}$	$B_{24\text{-TRP}}^{+E}$	C_{24+TRP}^{+E}	D_{24+TRP}^{-E}	$A_{24\text{-TRP}}^{-E}$	C_{12+TRP}^{+E}
Days of Gestation						
1-7	14±9	21±10	13±8	20±8	19±6	19±6
7-14	19±6	20±5	19±7	24±3	19±5	24±7
14-21	16±10	27±8	12±11	25±13	24±9	22±9
Mean Total Food Consumption During Gestation[1,2]	330±135[a]	490±101[a]	303±96[a]	483±27[a]	397±88[a]	432±130[a]
Feed Efficiency Ratio[1,2]	.05±.01[a]	.11±.04[b]	.05±.03[a]	.02±.02[a]	.05±.04[a]	.04±.02[a]

[1]Mean ± SD

[2]Means with different letters are significantly different, $P < 0.05$.

(Table 21). The mean weight gains (±SD) for the females during the first two weeks of gestation (Table 22) were consistantly greater for all groups during the second week of gestation.

Comparisons of the mean total weight gains (±SD) during gestation did show significant differences ($P > 0.05$). Mean total weight gains for group B_{24} were significantly larger than the other groups. Mean weight gains for groups A_{24}, C_{24}, D_{24}, A_{12} and C_{12} were not significantly different ($P > 0.05$).

E. Reproductive Data

Fertility, survival at parturition, and postnatal viability records are shown in Table 23. In general fertilization was less successful for the groups fed the 24% purified amino acids, with the exception of group B_{24}. No females died at parturition except in group A_{24}. However, postnatal viability was drastically affected in this experiment. Group B_{24} was the only group in which pups survived until weanling.

F. Weight Records for Pups

Weight records were not recorded weekly as in previous experiments, in the hope that survival rates would increase.

Table 21

Experiment III

Mean Body Weight Records For Females
(in g)

	Experimental Treatments[1]					
	24% Amino Acid				12% Amino Acid	
	$A_{24\text{-TRP}}^{-E}$	$B_{24\text{-TRP}}^{+E}$	C_{24+TRP}^{+E}	D_{24+TRP}^{-E}	$A_{12\text{-TRP}}^{-E}$	C_{12+TRP}^{+E}
Day of Gestation						
1	202±14	223±09	202±10	210±00	211±14	190±08
7	201±10	244±14	209±12	216±05	215±11	191±10
14	217±08	281±18	231±12	239±14	232±17	204±14

[1]Mean ± SD

Table 22

Experiment III

Mean Weight Gains For Females During Gestation
(in g)

	Experimental Treatments[1]					
	24% Amino Acid				12% Amino Acid	
	$A_{24\text{-TRP}}^{-E}$	$B_{24\text{-TRP}}^{+E}$	C_{24+TRP}^{+E}	D_{24+TRP}^{-E}	$A_{12\text{-TRP}}^{-E}$	C_{12+TRP}^{+E}
Days of Gestation						
1-7	.4±9	21±7	1±2	1±1	4±7	5±5
7-14	15±6	36±7	13±8	11±8	19±7	18±6
Mean Total Body Weight Gain During the 1-14th Days of Gestation	14±11[a]	58±9[b]	14±8[a]	12±9[a]	22±12[a]	25±8[a]

[1]Means ± SD

[2]Means with different letters are significantly different, $P < 0.05$.

Table 23

Experiment III

Reproduction Data

Fertility, Survival at Parturition

and Postnatal Viability

Diet	Females preg/ females mated		Females dying at partiurtion/ females mated		Pups surviving at weaning
	(no.)	(%)	(no.)	(%)	
$A_{24}{}^{-E}_{-TRP}$	7/10	70	1/7	14	0
$B_{24}{}^{+E}_{-TRP}$	7/8	87	0/7	0	55
$C_{24}{}^{+E}_{-TRP}$	5/9	55	0/5	0	0
$D_{24}{}^{-E}_{+TRP}$	5/8	62	0/5	0	0
$A_{12}{}^{-E}_{-TRP}$	7/8	87	0/7	0	0
$C_{12}{}^{+E}_{+TRP}$	8/8	100	0/8	0	0

The results previously reported indicate this was not the case.

The average weight per pup at weaning for the one surviving group, B_{24}, was 27 g. This figure can be compared to the body weights of pups at weanling in the previous experiments.

G. Incidence of Cataract in Weanling Rats

Group B_{24} had 55 pups from 6 litters which survived to weaning. Examination of these lenses revealed no incidence (0%) of cataracts. This figure should be compared to the 70 percent incidence of cataract for group B in experiments I and II with 18% purified amino acids which included a phenylalanine imbalance.

Five pups from Group C_{12} died several days prior to weaning and one was killed because it was too weak to survive. No cataracts were found in these lenses.

In addition five pups from group A_{24} were found dead or too weak to survive. No cataracts were discovered in these lenses. Before examination could proceed the eye lids had to be removed. Spontaneous cataracts have been observed when the lid is removed prior to the normal opening period.

H. G-200 Separation Patterns on Soluble Lens Homogenates from Weanling Rats

The protein lens pattern from group B_{24} (Figure 12) was obtained using a G-200 Sephadex column and fresh soluble lens homogenate. This pattern can be compared to those in experiments I and II and especially to group B from those experiments.

I. Plasma Amino Acid Analysis

Amino acid patterns for lens and plasma were obtained from weanling rats in group B_{24} (Table 24). Plasma amino acid patterns can be compared to those in experiments I and II. In each case lens and plasma tryptophan was detected. In the previous experiments this amino acid was not detectable in the plasma of some groups.

J. Urine Analysis for Total Nitrogen and Urea

The mean urea nitrogen ($\pm$SD) excretion patterns for females during the 7-14th days of gestation (Table 25) were significantly different ($P > 0.05$). Group B_{24} showed significantly greater excretions of urea nitrogen than the other groups. In general the groups containing the 24%

Figure 12

G-200 Separation Pattern of the Soluble Lens Homogenate from Weanling Rats in Group B_{24-TRP}^{+E}

The curve is a separation pattern of soluble lens homogenate from weanling rats in diet group B_{24}. The flow rate was 6-9 ml/hour and each fraction contained 2 ml. A total of 3.2 mg protein was applied to the column. No cataracts in lenses (20) from group B_{24}.

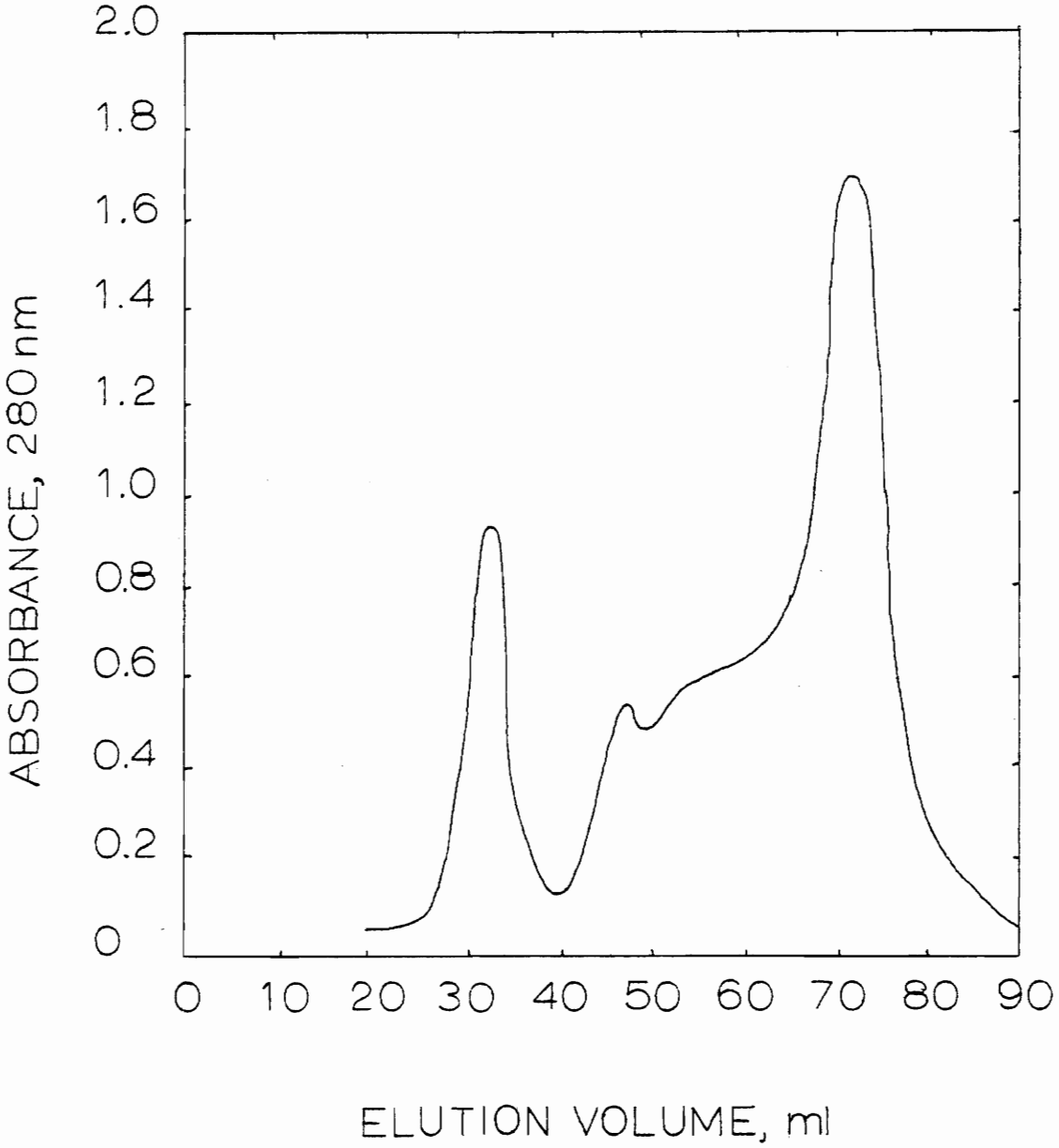

2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
ABSORBANCE, 280 nm
0
10
20
30
40
50
60
70
80
90
ELUTION VOLUME, ml

Table 24

Experiment III

Amino Acid Patterns For Lens Homogenate

And Plasma From Weanling Rats
(concentration nm/ml)

	Lens		Plasma	
Amino Acid	B_{24}^{+E} -TRP	B_{24}^{+E} -TRP	B_{24}^{+E} -TRP	B_{24}^{+E} -TRP
Tryptophan	74	41	5	6
Lysine				
Histidine	150	147	60	45
Arginine			100	111
Aspartic Acid	48	96	27	52
Threonine			50	67
Serine			105	105
Glutamic Acid	237	199	144	217
Proline				
Glycine			140	88
Alanine			140	63
Cysteine	60	59	154	92
Valine				
Methionine				
Isoleucine	155	101	22	19
Leucine	253	161	46	34
Tyrosine	112	88	64	67
Phenylalanine	107	77	37	34

Blank Space = Concentration greater than standard curve values.

Table 25

Mean Urinary Urea Nitrogen Excretion Patterns For Females During Gestation (mg/48 hrs)

	Experimental Treatments[1]					
	24% Amino Acid				12% Amino Acid	
	$A_{24\text{-TRP}}^{-E}$	$B_{24\text{-TRP}}^{+E}$	C_{24+TRP}^{+E}	D_{24+TRP}^{-E}	$A_{12\text{-TRP}}^{-E}$	C_{12+TRP}^{+E}
Days of Gestation						
7-8	472±179	597±142	265±180	353±27	179±42	142±47
9-10	413±146	680±50	487±250	266±150	199±130	211±108
11-12	330±85	785±98	320±75	591±21	196±54	285±169
13-14	311±300	725±338	296±156	587±191	198±53	208±158
Mean Urea Nitrogen[1,2] Excretion	381±74[a]	696±79[b]	342±99[a]	449±165[c]	193±9[d]	210±56[d]

[1]Mean ± SD

[2]Means with different letters are significantly different, $P < 0.05$.

amino acid mixture, with the exception of group B_{24}, showed significantly larger concentrations of urea nitrogen in the urine.

Mean total nitrogen (±SD) (mg nitrogen/mg creatinine/48 hrs) excretion patterns were obtained from females during the 7-14th days of gestation (Table 26). The mean nitrogen excretions between groups were significantly different ($P < 0.05$). Mean nitrogen excretion was significantly greater for group B_24 than the other groups. Groups A_{24} and D_{24} were not significantly different ($P > 0.05$) nor were groups A_{12} and C_{12}. Group C_{24} also differed significantly from the other groups.

Mean total nitrogen (±SD) (mg nitrogen/48 hrs) (Table 27) excretion patterns were significantly different. Nitrogen excretion was significantly greater for group B_{24} ($P < 0.05$). Groups consuming the 24% amino acid mixture showed significantly larger concentrations of total urinary nitrogen. Mean urinary creatinine (±SD) excretions (Table 28) were not significantly different ($P > 0.05$) between groups.

Table 26

Experiment III

Mean Urinary Total Nitrogen Excretion Patterns For Females During Gestation
(mg nitrogen/mg creatinine/48 hr)

	Experimental Treatments[1]					
	24% Amino Acid				12% Amino Acid	
	$A_{24\text{-TRP}}^{-E}$	$B_{24\text{-TRP}}^{+E}$	C_{24+TRP}^{+E}	D_{24+TRP}^{-E}	$A_{12\text{-TRP}}^{-E}$	C_{12+TRP}^{+E}
Days of Gestation						
7-8	24±2	31±4	31±8	22±2	12±2	13±1
9-10	26±2	35±3	30±6	31±16	14±3	14±2
11-12	30±1	44±1	35±6	30±3	15±1	16±2
13-14	30±7	41±10	29±3	26±5	14±1	17±4
Mean nitrogen excretion	28±5[a]	36±6[b]	31±6[c]	27±8[a]	13±2[d]	15±3[d]

[1]Mean ± SD

[2]Means with different letters are significantly different, $P < 0.05$.

Table 27

Experiment III

Mean Urinary Total Nitrogen Excretion Patterns For Females During Gestation (mg nitrogen/48 hrs)

	Experimental Treatments[1]					
	24% Amino Acid			12% Amino Acid		
	A -E 24-TRP	B +E 24-TRP	C +E 24+TRP	D -E 24+TRP	A -E 12-TRP	C +E 12+TRP
Days of Gestation						
7-8	649±118	617±49	513±225	495±72	217±69	292±30
9-10	545±145	749±66	767±163	683±223	331±137	307±171
11-12	409±110	940±14	759±53	786±27	336±30	359±181
13-14	887±323	759±224	605±107	709±137	312±49	386±68
Mean nitrogen Excretion[2]	662±201[a]	766±132[a]	661±123[a]	657±123[a]	299±55[b]	336±43[b]

[1]Mean ± SD

[2]Means with different letters are significantly different, $P < 0.05$.

Table 28

Experiment III

Mean Urinary Creatinine Excretion Patterns For Females During Gestation (mg/48 hrs)

	Experimental Treatments[1]					
	24% Amino Acid				12% Amino Avid	
	$A_{24\text{-TRP}}^{-E}$	$B_{24\text{-TRP}}^{+E}$	C_{24+TRP}^{+E}	D_{24+TRP}^{-E}	$A_{12\text{-TRP}}^{-E}$	C_{12+TRP}^{+E}
Days of Gestation						
7-8	23±3	21±2	17±5	22±3	20±5	24±3
9-10	20±5	22±2	25±2	19±2	22±5	23±13
11-12	21±4	21±2	22±2	26±1	22±8	22±10
13-14	23±7	19±5	21±3	24±3	23±3	21±5
Mean creatinine excretion[2]	22±1[a]	21±1[a]	21±3[a]	22±3[a]	22±1[a]	22±5[a]

[1]Mean ± SD

[2]Means with different letters are significantly different, $P < 0.05$.

Chapter V

DISCUSSION

Experimental Treatments

The diets used in these experiments were imbalanced diets. The imbalances were of two types. The first imbalance resulted from the addition of excess (5%) phenylalanine in experiments I and II and from an increase of dry purified amino acids in experiment III, and the second imbalance was due to dietary restrictions of vitamin E and/or tryptophan which occurred in each experiment.

Typically a diet containing an imbalance or restriction of one or several nutrients will cause a reduction in food intake resulting in reduced growth in the rat. Hier et al. (48) observed this when they fed 4% or more phenylalanine to rats on an adequate diet. Wrettend (49) observed this when phenylalanine was included in a diet inadequate in protein. It was hoped that the stress of pregnancy would overcome this appetite suppression response so frequently observed in non-pregnant rats. Lojkin (30) reports that nitrogen imbalanced diets fed to pregnant rats did not result in restricted food intake or growth.

It is logical to assume, then that diets containing the least imbalance will be consumed to a greater extent. In experiments I and II, diet C, the least imbalanced diet, containing supplements of vitamin E and tryptophan, would be more acceptable than diet A which was deficient in both these nutrients. However, as the results indicate this did not occur.

Addition of 5% phenylalanine, in both experiments I and II, may have been responsible for the alteration in the expected results. Similarly, in experiment III, the increased percentage of dry purified amino acids may have modified the expected food comsumption patterns. Rogers and Harper (30) in a report on amino acid diets indicate that dry purified amino acid diets are less acceptable than diets in which the amino acids are fed in an agar gel. Similar reports by Stucki and Harper (51) support these findings.

No significant differences were found between groups for weight gains during pregnancy, except for group B in experiments II and III. This difference was also reflected in the higher feed efficiency ratio seen for group B. In experiments I and II there was a tendency for both food consumption and weight gains to decrease just prior to parturition. Weight decreases are a natural reflection

of food consumption decreases. The most probable explanation for this occurrence was the females general lack of activity during this period and in some case the effect of the bleeding-like secretions.

Dietary imbalances, in experiments I and II, did not have an effect on the ability of the females to become pregnant. However, in experiment III increasing the purified amino acid content of the diets did result in slightly lower percentages of females which became pregnant, with the exception of group B_{24}. Success of fertilization for groups A_{12} and C_{12}, in experiment II, were similar to results reported by Bunce and Hess (4).

Reproductive Performance

The results of the repoductive data were unexpected. In experiment I group C, and not group A, had the most difficulty during gestation and postpartum as reflected in the number of females that died giving birth and the number of pups that survived to weaning. However, in experiment II, group D replaced group C as the group having the most difficulty. Reports by Boggs et al. (52) indicated that excess phenylalanine in the diet would not be a problem since female rats during pregnancy and lactation had normal litter sizes with no gross abnormalities.

One of the possible problems which they observed was that milk production was diminished in dams fed excess phenylalanine. To improve the nutritive status of the young they decreased the litter size to four pups per dam. Since the number of pups was critical to this project litter sizes were not reduced. While milk production may have been a problem, in experiments I and II, another more important problem was the failure of the dams to care for her progeny at birth.

An additional factor to consider is the tryptophan deficient diet. Tryptophan may not have been available in sufficient amounts to provide for milk production. Protein synthesis cannot occur if any of the amino acids required for construction of the protein are missing. But this would depend on whether the female is able to breakdown her own body stores to supply an adequate level of tryptophan for milk production. A possible complicating factor in these first two experiments is the excess (5%) phenylalanine. Reports by Airaksinen et al. (35) state that high levels of phenylalanine can inhibit not only the transport of tryptophan but also its incorporation into proteins. So even though the female may be capable of breaking down protein stores to supply adequate tryptophan

to the fetus this may be of questionable value when the diet contains excess phenylalanine.

In experiment III, pup survival was drastically affected in each group, except group B_{24}. The increased levels of dry purified amino acids may have been a factor, however, food consumption and weight gain records did not indicate any problem. Even more difficult to explain are the survival percentages (9%) of pups on the lower amino acid mixtures. Results by Bunce and Hess (4) indicate that the pup survival should be about 75% and 82% for groups A_{12} and C_{12} respectively.

Cataract Formation

An explanation for the incidence of cataract observed in experiments I and II, depends on the credibility of Nair's hypothesis that vitamin E is involved in heme systhesis. The expected results indicate that the groups fed a diet deficient in both vitamin E and tryptophan with a phenylalanine imbalance should have a higher incidence of cataract than the groups fed the supplemented diet. The actual observed results support this hypothesis. The double deficient diet A in experiments I and II, produced about twice the incidence of cataract as reported by Bunce and Hess (4). As predicated no cataracts were observed in

the progeny of females fed diet C, the supplemented diet. This indicates that there may be a protective action exerted by vitamin E and tryptophan, in spite of the phenylalanine imbalance, in cataract prevention.

The high incidence of cataract in the progeny of group B, was unexpected. Using a similar diet formula, without the excess phenylalanine, Bunce and Hess (4) reported no incidence of cataracts in the progeny of females fed this diet supplemented with vitamin E but deficient in tryptophan. With the addition of excess phenylalanine the level of vitamin E appears to be inadequate in protecting against cataract formation. Or the deficiency of tryptophan may have prevented an interaction, or a metabolite formation necessary for cataract prevention. In experiment III, to test this interaction diet D, the opposite of diet B was formulated. The incidence of cataracts produced by this diet was high (50%), however, due to poor survival percentages no significant conclusions could be drawn.

In experiment III an attempt was made to determine if exess phenylalanine, per se, or the nitrogen from the addition of excess phenylalanine was responsible for cataract formation. Although the survival of pups in this experiment was drastically affected, the experiment does

indicate that phenylalanine and not nitrogen, at least for group B_{24}, may have been responsible for cataract formation. Analysis of 110 lenses from pups in group B_{24} showed no signs of cataracts. In experiments I and II, diet B, containing approximately the same level of nitrogen as diet B_{24} but with an excess of phenylalanine, produced a 74% and a 48% incidence of cataracts, respectively. Even postmorteum and pre-weanling analysis of lenses from pups in group A_{24} did not reveal cataracts despite postmorteum changes or the danger of spontaneous cataract formation.

The type of cataracts observed in these experiments were small nuclear cataracts. Cataracts of this type are formed in the earliest lens fibers in the nuclear region. This reinforces the hypothesis that these cataracts were produced in the fetus, during early lens development, and were not the result of postnatal conditions.

The significance of diet in prevention of cataracts was also observed in experiment III. One female assigned diet A_{12}, a double deficient diet, developed bilateral cataracts. Upon returning the female to a standard rat chow diet it was possible to reverse the cataract formation and improve the condition noticeably. Similar results have been reported by Bunce et al. (28) on weanling rats which developed cataracts.

Lens Analysis

G-200 Sephadex lens separation patterns did not indicate any distinct differences in crystallin fractions from soluble lens homogenates obtained from weanling rats in either experiments I, II or III. It was hoped that the lenses with cataracts would reflect some visible alteration in the lens profiles when comparisons were made. Characteristic differences in lens profile patterns of cataractous lenses produced by feeding weanling rats a tryptophan deficient diet have been reported (53). The characteristically larger gamma peaks seen in these studies simply reflect the age of the lenses. In younger animals lens analysis usually shows larger gamma peaks. As an animal matures the alpha peak becomes larger and the gamma peak smaller.

During experiment I a 7.5 mm x 90 cm column was used, however, due to poor resolution of the beta fraction and this inpart explains the differences seen in the same patterns between experiments I and II.

Amino Acid Analysis

Plasma amino acid patterns for groups in experiments I and II did not indicate any apparent consistency. This was thought to be due to the lack of controls on feeding

and fasting of the pups before blood samples were collected. As a result, strict controls were imposed in experiment III to insure that the pups had consumed food, during the same period and that the food they consumed was only breast milk. Unfortunately due to the high mortality rates this aspect of the experiment could not be tested thoroughly. Group B_{24}, the one surviving group, did show consistently similar amino acid patterns between litters. It was hoped that this analysis would indicate imbalances of amino acids in the plasma, and possibly point to a cause of cataract formation between groups. Since tryptophan has the lowest free plasma concentration of all the essential amino acids it could easily become the limiting amino acid in the synthesis of tryptophan containing proteins.

Lens analysis was conducted further to determine if free amino acid patterns differed between groups. Again due to low survival figures this comparison was limited. Group B_{24}, however, did show similar amino acid patterns between litters tested. However studies (1) to date on amino acid patterns of lenses have been unable to show that significant difference exist.

Urine Analysis

In experiments I and II urinary keto acid analysis were performed to test the hypothesis that vitamin E was involved in heme synthesis. Phenylpyruvate a keto acid of phenylalanine was analyzed. If Nair's hypothesis is correct, groups deficient in vitamin E should show higher urinary phenylpyruvate concentrations. The results of experiment I and II do not support this hypothesis. No significant differences ($P > 0.05$) were found in phenylpyruvate concentrations between groups tested. Reports by Rowe *et al*. (54) indicate that phenylpyruvate is not the best indicator of phenylalanine metabolism. They suggest that hippuric acid be analyzed since it was detected in larger amounts.

Total nitrogen, urea and creatinine analyses were performed on urine samples collected from females during the 7-14th days of gestation. These parameters were used as a criteria for examining nitrogen metabolism in the female rat during gestation when various combinations of vitamin E, tryptophan and percentages of amino acids were fed. As expected the groups consuming the lower percentage (12%) amino acid mixture excreted less urea and nitrogen than groups consuming the higher percentage

(24%) amino acid mixture. However, there was no significant differences ($P > 0.05$) in excretion levels of either nitrogen or urea between groups A_{12} and C_{12}.

Mean urea nitrogen excretion for group B_{24} was significantly greater than the other groups. Mean total nitrogen excretion, however, was significantly greater for group B_{24}. This may have resulted from greater excretions of amino acids, ammonia and/or protein. Creatinine would not contribute to the increase since concentrations remained relatively constant throughout the collection period.

Increased diuresis was observed for groups fed diets containing 24% amino acid, with the exception of group B_{24}. The mean urine (ml/48 hr) volumes ($\pm$SD) for the collection period were: A_{24}, 115 ± 29; B_{24}, 33 ± 2; C_{24}, 119 ± 20; D_{24}, 112 ± 20; A_{12}, 60 ± 6; C_{12}, 87 ± 6. Increased diuresis has been reported by Ekland and Agran (55) when they fed male and female rats rapeseed and sunflower-seed protein concentrates (10 or 20% protein). Renal excretions of nitrogen, urea and protein remained unchanged. Studies by Stucki and Harper (51) indicate that feeding purified amino acids in the dry state causes renal and hepatic dysfunctions. Feeding increased percentage of dry purified amino acids in experiment III may have potentiated

the diuretic effect observed. The antidiuretic action of diet B_{24} is unknown but deserves further investigation. This diet, B_{24}, may have a protective effect on preventing renal dysfunction when dry purified amino acids are fed.

The use of dry purified amino acids, may also explain, in part, the physical condition and behavioral responses of the females during gestation, and the high mortality in experiment III. Similarly, the results of experiment I and II may have been affected by the use of dry purified amino acids, even though the percentage of amino acid was less in these experiments. Results similar to those reported here have been observed by Ekland (56). Using a rapeseed protein concentrate fed to female rats during gestation and lactation, he observed that at the 18th day of gestation the females showed a sudden loss of appetite, followed by a weight decrease and bleeding-like secretions at the nose and eyelids. Mortality was high for both the diseased animals and the progeny. One dissimilar observation, however, was delayed delivery which occurred in the diseased animals.

Liver Analysis

Phenylalanine hydroxylase activity in liver of weanling rats, from experiments I and II, was examined (43). Phenylalanine hydroxylase activity did not directly

correlate with the incidence of cataract in the lenses from these animals. Increased phenylalanine hydroxylase activity was observed for diet B, deficient only in tryptophan, which may reflect a decrease in protein snythesis. Decreased phenylalanine hydroxylase activity was observed for diet D, deficient only in vitamin E, this may also reflect a general depression of protein synthesis or a reduction of heme synthesis. No significant differences in phenylalaine hydroxylase activity were reported between groups A or group C, supplemented with vitamin E and tryptophan.

Conclusions

The results of these experiments lead to several conclusions concerning the incidence of cataract observed in weanling rats as a result of maternal diets containing a high level of phenylalanine but deficient in vitamin E and/or tryptophan. Phenylalanine in combination with a vitamin E and tryptophan deficient diet did result in a higher incidence of cataract than in studies previously reported (4), without excess phenylalanine (5%). Supplementing the deficient diet (A) with vitamin E and tryptophan (diet C) did prevent cataract formation. Supplementation with vitamin E alone (diet B) did not protect against cataract formation, in contrast to other studies

(4). This may point to the interaction of vitamin E and tryptophan in cataract formation.

The replacement of phenylalanine and gelatin with an approximate equivalent of nitrogen from purified amino acids did prevent cataract formation. However, this resulted in decreased pup survival. Further studies should be conducted to determine the optimum purified amino acid levels. Rather than feeding a dry amino acid mix, the diet may be made more acceptable by mixing the amino acids with an agar gel (50).

Further studies in this area should also include an examination as to the cause of diuresis observed when dry purified amino acids are fed. These studies should include blood analysis, urine analysis (specific gravity, ph, electrolytes, and other urinary metabolites), and detailed pathology of the liver and kidney tissues. Finally experiments should be conducted with other essential amino acids fed in excess to determine if the results observed are unique for phenylalanine.

Chapter VI

SUMMARY AND CONCLUSIONS

A series of three experiments were used to investigate the incidence ofcataract formation in young rats as a result of feeding a maternal diet, throughout gestation and lactation, containing an excess pf phenylalanine or a balanced free amino acid mixture but limited in vitamin E and/or tryptophan. Diets in the first two experiments contained a 5% excess phenylalanine and 6% excess protein (gelatin) in a defined diet containing a balanced protein equivalent of 12% protein in the form of free amino acids. Four groups of 10 females were maintained on the following diets: A, low in vitamin E and tryptophan; B, low in tryptophan; C (control) adequate levels of vitamin E and tryptophan; D, low in vitamin E. In experiment I only diets A, B, and C were tested.

In the third experiment excess phenylalanine and gelatin were replaced with a 12% balanced free amino acid mixture, making a total of 24% protein equivalent in the form of free amino acids. Six groups of eight female rats were maintained on the following diets: 24% amino acid mixtures--A, low in vitamin E and tryptophan; B, low in tryptophan; C (control) adequate levels of vitamin E and

tryptophan; D, low in vitamin E; 12% amino acid mixture--A, low in vitamin E and tryptophan; C, adequate levels of vitamin E and tryptophan.

The following parameters were examined: Experiments I and II--food consumption, weight gains (females and progeny), reproductive data, survival and viability of progeny, urinary keto acid (phenylpyruvate) analysis (7th-16th days of gestation), plasma amino acid analysis (progeny), and protein separation on soluble lens homogenate (progeny). Experiment III--the same parameters were examined as in experiments I and II with the following exceptions: urinary total nitrogen, urea, and creatinine analysis replaced keto acid analysis and amino acid analysis of soluble lens homogenates (progeny) were added.

The following conclusions may be drawn from these experiments:

1. Use of dry purified amino acid fed to female rats during gestation appears to cause diuresis, resulting in dehydration and subsequent bleeding-like secretions in some cases. This effect was observed just prior to parturition. Supplementing the 24% free amino acid mixture with vitamin E appeared to protect against diuresis.

2. Dietary imbalances, due to addition of excess phenylalanine or free amino acid mixture, did not result in significant differences ($P > 0.05$) in food consumption.

3. Dietary imbalances, due to addition of excess phenylalanine or free amino acid mixtures, did not result in significant differences ($P > 0.05$) in weight gained during gestation, with the exception of group B. Single supplementation with vitamin E (group B) appears to result in greater weight gain, in most cases.

4. The use of a 24% balanced free amino acid mixture (experiment III) resulted in decreased fertility and pup survival. Single supplementation with vitamin E (group B) had a protective effect.

5. Weight gains for surviving pups were not significantly different ($P > 0.05$).

6. Addition of 5% excess phenylalanine caused increased incidence of cataract formation than studies previously reported with diets limited in tryptophan and vitamin E alone or a single supplementation of vitamin E.

7. Use of 24% free amino acid mixture prevented cataract formation in the group supplemented with vitamin E alone (group B).

8. Urinary keto acid (phenylpyruvate) excretions for females during the 7th-16th days of gestation (experiments I and II) were not significantly different ($P > 0.05$).

9. G-200 lens protein separations did not show differences between cataractous and noncataractous soluble lens homogenates.

10. Plasma amino acid analysis did not show differences between cataractous and noncataractous animals.

11. Total nitrogen and urea nitrogen excretion for female rats during the 7th-14th days of gestation were greater for groups fed the 24% free amino acid mixture.

12. Single supplementation with vitamin E alone (group B) resulted in significantly different excretions of total nitrogen and urea than other groups fed 24% free amino acid mixtures.

13. Phenylalanine hydroxylase activity in liver of weanling rats from dams maintained on 5% excess phenylalanine diets throughout gestation and lactation did not correlate with the incidence of cataract in lenses from these animals.

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VITA

James Edward Hill was born in Burlington, Vermont on October 24, 1946. He attended South Burlington High school between the years 1961-1965. In the Fall of 1965 he entered the University of Vermont (UVM) at Burlington. In 1969 he graduated from UVM receiving a BSBA degree in Industrial Management. In 1970 he accepted employment as a programming-analyst for State Farm Insurance Company in Bloomington, Illinois. In 1972 he left State Farm and returned to the University of Vermont to continue his education. While enrolled as a continuing education student he completed the premedical course requirements. In 1974 he started graduate studies in Human Nutrition at Virginia Polytechnic Institute and State University (VPI). As a graduate student in the Human Nutrition and Foods Department he was assigned both graduate teaching and research responsibilities. He is also a member of Phi Tau Sigma, Phi Sigma and has been elected to Phi Upsilon Omicron. On August 20, 1976 he married Laura M. Gilbert. In the Fall of 1976 he will continue his studies in Nutrition at VPI at the PhD. level. This thesis completes the final requirement for his Masters Degree.

James Edward Hill

CATARACT FORMATION IN YOUNG RATS AS A CONSEQUENCE OF MATERNAL DIETS CONTAINING EXCESS PHENYLALANINE AND LOW IN TRYPTOPHAN AND/OR VITAMIN E

by

James Edward Hill

(ABSTRACT)

Diets containing 5.0% excess phenylalanine, 6.0% excess protein (gelatin), and limited in tryptophan (75mg/100g) and/or vitamin E (0.1mg/100g) were fed to female rats during gestation and lactation. Addition of 5.0% phenylalanine resulted in a greater incidence of cataract than studies previously reported with diets limited in tryptophan and vitamin E alone, or a single supplementation of vitamin E (40.0mg/100g). Supplementation with tryptophan (500mg/100g) and vitamin E (40.0mg/100g) prevented cataract formation. Replacing the 5.0% excess phenylalanine and 6.0% excess protein (gelatin) with an equivalent of a balanced free amino acid mixture prevented cataract formation in the group supplemented with vitamin E alone. Addition of balanced free amino acids resulted in decreased fertility, pup survival, and increased diuresis. Supplementation with vitamin E

(40.0mg/100g) alone prevented these effects. Urinary excretions of keto acid (phenylpyruvate), nitrogen, urea, and creatinine were examined during the 7-14th days of gestation. Amino acid analysis were performed on plasma and soluble lens homogenates from young progeny. Protein separations of soluble lens homogenates from young progeny were obtained. Analysis of these parameters did not indicate distinct differences between cataractous and noncataractous groups.