ANATOMICAL AND BIOCHEMICAL STUDIES OF A KERATOPATHY INDUCED IN RATS BY ADDED DIETARY TYROSINE

by

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A THESIS

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LIST OF ABBREVIATIONS

Å	Angstroms
cc	cubic centimeter
g	gravity, as in 4,000 x g
µl	microliter
mgm	milligram
ml	milliliter
mµ	millimicron
pHPPA	para-hydroxyphenylpyruvic acid
pHPPAO	para-hydroxyphenylpyruvic acid oxidase
PMN	polymorphonuclear leucocyte
TAT	tyrosine amino transferase

INTRODUCTION

I. General

Tyrosinemia, also referred to in the literature as tyrosinosis or tyrosyluria, is a disease characterized by elevated levels of serum tyrosine and by the presence of excess tyrosine, para-hydroxyphenylpyruvic acid (pHPPA), and other phenols in the urine. Among the disorders of amino acid metabolism, tyrosinemia has been studied more than others because of interest in the genetic defects of aromatic amino acid metabolism, e.g. alkaptonuria, albinism, and phenylketonuria. Attempts to simulate these disorders in animals have led to the development of experimental models for the study of tyrosinemia.

Sullivan, Hess, and Sebrell (44) described a specific syndrome in tyrosine toxicity of rats which included swollen and reddened legs, roughened corneas, a reddish-brown secretion about the eyes, a reddish-black material in the intestines, mottled kidneys, and rapid weight loss. Schweizer (41) described other aspects of tyrosine toxicity syndrome: alopecia, cheilitis, skin changes of the feet, edema of the legs and an occasional acute arthritis of the tibiotarsal joints. The severity of the symptoms, the weight loss and death rate increased with the concentration of tyrosine in the diet (16, 41). Presumably, the weight loss was a result and not a cause of tyrosine toxicity since the force feeding of a high tyrosine diet to rats neither diminished the weight loss nor lowered the death rate (10).

Although a tyrosine induced nephrotic disorder had previously been noted (41, 44), Lillie was the first to describe histopathologic

changes in the kidneys. He discovered mild parenchymatous degeneration, refractile crystals in the renal veins, and a diffuse glowing in the lumens of the cortical straight tubules when viewed with polarized light. Lillie also noted the presence of widespread ocular pathology, viz., lymphocytic infiltration of the iris and ciliary body, polymorphonuclear leucocyte infiltration of the cornea, and a breakdown of the center of the lens (27). Hueper and Martin (23) found hemorrhagic lesions of the brain, heart, bone marrow, and lungs, inflammatory and degenerative changes in the kidneys and pancreas, and engorgement of the adrenal medullary sinuses in rats fed tyrosine.

In 1947, Schweizer carefully studied the clinical manifestations of tyrosinemia in white rats. He reported the progressive nature of the eye changes including corneal ulceration, stromal edema, and a cloudiness of the cornea followed by vascularization. Although the feeding of tyrosine was continued, this cloudiness eventually diminished and the blood vessels disappeared; occasionally another episode of cloudiness developed. The bilateral character of the eye disease was emphasized, but a symmetrical evolution of the pathology in both eyes was not always found. On histological examination of corneal sections, edema, cellular infiltration, and capillary ingrowth were observed (41).

Several amino acids alter the response of rats to experimental high tyrosine diets. Phenylalanine, which is converted to tyrosine in the body, produces an increase in the toxicity of a 1.0% tyrosine diet (35). On the other hand, cystine, glycine, methionine or a mixture of leucine, isoleucine and valine prevent or diminish the severity of

adverse effects due to added dietary tyrosine (2, 21, 30, 44). A supplement of 1.25% L-threonine improves the growth of rats fed a 3% tyrosine diet but cataracts develop after prolonged feeding. If an additional supplement of 0.2% L-tryptophan is added to this tyrosine and threonine enriched diet, growth is improved further and these cataracts are prevented (3). The tyrosine toxicity is not affected by L-tryptophan alone (3) unless a higher dose (2.0%) is employed (35). The concentration of tyrosine in plasma, liver, eyes, and muscle is lower when 1.25% L-threonine is added to a 3% tyrosine diet (3,19). This is due to an increase in urinary excretion and protein incorporation of tyrosine (19).

Some investigators have found no alteration of the tyrosine induced pathology when vitamins are added in minimal doses to the high tyrosine diet (41), but others have found that the addition of 500 mgm% of nicotinic acid diminished the pathologic effects of added dietary tyrosine (35). Deficiencies of folic acid, pantothenic acid, thiamine (Vitamin B_1), pyridoxine (Vitamin B_6), Vitamin A, or Vitamin D apparently do not increase tyrosine toxicity, although a deficiency of riboflavin enhances the progression of the disease (30).

The nutritional adequacy and protein content of the experimental diet greatly influence the degree of tyrosine toxicity. A low protein diet increases the deleterious effect of added tyrosine, and this factor becomes more critical in a young, growing animal whose protein requirements are greater than that of the adult (21, 35).

High protein intake results in an elevated activity in many amino acid metabolizing enzymes (21). Serum tyrosine concentration

follows the daily rhythm of tyrosine amino transferase (TAT) (15), the first major enzyme of tyrosine catabolism. In rats fed ad libitum, the activity of TAT is lowest during the daylight hours and in darkness increases as much as threefold over the daytime level (9, 13, 18). Light itself does not seem to be the stimulus for increased TAT activity. Instead, the TAT rhythm is related to the nocturnal eating habits of rats because 1) the activity of TAT increases shortly after the onset of food intake (48); 2) in rats fed on a restricted schedule, the time of peak TAT activity is determined by the hours of feeding (18); 3) the TAT elevation follows the onset of darkness only if there is protein in the diet (53); and 4) the rise in TAT activity is proportional to the protein content of the food (48).

Shortly after tyrosine amino transferase was isolated and purified from the liver (12), Lin and Knox found that injections of L-tyrosine into rats would increase hepatic TAT activity (28). Feeding young rats added tyrosine in their diets also caused increased TAT activity and decreased para-hydroxyphenylpyruvic acid oxidase (pHPPAO), the enzyme which metabolizes pHPPA.

The enzyme, pHPPAO, is inhibited by the presence of an excess of its substrate, pHPPA. When tyrosine is fed to rats and metabolized by TAT, pHPPA is produced in greater than normal amounts, inhibits its enzyme, and causes a further increase in pHPPA concentration and its eventual appearance in the urine (24). The earliest reported patient with tyrosinosis had increased pHPPA in his urine (32) and other investigators have reported large increases

in other urinary simple phenols in this disease (4).

If an animal, e.g., guinea pig, which does not synthesize its own ascorbic acid, is made scorbutic and then given pHPPA, half of the pHPPAO activity is inhibited and there is urinary excretion of pHPPA and homogentisic acid. This enzyme can then be reactivated in vitro in the presence of ascorbate (52). Homogentisic acid is an inhibitor of TAT in vitro, but this inhibition can be blocked by prior addition of pyridoxal phosphate or α-ketoglutarate (14).

Knox, Linder, Lynch, and Moore (24), found that in young rats TAT activity increased immediately after tyrosine feeding but pHPPAO activity rose more slowly. These animals excreted tyrosine but dietary supplements of ascorbate did not prevent the tyrosyluria. Older rats fed added dietary tyrosine had a smaller increase in TAT activity than young rats, whereas pHPPAO was already highly active and homogentisic acid oxidase activity was low. In these rats homogentisic acid was excreted in the urine, producing alkaptonuria.

The general metabolic level of an animal has been implicated in the toxicity of tyrosine. Although added dietary tyrosine does not modify the oxygen consumption of rats and produces no pathological alterations in the thyroid gland (23, 27, 41), thyroxine given to rats fed a high tyrosine diet hastens the appearance of the pathology and increases the weight loss (41) and causes an increase in serum tyrosine concentration (21). Conversely, thiouracil, an inhibitor of thyroid hormone synthesis, prevents or delays the appearance of the syndrome, diminishes the weight loss, and increases the survival time of rats

fed a 1% or 2% tyrosine diet (41). Thyroxine/glucocorticoid ratio is important in tyrosine toxicity since thyroxine enhances the degradation of glucocorticoids (21) which are crucial in the daily rhythm of TAT (31).

There are many hormones known to influence tyrosine metabolism. Plasma tyrosine levels decrease following oral contraceptive therapy in human females (40). Pregnenolone-16α-carbonitrile, progesterone, ethylestrenol, estradiol, norbolethone, and spironolactone can prevent the signs of tyrosine toxicity (42). Glucagon induces TAT only if adrenal steroids are present (22).

Adrenal steroids are by far the most important hormones involved. Hydrocortisone can induce TAT (26, 28, 29, 51), and L-tyrosine causes an increase in TAT only if the adrenal glands are intact or in an adrenalectomized rat if hydrocortisone is replaced (13, 28). Specific glucocorticoid receptors which allow TAT induction by dexamethasone and cortisol have recently been identified in hepatoma tissue cultures (5).

A "superinduction" of TAT by Actinomycin D following induction by hydrocortisone or dexamethasone has been reported (45). Although there is a slight decrease in the synthesis of TAT after administration of Actinomycin D, there is significantly less degradation than normal, resulting in a net increase in TAT activity (38).

Phenobarbital, a non-specific inducer of the smooth endoplasmic reticulum of hepatocytes, protects against tyrosine poisoning (42).

The fact that diverse substances stimulate TAT activity implies that more than one mechanism may be involved in the induction of this enzyme. These multiple inductive pathways may in part be due to the presence of different forms of TAT. Valeriote, Auricchio, Tomkins, and Riley (47), found three components of TAT, two minor and one major, by chromatographic separation. Koler, Vanbellinghen, Fellman, Jones and Behrman (25), reported mitochondrial and soluble forms of TAT. Fellman, Vanbellinghen, Jones and Koler (17), found both of these forms in normal human heart, skeletal muscle and brain, but not in skin, erythrocytes, or leucocytes. In rat liver, both forms were stimulated in vivo by hydrocortisone (17). In monkeys the two forms of TAT become active at different times of development. The mitochondrial form is detectable in early fetal life but the soluble form does not become active until shortly after birth (25). Normally, the total activity of TAT in humans is low at birth and increases rapidly in the postnatal period. Premature infants often show elevations of serum tyrosine but lack other signs of tyrosinemia (50). This elevation is presumably due to an immaturity of one or both forms of TAT.

Amino acids can be accumulated by various tissues. The rat brain is capable of concentrating tyrosine intracellularly to four times that of the surrounding medium (20). Comparisons of amino acids in plasma and aqueous humor from rabbits, dogs, and various mammals indicate that some amino acids, including tyrosine, are actively transported into the anterior chamber of the eye (8, 36, 37).

Neither the level of serum tyrosine necessary to produce visible disease, nor the causative factor of the pathology has been determined. Among the metabolites of tyrosine, it is unlikely that homogentistic acid is the toxic agent, for it is excreted in the urine when elevated and causes alkaptonuria. The addition of tyramine to the diet of rats does not cause the signs of tyrosine toxicity (21), and an amine oxidase inhibitor did not make the signs worse in rats already tyrosinemic as would be expected if tyramine, epinephrine, or norepinephrine were implicated. Furthermore, an excess of pHPPA in the diet of rats did not cause the disease. Since these major compounds in the metabolic pathways of L-tyrosine do not produce the tyrosine-toxicity syndrome, it is possible that an accumulation of tyrosine itself causes the pathology.

II. Preliminary Studies

Because eye lesions such as referred to above have been evaluated in vivo only by gross inspection, the toxic effects of tyrosine on the cornea required further investigation and clarification. In a study preliminary to the present work (39), a diet was synthesized according to Schweizer (41) which contained sufficient protein and vitamins to maintain growth in rats and prevent eye lesions. This served as the control diet, and an appropriate amount of L-tyrosine was added to this to make experimental diets containing either 5% or 10% total tyrosine. Rats were fed one of these diets and the corneas were studied with a biomicroscope. Lesions developed only in those

rats receiving a tyrosine-supplemented diet. The corneas of these animals showed the first perceptible signs of tyrosine toxicity and progressed through definite stages. Figure 1A is a close-up photograph of a normal rat eye, and Figure 1B is a slit lamp (biomicroscope) picture of a normal rat cornea. Stage I (Figure 2) was defined by the appearance of a faint diffuse haze in the epithelium; Stage II (Figure 3) by discrete, snowflake-shaped opacities involving the full depth of the epithelium; Stage III (Figure 4) when the stroma became edematous and thickened; Stage IV (Figure 5) by the development of vascularization in all layers of the cornea, starting at the limbus and proceeding centrally; Stage V when the vessels covered the entire cornea; and Stage VI when the corneal opacity had regressed to a haze in the presence of centrally anastomosed blood vessels, although the dietary stimulus of excess tyrosine was maintained. Figure 6 illustrates the alopecia produced in a rat fed a 5% tyrosine diet for one week. A comparison of a normal rat paw and the paw of a tyrosine fed rat is shown in Figure 7. The kyphoscoliosis produced in a rat fed a 5% tyrosine diet is contrasted to a normal rat in the x-ray photograph of Figure 8.

Eye tissue for study by light microscopy was obtained from animals presenting each of the clinical stages. From these studies it was found that in Stage I (Figure 9) the basal cells of the corneal epithelium were vacuolated and slightly edematous; in Stage II (Figure 10), periodic acid Schiff staining was lost in the epithelium

FIGURE 1

NORMAL RAT

A. Close up view of a normal rat eye. Note the smooth corneal reflexion indicating an uninterrupted epithelium.

B. Biomicroscopic photograph of a normal rat cornea. Note the transparency of the cornea allowing a clear view of the iris.

FIGURE 2

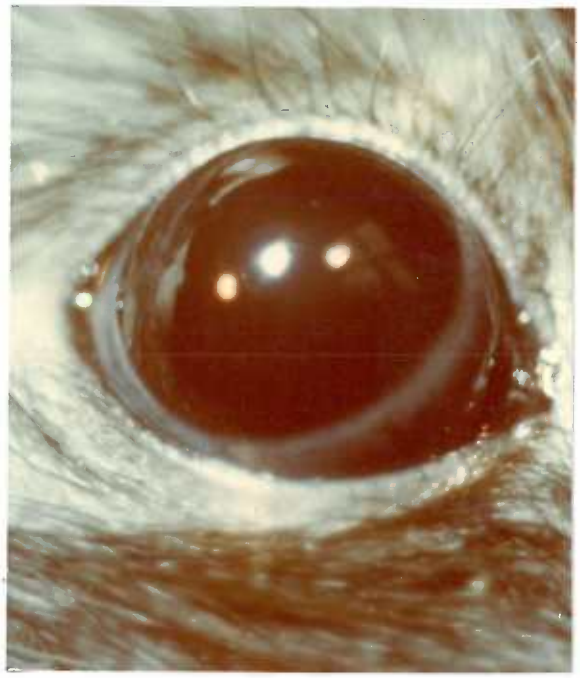
STAGE I

A. Close up view of a rat eye in Stage I of tyrosine induced keratopathy. Note the roughened reflexion indicating an interrupted epithelium.

B. Biomicroscopic photograph of a rat cornea in Stage I. With this instrument, a haze is evident in the cornea.

FIGURE 1

A



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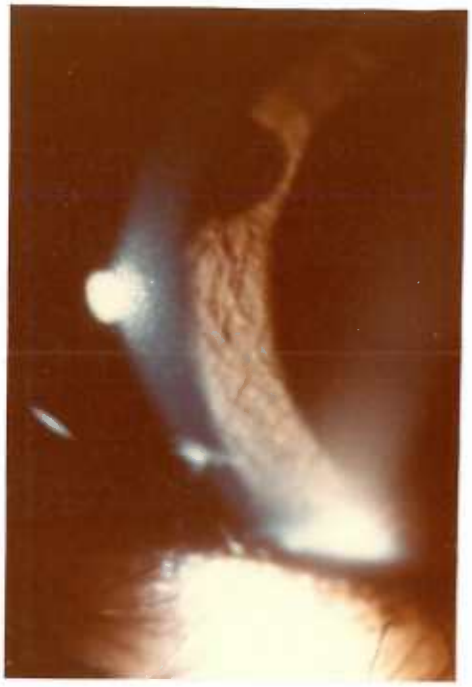
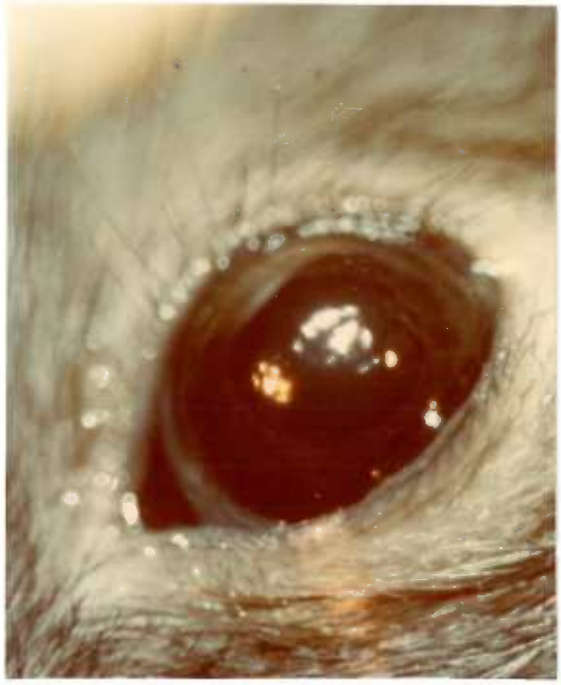


FIGURE 2

A



B

FIGURE 3

STAGE II

A. Close up view of a rat eye in Stage II of tyrosine induced keratopathy. Note the stellate shaped opacity in the center of the cornea.

B. Biomicroscopic photograph of a rat cornea in Stage II. The beginning of the stellate shaped opacity is shown.

FIGURE 4

STAGE III

A. Close up view of a rat eye in Stage III of tyrosine induced keratopathy. The opacity has enlarged to cover most of the central cornea.

B. Biomicroscopic photograph of the cornea in Stage III. Note the sharply defined saw-toothed border of the growing opacity.

FIGURE 3

A B

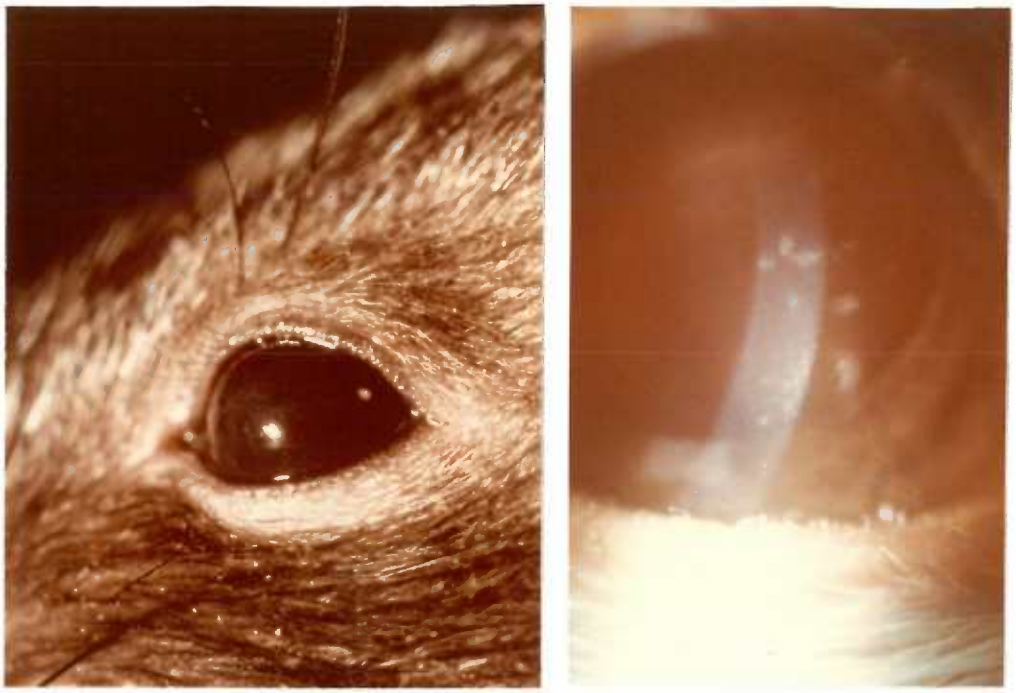


FIGURE 4

A B

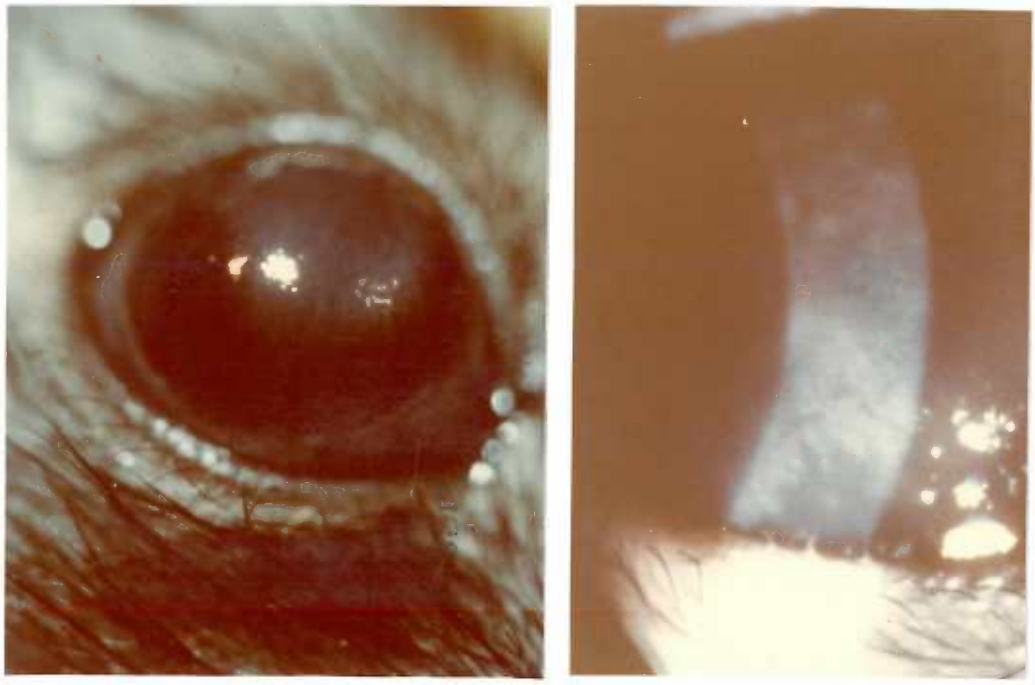


FIGURE 5

STAGE IV

A. Close up view of a rat eye in Stage IV of tyrosine induced keratopathy. Blood vessels are growing into the central corneal opacity.

B. Biomicroscopic photograph of the blood vessels starting to grow into the cornea from the corneal-scleral junction toward the center of the cornea.

FIGURE 6

Photograph of a rat which has been fed a 5% tyrosine diet for one week. Note the alopecia.

FIGURE 5

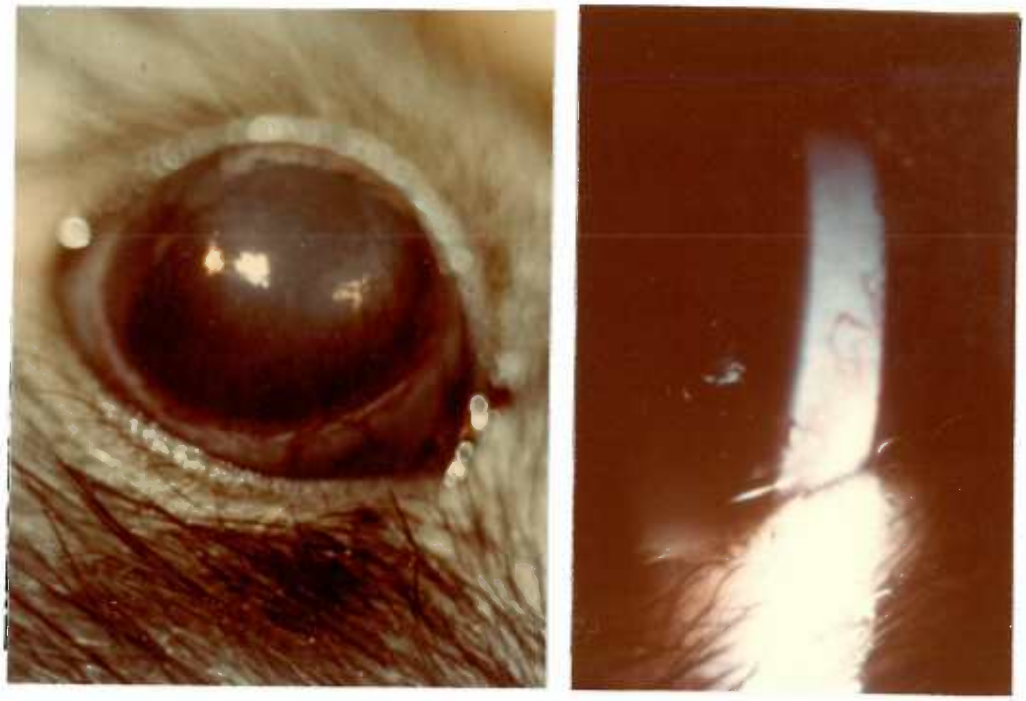


FIGURE 6

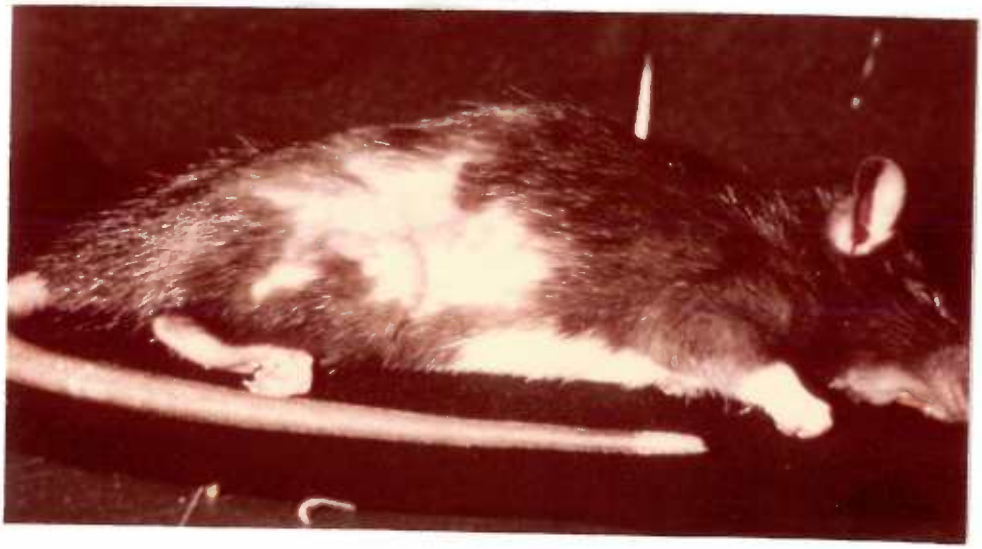


FIGURE 7

A. Normal rat paw.

B. Paw of a rat which has been fed a 5% tyrosine diet for one week. There is erythema, roughness, and scaling.

FIGURE 8

A. Photograph of an x-ray of a normal rat.

B. Photograph of an x-ray of a rat which was fed a 5% tyrosine diet for approximately one month. The weight loss and accentuated curvature of the vertebral column are evident.

FIGURE 7

A B

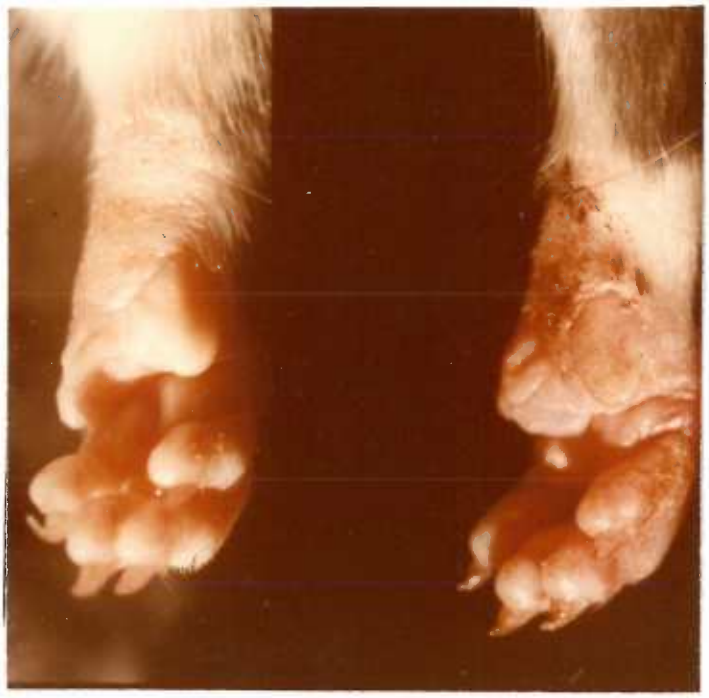


FIGURE 8

A B

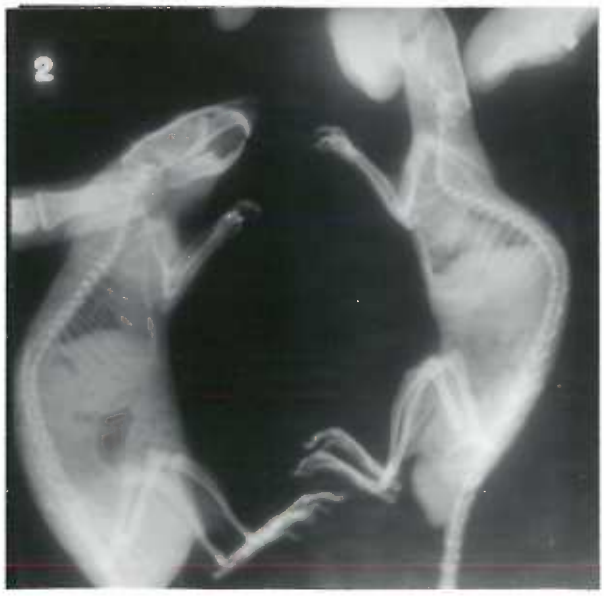


FIGURE 9

Cross sections of a rat cornea in Stage I of tyrosine induced keratopathy, 50 X magnification. The basal cells of the epithelial layer exhibit vacuolization and slight edema.

A. Hematoxylin and eosin stain.

B. Periodic acid Schiff stain.

FIGURE 10

Cross sections of a rat cornea in Stage II of tyrosine induced keratopathy, 50 X magnification. An infiltration of polymorphonuclear leucocytes is beginning.

A. Hematoxylin and eosin stain.

B. Periodic acid Schiff stain.

A

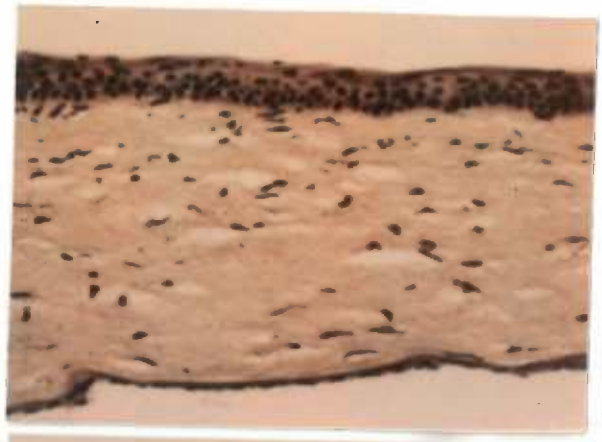
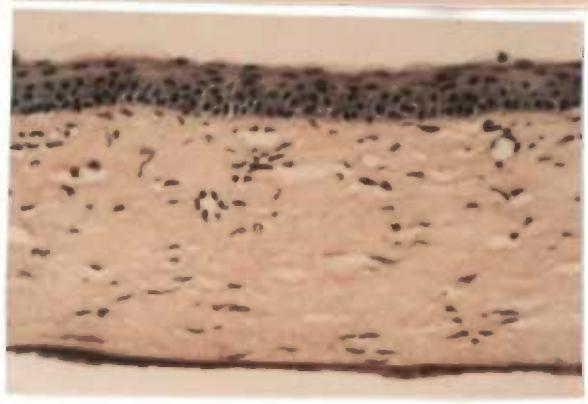


FIGURE 9

B



A

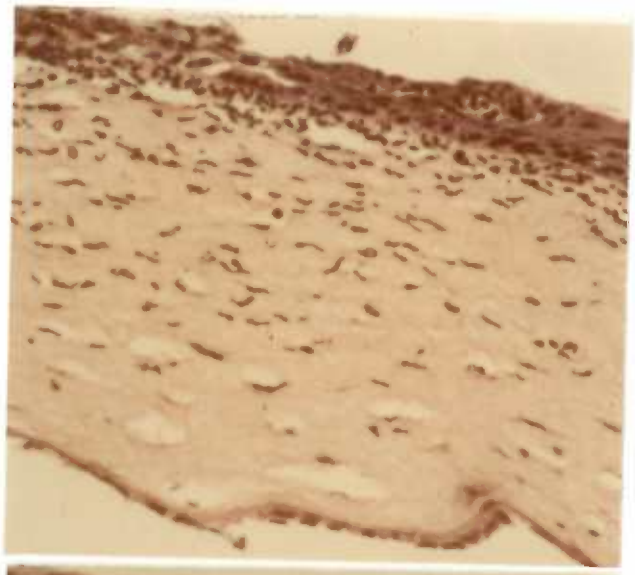


FIGURE 10

B

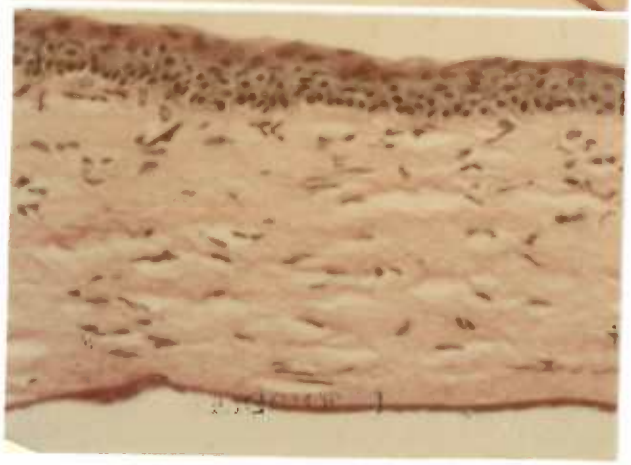


FIGURE 11

Cross sections of a rat cornea in Stage III of tyrosine induced keratopathy, 50 X magnification.

A. Hematoxylin and eosin stain of a section from the central cornea illustrating absence of the epithelial layer and increased corneal thickness.

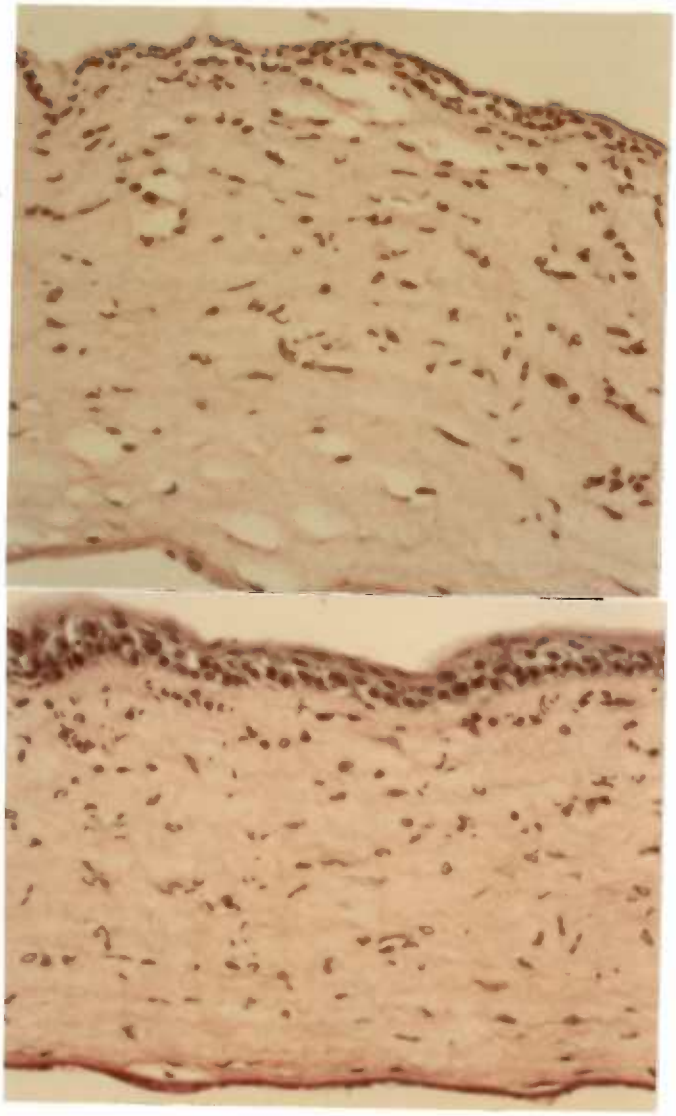
B. Periodic acid Schiff stain of a section from the peripheral area of the cornea.

FIGURE 12

Cross sections of a rat cornea in Stage IV of tyrosine induced keratopathy, 50 X magnification. Massive polymorphonuclear leucocyte infiltration, blood vessel ingrowth, and increased thickness are obvious.

A. Hematoxylin and eosin stain.

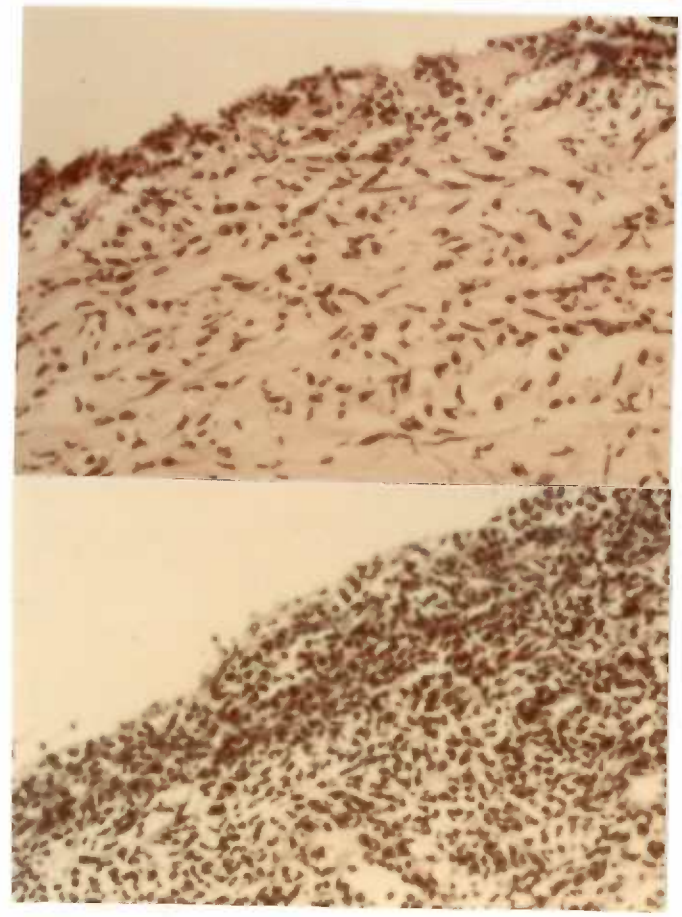
B. Periodic acid Schiff stain.



A

B

FIGURE 11



A

B

FIGURE 12

FIGURE 13

Cross sections of a rat cornea in Stage V of tyrosine induced keratopathy, 50 X magnification. The epithelial ulcers are beginning to heal. A single layer of squamous cells are beginning to form a new epithelium.

A. Hematoxylin and eosin stain.

B. Periodic acid Schiff stain.

FIGURE 14

Cross section of a rat cornea in Stage VI of tyrosine induced keratopathy, 50 X magnification. A return to near normal corneal architecture is demonstrated. Hematoxylin and eosin stain.

A

B

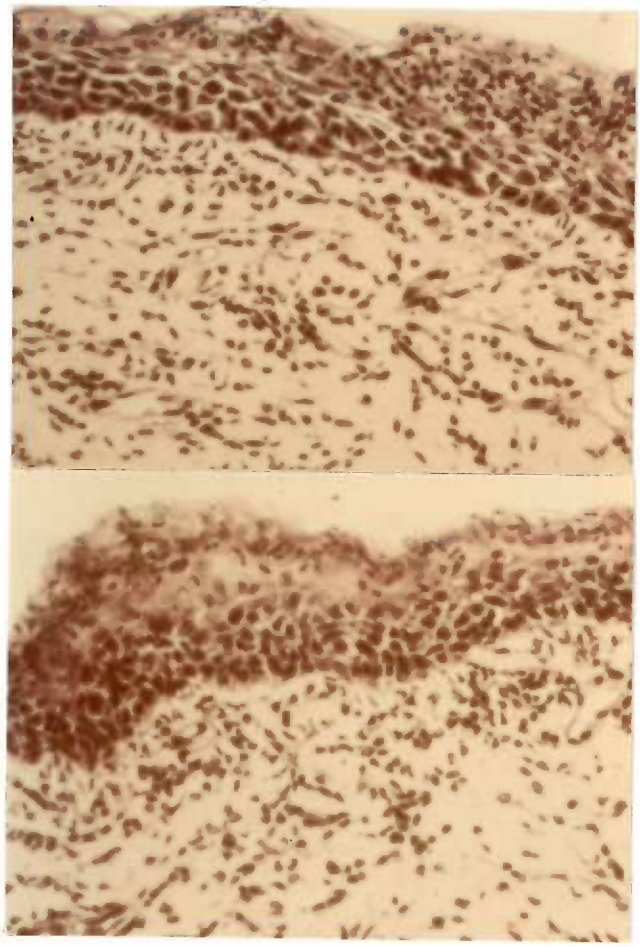


FIGURE 13

FIGURE 14

and its supporting basement membrane, and polymorphonuclear leucocytes (PMNs) infiltrated beneath the epithelium; in Stage III (Figure 11), the corneal epithelium began to desquamate peripherally and was absent centrally, more PMNs had invaded, and the stroma was markedly thickened; in Stage IV (Figure 12), numerous blood vessels were found in the stroma, and the PMNs had invaded massively; in Stage V (Figure 13), full thickness epithelial ulcers, sometimes covered by a single layer of squamous cells, were present, and epithelial regeneration was evident; and in Stage VI (Figure 14), the entire cornea had returned to a semblance of normal architecture.

III. Purpose

The present study was undertaken to characterize dose-response relationships to rats fed excess tyrosine by determining if:

(A). The development of the disease depends upon the dietary dose;

(B). Increasing dietary tyrosine causes a specific elevation in the tyrosine concentrations of blood and aqueous humor;

(C). The clinical stage of keratopathy depends upon either the serum tyrosine concentration or the length of time an animal is fed the added amino acid in its diet;

(D). There is any protective value of melanin in pigmented animals exposed to added dietary tyrosine since this amino acid is the precursor of melanin; and

(E). There is a phototoxic effect on the development of the lesion which might result from toxic, peroxide containing, free radicals liberated by ultraviolet light from pHPPA.

MATERIALS AND METHODS

I. Animals

The animals used were male and female young adult Simonsen albino and Long-Evans pigmented (agouti and black) laboratory rats. At the beginning of an experiment, each rat was labeled, weighed, and both of its eyes examined with a Haag-Streit 900 slit lamp to determine the initial condition of the eyes, particularly of the corneas. Food and water were available ad libitum; animals were housed in wire cages, 3 or fewer per cage. The ambient temperature was kept at 22 ° ± 2° and room lighting was maintained on a 12-hour on-12 hour off diurnal cycle.

II. Diet* (41)

The basic control diet consisted of the following:

1000 gms. boiled potatoes
652 gms. corn starch
75 gms. vitamin-free casein
50 gms. Brewer's yeast
40 gms. salt mixture W
50 ml. Cottonseed oil
20 ml. Cod liver oil

Either 99.7 gms. or 209.7 gms. of L-tyrosine were mixed with the above to make a 5% or 10% added tyrosine diet, respectively. The formula was then mixed well, pelleted, and dried at less than 150° F for 6-8 hours.

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* All ingredients except potatoes were obtained from Nutritional Biochemicals Co., Cleveland, Ohio.

III. Procedures

A. Experiment I

1). General

A total of 108 rats, weighing between 115-150 grams each, were separated into three groups of 36: Group A was fed the control diet without tyrosine added, Group B was fed the 5% tyrosine diet, and Group C was fed the 10% tyrosine diet. Daily, approximately six hours after the onset of light, two rats -- one pigmented and one albino, one male and one female, were selected at random from each of the three diet groups. These six animals were weighed, examined with the Haag-Streit slit lamp, and the eye disease graded according to the severity of the corneal pathology (see Preliminary Studies). The rat was then decapitated and blood collected without using anticoagulants. The blood cells were removed by centrifugation at 4,000 x g for 5 minutes and the serum carefully pipetted off, frozen, and stored until assayed.

Using a short beveled 30 gauge hypodermic needle attached to a 1 cc disposable tuberculin syringe, the anterior chamber of each eye was punctured and the aqueous humor aspirated, frozen and stored until assayed.

2). Tyrosine Assays

Tyrosine concentrations in the serum and aqueous humor were determined by the spectrofluorometric method of

Udenfriend (46) as modified by Wong, O'Flynn, and Inouye (49). This procedure is based upon the principle that tyrosine reacts with α-nitroso-β-naphthol to form a fluorescent product. The reaction is linear over the range of tyrosine concentrations found in this experiment. The validity of the fluorescence method as a measure of tyrosine in tissue fluids was confirmed when standard aqueous solutions and human serum, to which known amounts of tyrosine had been added, were assayed for total tyrosine (11) on the short column of a Biochrom Amino Acid Analyzer Model BD-200 (BioCal Instrument Co., Richmond, Calif.). Samples as small as 10μl. were assayed with a ± 5% accuracy.

The sera and aqueous humor samples were defrosted at 33° C and an aliquot of either 10, 15, 25 or 50 μl. was added to an equal quantity of 0.6 N trichloroacetic acid and centrifuged at 4,000xg for 5 minutes to remove the precipitated proteins. A measured quantity of supernatant was mixed with the α-nitroso-β-naphthol reagent* (1 part supernatant to 4 parts reagent) and incubated at 33° C for 20 minutes. This solution was diluted four times with water which was doubly distilled in glass and the unreacted reagent was removed by an

* The reagent was mixed before use by adding 2 volumes of a solution of α-nitroso-β-naphthol (200 mgm./100 ml. 95% ethyl alcohol) to 3 volumes of 3.0 N nitric acid and 3 volumes of 0.10 N sodium nitrite.

excess of ethylene dichloride. The aqueous phase was pipetted off and incubated at room temperature (about 23° C) for 60 minutes. The fluorescence at 545 mμ, using an activating wavelength of 460 mμ, was measured on a Farrand Mark I Spectofluorometer and expressed as milligrams % relative to a 20 mgm. % L-tyrosine standard solution.

B. Experiment II

Eighteen rats of random sex and pigmentation and weighing 180-210 grams apiece, were separated into three groups of six each. Group D was fed the control diet without tyrosine, Group E was fed the 5% tyrosine diet, and Group F was fed the 10% tyrosine diet. For the duration of the experiment, two animals of each group were exposed to either ultraviolet light (3250 Å wavelength), red incandescent light, or ordinary white fluorescent light in the normal diurnal cycle.

These lights were used to test the hypothesis that toxic free radicals released from pHPPA in the presence of oxygen by ultraviolet light might cause the disease or modify its progression. Red light was used in lieu of total darkness so that the normal diurnal cycle required by rats could be maintained. White light served as a control, being the normal environmental light source.

Using the Haag-Streit 900 slit lamp, the eyes of these rats were examined regularly and assigned a clinical stage based on the aforementioned criteria.

IV. Statistical Approach

Experiment I involved four independent (fixed) variables--dose of dietary tyrosine, sex, animal pigmentation, and time on diet--and two dependent (random) variables--serum tyrosine concentration and aqueous humor tyrosine concentration. Since any of the fixed variables or combinations of fixed variables could have had an effect on the independent variables, the experiment was designed to test all these possibilities. A multiple regression analysis was performed as an efficient method of statistically evaluating the contributions of these parameters (Appendix A). Table I indicates the designation given each variable. The validity of the clinical staging was tested by determining the correlation coefficient between assigned clinical stage versus the number of days on the experimental diet (Appendix B).

RESULTS

I. Experiment I

The experiments utilizing the 10% tyrosine diet could be carried out for no longer than 10 days because all the animals became severely ill and died prior to this time. Rats on the 5% diet survived through all six stages of the eye disease. Every rat on the control diet thrived, gained weight, and developed no eye changes even after as much as one month on the diet.

Figure 15 shows a plot of the clinical stage of keratopathy versus the number of days on the 5% or 10% tyrosine diet. The

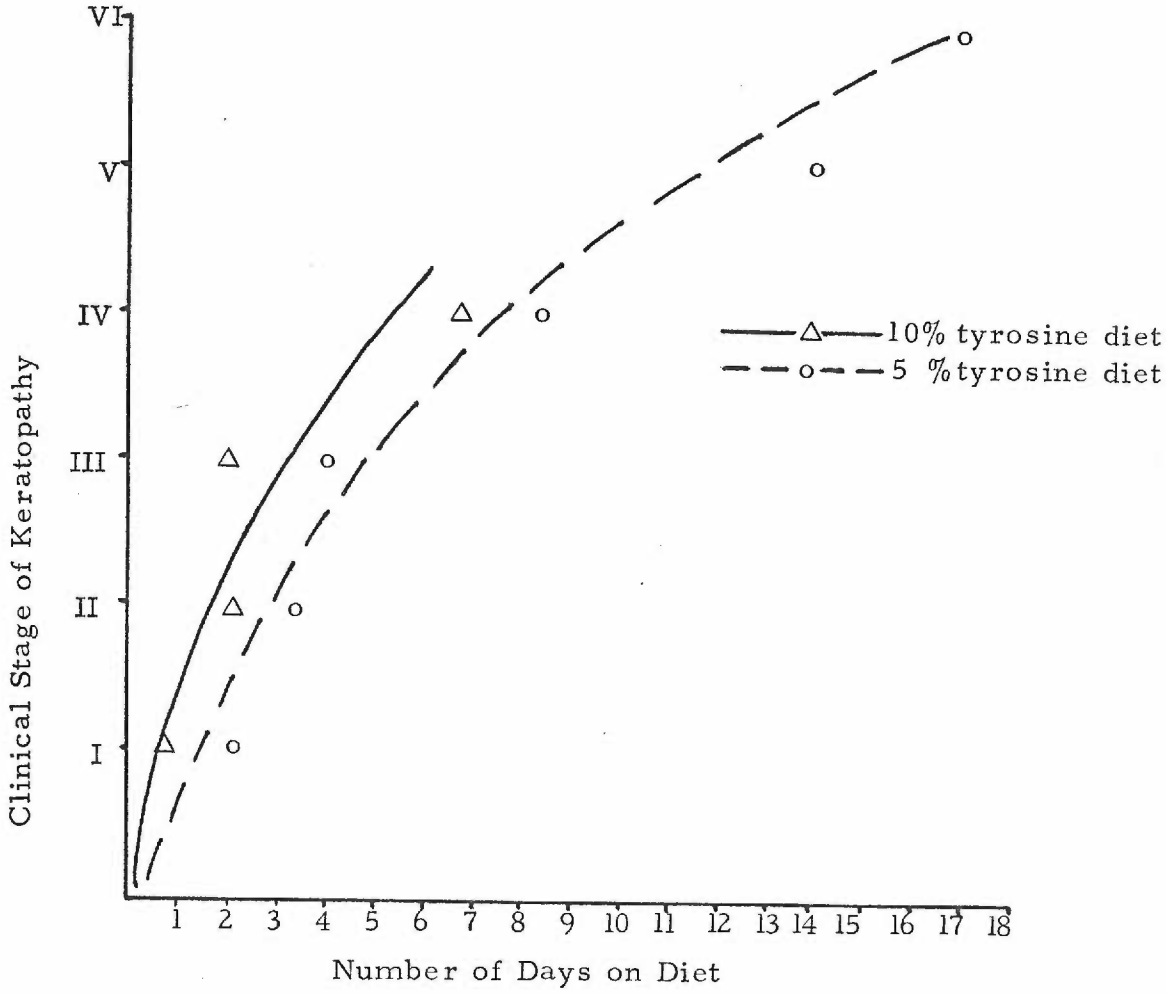
TABLE 1

Definition of Variables in the Multiple Regression Analysis

Variable Number	Variable
1	Dietary dose of tyrosine: 0%(control), 5%, or 10%
2	Animal pigmentation: albino or pigmented
3	Number of days on diet: 1-18
4	Serum tyrosine concentration in mgm. %
5	Aqueous humor tyrosine concentration in mgm. %
6	Stage of keratopathy: I-VI
7	Animal sex: male of female
8-17	Combinations of 1, 2, 3, 6, and 7

FIGURE 15

Graph of Clinical Stage of Keratopathy vs. Number of Days on Diet for rats fed either a 5% or 10% tyrosine diet.


VI
V
IV
III
II
I
Clinical Stage of Keratopathy
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Number of Days on Diet
10% tyrosine diet
5 % tyrosine diet

correlation coefficient of X (number of days) and Y (clinical stage) is 0.91 for both of these lines. This means that given the number of days on the high tyrosine diet, one can predict the clinical stage quite accurately. The corneal disease progresses through its clinical stages more rapidly in rats fed 10% dietary tyrosine than those fed 5%, but did not reach Stages V and VI because the animals did not survive beyond 10 days.

Although the severity of the disease, as indicated by clinical stage, increases with time on the diet, this is not a good predictor of serum tyrosine concentration (Figure 16). The mean serum tyrosine concentration rises and remains high throughout all stages.

The serum tyrosine concentration rises significantly ($p<0.001$) within 24 hours after the onset of feeding either experimental diet, but prolonging the number of days on the diet does not greatly improve the significance of this elevation (Table 2). Serum tyrosine continues to rise until death in rats fed the 10% tyrosine diet, but in those fed the 5% tyrosine diet, the initial rise levels off (Figure 17).

The dietary dose of tyrosine is the only independent variable which directly affects the serum tyrosine concentration. The presence or absence of pigmentation, the sex of the animal, and the amount of time the rat is fed an experimental diet did not add significantly to the elevated serum concentration caused by excess dietary tyrosine alone (Table 2). However, when all interactions of the variables were added in, there was an added significance (Table 3).

FIGURE 16

Graph of Serum Tyrosine Concentration in mgm % vs. Clinical Stage of Keratopathy.

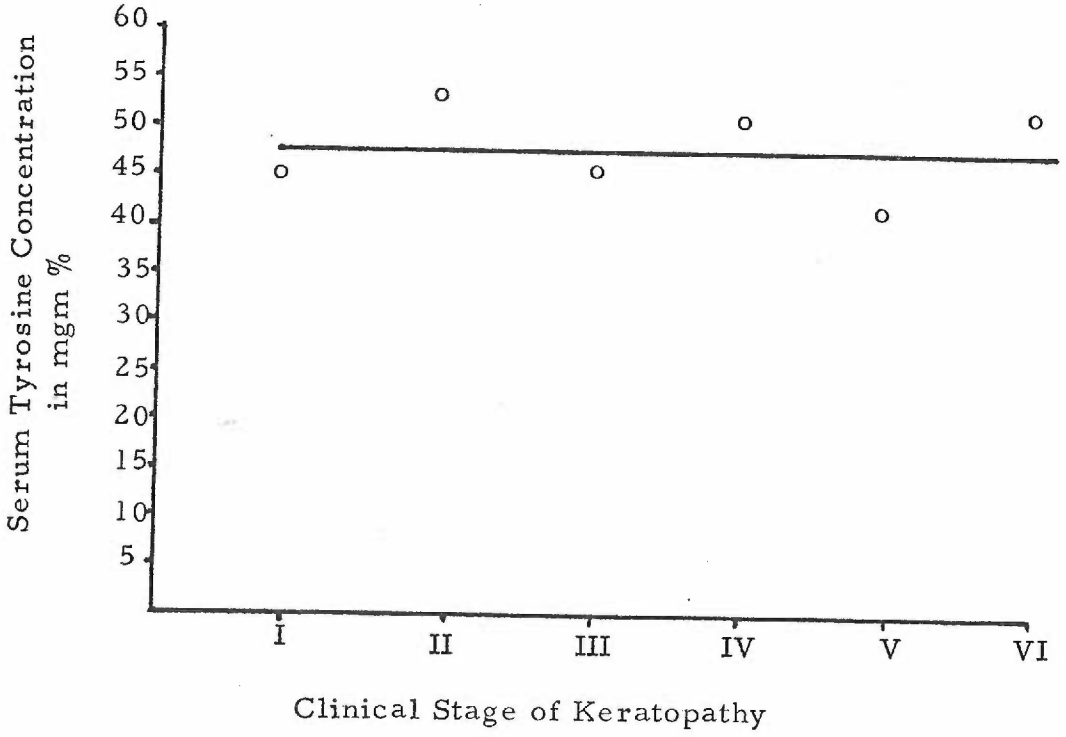

Serum Tyrosine Concentration
in mgm %
60
55
50
45
40
35
30
25
20
15
10
5
I
II
III
IV
V
VI
Clinical Stage of Keratopathy

TABLE 2

Analysis of Variance Table for Effect of Diet Dose on Serum Tyrosine

Source	DF	Sum of Squares	Mean Square	$F\left(\frac{\text{Regression Mean Square}}{\text{Residual Mean Square}}\right)$	Significance
Total	61	72203.8871	1083.67028		
Regression	1	40737.2327	40737.2327	$\frac{40737}{524} = 77.7$	$p<0.001$
Residual	60	31466.6544	524.444239		

r squared = .56419723

Analysis of Variance Table for Effect of Pigmentation on Serum Tyrosine

Source	DF	Sum of Squares	Mean Square	$F\left(\frac{\text{Regression Mean Square}}{\text{Residual Mean Square}}\right)$	Significance
Total	61	72203.8871	1183.67028		
Regression	1	20.451 4236	20.451 4236	$\frac{2.045}{1203.0} = 0.0016$	not significant
Residual	60	72183.4357	1203.05726		

r squared = .00028325

TABLE 2 Continued

Analysis of Variance Table for Effect of Number of Days Fed Diet on Serum Tyrosine

Source	DF	Sum of Squares	Mean Square	$F\left(\frac{\text{Regression Mean Square}}{\text{Residual Mean Square}}\right)$	Significance
Total	61	72203.8871	1083.67028		
Regression	1	280.839556	280.839556	$\frac{280}{1198} = 0.2337$	not significant
Residual	60	71923.0475	1198.71746		

r squared = .00388954

Analysis of Variance Table for Effect of Sex on Serum Tyrosine

Source	DF	Sum of Squares	Mean Square	$F\left(\frac{\text{Regression Mean Square}}{\text{Residual Mean Square}}\right)$	Significance
Total	61	72203.8871	1183.67028		
Regression	1	82.1497059	82.1497059	$\frac{8.21}{1202.0} = 0.0068$	not significant
Residual	60	72121.7374	1202.02896		

r squared = .00113775

FIGURE 17

Graph of Serum Tyrosine Concentration in mgm % vs. Number of Days on Diet for rats fed either the 5% or 10% tyrosine diet.

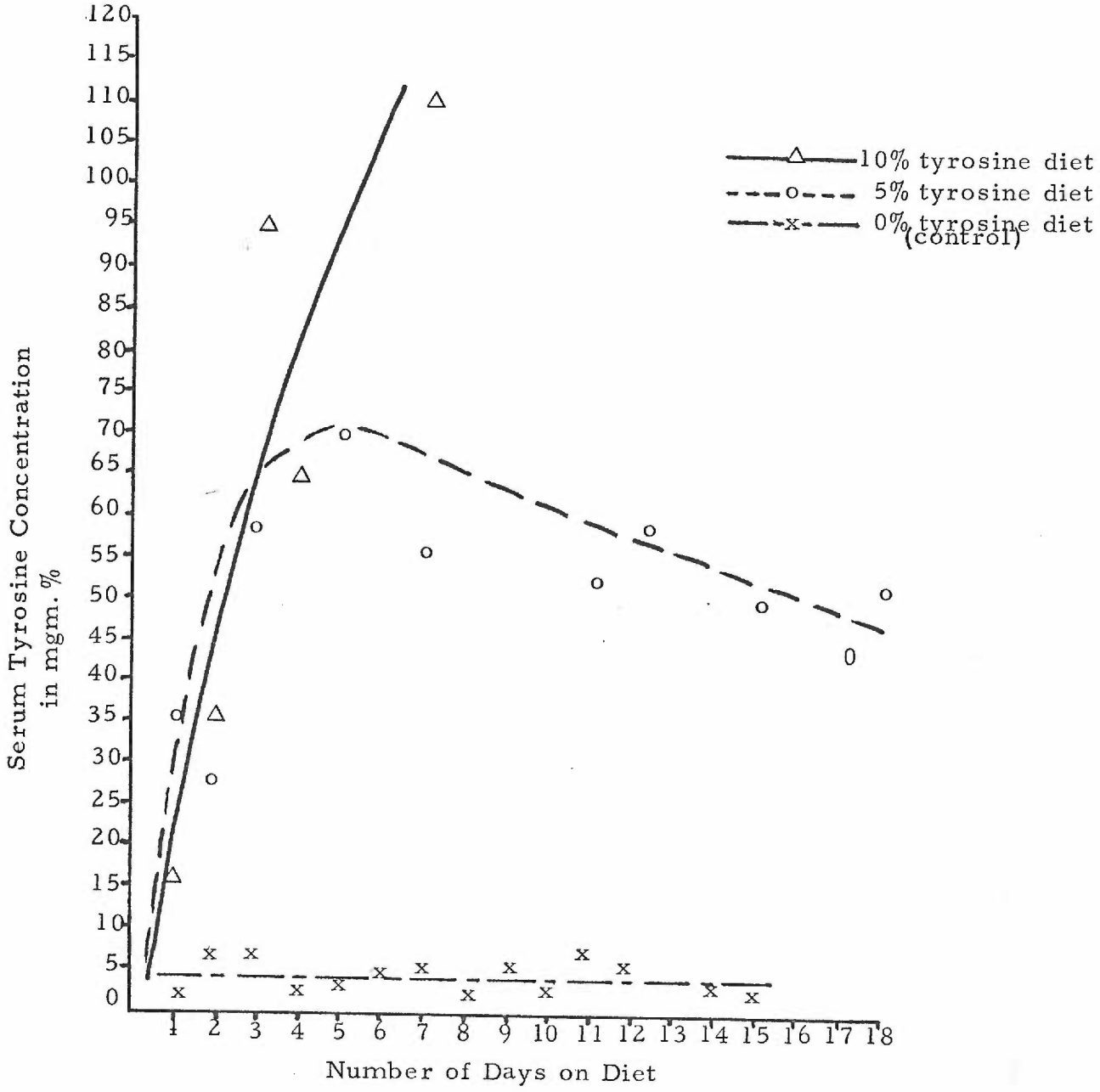

Serum Tyrosine Concentration in mgm. %
120
115
110
105
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Number of Days on Diet
10% tyrosine diet
5% tyrosine diet
0% tyrosine diet
(control)

TABLE 3

Analysis of Variance Table for Effect of Variables 1, 2, 3, 6, and 7 on Serum Tyrosine

Source	DF	Sum of Squares	Mean Square
Total	61	72203.8871	1183.67028
Regression	5	46745.0491	9349.00983
Residual	56	25458.838	454.622106

Analysis of Variance Table for Effect of All Variables and All Interactions of Variables on Serum Tyrosine

Source	DF	Sum of Squares	Mean Square
Total	61	72203.8871	1183.67028
Regression	15	56488.1898	3765.87932
Residual	46	15715.6973	341.645593

r squared = .78234278

For the Contribution of Interactions of Variables on Serum Tyrosine Concentration

$$F = \frac{56488.1898 - 46745.0491/10}{341.645593} = 2.8518 \qquad p < 0.01$$

(For method of calculation, see Appendix C).

The concentration of tyrosine in aqueous humor is affected in a manner similar to that of the serum (Figure 18). The initial rise in tyrosine concentration is followed by a leveling off effect, but here this phenomenon occurs with either the 5% or 10% tyrosine diets. With both diets, the increase in aqueous humor tyrosine is greater than that found in the serum.

Again, only the dietary dose of tyrosine significantly ($p<0.05$) elevates the tyrosine recovered from the aqueous humor. The other dependent variables (Table 4) had little or no effect on the tyrosine elevation. The interactions of these variables did add to the significance, however (Table 5).

II. Experiment II

Figure 19 shows the relationship between the clinical stage of keratopathy and the number of days on the diet (5% and 10% tyrosine diets are taken together) for the rats in each light environment. The disease progressed through the clinical stages at the same rate regardless of the light source illuminating the animals.

DISCUSSION

This investigation demonstrates that a dietary tyrosine load will cause an elevation in serum and aqueous humor tyrosine concentrations. That this rise occurs in a period of hours after the initiation of the dietary stimulus is evidence that the existing TAT acitvity is not sufficient to metabolize this huge excess. The elevation continues as long as the dietary stimulus remains, and the

FIGURE 18

Graph of Aqueous Humor Tyrosine Concentration in mgm % vs. Number of Days on Diet for rats fed either the 5% or 10% tyrosine diet.

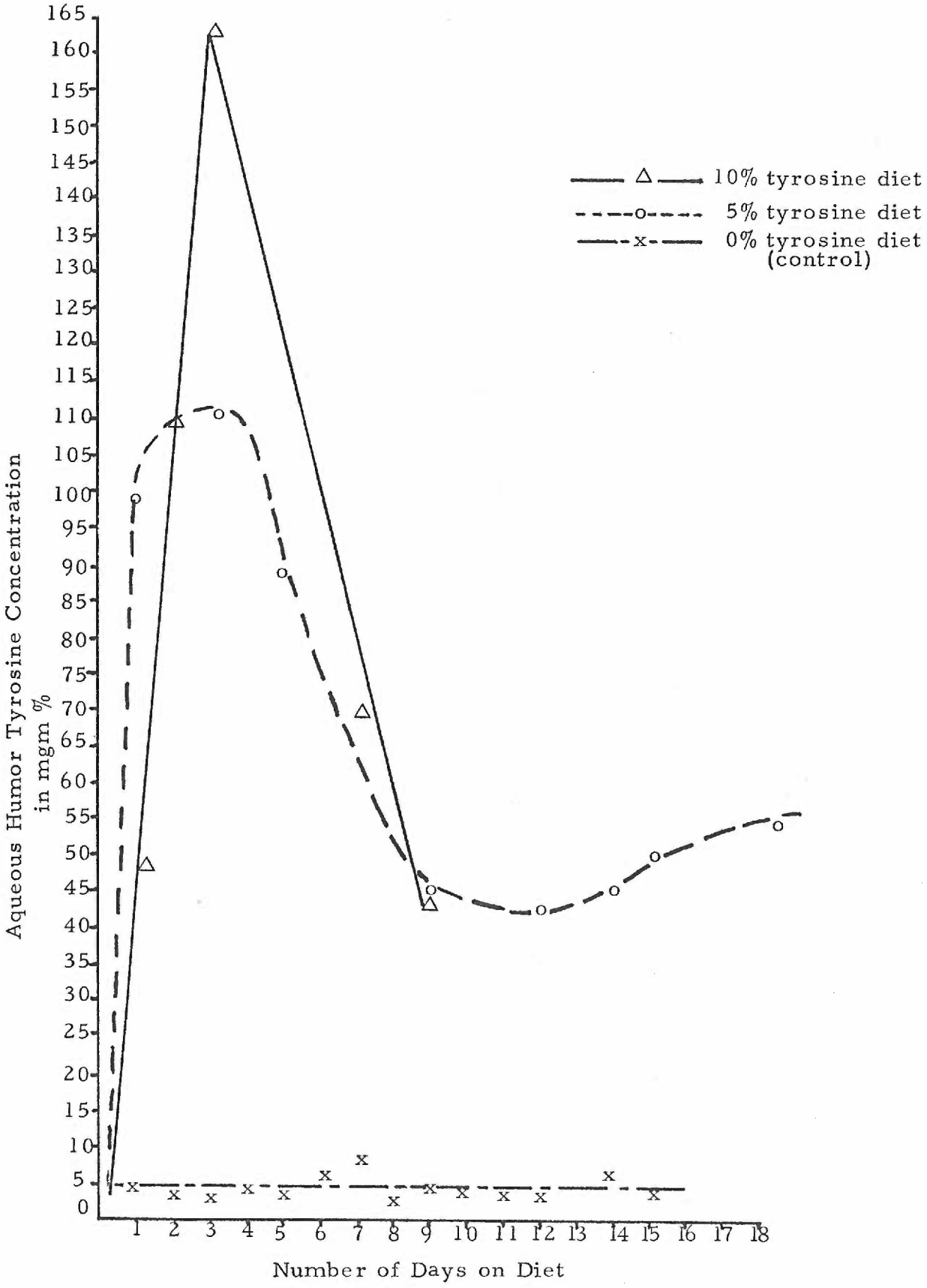

Aqueous Humor Tyrosine Concentration in mgm %
Number of Days on Diet
10% tyrosine diet
5% tyrosine diet
0% tyrosine diet (control)

TABLE 4

Analysis of Variance Table for Effect of Diet Dose on Aqueous Humor Tyrosine

Source	DF	Sum of Squares	Mean Square	$F\left(\frac{\text{Regression Mean Square}}{\text{Residual Mean Square}}\right)$	Significance
Total	51	100717.442	1974.85181		
Regression	1	54013.2076	54013.2076	57.8247	$p<0.001$
Residual	50	46704.2347	934.084693		

r squared = .53628454

Analysis of Variance Table for Effect of Pigmentation on Aqueous Humor Tyrosine

Source	DF	Sum of Squares	Mean Square	$F\left(\frac{\text{Regression Mean Square}}{\text{Residual Mean Square}}\right)$	Significance
Total	51	100717.442	1974.85181		
Regression	1	20.1059017	20.1059017	0.009	not significant
Residual	50	100697.336	2013.94673		

r squared = .00019963

TABLE 4 Continued

Analysis of Variance Table for Effect of Number of Days Fed Diet on Aqueous Humor Tyrosine

Source	DF	Sum of Squares	Mean Square	F $\left(\frac{\text{Regression Mean Square}}{\text{Residual Mean Square}}\right)$	Significance
Total	51	100717.442	1974.85181		
Regression	1	3007.40927	3007.40927	1.5389	not significant
Residual	50	97710.0330	1954.20066		

r squared = .02985987

Analysis of Variance Table for Effect of Sex on Aqueous Humor Tyrosine

Source	DF	Sum of Squares	Mean Square	F $\left(\frac{\text{Regression Mean Square}}{\text{Residual Mean Square}}\right)$	Significance
Total	51	100717.442	1974.85181		
Regression	1	433.483976	433.483976	0.2161	not significant
Residual	50	100283.958	2005.67917		

r squared = .00430396

TABLE 5

Analysis of Variance Table for Effect of Variables 1, 2, 3, 6, and 7 on Aqueous Humor Tyrosine

Source	DF	Sum of Squares	Mean Square
Total	51	100717.442	1974.85181
Regression	5	64347.1752	12869.435
Residual	46	36370.2671	790.657980

r squared = .63888810

Analysis of Variance Table for Effect of All Variables and All Interactions of Variables on Aqueous Humor Tyrosine

Source	DF	Sum of Squares	Mean Square
Total	51	100717.442	1974.85181
Regression	15	88071.9453	5871.46302
Residual	36	12645.4970	351.263805

r squared = .87444581

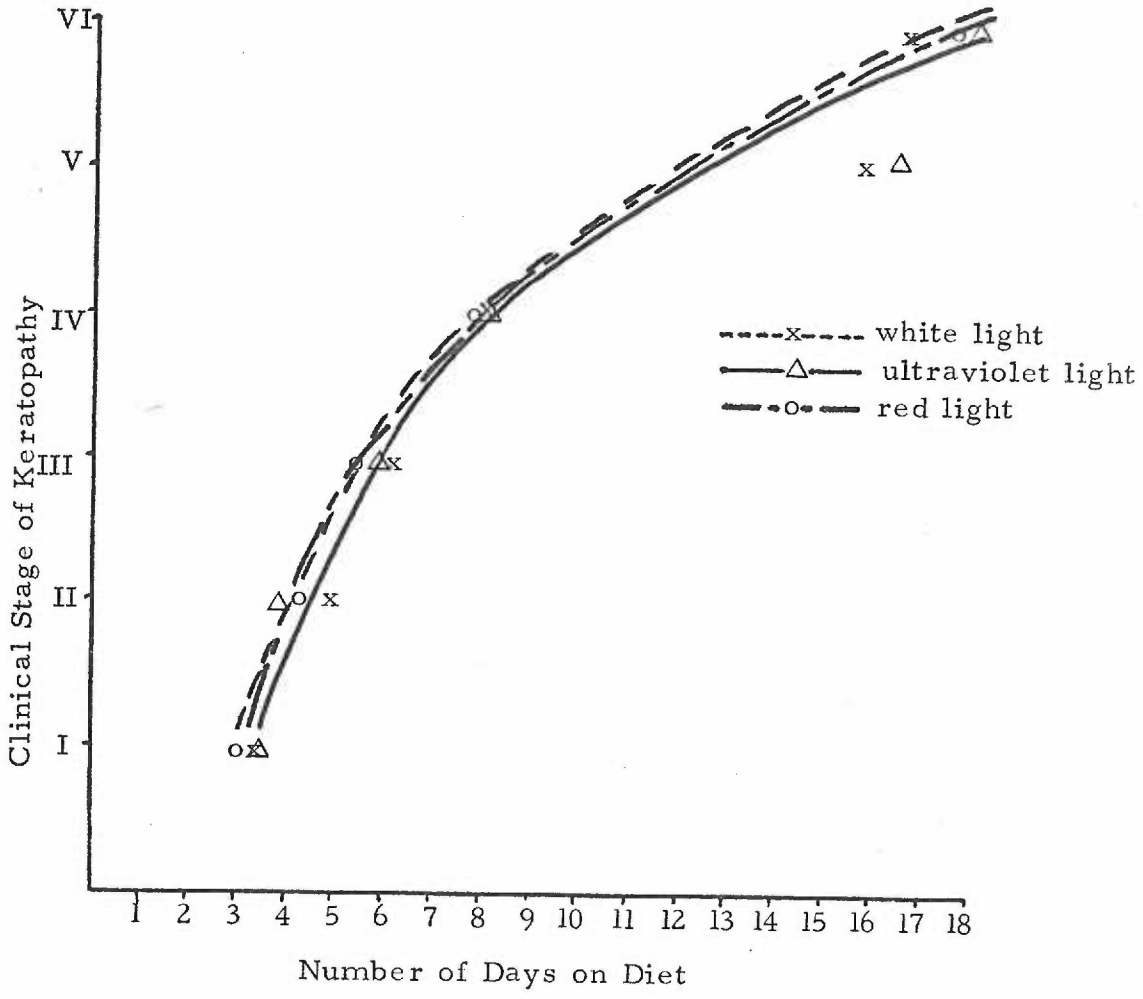
For the Contribution of Interactions of Variables on Aqueous Humor Tyrosine Concentration

$$F = \frac{88071.9453 - 64347.1752/10}{351.263805} = 6.7541 \qquad p < 0.001$$

(For method of calculation, see Appendix C).

FIGURE 19

Graph of Clinical Stage of Keratopathy vs. Number of Days on Diet for rats in three light environments: red, ultraviolet, or white light.


VI
V
IV
III
II
I
Clinical Stage of Keratopathy
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Number of Days on Diet
white light
ultraviolet light
red light

mean serum tyrosine concentrations are similar at all clinical stages. Therefore, it seems that once the serum tyrosine level reaches a critical value, the clinical signs of corneal disease become manifest but progress only if the high level of tyrosine is maintained.

Although the clinical stage of keratopathy could not predict the serum level of tyrosine, the close correlation ($r=0.91$) between the designated clinical stage and the number of days on the diet indicates that this staging system is an accurate means of determining the length of time the serum tyrosine has been elevated.

The statistical tests show conclusively that the dietary dose of tyrosine is the only important factor in elevating tyrosine in the aqueous humor and serum. Although tyrosine is a precursor of melanin (33, 34), a pigmented rat which possesses melanin-producing enzymes has no advantage over an albino which lacks these enzymes. This confirms what is already known about the preferred metabolic pathways of tyrosine, namely, that the melanin-forming pathway is not the major route for tyrosine degradation and cannot assimilate a large excess of that amino acid (33).

Interactions of pigmentation, sex, dietary tyrosine dose, and time on the diet added significance to the elevation in serum or aqueous humor tyrosine caused by dietary tyrosine excess alone, but this is not surprising considering that there were so many interaction terms. Nevertheless, the tyrosine concentration in the

serum or aqueous humor of a male pigmented rat fed a 5% tyrosine diet for 2 days has an equal probability of being elevated as that of a female albino rat fed the diet for 10 days.

Tyrosine is known to induce TAT in the presence of adrenal steroids (22, 24, 29). In rats fed the 10% tyrosine diet the serum tyrosine concentration continued to rise until death. Therefore, any TAT induced seems to have been insufficient to metabolize the excess amino acid. In contrast, those rats fed the 5% tyrosine diet seemed to have had successful adaptive enzyme response because the serum tyrosine concentration leveled off. Assays of hepatic enzymes in these rats indicate that TAT activity is elevated and pHPPAO is depressed in those animals fed excess dietary tyrosine.*

The ciliary body epithelium produces aqueous humor and is known to concentrate and secrete a variety of ions into this fluid (6). The quantity of tyrosine recovered from the aqueous humor compared with that from the serum in both normal and experimentally altered rats suggests but does not prove that the ciliary body is actively transporting tyrosine.

Experiment II shows that the corneal disease is not influenced by the wavelengths of light to which tyrosine fed rats are exposed. In particular, ultraviolet light had no effect. This indicates that the corneal disease is not the result of a phototoxic effect or of the presence of hydroperoxide containing free radicals liberated by ultraviolet light from pHPPA.

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*Fellman, J. H., Personal Communication. 1972

SUMMARY AND CONCLUSIONS

It has been shown that rats fed added dietary tyrosine have a significant elevation of serum and aqueous humor tyrosine concentrations. A clinical and microscopic staging system has been devised which is accurate in defining how long these tyrosine elevations have existed. With higher dietary doses of tyrosine, the clinical stage progresses more rapidly.

Neither sex, pigmentation, nor amount of time on the diet cause a significant increase in serum or aqueous humor tyrosine over that induced by added dietary tyrosine alone. The presence of pigmentation, furthermore, confers no protective value on an animal exposed to a high tyrosine diet. The toxic element in this corneal disease, although still unidentified, does not depend on the presence of ultraviolet light to cause the ocular pathology.

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APPENDIX A

Regression analysis attempts to describe the relationship between two variables X and Y. A mathematical function (f) is found which when applied to a given value of an independent variable X, predicts a value of the dependent variable Y. A simple regression function is a linear relationship between X and Y and would be expressed by the formula

$$Y = a + bX$$

in which a is the Y axis intercept and b is the slope of the function line. If several variables influence Y, then regression on Y is performed using all X's simultaneously. This is multiple regression analysis and it is accomplished by utilizing the equation

$$Y = a + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 \cdots$$

where again, a is the Y axis intercept, β_1 indicates the regression coefficient of Y on variable X_1 if the other variables ($X_2 X_3 \cdots$) were kept constant in the experiment, β_2 defines the regression coefficient of Y on variable X_2, etc. Thus, a more precise prediction of Y from all X's can be obtained. The statistical significance of this function (f) is then determined by applying Fisher's F test. The F value for each function is obtained by dividing the mean square due to regression by the residual mean square and comparing this quantity with values from standard F tables to obtain probability values.

APPENDIX B

The correlation coefficient r, otherwise known as the product moment correlation coefficient, is a statistical test used to analyze the significance of the relationship between two variables, X and Y. The value of r falls between +1 (perfect positive correlation) and -1 (perfect negative correlation) and a value of 0 represents no correlation. The calculation of r is

$$r = \Sigma x \cdot y / (n-1) S_x S_y$$

where x and y are individual values of X and Y, respectively; S_x and S_y are standard deviations of x and y, respectively; and (n-1) represents the number of degrees of freedom.