Effect of L-Phenylalanine and L-Tyrosine on The Toxicity of Mimosine to Rats*

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SUMMARY:

Mimosine is a toxic amino acid to general species of animal and to germination of mung bean. Both l-phenylalanine and l-tyrosine had been suggested that these two amino acids could reduce the toxicity of mimosine to rats. In this work, it was found that mimosine did not act as the competitive inhibitor of these two amino acids with regard to growth rate and cataract. Furthermore, the estimation of lens glutathione contents of all six groups of rats, could be used to support that the cataractogenic property of mimosine could no tbe reduced by l-tyrosine and l-phenylalanine.

L-Phenylalanine及 L-tyrosine 對於 Mimosine 毒性之影響:

Mimosine 係由銀合歡種子所提出的一種毒蛋白,它對於一般動物以及對於綠豆之發芽均有顯著的毒性存在。過去有人認爲 L-phenylalanine 及 L-tyrosine 可以阻抗 mimosine 之毒性,但在本篇報告中却證明 Mimosine 並非上述二種胺基酸在蛋白質合成對抗阻抑因子。同時老鼠眼球中之 Glutathione 之含量測定實驗來看亦足以支持如下之結論:mimosine 所引起之白內障,此種性質並非 L-tyrosine 或 L-phenyalanine 所能阻抗。

This work was submitted by the author to the Graduate Institute of Biochemistry of National Taiwan University in partial fulfillment of the requirements for the degree of Master of Science.

INTRODUCTION

Mimosine, 3-hydroxy-4-OXO-1 (4H)-Pyridinealanine, $C_8H_{10}N_2O_4$, is isolated from Leucena Glauca (Willd) Benth (L.G.B.) (1) (2). It is toxic to general species of animals and to germination of mung bean. The first report that animal was suffered from intoxication with symptoms of hair loss and growth retardation of the ingestion of the seeds of Leucana Glauca Benth was made by Norris in 1897.

Matsumoto. et al. (3) (1951) reported that the addition of ferrous sulfate to the ration containing leaves or seeds of the L.G.B. was effective in reducing the toxicity.

The clinical symptoms such as stunted growth, alopecia of varying degree and cataracts in young rats fed on mimosine as a dietary supplement was reported by Sallmann, et al. (4) (1959).

Lin, et al. (5) (1961) repored that a diet containing1% mimosine killed the mice in four weeks and at 0.5% mimosine resulted the same symptorms as described by Sallmann, et al. (4). If mimosine acts antimetabolite in amino acids metabolism, the toxicity by mimosine must be antagonized by aromatic amino acids, such as, phenylanine and tyrosine. The reason is that mimosine bears a structure resemblane to phenylalanine and tyrosine.

Lin, et al. (6) (1964) found that addition of 1% of DL-phenylalanine to the diet containing 0.5% mimosine could antagonize 37% of the growth inhibition caused by mimosine, and that addition of 0.5% L-phenylalanine to the basal ration containing 0.4% mimosine showed slight recovery (35%) from growth inhibition while 1% L-phenylalanine antagonize the growth inhibition caused by 0.4% mimosine. 1% L-tyrosine could completely recover the growth retardation.

Yang (7) (1968) also showed that L-tyrosine posessed anticataractogenic property when administered prophylactically and can alleviate the mimosine intoxication both hair loss and arrested growth, and that the ocular changes were manifest if 1% DL-phenylalanine was added to the diet containing 0.5% mimosine. Other symptoms also enhanced by addition of 1% DL-phenylanine.

On the contrary Chen (8) (1968) showed that basal ration supplemented with tyrosine could not reduce the toxicity of mimosine when the ground Tai-Tan diet was taken place by 18% casein diet. Owing to these contradict results, it is felt worth while to repeat the experiment on the effect of L-phenylalanine and L-tyrosine on the toxicity of mimosine to rats.

Determination of ascorbic acid and glutathione in lens was also carried out.

MATERIALS AND METHODS

I. Exp. I. Feeding Experiment

Isolation of mimosine from seeds of Leucaena Glauca Benth was followed as described previously. (8)

To 8 kg of L. G. B. seeds washed with tapwater, 24 litesrs of 80°C water was added to soften the waxy crest. After standing 6-8 hrs, with suitable amount of water seeds blended in a Waring Blendor for 1-1.5 mins. The sticky juice was put into a tin coated tank added with more water to make the final water volume to 60 liters following addition of 50 liters 95% alcohol. After standing at least 24 hrs. with occasional stirring the mixture was filtered. Recovered alcohol from filtrate with fractional distillation. The step is not only for the recovery of alcohol but also to insure the precipitation of protein and impurities from the filtrate which after cooling was concentrated to 1/10 of its volume by heating at 60-65°C in a rotary evaporator.

The crude mimosine precipitated (140 gm), which was dissolved in 300 ml of 3N HCl then with adding saturated NaOH solution to PH 4.7 (PI of mimosine) mimosine precipitated again. After standing at 4°C for 1-2 hrs, it was filtered. The acid-alkali treatment repeated once more,then recrystallized from boiling water twice, the 27 gm. of almost colorless mimosine was obtained. Twenty grams more mimosine was recovered from the mother liquid.

Total yield of mimosine was 0.59%. Mimosine recrystallized from water five times gives colorless needles, m.p. 226-228°C (Cor.) with decomposition. The U.V. absorption spectrum is the same as drawn by Adams, et al. The absorption maximum of its purple ferric complex is at 535mμ.

L-phenylanine: Purchased from Nutritional Biochemicals Corporation, Ohio, U. S. A.

L-Tyrosine: Purchased from Mead Johnson & Co. Indiana, U. S. A.

Salt mixure: shown in Table II

Vitamine mixture: shown in Table III

Dasal Diet: shown in Table IV

From 50 male weanling rats of Long-Evans R weighing 44.3-69.7g, donated by Animal Quarter ; US-NAMRU-2 Taipei, thirty rats were selected and divided into 6 groups after feeding on the basal diet for three days. Each group was raised in one cage. In this experiment, one group was fed on the basal diet (Control). The remaining groups were fed on the basal ration with addition of various amount of mimosine and other amino acids (indicated in Table I). Food and distilled water were provided *ad libidum*. The rats were weighed every other day. Food consumption was counted every day. Water intake was measure twice a week. Ocular change was examined with Goldmann slit lamp biomicroscope weekly in he Department of Ophthalmology, University Hospital National Taiwan University, by

Dr. W.F. Tsai and Dr. Y.T. Shen. One percent of atropine solution was applied for the preparation of mydriasis. And the rats were examined at consciousness.

Table I. The Groupings of Rats and Composition of Diet in Exp. I.

Group	Rats/Group	Initial Body wt.(aveage)	Diet
B	5 male	52.1	Basal diet
M	5 male	61.5	Basal diet containing 0.5% mimosine
P	5 male	54.8	Basal diet containing 1.0% L-phenylalanine
PM	5 male	56.5	Basal diet containing 1.0% L-phenylalanine and 0.5% mimosine
T	5 male	54.6	Basal diet containing 1.0%L-tyrosine
TM	5 male	58.7	Basal diet containing 1.0% L-tyrosine and 0.5% mimosine

Table II. Hawk-Oser Salt Mixture*

*Science, **74**, 369. 1931

Ca-Citrate.$4H_2O$		308.2
$Ca(H_2PO_4)_2.H_2O$		112.8
K_2HPO_4		218.7
KCl		124.7
NaCl		77.0
$CaCO_3$		68.5
3 $MgCO_3.Mg(OH)_2.3H_2O$		35.1
$MgSO_4$(anhydrous)		38.3
Fe (NH_4) Citrat (U.S.P)	91.41	
$CuSO_4.5H_2O$	5.98	
NaF	0.76	
$MnSO_4.2H_2O$	1.07	16.7
$KAl(SO_4)_2.12H_2O$	0.54	
KI	0.24	
	100.00	1000.00 gm.

Table III. Componsition of Vitamin mixture

Corn Starch	442.5 g
Thiamine-HCl	100 mg
Riboflavine	150 mg
Pyridoxine-HCl	125 mg
Nicotinic acid amide	1 g
Ca-pantothenate	1 g
Inositol	5 g
Choline chloride	50 g
Menadione	50 mg
Biotin	5 mg
Folic aoid	50 mg
Cyano cobalamine	5 mg

Table IV. Compsition of Basal Diet in Experiment I

Casein	18%
Corn starch	62%
Peanut Oil	10%
Methyl cellulose	4%
Salt mixture	4%
Cod-liver oil	1%
Vitamin mixture	1%

Exp.II

Determination of Lens Ascorbic Acid and Lens Glutathione of Rats.

Eyes of rats were taken at the end of Exp. I. and were divided two groups, e.g. left and right eyes group. Determinations of ascorbic acid and glutathione concentration were run for each group. Then average values were calculated.

A) Determination of Ascorbic Acid by Photometric Method (9). The method is based on measurment of decolorization of 2, 6-dichlorophenolindophenol solution by ascorbic acid. Optical density was read with Beckman model DU Spectrophotometer.

1. Sample Preparation
   a) Homogenize five lens with 2 ml 6% HPO_3 in a Teflon homogenizer.
   b) Add 1 ml citrate buffer*1 and adjust PH to 3.5-3.7 with 6% HPO_3, and make the final volume to 10 ml with distilled water.
   c) Centrifuge off the precipitate.
   d) A reagent blank was made by diluting 5 ml of 6% HPO_3 and 1 ml of citrate buffer to 10 ml with water.
2. Photometric Determination of Ascorbic acid.
   a) Place a cuvette containg 1 ml of dilute dye solution*2 in the instrument and run 1 ml of the buffer sample extract rapidly into the dye solution. Record the optical density after 5 seconds against distilled water (0.D=O) at 520mμ.
   b) Place another cuvette in the instrument, add 1 ml of dilute dye solution and 1 ml of reagent blank. Read the optical density after 5 seconds, againt distilled water(0.D=0).
   c) Repeat the same procedure with the standard ascorbic acid to constructe the standard curve is the range from 1 μg/ml to 4 μg/ml.

B) Determination of Glutathione by Nitroprusside Reaction:
The method of Grount and Phillips(10) modified by R.W. von Korff's was used.

1. Sample Preparation:
   Homogenize 5 eyes of each group with 2 ml H_2O in a Teflon homogenizer, then the homogenate was transfered to a test tube and 3 ml H_2O was added to the homogenizer for washing, which was combined with homogenate. Add

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*1 Citrate buffer:29.4 g. of citric acid dissolved in 140 ml of 2.0N NaOH (carbonate-free) and make the final volume to 250 ml. with redistilled water.

*2 Dilute dye solution:
Dissolve 200mg of Sodium Salt of 2,6-dichlorophenol indophenol (Distillation Products Industries, Eastman Organic Chemicals Dept., Rochester 3,N.Y. in approximately 150 ml of hot redistlled water to 200 ml and dilute to the stook solution into 250-ml columetic flask and dilute to volume with redistilled water.

0.8ml of 10% Trichloroacetic acid to 0.8 ml of homogenate, and centrifuge for 10 min to remove the precipitate.
The supernatant was used for specimen.

2) Photometric Method:
Beckman Model DU Spectrophotometer was used for measurements.
Mix 2 ml. of staurated NaCl solution, 0.4 ml of N $NaCO_3$-NaCN solution*, and 0.4 ml of Na-nitropusside solution (27 mg/ml). Immediately add 0.2 ml specimen, mix and read against distilled water at 520mμ.
It is necessary to take the reading at a constant interval after mixing, say 20—30 sec.

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* Na_2CO_3-NaCN solution:
Add 21.2g. Na_2CO_3(anhy,)and 0.44 gNCN in 100ml distilld water.

RESULTS

I) *Feeding Experiment*

The growth curves of rats on variuos diets in Expt. I. are indicated in Fig 1,2, and 3. The differences of growth rates between each other group were significant in statistical view. Growth retardations were obtained in Group M, TM, and PM which were fed on the diet containing mimosine or mimosine plus L-tyrosine, or mimosine plus L-phenylalanine in comparing with the growth of the group of their counter part, which namely fed the basal or basal plus L-tyrosine or basal plus L-phenylanine. The growth of the group fed on L-tyrosin plus basal (Group T) was better than that fed on the basal diet (Group B). The growth of the latter, on the other hand, was better than that fed on the basal plus L-phenylanine (Group P). The growth of the group fed on L-tyrosine plus mimosine (Group TM) was better than that fed on the mimosine diet (Group M) and the growth of the latter group was better than that fed of L-phenylalanine plus mimosine (Grtoup PM).

Addition of mimosine to the diet of Group B or Group T or Group P decreased the intake of both food and water in comparing with their counter part group, namely, B, T, P Group, respectively. Addition of L-tyrosine could increase the uptake of both food and water, in comparing with the group fed on basal diet. Although addition of L-phenylalaninine showed the growth retardation in comparing with that fed on basal diet, the uptake of food and water was not affected.

II. *Observation of Alopecia and Ocular Change in Exp.I.*

Alopecia and cataract, symptoms of the toxicity of mimosine to rats, occured after feeding on the diet which mimosine added after 12 days, or one week respectively. Results are shown in Table VI and VII. In additon of the growth inhibition caused by mimosine, both alopecia and cataract were not recovered by the addtition of L-tyrosine or L-phenylalanine.

III. *Determination of Ascorbic Acid and Glutathione Content in Lens of Rats.*

The measurements of the optical density with spectrophotometer for determinating the ascorbic acid content in lens of rats were shown in Table VIII. It was failure to estimate the contents of ascorbic acid for each group.

The average content of glutathione in lens of rats are indicated in Table IX. L-phenylalane and L-tyrosine could increase the glutathione content of rats after administering mimosine, but could not recover to the normal level.

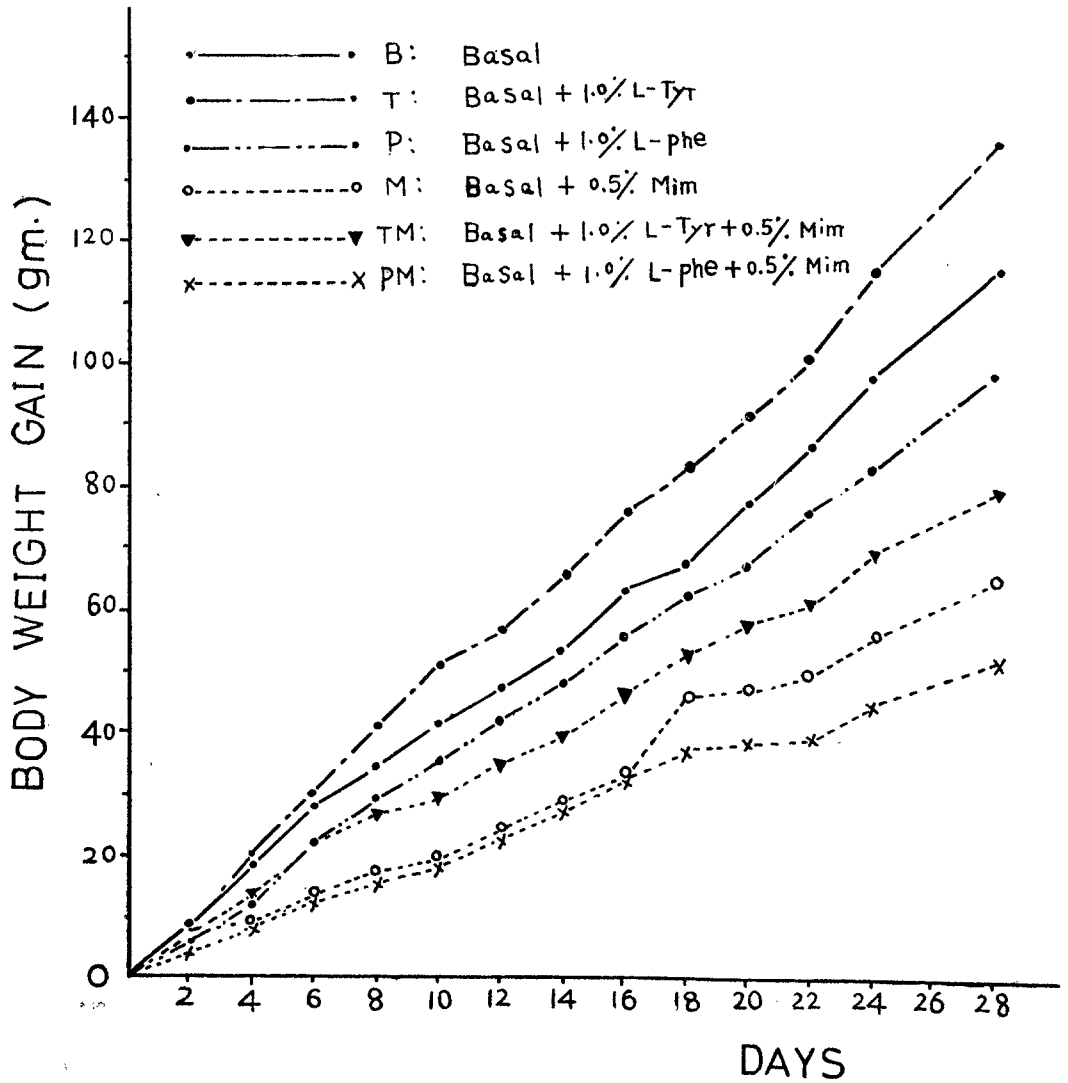

Fig. I Growth Curves Of Exp. I

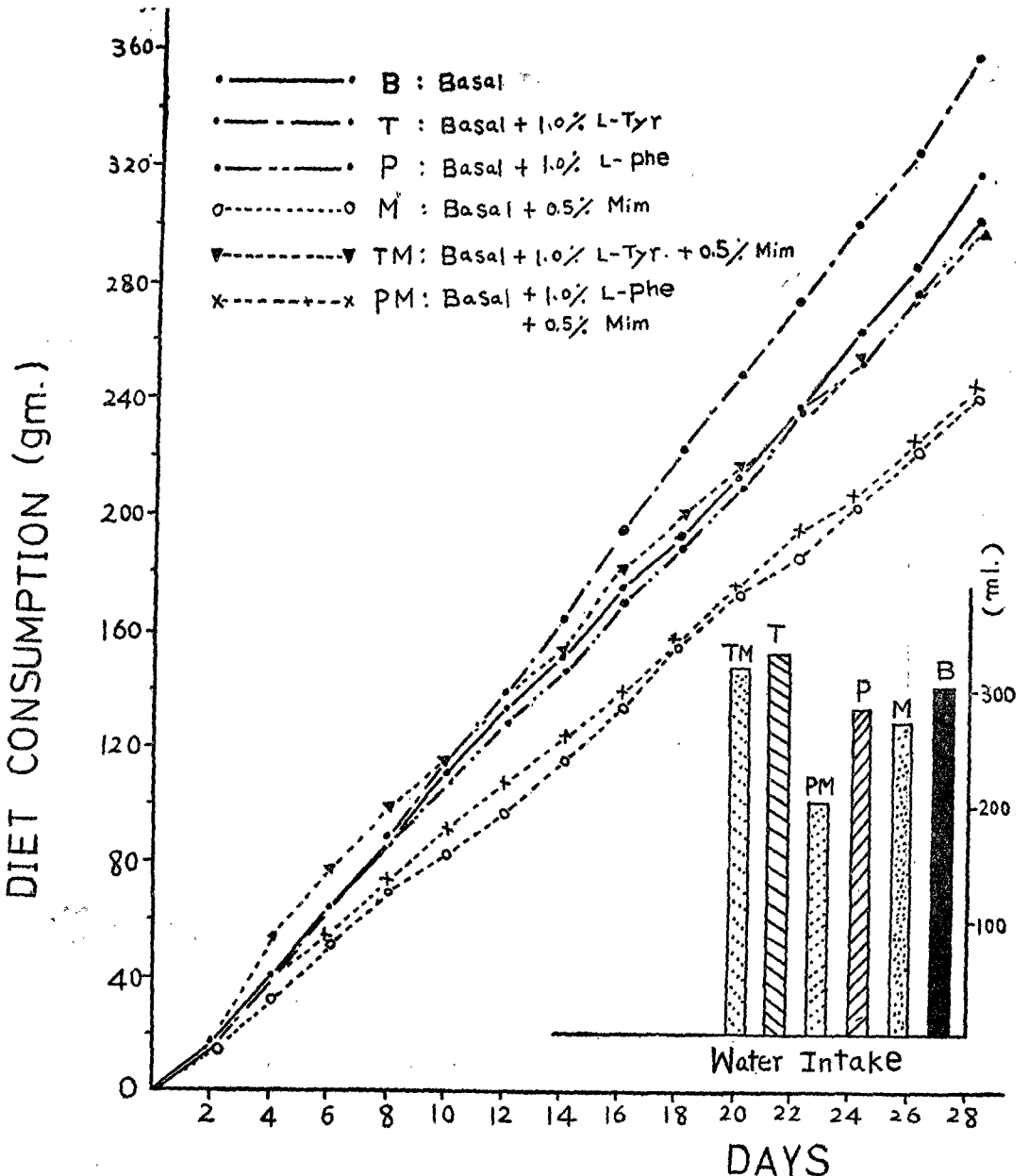

Fig. 2 Diet Consumption & Water Intake In Exp. I

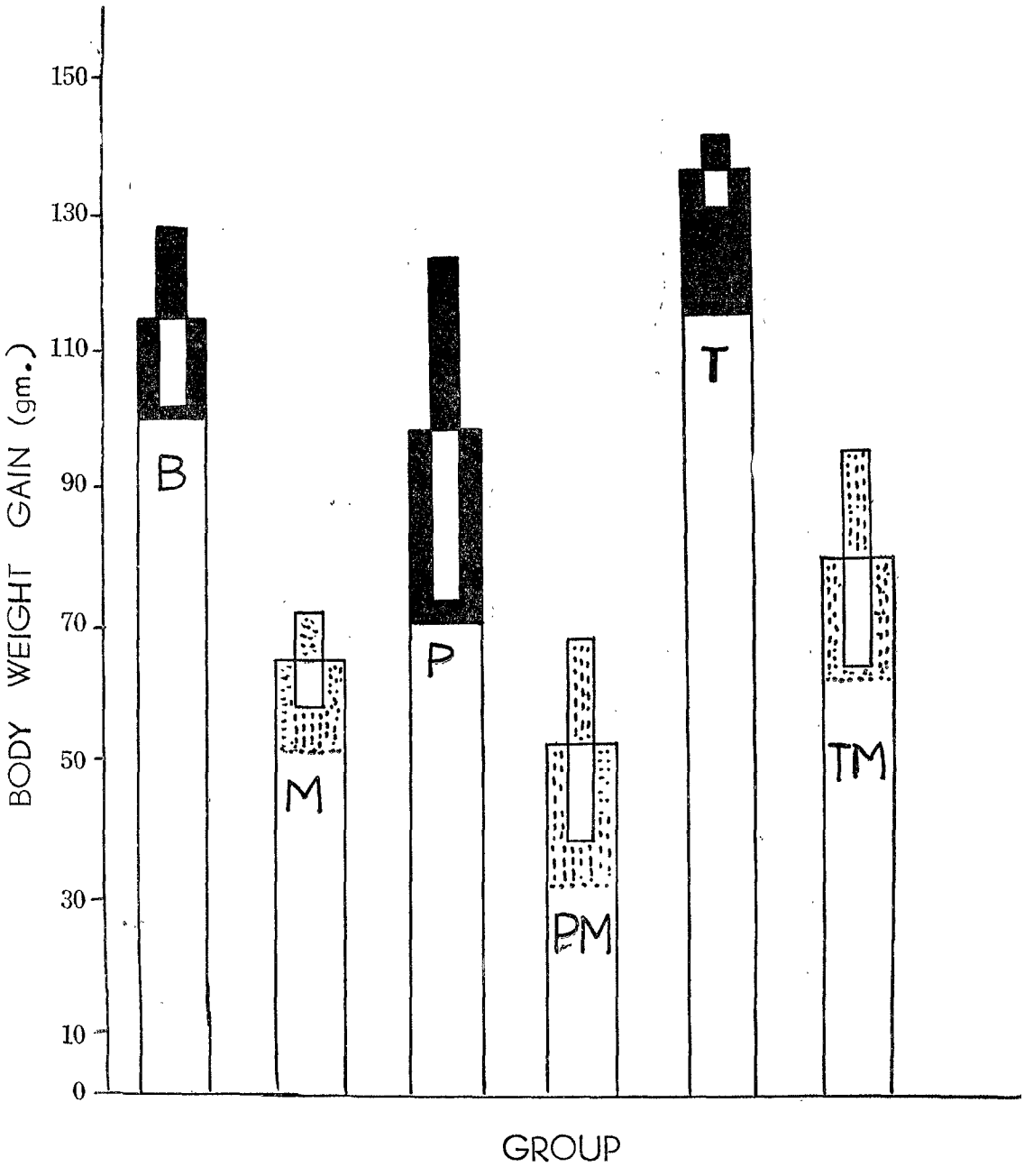

Fig.3 Total Body Weight Gain Per Rat In Exp. I

Table VI. Cataract Examination of Rats in Exp. 1.

Rat

Group	Day	1	2	3	4	5
B,P,T,	8	−	−	−	−	−
	15	−	−	−	−	−
	21	−	−	−	−	−
M	8	±−	−−	±±	±±	±−
	15	+±	±±	++	++	+±
	21	##	++	++	++	++
PM	8	−±	++	+±	−−	±−
	15	++	++	++	++	++
	21	++	++	++	++	++
TM	8	+±	±−	−±	−−	±−
	15	++	++	±+	++	+±
	21	++	++	++	++	++

Table VII. Occurance of Alopecia of Rats in Exp. I.

Group	Day	1	2	3	4	5
B,P,T,				all were negative in Exp.		
M	14	±	±	+	+	+
PM	14	±	±	+	+	±
M	28	+++	++	++++	+++++	++++
PM	28	+++	+++	++++	++++	+
TM	28	++	+	++++	++	++

Table VIII. Ascorbic Acid Content in Lens of Rats

Sample	Opetical Density	
Standard Ascorbic Acid	1	2
4 μg/ml	0.053	0.395
2 μg/ml	0.082	0.470
1 μg/ml	0.110	0.495
Reagent blank	0.129	0.500
B	0.184	0.462
M	0.130	0.485
P	0.234	0.453
PM	0.155	0.525
T	0.317	0.512
TM	0.313	0.535

Table IX. The Average Content of Clutathione in Lens of Rats

Group	A left eyes mg/g	B right eyes mg/g	(A+B)/5 average/rat mg/g
B	4.62	5.82	2.09
M	1.43	1.26	0.54
P	2.62	4.92	1.71
PM	2.00	4.24	1.25
T	4.75	5.29	2.01
TM	1.79	4.47	1.25

DISCUSSION

If we only compare the results of the growth rate of Group M,PM, and TM we may concluded as Lin, et al(1) and Yang, et al (7) did, that tyrosine antagonized the growth retardation caused by mimosine and that addition of phenylalanine alleviated the toxicity of mimosine. However, addition of tyrosine to the basal diet increased the rate of growth. On the other hand, the addition of phenylalanine to the basal diet decreased the rate of growth in one experiment. Therefore, it is concluded that both L-phenylalanine and L-tyrosine did not affect on the growth retardation caused by mimosine. Probably mimosine does not act as amino acid antagonist. This conclusion is harmonious with the results of the experiment of mung beans, tissue culture, and the amino acids transport of intestine. Addition of one precent of L-phenylalanine to the basal diet may cause amino aoid imbalance in the casein diet. On the other hand, addition of one percent of tyrosine may correct the amino acid imbalance of the casein diet.

The concentration of ascorbic acid in lens is normally very high. Huysams and Fischer (11) have made determinations in which they find that more than 90 per cent of the total concentration of vitamin C is present as ascorbic acid. Borsook and his associates(12) investigated the reduction of dehydro-ascorbiced glutathione was necessary.

The presence of a high concentration of glutathione in the lens and the observation that the concentration of ascorbic acid in the lens and aqueous humour falls with the decrease in glutathione in the lens. Miller(13),1937, suggested that the lens contains an enzyme responsible for the transfer of hydrogen between glutathione and Vitamine C system. In Exp. II, the method for measuring ascorbic acid content of the lens was failure because even the normal content (27-570 mg/kg wet weight) (14) is too low and beyond the sensitivity of the instrument.

In general, the glutathione content of the lens falls in all types of cataract (15). The fall is precedent and causative. Certain catacactogenic agents, however, such as galactose and hephthalene, have been shown to cause a fall in the glutathione content before the development of cataract. Herrman and Moses (16), who found all the glutathione present in the reduced form, estimated that there were 388-570 and 64-100 mg. per 100 gm fresh tissue in the outer cortex and nucleus respectively. Piri and Heyningen(17) estimated that there were 1.7-3.2 mg. glutathione per gram wet weight of tissue in the lens of rats. Results, in the Table IX, showed that mimosine caused the decrease of the glutathione content below the normal range. Both L-phenylalanine and L-tyrosine presented in the diet could not bring the glutathione content to the normal range. The results were coincided with the symptom of cataract which indicated in Table VI.

ACKNOWLEDGEMENTS

I am greatly indebted to Dr. Kuo-Huang Ling for his guidance, and constructive criticism during this study. Thanks are extended to Dr Po-Chao Huang for his invaluable instruction for enabling me to carry out biological study. I also express my gratitude to both Dr. Y. T. Shen and Dr. W. F. Tsai for their detecting the ocular change of rats.

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