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A Study Of The Relationships Of Xanthurenic Acid, Pyridoxal Phosphate And Glucose Metabolism In The Rabbit (Tryptophan, Deficiency, Glucose Tolerance, Insulin, High Performance Liquid Chromatography).

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A STUDY OF THE RELATIONSHIPS OF XANTHURENIC ACID, PYRIDOXAL PHOSPHATE AND GLUCOSE METABOLISM IN THE RABBIT

The University of Alabama in Birmingham PH.D. 1985

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A STUDY OF THE RELATIONSHIPS OF XANTHURENIC ACID, PYRIDOXAL PHOSPHATE AND GLUCOSE METABOLISM IN THE RABBIT

by

Shirley A. Williams

A DISSERTATION

Submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy in the Department of Physiology and Biophysics in the Graduate School The University of Alabama at Birmingham

Birmingham, Alabama

1985

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1985

ABSTRACT OF DISSERTATION
GRADUATE SCHOOL, UNIVERSITY OF ALABAMA AT BIRMINGHAM

Degree Ph.D. Major Subject Physiology and Biophysics

Name of Candidate Shirley Ann Williams

Title A Study of the Relationships of Xanthurenic Acid, Pyridoxal Phosphate and Glucose Metabolism in the Rabbit

The goals of this study were to confirm, in a rabbit model, that glucose intolerance is associated with dietary restriction of vitamin B-6 and to ascertain whether xanthurenic acid (XA), a tryptophan metabolite excreted in high amounts during B-6 deficiency, is the causative agent. For this study a HPLC method was developed to measure blood levels of XA. Vitamin B-6 status was determined by measurement of erythrocyte aspartate aminotransferase activity and observation of physical symptoms.

The appearance and disappearance of XA from serum was similar in all groups of rabbits. The volume of distribution (V_D) decreased in B-6 deficient rabbits when compared to pretreatment or normally fed rabbits (group 1).

As the rabbits in group 2 (purified diet plus B-6) and group 3 (purified diet minus B-6) became increasingly B-6 deficient, plasma XA levels rose from baseline (200 ng/ml) to values in excess of 1500 ng/ml, but returned to

baseline after B-6 supplementation. Glucose levels in B-6 deficient rabbits rose significantly ($p < 0.05$) in response to glucose challenge when compared to normal rabbits and had accompanying depressed insulin responses. Glucose and insulin responses returned to normal with B-6 supplementation. Xanthurenic acid, in levels comparable to those in B-6 deficient rabbits, was infused subcutaneously for 12 days into normal rabbits in an effort to reproduce the glucose and insulin responses observed during B-6 deficiency. Glucose intolerance in association with depressed insulin response to a glucose challenge was observed.

These data confirm that glucose intolerance occurs in association with B-6 deficiency, demonstrate that depressed insulin response and elevated XA levels occur concomitant with glucose intolerance and strongly suggest that XA is the causative agent. The exact mechanism(s) by which XA alters insulin response are unknown. However, XA may act as an organic stressor similar to other drugs, eliciting humoral and/or hormonal alterations which interplay to cause hyperglycemia and hypoinsulinemia.

Abstract Approved by: Committee Chairman Larry Boyd

Program Director

Date 3/15/85 Dean of Graduate School

DEDICATION

If it wasn't for kids have you ever thought,
There wouldn't be no Santa Claus.
But look what the stork has brought,
Thank God for kids.

And we don't live in a quiet house without
Big Bird and a Mickey Mouse, Kool-aid on the
couch,
Thank God for kids.

Thank God for kids, there's a magic for awhile,
A special kind of sunshine in their smile.
If you ever stop and think, or wonder why,
The dearest thing to heaven is a child.

When I look down in their trusting eyes
That look to me,
I realize, there's love that I can't buy.
Thank God for kids.

When I get down on my knees at night,
And thank the Lord for his guiding Light,
I pray they turn out right.
Thank God for kids.

Thank God for kids,
Thank God for Denise, Jonathan, Megan and Nolfris
And for my Mom and Dad, Sarah Ellis and James
Nolfris Williams.

ACKNOWLEDGEMENTS

> I will lift up mine eyes unto the hills,
> from whence cometh my help. My help cometh
> from the Lord, which made heaven and earth...
> Behold, he that keepeth Israel (me) shall
> neither slumber nor sleep.
>
> Psalm 121:1-2, 4
> (King James Version)

I would like to express my deepest appreciation to Dr. Steve Barker for his assistance in finding means and instruments to develop a method to quantify xanthurenic acid in blood. For without this information, the completion of my dissertation research would have been, at best, much prolonged.

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LIST OF ABBREVIATIONS

AST - aspartate aminotransferase

C^{O} - concentration extrapolated to zero time

CV - coefficient of variance

GTT - glucose tolerance test

HPLC - high performance liquid chromatography

IP - intraperitoneally

IV - intravenously

K^{-min} - elimination rate constant

NA - normalized area

OCA - oral contraceptive agent(s)

OD - optical density

PLP - coenzyme form of pyridoxal phosphate

RIA - radioimmunoassay

SEM - standard error of mean

$T_{1/2}$ - half-life

uIU - micro units

UV - ultraviolet

V_D - volume of distribution

XA - xanthurenic acid

INTRODUCTION

Tryptophan is one of the most widely distributed indole derivatives in nature and is converted to several biologically active substances including serotonin, indoleacetic acid and nicotinic acid. Among the factors influencing tryptophan metabolism is the coenzyme form of vitamin B-6, pyridoxal phosphate (PLP) (81). Several pathways are available for the metabolism of tryptophan, but during vitamin B-6 deficiency the tryptophan-niacin pathway is most important. This pathway requires PLP and since the demonstration that a dietary restriction of vitamin B-6 in man caused an increased excretion of xanthurenic acid (XA) via this pathway, there has been interest in the relationship between tryptophan metabolism and vitamin B-6 (64). The production of excessive levels of XA has been implicated as a factor in causing deteriorating glucose metabolism in man and animals deficient in B-6. In the following paragraphs, the history of the developing relationships among vitamin B-6, tryptophan metabolism and glucose regulation are described.

Isolation and Identification of Vitamin B-6

In 1934, Gyorgy proposed that the fraction of food extracts which prevented skin lesions in rats be called

vitamin B-6 (36). Several groups studied this substance and its isolation was reported by several different laboratories (37, 52, 53, 63). However, the isolation of the pure crystalline vitamin B-6 was first reported by Lepkovsky in 1938 (63) and within a year the correct chemical structure was elucidated and termed 'pyridoxine' and vitamin B-6 by Gyorgy and Eckhardt (38). Snell and associates (120) recognized the existence of the different forms of the vitamin, i.e., pyridoxal and pyridoxamine, and their phosphorylated forms which act as coenzymes as early as 1942. Since that time many enzymes have been identified which require pyridoxal phosphate (PLP) for activity including transaminases, phosphorylases, decarboxylases and desulfurases (111).

Mechanism of Action and Dietary Requirements of Vitamin B-6

The mechanism by which pyridoxal phosphate functions as a coenzyme has received intensive study and is the subject of numerous reviews (112). It is generally agreed that pyridoxal phosphate functions in many of these enzymes in the manner of a Schiff base. The enzyme systems most investigated are the transaminases (64, 113), and in these cases, all evidence appears consistent with the Schiff base mechanism in that linkage between pyridoxal phosphate and an amino group of lysine in the apoenzyme occurs, with additional interactions between the functional group of the coenzyme and the apoenzyme.

The transaminases represent a major group of the pyridoxal phosphate- dependent enzymes. Aspartate aminotransaminase (E.C.2. 6.1.1), also known as glutamate-oxalacetate transaminase or GOT is the most abundant of the transaminases in mammalian tissue. The abundance of this apoenzyme provides an accessible means of determining the B-6 nutritional status of individuals (which will be described later). The recommended daily requirement of vitamin B-6 for humans ranges from 430.5 to 3075 ug per day (72), while the requirement for rabbits as recommended by the National Research Council, is 40 ug per day (47, 84). Inadequate intake of the vitamin results in a vitamin B-6 deficiency which has been reported to result in pathological conditions affecting the skin, nervous system, hematopoietic, adrenal and reproductive systems (140). Severinghaus (117) reported that a B-6 deficiency results in seborrheic types of dermatitis in experimental dietary deprivation, convulsive attacks in infants, irritability, depression, peripheral neuritis, somnolence and skin lesions in deficient human adults. Typical arteriosclerotic lesions were also described in B-6 deficient human adults and in B-6 deficient monkeys (98), while accelerated arteriosclerosis has been found in children with a vitamin B-6 deficiency (31, 70).

Apart from these clinical observations, the earliest studies on the physiological role of pyridoxal phosphate dealt with the requirement for this coenzyme in pregnancy and the relationship of decreased metabolic production of niacinamide from the amino acid tryptophan.

Isolation and Dietary Requirements of Tryptophan

The isolation and recognition of tryptophan by Hopkins and Cole in 1901, in tryptic digests of proteins (43), stimulated considerable interest in the metabolism of this amino acid. Since that time tryptophan has been shown to be essential for proper growth and development in most animals and humans and has been shown to be the precursor of serotonin, indoleacetic acid, pigments and nicotinic acid (73).

The average daily intake of tryptophan by Americans is about 0.9 g, while the minimum daily requirement for women is about 0.16 g and for men about 0.25 g (146) . Therefore, a considerable quantity of dietary tryptophan is available for conversion to products other than protein through several metabolic pathways.

It has been shown that the level of tryptophan in plasma is usually higher than in other body compartments (e.g., brain and cerebrospinal fluid), and plasma concentrations of the amino acid vary from species to

species and fluctuate diurnally (146). Tryptophan in plasma is derived from two sources, i.e., diet and secretion from the free amino acid pools of various tissues (146). Plasma levels of the amino acid exhibit characteristic diurnal rhythyms generated largely in response to eating (30,147). Wurtman et al. (147) found that plasma samples collected from untreated humans or rats at various times of day or night exhibited characteristic fluctuations in their concentrations of tryptophan during each 24 hour period. Among human subjects who ate three meals per day, tryptophan levels were lowest from 2 to 5 a.m.; then rose 50-80% to attain a plateau in the late morning or early afternoon. The tryptophan levels ranged from 6.30 $\pm$0.49 to 9.12 $\pm$ 1.22 ug/ml between 2 and 5 am and increased to approximately 11 $\pm$ 8.39 ug/ml at or after 10:30 am. In rats the peak values of tryptophan occur during the night, coinciding with the pattern of food intake.

Metabolism of Tryptophan

Efforts to elucidate the biological importance of tryptophan have resulted in the discovery of several metabolic schemes, one of which is referred to as the "tryptophan-niacin pathway" (93). It is estimated that approximately 60 mg of catabolized dietary tryptophan may give rise to 1 mg of niacin via this pathway. Although

tryptophan is preferentially used for the maintenance of body protein and serotonin production, the tryptophan-niacin pathway becomes quantitatively more important (2, 18, 25, 54, 75, 83, 136) when the ingestion or parenteral administration of tryptophan is in excess of 50 mg/kg. Approximately 98% of tryptophan is metabolized in the liver, via the tryptophan-niacin pathway, which is initiated by the enzyme tryptophan pyrrolase. The amount of kynurenine produced increases proportionately as the plasma concentration of tryptophan increases. The synthesis of serotonin from tryptophan also increases proportionately, up to 50 mg/kg of tryptophan in the rat (30, 146) and humans (30, 33, 146, 151). Fifty mg/kg I.P. of tryptophan in the rat yield approximately 0.68 ug/g of brain serotonin. However 50-1600 mg/kg tryptophan produce no further increase in brain serotonin (31, 33, 146, 151). High doses of tryptophan induce tryptophan pyrrolase, increase the catabolism of tryptophan via the kynurenine pathway and decrease the amount of tryptophan available for serotonin synthesis.

Role of Vitamin B-6 in Tryptophan Metabolism

It is most important to recognize that vitamin B-6 in coenzyme form is required in at least four reactions of the tryptophan-niacin pathway (Figure 1) in the conversion of kynurenine to anthranilic acid; 2) in kynurenine

Figure 1. An abbreviated outline of the metabolic pathway of tryptophan. The reactions in which vitamin B-6 is of particular importance have been indicated by the symbol, B-6, adjacent to the arrows. (Taken from Price, J.M., R.R. Brown and N. Yess. Advan. Metab. Disorders 2:159-225, 1965).

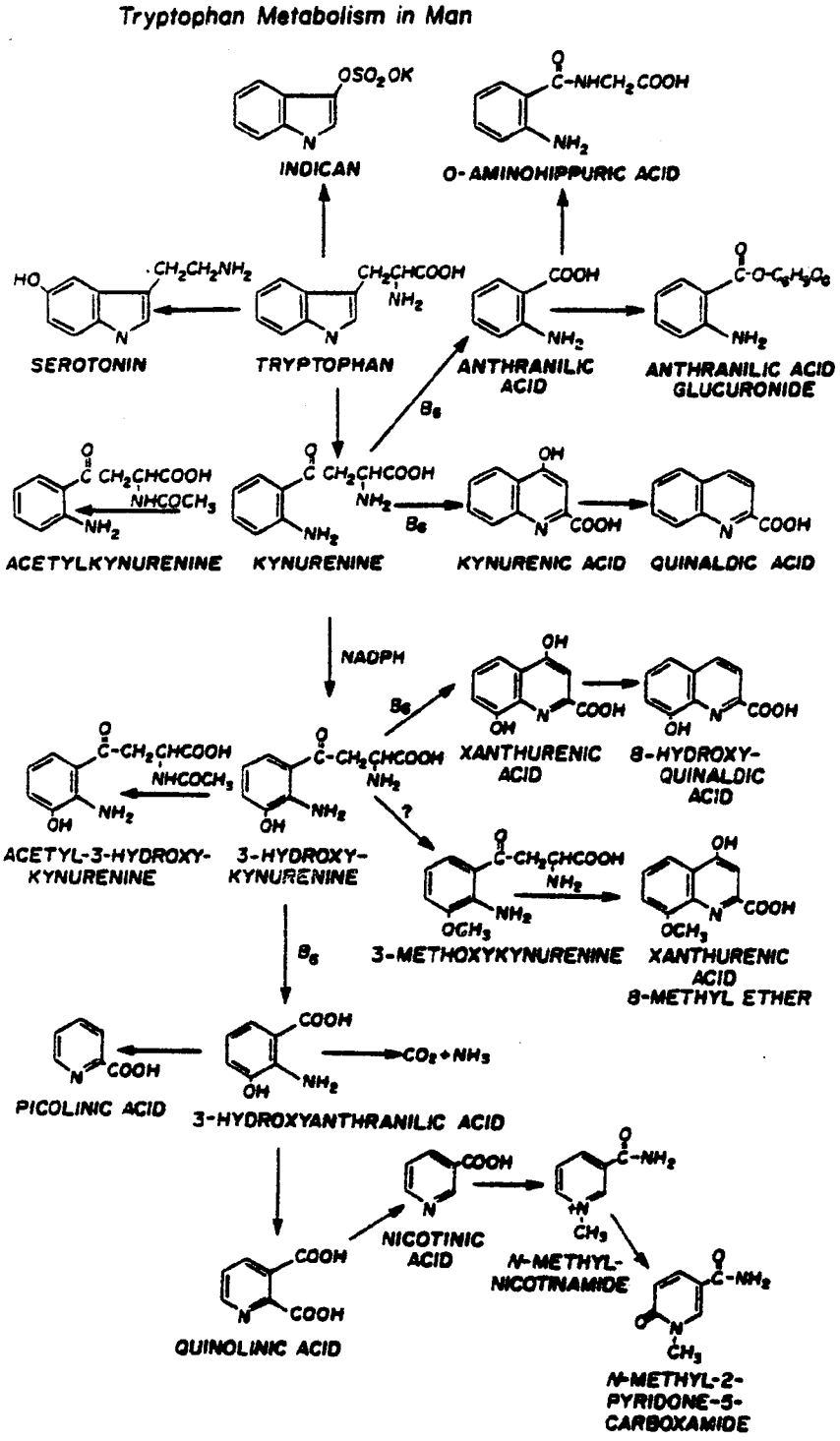
Tryptophan Metabolism in Man
INDICAN
O-AMINOHIPPURIC ACID
SEROTONIN
TRYPTOPHAN
ANTHRANILIC ACID
ANTHRANILIC ACID GLUCURONIDE
ACETYLKYNURENINE
KYNURENINE
KYNURENIC ACID
QUINALDIC ACID
NADPH
XANTHURENIC ACID
8-HYDROXY-QUINALDIC ACID
ACETYL-3-HYDROXY-KYNURENINE
3-HYDROXY-KYNURENINE
3-METHOXYKYNURENINE
XANTHURENIC ACID 8-METHYL ETHER
PICOLINIC ACID
3-HYDROXYANTHRANILIC ACID
NICOTINIC ACID
N-METHYL-NICOTINAMIDE
QUINOLINIC ACID
N-METHYL-2-PYRIDONE-5-CARBOXAMIDE

Figure 1

conversion to kynurenic acid; 3) in 3-hydroxy-kynurenine conversion to 3-hydroxyanthranilic acid and 4) in the conversion of 3-hydroxykynurenine to xanthurenic acid. In addition, the turnover rate for kynurenine conversion to nicotinic acid increases with vitamin B-6 supplementation (33). However, vitamin B-6 deficiency results in decreased formation of the pyridine derivatives' N-methyl nicotinamide, quinolinic acid and pyridine nucleotides and increased formation and excretion of kynurenine, kynurenic acid, 3-hydroxykynurenine and xanthurenic acid (107,135). The increased formation of the latter tryptophan metabolites, in particular xanthurenic acid, indicates that a deficiency in vitamin B-6 results in decreased conversion of 3-hydroxykynurenine to 3-hydroxyanthranilic acid. This reaction is catalyzed by B-6 dependent kynureninase (85), whose activity is more sensitive to and reduced by B-6 deficiency than is kynurenine aminotransferase. The latter enzyme produces xanthurenic acid from 3-hydroxykynurenine during B-6 deficiency (133,148). This difference in sensitivity to a decrease of B-6 coenzyme explains why the urinary excretion of XA is elevated during a vitamin B-6 deficiency. However, under prolonged or severe deficiency the formation of XA is also reduced (103).

Tryptophan Metabolism During Pregnancy and Oral Contraceptive Use

One of the first reports of high levels of urinary xanthurenic acid as a result of a vitamin B-6 deficiency was based on a study of pregnant women. In 1951, Vandelli (82) reported that pregnant women excreted greater amounts of XA than nonpregnant women after ingestion of L-tryptophan. This was interpreted as signifying a pyridoxine deficiency during pregnancy, inasmuch as supplementation of the diet with vitamin B-6 restored the excretion of XA to control levels. Similar conclusions were reached by Sprince et al. (128) and Wachstein and Gudaitis (137). Brown et al. (19) subsequently confirmed the existence of a B-6 deficiency during pregnancy and reported increased urinary excretion of XA, kynurenine, and 3-hydroxykynurenine. Other investigators have confirmed the presence of a B-6 deficiency during pregnancy and the reversal of high levels of excretion of tryptophan metabolites by administration of pyridoxine (23, 26, 39, 41). It has been suggested that the B-6 deficiency observed during pregnancy is caused by: 1) a sequestering and utilization of maternal B-6 by the fetus and 2) high levels of circulating estrogens which increase the B-6 requirement by stimulating B-6 dependent pathways in a manner similar to that of synthetic estrogens in contraceptive users (16, 104).

An extensive literature suggests that 70-80% of women using oral contraceptives containing 50 ug or more of estrogen develop altered tryptophan metabolism, i.e. increased tryptophan pyrrolase activity and increased excretion of metabolites, while approximately 15% of users develop vitamin B-6 deficiency (104, 106). In addition, there is evidence that the elevated levels of estrogens, progestins and placental hormones, e.g. human chorionic gonadotropin and human placental lactogen during pregnancy could modify tryptophan metabolism. Rose and Braidman (105) demonstrated that hydrocortisone produced an increase in the urinary excretion of kynurenine, 3-hydroxykynurenine, xanthurenic acid and 3-hydroxyanthranilic acid. In addition, they reported that pregnant women, those using oral contraceptive agents and patients receiving estrogens alone, all excreted elevated levels of these metabolites. These observations have also been confirmed in baboons receiving oral contraceptives (13).

In a recent review, Rose (104) reported that some esters of estrogen inhibited kynurenine aminotransferase and kynureninase activity by forming a complex with the apoenzymes and thus prevented association with the coenzyme, PLP. It was subsequently observed that sulfate esters of estradiol, estrone and diethylstilbestrol all

interfered with pyridoxal phosphate binding with vitamin B-6 dependent enzymes (67). These reactions occurred at very low levels (0.5 uM) of steroid sulfates in vitro, which suggested that the levels of steroid conjugates present in the tissue might also be effective. Saad et al. (108) further reported that in liver homogenates, estradiol and ethinylestradiol inhibited kynurenine aminotransferase and kynureninase while mestranol and progesterone were without any effect. Inhibition of these apoenzymes by estradiol was competitive while that of ethinylestradiol was non-competitive. It was also noted that estrogens induced a number of B-6 dependent enzymes in liver, erythrocytes and in the uterus (14, 104).

In the late 1960's and early 1970's, additional evidence supporting a role for estrogen in the occurrence of altered tryptophan metabolism was reported. Oral contraceptive agents were implicated as causative agents in the production of elevated levels of tryptophan metabolites (15, 62, 65, 68, 95, 109, 144). The results of investigation in this area (15, 62, 65, 68, 95, 109, 144) can be summarized as follows: estrogen preparations increased hepatic tryptophan oxygenase activity and inhibited the conversion of 3-hydroxyanthranilic acid, resulting in the accumulation of metabolites of this pathway, including xanthurenic acid.

The Role of Xanthurenic Acid, Tryptophan and Vitamin B-6 in Carbohydrate Metabolism

Investigations of vitamin B-6 metabolism and glucose tolerance in women taking oral contraceptive agents (99, 106, 119) showed that in some women, development of glucose intolerance was related to estrogen-containing oral contraceptives (9, 29, 122-124, 127, 149). Wynn and Doar (148, 149) reported "relative" impairment of glucose tolerance in over 70% of women taking an oral contraceptive, while 13% had frankly abnormal glucose tolerance that was classified as subclinical diabetes. In another study, Spellacy et al. (126) reported only slight increases in peak blood glucose concentrations during glucose tolerance tests, but observed pronounced elevation in plasma insulin levels. Javier et al. (49) noted that plasma insulin levels rose during the first months of oral contraceptive use, but with prolonged use the plasma insulin levels decreased even though further deterioration of glucose metabolism occurred.

Excessive urinary levels of xanthurenic acid were found in 13.5% of 1000 diabetic patients by Montenero (78). Parenteral administration for one week with 500-600 mg per day (sic) of pyridoxine caused almost complete disappearance of urinary xanthurenic acid. Montenero suggested that, if indeed xanthurenic acid was

considered an index of vitamin B-6 deficiency, normalized levels after B-6 therapy may be a confirmation that in some diabetic patients there exists an alteration in the catabolism of tryptophan via the tryptophan-niacin pathway due to a possible deficiency of vitamin B-6. Loading diabetics with 5g of tryptophan, as cited by Musajo et al. (82), increased excretion of XA with a simultaneous glycemic rise. In addition, pyridoxine administration resulted in a lessening of xanthenuria. It was also reported by these same investigators that the addition of vitamin B-6 to insulin therapy allowed the use of lower doses of insulin and, in one subject, the total cessation of insulin administration. Oka and Leppanen (86) noted an increase in the excretion of XA as well as other tryptophan metabolites after loading diabetic patients with tryptophan and attributed the metabolic derangement partly to a B-6 deficiency.

On the other hand, Wohl et al. (143) and Musajo and Benassi (82) found that diabetic patients and controls excreted, before and after tryptophan loading, low levels of XA and that this is not modified by the administration of pyridoxine or by a second test load of tryptophan. These investigators concluded that pyridoxine deficiency

is not metabolically peculiar to diabetes and that XA excretion is comparable to that found in controls.

Additionally, it was shown that tryptophan produced hypoglycemia in fasting rats (77) due to inhibition of gluconeogenesis via a decrease in phosphoenolpyruvate carboxykinase (96). Further study indicated that inhibition of this enzyme was due to quinolinic acid (71), a normal precursor of nicotinic acid in the tryptophan-niacin pathway. Studies by Mirsky (76) of insulinase, diabetes mellitus, and tryptophan revealed that various tryptophan metabolites, e.g., anthranilic acid, niacin, indole-3-acetic acid, 5-hydroxytryptophan, and serotonin, produced significant hypoglycemia in normal and diabetic rats. A hypoglycemic response followed by a hyperglycemic response was produced by kynurenine, whereas kynurenic acid and nicotinamide gave a significant hyperglycemic response only.

Evidence in the literature is supportive of a relationship between vitamin B-6 metabolism, deteriorating glucose utilization and tryptophan metabolism. However, the mechanism(s) are unclear. In view of the complex and equivocal data presented in relation to diabetes and other clinical problems no firm conclusions can be drawn about the connection between tryptophan metabolism,

diabetes or glucose metabolism. However, it should be considered that diabetes is a pathological disorder which has several possible etiologies, manifesting as insulin insufficiency, insulin resistance, decreased receptor concentration volume or altered post-receptor mechanisms. Each of the metabolic states is characterized by a decreased tolerance for glucose (116). Each of these factors might well constitute variability in the results obtained by the cited investigators.

Although the measurement of urinary XA is a sensitive index of a vitamin B-6 deficiency which correlates well with other indices of vitamin B-6 status, subjects having serious diseases, such as the diabetics with hyperthyroidism reported by Wohl et al. (143), make interpretation of the tryptophan load test difficult. In these instances, the possibility that tryptophan pyrrolase is induced by the stress of the disease must also be considered. The increased xanthurenic acid levels in this situation are the result of an induction of tryptophan pyrrolase rather than the result of a vitamin B-6 deficiency.

Stress, whether caused by physical stressor agents (e.g., disease, injury, or exercise), or psychological trauma, causes change in the internal environment. These changes elicit responses from the neuroendocrine system and result in the increased production of several

hormones including epinephrine, growth hormone, glucagon and in particular, cortisol. Altman and Greengard (3) and Greengard (35) reported that the administration of 250 mg of hydrocortisone (hemisuccinate) intramuscularly to male and female patients caused a two- to four-fold increase in tryptophan pyrrolase activity in liver and increased urinary kynurenine excretion. Under these conditions, increased levels of tryptophan metabolites may be due to the actual diseased state which triggers an increase in cortisol and not due to a vitamin B-6 deficiency.

Of particular interest to this research is the work of Kotake and co-workers (55-61) and Murakami (81). Reports from these investigators supported the hypothesis that deteriorating glucose regulation in oral contraceptive users and pregnant women might be related to vitamin B-6 deficiency and abnormal tryptophan metabolism (125). As early as 1953, Kotake and Inada (56) reported that hyperglycemia occurred in rats as the result of administration of XA. By 1957, Kotake (55) concluded XA was related to the development of diabetes in humans, that XA was hyperglycemic in rats, that XA was excreted in the urine of diabetics but not of normal patients and that XA inhibited hexose phosphorylation, thereby inhibiting glucose metabolism and giving rise to hyperglycemia and glucosuria in rats. Further investigation on the mechanism

of action of XA, as reported by Kotake et al. (58-60) and Murakami (81) suggested that the excessive levels of XA formed as a result of a B-6 deficiency combined with insulin and that this complex was only 50% as active as native insulin in decreasing blood glucose levels in rabbits and dogs.

Adams et al. (1) and Cornish and Tesoriero (24) reported that the pattern of excretion of tryptophan metabolites observed in nutritional B-6 deficiency also occurred in gestational diabetics and women on combination oral contraceptive agents. Administration of vitamin B-6 restored the pattern of excretion of metabolites to normal and decreased XA levels. Adams et al. (1) assessed carbohydrate and vitamin B-6 metabolism before and after pyridoxine administration in 46 women taking combined estrogen-progestogen oral contraceptives. Eighteen women had evidence of tissue depletion of vitamin B-6. However, all women had abnormal tryptophan metabolism, including increased urinary XA excretion. Administration of vitamin B-6 to women with a B-6 deficiency caused elevation of fasting blood pyruvate levels and reduction in plasma glucose and insulin in response to an oral glucose load. The 28 women considered to be vitamin B-6 replete did not exhibit a similar response nor changes in carbohydrate metabolism. Adams et al. (1) suggested that the

results indicated that carbohydrate intolerance in women on oral contraceptives is unlikely to be mediated by the proposed hypothesis of Kotake and offered an alternative explanation of quinolinic acid involvement in carbohydrate metabolism.

Cornish and Tesoriero (24) suggested that estrogen induced glucose intolerance independently of any abnormalities in XA excretion and reported that XA given one hour before an oral glucose load did not impair glucose tolerance. In addition, these investigators reported that XA given intraperitoneally to rats failed to alter blood glucose levels, and, in a single experiment with one rabbit, XA given intravenously did not demonstrate changes in blood glucose concentration over a three hour period.

Neither group of these investigators believed that increased XA levels were causally related to glucose intolerance and they ruled out the formation of a XA-insulin complex. The data presented by Adams et al. (1) to substantiate this belief are unclear and no firm conclusion can be drawn on the basis of information given. Cornish and Tesoriero (24) utilized male rats and rabbits on special high fat diets and did not measure insulin levels during the administration of glucose tolerance tests; nor were the criteria used to determine B-6 status clear. On the basis of the data presented no conclusions

on carbohydrate metabolism in relation to XA or vitamin B-6 could be drawn.

On the other hand, Bennink and Schueres (10, 11) reported increased urinary levels of XA after a tryptophan load in gestational diabetics and women using combination estrogen and progestogen oral contraceptives. Oral administration of vitamin B-6 improved glucose tolerance and decreased urinary XA levels of these individuals. These investigators suggested that XA acts, in an unknown manner, as an insulin antagonist.

Preliminary Experiments

As mentioned earlier, the information cited provided a basis for the relationship between vitamin B-6 and glucose metabolism. However, the factor responsible for impaired glucose tolerance and altered insulin responses in gestational diabetes and OCA users, is undetermined. The results reported do not support the existence of a XA complex, but neither do they rule it out. In an attempt to develop an animal model in which the roles of XA and vitamin B-6 in glucose metabolism could be determined, we (Williams et al., 141), in preliminary experiments, studied glucose dynamics in vitamin B-6 deficient rabbits by administering glucose tolerance tests to mature normal female rabbits and again when they were subsequently fed

a vitamin B-6 deficient diet and determined to be B-6 deficient. As seen in Figure 2, the B-6 deficient rabbits had higher peak levels of glucose and those levels came down at a slower rate than in normal rabbits. In relating the insulin response to a glucose load, B-6 deficient rabbits' insulin did not rise significantly above baseline levels while in normal rabbits a significant elevation in insulin levels occurred (Figure 3). Based on these data, it was concluded that the B-6 deficient rabbits exhibited glucose intolerance and an altered insulin response to a glucose load. A series of studies was then initiated to determine whether or not XA might be responsible.

There are a number of possible explanations for the apparent relationship between vitamin B-6 status and deteriorating glucose metabolism. Because Kotake and Murakami (58) and Murakami (81) reported that xanthurenic acid was capable of combining with insulin, thereby reducing its biologic activity, an attempt was made to replicate the results of these investigators. The XA-insulin complex was prepared according to the methods of these investigators and subjected to Sephadex G-50 gel filtration. The U.V. elution pattern obtained after passing the complex through a 1.5 cm x 40 cm Sephadex G-50 column (Figure 4) suggested that a complex was

Figure 2. Comparison of mean (± SEM) glucose levels after the administration of IV glucose tolerance tests in normal (●) and B-6 (■) deficient rabbits.

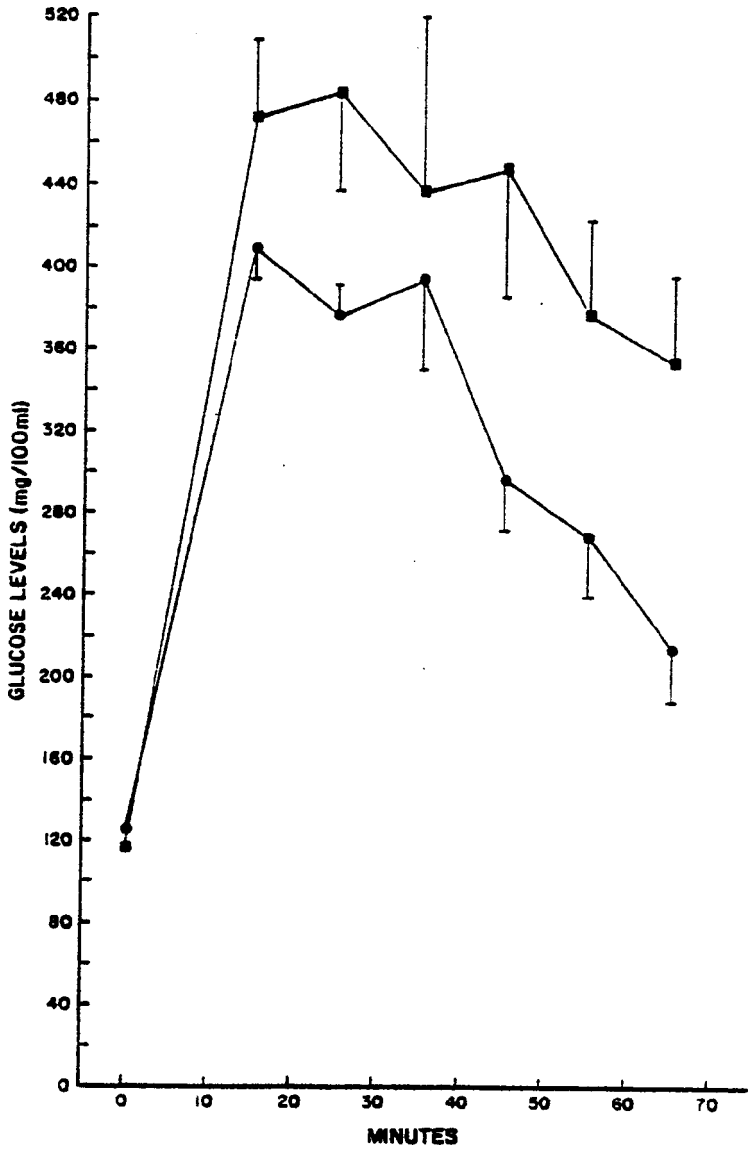
GLUCOSE LEVELS (mg/100ml)
520
480
440
400
360
320
280
240
200
160
120
80
40
0
0
10
20
30
40
50
60
70
MINUTES

Figure 2

Figure 3. Comparison of mean (± SEM) insulin response to a glucose load in normal (●) and B-6 deficient rabbits (■).

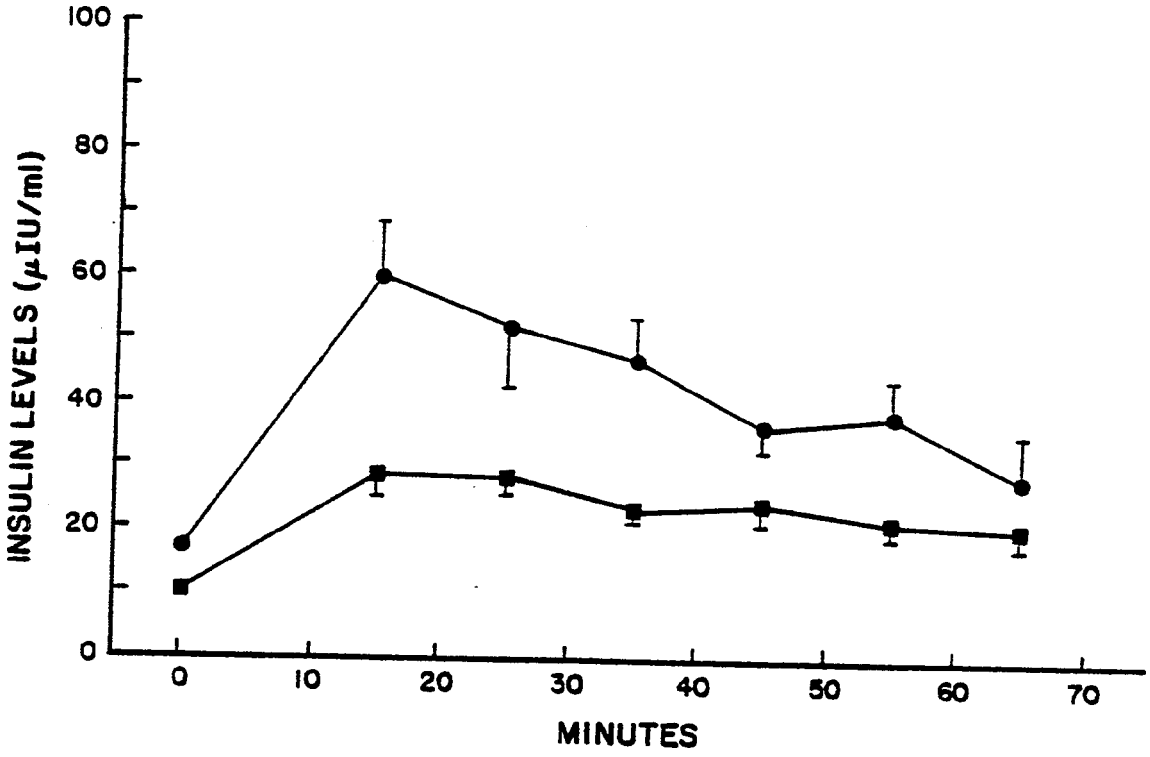
INSULIN LEVELS (μIU/ml)
100
80
60
40
20
0
0
10
20
30
40
50
60
70
MINUTES

Figure 3

eluted in the first six fractions (5 ml each). Comparison with elution patterns of XA or insulin alone (Figure 5) appeared to demonstrate the uniqueness of this peak (peak A).

However, fluorescence emission and nuclear magnetic resonance spectra of peak A were indistinguishable from that of native insulin. This information suggested that there was no complex formed between XA and insulin *in vitro*.

Kotake et al. (58-60) and Murakami (81), however, demonstrated that the material comprising peak A was only 50% as active as native insulin in lowering blood glucose when injected into rabbits and dogs. In attempting to repeat these observations, 21 and 42 ug/kg body weight of peak A were injected into normal female rabbits. Blood glucose (Figure 6) and insulin (Figure 7) levels were measured and compared to responses from similar amounts of native insulin. The effect of peak A on glucose levels was the same as if pure insulin were injected. Plasma insulin responses (Figure 7) could not be assessed since the insulin assay measured peak A material as well as endogenous insulin. Therefore, the two uppermost curves in Figure 7 are disappearance curves for the insulin portion of peak A and the higher levels seen with injection

Figure 4. Gel filtration of the XA-insulin complex with Sephadex G-50. By dissolving 200 mg crystalline insulin in 5 ml distilled water and adjusting the pH to 8.0. Xanthurenic acid (40 mg) was dissolved in 5 ml distilled water, then adjusted to pH 8.0, and poured into the insulin solution. Sodium diphosphate (10 ml of 0.15 M solution) was added to the mixture, and the solution was incubated at 37^{o} C for 30 minutes.

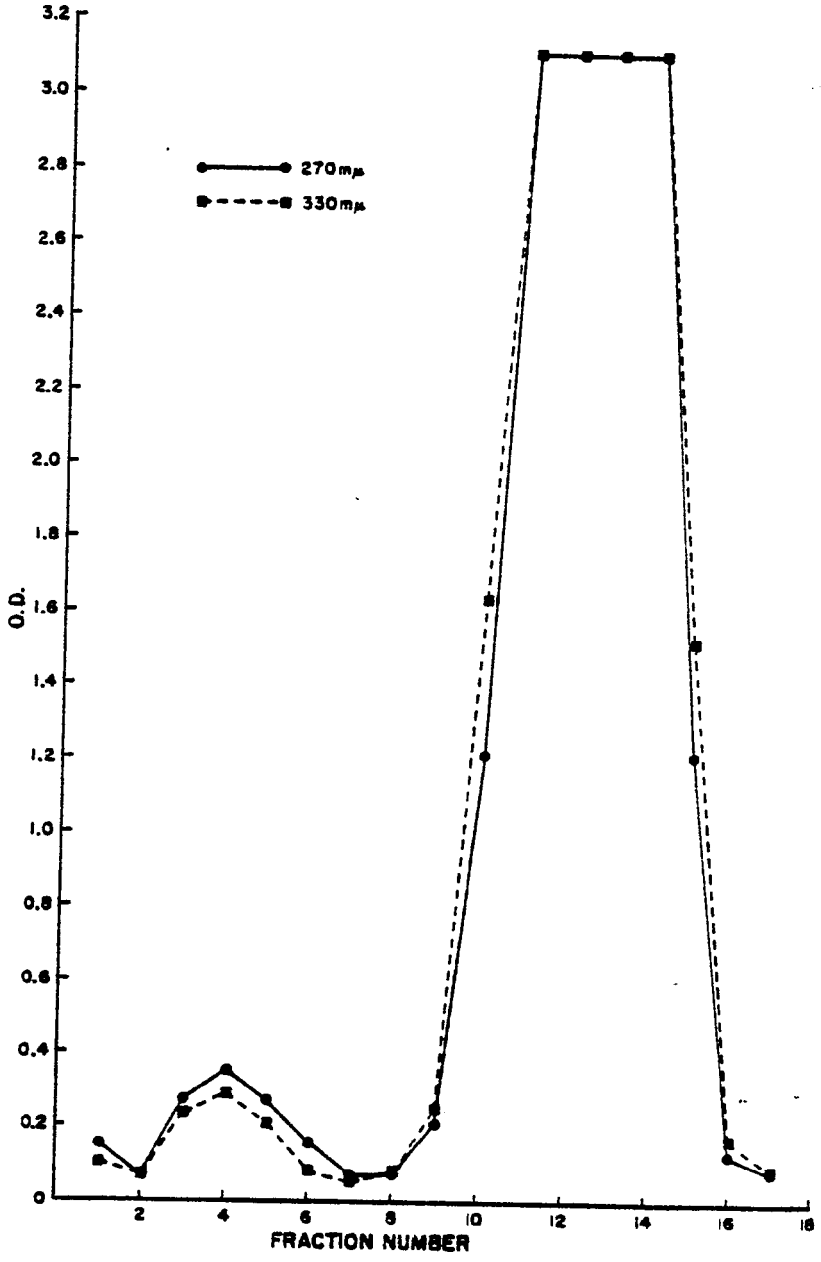
270mμ
330mμ
O.D.
FRACTION NUMBER
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
2.4
2.6
2.8
3.0
3.2
2
4
6
8
10
12
14
16
18

Figure 4

Figure 5. Gel filtration of authentic insulin and XA with Sephadex G50. Optical density (O.D.) at 270 mu.

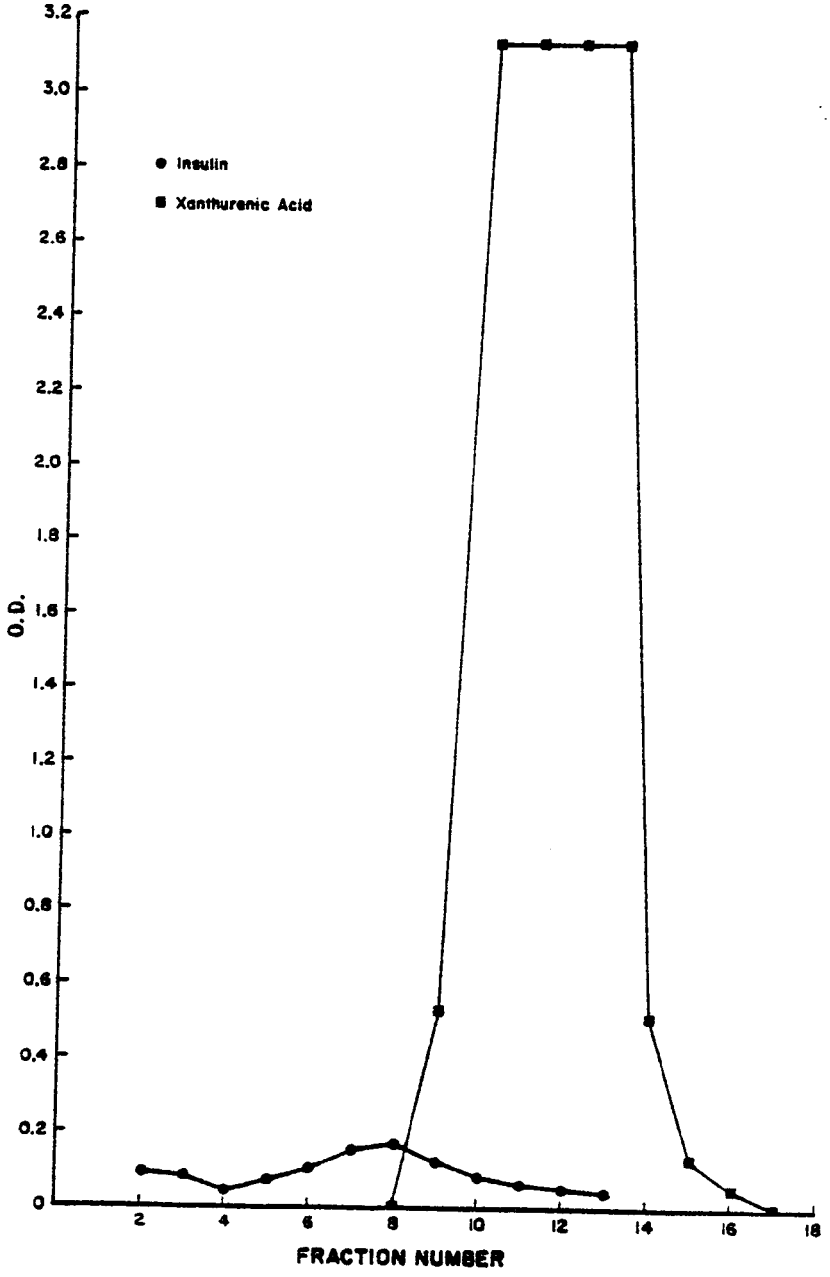
3.2
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
O.D.
Insulin
Xanthurenic Acid
2
4
6
8
10
12
14
16
18
FRACTION NUMBER

Figure 5

Figure 6. Effects of native insulin, XA and peak A on glucose levels (means± SEM) of rabbits. Each rabbit was injected with 21 or 42 ug of sample/kg body weight.

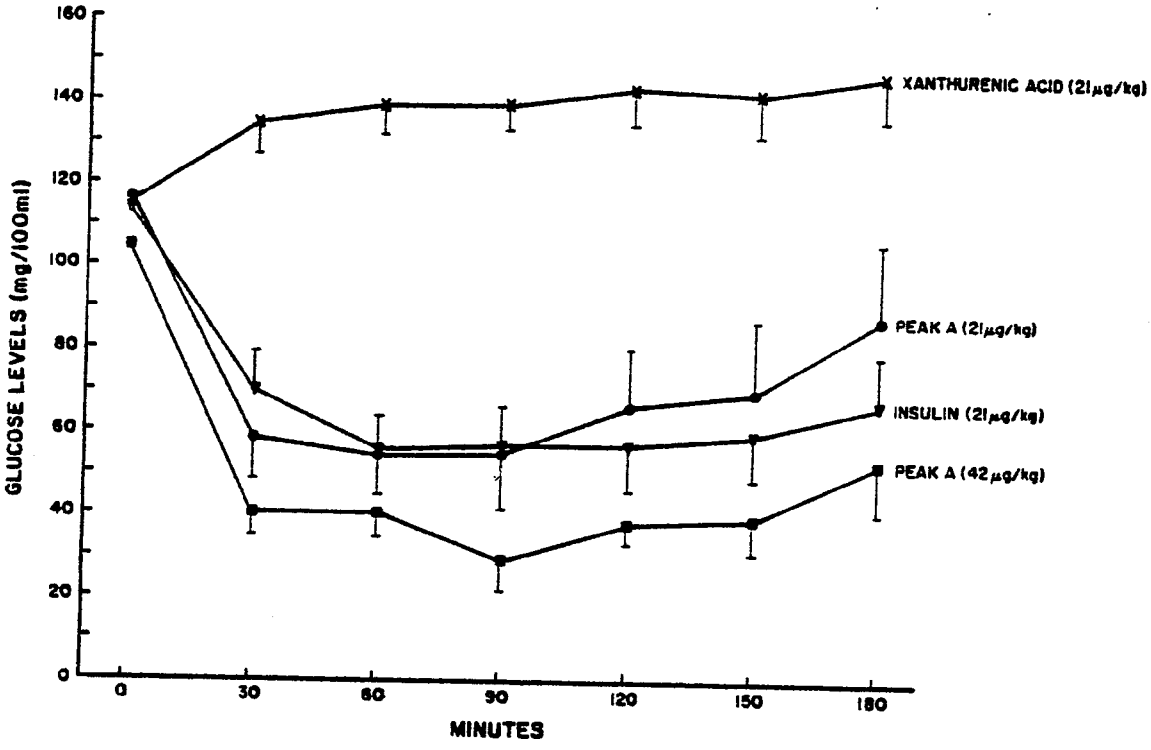
160
140
120
100
80
60
40
20
0
GLUCOSE LEVELS (mg/100ml)
0
30
60
90
120
150
180
MINUTES
XANTHURENIC ACID (21μg/kg)
PEAK A (21μg/kg)
INSULIN (21μg/kg)
PEAK A (42μg/kg)

Figure 6

Figure 7. Plasma insulin (means ± SEM) levels after subcutaneous injection of 21 or 42 ug/kg of body weight of XA or peak A.

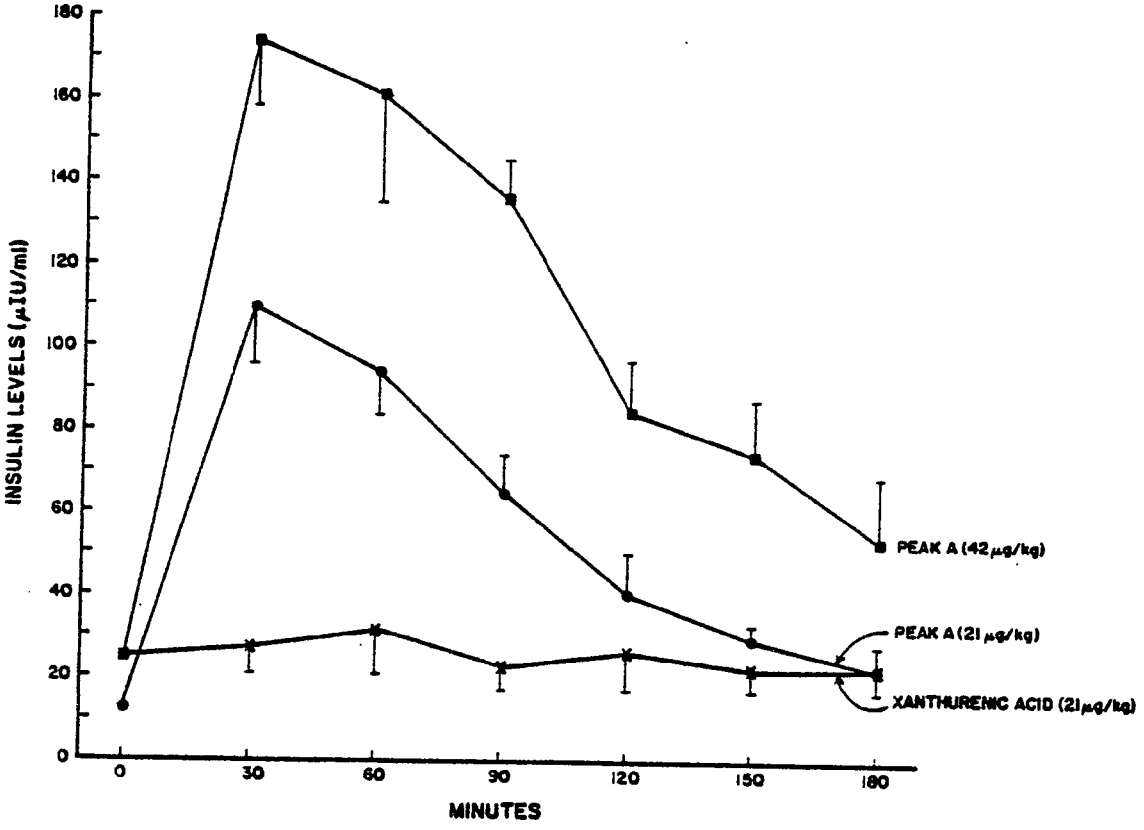
INSULIN LEVELS (μIU/ml)
180
160
140
120
100
80
60
40
20
0
0
30
60
90
120
150
180
MINUTES
PEAK A (42 μg/kg)
PEAK A (21 μg/kg)
XANTHURENIC ACID (21 μg/kg)

Figure 7

of 42 ug/kg body weight reflect the fact that there is twice as much insulin in the 42 ug dose as the 21 ug dose of peak A. Injection with 21 ug XA alone caused a hyperglycemic response but elicited no insulin response. These data suggest that complexes between XA and insulin do not form in vitro.

Rationale and Objectives of Dissertation Research

The proposed role of XA in glucose metabolism resulting from a vitamin B-6 deficiency has been primarily based on speculation and assumptions. Very little is actually known of the biological function of XA. In order to begin to understand the possible physiological role of XA, new approaches must be undertaken, including measurement of XA in the circulation.

In previous studies, it has been repeatedly demonstrated that a vitamin B-6 deficiency results in increased urinary excretion of XA. Measurement of increased levels of XA in the urine has been considered a sensitive and reliable index of the B-6 nutritional status of individuals (17). Several methods have been developed to quantitate XA and other tryptophan metabolites in the urine. One of the first methods assayed XA by a colorimetric procedure described by Miller and Bauman (74). Since that time several procedures have been developed to measure urinary levels of the metabolite, including the

use of fluorimetry (94, 110), paper or thin-layer chromatography (18, 92, 139, 150), ion exchange chromatography (17, 145), high performance liquid chromatography and gas chromatography (97, 107, 129, 132).

Even though these methods of isolating XA in urine have become increasingly sophisticated and sensitive, a method must be developed which would allow for the detection of very small quantities of XA in plasma, take into consideration its basic insolubility in most aqueous solutions and exclude the detection of similar indole compounds. Development of such a quantitative procedure to determine plasma levels of XA in normal and vitamin B-6 deficient rabbits is crucial to designing appropriate experiments to study potential physiologic and pharmacologic interrelationships of XA, vitamin B-6 deficient status and glucose metabolism.

As reviewed by Rose (104), supplementation with vitamin B-6 improves glucose dynamics and decreases excretion of XA by B-6 deficient individuals. However, circulatory levels of XA in response to vitamin B-6 therapy have not been determined. In addition, the demonstration that XA, when injected alone, is hyperglycemic, was done without knowledge of the levels of XA present in the circulation of normal or B-6 deficient individuals. The rate at which XA disappears from the circulation as a result of

the B-6 nutritional status of the individual is also unknown. Experiments designed to study these parameters will help to determine whether XA is indeed a causal agent in deteriorating glucose tolerance, and will provide the information necessary to reproduce the insulin and glucose characteristics seen in the B-6 deficient state.

As a result of the literature review and preliminary data, the following hypothesis was formulated: that deterioration of glucose metabolism observed in conjunction with vitamin B-6 deficient states is causally related to a concomitant increase in circulating levels of XA. The objectives defined to test this hypothesis were to: 1) develop an assay for quantitating XA in serum; 2) determine circulating levels and the disappearance rate of XA in normal and B-6 deficient rabbits; and 3) study causal relationships between XA and glucose metabolism by a) studying plasma glucose and insulin levels in B-6 deficient and B-6 supplemented rabbits and b) by chronically infusing normal rabbits with XA in an attempt to reproduce the glucose and insulin responses observed in B-6 deficient rabbits.

MATERIALS AND METHODS

Animal Model

Normal, mature female New Zealand White rabbits with an average body weight of 3.8 kg were used in the experiments. Prior to the beginning of the experiments, all rabbits were maintained one week on the standard rabbit diet provided by UAB Animal Services and obtained from Cosby-Hodges Milling Company, Birmingham, Alabama. After acclimatization, rabbits were then grouped and fed one of the following: (a) standard rabbit diet (group 1, n=5), (b) ICN purified diet with added B-6 (group 2, n=6), or (c) ICN purified diet without B-6 (group 3, n=5), according to the diet regimen described in Figure 8. The purified diets were obtained from ICN Nutritional Biochemicals, Cleveland, Ohio and their approximate analyses are given in Appendix I. Unless otherwise indicated, all other rabbits used in these studies were maintained on the standard rabbit diet.

Blood samples were collected between 9-11 a.m. from groups 2 and 3 rabbits at 0, 2, 4, 6, 8, 12, 16, and 24 weeks and from group 1 rabbits at 0, 8, and 24 weeks of

Figure 8. Description of diet regimen for treatment groups. Each group of rabbits was maintained on standard rabbit diet, ICN purified diet plus B-6 and/or ICN Purified Diet Without B-6 for a period of 24 weeks according to the following schemes.

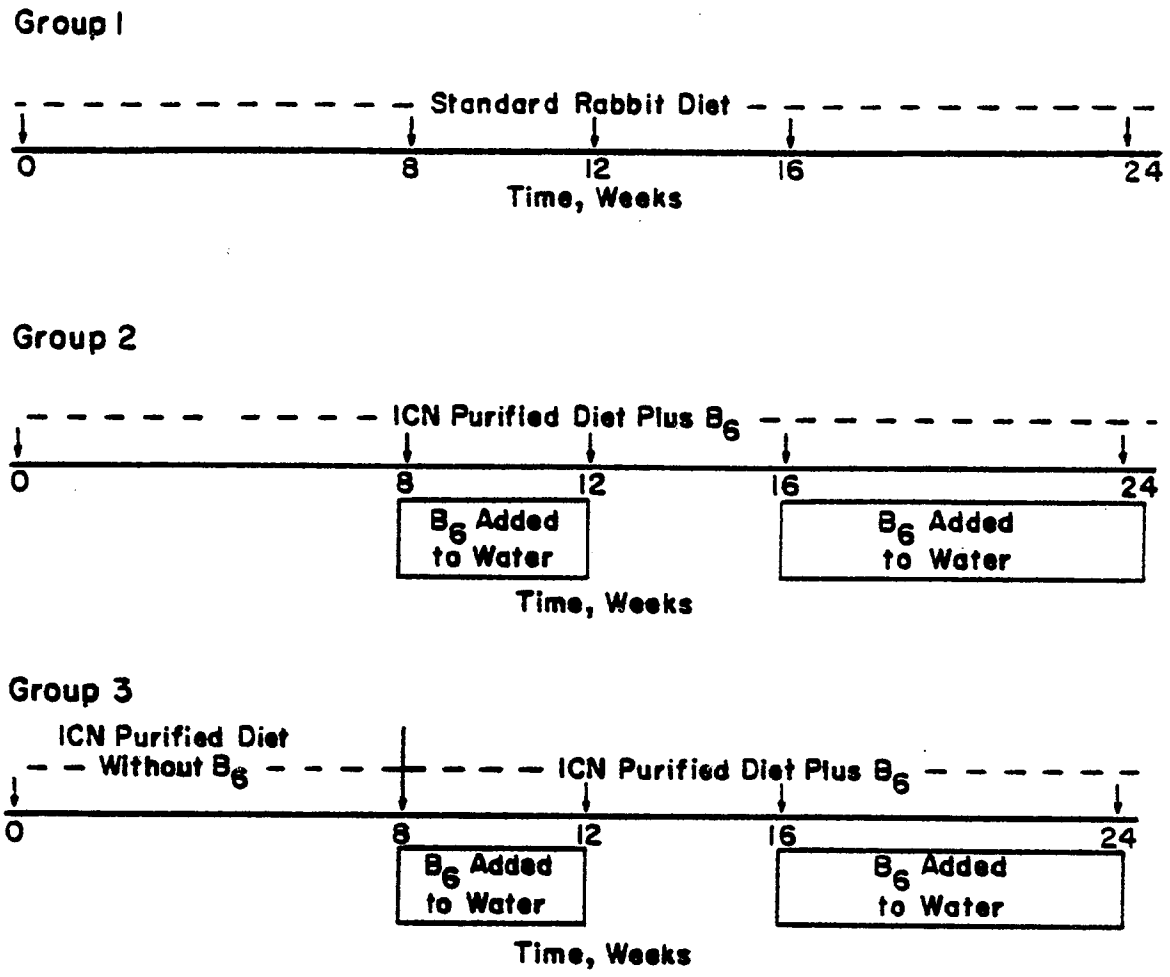
Group I
Standard Rabbit Diet
0
8
12
16
24
Time, Weeks
Group 2
ICN Purified Diet Plus B_6
0
8
12
16
24
B_6 Added to Water
B_6 Added to Water
Time, Weeks
Group 3
ICN Purified Diet Without B_6
ICN Purified Diet Plus B_6
0
8
12
16
24
B_6 Added to Water
B_6 Added to Water
Time, Weeks

Figure 8

the treatment period. Blood samples were collected from from five new rabbits used in the chronic infusion study prior to the infusion and 7 and 12 days during infusion.

Analysis of Rabbit Diets for B-6

Two separate analyses of each batch of rabbit diet were performed for pyridoxine-HCl (B-6) by Hazelton Laboratories, Madison, Wisconsin, according to the method of Atkins, et al. (5). The standard rabbit diet contained 10.0 ug/g of pyridoxine-HCl while the ICN purified diet with added B-6 and ICN purified diet without B-6 contained 20.2 ug/g and 0.08 ug/g, respectively.

Vitamin B-6 Nutritional Status Assessment

The criteria used to determine a B-6 deficient state in the rabbits were modified from the criteria for assessing B-6 nutritional status as outlined by Sauberlich (112). The rabbits were monitored every two to three days over a 24 week period to determine B-6 deficiency symptoms and the alleviation of these conditions as a result of B-6 therapy. The criteria for assessing B-6 nutritional status included:

1. Measurement of aspartate aminotransferase (AST) activity and activity coefficient (degree of saturation of enzyme by PLP),
2. Increase in the concentration of plasma xanthurenic acid (XA) levels,

3. Physical symptoms including: loss of hair, seborrheic dermatitis, conjunctivitis, incrustations of nose and paws, irritability on being handled, decreased motor function (diminished hind limb movement), and muscular weakness.

The rabbits were said to be B-6 deficient when the stimulated activity of the enzyme decreased 35-50% and the A/C increased above 1.3 and when the above physical symptoms of a B-6 deficiency were evident.

Aspartate aminotransferase activity

Red cell hemolysates were assayed for aspartate aminotransferase enzyme (AST: E.C.2.6.1.1) according to the procedure of Bayoumi and Rosalki (7). Enzyme activity was determined on a Hitachi 100-80 double beam spectrophotometer by measuring NADH oxidation at 340 nm, both in the presence and absence of added pyridoxal-PO_4 (PLP) according to the following coupled reactions:

L-Aspartate + 2-oxoglutarate --> Glutamate + Oxaloacetate

Oxaloacetate + NADH ----> Malate + NAD^+

The enzyme data were recorded either as the activity coefficient, A/C (the quotient of the activities obtained with and without excess PLP) or activity per ml RBC.

Calculations:

1. Find ΔOD @ 340 nm/min for both unstimulated and PLP stimulated reactions.

2. Divide $\frac{+\text{PLP } \Delta\text{OD } 340\text{nm}}{-\text{PLP } \Delta\text{OD } 340\text{nm}} \times 100 =$ % stimulation or A/C (Activity Coefficient)

Plasma XA Levels

The plasma samples were prepared and analyzed according to the procedure described in Appendix III. Briefly the freshly drawn blood was centrifuged at 2000xg for 5 minutes. Plasma protein was precipitated by the addition of 0.6 ml of cold 20% (w/v) TCA to each 2 ml of serum. The samples were recentrifuged and the supernatant was lyophylized and reconstituted with 2 ml of absolute methanol. The samples were then centrifuged (2000 xg) and filtered through 0.2 uGelman acrodiscs and analyzed by High Performance Liquid Chromatography (HPLC) using a Varian Model 5000 liquid chromatograph and a 25 cm x 0.5 cm i.d. reversed phase, five micron column. Column eluents were monitored at 243 nm, using a Laboratory Data Control (LDC) spectromonitor III.

Chronic Infusion of XA

Xanthurenic acid, supplied by Sigma Chemical Company, St. Louis, Missouri, was dissolved in distilled water (10 mg/ml) and adjusted to a pH of 7.6 with saturated NaOH. Continuous subcutaneous infusion of XA for two weeks was accomplished by means of Model 2002 Alzet osmotic minipumps (Alza Corporation, Palo Alto, California), implanted subcutaneously in the dorsal region of the neck.

Implantation was carried out under anesthesia with ketamine (35 mg/kg IV in marginal ear vein). The constancy of the rate of delivery of the osmotic pumps was investigated in preliminary studies by implanting osmotic pumps, filled with solutions of XA in normal rabbits (n=5) and measuring serum levels of XA at days 7 and 12. Each pump, according to the manufacturer, had a reservoir volume of 231 ul and would deliver 0.5 ul/hr in vitro. Each pump was loaded with approximately 2300 ug of XA. A total of eight pumps was placed in each rabbit.

Disappearance of XA from Serum

Rabbits received subcutaneous injections of 160 ug XA/kg body weight in the dorsal region of the neck. Blood samples were drawn immediately before and 4, 6, 10 and 15 minutes post-injection, from the central artery of the ear. Samples were prepared and analyzed by HPLC for XA levels as described earlier.

Glucose Tolerance Test

This procedure was based on the method of Maracek and Feldman (66). Two milliliters of 50% dextrose solution per kg body weight (1 gm glucose/kg body weight) were infused IV over a 3-5 minute period into the marginal ear vein. Blood samples were collected from the central artery of the opposite ear at 0, 15, 30, 45, 60, 90, and 120 minutes and analzyed for glucose and insulin.

Glucose Determinations

Serum glucose levels were determined according to procedures outlined by Sigma Chemical Company, St. Louis, Missouri in technical Bulletin No. 510. Rabbit serum was added to a mixture containing glucose oxidase, peroxidase and o-diansidine and allowed to incubate for 45 minutes at 20-25^{o}C. The optical density of each reaction was measured on a Bausch and Lomb Spectronic 20 at 450 nm. The final color intensity of each sample was proportional to the glucose concentration. Glucose concentrations were calculated as follows:

$$\text{Serum glucose (mg/100 ml)} = \frac{\text{Test A}}{\text{Standard A}} \times 100$$

The levels of blood glucose were plotted against time to estimate the glucose disposal rate.

Insulin Levels

Insulin levels were determined by using a RIA kit obtained from Immuno Nuclear Corporation, Stillwater, Minnesota. Serum samples were combined with guinea pig anti-porcine insulin serum and ^{125}I porcine insulin and incubated at 4^{o}C overnight. Rabbit anti guinea pig precipitating complex (second antibody) was then added to each sample. The samples were centrifuged at 760 xg for 20 minutes, decanted and counted on a Micromedic System 4/200 Automatic Gamma Counter. The data were reduced by a Hewlett-Packard 9815A/S Calculator utilizing a weighted linear regression analysis.

A parallel dose response (Figure 9) was obtained between serial dilutions of rabbit plasma and porcine insulin in the RIA system. Micro units/ml of insulin can be converted to nanograms per ml by using the conversion factor of 25 units/mg of insulin.

Data Analysis

All data are expressed as means ± SEM. Analyses used a general linear models procedure and unpaired and paired t tests as options of the Statistical Analysis System (40). Comparisons are described with the presentation of data in the "Results" section.

Figure 9. The dose response curve comparing cross reactivity of rabbit serum (curve B) to the standard curve of human insulin (curve A) when rabbit insulin levels were assayed in a human insulin radioimmunoassay (Immunonuclear Corporation, Stillwater, MN).

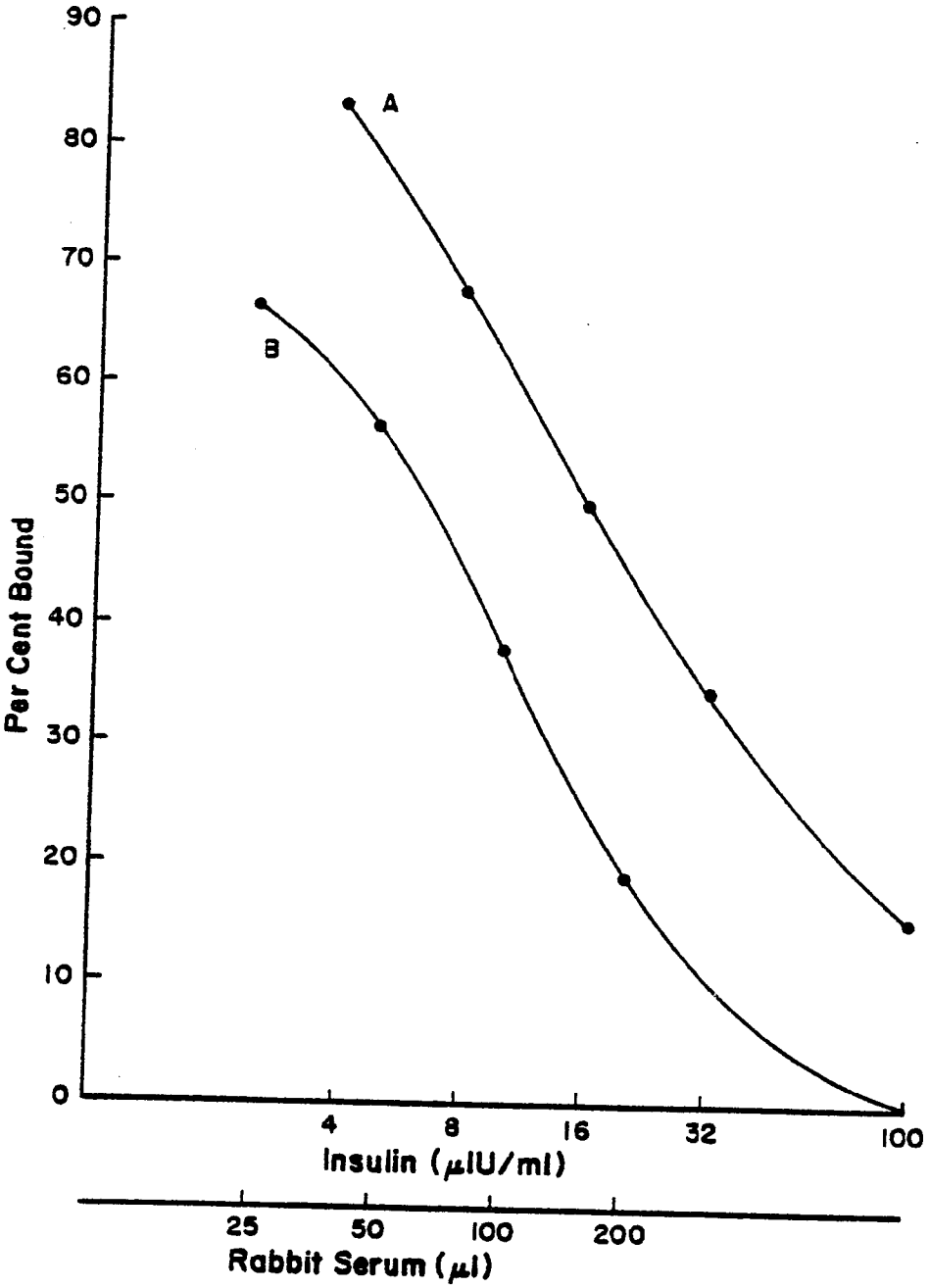
90
80
70
60
50
40
30
20
10
0
Per Cent Bound
A
B
4
8
16
32
100
Insulin (μIU/ml)
25
50
100
200
Rabbit Serum (μl)

Figure 9

RESULTS

Vitamin B-6 Nutritional Status

Group 1 rabbits (standard rabbit diet) maintained a healthy state throughout the study and had a 28% increase in body weight by the 24th week of treatment (Figure 10). Rabbits in group 3 (ICN purified diet without B-6) were the first to exhibit B-6 deficiency symptoms with hair loss just two weeks into the study period. At four to six weeks seborrheic dermatitis, decreased motor function diminished hind limb movement, irritability on being handled and muscular weakness were noted and by six weeks two of the animals contracted conjunctivitis. Group 2 (ICN purified diet with added B-6) animals began losing hair at 6 weeks of the study and exhibited seborrheic dermatitis and some irritability by 8 weeks of the study. Both groups 2 and 3 lost weight (Figure 10) and by the 8th week, had lost 14% to 17% of their original weight. When supplemented with vitamin B-6 (4.4 mg/kg body weight), from the 8th to 12th weeks of the study, groups 2 and 3 maintained their weights. A second period of B-6

Figure 10. A comparison of mean percent change in body weight of rabbits in treatment Groups 1 (▲), 2 (●) and 3 (■) over a period of 24 weeks. It is essential to note the treatment plan as described in Figure 8.

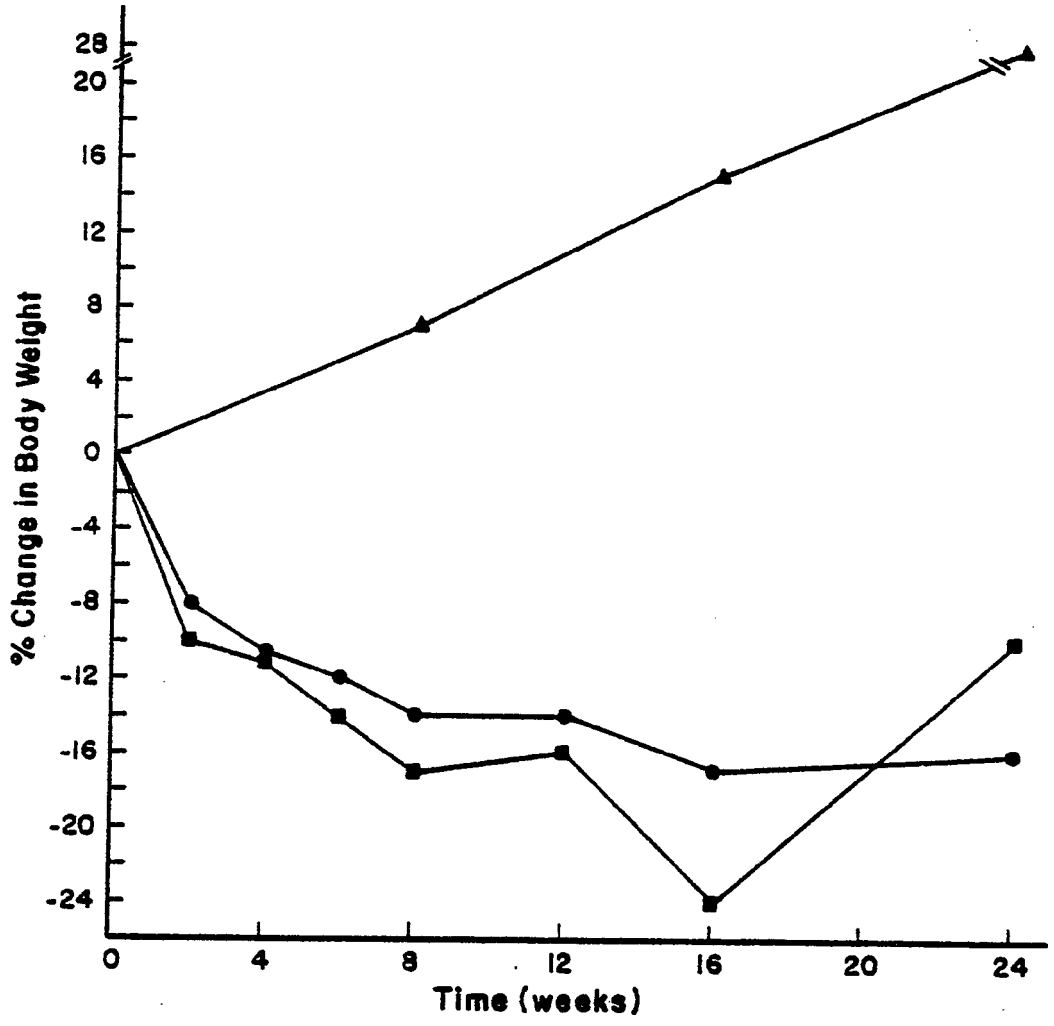
28
20
16
12
8
4
0
-4
-8
-12
-16
-20
-24
% Change in Body Weight
0
4
8
12
16
20
24
Time (weeks)

Figure 10

supplementation from 16 to 24 weeks resulted in both groups 2 and 3 gaining weight. The weight gain in group 3 was statistically significant ($p < 0.05$).

As a biochemical assessment of the B-6 nutritional status of the rabbits, aspartate aminotransferase (AST) stimulated activity by excess coenzyme and activity coefficients (A/C) were determined. By the second week of the study the activity coefficients approached values of 2.0 and greater (normal = 1.0 to 1.3). Both the unstimulated and stimulated activity of AST decreased in groups 2 and 3 by the second week (Table 1). By six weeks, the activity coefficients became excessively variable, probably as a result of decreased protein synthesis and low total enzyme in the body pool and were considered to be unreliable as an index of B-6 status. At the end of the 8 week study period, the unstimulated and stimulated enzyme activity of AST remained significantly decreased ($p < 0.05$) in groups 2 and 3. As a result of B-6 therapy from 8 to 12 weeks, the enzyme concentration and stimulated activity increased in groups 2 and 3. The enzyme concentration and stimulated activity decreased again between 12 and 16 weeks as a result of the withdrawal of the B-6 supplement. After B-6 supplementation between 16 and 24 weeks, the unstimulated as well as the stimulated activity of the enzyme increased to levels somewhat above pretreatment values. Group 1 rabbits (standard diet) had

Table 1. Aspartate aminotransferase activity (unstimulated* and stimulated**- uMoles NADH oxidized/min) for each group of rabbits for the complete study period. Group 1 was maintained on the standard rabbit diet throughout the study. Group 2 and 3 were fed according to the dietary regimen noted in Figure 1. Values represent the mean ± SEM.

Group	Pretreatment		2		4		6		8		12		16		24		weeks
	US	SA	US	SA	US	SA	US	SA	US	SA	US	SA	US	SA	US	SA	
1	215±17	273±15													348±9	356±26	
2	205±15	228±16	92±8	142±15	143±15	178±18	48±8	63±7	52±12	81±14	161±9	202±1	77±29	117±21	398±92	453±101	
3	198±12	229±20	75±12	134±8	86±10	118±12	82±9	107±9	76±3	101±6	142±25	222±36	76±11	107±11	290±9	371±34	

* Unstimulated activity abbreviated as US
* Stimualted activity abbreviated as SA - represents red cell hemolysate with 100 ul added PLP.

a relatively constant activity of 273 ± 15 uMoles NADH oxidized/min at the pretreatment period and 356 ± 26 uMoles NADH oxidized/min at 24 weeks.

Plasma Xanthurenic Acid Levels

Figure 11 displays the fluctuation of plasma XA levels (ng/ml) as the diet regimen and B-6 nutritional status of each group changed. In both groups 2 and 3, plasma XA levels were significantly ($p < 0.05$) elevated (900 and 1250 ng/ml respectively) when compared to pretreatment values (248 and 320 ng/ml, respectively) at 4 weeks into the treatment period. From 6 to 8 weeks, XA levels began to decline in groups 2 and 3 and when supplemented with vitamin B-6, plasma XA levels approached pretreatment levels in both groups. From 12 to 16 weeks vitamin B-6 therapy was withdrawn and the plasma XA levels rebounded to levels equal to and above 1350 ng/ml in group 2 and 1600 ng/ml in group 3. From 16 to 24 weeks the two groups were again given vitamin B-6 supplement and the plasma XA levels decreased to levels near pretreatment values. Plasma XA levels in rabbits fed the standard diet (group 1), remained substantially the same during the first 8 weeks of the study (190 ± 47 ng/ml to 234 ± 4 ng/ml). An increase to 419 ± 128 ng/ml in plasma levels was noted in group 1 by 24 weeks.

Figure 11. Mean xanthurenic acid levels (ng/ml ± SEM) in plasma for each group of rabbits for the complete study period. Group 1 (▲) was maintained on the standard rabbit diet throughout the study. Group 2 (●) and 3 (■) were fed according to the dietary regimen noted in Figure 8.

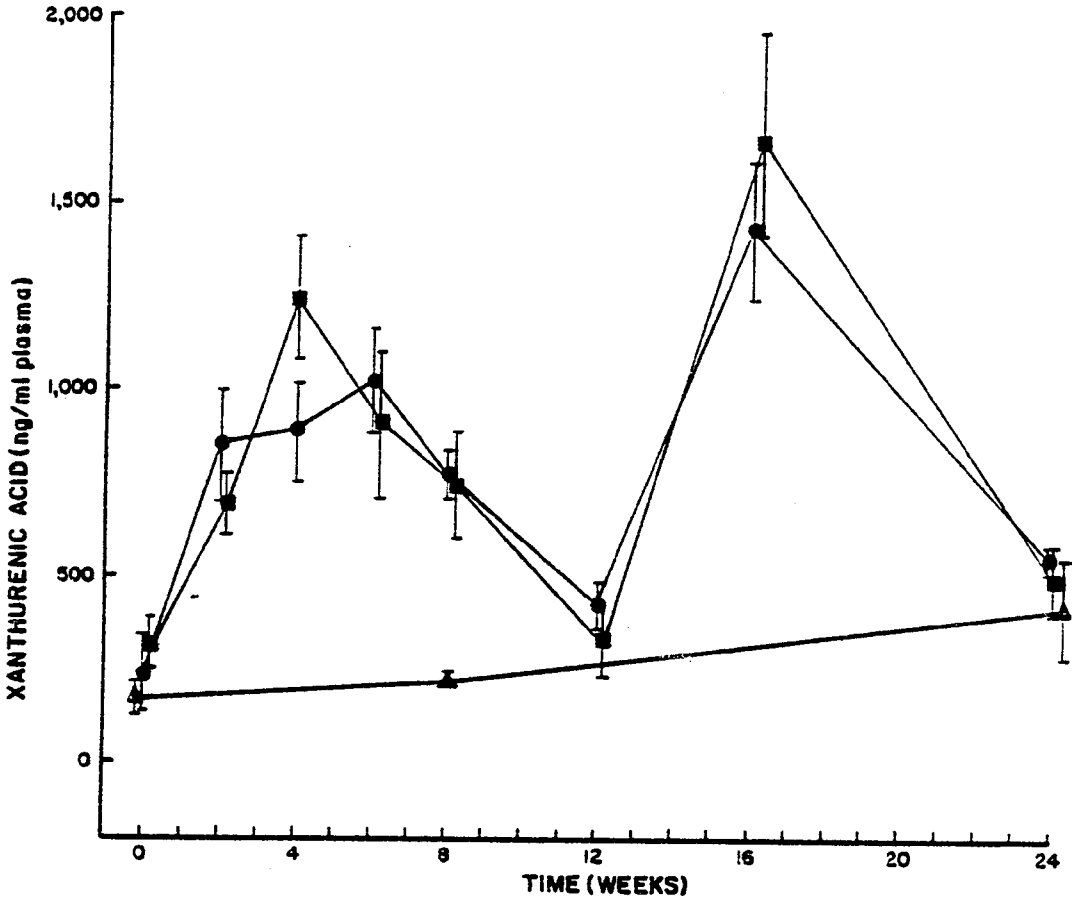
2,000
1,500
1,000
500
0
XANTHURENIC ACID (ng/ml plasma)
0
4
8
12
16
20
24
TIME (WEEKS)

Figure 11

Disappearance of XA From Serum

In order to understand the pharmacokinetics of XA, a bolus of XA (160 ug/kg body weight) was injected subcutaneously into all rabbits during the pretreatment period and 8 weeks into the study and the pattern of disappearance from serum was studied. Regardless of treatment or B-6 nutritional status, the absorption phase, after injection of XA, was between 0 and 4 minutes (Table 2). Peak values occurred at 4 to 6 minutes. The post-absorptive phase was between 6 and 15 minutes. As shown in Figure 11, the baseline concentration of XA remained the same for group 1 rabbits, fed the standard diet, at pretreatment and 8 weeks. Meanwhile the concentration of XA in the plasma of rabbits on the purified diets with added vitamin B-6 (group 2) and without added vitamin B-6 (group 3), increased to 803 ng/ml and 745 ng/ml, respectively, by 8 weeks of the study.

Figures (12-15, Table 3) show serum concentrations of XA over time for each group after a subcutaneous injection of a bolus of 160 ug XA/kg body weight. The disappearance of XA from serum could be described by a one compartment open model with elimination from the central compartment. The XA concentration at any time (c) can be described by the equation $C_o=Ce^{-kt}$ where C_o is the zero intercept, t is time and k is the rate constant for decay.

TABLE 2. Xanthurenic acid in serum (ng/ml±SEM) of rabbits after subcutaneous injections of 160 ug XA/Kg body weight.

Group	Period	0 min	4 min	6 min	10 min	15 min
	*Pretreatment n=18	248±47	406±48	351±52	256±33	172±31
1	8 weeks n=5	234±41	444±80	591±139	328±87	313±98
2	8 weeks n=6	803±65	672±91	850±111	739±79	542±70
3	8 weeks n=5	745±147	612±109	1160±81	587±96	455±68

* This group consists of all rabbits used in the entire study prior to being separated into treatment groups.

Figure 12. The initial serum profile (means ± SEM) of xanthurenic acid (XA) and the best-fit line derived from linear regression analysis after injection of 160 ug XA/kg body weight to all rabbits used in the entire study prior to being separated into specific treatment groups.

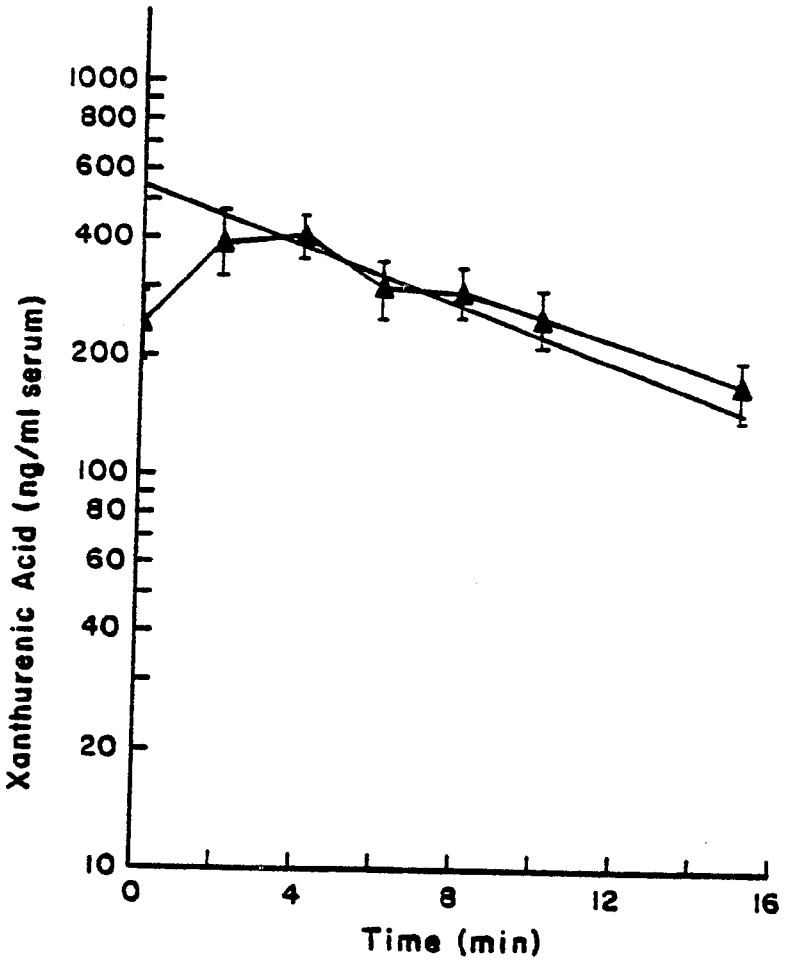
1000
800
600
400
200
100
80
60
40
20
10
Xanthurenic Acid (ng/ml serum)
0
4
8
12
16
Time (min)

Figure 12

Figure 13. The serum profile (means ±SEM) of xanthurenic acid (XA) and the best-fit line derived from linear regression analysis after injection of 160 ug XA/kg body weight in Group 1 rabbits. The rabbits had been maintained for 8 weeks on the standard diet.

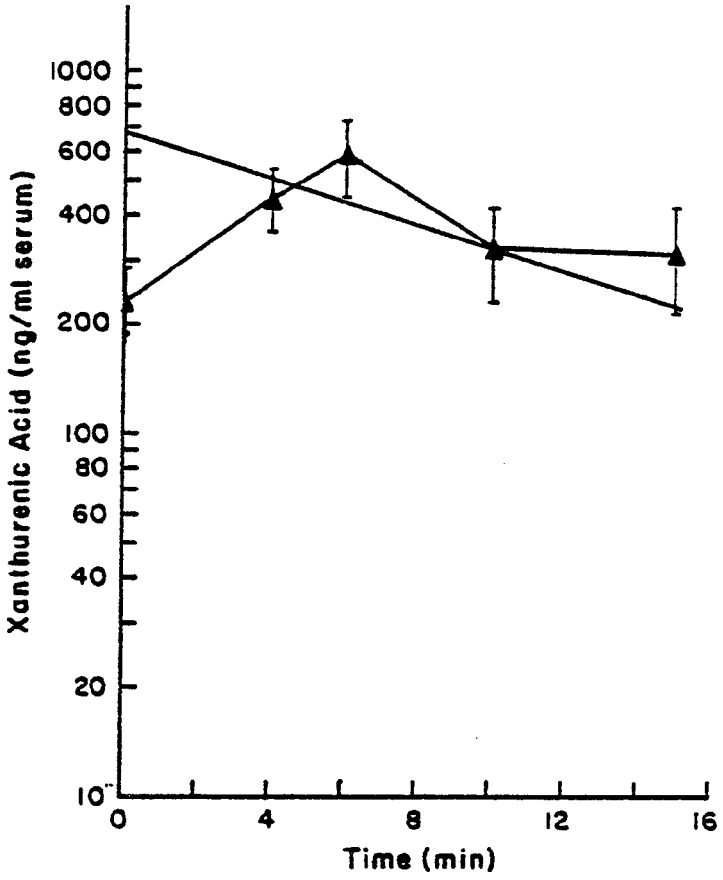
1000
800
600
400
200
100
80
60
40
20
10
Xanthurenic Acid (ng/ml serum)
0
4
8
12
16
Time (min)

Figure 13

Figure 14. The serum profile (means ±SEM) of xanthurenic acid (XA) and the best-fit line derived from linear regression analysis after injection of 160 ug XA/kg body weight in group 2 rabbits. The rabbits had been maintained for 8 weeks on ICN purified diet containing vitamin B-6.

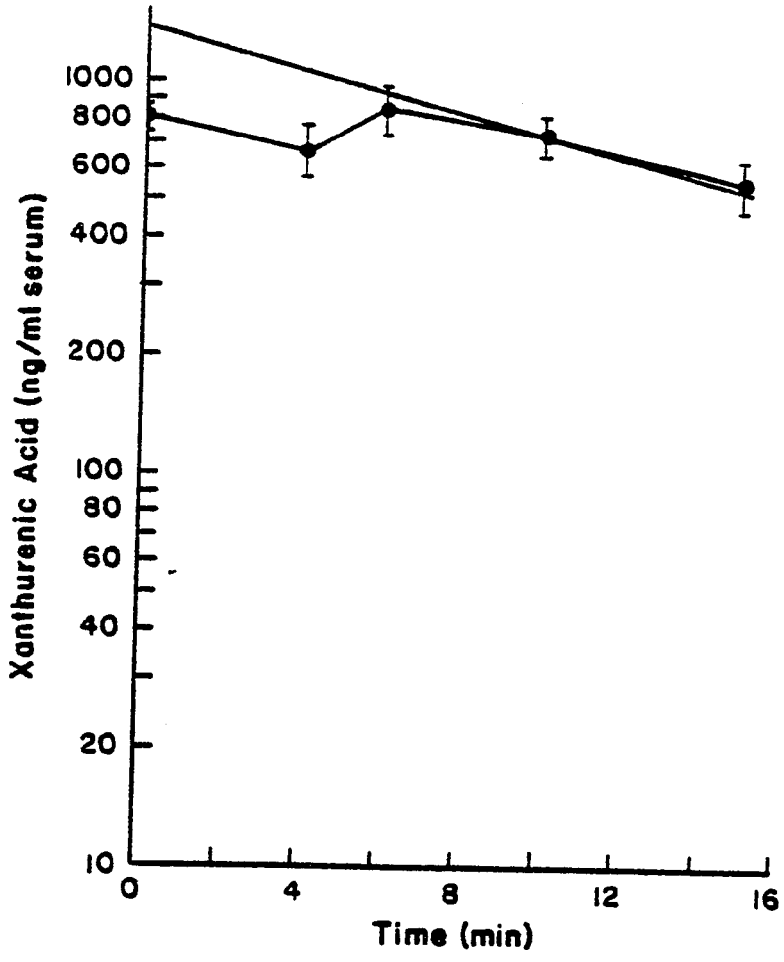
Xanthurenic Acid (ng/ml serum)
1000
800
600
400
200
100
80
60
40
20
10
0
4
8
12
16
Time (min)

Figure 14

Figure 15. The serum profile (means ± SEM) of xanthurenic acid (XA) and the best-fit line derived from linear regression analysis after the injection of 160 ug XA/kg body weight in group 3 rabbits. The rabbits had been maintained for 8 weeks on ICN purified diet with vitamin B-6 omitted.

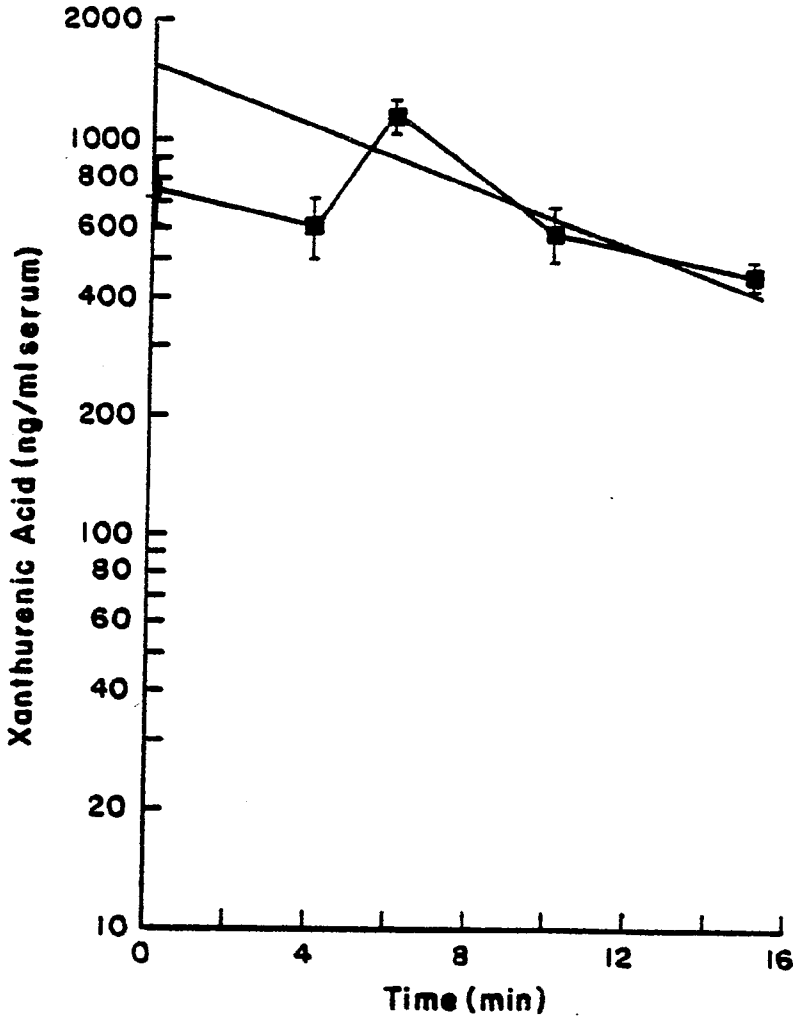
2000
1000
800
600
400
200
100
80
60
40
20
10
Xanthurenic Acid (ng/ml serum)
0
4
8
12
16
Time (min)

Figure 15

TABLE 3. Comparative disappearance data (means) for XA in rabbit serum after subcutaneous injections of 160 ug XA/kg body weight as determined by linear regression analysis.

Group	#Animals	#Data Pairs	$T_{1/2}$*	K-^{min}**	C_o ng/ml***
Pretreatment 1	18	68	7.89	.0878	537
8 weeks 1	5	16	9.19	.0754	682
8 weeks 2	6	17	10.99	.0630	1349
8 weeks 3	5	16	7.70	.0900	1561

* $T_{1/2}$ - Half Life of XA in serum ($T_{1/2} = 0.693/K$)

** K - Elimination rate constant ($K = 0.693/T_{1/2}$)

***C_o - Concentration of XA extrapolated to time zero on a serum concentration: time-profile.

Converting to the log form and rearranging results gave the equation: $\ln C = \ln C_o - kt$, which was analyzed by linear regression to determine the slope and zero time intercept. Values ranging from the peak levels at 4 to 6 minutes to levels at 15 minutes were used in the analysis. No significant difference in the apparent half-life of XA in serum was noted between treatment groups. The apparent half-life time ranged from 7.70 minutes to 10.99 minutes.

The zero time concentration (C_o) of XA in serum for group 1 rabbits on the standard diet was essentially the same (537 and 682 ng/ml) at pretreatment and 8 weeks of the study. The C_o for groups 2 and 3 rabbits, increased to values approximately twice those of group 1 (1349 and 1,561 ng/ml, respectively) by 8 weeks of treatment.

To further understand the disposition of XA in normal and B-6 deficient rabbits, the apparent volume of distribution (V_D) was computed for each group of rabbits (Table 4). Based on the assumption that the body acts as a single compartment with respect to XA, the V_D is defined as the volume of fluid into which XA appears to distribute with a concentration equal to that in plasma. The V_D of Group 1 remained the same (1171 ml and 996 ml) for the pretreatment and 8 week period of the study,

TABLE 4. The averaged volume of distribution (V_D) of XA in rabbits injected subcutaneously with 160 ug XA/kg of body weight.

Group	#Animals	C_o ng/ml	Weight (kg)	Dosage XA ug	*$V_D$ml ± SEM	
Pretreatment	18	537	3.94	630	1171	149
8 weeks 1	5	682	4.24	679	996	280
8 weeks 2	6	1349	3.32	531	**394	48
8 weeks 3	5	1561	3.30	528	**338	45

*V_D - $\frac{\text{Total Dose ng/ml}}{\text{Total ng}}$

**V_D significantly lower ($p < 0.05$) than for pretreatment group and Group 1.

while the V_D's of groups 2 and 3 significantly decreased ($p < 0.05$) to 394 ml and 338 ml, respectively. A decrease in body fat and weight would decrease the available volume of distribution. An expected or probable linear relationship between the V_D and percent change in body weight could not be established because of insufficient data. However, Figure (16) shows that the V_D decreased along with a decrease in the percent change in body weight in groups 2 and 3.

Glucose Tolerance Studies

Glucose tolerance tests (GTT) were administered to each group at pretreatment, 8 weeks and 24 weeks of the study. Within group analysis for group 1 (standard diet) results demonstrated the same response to a glucose load for the pretreatment and 8 week periods (Figure 17, Table 5). The peak values (373 $\pm$ 11 and 337 $\pm$ mg/100 ml) occurred 15 minutes following the glucose load at pretreatment and 8 weeks, respectively. By 120 minutes, values had decreased and began to approach baseline glucose levels of the pretreatment GTT and 8 week GTT. A deterioration in glucose tolerance was noted at 24 weeks. At 15 minutes following administration of a glucose load, group 1 had significantly increased ($p < 0.05$) glucose levels (424 mg/100 ml) compared to the pretreatment and 8 week periods.

Figure 16. The volume of distribution for xanthurenic acid plotted against the percent change in body weight 8 weeks after the rabbits began their respective treatment diets. The bars represent the mean ± SEM for each group. Groups 1, 2 and 3 are represented by the symbols, ▲, ●, ■, respectively.

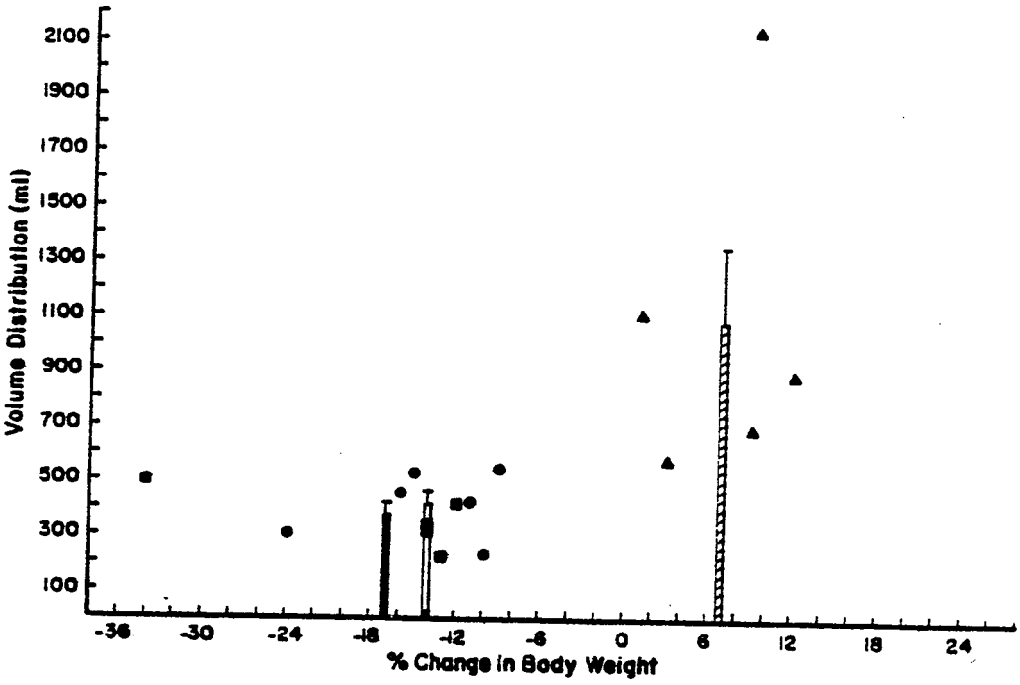
Volume Distribution (ml)
2100
1900
1700
1500
1300
1100
900
700
500
300
100
-36
-30
-24
-18
-12
-6
0
6
12
18
24
% Change in Body Weight

Figure 16

Figure 17. Serum glucose levels (means ± SEM) in response to glucose tolerance tests of rabbits fed standard diet for 24 weeks. Glucose tolerance tests were performed pretreatment and after 8 and 24 weeks on the diet by injecting, IV, 2 ml of 50% dextrose solution per kg body weight (1 gm glucose/kg body weight). The number of rabbits tested pretreatment and after 8 and 24 weeks of dietary treatment was 5, 5 and 3, respectively.

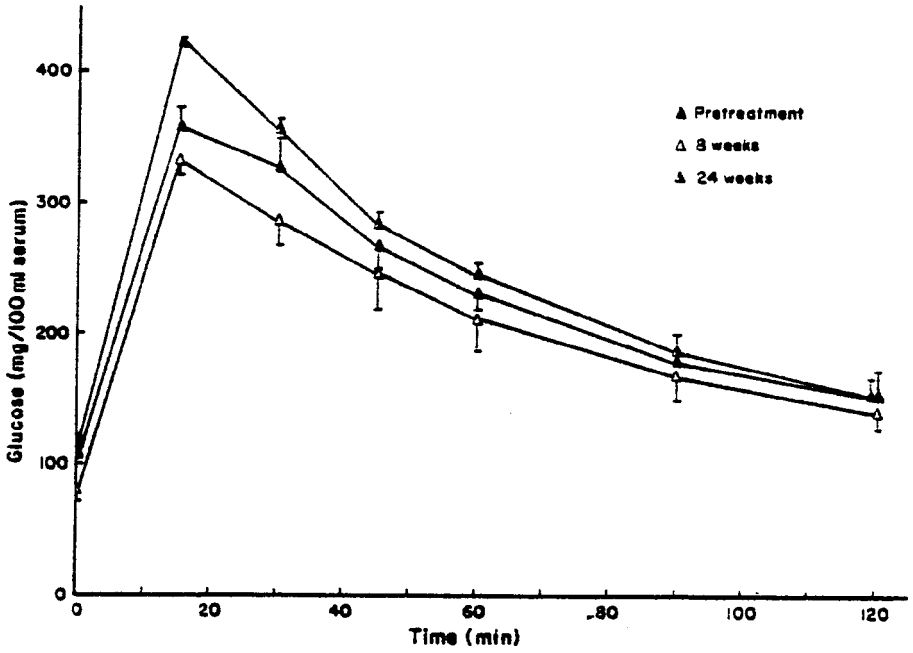
▲ Pretreatment
△ 8 weeks
▲ 24 weeks
Glucose (mg/100 ml serum)
400
300
200
100
0
0
20
40
60
80
100
120
Time (min)

Figure 17

TABLE 5. Serum glucose (mg/100 ml) measured during IV glucose tolerance tests (described in Figure 17) in Group 1 rabbits (standard rabbit diet). Values are expressed as means ± SEM.

Test (minutes)	0	15	30	45	60	90	120
Pretreatment n=5	112±3	373±11	329±20	368±17	233±13	183±16	153±11
8 weeks n=5	80±5	337±9	289±17	249±25	214±25	171±23	145±18
24 weeks n=3	113±5	424*	359±5	286±7	248±6	190±9	154±12

* Value is significantly higher ($p < 0.05$) compared to pretreatment and 24 week values.

Rabbits fed the ICN purified diet with added vitamin B-6 (Group 2), exhibited deteriorated glucose tolerance at 8 weeks of the study period (Figure 18, Table 6). The basal level of glucose increased from 88±7 to 118±5 mg/100 ml. After a glucose load, glucose levels increased significantly ($p < 0.05$) above the values noted in the pre-treatment GTT. At 15 minutes, the levels peaked at 420± 5 mg/100 ml and decreased slowly at 15, 30 and 45 minutes. At 120 minutes the values returned to the basal level noted at the pretreatment period. After supplementation with vitamin B-6, glucose tolerance improved in this group of rabbits. There was no significant difference in the glucose levels observed at the 24-week period from those noted during the pretreatment period.

Group 3 rabbits (ICN purified diet without vitamin B-6) also exhibited decreased glucose tolerance at 8 weeks (Figure 19, Table 7). A significant increase ($p < 0.05$) was noted in glucose levels at 15 and 30 minutes at 8 weeks when compared to glucose levels at 15 and 30 minutes during the pretreatment GTT. After the group was supplemented with vitamin B-6 (4.4 mg/kg) for 8 weeks and then administered a glucose load, the peak levels (282 ± 21 mg/ 100 ml) of glucose present at 15 minutes was significantly less ($p < 0.05$) than the peak values at the pretreatment or 8 week study period.

Figure 18. Serum glucose levels (means ± SEM) in response to glucose tolerance tests of rabbits fed ICN purified diet with added B-6 (Group 2), according to the diet regimen noted in Figure 8. Glucose tolerance tests were performed pretreatment and after 8 and 24 weeks on the diet by injecting, IV, 2 ml of 50% dextrose solution per kg body weight (1 gm glucose/kg body weight). There were 6 rabbits at each treatment period.

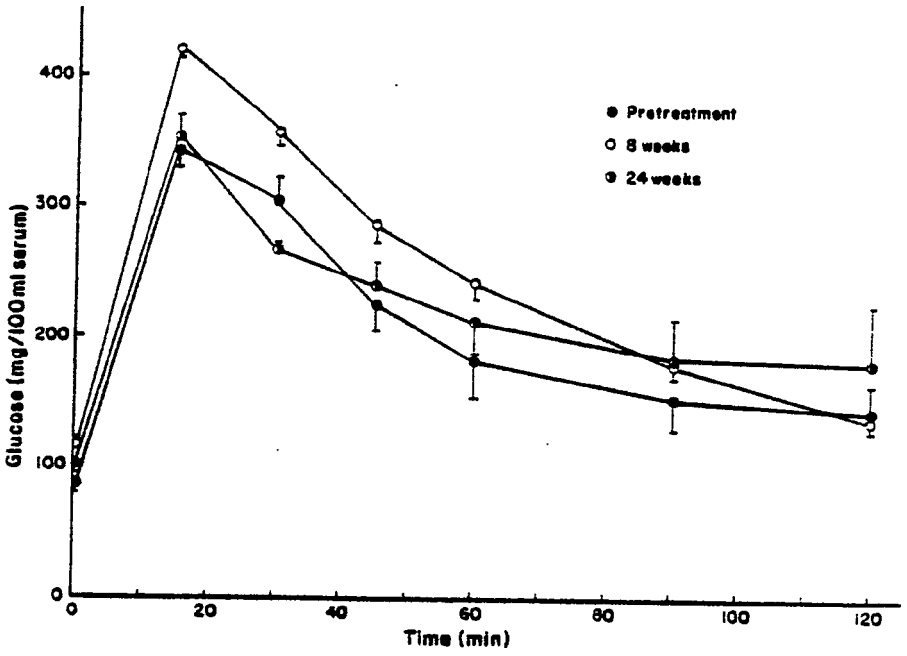
Pretreatment
8 weeks
24 weeks
Glucose (mg/100ml serum)
Time (min)
0
100
200
300
400
0
20
40
60
80
100
120

Figure 18

TABLE 6. Serum glucose (mg/100 ml) measured during IV glucose tolerance tests (described in Figure 18) in Group 2 rabbits (ICN purified diet with added vitamin B-6). Values are expressed as means ± SEM.

TIME	0	15	30	45	60	90	120
Pretreatment n=6	90±5	343±11	306±18	225±17	183±27	152±22	143±21
8 weeks n=6	115±7*	421±5*	356±6*	286±9*	244±10	182±8	139±5
24 weeks n=6	103±5	352±15	267±6	238±17	214±27	185±30	182±43

* These values are significantly elevated ($p < 0.05$) when compared to both pretreatment and 24 week periods.

Figure 19. Serum glucose levels (means ± SEM) in response to glucose tolerance tests of rabbits fed ICN purified diet without vitamin B-6 (Group 3), according to the schedule in Figure 8. Glucose tolerance tests were performed pretreatment and after 8 and 24 weeks of the treatment period by injecting, IV, 2 ml of 50% dextrose solution per kg body weight (1 gm glucose/kg body weight). There were 5 rabbits pretreatment and at 8 weeks and 2 rabbits at 24 weeks.

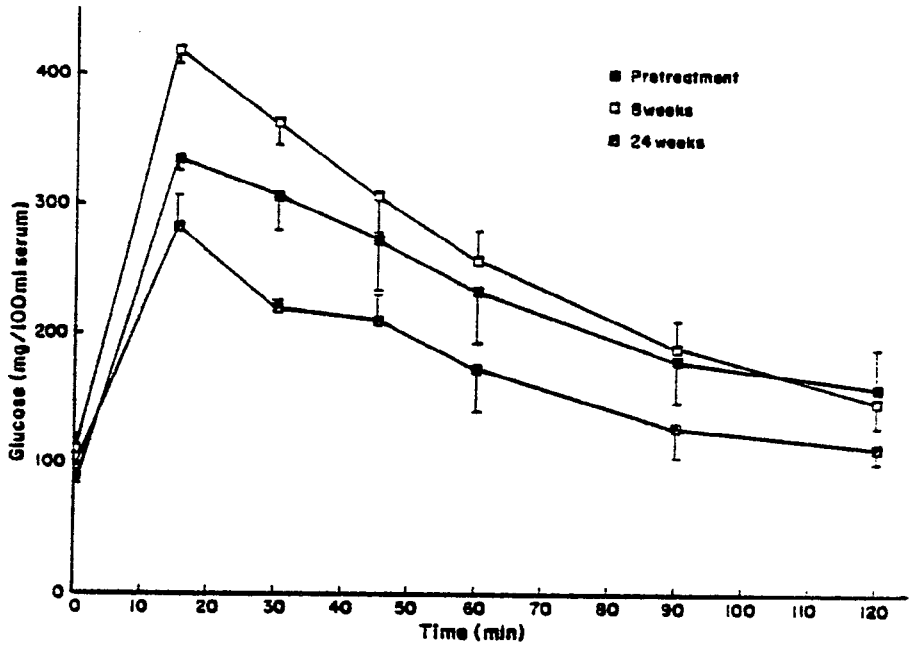
Pretreatment
5weeks
24 weeks
Glucose (mg/100ml serum)
400
300
200
100
0
0
10
20
30
40
50
60
70
80
90
100
110
120
Time (min)

Figure 19

TABLE 7. Serum glucose (mg/100 ml) measured during IV glucose tolerance tests in Group 3 rabbits (ICN purified diet without added vitamin B-6). Values are expressed as means ± SEM.

TIME	0	15	30	45	60	90	120
Pretreatment n=5	93±6	333±5	305±25	275±37	234±36	179±31	158±25
8 weeks n=5	113±9	419±6*	363±14*	307±28	254±23	187±21	149±16
24 weeks n=2	97±0	282±21#	223±1^	210±129	174±29	127±21	112±9

* These values are significantly higher ($p < 0.05$) than values at pretreatment or 24 week periods.

\# This value is significantly lower ($p < 0.05$) than values at pretreatment and 8 weeks.

^ This value is significantly lower ($p < 0.05$) than the corresponding value at 8 weeks.

Between group analysis showed that glucose utilization after a glucose load was essentially the same in each treatment group at the pretreatment period. In addition, there was no significant difference in glucose tolerance between group 2 (ICN purified diet with added vitamin B-6) and group 3 (ICN purified diet without added B-6) after the administration of a glucose load at 8 weeks of the study period. However, their response was impaired as compared to glucose tolerance observed in group 1 (standard diet). At 24 weeks, group 1 glucose levels in response to a glucose load were significantly elevated ($p < 0.05$) at 15 and 30 minutes above the levels in group 2. Meanwhile, the glucose levels in group 1 were also significantly greater than group 3 levels at 15, 30 and 45 minutes. Group 3 glucose levels in response to a glucose load was significantly lower than either group 1 or 2 at 15, 30 and 45 minutes.

Serum Insulin Response to a Glucose Challenge

The insulin level of the rabbits in group 1 (standard diet) prior to any treatment was 10 $\pm$ 2 uIU/ml. In response to a glucose load, insulin levels increased to peak values of 38 $\pm$ 7 uIU/ml at 15 minutes (Figure 20, Table 8). In 120 minutes, insulin levels approached baseline levels. At 8 weeks, the insulin response to a

Figure 20. Serum levels of insulin (means ± SEM) in Group 1 rabbits (normal diet) during glucose tolerance tests in which insulin responses to glucose were determined at pretreatment, 8 weeks and 24 weeks of the study. Five rabbits were tested pretreatment and at 8 weeks and 3 at 24 weeks.

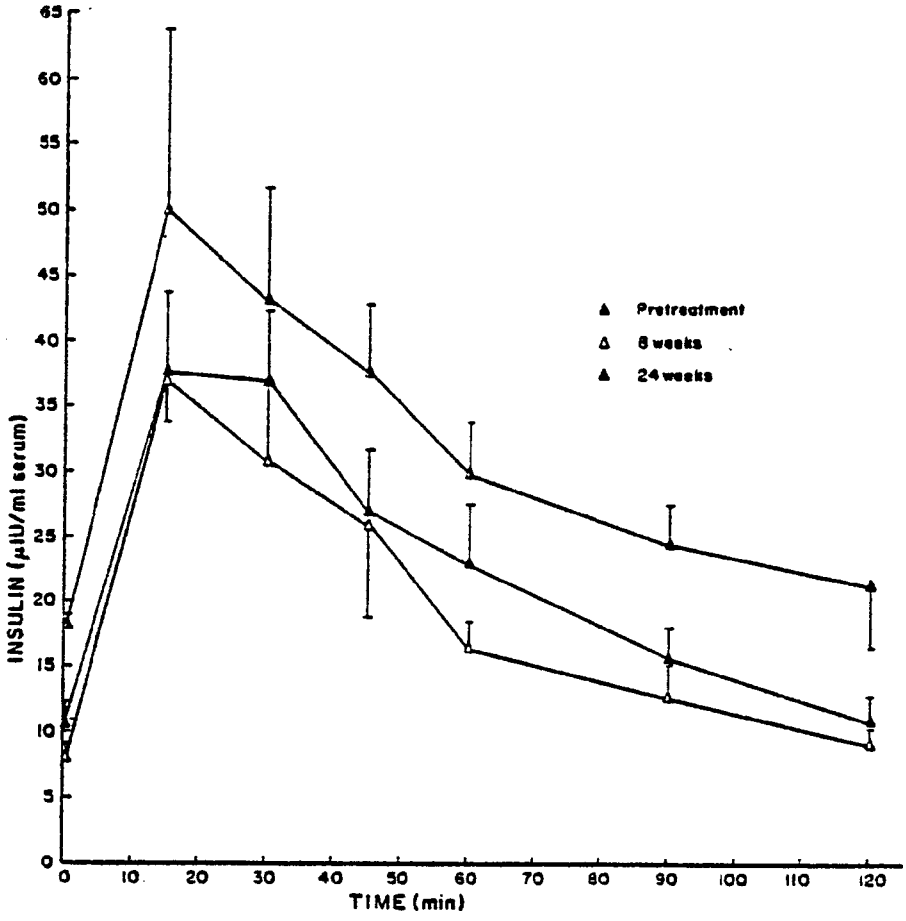
INSULIN (μIU/ml serum)
TIME (min)
Pretreatment
8 weeks
24 weeks
0
5
10
15
20
25
30
35
40
45
50
55
60
65
0
10
20
30
40
50
60
70
80
90
100
110
120

Figure 20

TABLE 8. Mean (± SEM) serum insulin levels (uIU/ml) measured in Group 1 rabbits (standard diet) during IV glucose tolerance tests.

TIME	0	15	30	45	60	90	120
Pretreatment n=5	11±2	38±7	37±5	27±5	23±5	16±2	11±2
8 weeks n=5	8±1	37±3	31±6	26±7	17±2	13±3	9±2
24 weeks n=3	18±0.4*	50±14	43±9	38±5**	30±4*	25±3**	21±4**

* Values are significantly higher ($p < 0.05$) than values at eight weeks.

** Values are significantly higher ($p < 0.05$) than values at pretreatment and 8 weeks.

glucose load was comparable to values of the pretreatment period. The baseline insulin levels at 24 weeks were significantly ($p < 0.05$) elevated. As a result of a glucose load, the peak levels of insulin were increased to 50 $\pm$ 14 uIU/ml at 15 minutes and were significantly higher at 45, 60, 90 and 120 minutes ($p < 0.05$) than values at pretreatment or 8 weeks. In addition, the insulin levels decreased at a slower rate and returned to baseline levels at 120 minutes.

In group 2 rabbits (ICN purified diet, with added vitamin B-6), the initial baseline insulin level was 15 $\pm$ 2 uIU/ml (Figure 21, Table 9). The insulin levels increased to 40 $\pm$ 1 uIU/ml at 15 minutes as a result of a glucose load. Thereafter, the insulin levels decreased and returned to baseline levels at 120 minutes. At 8 weeks, baseline insulin levels decreased to 7 $\pm$ 1 uIU/ml and did not rise significantly above baseline levels in response to a glucose load. The levels remained approximately 11 $\pm$ 3 uIU/ml from 60 to 90 minutes of the GTT and returned to baseline at 120 minutes. At 24 weeks and after 8 weeks of vitamin B-6 supplementation, the baseline insulin levels returned to pretreatment levels. The insulin response to a glucose load was comparable to the response at the pretreatment period.

Figure 21. Serum insulin levels (means ± SEM) in Group 2 rabbits (ICN purified diet with vitamin B-6) in response to a glucose load during glucose tolerance tests at pretreatment, 8 and 24 weeks of the study. There were 6 rabbits at each treatment period.

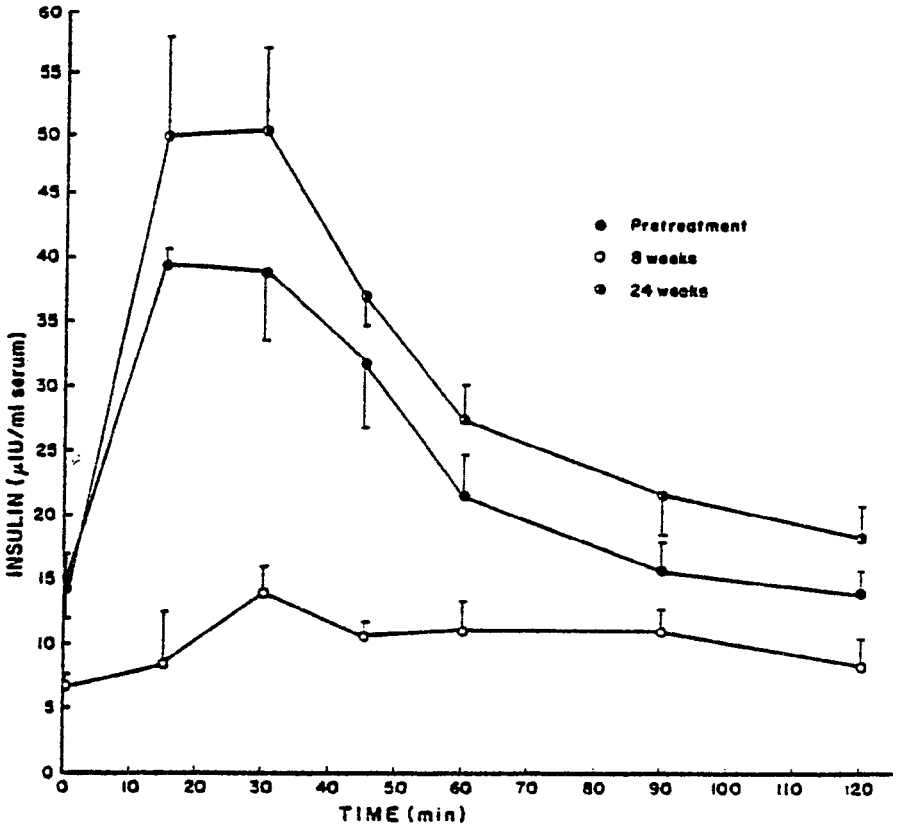
INSULIN (μIU/ml serum)
TIME (min)
Pretreatment
8 weeks
24 weeks
0
5
10
15
20
25
30
35
40
45
50
55
60
0
10
20
30
40
50
60
70
80
90
100
110
120

Figure 21

TABLE 9. Mean (± SEM) serum insulin levels (uIU/ml) measured in Group 2 rabbits (ICN purified diet with added vitamin B-6) during IV glucose tolerance tests.

TIME	0	15	30	45	60	90	120
Pretreatment n=6	15±2	40±1	39±6	40±5	22±3	16±2	14±2
8 weeks n=6	7±1*	11±3*	14±2*	11±1*	11±2*	11±2	8±2
24 weeks n=6	15±2	49±8	50±7	37±2	28±3	22±3	18±2

* Values are significantly lower ($p < 0.05$) than values at pretreatment and 24 weeks.

Pretreatment baseline insulin levels in group 3 (ICN purified diet without vitamin B-6) averaged 9 $\pm$ 2 uIU/ml (Figure 22, Table 10). As a result of a glucose load, insulin levels peaked at 15 minutes at 39 $\pm$ 5 uIU/ml. At 30 minutes the levels began to decline and by 120 minutes the levels returned to baseline. However, at 8 weeks, the baseline insulin levels decreased and insulin levels did not rise above 14 $\pm$ 4 uIU/ml in response to a glucose load. The insulin response to the glucose load was significantly decreased ($p < 0.05$) when compared to the pretreatment values. After the administration of vitamin B-6 supplementation, the insulin response to a glucose load returned to pretreatment levels.

Between-group analysis showed that the insulin response to a glucose load was the same at pretreatment in all groups. However, after being demonstrably vitamin B-6 deficient, baseline insulin values and the response to a glucose load at 8 weeks in both groups 2 and 3 were significantly ($p < 0.05$) decreased, when compared to both their pretreatment values and group 1 insulin response. After vitamin B-6 therapy for Groups 2 and 3, at 24 weeks, the insulin response after a glucose load was again the same for all treatment groups. The insulin response in groups 2 and 3 increased and was comparable to pretreatment values and group 1 response at this time period.

Figure 22. Serum insulin levels (means ± SEM) in Group 3 rabbits (ICN purified diet with vitamin B-6 omitted) in response to a glucose load during glucose tolerance tests at pretreatment, 8 and 24 weeks of the study. Five rabbits were tested pretreatment and at 8 weeks and 2 were tested at 24 weeks.

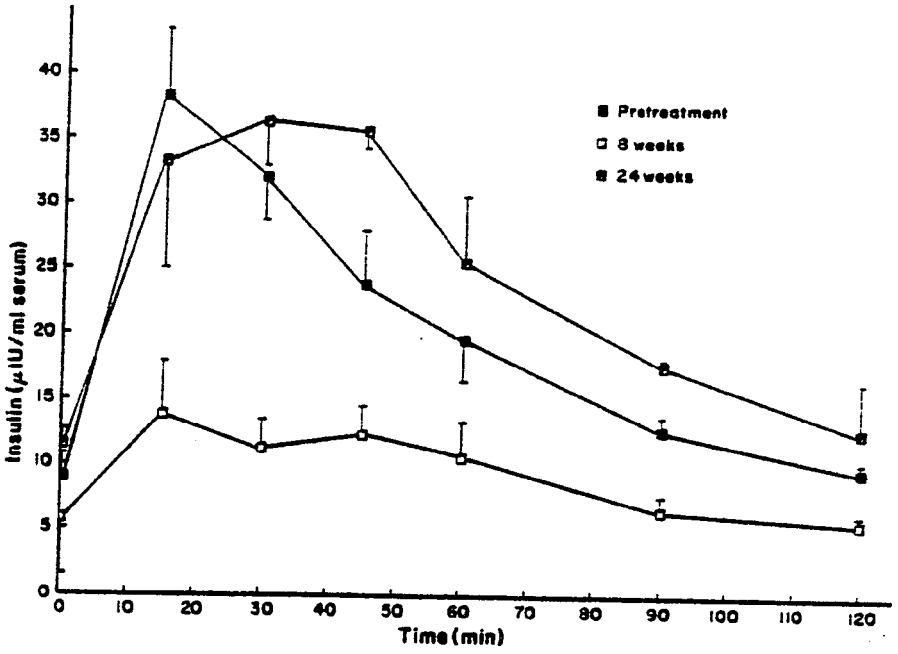
Pretreatment
8 weeks
24 weeks
Insulin (μIU/ml serum)
Time (min)
0
5
10
15
20
25
30
35
40
0
10
20
30
40
50
60
70
80
90
100
110
120

Figure 22

TABLE 10. Mean (± SEM) serum insulin levels (uIU/ml) measured in Group 3 rabbits (ICN purified diet without vitamin B-6) during IV glucose tolerance tests.

TIME	0	15	30	45	60	90	120
Pretreatment n=5	9±2	39±5	32±3	26±4	20±3	13±1	10±1
8 weeks n=5	6±4	14±4*	11±2*	12±2*	11±3*	7±1*	6±1*
24 weeks n=2	12±1	34±8	36±3	36±1	26±5	18±1	13±4

* Values are significantly lower ($p < 0.05$) than values at pretreatment and 24 week periods.

Chronic Infusion of XA

Prior to chronic infusion with XA for 12 days, the five new rabbits used specifically for this study had an average baseline plasma XA concentration of 130 ± 11 ng/ml. At days 7 and 12 after subcutaneous implantation of the Alzet osmotic pumps plasma XA levels averaged 1650 ± 253 ng/ml and 1764 ± 207 ng/ml, respectively.

Administration of a glucose load prior to infusion of XA resulted in peak glucose levels of 383 ± 22 mg/100 ml at 15 minutes and a return to baseline glucose levels (112 ± 7 mg/100 ml) at 120 minutes (Figure 23, Table 11). A glucose load, administered after 12 days of XA infusion, resulted in glucose levels reaching 425 ± 29 mg/100 ml at 15 minutes. In addition, the glucose levels decreased at a slower rate than pretreatment levels and did not approach baseline levels at 120 minutes. There was a significant ($p < 0.05$) elevation in glucose levels at 45, 60, 90 and 120 minutes when compared to the pretreatment levels at similar periods.

The insulin response to the glucose load during pretreatment resulted in peak levels of 36 ± 3 uIU/ml at 15 minutes (Figure 24, Table 12). Insulin values decreased to baseline levels (11 ± 3 uIU/ml) at 120 minutes.

Figure 23. Serum glucose levels (means ± SEM) in response to glucose tolerance tests of five normal rabbits infused with XA (with Alzet osmotic pumps) for a period of 12 days. Glucose tolerance tests were performed by injecting, IV, 2 ml of 50% dextrose solution per kg body weight (1 gm glucose/kg body weight).

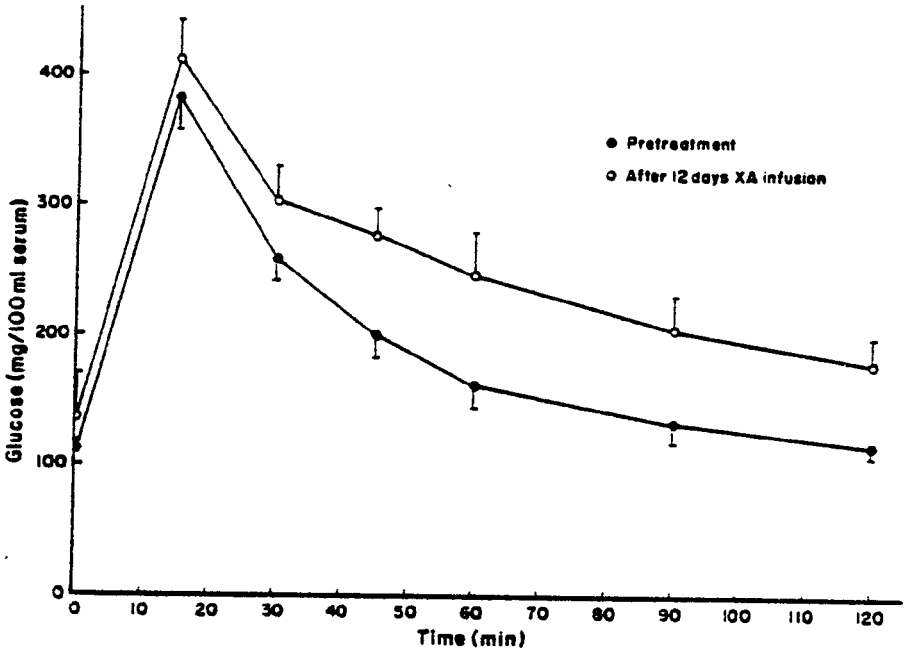
Glucose (mg/100 ml serum)
400
300
200
100
0
0
10
20
30
40
50
60
70
80
90
100
110
120
Time (min)
● Pretreatment
○ After 12 days XA infusion

Figure 23

TABLE 11. Mean (± SEM) serum glucose (mg/100 ml) measured during IV glucose tolerance tests in rabbits chronically infused with XA.

TIME	0	15	30	45	60	90	120
Pretreatment	112±5	383±22	259±14	201±18	164±17	134±12	119±8
12 days XA Infusion	138±31	425±29	305±25	277±22*	248±32*	205±27*	181±19

* Values are significantly higher ($p < 0.05$) than values at pretreatment period.

Figure 24. Serum insulin levels (means ± SEM) in response to glucose tolerance tests in five normal rabbits infused with XA (with Alzet osmotic pumps) for a period of 12 days. Glucose tolerance tests were performed by injecting, IV, 2 ml of 50% dextrose solution per kg body weight (1 gm glucose/kg body weight).

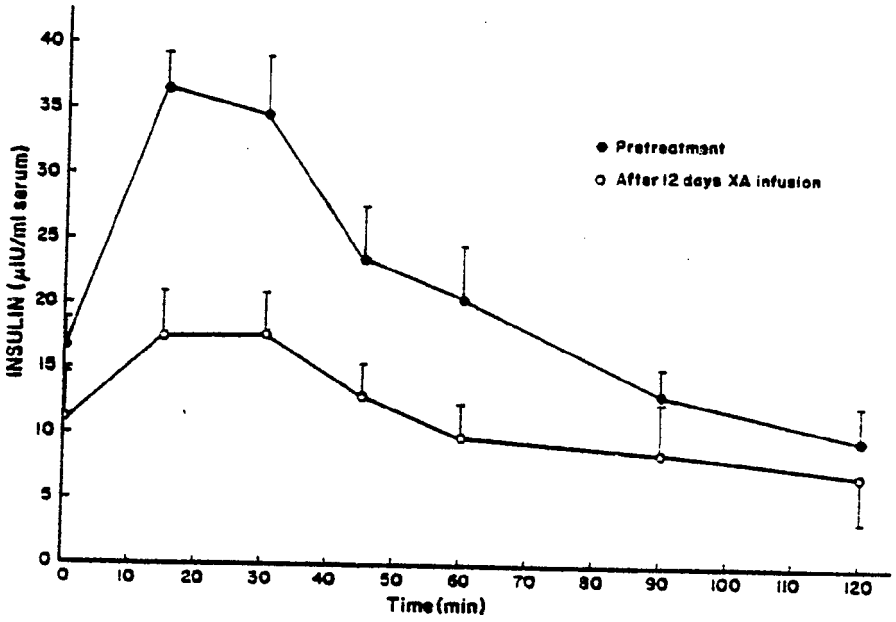
INSULIN (μIU/ml serum)
Time (min)
Pretreatment
After 12 days XA infusion
0
5
10
15
20
25
30
35
40
0
10
20
30
40
50
60
70
80
90
100
110
120

Figure 24

TABLE 12. Mean (± SEM) serum insulin levles (uIU/ml) measured during IV glucose tolerance tests in rabbits chronically infused with XA

TIME	0	15	30	45	60	90	120
Pretreatment	17±2	37±3	35±4	24±4	21±4	13±2	10±2
12 days XA Infusion	11±3	17±3*	18±3*	13±2*	10±2*	10±4	11±3

* Values are significantly lower ($p < 0.05$) than values at pretreatment periods.

After the infusion of XA for 12 days, the insulin response to a glucose load was significantly ($p < 0.05$) decreased at 15, 30, 45 and 60 minutes. The peak values at 15 minutes averaged 18 $\pm$ 4 uIU/ml and decreased to 10 $\pm$ 3 uIU/ml between 30 and 60 minutes.

DISCUSSION

The goal of this research was to study the possible interrelationships among vitamin B-6, xanthurenic acid (XA) and glucose metabolism. Since the discovery of vitamin B-6 and the subsequent elucidation of its many roles in metabolism, numerous methods have been developed and utilized to determine the B-6 nutritional status of individuals. Among the methods used, including measurement of plasma pyridoxal 5'-phosphate levels, evaluation of erythrocyte transaminase activity, and determination of urinary 4-pyridoxic acid (a metabolite of vitamin B-6), the measurement of urinary tryptophan metabolites and in particular XA is considered to be one of the most feasible and reliable. Relying totally on the measurement of increased urinary levels of XA in a B-6 deficient state can have its pitfalls, however, in certain pathological or drug related conditions. Elevated urinary levels of XA or other tryptophan metabolites may be a reflection of the diseased or altered states and not merely a consequence of the B-6 nutritional status of an individual.

In addition, this mode of assessment gives no indication of the circulating levels of XA in a healthy or diseased state nor if the changes are a reflection of a decreased vitamin B-6 pool.

A review of the literature has established that XA, a tryptophan metabolite, which is excreted in excess amounts in response to a deficiency of vitamin B-6, might be the causative agent in deteriorating glucose tolerance. It was also concluded that any study of the effects of XA should be done on blood levels of XA rather than urinary levels. To these ends, the rabbit was selected as the model for study and the development of a method sufficiently sensitive to assay XA in blood was undertaken.

The development of a high performance liquid chromatographic procedure (142) to measure XA in serum has improved our capabilities for studying the relationship of vitamin B-6 and XA and their possible physiological roles in metabolism. The method is sensitive and selective in the determination of XA and affords the detection of nanogram quantities of reference XA, adequate for analysis in serum. This procedure has been published and is presented as Appendix II of this dissertation.

The original experimental design for this dissertation called for three treatment groups of rabbits. The first of these known hereafter as group 1 was to be fed a commercially available rabbit diet commonly used in rabbit husbandry. The second group, group 2, was fed a purified diet which included vitamin B-6 and was reportedly adequate for normal growth and maintenance. The third group, group 3, was fed the same purified diet but from which vitamin B-6 had been omitted. The primary comparison was to be between groups 2 and 3 where a clear distinction would be possible concerning the effects of a vitamin B-6 deficient diet and blood levels of XA on glucose tolerance and on insulin response to a glucose challenge. After a vitamin B-6 deficient state had been achieved, group 3 would be supplemented with sufficient vitamin B-6 to reverse the deficiency state and demonstrate conclusively the relationships of vitamin B-6 to blood XA levels, glucose tolerance and insulin response to glucose challenge.

While performing the above studies, XA levels characteristic of B-6 deficient rabbits would be determined. After ascertaining whether the disappearance of XA from the circulation was or was not altered during the vitamin B-6 deficient state, XA, in levels characteristic of B-6 deficient rabbits, would be infused into rabbits for a 12 day period. If this treatment produced glucose intolerance and diminished insulin response to a glucose

challenge, this would be additional evidence that XA was functioning as the intermediary in vitamin B-6 deficiency induced glucose intolerance.

Within a few weeks of initiating the dietary treatment studies it was noted that both groups 2 and 3 were exhibiting biochemical and physical symptoms of a B-6 deficiency. It was determined, in retrospect, that both purified diets were inadequate, and henceforth both groups 2 and 3, while presented as separate groups, are considered to be B-6 deficient.

The reasons for a vitamin B-6 deficiency occurring in both groups fed purified diets are complex. Analysis of the rabbit diets for vitamin B-6 content revealed the following levels for the ICN purified diets with added B-6 or with B-6 omitted, and for standard commercial diet, respectively: 20 ug/gm, 0.08 ug/gm and 10 ug/gm. The amount of B-6 present in the ICN purified diet with added B-6 and the standard diet was adequate for normal maintenance and growth of rabbits (44, 45, 47, 84). However, since both groups of rabbits on the purified diets exhibited B-6 deficiency symptoms, it was obvious that other factors must be involved which resulted in group 2 becoming B-6 deficient.

The body's pool of vitamin B-6 can be depleted very rapidly when diets are essentially devoid of the nutrient (8, 50, 112, 131).

A major reduction in food consumption, due to poor palatability of the diet, could be a factor in the development of a B-6 deficiency. During the first eight weeks of treatment, consumption of both purified diets was 50% of Group 1's consumption of the standard diet (160-180 g/day). However, this factor alone could not account for a portion of the problem, since group 2 received approximately 1721 ug of vitamin B-6 per day, while group 3 received approximately 6.2 ug of vitamin B-6 per day. After eight weeks, consumption increased and by 12 weeks on the various diet regimens, consumption was similar in all three groups of rabbits. Because weight loss and advancement of B-6 deficiency symptoms continued after this time, it is unlikely that decreased food consumption was the sole cause of B-6 deficiency in group 2.

The cessation of coprophagy in groups 2 and 3 rabbits might have been an additional factor contributing to their vitamin B-6 deficiency. Coprophagy is considered an important dietary habit of rabbits which provides protein and other nutrients in addition to that derived from the actual food eaten. Poor palatability of feces while eating purified diets has been reported to prevent this process and decrease the total utilization of the diet (34, 46, 130). In addition, the amount of soft feces, which contains 3-4 times as much B complex vitamins and increased levels of protein and other

nutrients than the actual diet, is lessened because of a decrease in mucoidal secretions by the cecum. Thacker et al. (130) suggested that mucoidal secretions from the cecal walls increase the protein composition of the soft feces and also contain lipid and mineral constituients. The mucoidal secretion rate is lower in rabbits on purified diets than on roughage or standard diets. Finally, cessation of coprophagy decreases the time fiber and cellulose residues in the digestive tract (46, 130). This lessens the digestion of cellulose and the production of nutrients derived from bacterial digestion of cellulose and further decreases the amount of soft feces produced.

As stated earlier, both groups 2 and 3 exhibited decreased aspartate aminotransferase activity, and physical symptoms--loss of hair and weight, dermatitis, conjunctivitis, irritability when handled, decreased motor function (diminished hind limb movement) and muscular weakness--of a vitamin B-6 deficiency. Oral supplementation with vitamin B-6 corrected these symptoms but they recurred as a consequence of withdrawing the vitamin B-6 supplement. When supplemented with vitamin B-6 the final eight weeks of the study, the physical and biochemical alterations improved and the rabbits began to thrive. These changes, which occurred as a result of

decreased vitamin B-6 intake and later B-6 supplementation, substantiate the premise that both groups 2 and 3 experienced vitamin B-6 deficiency.

The fluctuations in circulating XA levels observed in response to the changes in dietary regimen and vitamin B-6 nutritional status of groups 2 and 3 were similar to reported changes in urinary levels of XA under similar conditions (17, 32, 64, 65). As the degree of vitamin B-6 deficiency progressed, levels of plasma XA increased and in response to vitamin B-6 repletion, plasma XA levels declined. The increased production of XA is explained as the result of differential depletion of two specific enzymes in the tryptophan metabolic pathway of their coenzyme, pyridoxal phosphate (PLP). As vitamin B-6 levels become deficient, the levels of the coenzyme form of vitamin B-6, PLP, also decline. The first enzyme affected is the PLP-dependent enzyme, kynureninase, which metabolizes 3-hydroxykynurenine to 3-hydroxyanthranilic acid and permits completion of the entire pathway. Kynurenine aminotransferase, also PLP-dependent, is much less sensitive to declining levels of PLP and readily transaminates the increased levels of 3-hydroxykynurenine to XA, resulting in increased levels of plasma XA.

It was observed that after four to six week of the B-6 deficient diet, XA levels no longer continued to increase but rather plateaued or decreased. Even though the affinity of kynurenine aminotransferase for PLP is significantly greater than for kynurenine (119, 140), a severe vitamin B-6 deficiency results in impaired apoenzyme:coenzyme function throughout the tryptophan pathway, resulting in lower levels of all metabolites including XA (103, 104, 140).

While the exact relations between PLP levels and tryptophan metabolism are unkown, these data substantiate the claim that plasma XA levels are indicative of vitamin B-6 status. Whether plasma levels provide a more sensitive indicator of B-6 deficiency than urinary levels has, however, not been determined.

Changes in physiological and pathological conditions exert major influences on the biological half-lives of drugs and other chemical agents (138). Because vitamin B-6 deficiency, which results in severely disturbed metabolism and weight loss, constitutes even a life-threatening condition or "stress", it was important to study half-life patterns in rabbits of all three groups. These data demonstrated that neither the rate of absorption nor the rate of disappearance of XA from

the circulation was affected by vitamin B-6 status. The time required for serum levels of XA to decline to one-half of its concentration after the absorptive and pseudoequilibrium phase, was the same in both B-6 deficient and normal rabbits.

However, the distribution of XA was altered in vitamin B-6 deficient rabbits when compared to rabbits maintained on commercial diets. When the pharmacokinetic parameter, volume of distribution (V_D) of XA was determined, groups 2 and 3 each had V_D values greater than either the volume of plasma or whole blood on a body weight basis. The decreased V_D compared to pretreatment or normally fed rabbits, infers a change in distribution and/or that tissue sites for XA were possibly saturated. Also, the loss of weight and, in particular, the loss of fatty tissues likely contributed to the decreased V_D of XA in both vitamin B-6 deficient groups.

The primary purpose of these studies was to demonstrate that glucose intolerance develops in association with vitamin B-6 deficiency and in turn is related to rising levels of XA. To obtain evidence testing this hypothesis, glucose and insulin responses to a glucose load (glucose tolerance test or GTT) were studied before, during the development of vitamin B-6 deficiency and

after B-6 therapy as described earlier. The comparisons were between groups 1, 2, and 3, also as described earlier.

Group 1 rabbits, fed standard rabbit diet, displayed normal glucose and insulin responses to a glucose load, pretreatment and after 8 weeks, but by the end of the experimental period of 24 weeks, a slight glucose intolerance with an exaggerated insulin response occurred. Both groups 2 and 3 (fed ICN purified diets with and without vitamin B-6 respectively) exhibited glucose intolerance and a marked reduction in insulin response to a glucose load in association with B-6 deficiency. Both parameters were reversed in response to a period of vitamin B-6 supplementation (4.4 mg/kg). These data further support the supposition that a deficiency of vitamin B-6 results in a decreased glucose tolerance, probably via interference in insulin action and/or secretion.

Several aspects of the glucose tolerance testing data in this study require more discussion. Group 1 rabbits, fed normal diet throughout the study, developed mild glucose intolerance by the end of 24 weeks. These observations might be attributed to the significant weight increase of 28 percent observed during the study. Obesity is characterized by hyperinsulemia and partial resistance to the effects of both endogenous and exogenous

insulin. Overweight animals and man have a number of biochemical and physiological alterations which distinguish them from normal weight animals. Cahill (20) has reported increased insulin levels as well as an exuberant insulin response to several secretogues, decreased levels of growth hormone and diminished growth hormone responsiveness. In addition, obesity is closely related to diabetes in that it induces resistance to one's own insulin. Abundant evidence has emerged which indicates that the hyperinsulemia which attends obesity is initially a compensatory response, and ultimately a contributory factor to the development of insulin resistance (4, 115).

The most common syndrome associated with obesity includes hyperglycemia as well as hyperinsulemia and insulin resistance (89). Mordes and Rossini (80) have reported that decreased glucose tolerance and spontaneous hyperglycemia are common occurrences in many animal species and that insulin resistance is one of the earliest detectable metabolic anormalities in most obese humans (28) and animals (22, 79).

A large body of data indicates that the insulin resistance of obesity is associated with specific abnormalities of the cellular receptors for insulin. When insulin binding to the cells from obese patients is carefully examined, the capacity to bind insulin is usually

decreased. Analysis shows this is related to a decreased number of insulin receptors (121), which is considered to be negative cooperativity or down regulation of insulin receptors (87, 88, 118). Insulin resistance is also reversible in that decreased body weight improves tissue sensitivity to insulin, lowers plasma levels of insulin and improves binding of insulin to receptors. Consequently, an increase in the number of cellular receptors occurs, since insulin can regulate the concentration of its own receptors.

The decreased glucose tolerance observed in groups 2 and 3 were definitely not related to increased body weight since the reverse occurred. In addition, the glucose intolerance observed in response to vitamin B-6 deficiency differed in one important aspect from the normally fed rabbits. The increased levels of blood glucose in response to a glucose load were not accompanied by an increase in insulin response, but rather, by a very subnormal level of insulin secretion.

The control of glucose metabolism is very complex. Hyperglycemia with its consequent decreased utilization of glucose, increased glycogenolysis, decreased glycogenesis, and increased gluconeogenesis are all physiological descriptions which characterize a basic defect in carbohydrate metabolism (116). The changes in carbohydrate

metabolism usually become evident with symptoms of glucosoria, polyuria, polydipsia and polyphagia (114). However, in the absence of these symptoms, at least two abnormal plasma glucose values, either fasting or post absorption, establish diagnosis of deteriorating glucose tolerance and carbohydrate metabolism (32). Considering the basic feedback loop for the regulation of plasma glucose, which in the basal state involves liver production of glucose and peripheral tissue utilization as regulated by the pancreas, two potential mechanisms for the production of hyperglycemia are possible (21, 69, 91): 1) an increased rate of glucose entry into the blood from the liver and/or 2) decreased glucose utilization in peripheral tissues. Many factors including drugs, toxins, hormones, disease and the stress of trauma, pain, surgery, anesthesia, sepsis, burns, hypoxia, cardiovascular diseases, hypothermia, cold and hypoglycemia (91) can cause alterations in carbohydrate metabolism and thus hyperglycemia and decreased glucose tolerance which may involve either an absolute or relative lack of insulin.

This study has provided evidence that impairment of glucose tolerance as a result of a vitamin B-6 deficiency with subsequent improvement after vitamin B-6 supplementation occurs in the rabbit. Occurrence of glucose intolerance during a B-6 deficient state and its correction by

B-6 supplementation has been previously reported in other species (17, 19, 104, 107), but the nature of the disturbance has not been addresssed. It has also been observed in this study that increased circulating levels of XA occur as a result of a B-6 deficiency and that XA levels decrease with B-6 supplementation. The observation that chronic injection of XA mimics the insulin and glucose data obtained in the dietary treatment studies may provide a link between a vitamin B-6 deficiency and elevated plasma levels of XA.

This study has shown that when XA is acutely injected into rabbits, plasma glucose levels increase with virtually no glucose-stimulated insulin secretion (preliminary studies). In addition, the glucose intolerance and blunted insulin response observed in rabbits chronically infused with XA at levels observed in B-6 deficient rabbits, mimicked the glucose and insulin responses observed in groups 2 and 3 rabbits at 8 weeks of the study. This would suggest that XA is a causative agent in decreased glucose tolerance and altered insulin response to a glucose load.

Xanthurenic acid may influence carbohydrate metabolism either directly by binding to glucose receptors in the pancreas and inhibiting the glucose stimulated insulin response or by causing a reduction in the conversion

of proinsulin to insulin. Zinc is a required element in the insulin molecular structure and with the discovery that XA binds to zinc in vitro (48, 133), it is possible that XA may bind to intracellular zinc molecules in the pancreas and prevent insulin formation as well as secretion. The subsequent decrease in the secretion of insulin would cause hyperglycemia and decreased glucose utilization.

It is also conceivable that XA indirectly causes decreased glucose tolerance by influencing other humoral or hormonal factors which affect insulin and glucose metabolism. A substantial body of experimental work indicates that hepatic glucose production is controlled by interplay between insulin and other hormones such as glucagon and epinephrine (6, 12, 21, 27, 51, 69). The general mechanism is that an increase in plasma insulin and glucose inhibit hepatic glucose production. On the other hand, glucagon, glucocorticoids and catecholamines, with a concomitant decrease in plasma insulin and glucose, promote gluconeogenesis and glycogenolysis in the liver. Whether this is the path in which XA directs its influence in promoting decreased insulin levels and glucose production has not been determined. However, it is possible that XA causes the increased production of other hormones that would result in glucose intolerance. Somatostatin, which

is secreted from the D cells of the pancreas, inhibits insulin secretion (12, 69). A change in the secretion of this hormone elicited by increased XA levels could directly inhibit the insulin secretion from the B cells.

Pancreatic glucagon, adrenomedullary epinephrine, adrenocortical cortisol and adenohypophyseal growth hormone are all capable of raising plasma glucose levels. However, glucagon, cortisol and growth hormone are thought to be insulin antagonists (6, 88, 27) and result in insulin resistance. Epinephrine effects may be more complex, in that hepatic glucose production through glycogenolysis and gluconeogenesis is increased and glucose utilization is limited in several tissues (6, 27, 51). In addition, epinephrine suppresses insulin secretion. Epinephrine causes hyperglycemia in man both directly and indirectly through α-and β-adrenergic mechanisms (90, 100, 101, 102). Stimulation of β-adrenergic receptors increases glucose production and decreases glucose uptake and clearance (100, 102). Stimulation of α-adrenergic receptors prevents an increase in insulin appropriate for a concomitant hyperglycemia (100). In addition, α-adrenergic stimulation may increase glucose production by stimulating glucagon secretion (100). Since XA is of low

molecular weight and may have other actions as a neurotransmitter molecule, the possibility that XA may interact with α-and β-adrenergic receptors exists.

Finally, XA may act as an organic stressor similar to other drugs or chemicals, eliciting humoral and/or hormonal alterations which interplay to cause hyperglycemia and hypoinsulinemia.

SUMMARY AND CONCLUSIONS

Vitamin B-6 deficiency is reportedly associated with glucose intolerance. The goals of this study were to 1) confirm, in a rabbit model, that glucose intolerance was related to vitamin B-6 deficiency, 2) determine if the insulin response to a glucose challenge was also altered and 3) if xanthurenic acid (XA), a tryptophan metabolite excreted in high levels during a B-6 deficiency, was causing the glucose intolerance. Xanthurenic has traditionally been measured as an excretory metabolite, but the nature of this study required that circulating levels be assayed. Since there were no procedures available that were sufficiently sensitive to assay blood levels of XA, a HPLC technique was developed.

Xanthurenic acid was then measured in the blood of three groups of rabbits. Group 1 rabbits were maintained on normal commercial rabbit diet throughout the study period. Groups 2 and 3 rabbits were fed purified diets containing adequate vitamin B-6 for normal growth and maintenance or with vitamin B-6 omitted, respectively. Within two to three weeks, clinical symptoms of vitamin B-6 deficiency appeared in both groups 2 and 3 and XA

levels began to rise. It was subsequently determined that feeding of both purified diets resulted in inadequate vitamin B-6 intake and for the balance of this study, groups 2 and 3 are both considered as B-6 deficient. These two groups of rabbits also developed glucose intolerance and demonstrated depressed insulin responses to a glucose challenge when compared to group 1 rabbits. All of the physical symptoms of B-6 deficiency, XA levels, glucose intolerance and depressed insulin responses returned to normal when vitamin B-6 supplementation was implemented.

These data clearly demonstrated temporal relationships between the occurrence of vitamin B-6 deficiency, rising blood levels of XA and the existence of deteriorating glucose metabolism. The next step was to determine whether XA by itself would mimic the glucose and insulin parameters reported in the dietary study. It was first determined that XA disappeared from the circulation in the same time frame for normal or B-6 deficient rabbits. Levels of XA equivalent to those seen in B-6 deficient rabbits were then infused over a 12-day period. These rabbits did indeed develop glucose intolerance and blunted insulin response to glucose challenge which mimicked the diet-induced hyperglycemia and hypoinsulinemia seen earlier.

Several important conclusions can be drawn from these studies. For the first time, the method has been evolved which is capable of measuring circulating levels of XA. Additionally, this is the first report demonstrating increased circulating levels of XA in association with vitamin B-6 deficiency. The occurrence of glucose intolerance in association with B-6 deficiency was confirmed with the additional observation of depressed insulin response to glucose challenge and increased circulating levels of XA. Finally, it was demonstrated that XA, infused at levels observed during B-6 deficiency, recreated the glucose and insulin responses, thus strongly suggesting that XA is the causative agent in altering glucose metabolism in association with deficient dietary vitamin B-6. The mechanisms by which XA acts to suppress insulin response to glucose challenge remain unknown at this time.

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APPENDIX I

Approximate Analyses of ICN Purified Diets and Standard Rabbit Diet

Table 1. Composition of Cosby-Hodges rabbit marketer standard rabbit diet.

Components	Content
protein	not less than 16%
fat	not less than 2.5%
fiber	15%
phosphorus	6%
calcium	1%
lysine	0.68%
methionine	0.23%
vitamins	
A in excess of 33 IU/kg	
D in excess of 1100 IU/kg	
E in excess of 52.8 mg/kg	
B-6 in excess of 51.1 mg/kg	
choline	3.3 gm/kg
potassium	0.3%
sodium	0.15%
chlorine	0.25%
iodine	0.44 mg/kg
copper	4.4 mg/kg
magnesium	8.8 mg/kg
zinc	61 PPM

2640 + digestible energy calories per kg.

Table 2. Composition of ICN purified rabbit diets.

Components	Contents
Casein	25%
Sucrose	40%
Coconut Oil Hydrogenated	4%
Salt Mix XIV	5%
Alphacel	15%
Vitamin Diet Fortification Mixture:	1%
Dry Vitamin A Acetate	39.6 mg/kg
Calciferol (gran) 850,000 /gm	21 mg/kg
Alpha Tocopherol Powder 250 IU/gm	0.48 mg/kg
Inositol	0.11 mg/kg
Choline Chloride	10.7 gm/kg
Menodione	0.05 gm/kg
Biotin	0.44 mg/kg
P Amino Benzoic Acid	0.11 gm/kg
Ascorbic Acid	1.0 gm/kg
Niacin	0.1 gm/kg
Riboflavin	22 mg/kg
Thiamine Hydrochloride	22 mg/kg
Calcium Pantothenate	0.07 gm/kg
Folic Acid	2 mg/kg
B-12 Trituration (0.1%)	30 mg/kg
* Pyridoxine - HCl	22 mg/kg

* Pyridoxine-HCl was omitted from ICN purified diet without added vitamin B-6.

APPENDIX II

Reprint of Quantitation of Xanthurenic Acid in Rabbit Serum Using High Performance Liquid Chromatography. By Shirley A. Williams, John A. Monti, Larry R. Boots and Phillip E. Cornwell

The University of Alabama in Birmingham
Department of Obstetrics and Gynecology
Division of Reproductive Biology and Endocrinology
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205/934-4824

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Ben Gilda Knight

November 13, 1984

Ms. Gilda Knight
American Journal of Clinical Nutrition
9650 Rockville Pike
Bethesda, MD 20014

Dear Ms. Knight:

In reference to our brief chat on the telephone today, this constitutes our formal request for permission to use the copyrighted material contained in the following publication for use in the senior author's dissertation:

"Quantitation of Xanthurenic Acid in Rabbit Serum Using High Performance Liquid Chromatography: by Shirley A. Williams, M.S., John A. Monti, Ph.D., Larry R. Boots, Ph.D. and Phillip E. Cornwell, Ph.D. Am J Clin Nut 40: JULY 1984, pp. 159-167.

Attached is a copy of page 3 of the "Theses and Dissertations, A Guide to Preparation" from the Graudate School, The University of Alabama in Birmingham. It appears that at the bottom of page 3 certain verbage is specified in relation to microfilm privileges for the doctoral dissertation.

Ms. Williams' graduate committee has been delayed during this past Summer for various reasons, so she is especially anxious to finish her final dissertation draft as soon as possible. We certainly do appreciate your help with this. Enclosed is a stamped, return-addressed envelope for your convenience.

Sincerely,

Barbara G. Quinnelly
Secretary to
Larry R. Boots, Ph.D.

Attachment

QUANTITATION OF XANTHURENIC ACID IN RABBIT SERUM USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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Running head: Serum xanthurenic acid

ABSTRACT

Xanthurenic Acid (XA) has been quantified in the serum of normal and B-6 deficient rabbits using high performance liquid chromatography. The concentration of XA in the serum of normal and B-6 deficient rabbits was 141 and 2275 ng/ml, respectively. The coefficient of variation for a series of dilutions of standard XA (3.9 ng - 1000 ng) ranged from 45.5% at the lower limit of the curve to 10.9% at the higher ranges of the curve. The minimum detectable level was 3.9 ng/ml. Serum samples spiked with reference XA exhibited a parallel dose response. The percent recovery of XA from serum samples was 80.8%. The procedure, which requires 1-2 ml of serum, is sensitive and may be a useful tool for assessing B-6 nutritional parameters as well as the physiological role of XA. It offers advantages over urinary procedures in being more sensitive and more specific and allows the study of blood levels of XA.

KEY WORDS: High Performance Liquid Chromatography, Xanthurenic Acid, B-6 Deficiency, Rabbit Serum, Gas Chromatography/Mass Spectrometry

INTRODUCTION

Tryptophan was first isolated in the tryptic digests of proteins (1). Since that time the amino acid has been shown to be indispensible, having various important biological functions. Studies of tryptophan metabolism have resulted in the discovery of several metabolic schemes, one of which is referred to as the tryptophan-niacin pathway (2). It is estimated that approximately 60 mg of dietary tryptophan may give rise to 1 mg of niacin, which may vary according to the amount of ingested tryptophan. This amino acid has also been shown to be preferentially used for the maintenance of body protein and serotonin production. However, when the ingestion of tryptophan is in excess, the tryptophan-niacin pathway become quantitatively more important (3-9).

In addition to the production of niacin and serotonin, a number of metabolic intermediates and/or conjugates have been shown to result either from the activity of microorganisms or from enzyme activity in tissue. These metabolites include a variety of indolic compounds, aromatic amino acids, amino phenols, pyridines and quinoline compounds (10).

The metabolic scheme depicted in Figure 1 indicates that vitamin B-6 is a required coenzyme in at least four reactions in the conversion of tryptophan to niacin. Measurements of the levels of tryptophan metabolites have been used to assess the vitamin B-6 nutritional status of individuals. This mode of assessment stemmed from the observation that rats and mice deficient in vitamin B-6 excreted urine that turned green on contact with metal cages (11, 12). The green color was the result of increased urinary levels of xanthurenic acid (XA) which had complexed with the iron from the metal cages. It was then concluded that XA along with other tryptophan metabolites, occurred in elevated amounts in the urine as a result of a B-6 deficiency.

Vitamin B-6 is a required cofactor for kynureninase and kynurenine-aminotransferase involved in the tryptophan-niacin pathway. These enzymes act on kynurenine and 3-hydroxykynurenine to produce anthranilic and 3-hydroxyanthranilic acids, and kynurenic and xanthurenic acids. Therefore, an alteration in kynureninase activity as a result of a B-6 deficiency leads to the excretion of high urinary levels of the tryptophan metabolites, in particular XA as evidenced in B-6 deficient humans, rats, dogs and rabbits (13-17).

Even though it has been repeatedly demonstrated that a deficiency in vitamin B-6 results in increased urinary excretion of xanthurenic acid and other tryptophan metabolites, little is known of their biological function. Several investigators (22, 23, 24) have observed that glucose metabolism and insulin secretion are altered with a concomitant increase in urinary levels of XA and, in turn, have suggested that increased levels of XA are directly responsible for these metabolic changes. Some of the studies (25) have proposed that XA binds to insulin, reducing its biologic potency and thereby altering glucose metabolism.

Until now, the above postulations were made without knowledge of circulating levels of XA. If indeed there is a difference between the levels of the metabolite in serum under normal versus pathological states, development of a quantitative procedure to measure serum levels of XA is crucial to devising appropriate experimental protocols in order to study its physiological and pharmacological effects. Several methods have been developed to isolate and measure tryptophan metabolites in urine, including paper or thin-layer chromatography (7, 13, 14, 18), ion exchange chromatography (10, 19), high performance liquid chromatography and gas chromatography (20, 21). This study was designed to develop an assay procedure for the measurement of XA in rabbit serum.

MATERIAL AND METHODS

Reagents

All reagents used were of highest purity (ACS Certified grade). All buffers and aqueous solutions were prepared in distilled deionized water and HPLC grade methanol and acetonitrile.

Potassium dihydrogen phosphate, XA and trichloroacetic acid (TCA) were obtained from Sigma Chemical Company, St. Louis, Missouri. Methanol and acetonitrile were obtained from Fisher Scientific Company, Springfield, New Jersey. Trimethylchlorosilane (TMS) was purchased from Suppelco, Inc., Bellefonte, PA. Solutions of reference xanthurenic acid were prepared in 100% methanol and made basic (pH 8.0) with saturated NaOH.

Instrumentation

A Varian Model 5000 liquid chromatograph and a 25 cm x 0.5 cm i.d. reversed phase column (5u) were used in all determinations. Column eluents were monitored at 243 nm, using a LDC spectromonitor III.

Mass Spectral analyses were performed using a Hewlett-Packard 5985A gas chromatograph-mass spectrometer (GC/MS) equipped with a data analysis system.

Animal Model

Mature New Zealand White female rabbits, averaging 3.76 kg were obtained from Myrtle Rabbitry, Thompson Station, Tennessee. Normal rabbits were maintained on regular rabbit chow (Wayne Feeds, Chicago, IL). Vitamin B-6 deficient rabbits were maintained on a pyridoxine-deficient purified diet purchased from ICN Nutritional Biochemicals, Cleveland, Ohio and were determined to be B-6 deficient when the activity coefficient (A/C) of the B-6 dependent enzyme aspartate aminotransferase averaged 2 or greater.

Vitamin B-6 Status

A number of enzymes are dependent upon pyridoxal phosphate as their cofactor. One such enzyme is aspartate aminotransferase (AST). Lowered vitamin B-6 levels reduce the level of saturation of the apoenzyme as well as the activity of the holoenzyme. The AST enzyme was assayed according to the procedure of Bayoumi and Rasalki (26) using red cell hemolysates.

Sample Preparation

Freshly drawn blood was collected and centrifuged at 2000 xg for 5 minutes. Serum protein was precipitated by the addition of 0.6 ml of cold (20% w/v) TCA to each 2 ml of serum. (Smaller volumes of serum may be used by keeping the ratio of serum and TCA volumes constant).

The samples were recentrifuged and the supernatant was lyophylized and reconstituted with 2 ml of absolute methanol. The samples were then centrifuged (2000 xg) and filtered through 0.2 u Gelman acrodiscs.

Chromatographic Conditions

For HPLC analysis, samples were analyzed using a reversed phase partition mode. For the identification of reference and serum XA, the low concentration eluent was 0.1M KH_2PO_4, pH 5.5 while the high concentration eluent was 0.1M KH_2PO_4/ acetonitrile (70/30%). A linear gradient from 100-0% of the low concentration eluent in 40 minutes was used. The flow rate was 1.0 ml/min with operating pressures ranging from 140-240 ATM over the course of the gradient. The temperature was ambient in all cases. Elutions were monitored at 243 nm using a UV detector. All buffers were degassed under vacuum before use.

Calculation of Peak Area

The amounts of XA present in serum samples were determined by comparing the normalized (AUFS of 0.02) area of serum extract chromatographic peaks to a standard curve of reference XA, constructed by plotting the normalized area of reference XA chromatographic peaks against corresponding quantities of the reference compound ranging from 3.9 to 1000 nanograms. The chromatographic peaks were measured manually by triangulation.

GC/MS Conditions

GC/MS samples were analyzed on a column four feet in length containing 2% SP-2250, 100-120 mesh, combined with W-HP. Temperature was increased 180-270^{o} at a rate of 30^{o}/min.

Derivatization

0.5 ml of trimethylchlorosilane (TMS) and lyophilized column effluent were mixed at room temperature for 5 minutes. Three microliters of the mixture were injected into the GC/MS. Standard XA was treated in the same manner.

Identification of Peaks in Biological Extracts

Initial identification of the chromatographic peaks from the serum samples was accomplished by comparing the elution pattern and retention time with reference XA, and by co-injection with the reference compound. Further identification of the serum peaks was accomplished by GC/MS. The gas chromatographic peaks and fragmentation patterns of the TMS derivatives of reference XA and serum samples separated using HPLC were observed and compared.

RESULTS

The minimum detectable amount of reference XA was 3.9 nanograms. This sensitivity was adequate for the analysis of the metabolite in serum. Reference XA at levels of 500 ng and 1000 ng eluted with a retention time of 12.9 minutes (Figure 2). The gradient composition at the onset of the elution was 67% of the low concentration eluent, 70/30% KH_2PO_4/CH_3CN. The solvent and/or unidentified components eluted at 6.1 minutes. Under similar conditions, injections of lyophilized methanol extracts of normal and B-6 deficient rabbit serum resulted in peaks with a retention time of 12.9 minutes which is identical to the retention time of reference XA (Figure 3).

The standard curve (Figure 4) for XA expressed as normalized peak area versus the amount of xanthurenic acid injected was linear from 3.9 ng to 1 ug with a correlation coefficient (r) of 0.992. The coefficient of variance for the standard curve was greatest as the assay approached the minimal detectable levels of xanthurenic acid (Table 1).

Additions of varying amounts (3.9-500 ng) of authentic XA to rabbit serum showed a linear dose-response relationship as indicated in Figure 5. The slope of the curve was 0.93 with an intercept of -1.26 and a correlation of 0.996. The coefficient of variance was higher at lower levels of measured XA peaks.

Serum from normal and B-6 deficient rabbits was extracted and concentrated and 10, 20 and 40 microliter equivalents of serum were injected in 10 ul of methanol on the column. The dose response curve (Figure 6) for the normal serum had a slope of 1.018 and an intercept of -1.712 with a correlation of 0.999. The slope of the B-6 deficient serum dose response curve was 0.988 with an intercept of -1.198. The correlation for this curve was 0.998. Both dose response curves were essentially parallel to the standard curve obtained from reference XA.

The recovery of added XA (1 ug/10 ml of serum) ranged from 74 to 94% with average recovery of approximately 80% (Table 2). The coefficient of variance (%) for recovery values was 9.86.

To further confirm the identity of the compound eluted from the HPLC, GC/MS data were obtained for TMS derivatives of both reference XA and HPLC column eluents. Mass spectra (Figure 7) of TMS derivative of reference XA and the column eluent show ion fragments at 406, 407, and 408.

The effectiveness of trichloroacetic acid as an extractant was tested by adding 0.66 ml of the reagent to 2.0, 2.5, 3.0 and 3.5 ml of serum. Each quantity was spiked with equal volumes of reference XA solution (1 ug/10 ul). The percent recovery for each sample was 77.5, 60.0, 49.1 and 35.7, respectively.

Serum from vitamin B-6 deficient rabbits was extracted and XA levels determined. The B-6 deficient rabbits had serum levels approximately 16 times those of normal rabbits (Table 3).

DISCUSSION

The reversed phase mode of HPLC with gradient elution was useful in the separation of XA from other serum constituents. The sensitivity of the method affords the detection of nanogram quantities of reference XA which is adequate for analysis in serum. One ml of serum is sufficient for the measurement of the metabolite. However, to insure maximum extraction and recovery of the compound, the ratio of the TCA to serum should remain constant (1 ml serum: 0.3 ml TCA).

Similiar retention times for the serum extracts and reference XA on the HPLC, the average recovery of 80% in spiked serum with a coefficient of variance of approximately 9.86%, and the curves obtained in the dose relationship further support the validity of the procedure.

The GC/MS of TMS derivatives of both reference XA and serum extracts eluted from the HPLC showed single GC peaks and had the same major ion fragments (i.e. m/x of 406, 407 and 408). These data provide additional evidence for the identification of XA as the substance present in serum extracts which eluted from the HPLC column with a retention time of 12.9 minutes.

The observed increase in serum levels of XA in B-6 deficient rabbits versus normal animals is in accordance with earlier observations of increased urinary levels of the metabolite as a result of a vitamin B-6 deficiency. However, since the control animals received standard rabbit chow, these observations must be considered as preliminary. Experiments are currently underway to determine serum levels of XA in animals which received synthetic complete and B-6 deficient diets.

In conclusion, the HPLC method described is sensitive and selective in the determination of XA in rabbit serum and may be a useful tool in the assessment of B-6 nutritional parameters, as well as in studies on the physiological role of this metabolite.

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Table 1. Normalized area (NA) and coefficient of variance (CV%) for HPLC peaks of reference xanthurenic acid (XA).

ng XA	x NA	(±)1 SD	CV(%)
1000	56.92	6.2	10.1
500	27.02	1.77	6.6
250	15.6	2.11	13.5
125	6.56	0.86	13.1
62.5	3.9	0.585	15.0
31.2	2.33	0.475	20.4
15.6	1.16	0.227	19.6
7.8	0.61	0.268	43.9
3.9	0.44	0.200	45.5

Table 2. Recovery profile of authentic xanthurenic acid in rabbit serum. The correct N.A. = N.A. per sample - avergaed N.A. (.31) of normal serum.

SAMPLE	N.A.	CORRECTED N.A.	& RECOVERY
1 ug reference	56.92	----	----
Serum			
1	49.5	49.2	86.4
2	54.0	53.7	94.3
3	52.5	52.2	91.7
4	41.5	41.2	72.3
5	45.0	44.7	78.5
6	45.0	44.7	78.5
7	42.5	42.2	74.1
8	44.0	43.7	76.7
9	43.0	42.7	75.0

Table 3. Xanthurenic acid in serum of normal and B-6 deficient rabbits. The activity coefficient (A/C) for asparatate aminotransferase enzyme was used to determine defiency in vitamin B-6.

	n	A/C	ng XA/ml serum
Normal	6	1.1	141
B-6 deficient	3	2.4	2275

Figure 1. An abbreviated outline of the metabolic pathway of tryptophan. The reactions in which vitamin B-6 is of particular importance have been indicated by the symbol, B-6, adjacent to the arrows.

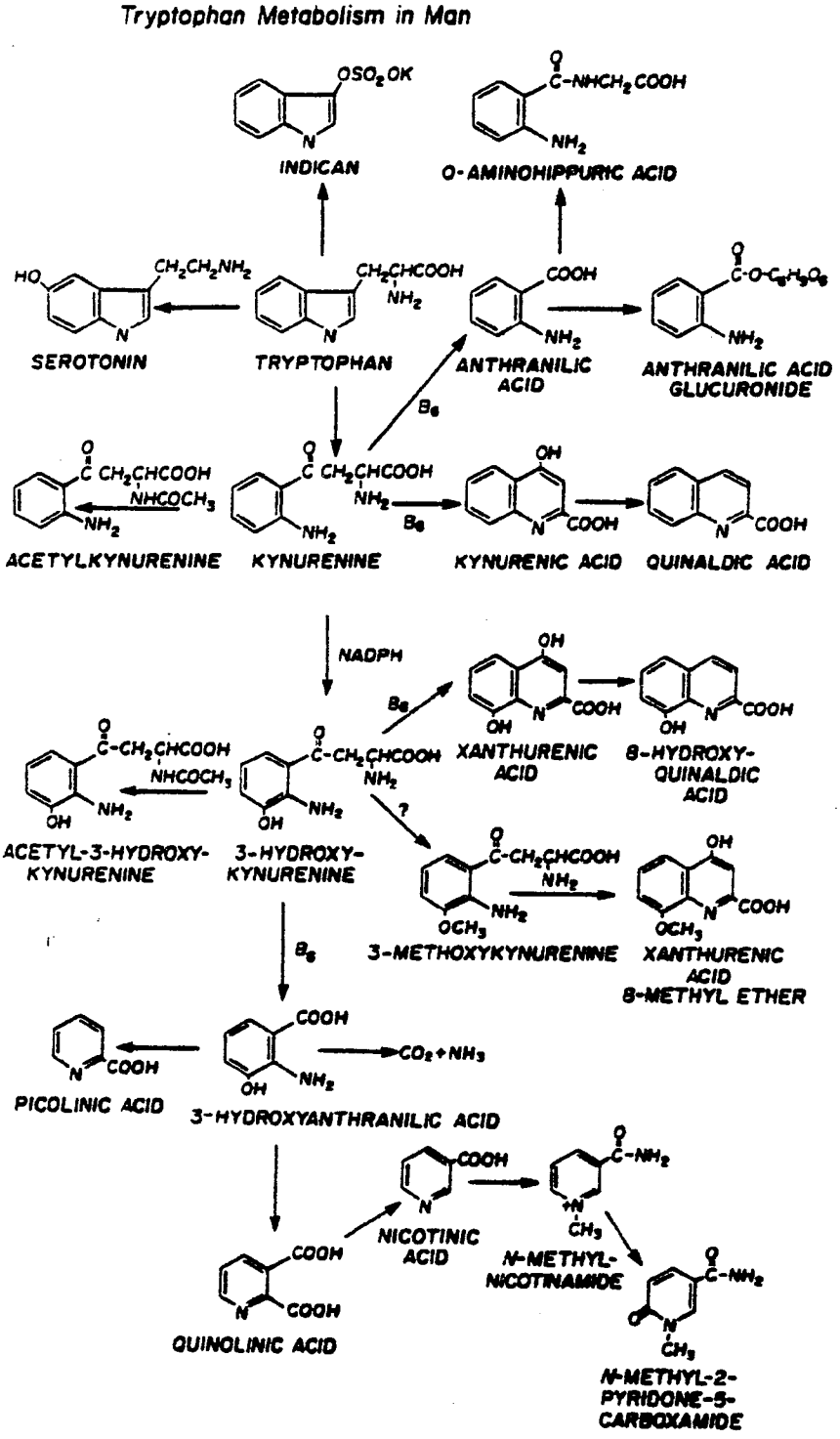
Tryptophan Metabolism in Man
INDICAN
O-AMINOHIPPURIC ACID
SEROTONIN
TRYPTOPHAN
ANTHRANILIC ACID
ANTHRANILIC ACID GLUCURONIDE
ACETYLKYNURENINE
KYNURENINE
KYNURENIC ACID
QUINALDIC ACID
NADPH
XANTHURENIC ACID
8-HYDROXY-QUINALDIC ACID
ACETYL-3-HYDROXY-KYNURENINE
3-HYDROXY-KYNURENINE
3-METHOXYKYNURENINE
XANTHURENIC ACID 8-METHYL ETHER
PICOLINIC ACID
3-HYDROXYANTHRANILIC ACID
NICOTINIC ACID
N-METHYL-NICOTINAMIDE
QUINOLINIC ACID
N-METHYL-2-PYRIDONE-5-CARBOXAMIDE

Figure 1

Figure 2. Chromatograms of 500 ng and 1000 ng of reference xanthurenic acid, monitored at 0.5 absorbance units full scale (AFS). Chromatographic conditions are as given in the text.

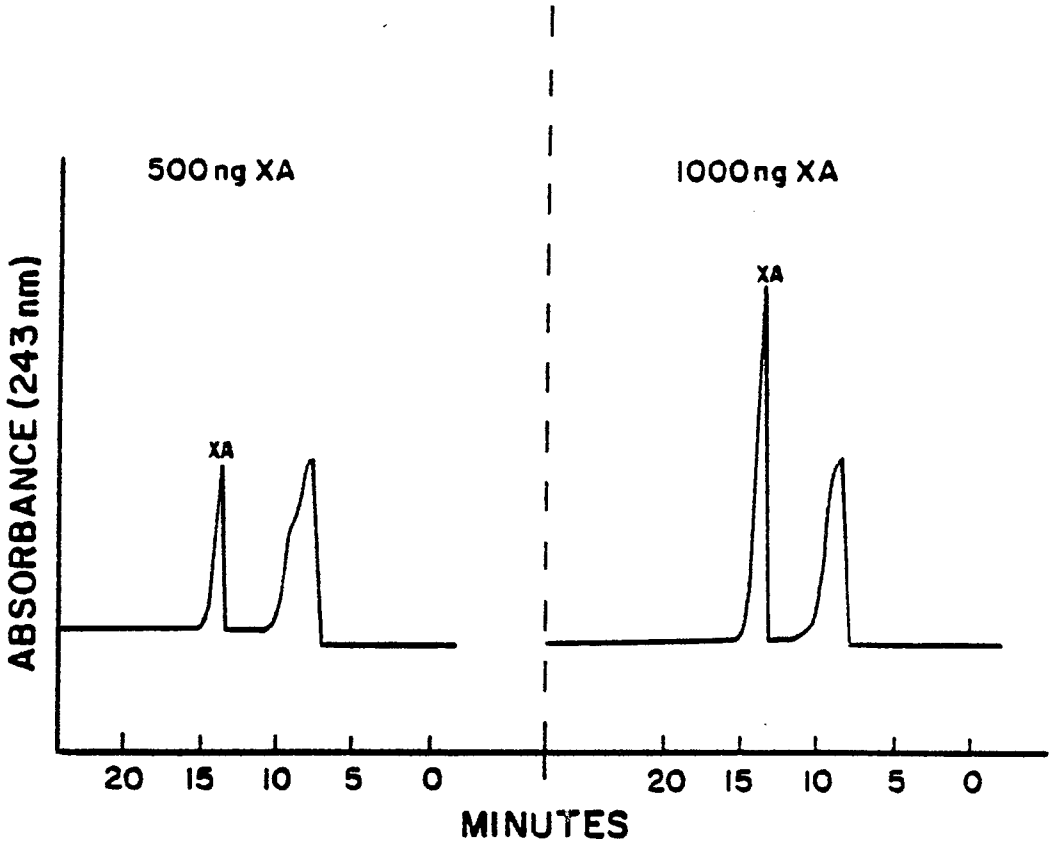
500 ng XA
1000 ng XA
XA
XA
ABSORBANCE (243 nm)
20 15 10 5 0
20 15 10 5 0
MINUTES

Figure 2

Figure 3. Chromatograms of 7.8 ng reference xanthurenic acid and lyophilized methanol extracts of normal and B-6 deficient rabbit serum. The chromatograms were recorded at 0.2 absorbance units full scale (AFS).

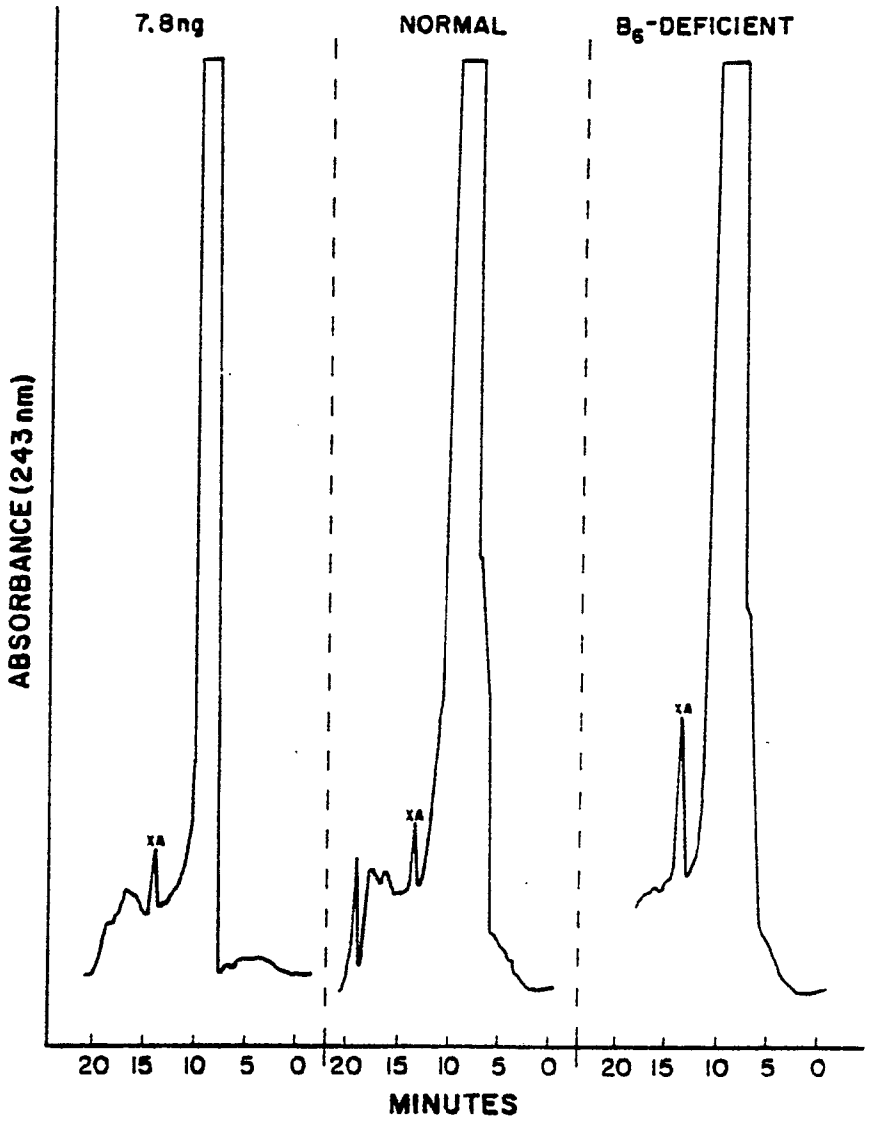
7.8ng
NORMAL
B6-DEFICIENT
ABSORBANCE (243 nm)
XA
XA
XA
20 15 10 5 0
20 15 10 5 0
20 15 10 5 0
MINUTES

Figure 3

Figure 4. Proportionality between the amount of reference xanthurenic acid injected and the normalized area of the resulting peaks. Parenthetical values represent the coefficient of variance (%) for each point.

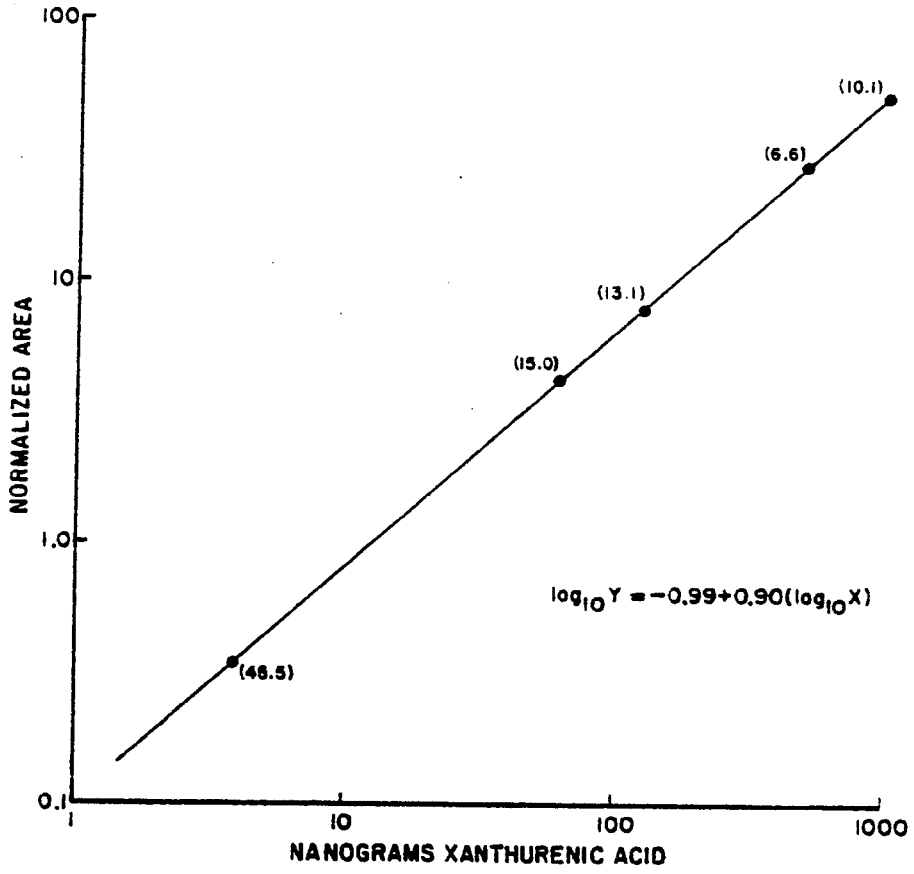
100
10
1.0
0.1
NORMALIZED AREA
(10.1)
(6.6)
(13.1)
(15.0)
(46.5)
$\log_{10} Y = -0.99 + 0.90(\log_{10} X)$
1
10
100
1000
NANOGRAMS XANTHURENIC ACID

Figure 4

Figure 5. Dose response curve of rabbit serum spiked with 3.9, 7.8, 62.5, 250 and 500 nanograms of reference xanthurenic acid. The averaged normalized area of serum peaks was substracted from the total values obtained from normalized peak area of each spiked injection. The coefficient of variance (%) is given at each point.

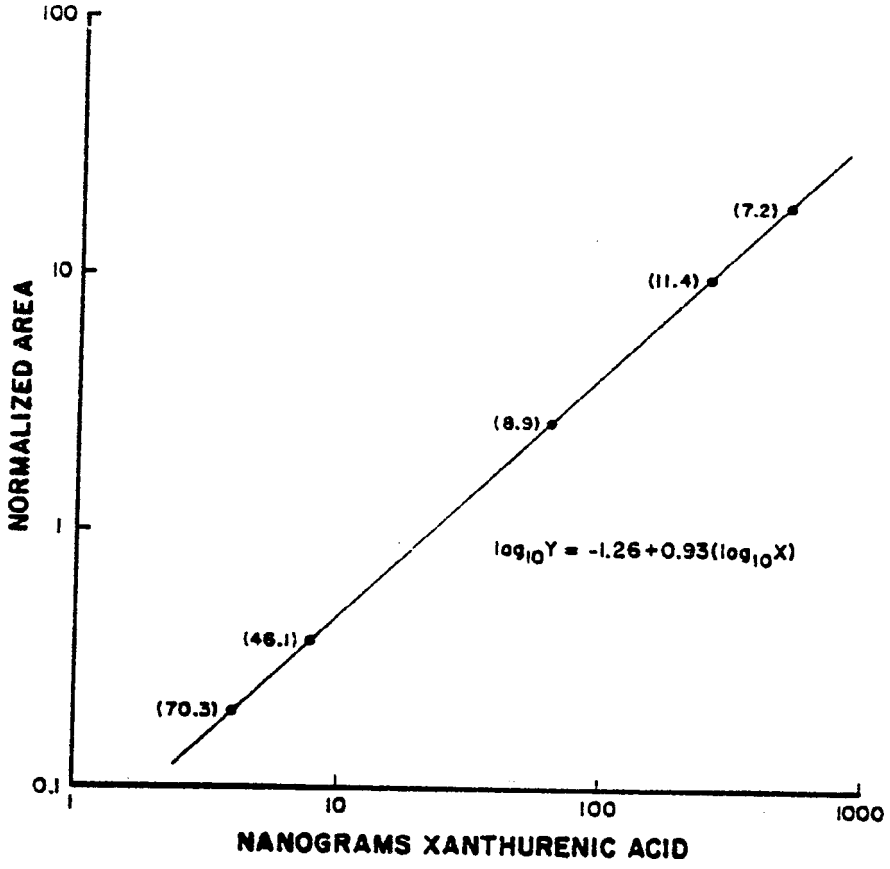
100
10
1
0.1
NORMALIZED AREA
(7.2)
(11.4)
(8.9)
(46.1)
(70.3)
$\log_{10}Y = -1.26+0.93(\log_{10}X)$
1
10
100
1000
NANOGRAMS XANTHURENIC ACID

Figure 5

Figure 6. Dose response curves of XA based on the normalized areas of 10, 20 and 40 ul equivalents of serum from normal and vitamin B-6 deficient rabbits and compared to standard XA. Chromatographic conditions are given in the text.

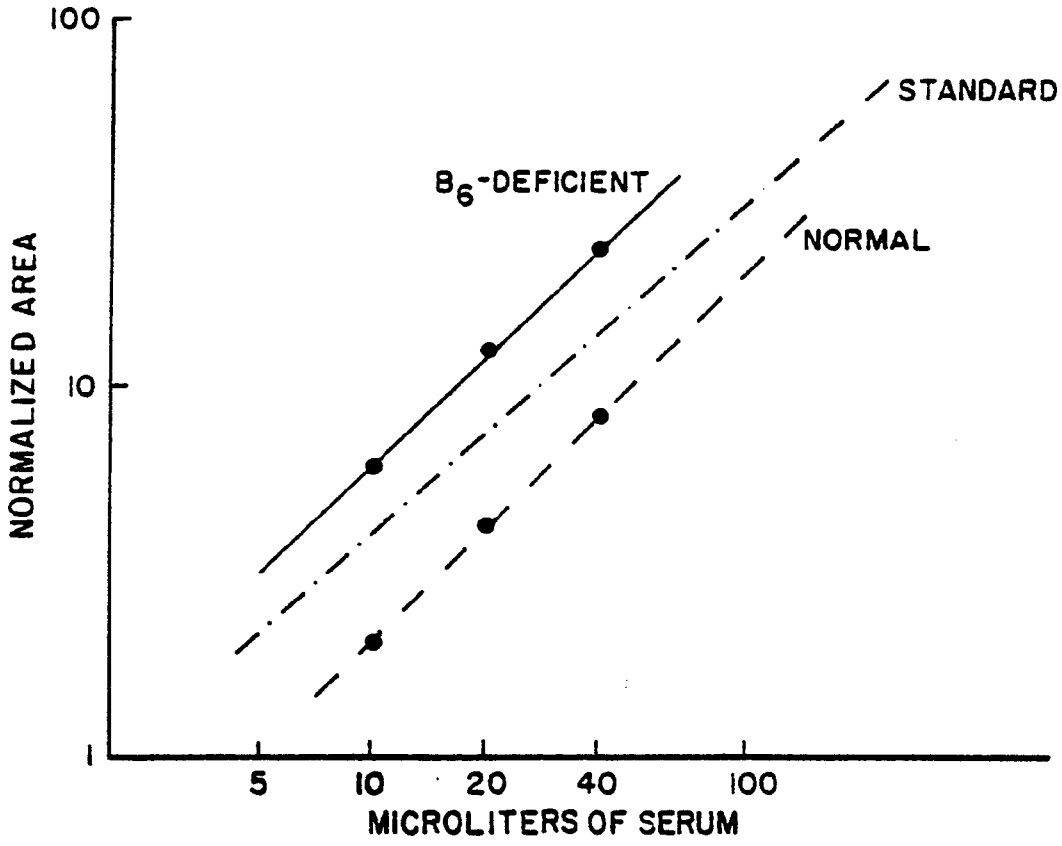
100
10
1
NORMALIZED AREA
STANDARD
B_6-DEFICIENT
NORMAL
5
10
20
40
100
MICROLITERS OF SERUM

Figure 6

Figure 7. Mass spectral analysis of TMS derivatives of authentic xanthurenic acid and peaks collected from the HPLC column.

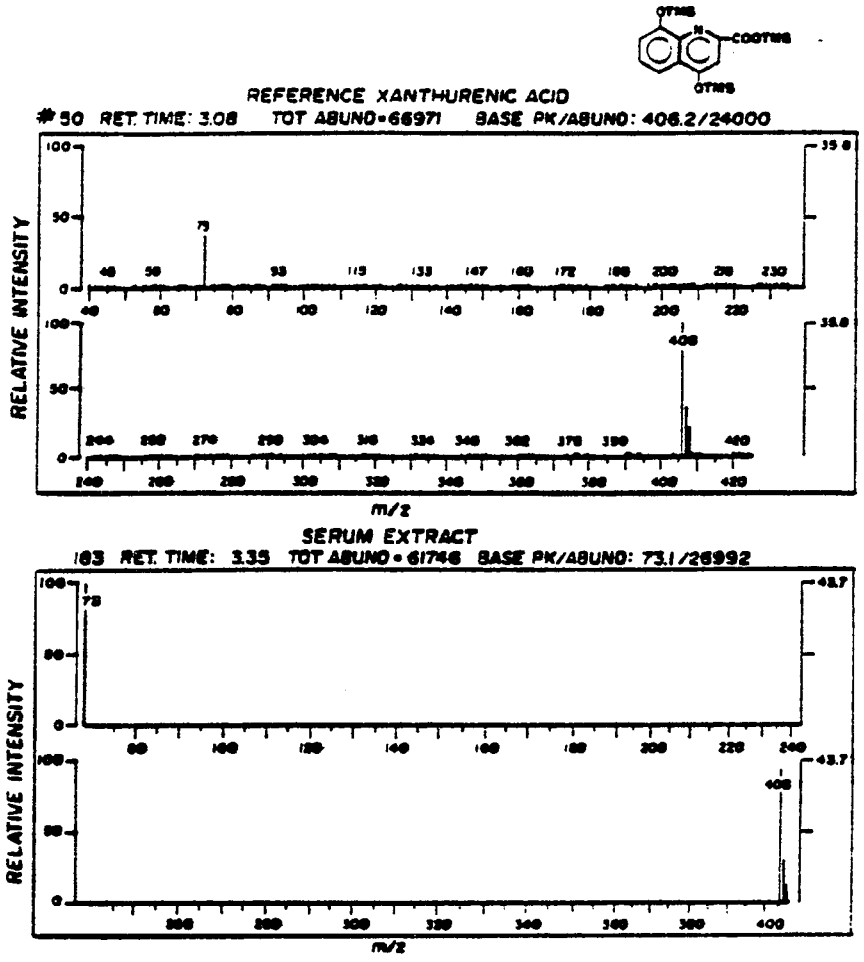
REFERENCE XANTHURENIC ACID
#50 RET. TIME: 3.08 TOT ABUND=66971 BASE PK/ABUND: 406.2/24000
RELATIVE INTENSITY
m/z
SERUM EXTRACT
183 RET. TIME: 3.35 TOT ABUND=61746 BASE PK/ABUND: 73.1/26992
RELATIVE INTENSITY
m/z

Figure 7

GRADUATE SCHOOL
UNIVERSITY OF ALABAMA IN BIRMINGHAM
DISSERTATION APPROVAL FORM

Name of Candidate Shirley Ann Williams

Major Subject Physiology and Biophysics

Title of Dissertation A Study of the Relationships of Xanthurenic Acid, Pyridoxal Phosphate and Glucose Metabolism in the Rabbit

Dissertation Committee:

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Date 3/15/85

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