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Title: URINARY EXCRETION OF 4-PYRIDOXIC ACID BY WOMEN USING STEROID CONTRACEPTIVES AND BY MENTAL RETARDATES WITH AND WITHOUT DOWN'S SYNDROME

Abstract approved: _____
Lorraine Miller

The urinary excretion of 4-pyridoxic acid (PIC), the principle end-product of vitamin $B_6$ metabolism found in human urine, was measured in two populations in whom altered vitamin $B_6$ metabolism has been reported: in women who use oral contraceptives and in mental retardates with and without Down's syndrome.

In the first study 4 women who had been taking an oral contraceptive for 2 to 12 months served as experimental subjects; two women who did not use an oral contraceptive pill served as control subjects. They were placed on a constant diet that met the requirements for all essential nutrients. The study lasted for 11 days. During this period five 24-hr urine speciments were collected from each subject and were analyzed for 4-pyridoxic acid as well as for total creatinine.

The urinary excretion of PIC by the subjects using an oral

contraceptive was similar to that by the control group in this study and that by normal women subjects reported in the literature. The results of this study suggest that the alteration in vitamin $B_6$ metabolism observed in women using steroid contraceptives is not reflected by any change in urinary excretion of PIC. In general all subjects excreted more PIC during the latter part of the study than the initial stage, probably reflecting an adjustment to the higher intake of the vitamin supplied by the diet. Also, there was an inverse relationship between the urinary excretion of PIC and the body weight of the participants.

In another study the excretion of PIC before and after pyridoxine (PIN) loading was studied in 12 patients with Down's syndrome and 12 mentally retarded controls without Down's syndrome. Three mongoloids and three non-mongoloids of the same sex and matched for age and weight were studied at a time for 6 days. They received a constant diet that was adequate in all essential nutrients for man. PIC was determined in urines collected on days 1, 5, and 6, 50 mg of pyridoxine being orally administered on day 5. Data show that the basal urinary excretion of PIC is similar in the mongoloid and control subjects. The total increase in PIC excretion by patients with Down's syndrome during the two days following the ingestion of the test dose of pyridoxine averaged 22.03 mg, while the excretion by the controls averaged 19.34 mg. The larger excretion of PIC

by Down's syndrome patients compared with controls following pyridoxine loading was significant ($P < 0.05$). An explanation for this greater excretion by mongoloids is proposed.

# Urinary Excretion of 4-Pyridoxic Acid by Women Using Steroid Contraceptives and by Mental Retardates With and Without Down's Syndrome

by

Joey Nim-Cho Young

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Associate Professor of Foods and Nutrition
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Head of Department of Foods and Nutrition

Dean of Graduate School

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This thesis is respectfully dedicated to my mother, who has made possible my graduate study at Oregon State University.

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## INTRODUCTION

Vitamin $B_6$ comprises a group of closely related compounds: pyridoxine, pyridoxal and pyridoxamine, all of which are equally effective in animal nutrition (Leitch and Hepburn, 1961). The phosphorylated forms, pyridoxal and pyridoxamine phosphates, function as coenzymes in about 50 known enzymatic reactions (Braunstein, 1961). The chief metabolic role of pyridoxal phosphate is in the degradation, interconversion, and biosynthesis of amino acids (Meister, 1965). Pyridoxal phosphate is also involved in carbohydrate metabolism, notably being an essential constituent of glycogen phosphorylase (Krebs and Fischer, 1964), and in fat metabolism (Spitzer, Newcomb and Noyes, 1966).

The various biochemical techniques that are employed to assess vitamin $B_6$ status in the human have been reviewed recently by Sauberlich et al. (1972). These include the measurement of the free and phosphorylated forms of the vitamin in blood and urine as well as the determination of urinary 4-pyridoxic acid (PIC), the major end-product of vitamin $B_6$ metabolism which accounts for about half of the dietary intake of the vitamin (Reddy, Reynolds and Price, 1958). Several functional tests which are indirect indicators of vitamin $B_6$ status are based on changes in blood or urinary levels of metabolites requiring vitamin $B_6$ for their production or breakdown. Of these the

tryptophan load test is the most commonly used. Other indirect indices of vitamin $B_6$ status include urinary taurine, oxalate, urea, as well as plasma and urinary amino acids. The activities of transaminases, which are vitamin $B_6$-dependent, are also indicative of vitamin $B_6$ nutriture.

The requirement for vitamin $B_6$ by man appears to be related to the protein content of his diet. Subjects fed a vitamin $B_6$-deficient diet high in protein developed abnormal tryptophan metabolism much sooner and to a greater degree than subjects fed a vitamin $B_6$-deficient diet which was lower in protein (Miller and Linkswiler, 1967). Canham et al. (1969) found that in subjects receiving 100 g of protein daily the requirement for vitamin $B_6$ was 1.5 to 2.0 mg, whereas in those receiving 30 g of protein the requirement was 1.25 to 1.5 mg.

Important influences of oral contraceptive drugs on the metabolism of vitamin $B_6$ have been reported recently. Women who use this drug appear to have an increased need for vitamin $B_6$ (Rose, 1966; Brin, 1970). Studies on which this suggestion is based show that users of oral contraceptives exhibit abnormal tryptophan metabolism which can be corrected by vitamin $B_6$ (Price, Thornton and Mueller, 1967; Rose et al., 1972). The activity of erythrocyte glutamic-pyruvic transaminase is lower in women using oral contraceptives than women not using this drug (Doberenz et al., 1971).

The metabolism of vitamin $B_6$ may also be altered in patients with Down's syndrome. McCoy, Anast and Naylor (1965) by using the vitamin $B_6$ antagonist deoxypyridoxine to produce a deficiency

of the vitamin in their subjects, showed that mongoloids excreted significantly more urinary oxalic acid and xanthurenic acid in response to a tryptophan load test than did non-mongoloids. They suggested that there is a greater tendency for pyridoxine depletion to occur in the mongoloid than in the non-mongoloid subjects. This is supported by the finding that mongoloids excreted more urinary 4-pyridoxic acid following the ingestion of a vitamin $B_6$ supplement compared with non-mongoloids, implying that the mongoloids oxidize pyridoxine to PIC more rapidly than do the non-mongoloids (Gershoff, Mayer and Kulczycki, 1959).

Urinary 4-pyridoxic acid has been used in only a few investigations to study the metabolism of vitamin $B_6$. Wachstein (1964) proposed that the concentration of this substance in urine can be used to judge the quantity of metabolized vitamin $B_6$. In this regard, Storvick and Peters (1964) suggested that accurate determination of urinary PIC enables one to follow more closely the metabolic patterns involving the vitamin in cases of vitamin $B_6$ deficiency, vitamin $B_6$ dependency, or in other abnormal conditions.

Thus, the purpose of the research reported in this thesis was to determine whether or not changes in the urinary excretion of PIC occurs in two populations in whom altered vitamin $B_6$ metabolism has been reported: (1) women using steroid contraceptives for whom an increased need for vitamin $B_6$ is indicated and (2) patients with Down's syndrome who may be more subject to vitamin $B_6$ depletion.

Part I of this thesis was devoted to the investigation of the

effect of steroid contraceptives on the urinary excretion of PIC. This study was conducted as part of a larger project in which the influence of steroid contraceptives on the metabolism of tryptophan as well as urinary and blood vitamin $B_6$ was studied in 6 young women.

In an attempt to evaluate how vitamin $B_6$ metabolism is altered by the chromosome aberration in Down's syndrome, the study reported in Part II was undertaken to determine the urinary excretion of PIC by mental retardates with and without Down's syndrome before and after they received a loading dose of pyridoxine. Because vitamin $B_6$ is involved in amino acid metabolism, this study was performed in conjunction with one on the measurement of free amino acids in plasma and urine and the activities of erythrocyte transaminases.

In both of these investigations, the subjects were studied under controlled dietary conditions.

# REVIEW OF LITERATURE

## Metabolism of Vitamin $B_6$

Vitamin $B_6$ was identified by György (1934) as that part of the B complex responsible for the cure of a specific dermatitis developed by rats on a vitamin-free diet supplemented with vitamin $B_1$ and lactoflavin. Vitamin $B_6$ was later found to include three compounds, namely, pyridoxine (PIN) (2-methyl-3-hydroxy-4, 5-hydroxymethyl pyridine) and its 4-formyl and 4-aminomethyl analogues, pyridoxal (PAL) and pyridoxamine (PAM), respectively (György, 1971). PAL and PAM are the major free forms of vitamin $B_6$ that occur in animal tissues, whereas PIN is the predominant free form in plants (Toepfer and Polansky, 1964).

The metabolic interconversions of vitamin $B_6$ occurring in animals, as summarized by Snell (1964), are shown in Figure 1. The free forms of vitamin $B_6$ can be converted with approximately equal efficiency to the corresponding 5-phosphates by pyridoxal kinase. The major coenzymatically active forms of vitamin $B_6$ are pyridoxal phosphate (PALP) and pyridoxamine phosphate (PAMP) (McCormick, Gregory and Snell, 1961). Pyridoxine phosphate (PINP) and PAMP can be oxidized to PALP by pyridoxine phosphate oxidase (Wada and Snell, 1961).

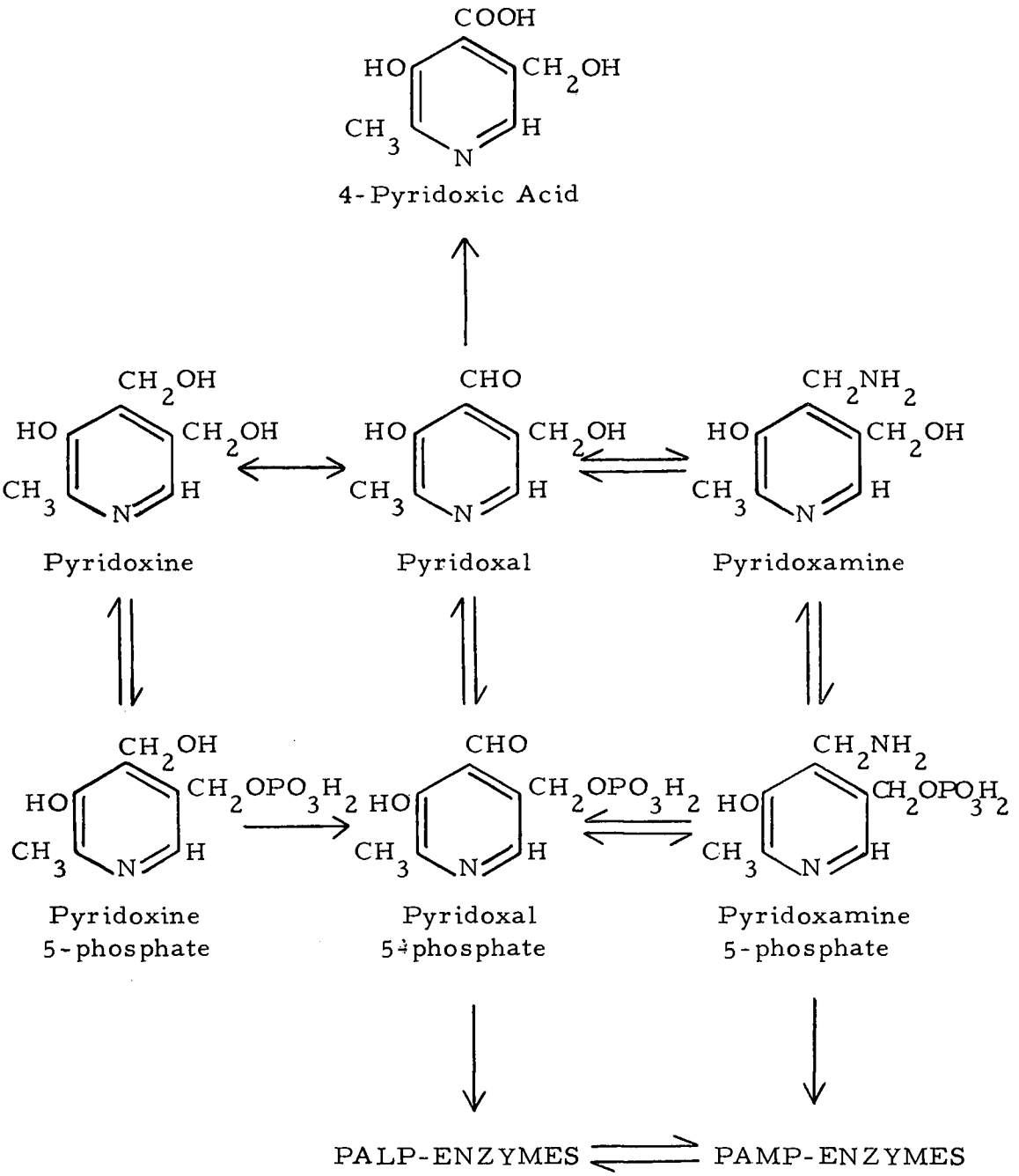



Figure 1. A summary of the metabolic interconversions and functions of vitamin $B_6$ (after Snell, 1964).

PAL can be formed by the oxidation of PIN as well as by the deamination of PAM. The oxidation of PAL by aldehyde oxidase results in the direct formation of 4-pyridoxic acid (Schwartz and Kjeldgaard, 1951). Based on the evidence of in vitro experiments, the formation of PIC in animal tissues has been postulated to be via the hydrolysis of PALP to PAL, which in turn is oxidized to PIC (Snell, 1964).

The exact site and mode of conversion of PIN and the other vitamin $B_6$ compounds in the body, however, have not been fully established. Recently Contractor and Shane (1970a) identified 4-pyridoxic acid 5-phosphate (PICP), a compound which had not been detected before, in urine and tissues of rats. The rapid formation of this compound following an intraperitoneal injection of PIN tagged with carbon 14 suggested to Contractor and Shane that PICP may be a direct oxidation product of PALP.

In rats that had been given an intraperitoneal injection of $^{14}C$-PIN Contractor and Shane (1971) later demonstrated a build up of PICP and some PIC after the appearance of PALP in liver, kidney and brain. They found, however, that PIC was more prominent in blood than PICP. From these observations Contractor and Shane (1970a) proposed that the predominant pathway for the catabolism of PALP in animal tissues is: pyridoxal 5-phosphate $\longrightarrow$ 4-pyridoxic acid 5-phosphate $\longrightarrow$ 4-pyridoxic acid.

Furthermore, in these same experiments, Contractor and Shane (1971) observed that only a small amount of the injected $^{14}C$ appeared in the brain in 24 hours and that the major part of the radioactivity was in PALP and PAMP. Since the amount of $^{14}C$ in PIN and PAL appeared to be too low to serve as a precursor of $^{14}C$-pyridoxal 5-phosphate, they speculated that PALP was transported to the brain, rather than formed there by phosphorylation.

A pathway for the conversion of PIN to the active form in red cells has been proposed by Anderson et al (1971). When normal subjects were given orally 50 mg of pyridoxine hydrochloride (PIN·HCl), PALP and PAL in the erythrocytes increased to a peak within an hour, while PAL in plasma reached a plateau during the second hour. This suggested to Anderson et al. that PIN was taken up by the red cells, converted to PALP and hydrolyzed to form PAL, which was then partially and gradually released into the plasma. The pathway they proposed is presented in Figure 2. Their experiments on in vitro incubation of blood with PIN confirmed this observation.

## Chemical and Physical Properties of 4-Pyridoxic Acid

The chemical and physical properties of 4-pyridoxic acid (2-methyl-3-hydroxy-4-carboxy-5-hydroxymethylpyridine) were studied by Huff and Perlzweig (1944). Four-pyridoxic acid is a white crystalline solid, having a melting point of 247°- 248°C. It is

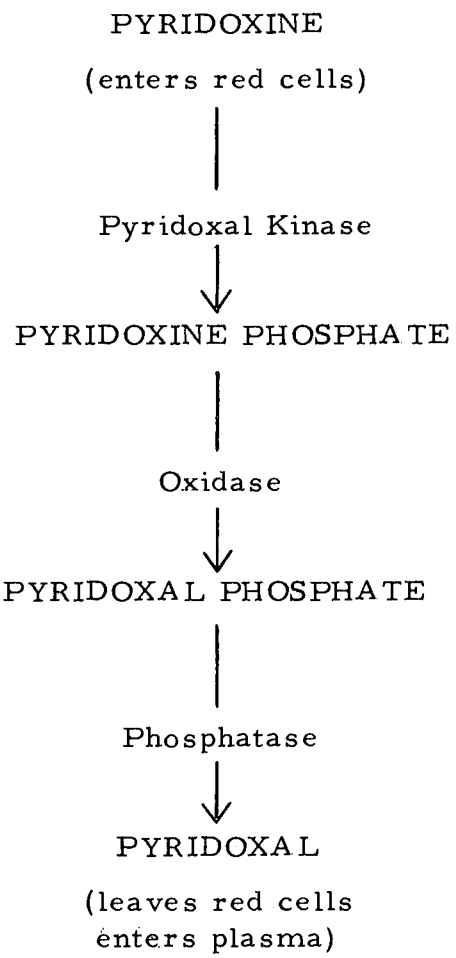
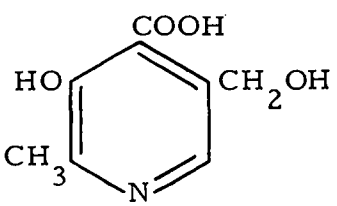
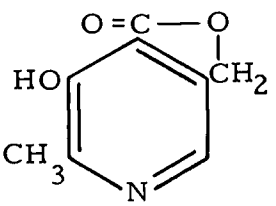



Figure 2. Proposed pathway for the conversion of pyridoxine to pyridoxal in blood (from Anderson et al., 1971).

slightly soluble in water, alcohol, and pyridine. It is insoluble in ether or aqueous acid solutions, but completely soluble in alkaline solutions. When exposed to ultraviolet light, an aqueous solution of PIC exhibits a characteristic blue fluorescence, the intensity of which reaches a maximum at pH 3 to 4. Since the lactone of PIC is 25 times more fluorescent than the metabolite, PIC is usually determined in that form. The lactone of PIC has a maximum fluorescence at pH 9.0 ± 0.3. The chemical structures of PIC and its lactone derivative are shown below:




4-Pyridoxic Acid




Lactone of PIC

## Urinary Excretion of 4-Pyridoxic Acid by Humans Receiving Adequate Vitamin $B_6$

Under normal conditions, the average values of urinary PIC for women consuming self-selected diets ranged from 3.8 to 6.9 μmoles (0.70 to 1.26 mg) per 24 hour (Woodring, Fisher and Storvick, 1964). Urinary excretion of PIC by men was determined by Kelsay,

Baysal and Linkswiler (1968). Their male subjects, who received a constant daily intake of 1.66 mg of vitamin $B_6$, excreted an average of 1.01 ± S.D. 0.32 mg of PIC per day. The men and women studied by Contractor and Shane (1970b) excreted, respectively, an average of 1.32 ± 0.78 mg and 1.21 ± 0.85 mg of PIC daily. In subjects receiving 8.6 μmoles (1.57 mg) of vitamin $B_6$ from a constant diet of natural foods, the amount of PIC excreted ranged from 3.9 to 4.2 μmoles (0.71 to 0.77 mg) per day (Reddy et al., 1958). The men and women studied by Mikac-Devic and Tomanic (1972) excreted ranges of 3.6 to 7.8 μmoles of PIC (0.66 to 1.43 mg) and 3.1 to 5.3 μmoles (0.57 to 0.97 mg) per day, respectively.

# PART I: THE URINARY EXCRETION OF 4-PYRIDOXIC ACID BY WOMEN USING STEROID CONTRACEPTIVES: THE "$B_6$-ORAL CONTRACEPTIVE STUDY"

## REVIEW OF LITERATURE

### Usage and Composition of Steroid Contraceptives

Oral contraceptives were introduced in the United States in 1960. Since that time synthetic steroid hormone preparations for fertility control have gained wide acceptance. Recently it was estimated that about 10,000,000 women in the United States use oral contraceptives (Hodges, 1971).

Steroid contraceptives available in this country are mixtures of an estrogen and a progestogen. Two synthetic estrogens, ethinyl estradiol and mestranol, and 8 progestational agents, which possess widely differing properties are used (AMA Council on Drugs, 1971). The total effect of the combination of these two steroids depends upon the compounds used, the absolute dose, the relative proportions of estrogen and progestogen, and whether a sequential or combination type[1/] of oral contraceptive is used.

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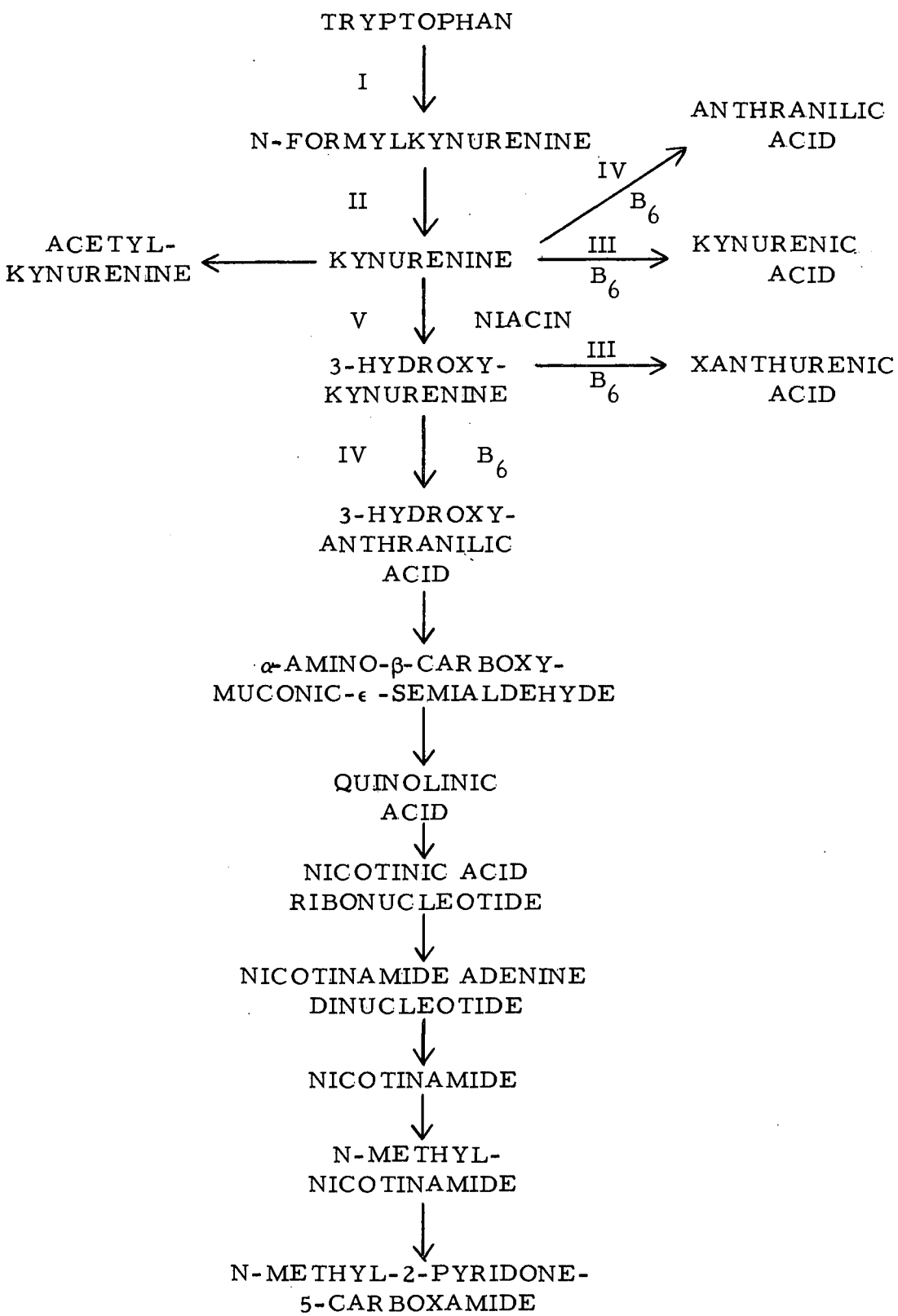
[1/] Two types of oral contraceptives are available. Each tablet of the combination type contains an estrogenic and a progestational substance. In the sequential type, tablets taken during the first part of the menstrual cycle contain only estrogen while those taken during the latter part contain both estrogen and progestogen. For both types, a tablet is taken daily for 21 days and then discontinued for 7 days for the menses interval.

Oral contraceptive agents appear to produce their effects by similar mechanisms. A suppression of the pituitary gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), seems to be the primary mode of action. The estrogen component is apparently mainly responsible for preventing the sharp rise of LH in the mid-cycle, which would otherwise cause ovulation to occur. Release of LH is probably controlled by an estrogen inhibition of LH-releasing factor in the hypothalamus, which in turn controls the secretion of LH by the pituitary gland. The primary effect of the progestogen is to dictate the amount and length of the menstrual flow by artificially building up the endometrial blood supply during the period after ovulation has occurred. When the artificial progesterone support is withdrawn, menstruation begins (Chinn et al., 1968).

## Metabolism of Vitamin $B_6$ and Tryptophan in Women Using Steroid Contraceptives

Women using oral contraceptives exhibit abnormal tryptophan metabolism, as indicated by increased urinary excretion of metabolites of the tryptophan-kynurenine pathway in response to a loading dose of L- or DL-tryptophan. (An outline of the pathway is shown in Figure 3). The test dose "stresses" this tryptophan pathway and emphasizes small abnormalities otherwise not detectable (Leklem, 1971). Without a test dose of this amino acid it is difficult to


TRYPTOPHAN
I
N-FORMYLKYNURENINE
ANTHRANILIC ACID
IV
II
B6
ACETYL-KYNURENINE
KYNURENINE
III
B6
KYNURENIC ACID
V
NIACIN
3-HYDROXY-KYNURENINE
III
B6
XANTHURENIC ACID
IV
B6
3-HYDROXY-ANTHRANILIC ACID
α-AMINO-β-CARBOXY-MUCONIC-ε-SEMIALDEHYDE
QUINOLINIC ACID
NICOTINIC ACID RIBONUCLEOTIDE
NICOTINAMIDE ADENINE DINUCLEOTIDE
NICOTINAMIDE
N-METHYL-NICOTINAMIDE
N-METHYL-2-PYRIDONE-5-CARBOXAMIDE

measure accurately the small amounts of most tryptophan metabolites normally excreted in urine.

Since several PALP-dependent enzymes are involved in the tryptophan-kynurenine pathway, the tryptophan load test is commonly used to assess vitamin $B_6$ nutriture in humans. When humans deficient in vitamin $B_6$ are given a test dose of this amino acid, they excrete increased amounts of xanthurenic acid, kynurenine, kynurenic acid, 3-hydroxykynurenine and acetylkynurenine in their urine (Miller and Linkswiler, 1967).

Rose (1966) was the first to report that women who used steroid contraceptives excreted grossly increased amounts of xanthurenic acid in urine after an oral load of 5 g of L-tryptophan. Price, Thornton and Mueller (1967) studied the metabolism of tryptophan in ten women who were taking a contraceptive containing 2.5 mg of norethynodrel and 0.1 mg of mestranol (Enovid-E) and in 18 control subjects. The oral contraceptive had no significant effect on the basal excretion of urinary tryptophan metabolites. But after a loading dose of 2 g of L-tryptophan, the users of oral contraceptives excreted significantly more xanthurenic acid as well as several other tryptothan metabolites than the control subjects. These abnormal excretions were corrected by the oral administration of 100 mg of pyridoxine hydrochloride (PIN·HCl) daily during the two days required to repeat the tryptophan load test.

Luhby and his coworkers (1971) undertook a systematic study to determine the minimum daily oral dose of PIN·HCl necessary to correct the abnormal tryptophan metabolism in women using oral contraceptive agents. Abnormal tryptophan metabolism was observed in about 75% of the women tested. They found that a daily supplement of 25 mg of PIN·HCl was necessary to normalize the urinary excretion of xanthurenic acid in all subjects. Based on the small number of subjects studied they recommended a daily intake of 30 mg of PIN·HCl for women taking oral contraceptives.

Toseland and Price (1969) reported finding high levels of urinary 3-hydroxyanthranilic acid (3HA) without tryptophan loading in women taking oral contraceptives. Their data indicated that the spontaneous urinary excretion of 3HA increased progressively with the length of time that estrogen-progestogen compounds were used.

To investigate the metabolic interrelationships and the nutritional implications of the long-term use of oral steriod hormones, Aly et al. (1971) studied several indices associated with vitamin $B_6$ metabolism in 5 women who were using an oral contraceptive and in 5 women who were not. Receiving a constant diet that was adequate in all nutrients except iron, the subjects were studied at the time of ovulation when estrogen production is normally at a peak. The users of oral contraceptives exhibited abnormal tryptophan metabolism, but excreted the same amount of vitamin $B_6$ in urine

as the controls. The activity of erythrocyte glutamic-oxaloacetic transaminase (EGOT) was higher in the women taking oral contraceptives than in those who were not, while the activity of erythrocyte glutamic-pyruvic transaminase (EGPT) was the same for both groups. Total plasma amino acid levels were significantly lower in the subjects using the oral contraceptive. This difference was due to a marked decrease in the level of nonessential amino acids in the plasma of the experimental subjects.

Doberenz and his coworkers (1971) reported that the activity of EGPT was lower and percentage of in vitro stimulation by PALP was higher in women taking oral contraceptives than in control females. Results of their investigation appear to support evidence of a vitamin $B_6$ depletion in women using oral contraceptive agents.

## Effect of Estrogen on the Activity of Some Vitamin $B_6$-Dependent Enzymes in Rats

Rose and Brown (1969) have shown that the activity of kynureninase, a vitamin $B_6$-dependent enzyme, was higher in the livers of male rats than in those of the female ones. They also found that the administration of estradiol benzoate to the males reduced the activity of hepatic kynureninase to that in the females. These results provide evidence that sex differences in the activity of this enzyme is due to the higher levels of estrogens in the female animals. They

were unable to demonstrate any sex differences in the activity of hepatic kynurenine transaminase. Estradiol benzoate had no influence on this enzyme either.

On the other hand, Mason and Gullekson (1960) found that the sulfate esters of some estrogens inhibit the activity of renal kynurenine transaminase in rates. This effect seems to result from their competition with PALP for binding sites on the apoenzyme.

Comparison of data of studies by different investigators, however, does not favor the view that estrogen produces a simple pyridoxine deficiency. Mason and Manning (1971) suggested that estrogen promotes a redistribution of PALP in the cells.

## Proposed Mechanism for Abnormal Tryptophan Metabolism and Increased Needs for Vitamin $B_6$ in Women Using Oral Contraceptives

Brin (1971) suggested that in women who use the contraceptive pill there is an increased activity in tryptophan oxygenase, which has been induced by the increased levels of plasma glucocorticoids. These glucocorticoids also induce certain vitamin $B_6$-requiring enzymes, such as tyrosine aminotransferase and alanine aminotransferase (Braidman and Rose, 1971), resulting in increased binding sites for PALP. Also, kynurenine transaminase is desaturated due to decreased PALP binding (Mason, Ford and Wu, 1969). Brin concluded that these factors combine to result in the abnormal excretion of tryptophan

metabolites following a loading dose of the amino acid. He suggested that these findings probably explain the increased vitamin $B_6$ needs for women taking steroid contraceptives.

# MATERIALS AND METHODS

## Subjects

The study was performed during Spring term, 1971 and lasted for 11 days. Six women served as subjects. Four young women who were using oral contraceptive pills for ovulation control served as experimental subjects and two other young women who were not taking steroid contraceptives served as control subjects. Five of the subjects were students at Oregon State University and the sixth was an employee of that institution. The experimental subjects ranged in age from 24 to 27 years, with a mean age of 25 years, and the control subjects were 22 and 31 years old, respectively. Their vital statistics are presented in Table 1.

The oral contraceptives used by the experimental subjects and the length of time they had used them are included in Table 1. All experimental subjects used the combination type containing an estrogen and a progestogen in each tablet.

## Diet

During the study all subjects consumed the constant diet which is presented in Table 2. The diet was prepared under the supervision of a nutritionist and served to the subjects in the Nutrition

Table 1. Vital statistics of the six women subjects participating in the "$B_6$-Oral Contraceptive Study."

| Subjects | Age years | Body Weight kg | Height cm | Contraceptive[a/] | Length of time on contraceptive months |
|---|---|---|---|---|---|
| Control | | | | | |
| 1 | 22 | 73.7 | 165.1 | none | |
| 2 | 31 | 51.4 | 176.5 | none | |
| Experimental | | | | | |
| 3 | 24 | 71.4 | 177.8 | Ortho-Novum 1 | 5 |
| 4 | 27 | 61.7 | 172.7 | Ortho-Novum 2 | 12 |
| 5 | 24 | 80.0 | 162.6 | Ovulen 21 | 12 |
| 6 | 26 | 64.2 | 168.9 | Ovulen 21 | 2 |

[a/] Composition per tablet of steroid contraceptives used: (E = estrogen; P = progestogen) Ortho-Novum 1, E = 50 μg mestranol and P = 1 mg norethindrone; Ortho-Novum 2, E = 100 μg mestranol and P = 2 mg norethindrone; Ovulen 21, E = 100 μg mestranol and P = 1 mg ethynodiol diacetate.

Table 2. Diet[a] used in the "$B_6$-Oral Contraceptive Study."

| | Food items | Wt, g |
|---|---|---|
| Breakfast (7 am) | Ground beef | 100[b] |
| | Bread, white | 25 |
| | Instant Breakfast[c/d], chocolate | 30 |
| | Non-fat dry milk[d] | 20 |
| | Orange juice, frozen reconstituted | 100 |
| | Ice cream, vanilla | 100 |
| | Butter[e] | |
| | Carbohydrate foods[e/f] | |
| | Folic acid supplement[g] | |
| Lunch (12 noon) | Ground beef | 100[b] |
| | Bread, white | 25 |
| | Instant Breakfast[c/d], chocolate | 30 |
| | Non-fat dry milk[d] | 20 |
| | Pears, drained | 80 |
| | Pears, syrup | 20 |
| | Ice cream, vanilla | 100 |
| | Butter[e] | |
| | Carbohydrate foods[e/f] | |
| | Folic acid supplement[g] | |
| Dinner (5 pm) | Ground beef | 100[b] |
| | Bread, white | 25 |
| | Instant Breakfast[c/d], chocolate | 30 |
| | Non-fat dry milk[d] | 20 |
| | Peaches, drained | 80 |
| | Peaches, syrup | 20 |
| | Ice cream, vanilla | 100 |
| | Butter[e] | |
| | Carbohydrate foods[e/f] | |
| | Folic acid supplement[g] | |

[a] Nutrient content of the diet as calculated by using "Composition of Foods" (Watt and Merrill, 1963): protein, 127 g; calcium, 1.6 g; iron, 17.4 mg; vitamin A, 4665 I.U.; thiamine, 1.3 mg; riboflavin, 2.7 mg; niacin, 25 mg; ascorbic acid, 127 mg. The vitamin $B_6$ content was 1.9 mg as determined by microbiological assay using Saccharomyces carlsbergensis. Butter was not included in this calculation.

Table 2. Continued.

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[b/] Raw weight.

[c/] Carnation Company, Los Angeles, California.

[d/] Instant Breakfast and non-fat dry milk were placed in a cup and mixed. Hot water was added to make a beverage.

[e/] Amounts were adjusted for each subject so that her initial weight was maintained.

[f/] This included sugar, 7-Up, gum drops and hard candies.

[g/] 0.033-mg supplement given at each meal after day 3.

Research Laboratory, School of Home Economics, Oregon State University. By means of Agriculture Handbook No. 8 (Watt and Merrill, 1963), the diet was calculated to contain 127 g of protein and approximately 2000 kcal. A supplement of folic acid was given to each subject in order to make the diet meet the National Research Council's (1968) Recommended Dietary Allowances for all nutrients. The calculated iron intake was 17.4 mg and was not supplemented because of the short duration of the study. The three meals were equal in vitamin $B_6$ and tryptophan content. The vitamin $B_6$ content of the diet was estimated to be 1.9 mg as determined by microbiological assay employing Saccharomyces carlsbergensis of representative samples of the diet. The requirement of vitamin $B_6$ by young women has been found to be about 1.5 mg per day (Donald et al., 1971).

## Urine Collection

Continuous 24-hr urine collections were made throughout the study. Urine for the determination of 4-pyridoxic acid was collected on those days when no tryptophan load test was given (Table 3). All daily urine samples were kept under a layer of toluene in amber bottles and were kept under refrigeration. The following morning the urine was mixed and measured. Fifty to 100-ml portions of urine for the determination of 4-pyridoxic acid and creatinine were

Table 3. Schedule for urine collection[a/] - "$B_6$-Oral Contraceptive Study".

| Subjects | Day of Experiment | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
| Control | | | | | | | | | | | |
| 1 | O | X | - | - | X | X | - | X | X | - | - |
| 2 | O | X | - | - | X | X | - | X | X | - | - |
| Experimental | | | | | | | | | | | |
| 3 | O | X | X | - | X | X | - | - | X | - | - |
| 4 | O | X | X | - | X | X | - | - | X | - | - |
| 5 | O | X | - | - | X | X | - | X | X | - | - |
| 6 | O | X | - | - | X | X | - | X | X | - | - |

[a/] "X" indicates days on which 24-hour urine specimens were collected for the determination of 4-pyridoxic acid and vitamin $B_6$. "-" indicates days on which urine was collected following the tryptophan load test. "O" indicates that no analyses were made on urine collected on day 1. Blood for the determination of vitamin $B_6$ was drawn shortly before the subjects ingested the 2 g dose of L-tryptophan. Results from tryptophan loading and the determination of blood and urinary vitamin $B_6$ will be published elsewhere.

transferred to bottles and kept frozen until analyzed.

## Determination of Urinary 4-Pyridoxic Acid

The microprocedure for the determination of urinary PIC, which was developed by Woodring, Fisher and Storvick (1964), was used with minor modifications. This method is sensitive, specific and readily reproducible. For each analysis an aliquot of urine sample representing 1% of the 24-hr excretion was adjusted to pH 10.6 with 2 N NaOH, followed by the addition of 1.5 ml of 1.5 N $NH_4OH$. The urine was applied to a column of Dowex 1 x 10 ($Cl^-$). The PIC was released from the Dowex 1 column with 0.05 N HCl. This eluate was applied directly to a Dowex 50W x 12 ($H^+$) column, from which the PIC was eluted with 2 N HCl. The assay of a recovery sample (urine plus 5 μg of PIC) was run concurrently and was handled the same as the sample. Analysis of the eluate from the Dowex 50W column was made in triplicate; a blank was also made for each set. The quantitative estimation of PIC in the eluate consisted of 4 steps: (1) delactonization, by the addition of 0.4 N NaOH and heating in a boiling water bath, (2) lactonization, by adding 6 N HCl and heating, (3) adjustment with 6 N $NH_4OH$ to a pH between 9 and 10 for maximum fluorescence of the lactone, and (4) fluorescence measurement with a Model A Farrand fluorometer with Corning No. 5860 as a primary filter and with a secondary filter

composed of Corning No. 3389 and No. 4308 separated by a Wratten gelatin filter 2A. The blank was prepared by using the same procedure except that the heating in step (2) was omitted. The amount of PIC in the sample was determined through the use of a standard curve.

## Determination of Urinary Creatinine

Creatinine was determined by a micromodification of the method by Folin (Oser, 1965). For the estimation of total creatinine in a 24-hr urine sample, 0.5 ml of urine and 0.5 ml of $H_2O$ were transferred into a 100-ml volumetric flask. A red color was obtained by the addition of picric acid, followed by 15% NaOH. The resulting mixture was allowed to stand for 15 minutes and then diluted to volume with water and mixed. Absorbance was measured in a Bausch and Lomb Spectronic 20 colorimeter at 520 nm. The true reading was obtained by subtracting the reading of the blank (water used in place of urine) from that of the sample. The creatinine value of the 24-hr specimen was determined through the use of a standard curve.

# RESULTS AND DISCUSSION

The urinary excretion of PIC and creatinine by the 6 women subjects participating in the "$B_6$-Oral Contraceptive Study" are presented in Tables 4 and 5, respectively.

On the 5 days that urinary PIC was measured, all of the subjects except subject 4 excreted amounts of the metabolite that were within or slightly above the range reported by Woodring et al (1964) for 7 women who were consuming self-selected diets. In this study the two control subjects excreted from 0.73 to 1.34 mg of PIC per 24 hr (mean 1.07 ± S.D. 0.24). Experimental subjects 3, 5 and 6 excreted from 0.71 to 1.23 mg per 24 hr (mean 0.95 ± S.D. 0.16). The difference in the urinary excretion of PIC between the two groups is not statistically significant by the Student t test.

Experimental subject 4 consistently excreted less PIC than either the control or experimental subjects. She excreted from to 0.64 mg of PIC per 24 hr. Whether or not the values obtained from subject 4 are accurate is questionable. Her daily urinary excretion of creatinine was variable, which may be normal (Bleiler and Schedl, 1962). But, in addition, her creatinine coefficient was lower than the normal range of 15 to 25 mg per kg of body weight per 24 hr (Conn, 1970). This most likely indicates that her 24-hr urine specimens were incomplete and hence her PIC values are not correct.

Table 4. Urinary excretion of 4-pyridoxic acid by six women participating in the "$B_6$-Oral Contraceptive Study" (mg/24 hr).

| Subjects | Day of Experiment | | | | | | Average of last 4 samples |
|---|---|---|---|---|---|---|---|
| | 2 | 3 | 5 | 6 | 8 | 9 | |
| Control | | | | | | | |
| 1 | 0.89 | | 1.04 | 0.74 | 1.01 | 1.18 | 0.99 |
| 2 | 0.73 | | 1.31 | 1.31 | 1.34 | 1.32 | 1.32 |
| Experimental | | | | | | | |
| 3 | 0.71 | 1.02 | 1.04 | 0.76 | | 1.01 | 0.96 |
| 4 | 0.40 | 0.39 | a/ | 0.64 | | 0.59 | 0.54 |
| 5 | 0.72 | | 0.83 | 0.96 | 0.97 | 0.97 | 0.93 |
| 6 | 0.79 | | 1.13 | 1.09 | 1.07 | 1.23 | 1.13 |

a/ Sample was discarded because of incomplete collection.

Table 5. Total creatinine in urine - "$B_6$-Oral Contraceptive Study" (g/24 hr).

| Subjects | Day of Experiment | | | | | |
|---|---|---|---|---|---|---|
| | 2 | 3 | 5 | 6 | 8 | 9 |
| Control | | | | | | |
| 1 | 1.26 | | 1.38 | 1.38 | 1.36 | 1.40 |
| 2 | 1.18 | | 1.27 | 1.16 | 1.13 | 1.17 |
| Experimental | | | | | | |
| 3 | 1.28 | 1.34 | 1.63 | 1.39 | | 1.59 |
| 4 | 0.64 | 0.47 | -[a] | 0.91 | | 0.84 |
| 5 | 0.96 | | 1.21 | 1.23 | 1.18 | 1.09 |
| 6 | 1.03 | | 1.03 | 1.17 | 1.08 | 1.17 |

[a] Sample was discarded because of incomplete collection.

The findings of the present study are consistent with those reported by Rose et al. (1972). They studied the urinary excretion of PIC by 28 control subjects and 31 women who had been taking an oral contraceptive for 6 to 36 months. There was no significant difference between the concentration of PIC in urine of the control subjects (mean 0.71 mg ± S.D. 0.13) and that of the users of oral contraceptives (0.62 mg ± 0.22). Of the women using oral contraceptives, only 7 had a decreased excretion of PIC. Besides, in 6 of these 7, a diagnosis of subclinical vitamin $B_6$ deficiency was supported by a raised ratio in the urinary excretion of hydroxykynurenine to hydroxyanthranilic acid in response to a tryptophan load test. It should be pointed out that the subjects studied by Rose et al. were consuming uncontrolled diets, which may or may not have been adequate in vitamin $B_6$.

In general the amount of PIC excreted in urine by both the control and experimental subjects in this study increased with time and stabilized at a higher level (Table 4). The average daily excretion of all subjects, excluding subject 4, on day 2 was 0.77 mg; on day 6, 0.97 mg; and on day 9, 1.14 mg. The excretion on day 2 would probably reflect the previous dietary intake of vitamin $B_6$ and would also be indicative of the body stores of the vitamin at that time. This progressive increase in urinary PIC probably can be explained by the fact that the experimental diet in this study, which supplied

1.9 mg of vitamin $B_6$, may have been higher in vitamin $B_6$ than the subjects' diets before their participation in this study. Thus the higher values during the latter part of the study could indicate an adjustment to the increased dietary intake of the vitamin. Similar observations were made by Reddy et al. (1958). Their subjects were fed a diet containing 7.3 μmoles (1.23 mg) of vitamin $B_6$ for 6 days before being changed to one containing 14.7 μmoles (2.46 mg). During the 14-day period the subjects received the higher level of the vitamin, their daily urinary excretion of PIC increased progressively from an average of 7.05 μmoles on day 1 to 14.7 μmoles on day 14.

Because of the small number of subjects participating in the present study no definite conclusion can be made as to the effect of oral contraceptives on the excretion of PIC by women who are taking this kind of drug. Besides, from the results of this study that the abnormality of vitamin $B_6$ metabolism in women using oral contraceptives, as suggested by an altered tryptophan metabolism, cannot be demonstrated by the measurement of PIC alone, which is the major excretory product of vitamin $B_6$. Besides, these results lend further support to the view that a simple vitamin $B_6$ deficiency does not occur in users of oral contraceptives. Studies indicate that a relative deficiency of the vitamin exists in users of oral contraceptives (Brin, 1971). Under these conditions, the excretion of PIC can still be normal. In the subjects studied by Luhby et al. (1971),

only 75% of the oral contraceptive users exhibited a derangement in tryptophan metabolism. It would be interesting to see the pattern of PIC excretion among this group of people.

There is an inverse relationship between the weight of the subjects and the amount of PIC they excreted in urine (Figure 4). (Data from subject 4 were excluded). Two explanations can be offered for this relationship which involve either the vitamin $B_6$ alone or the vitamin $B_6$-protein interrelationship. In this study each subject received a diet containing 1.9 mg of vitamin $B_6$ and 127 g of protein. This amount of vitamin $B_6$ is slightly below the National Research Council's Recommended Dietary Allowance (1968), but higher than the minimum requirement of 1.5 mg proposed by Donald et al. (1971) for women receiving 54 g of protein and that of 1.64 mg found by Baker et al., (1964) for men consuming 100 g of protein. Since the requirement for vitamin $B_6$ depends upon the protein content of the diet (Baker et al., 1964; Miller and Linkswiler, 1967), the subjects in this study may have received a marginal intake of the vitamin. The protein intake of the subjects in this study was in excess of the recommended allowance of 0.9 g per kg of body weight. The lightest subject (51.4 kg) was receiving 2.7 times her recommended allowance of protein; the heaviest subject 1.7 times.

Considering vitamin $B_6$ intake alone, one explanation for this negative relationship between body weight and urinary PIC in this

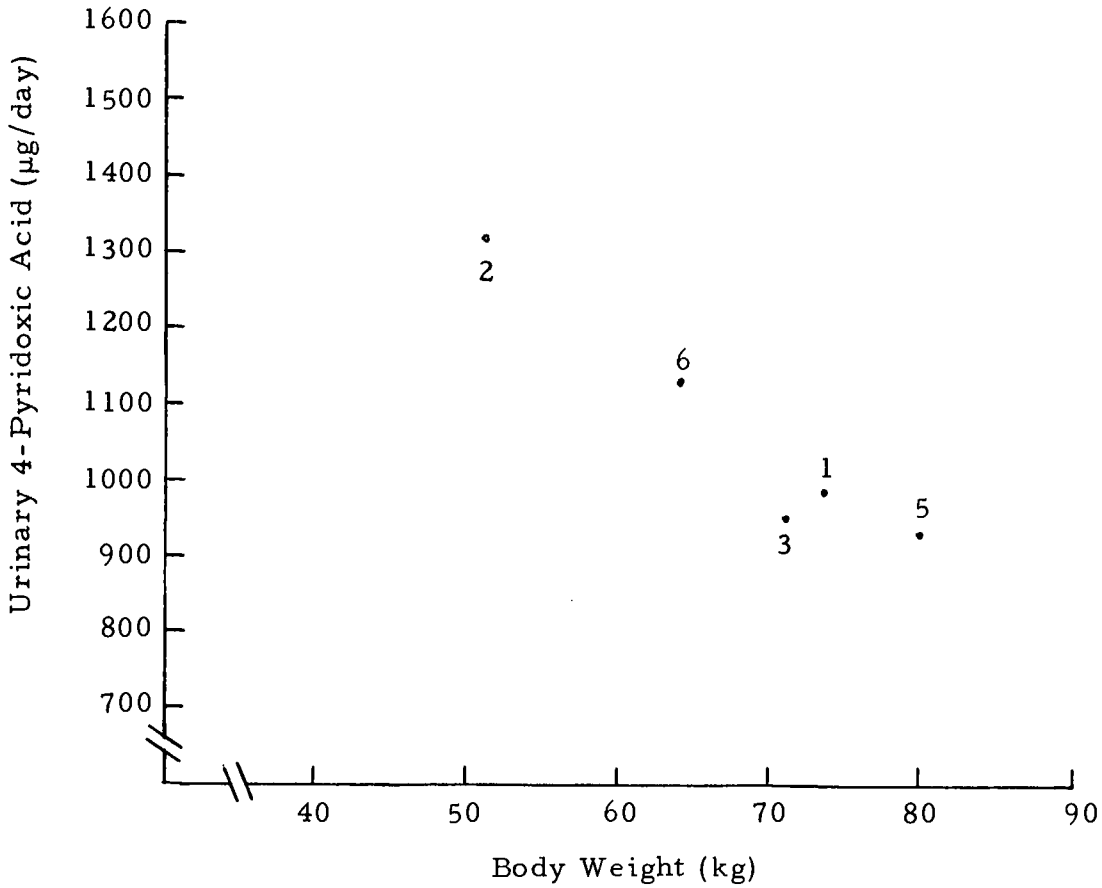


Figure 4. Mean urinary excretion of PIC versus body weight "$B_6$-Oral Contraceptive Study". These values represent the mean for 4 days and do not include data from urine collected on day 2. Control subjects are 1 and 2; experimental subjects are 3, 5, and 6. The mean for experimental subject 4 is not included in this figure because of the possibility of incomplete urine collections.

study is that the lighter subject may have a smaller body pool of the vitamin, thereby making more vitamin $B_6$ available for conversion to the metabolite.

Another explanation is that under these conditions of high protein intake, urinary PIC may also represent the vitamin $B_6$ that had been utilized for the catabolism of excess protein. Contractor and Shane (1971) observed that in rat tissues large amounts of PALP were oxidized to PICP which was retained in the tissues until hydrolysis to PIC. Could PICP possibly have been formed from PALP that was used up as a coenzyme in protein catabolism?

Since all subjects in the present study were receiving the same amount of protein regardless of body size, the lighter subjects had a higher requirement for vitamin $B_6$ because they were receiving a greater excess of protein than the heavier ones. Cinnamon and Beaton (1971) observed that of their subjects fed a vitamin $B_6$-deficient diet containing 100 g of protein, the lightest subject developed abnormal tryptophan metabolism sooner and to a greater degree than their heavier subjects. They noted a relatively linear relationship between the number of days required to attain an excretion of 35 mg of xanthurenic acid in response to 2 g of L-tryptophan and the "excess" protein (protein in excess of 0.9 g per kg of body weight) the subjects were fed. Miller and Linkswiler (1967) studied the development of abnormal tryptophan metabolism in subjects

receiving a diet containing 150 g of protein and 0.166 mg of vitamin $B_6$. After 14 days of vitamin $B_6$ deprivation their smaller subjects excreted larger quantities of tryptophan metabolites in response to a tryptophan load test than their heavier ones. Animal studies show that an increase in nonutilizable amino acids, which occurs when the intake of protein is greater than the requirement, will increase the need for vitamin $B_6$ for amino acid catabolism (Williams, 1964).

# PART II: THE URINARY EXCRETION OF 4-PYRIDOXIC ACID IN RESPONSE TO PYRIDOXINE SUPPLEMENTATION BY MENTAL RETARDATES WITH AND WITHOUT DOWN'S SYNDROME

## REVIEW OF LITERATURE

### Down's Syndrome

Down's syndrome is also known as mongolism, or Trisomy 21, Trisomy 22 and Trisomy $G_1$ syndromes. It affects both sexes and all races of mankind. That Down's syndrome has a direct relationship to the age of the mother has been well established (Benda, 1969). At a maternal age of 25 years, the incidence of Down's syndrome is estimated to be 1 in 2,000 births. At a maternal age of 35 years, the incidence may be 5 in 1,000 births (Magalini, 1971). After a maternal age of 40, 10 to 20 children with Down's syndrome may be expected per 1,000 births. The number doubles after the maternal age of 45 years (Benda, 1969).

There are two populations of mongoloids: those who are stillborn or die within the first year of life, and those who survive infancy. Down's syndrome is characterized by mental retardation (I.Q. 20 to 60), certain physical characteristics, delay in walking and speaking, generalized hypotonia, and delayed puberty. Early menopause occurs in the female. There is no specific therapy for

disease. The average life span of survivors is around 30 years (Magalini, 1971).

Down's syndrome is caused by chromosome anomalies which can be categorized into four groups: (1) Non-disjunction trisomy 21 or "regular mongol" (most common type), somatic cells with 47 chromosomes due to faulty meiosis during the maturation division of the female or male gonads; (2) "de novo" translocation, caused by attachment of a chromosome fragment to a nonhomologous chromosome, arising from an unknown source; (3) inherited translocation, a chromosome aberration involving an interchange between nonhomologous chromosomes transmitted from the parents to the offspring; (4) mongol mosaicism, a variety of chromosome counts occurring with chromosome assortments of 45, 46 and 47.

The presence of a small extra acrocentric chromosome in Down's syndrome (group 1 above) was described more than a decade ago. There is little reliable information on how the chromosomal imbalance produces multiple physical and chemical changes described in the syndrome. A number of enzyme changes have been reported. Hsia and his coworkers (1964) were able to show an increase of galactose-1-phosphate uridyl transferase in leukocytes obtained from patients with Down's syndrome. An increase in leukocyte alkaline phosphatase was also demonstrated by Rosner et al. (1965). Furthermore, erythrocytes in patients with Down's syndrome show

an increase in several enzymes, including phosphohexokinase and serum glutamic-oxaloacetic transaminase (Hsia et al., 1971).

## Metabolism of Vitamin $B_6$ in Patients with Down's Syndrome

Several studies on patients with Down's syndrome indicate that they are more subject to depletion of vitamin $B_6$ than normal controls or non-mongoloid mental retardates. McCoy and Chung (1964) studied tryptophan metabolism in mongoloids and suitable non-mongoloid controls who were made vitamin $B_6$-deficient by the administration of deoxypyridoxine, a vitamin $B_6$ antagonist. The mongoloids excreted significantly more 3-hydroxykynurenine and xanthurenic acid in response to a test dose of L-tryptophan than the controls.

The findings of Tu and Zellweger (1965) suggest that the activity of the PALP-dependent decarboxylase involved in the decarboxylation of 5-hydroxytryptophan to serotonin (5-hydroxytryptamine) is depressed in the mongoloid. Their patients with Down's syndrome had abnormally low levels of blood serotonin. L-tryptophan was ineffective in raising the level of this metabolite. In addition, DL-penicillamine, an antagonist of vitamin $B_6$, caused a significant fall in blood serotonin. A supplement of L-tryptophan and PIN·HCl increased the levels of serotonin in the blood of these subjects.

In contrast, Naiman and Oski (1965) who studied

12 institutionalized male mongoloids obtained normal values for erythrocyte glutamic-oxaloacetic transaminase stimulated with PALP in vitro.

McCoy and England (1968) postulated that the activity of enzymes which metabolize vitamin $B_6$ is greater in mongoloids than in non-mongoloids. When mongoloids were given 100 mg of PIN·HCl intramuscularly, they excreted significantly more urinary PIC than the non-mongoloid retardates.

However, in another study, McCoy, Colombini and Ebadi (1969) observed no significant difference in the urinary excretion of PIC by 6 mongoloid and 6 non-mongoloid male subjects who had received orally a 100-mg dose of pyridoxal hydrochloride. This suggested to these authors that the activity of aldehyde oxidase, which oxidizes PAL to PIC, is similar in the two groups.

Leukocyte PALP in patients with Down's syndrome was determined by Coburn and Seidenberg (1968). The level in patients with Down's syndrome (9.8 ng/$10^8$ cells) was significantly lower than in the retarded controls (11.9 ng) which in turn was lower than that in the normal controls (18.7 ng).

# MATERIALS AND METHODS

The study on the effect of pyridoxine loading on the urinary excretion of 4-pyridoxic acid by mongoloid and non-mongoloid mental retardates was carried out at Fairview Hospital and Training Center, Salem, Oregon during June and July of 1971. This was one phase of a larger study on the effect of pyridoxine supplementation on plasma and urinary amino acids as well as erythrocyte transaminases in mental retardants with and without Down's syndrome.

## Subjects

Twelve mongoloid and 12 non-mongoloid mental retardates ranging in age from 17 to 24 years participated in this study. An attempt was made to match each mongoloid with a non-mongoloid control as closely as possible with respect to age, weight and height. This was not entirely possible, however, because patients with Down's syndrome are characteristically shorter and lighter than non-mongoloids. Down's syndrome was diagnosed in subjects by their physical characteristics and, in some, by chromosome karyotype. Vital statistics of the subjects is presented in Table 6. The physical condition of the subjects was good as judged by the cooperating physician. None of the participants took any medication during the course of the study.

The study lasted for a total of four weeks. Six subjects were studied at a time for a one-week period. Each week three matched

Table 6. Vital statistics of mental retardates[1/] with and without Down's syndrome.

| Subjects | Age years | Body weight kg | Height cm | Diagnosis and comments |
|---|---|---|---|---|
| Group 1: Women (studied June 17 to June 23) | | | | |
| JG[a] | 17 | 45.0 | 148.6 | Down's syndrome |
| SH[b] | 20 | 48.4 | 144.8 | Down's syndrome; karyotype 47, XX G+ |
| CP[c] | 18 | 47.7 | 146.1 | Down's syndrome |
| BL[c] | 20 | 52.3 | 167.6 | Idiopathic encephalopathy; history of convulsion |
| DM[a] | 18 | 48.4 | 146.1 | Idiopathic mental retardation |
| GT[b] | 21 | 44.3 | 153.7 | Bilirubin encephalopathy; deaf; spastic |
| Group 2: Women (studied July 8 to July 14) | | | | |
| JC[d] | 18 | 47.0 | 147.3 | Down's syndrome; G trisomy |
| NH[e] | 19 | 53.2 | 149.8 | Down's syndrome |
| JM[f] | 22 | 44.6 | 157.5 | Down's syndrome; trisomy 21 |
| SH[f] | 21 | 50.9 | 147.3 | Encephalopathy due to anoxemia at birth; birth after 8 months of pregnancy, due to injury of mother |
| SR[d] | 18 | 54.1 | 153.7 | Idiopathic encephalopathy; cyanotic at birth |
| FT[e] | 19 | 47.3 | 154.9 | Idiopathic encephalopathy; speech defect |

[1/] Matches for mongoloid and non-mongoloid subjects are indicated by the same superscript.

Table 6. continued

| Subjects | Age years | Body weight kg | Height cm | Diagnosis and comments |
|---|---|---|---|---|
| Group 3: Men (studied July 15 to July 21) | | | | |
| AL[g] | 21 | 58.4 | 151.8 | Down's syndrome; trisomy 21; acute malnutrition in early infancy |
| JM[h] | 20 | 66.6 | 165.7 | Down's syndrome; trisomy 21 |
| GV[i] | 21 | 63.4 | 156.2 | Down's syndrome |
| RL[i] | 22 | 63.4 | 162.6 | Idiopathic mental retardation |
| JS[h] | 21 | 70.0 | 172.7 | Encephalopathy due to postnatal injury (subdural hematoma) |
| RT[g] | 21 | 61.8 | 161.3 | Encephalopathy, congenital (associated with toxemia of pregnancy) |
| Group 4: Men (studied July 22 to July 28) | | | | |
| CB[j] | 21 | 48.6 | 149.9 | Down's syndrome; G trisomy; probable mosaicism |
| RF[k] | 24 | 63.6 | 160.0 | Down's syndrome |
| DW[l] | 22 | 53.2 | 166.4 | Down's syndrome |
| DE[k] | 23 | 53.6 | 177.2 | Encephalopathy due to mechanical injury at instrumental birth |
| FH[j] | 22 | 53.6 | 167.6 | Idiopathic mental retardation |
| EM[l] | 21 | 65.0 | 165.1 | Encephalopathy due to anoxemia at birth |

pairs of subjects of the same sex, i.e., 6 subjects in all, were admitted to the hospital ward in the afternoon before day 1.

## Diet

Starting on day 1 the subjects consumed a constant diet, which was prepared by the main kitchen staff of the Center and by the diet kitchen staff of the Hospital. The diet was adequate in all nutrients essential for man. The men received a diet containing 93 g of protein and approximately 3000 kcal; the women received one containing 86 g of protein and about 2500 kcal (Watt and Merrill, 1963). The calculated vitamin $B_6$ content (Orr, 1969) of these two diets are 1.60 mg and 1.58 mg respectively. The diets for male and female subjects are shown in Table 7.

## Urine Collection

All subjects were catheterized to insure complete collection of urine. They remained in their beds during the days urine was collected. On completion of the 24-hr collection urine was measured, mixed and frozen until assayed for 4-pyridoxic acid and creatinine. Twenty-four hr urine collections were made on days 1, 5 and 6. On day 5, 50 mg of pyridoxine[1/] was administered orally just before

[1/] Prepared by dissolving 0.4255 g of pyridoxine hydrochloride in 14 ml of twice distilled water. Two-ml portions of this solution, which contained 50 mg of pyridoxine, were stored in red colored flasks and kept frozen until used.

Table 7. Diet fed mental retardates with and without Down's syndrome.

| | Food Items | Women[a] wt, g | Men[b] wt, g |
|---|---|---|---|
| Breakfast | Blended orange and grapefruit juice, canned | 100 | 100 |
| | Cornflakes | 25 | 30 |
| | Boiled egg | 50 | 50 |
| | Bread, white enriched | 25 | 50 |
| | Butter | 10 | 20 |
| | Jelly | 20 | 40 |
| | Milk | 240 | 240 |
| | Sugar (as desired) | | |
| Lunch | Hamburger patty, cooked wt. | 65 | 65 |
| | Hamburger bun, enriched | 60 | 60 |
| | Hamburger relish | 30 | 30 |
| | Potato chips | 14 | 14 |
| | Carrot, raw | 25 | 20 |
| | Ice cream | 114 | 114 |
| | Milk | 240 | 240 |
| Afternoon | Punch[c] (1 cup) | | |
| Dinner | Frankfurters | 120 | 120 |
| | Buns | 40 | 80 |
| | Mustard | 5 | - |
| | Tomato catsup | 15 | 40 |
| | Butter | - | 20 |
| | Sweet pickle relish | 15 | 20 |
| | Celery sticks | 20 | 20 |
| | Banana (edible portion) | 125 | 120 |
| | Milk | 240 | 240 |
| Before bed | Chocolate pudding[d] | 128 | 128 |

[a] Calculated nutrient content of the diet: protein, 86 g; calcium, 1.347 g; iron, 12.7 mg; vitamin A, 5990 I.U.; thiamine, 1.2 mg; riboflavin, 2.6 mg; vitamin $B_6$, 1.58 mg; ascorbic acid, 103 mg.

Table 7. Continued

[b] Calculated nutrient content of the diet: protein, 93 g; calcium, 1.382 g; iron, 15.1 mg; vitamin A, 6776 I.U.; thiamine, 1.4 mg; riboflavin, 2.7 mg; vitamin $B_6$, 1.60 mg; ascorbic acid, 107 mg.

[c] Allen Foods, Inc., St. Louis, Missouri 63116.

[d] Rich Products Corp., Buffalo, New York 14213.

breakfast and shortly after blood was drawn for other biochemical studies. The determination of complete blood counts, differential counts, and hemoglobin and hematocrit values were done by the medical technologists at the Hospital. Other analyses done on the blood and urine collected from the subjects include erythrocyte glutamic-oxaloacetic transaminase and glutamic-pyruvic transaminase, and plasma and urinary amino acids. Results from these will be reported elsewhere.

## Determination of Urinary 4-Pyridoxic Acid and Creatinine

The same procedures were used as given under Materials and Methods in the "$B_6$-Oral Contraceptive Study".

# RESULTS AND DISCUSSION

The urinary excretion of PIC by the 12 mongoloid and 12 non-mongoloid mental retardates before (day 1) and after (days 5 and 6) supplementation with 50 mg of pyridoxine is presented in Table 8.

There was no significant difference in the urinary excretion of PIC by these two groups of mental retardates while they were receiving a diet containing 1.6 mg of vitamin $B_6$ (day 1). The mean urinary excretion of PIC by the mongoloid subjects was 0.91 mg per 24 hr and that by the non-mongoloid subjects was 0.93 mg. The amount of PIC excreted by each subject on day 1 (Table 8) was within the ranges for normal subjects reported by Woodring et al. (1964), Contractor and Shane (1970b) and Kelsay et al. (1968). These results suggest that the degradation of vitamin $B_6$ to PIC was similar in these two groups of mental retardates and was no different from that of normal subjects. The findings in this study are in agreement with those reported by McCoy and England (1968), who found no significant difference between mongoloids and non-mongoloids in the urinary excretion of this metabolite.

Vitamin $B_6$ status in patients with Down's syndrome was investigated because of previous reports of impaired tryptophan metabolism in these individuals (McCoy and Chung, 1964; Tu and Zellweger, 1965).

Table 8. Urinary excretion of 4-pyridoxic acid by mental retardates (mg/24 hr).

| Non-Mongoloid | | | | Mongoloid | | | |
|---|---|---|---|---|---|---|---|
| Subjects | Day 1 | Day 5 [a] | Day 6 | Subjects | Day 1 | Day 5 | Day 6 |
| Group 1: Women | | | | | | | |
| BL | 1.13 | 17.60 | 4.20 | JG | 0.83 | 16.00 | 2.45 |
| DM | 0.88 | 21.20 | 3.60 | SH | 0.70 | 18.00 | 4.30 |
| GT | 1.03 | 17.20 | 3.40 | CP | 0.80 | 19.70 | 3.80 |
| Group 2: Women | | | | | | | |
| SH | 0.97 | 20.00 | 3.53 | JC | 0.91 | 21.10 | 4.55 |
| SR | 1.00 | 18.85 | 3.80 | NH | 0.72 | 19.90 | 4.33 |
| FT | 0.86 | 20.20 | 3.23 | JM | 1.09 | 19.30 | 3.93 |
| Group 3: Men | | | | | | | |
| RL | 1.05 | 11.10 | 3.48 | AL | 0.71 | 20.75 | 4.15 |
| JS | 0.89 | 19.90 | 3.75 | JM | 1.19 | 20.80 | 4.15 |
| RT | 1.04 | 18.30 | 3.38 | GV | 1.15 | 19.60 | 4.33 |
| Group 4: Men | | | | | | | |
| DE | 0.94 | 15.40 | 3.10 | CB | 0.92 | 21.15 | 4.40 |
| FH | 0.76 | [b] | 2.73 | RF | 0.93 | 15.25 | 3.80 |
| EM | 0.64 | 15.45 | 2.93 | DW | 0.92 | 25.70 | 4.60 |
| Mean | 0.93 | | | | 0.91 | | |

[a] An oral dose of 50 mg of PIN was given just before the 24-hr urine collection was started.

[b] Specimen lost by accident.

In view of the normal urinary excretion of PIC by the mongoloids on day 1, the aberration in vitamin $B_6$ metabolism in this condition is not caused by an enhanced conversion of vitamin $B_6$ to PIC or by a deficiency of the vitamin. The urinary excretion of PIC drops appreciably in human subjects depleted in vitamin $B_6$ (Donald, McBean and Simpson, 1971). Naiman and Oski (1965) found no evidence of vitamin $B_6$ deficiency in males with Down's syndrome as determined by measuring the activity of EGOT stimulated with added PALP in vitro.

There was much variation in the excretion of PIC among the subjects of both groups on day 1 (Table 8; Figure 5). The excretion by the mongoloids ranged from 0.70 mg to 1.19 mg per 24 hr, while the non-mongoloids excreted from 0.64 to 1.13 mg. This variation in urinary PIC could be due to differences in the subjects' dietary intake of vitamin $B_6$ prior to their participation in this study (Donald, McBean and Simpson, 1971). Also, as in this thesis on the urinary excretion of PIC by women using steroid contraceptives, several days were required for the subjects to adjust to the level of vitamin $B_6$ they received in their diet. Possibly if urinary PIC in the present study had been determined on day 3 or 4 after the subjects had adjusted to the diet they were receiving, there would have been less variation among them in the excretion of this metabolite. Variation in PIC excretion by the non-mongoloid subjects could be attributed also to heterogeneity in the etiology of their mental retardation

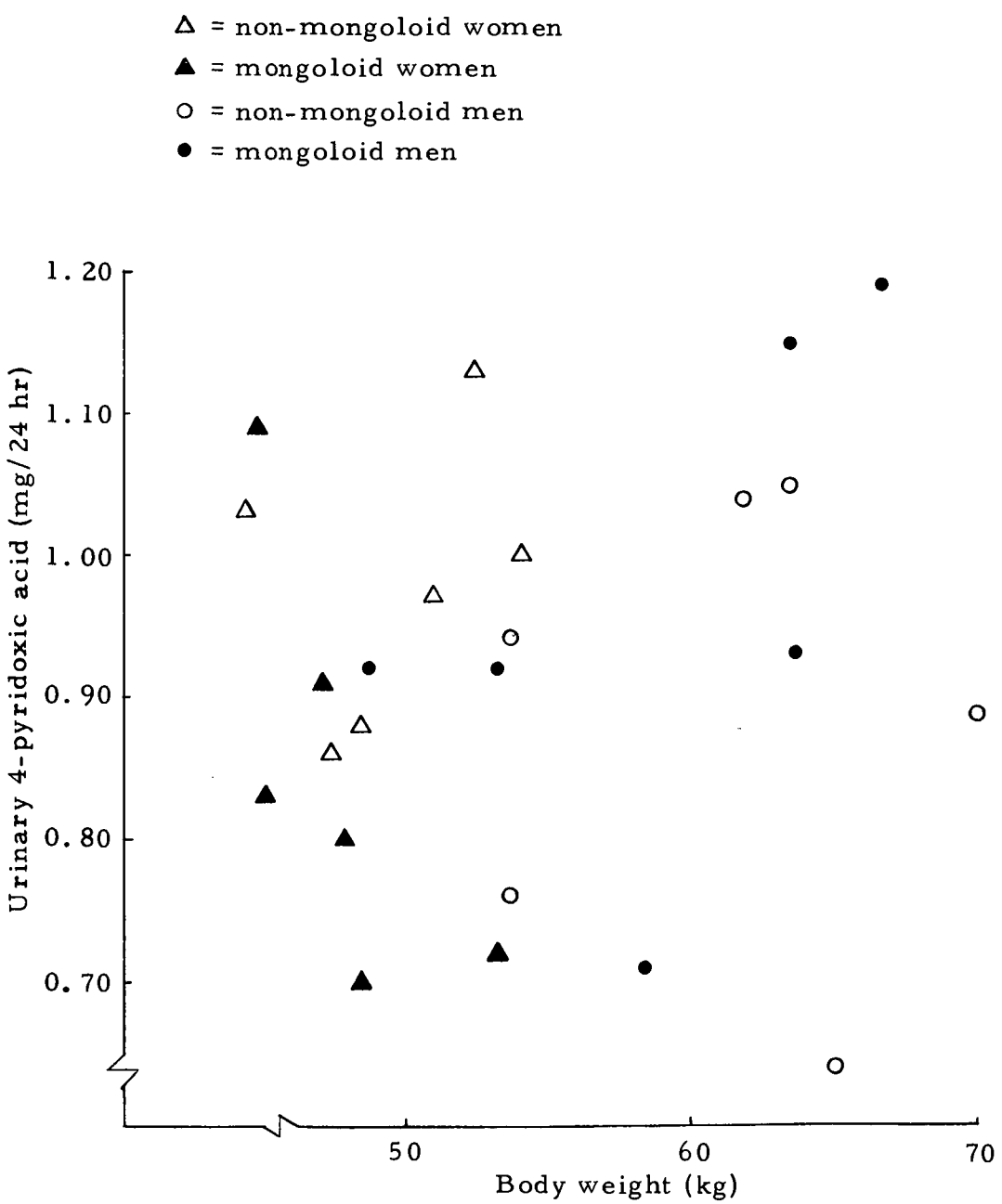


Figure 5. Urinary 4-pyridoxic acid excretion versus the body weight of mongoloid and non-mongoloid mental retardates receiving 1.6 mg of dietary vitamin $B_6$ (day 1).

(Table 6). In addition, individual variation may well be an element that influence the excretion of this metabolite. Kelsay et al. (1968) and Reddy et al. (1958) also reported a variation in the excretion of PIC among their subjects who were receiving a constant diet.

In Part I of this thesis it was suggested that the concentration of PIC in urine is inversely related to the body weight of the subjects (Figure 4). This relationship was not observed among the subjects of either group of mental retardates (Figure 5). Lack of adjustment to the experimental diet may be a reason why there was no relationship between urinary PIC and body weight. In Study I, a mean of PIC concentration in urine was used to show this relationship, whereas the PIC values in the present study at this level of vitamin $B_6$ intake are for only one day. The amounts of protein the subjects received, in grams of body weight, in Parts I and II were similar.

The urinary excretion of PIC by all subjects in response to an oral dose of 50 mg of PIN is presented in Table 9. These values were obtained by subtracting the excretion of PIC on day 1 from that excreted on days 5 and 6, the two days following the administration of the test dose of PIN. On the second day following the loading with PIN (day 6), the urinary PIC levels still had not returned to the levels excreted on day 1. Reddy et al. (1958) reported a similar observation.

In response to the 50-mg dose of PIN the patients with Down's syndrome excreted slightly more PIC on days 5 and 6 than the

Table 9. Increase[a/] in urinary excretion of 4-pyridoxic acid by mental retardates in response to 50 mg of pyridoxine (mg/24 hr).

| Non-Mongoloid | | | | Mongoloid | | | |
|---|---|---|---|---|---|---|---|
| Subjects | Day 5 | Day 6 | Total | Subjects | Day 5 | Day 6 | Total |
| Group 1: Women | | | | | | | |
| BL | 16.48 | 3.08 | 19.55 | JG | 15.17 | 1.62 | 16.79 |
| DM | 20.32 | 2.72 | 23.04 | SH | 17.30 | 3.60 | 20.90 |
| GT | 16.17 | 2.37 | 18.54 | CP | 18.90 | 3.00 | 21.90 |
| Group 2: Women | | | | | | | |
| SH | 19.03 | 2.56 | 21.59 | JC | 20.19 | 3.64 | 23.83 |
| SR | 17.86 | 2.81 | 20.66 | NH | 19.19 | 3.61 | 22.80 |
| FT | 19.35 | 2.37 | 21.72 | JM | 18.21 | 2.84 | 21.05 |
| Group 3: Men | | | | | | | |
| RL | 10.05 | 2.43 | 12.48 | AL | 20.04 | 3.44 | 23.48 |
| JS | 19.02 | 2.87 | 21.88 | JM | 19.61 | 2.86 | 22.57 |
| RT | 17.27 | 2.34 | 19.61 | GV | 18.45 | 3.18 | 21.63 |
| Group 4: Men | | | | | | | |
| DE | 14.47 | 2.17 | 16.63 | CB | 20.23 | 3.48 | 23.71 |
| FH | b/ c/ | 1.97 | - | RF | 14.33 | 2.88 | 17.20 |
| EM | 14.82 | 2.29 | 17.11 | DW | 24.78 | 3.68 | 28.46 |

Average of the sum for day 5 and day 6 (mean ± S. D.)
Non-mongoloids: 19.34 ± 3.05 mg. Mongoloids: 22.03 ± 3.04 mg ($P < 0.05$)

a/ Obtained by subtracting PIC excreted on day 1 from that excreted on days 5 and 6 (Table 8).
b/ Specimen lost by accident c/ Value not included in mean.

non-mongoloid controls. The average increase in the urinary excretion of PIC for the two consecutive 24-hr periods following the ingestion of the test dose was 22.03 mg in the mongoloid subjects and 19.34 mg in the non-mongoloid subjects. This difference is statistically significant ($P < 0.05$). McCoy and England (1968) also reported that, following an intramuscular administration of 100 mg of PIN·HCl, the mongoloids excreted significantly more PIC in urine than the mentally retarded controls.

The sum of the increased excretion of PIC on days 5 and 6 accounted for 35.7 and 31.0% of the supplement administered to mongoloid and non-mongoloid subjects, respectively. These results are similar to those reported by Rabinowitz and Snell (1949) who found that about 30% of a 82-mg supplement of pyridoxine was excreted in urine as PIC. In the study by Reddy et al. (1958) urinary PIC in two subjects accounted for 51 and 48 percent, respectively, of a 10-mg dose of PIN·HCl. The differences in results obtained in the present study and the others just mentioned may have been due to the various levels of vitamin $B_6$ given to the subjects and the different procedures that were employed to determine urinary PIC.

Several studies have suggested the pathways for the degradation and interconversion of vitamin $B_6$ compounds in mammalian tissues. McCoy and Colombini (1972) after having given $^{14}C$-labeled PIN intravenously to rats found that the major route of synthesis of

phosphorylated forms of vitamin $B_6$ in liver and brain is through PINP, which is in turn converted to PALP. In muscle, however, the preferred route is the conversion of PIN to PAL (refer to the scheme on page 5). Contractor and Shane (1970a) proposed that the formation of PIC from PALP via PICP predominates over the formation via PAL. McCoy et al. (1969), who demonstrated that the excretion of PIC following the intramuscular injection of 100 mg of PAL·HCl was lower, but not significantly, in the mongoloid subjects than in the non-mongoloid subjects, suggested that aldehyde oxidase activity in mongoloids and non-mongoloids is similar. In view of these studies the higher urinary excretion of PIC by the mongoloids following PIN loading in the present study suggests that one or more enzymes involved in the degradation of PIN to PIC via PICP are more active in the mongoloid subjects than in the non-mongoloid controls.

As it can be seen from Table 9 and Figure 6, the urinary excretion of PIC by subjects in the two groups in response to PIN loading are fairly variable. The values for the mongoloids ranged from 16.79 to 28.46 mg and those for the non-mongoloids ranged from 12.48 to 23.04 mg. One explanation for this variation is that the ability of the small intestine to absorb vitamin $B_6$ may differ among individuals (Brown, 1972). Also the activities of the enzymes involved in the degradation for pyridoxine to PIC may vary from person to person. A study by Gaynor and Dempsey (1971) showed that the activities of

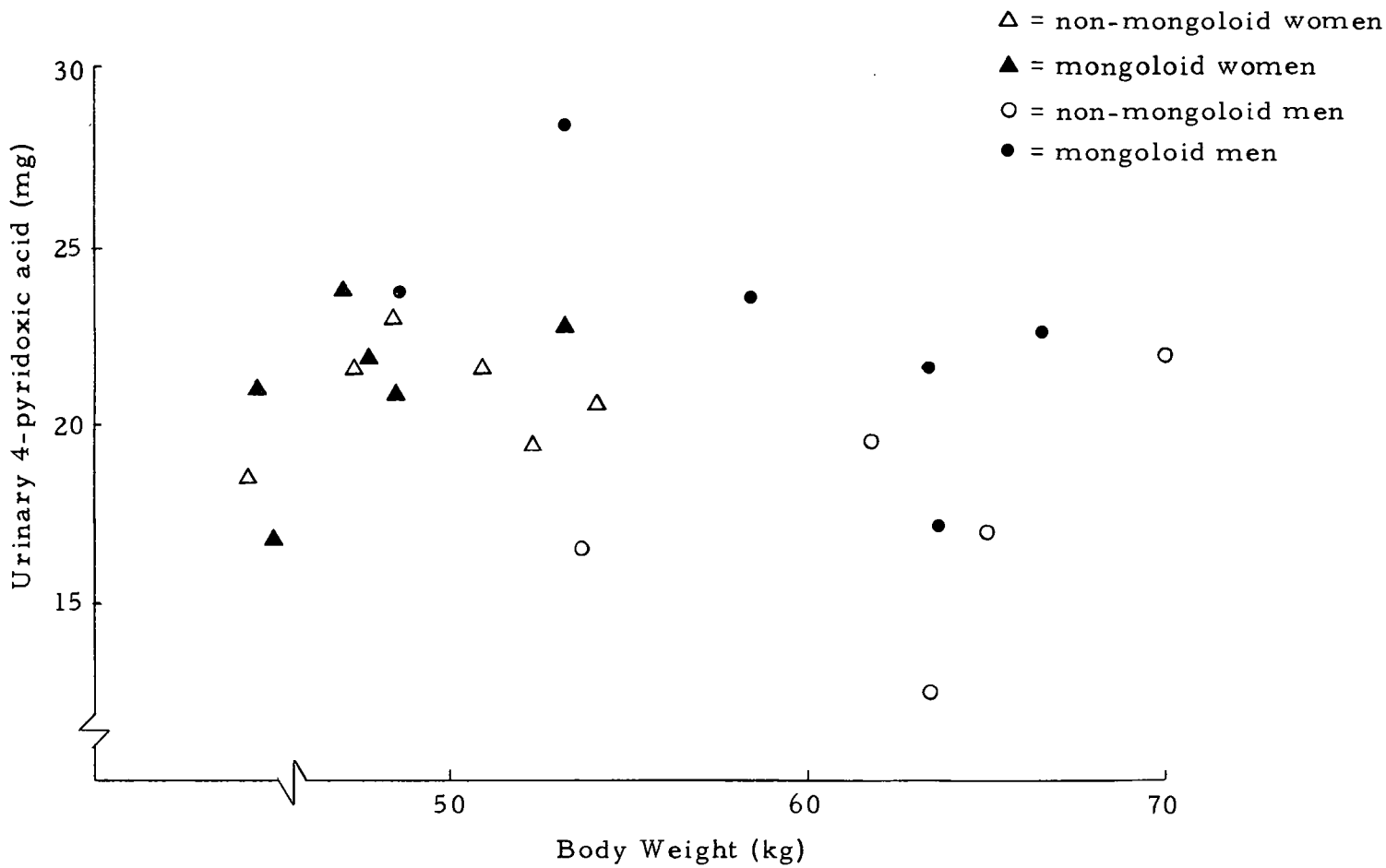


Figure 6. Urinary 4-pyridoxic acid excretion of mongoloid and non-mongoloid mental retardates in response to 50-mg pyridoxine loading versus the body weight of the subjects.

pyridoxal kinase and pyridoxine phosphate oxidase were lower in placentae from pregnant women with preeclampsia, a condition which may result from failure of the toxemic placenta to convert adequate amounts of pyridoxine to phosphate. Moreover, other unidentified metabolites of vitamin $B_6$ besides PIC have been found in urine. By injecting rats intraperitoneally with 4, 5-$^{14}$C-pyridoxine for several months, Bernett and Pearson (1968) detected many (±9) metabolites in the urine of these rats by thin layer chromatography and autoradiography. Conceivably, these unidentified metabolites could be excreted in urine in various quantities along with PIC. Another reason for variation may be due to difference in the body pools of vitamin $B_6$. Boxer, Pruss and Goodhart (1957) demonstrated that ingestion of more than 7 mg of pyridoxine per day did not cause further elevation of pyridoxal phosphate levels in blood. Experiments have shown that the total body pool of pyridoxine is 16 to 25 mg (Baker et al., 1966). Approximate values for the body content of vitamin $B_6$ have been calculated to be 40 to 150 mg (Johansson et al., 1966).

Table 10 shows the urinary excretion of creatinine by the 24 mental retardates with or without Down's syndrome. When these values are expressed in terms of body weight, the creatinine coefficients of these subjects are within the normal range of 15 to 25 mg per kg of body weight per 24 hr (Conn, 1970). It is noteworthy that each subject excreted an approximately equal amount of this substance

Table 10. Urinary excretion of creatinine by mental retardates (g/24 hr).

| Non-Mongoloid | | | | Mongoloid | | | |
|---|---|---|---|---|---|---|---|
| Subjects | Day 1 | Day 5 | Day 6 | Subjects | Day 1 | Day 5 | Day 6 |
| Group 1: Women | | | | | | | |
| BL | 1.27 | 0.96 | 1.00 | JG | 0.92 | 0.83 | 0.86 |
| DM | 0.92 | 0.98 | 0.82 | SH | 1.02 | 0.96 | 0.97 |
| GT | 1.51 | 0.85 | 0.91 | CP | 0.93 | 0.81 | 0.79 |
| Group 2: Women | | | | | | | |
| SH | 0.77 | 0.91 | 0.94 | JC | 0.96 | 1.03 | 1.00 |
| SR | 1.09 | 1.08 | 1.14 | NH | 1.13 | 1.17 | 1.20 |
| FT | 1.00 | 1.05 | 1.06 | JM | 1.02 | 1.14 | 1.09 |
| Group 3: Men | | | | | | | |
| RL | 1.57 | 1.55 | 1.59 | AL | 1.40 | 1.38 | 1.41 |
| JS | 1.77 | 1.68 | 1.74 | JM | 1.43 | 1.76 | 1.82 |
| RT | 1.42 | 1.49 | 1.49 | GV | 1.57 | 1.49 | 1.55 |
| Group 4: Men | | | | | | | |
| DE | 1.37 | 1.34 | 1.42 | CB | 1.11 | 1.18 | 1.15 |
| FH | 1.43 | - [a] | 1.41 | RF | 1.63 | 1.43 | 1.49 |
| EM | 1.48 | 1.56 | 1.52 | DW | 1.49 | 1.49 | 1.30 |

[a] Specimen lost by accident.

during the 3 days urine was collected. This indicates that complete collection of 24-hr specimens had been achieved through catheterization. Constancy in the urinary excretion of creatinine may also be attributed to restricted activity of the subjects during the study.

# SUMMARY

The urinary excretion of 4-pyridoxic acid (PIC), the major metabolite of vitamin $B_6$ in urine, was measured in two populations in whom altered $B_6$ metabolism has been reported: women who use steroid contraceptives and mental retardates with and without Down's syndrome.

In one study, 6 young women were placed on a constant diet containing 1.9 mg of vitamin $B_6$ for 11 days. Four of them had been using an oral contraceptive for 2 to 12 months while two others had not used any. Five 24-hr urine specimens were obtained from each subject for the determination of 4-pyridoxic acid and creatinine. The mean daily urinary excretion of PIC in the women who used oral contraceptives (1.07 mg) was not statistically different from that excreted by the control subjects (0.95 mg). The results in this study suggest that steroid contraceptives do not have any apparent effect on the extent to which vitamin $B_6$ is metabolized to PIC.

In the second study, the urinary excretion of PIC was measured in 12 mongoloid subjects and 12 non-mongoloid mental retardates before and after an oral administration of a 50-mg dose of pyridoxine. The basal (pre-loading) urinary excretion of PIC in patients with Down's syndrome (mean 0.91 mg/24 hr) was similar to that in the mentally retarded controls (mean 0.93 mg/24 hr), suggesting that with a normal

intake of vitamin $B_6$ degradation of the vitamin in Down's syndrome patients is no different from that in the non-mongoloid subjects. However, during the two days following the loading with 50 mg of pyridoxine the mongoloids excreted significantly more PIC than the non-mongoloids ($P < 0.05$) in spite of considerable variation among the subjects of each group. These data suggest that the enzymes of vitamin $B_6$ catabolism may have greater activity in the mongoloid subjects than in the non-mongoloid mental retardates.

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# APPENDIX

# APPENDIX

## LIST OF ABBREVIATIONS

| | |
|---|---|
| EGOT | Erythrocyte glutamic-oxaloacetic transaminase |
| EGPT | Erythrocyte glutamic-pyruvic transaminase |
| FSH | Follicle-stimulating hormone |
| 3HA | 3-Hydroxyanthranilic acid |
| LH | Luteinizing hormone |
| NADPH | Reduced nicotinamide adenine dinucleotide phosphate |
| PAL | Pyridoxal |
| PALP | Pyridoxal 5-phosphate |
| PAM | Pyridoxamine |
| PAMP | Pyridoxamine 5-phosphate |
| PIC | 4-Pyridoxic acid |
| PICP | 4-Pyridoxic acid 5-phosphate |
| PIN | Pyridoxine |
| PIN·HCl | Pyridoxine hydrochloride |
| PINP | Pyridoxine 5-phosphate |