AN ABSTRACT OF THE THESIS OF

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in FOODS AND NUTRITION (Major Department) presented on MAY 27, 1975 (Date)

Title: METABOLISM OF METHIONINE IN WOMEN USING ORAL CONTRACEPTIVES

Abstract approved: Lorraine T. Miller

The effect of oral contraceptives on the urinary excretion of several methionine metabolites was determined in women before and after they had received a 3-g dose of L-methionine. Nine women between the ages of 20-29 years served as subjects: five had been using a combination-type oral contraceptive for six months or more (experimental group), and four had not been using these drugs (control group). Cystathionine excretion by both groups before and after the methionine loading was in the range reported for normal female subjects who were not deficient in vitamin B_6 (Krishnaswamy, 1972; Shin and Linkswiler, 1974). Changes in urinary methionine metabolites that were apparently produced by oral contraceptive druge are: (1) homocysteine was detected in the basal urine of three of the oral contraceptive users. After methionine loading, it was found in the urine of four of these subjects, two of whom excreted

measurable quantities. In contrast, three of the control subjects excreted traces of homocysteine only after methionine loading. (2) The mean excretion of taurine by oral contraceptive users was only one-tenth of that excreted by the control subjects.

The activity of erythrocyte glutamic oxaloacetic transaminase (EGOT) before and after in vitro stimulation with added pyridoxal phosphate was similar in both groups. Basal activity of erythrocyte glutamic pyruvic transaminase (EGPT) was lower in oral contraceptive users, although the mean values for both groups were within the normal range reported by Miller et al. (1975) and Woodring and Storvick (1970). The percent in vitro stimulation after addition of pyridoxal phosphate was somewhat higher in oral contraceptive users, but the difference was not statistically significant.

Thirteen free acidic and neutral amino acids (including metabolites of the methionine pathway) were measured in the urine specimens. The sum of the urinary excretion of these 13 amino acids was significantly lower ($p < 0.01$) for oral contraceptive users than for control subjects. However, total α-amino nitrogen excretion, measured in the same urine specimens, was similar for both groups.

Metabolism of Methionine in Women Using Oral Contraceptives

by

Marjorie J. Dow

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Completed May 1975

Commencement June 1976

APPROVED:

Associate Professor of Foods and Nutrition
in charge of major

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Dean of Graduate School

Date thesis is presented May 27, 1975

Typed by Mary Jo Stratton for Marjorie J. Dow

ACKNOWLEDGEMENTS

I wish to express my appreciation to Dr. Lorraine T. Miller for her willing help in designing and implementing this study as well as in writing this thesis.

The encouragement of Dr. Margy Woodburn, Head of the Food and Nutrition Department, and Dr. Elizabeth Yearick, in my graduate work is greatly appreciated.

I am also grateful to Mrs. Margaret Edwards for assistance in the laboratory and to Shelly Kokkeler for her assistance in the calculation of the dietary data of the subjects.

I wish to thank the subjects for their cheerful participation in the study in spite of personal inconvenience. I am indebted to the staff of the Family Planning Clinic associated with the Corvallis Public Health Department for their interest and assistance in selecting subjects for this study.

I am especially grateful for the encouragement and support given by my husband throughout this study.

Financial support was given by the Ruth Kennedy Tartar award and in part by the Oregon Agricultural Experiment Station Project No. 153.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDIX TABLES

METABOLISM OF METHIONINE IN WOMEN USING ORAL CONTRACEPTIVES

INTRODUCTION

Since the advent of oral contraceptives, the number of women taking these drugs has increased until it has been estimated that more than ten million women are now using them (Hodges, 1971). Concern has been expressed that, over long periods of usage, seemingly insignificant alterations in metabolism caused by these drugs may be harmful. For this reason research has been conducted to determine if there are any harmful effects from the long-term usage of oral contraceptives.

A reduction of total plasma amino acids in oral contraceptive users (Craft, Wise and Briggs, 1970; Craft and Peters, 1971) has been demonstrated to be caused by both the progesterone and estrogen components of the drug (Zinneman, Seal and Doe, 1967; Seal and Doe, 1969). Aly, Donald and Simpson (1971) reported a significant reduction in nonessential amino acids, but found that some essential amino acids were lower also in the plasma of these women. The effect of estrogen and progesterone on urinary amino acids is not as well documented. Seal and Doe (1969) state that estrogen and estrogen-progesterone administration produces a mild aminoaciduria.

Armstrong (1973) reports taurine excretion to be significantly lower in oral contraceptive users.

Vitamin B_6 metabolism seems to be affected by oral contraceptive use (Theuer, 1972). Pyridoxal phosphate, the metabolically active form of vitamin B_6, is necessary as a cofactor for 60 enzymes involved in amino acid metabolism (Lehninger, 1972).

In response to an oral dose of tryptophan, oral contraceptive users excrete unusually high amounts of xanthurenic acid and other metabolites of the tryptophan-niacin pathway (Brown et al., 1969; Lubby et al., 1971; Rose et al., 1972). This abnormality in tryptophan metabolism is similar to that in persons deficient in vitamin B_6 (Miller and Linkswiler, 1967) and is observed in 75 to 80 percent of the women using these drugs (Lubby et al., 1971). These results indicate that a subclinical vitamin B_6 deficiency may exist in women using oral contraceptives.

Other tests that are used to detect subclinical vitamin B_6 deficiency often give inconsistent results and are not conclusive in defining a subclinical deficiency in these women (Theuer, 1972).

The use of the methionine load test as a different means of assessing the vitamin B_6 status of oral contraceptive users is the subject of this study. This has been suggested as a reliable test of subclinical vitamin B_6 deficiency. Cystathionine, an intermediate metabolite in the methionine-cysteine pathway, is excreted in

abnormal amounts by vitamin B_6-deficient persons (Park and Linkswiler, 1970; Krishnaswamy, 1972, 1974; Shin and Linkswiler, 1974). There has been no word in the literature up to this point of using this test to detect subclinical vitamin B_6 deficiency in women using oral contraceptives. It was used in this study because it gives a means of assessing another pathway which utilizes vitamin B_6-dependent enzymes, thus indicating whether the apparently increased need is affecting other pathways besides the tryptophan-niacin pathway. Erythrocyte transaminases were also measured in the subjects in order to provide another test of vitamin B_6 status to which the results from the methionine load test could be compared.

REVIEW OF LITERATURE

Altered Vitamin B_6 Metabolism in Women Using Oral Contraceptives

Tryptophan Load Test

The tryptophan load test is responsive to experimentally-produced vitamin B_6 deficiency and repletion and is considered a good diagnostic tool (Sauberlich et al., 1972). It tests the efficiency of the conversion of tryptophan to niacin (Figure 1). Kynureninase, which has as its cofactor pyridoxal phosphate, is more sensitive to vitamin B_6 deficiency than the pyridoxal phosphate-dependent transaminases in this pathway (Mason, Ford and Wu, 1969). When an oral dose of tryptophan is given to an individual deficient in vitamin B_6, abnormal amounts of intermediates in the pathway are formed and are excreted in the urine in increasing amounts as the deficiency progresses. These metabolites are: xanthurenic acid, kynurenic acid, kynurenine, acetyl kynurenine, 3-hydroxykynurenine and 3-hydroxyanthranilic acid (Price, Rose and Toseland, 1972; Sauberlich et al., 1972).

Pregnant women respond abnormally to a tryptophan load test (Sprince et al., 1951; Wachstein and Gudaitis, 1953; Wachstein and Lobel, 1954; Brown, Thorton and Price, 1961; Wachstein, 1964). Although the significance of abnormal tryptophan metabolism in

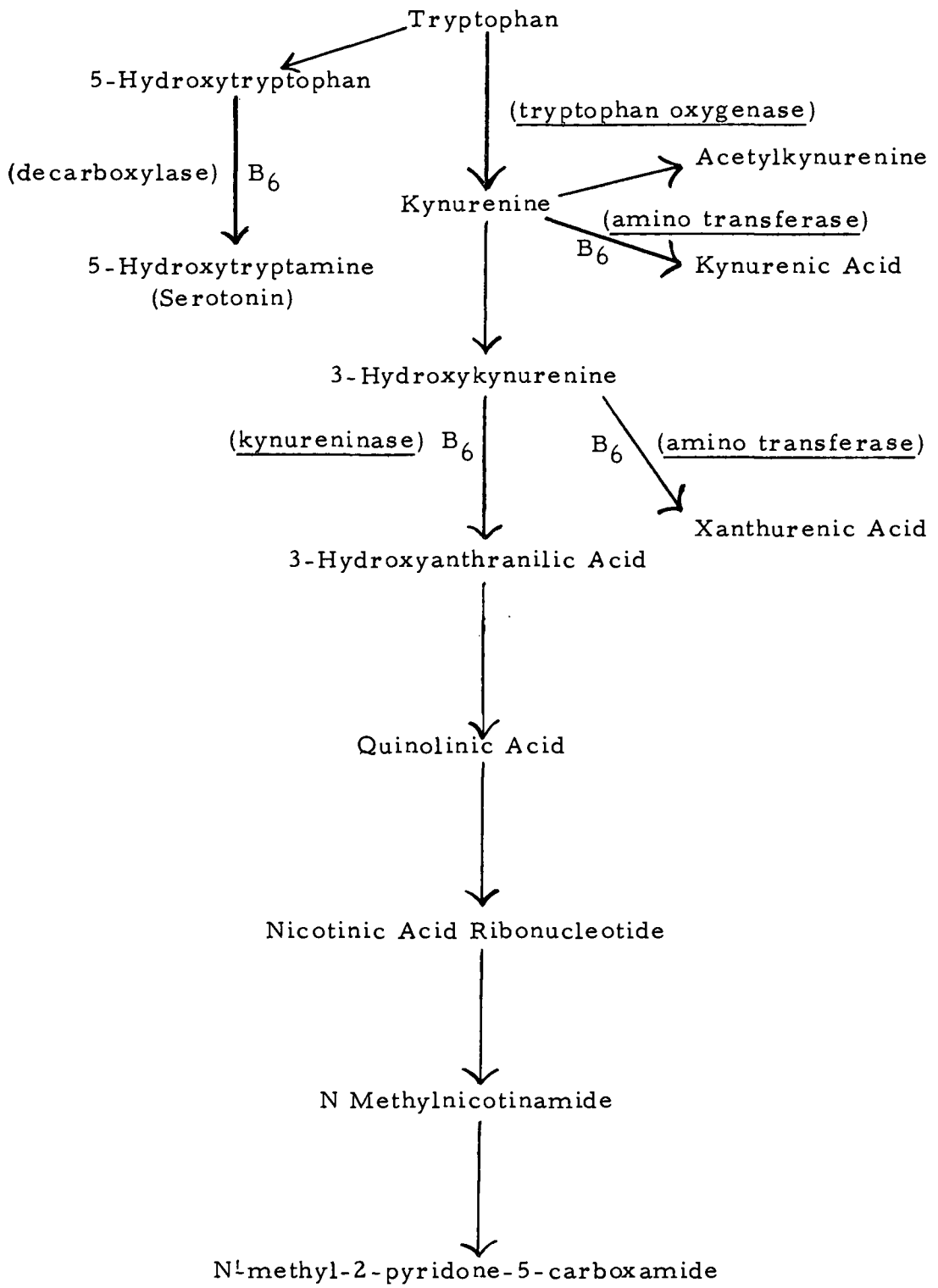

Figure 1. Tryptophan-niacin pathway (adapted from Rose et al., 1972).

pregnancy is unknown, this abnormality is corrected by the administration of 10 mg pyridoxine hydrochloride daily (Wachstein, 1964).

Studies conducted on women using oral contraceptives have produced results similar to those in pregnant women. After an oral dose of tryptophan, women taking these drugs excrete abnormal amounts of xanthurenic acid, kynurenine, kynurenic acid and acetyl kynurenine (Price, Thorton and Mueller, 1967; Brown et al., 1969; Rose, 1969; Aly et al., 1971; Lubby et al., 1971; Miller et al., 1974). Price et al. (1972) found 3-hydroxyanthranilic acid elevated at the basal level as well as after a tryptophan load, but Rose and Adams (1972) and Miller et al. (1974) found no significant difference between control women and oral contraceptive users in the excretion of this metabolite.

Pyridone (N'methyl-2-pyridone-5-carboxamide) and niacin, the end products of the pathway, are elevated in the urine of pregnant women and oral contraceptive users. This abnormality is not corrected by pyridoxine hydrochloride (Brown et al., 1969; Rose, 1969). The total yield of niacin and pyridone is 60 percent greater in the hormone-treated subjects than in the untreated control subjects (Brown et al., 1969). Wolf et al. (1970) established that this effect is produced by the estrogen component of the drug. These results suggest that the amount of tryptophan metabolized by this pathway is increased by use of oral contraceptives (Brown et al., 1969).

Erythrocyte Transaminase Activity

In vitamin B_6 deficiency, the activities of erythrocyte glutamic pyruvic transaminase (EGPT) and glutamic oxaloacetic transaminase (EGOT) decline rapidly. Activities of these enzymes increase when the subject is repleted with the vitamin. The percent saturation of these enzymes with their cofactor is measured by the in vitro addition of pyridoxal phosphate to the assay medium. As the enzyme becomes less saturated with the cofactor during vitamin B_6 deficiency, the percent stimulation by pyridoxal phosphate added in vitro is increased. This is a good diagnostic tool for detecting subclinical vitamin B_6 deficiency (Baysal, Johnson and Linkswiler, 1966; Linkswiler, 1967; Sauberlich et al., 1972).

The results obtained by using EGPT and EGOT as a measure of subclinical vitamin B_6 deficiency in women taking oral contraceptives are conflicting. In some studies there was no significant difference between oral contraceptive-treated women and untreated ones in the basal activity of EGPT or stimulation with pyridoxal phosphate added in vitro (Aly et al., 1971; Rose et al., 1973; Brown et al., 1975; Miller et al., 1975). On the other hand, Doberenz et al. (1971) found that the basal activity of EGPT was significantly lower and the in vitro stimulation was higher in women using oral contraceptives than in the control women.

Some workers reported higher EGOT activity in oral contraceptive users than in untreated women. Stimulation by pyridoxal phosphate added *in vitro* was the same for both groups, suggesting that the total amount of EGOT apoenzyme is increased by these drugs (Aly *et al.*, 1971; Rose *et al.*, 1973; Miller *et al.*, 1975).

Brown *et al.* (1975) report no difference between oral contraceptive users and control subjects during depletion and repletion of vitamin B_6 in EGPT or EGOT levels with or without *in vitro* stimulation with pyridoxal phosphate.

Plasma Pyridoxal Phosphate and Blood Vitamin B_6

Pyridoxal phosphate in plasma is another means of determining vitamin B_6 status (Kelsay *et al.*, 1968). Lumeng, Cleary and Li (1974) found significantly lower levels of plasma pyridoxal phosphate in oral contraceptive users during their first few months of use than before they started the drug. However, many of their subjects' plasma pyridoxal phosphate returned to normal levels after six months of oral contraceptive use. Brown *et al.* (1975) and Miller *et al.* (1975) found no difference between oral contraceptive users and control women in this measurement.

Miller *et al.* (1975) measured total vitamin B_6 in whole blood

and found no significant difference between oral contraceptive users and control subjects.

Suggested Mechanisms for Altered Vitamin B_6 Metabolism

Two effects of oral contraceptives on pyridoxal phosphate-dependent enzymes have been demonstrated experimentally.

Increased Glucocorticoid Levels. Elevated levels of protein-bound and unbound cortisol in the blood of estrogen-treated subjects have been reported (Sandberg and Slaunwhite, 1959; Plager, Schmidt and Staubitz, 1964; Pulkkinen and Pekkarinen, 1967). The effect is caused entirely by the estrogen component of the oral contraceptive agents (Layne et al., 1962; Zinneman et al., 1967). Plager et al. (1964) report that, although blood levels of bound and unbound cortisol were raised in male subjects treated with estrogen, the cortisol production was not changed. Decreased secretion of cortisol in the renal tubules may explain the increased blood levels of this substance (Layne et al., 1962).

Studies with rats[1] have shown that estrogen causes a redistribution of pyridoxal phosphate in hepatic enzymes that require this

[1] The problem of using rats for evaluation of the effects of oral contraceptive hormones in humans is demonstrated by Beare-Rogers et al. (1969). Envoid, a popular contraceptive preparation, was given to rats during an imposed vitamin B_6 deficiency. The level of xanthurenic acid excreted in the urine was depressed by the drug rather than elevated as in humans, suggesting that the effect is different in rats.

coenzyme. Although the total pyridoxal phosphate concentration remains the same, there is a decrease in the activity of kynureninase (Mason and Manning, 1971), homoserine dehydratase and cysteine sulfinic acid decarboxylase (Lefauconnier et al., 1973). Estrogen causes an increase in the activities of: alanine aminotransferase (Beare-Rogers, Rogers and Sarby, 1969; Brin, 1971; Lefauconnier et al., 1973), tyrosine aminotransferase (Braidman and Rose, 1971; Brin, 1971; Lefauconnier et al., 1973), serine dehydratase (Lefauconnier et al., 1973) and tryptophan oxygenase (Braidman and Rose, 1971; Brin, 1971). Lefauconnier et al. (1973) found no change in cystathionine synthase activity after estrogen administration. However, the redistribution of pyridoxal phosphate among hepatic enzymes caused by estrogen is not the same as that occurring during vitamin B_6 deficiency (Mason and Gullerson, 1960; Mason et al., 1969; Mason and Manning, 1971).

Direct Effect of Estrogen. Braidman and Rose (1971) and Brin (1971) used adrenalectomized rats to determine the effect of estrogen on the activity of certain enzymes. Activities of tryptophan oxygenase and tyrosine aminotransferase were increased by estrogen administration, but not as much as in rats that had intact adrenal glands, suggesting that both the adrenal glands and estrogen alone have an effect on these enzymes. Alanine aminotransferase was not increased in adrenalectomized rats after estrogen administration,

suggesting that its increase in activity in normal rats is mediated solely through the adrenal gland.

Supplementation with Pyridoxine Hydrochloride

Supplementing the diet with vitamin B_6 apparently corrects the metabolic abnormalities that are observed in oral contraceptive users. Rose et al. (1972) found that treatment with 20 mg of pyridoxine hydrochloride per day for one month corrected abnormal tryptophan metabolism in oral contraceptive users. Lubby et al. (1971) concluded that a daily supplement of 30 mg of pyridoxine hydrochloride would normalize this test in all oral contraceptive users and allow for a margin of safety. Brown et al. (1975) and Leklem et al. (1975) studied oral contraceptive users and controls during a period of depletion and repletion of vitamin B_6. They report that, after a period of depletion, 2.0 mg of pyridoxine hydrochloride per day for one week was sufficient to normalize EGOT, EGPT, urinary 4-pyridoxic acid, and plasma pyridoxal phosphate in oral contraceptive users as well as in control women. The urinary excretion of tryptophan-pathway metabolites after tryptophan loading returned to normal except for xanthurenic acid, which remained elevated in oral contraceptive users. Twenty mg of pyridoxine hydrochloride per day corrected this abnormality within one week.

Oral Contraceptives and Depression

One of the clinical complaints found in women using oral contraceptives is that of depression. Only a small percentage of users have this problem, however. Adams et al. (1973) report that low levels of 5-hydroxytryptamine (serotonin) and its metabolites are found in brain and cerebral-spinal fluid in depressed and suicidal persons. A disturbance of tryptophan metabolism may cause lowering of brain serotonin levels and thus give a biochemical reason for the depression. Possible causes of this disturbance are: a decrease in tryptophan available for serotonin formation due to increased niacin production, or a shortage of pyridoxal phosphate available as cofactor for 5-hydroxytryptophan decarboxylase, the enzyme necessary for serotonin formation (Figure 1). Adams et al. (1973) found that a significant number of the women complaining of depression were suffering from a subclinical vitamin B_6 deficiency. The administration of large doses of pyridoxine hydrochloride (20 mg twice a day) reduced the depression symptoms in those persons having a subclinical vitamin B_6 deficiency, but not in other depressed women who were not deficient (Winston, 1969, 1973; Adams et al., 1973).

Methionine Metabolism

Methionine Load Test as a Diagnostic Tool

A problem in using the tryptophan load test as a means to measure vitamin B_6 status is that tryptophan oxygenase is subject to hormonal influence (Figure 1) (Mason et al., 1969). Therefore, interest has been shown in the pyridoxal phosphate-dependent enzymes responsible for transsulfuration as a basis for testing vitamin B_6 status in humans. A methionine load test, using the same principle as the tryptophan load test, is effective in detecting subclinical vitamin B_6 deficiency. The urine is collected for a period immediately before and after ingestion of a test dose of methionine and several metabolites of methionine are determined in the urine (Park and Linkswiler, 1970; Shin and Linkswiler, 1974).

Metabolic Pathways

Methionine is an essential amino acid having three major metabolic functions in the body (Finkelstein, 1974): (1) utilization for protein synthesis; (2) conversion to S-adenosyl-L-methionine, which is the primary methyl group donor; and (3) conversion to cysteine and its derivatives through the intermediate, cystathionine, by the "transsulfuration" pathway (Frimpter, 1972; Finkelstein, 1974).

Approximately 80 to 90 percent of the methionine in the diet is metabolized by this pathway (Rose, 1955). Most of the evidence for this pathway has come from studies using rats (Frimpter, 1972). Methionine is essential for growth and as long as there is enough of it in the diet, it can totally substitute for cysteine.

The transsulfuration pathway is shown in Figure 2. Methionine is first converted to S-adenosyl-L-methionine. After removal of the methyl group, the resulting product, S-adenosyl-L-homocysteine, is converted to L-homocysteine. Cystathionine synthase catalyzes the formation of cystathionine from homocysteine and serine, which is essentially an irreversible reaction in man. Homocysteine that has been converted to cystathionine can no longer serve as a precursor for methionine[2] (Finkelstein, 1974; Finkelstein, Kyle and Harris, 1974). In normal persons, cystathionine is rapidly converted to cysteine and homoserine by the action of cystathionase (cystathionine-cleaving enzyme). Cystathionine, an intermediate, serves in the function of transferring sulfur from methionine to cysteine (Frimpter, 1972). The other product of the cleavage reaction,

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[2] As shown in the diagram, homocysteine can be remethylated to form methionine by two different pathways: (a) betaine homocysteine methyltransferase catalyzes the transfer of a methyl group from betaine, a choline derivative, to homocysteine, forming methionine; (b) N^5-methyltetrahydrofolate donates a methyl group, forming methionine from homocysteine. Vitamin B_{12} is used as a cofactor.

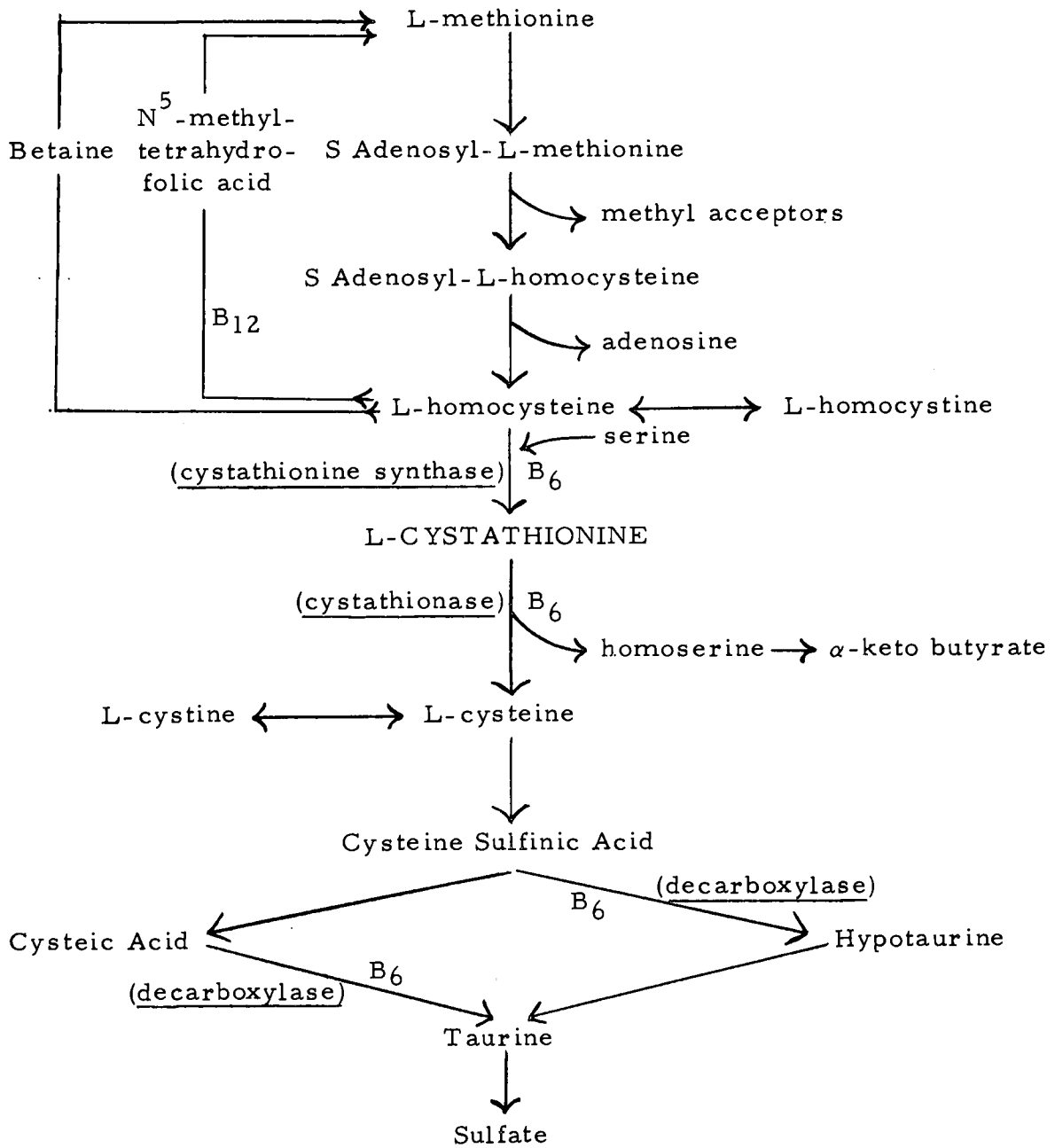

Figure 2. Transsulfuration pathway (adapted from Finkelstein (1974) and Krishnaswamy (1972)).

homoserine, is converted to α-ketobutyrate (Carroll, Stacy and DuVineud, 1949), which is then converted to succinyl Co-A and enters the TCA cycle as succinate (Lehninger, 1972).

Transsulfuration Enzymes Requiring Pyridoxal Phosphate

Both cystathionine synthase and cystathionase require pyridoxal phosphate as their cofactor. Binkley, Christensen and Jensen (1952) found that rats deficient in vitamin B_6 had greatly reduced levels of both of these enzymes in their livers. The percent stimulation when pyridoxal phosphate was added to the assay mixture was markedly increased showing that the enzymes had a greater degree of unsaturation during deficiency. Sturman, Cohen and Gaull (1969) studied vitamin B_6-deficient rats and found that the activity of cystathionase was greatly reduced in the liver, pancreas and kidney tissue, and the percent stimulation after in vitro addition of pyridoxal phosphate was increased. Cystathionine synthase, however, was not affected by deficiency as much as cystathionase in these tissues.

Finkelstein and Chalmer (1970) found that cystathionine synthase was completely saturated with coenzyme in liver tissue of normal rats receiving an adequate vitamin B_6 intake, but cystathionase was not saturated in the same extracts. These studies suggest that pyridoxal phosphate is bound more strongly to cystathionine synthase than to

cystathionase, which gives up its cofactor more readily during vitamin B_6 deficiency.

In humans, Mudd et al. (1965) found a much greater activity of cystathionase in liver than in brain.

Cystathionine in Tissues During Vitamin B_6 Deficiency--Rat Studies

Blaschko, Datta and Harris (1953) noted that rats having a diet deficient in vitamin B_6 excreted a ninhydrin-positive substance in their urine that was not found in urine of control rats. Hope (1957) identified this compound as cystathionine. Sturman and Cohen (1971) reported that rats deficient in vitamin B_6 excreted 5.63 μmoles of cystathionine per 24 hours in urine as compared with none by the control animals. The plasma of the deficient rats contained 0.93 μmoles of cystathionine per 100 ml whereas none was detected in the plasma of the control animals. Swenseid, Villalobas and Freidrich (1964), in a similar study, reported plasma cystathionine values of 1.3 μmoles per 100 ml in vitamin B_6-deficient rats compared with 0.2 μmoles in control animals. The concentration of cystathionine in rat muscle, liver, pancreas, kidney and brain increases during deficiency (Sturman et al., 1969; Sturman and Cohen, 1971). Hope (1964) found a ten-fold increase in brain tissue of deficient rats as compared with control rats. Tews (1969) reported that the levels of

cystathionine + methionine in mouse brain tissue increased as deficiency progressed. The amounts reportedly found in plasma and urine are relatively modest compared with changes in other tissues, thus showing the problem in using plasma and urine values as an indication of what is happening in other tissues (Sturman et al., 1969).

Radioactive Tracer Studies in Rats

Studies using radioactive methionine or one of its metabolites have been done to trace the fate of these substances during vitamin B_6 deficiency. Brown and Gordon (1971) injected ^{14}C-cystathionine into vitamin B_6-deficient rats as well as into control animals. The deficient rats excreted large amounts of the ^{14}C-cystathionine in their urine while the control rats excreted small amounts of radioactivity, of which very little was in the form of cystathionine. ^{14}C-cystathionine accumulated in the deficient animals in the kidney but not in the liver or brain, suggesting that the kidneys play a greater role in cystathionine metabolism than had been considered previously.

Sturman, Cohen and Gaull (1970) injected L-^{35}S-methionine into animals deficient in vitamin B_6 and control animals. They found that the radioactive methionine was taken up rapidly by the organs of both control and deficient animals. The pancreas was found to contain by far the most radioactivity of the organs measured in both groups. The total radioactivity in the form of cystathionine found in liver,

kidney and pancreas was higher in the deficient animals than the controls. Radioactive cystathionine was found in the urine of both groups but only in the kidney of the deficient animals. The absence of cystathionine in the kidneys of the control rats was attributed to the rapid metabolism or urinary excretion of this compound. No ^{35}S-cystathionine was found in the plasma of either group, in spite of the fact that there was some unlabeled cystathionine in the plasma of the deficient animals which suggests that the appearance of cystathionine in the plasma is a gradual process.

Cystathionine in Human Tissue

Cystathionine is normally found in low concentrations in liver, kidney and muscle of man, and several other species. A higher concentration has been found in human brain compared with other species, but the significance of this is not known at present (Tallen, Moore and Stein, 1958). Cystathionine is found in trace amounts, if at all, in plasma and urine of normal subjects, but in circumstances where cystathionase activity is limited by metabolic disorder or vitamin B_6 deficiency, it is found in greater amounts in these biological fluids (Park and Linkswiler, 1970; Frimpter, 1972). Cystathionine is rapidly cleared from the plasma by the kidney due to a low tubular maximum. If the concentration of plasma cystathionine exceeds 1 μmole per 100 ml, reabsorption is less than 20 percent of the filtered

load. Therefore, the measurement of urinary cystathionine is considered a good diagnostic tool to detect an abnormal metabolism of methionine (Frimpter and Greenberg, 1967).

Methionine Metabolism during Vitamin B_6 Deficiency

Methionine load tests conducted on human subjects who were fed a vitamin B_6-deficient diet showed that urinary excretion of cystathionine is greatly increased in deficient subjects at the basal level (no methionine) and after a loading dose of 3 g of L-methionine (Park and Linkswiler, 1970; Shin and Linkswiler, 1974). When receiving adequate vitamin B_6 intake, five women studied excreted 122 μmoles cystathionine at the basal level and 226 μmoles after a methionine loading dose. After two weeks of vitamin B_6 deficiency, they excreted 517 μmoles before and 1877 μmoles of cystathionine after a methionine loading dose (Shin and Linkswiler, 1974). Comparable results were obtained with six men after two weeks of deficiency. After three weeks, their values for cystathionine excretion were 1508 μmoles at the basal level and 3710 μmoles after methionine loading (Park and Linkswiler, 1970). Homocysteine was not detected in the urine until the deficiency had proceeded for at least two weeks, and then only small amounts were found after a methionine load test. Methionine excretion was apparently not affected by vitamin B_6 deficiency.

Repleting the subjects with 2 mg of pyridoxine hydrochloride for one week was sufficient to correct the abnormal methionine metabolism (Park and Linkswiler, 1970).

The methionine load test was used to diagnose vitamin B_6 deficiency in six pregnant women exhibiting symptoms of angular stomatis, glossitis and cheilosis, and in five men having symptoms of peripheral neuropathy. These symptoms were judged by the investigator to be caused by vitamin B_6 deficiency because of previous experience in treating similar disorders. The urinary excretion of cystathionine after a methionine loading dose in these subjects was approximately three times greater than in nondeficient controls. In the basal urine, the pregnant women had a greater amount of cystathionine than the control subjects, but there was no difference between the male experimental subjects and their controls. Other methionine metabolites were not affected appreciably by vitamin B_6 deficiency. Treatment with 20 to 50 mg pyridoxine hydrochloride for 15 to 30 days corrected the abnormal cystathionine excretion after a methionine load and relieved the clinical symptoms exhibited by the subjects at the beginning of the experiment (Krishmaswamy, 1972, 1974).

Taurine Excretion as a Measure of Methionine Metabolism

Taurine, a sulfur-containing amino acid, is a major end-product of the transsulfuration pathway and is found in the urine in relatively

large quantities. There is a wide range in the amount excreted between individuals as well as by the same individual on different days. Jacobson and Smith (1968) in reviewing taurine studies, report values ranging from 140 to 2650 μmoles taurine excreted per 24 hours in normal subjects.

There are several pathways by which taurine may be formed (Figure 2). The decarboxylase enzymes catalyzing the formation of hypotaurine from cystine sulfinic acid and taurine from cysteic acid require pyridoxal phosphate as their cofactor and are decreased in activity in vitamin B_6-deficient rats (Hope, 1955; Greengard and Gordon, 1963).

In rats, taurine excretion is markedly decreased during vitamin B_6 deficiency (Hope, 1957; McAfee and Williams, 1962; Sturman et al., 1969; Sturman and Cohen, 1971). Measurement of taurine in other tissues of the body during deficiency, however, shows that taurine is not necessarily decreased in the organs when it decreases in the urine. Metabolic studies have given evidence suggesting that there are storage pools of taurine in each organ which turn over slowly and are not subject to sudden dietary changes in vitamin B_6 (Hope, 1957; Boquet and Fromageat, 1965; Merrow et al., 1966; Sturman et al., 1969; Sturman and Cohen, 1971).

Sturman and Cohen (1971) injected ^{35}S-cysteine into deficient rats as well as controls and noted that the deficient rats excreted

more radioactivity in the form of sulfate and less as taurine than the control animals. This suggests that a pathway not involving taurine or vitamin B_6 may be used during deficiency. Taurine is known to have several biological functions, the most important of which is the conjugation with bile acids to form bile salts (Jacobson and Smith, 1968). It has been suggested that there may be a mechanism for conserving taurine during a vitamin B_6 deficiency, such as increased renal reabsorption (Sturman et al., 1969).

In human studies, interest has been shown in using taurine excretion as a test for subclinical vitamin B_6 deficiency (Swan, Wentworth and Linkswiler, 1964). Merrow et al. (1966), after giving 6 g of D, L-methionine to human subjects having a vitamin B_6-deficient diet, found decreased taurine levels in the plasma, but due to wide variation in individual excretion, no consistent changes in urinary excretion of taurine was noted during depletion or repletion with pyridoxine hydrochloride. Swan et al. (1964) gave 3.5 g of L-cysteine on selected days during a period of deficiency and repletion of vitamin B_6 to six young men. Taurine excretion dropped during deficiency in four of the subjects before and after a cysteine load, but in four subjects it continued to drop during the repletion period. Two of the subjects increased their taurine excretion during the repletion period.

Scriver and Hutchesin (1963) reported a child having vitamin B_6 deficiency for undetermined reasons had increased taurine levels in

plasma and urine after vitamin B_6 therapy was given. Comparable increases in other plasma amino acids did not occur, so the increase was not due to a general trend. Krishnaswamy (1972) reported pregnant women deficient in vitamin B_6 had a taurine excretion that was similar to that of nondeficient, nonpregnant controls. Men having a vitamin B_6 deficiency who were studied by the same author excreted less taurine than the control men before treatment with pyridoxine hydrochloride. Their values increased after supplementation with pyridoxine hydrochloride, but these changes were not statistically significant due to individual variation (Krishnaswamy, 1974). Other studies have shown that taurine excretion decreased during a period of induced vitamin B_6 deficiency. However, it continued to decrease during repletion, suggesting that there is not a direct relationship between vitamin B_6 deficiency and taurine excretion (Park and Linkswiler, 1970; Shin and Linkswiler, 1974).

Other factors have been shown to influence the rate of taurine excretion. Block, Markovs and Steele (1965) found taurine excretion increased when methionine was added to a low protein diet, but decreased when added to a high protein diet. A high intake of alcohol as well as ACTH or other adrenal cortical hormones having a high glucocorticoid activity increase taurine excretion (Pentz, Moss and Denko, 1959). Progesterone or a combination of progesterone and diethylstilbestrol administered to men increased taurine excretion

significantly, but estrogen alone had little effect (Zinneman, Seal and Doe, 1967). Armstrong (1973) reported a low taurine excretion in women four weeks postpartum and in women taking an estrogen-progesterone medication for birth control. The amount of taurine in the diet may also have an appreciable effect on the amount excreted in the urine (Jacobson and Smith, 1968).

MATERIALS AND METHODS

Experimental Design

To determine the effect of oral contraceptives on vitamin B_6 status, several methionine metabolites were measured in the urine before and after the subjects had ingested an oral loading dose of the amino acid. Twenty four-hour urine specimens were collected for two consecutive days. Urine collected on the first day was used to establish the basal excretion of these metabolites and that collected on the second day was used to measure the effect of a 3-g oral dose of L-methionine. Vitamin B_6 status of the subjects was also measured by the activities of two erythrocyte transaminases. The subjects kept a dietary record on the two days that they collected urine and on one other day to determine the amount of vitamin B_6 in their diets as well as their general dietary intake.

Subjects

Nine women between the ages of 20 and 29 years participated in the study. They were free of metabolic disorders as determined by their medical history. Their hemoglobin and hematocrit values were within the normal range for women of their age. None of the subjects was taking supplementary vitamins or drugs, except for oral

contraceptives by the experimental group. Four of the women (subjects A, B, C and D) had taken no oral contraceptives for at least six months. They served as controls for the experimental group, which consisted of five women (subjects K, S, P, Q and N), who had been taking a combination-type oral contraceptive for six months or more. The two days of the experiment for each person were chosen so that they did not coincide with the menses in the control group and were at least seven days after the subjects in the experimental group had started a series of birth control pills. Table I gives the subjects' vital statistics as well as the time of menstrual cycle during the study, their hemoglobin and hematocrit, and type of oral contraceptive used.

Procedure

Details of the experiment were explained to the subjects, who freely gave their consent to participate in the study.

On the first day, the subjects collected a complete urine specimen and kept a dietary record of all food and beverages consumed during that 24-hour period. On the second day, while the subjects were in the post-absorptive state (between 7:00 and 8:00 A.M.), 10 ml of venous blood were obtained by a medical technician at the Good Samaritan Hospital. Immediately following this, the subjects received, with breakfast, a loading dose of 3 g of L-methionine that had been dissolved in 100 ml of water and added to frozen orange juice

Table I. Vital statistics of subjects.

Subject	Age (years)	Height (inches)	Weight (lbs)	Hemoglobin (g/100 ml)	Hematocrit (%)	Time of cycle	Days of pill cycle
				Control			
A	28	62	109	12.8	36.8	luteal	
B	25	61-1/2	109	12.6	36.0	follicular	
C	23	68	135	15.0	42.8	luteal	
D	25	66	160	14.8	43.2	follicular	
				Experimental			
K	29	62	112	13.5	39.0		19 & 20
N	21	72	140	14.0	39.8		7 & 8
S	20	63-1/4	112	13.9	40.7		7 & 8
P	23	65	129	15.5	43.5		13 & 14
Q	21	64	145	13.6	39.3		13 & 14

The brand name and content of oral contraceptive "pills" taken by experimental subjects are:

Subject: K - Norlestrin (1.0 mg norethindrone acetate + 0.05 mg ethinyl estradiol)
N - Ortho Novum I/50 (1.0 mg norethindrone + 0.05 mg mestranol)
S - Demulen (1.0 mg ethynodiol diacetate + 0.05 mg ethinyl estradiol)
P - Norinyl 1-80 (1.0 mg norethindrone + 0.08 mg mestranol)
Q - Norinyl 1+50 (1.0 mg norethindrone + 0.05 mg mestranol)

concentrate. Another 24-hour urine specimen was collected on this day and the dietary record of this and one other day was completed.

Methods

The urine was collected under toluene and kept in a cool place during the 24-hour collection periods. The total volume was determined, and after mixing, an aliquot was frozen for a short period of time until analysis. On the day of analysis, 20 ml of filtered urine was acidified to pH 2.0-2.2 with 6 N HCl and diluted to 25 ml with 0.2 M sodium citrate buffer, pH 2.2. The urine was analyzed for 12 acidic and neutral amino acids on a Beckman model 116 amino acid analyzer by the method of Spackman, Stein and Moore (1958).

Urinary creatinine was measured by a modified micro-adaptation of the Folin method (Oser, 1964). Total α-amino nitrogen in the urine samples was determined by using p-benzoquinone (Lorentz and Flatter, 1974).

Ten ml of blood were collected on day 2 while the subject was in the post-absorptive state. It was drawn into a heparinized vacutainer tube and placed immediately in ice water. An aliquot was removed for hemoglobin and hematocrit determinations. The blood was then centrifuged for 30 min. at 0 C and the plasma was removed. The red cells were washed twice with 0.85 percent saline solution and frozen for subsequent transaminase determinations.

EGPT and EGOT activity and in vitro stimulation when 100 μg of pyridoxal phosphate was added to the assay mixture was measured in the rood blood cells[1] by the method proposed by Woodring and Storvick (1970).

Hemoglobin concentration was determined by the cyanomethemoglobin method (Henry, 1964). Hematocrit was measured by the microhematocrit method (Wintrobe, 1967).

Nutrient content of the diets was calculated by computer at Oregon State University using a nutrient data bank (tape no. 3610) compiled at Ohio State University (Schaum, Mason and Sharp, 1973). Vitamin B_6 content of the diets was calculated by hand using information on the nutrient data bank and substituting foods where necessary because some had not been analyzed for this nutrient.

Statistical analysis of the data was obtained through the counseling service of the Statistics Department, Oregon State University.

Experiment Approval

This project was submitted to the Human Subjects Committee by Dr. Lorraine T. Miller and was approved on July 23, 1974. According to their recommendation, the subject signed a consent form before participating in the study.

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[1] Performed by L. T. Miller.

RESULTS AND DISCUSSION

Three-Day Dietary Intake

The calculated nutrient content of the diet that the subjects recorded for three days is reported in Appendix Table I. In general, the subjects' diets met or were above the Recommended Dietary Allowances (National Research Council, 1974) for most nutrients except iron and vitamin B_6. The subjects' vitamin B_6 intake on the days of the experiment are given in Table II. It will be noted that subject S was the only one who had an average intake of vitamin B_6 that was above the 2.0 mg recommended by the National Research Council (1974). Two subjects had intakes that were half this amount in spite of the fact that their diets were quite adequate in all other nutrients except iron. The dietary intake of vitamin B_6 by the subjects in this study was proportional to their protein intake (Figure 3). Although vitamin B_6 is found in many foods, the more concentrated sources of this nutrient are also good sources of protein, i.e., meat, legumes and whole grains (Home Economics Research Report No. 36, 1969).

Metabolism of Methionine

The effect of the 3-g loading dose of L-methionine on the urinary

Table II. Subjects' dietary intake of vitamin B_6[a].

Subject	Day 1[b] (mg)	Day 2[c] (mg)	Day 3[d] (mg)	3-Day average (mg)	% of RDA[e] (3-day average) (%)
			Controls		
A	1.8	2.6	1.2	1.9	95
B	1.4	1.7	1.7	1.6	80
C	1.8	1.6	1.9	1.8	90
D	0.61	1.4	1.0	1.0	50
			Experimentals		
K	1.1	1.6	2.1	1.6	80
N	1.1	1.2	1.5	1.3	65
S	2.8	2.0	2.4	2.4	120
P	1.0	1.6	1.4	1.3	65
Q	1.0	0.73	1.3	1.0	50

[a] Calculated from the nutrient data bank (tape no. 3610) on file at OSU Computer Center.

[b] Day when basal urine excretion was measured.

[c] Day when methionine loading dose was given.

[d] One other day not included in the methionine load test.

[e] Recommended daily dietary allowance for adults is 2.0 mg vitamin B_6 (National Research Council, 1974).

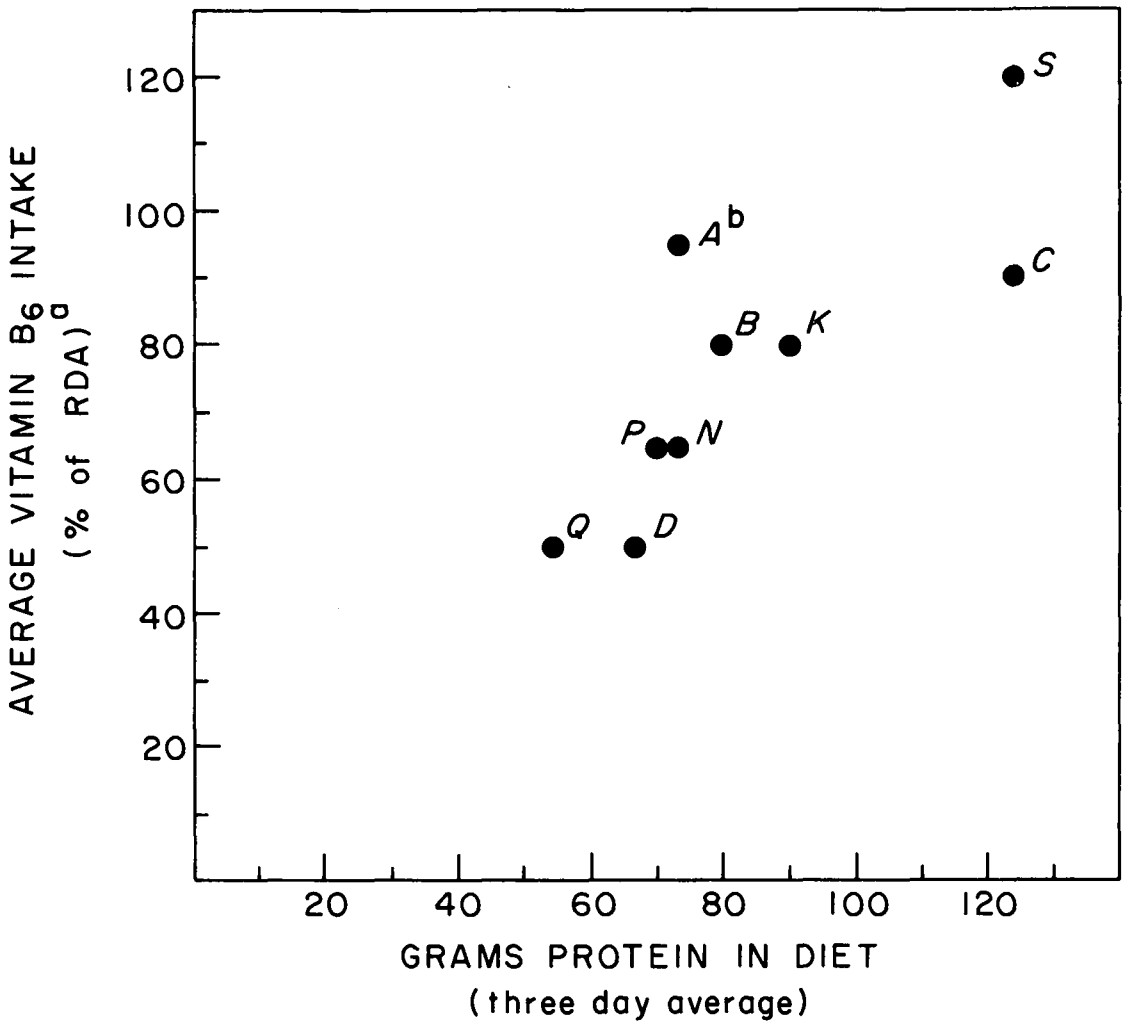

Figure 3. Relationship between subjects' dietary protein and vitamin B_6 intake (3-day average).

[a]Recommended Daily Dietary Allowances (National Research Council, 1974).

[b]Letters represent subjects in the study.

excretion of certain metabolites of the transsulfuration pathway is shown in Table III. In general, the experimental subjects excreted smaller amounts of these metabolites than did the control subjects.

Cystathionine Excretion

The mean urinary excretion of cystathionine was less in the experimental than in the control group, both before and after the methionine loading dose. However, all values were within the range for normal female subjects having an adequate vitamin B_6 intake (Krishnaswamy, 1972; Shin and Linkswiler, 1974). The mild increase in cystathionine excretion following a methionine loading dose has been observed in normal subjects (Park and Linkswiler, 1970; Krishnaswamy, 1972, 1974; Shin and Linkswiler, 1974). Shin and Linkswiler (1974) report that the excretion of cystathionine by women who had received a vitamin B_6-deficient diet for two weeks was, before and after methionine, four and eight times that of pre-depletion levels, respectively. After three weeks of dietary vitamin B_6 deficiency, men excreted, before and after methionine loading, 13 and 23 times the amount of cystathionine that they had excreted before depletion (Park and Linkswiler, 1970). Persons studied by Krishnaswamy (1972, 1974) who exhibited symptoms of vitamin B_6 deficiency excreted three times the amount of cystathionine excreted by control subjects after a loading does of 3 g L-methionine.

Table III. Excretion of methionine metabolites (μmoles/24 hr) before and after the subjects received a 3 gram oral dose of L-methionine.

Subject	Cystathionine		Cysteine sulfinic acid[a]+phosphoserine		Methionine		Homocysteine		Taurine	
	pre	post	pre	post	pre	post	pre	post	pre	post
					Control					
A	81.3	95.8	60.2	61.9	-[b]	111.3	0	0	3760.5	3384.6
B	67.6	80.5	38.6	44.2	-	96.8	0	trace	498.6	919.8
C	79.2	86.0	71.0	86.8	-	105.9	0	trace	2158.5	2190.2
D	50.3	88.6	49.7	65.2	-	87.6	0	trace	257.6	273.7
Mean	69.6	87.7	54.9	64.5	-	100.4	-	-	1668.8	1692.1
St. dev.	±14.2	± 6.4	±13.9	±17.5		± 10.4			±1630.5	±1380.0
					Experimental					
K	68.2	72.5	51.8	53.9	-	100.8	trace	trace	354.6	474.5
N	57.9	63.9	40.9	68.2	-	99.8	trace	9.2	141.5	117.9
S	64.5	60.6	55.1	60.8	-	114.6	trace	trace	188.7	145.8
P	45.9	68.9	55.7	66.3	-	107.9	0	15.0	73.4	76.3
Q	28.4	61.9	32.9	50.1	-	77.0	0	0	31.3	73.5
Mean	53.0	65.6	47.3	59.9	-	100.0	-	-	157.9	177.6
St. dev.	±16.1	± 5.0	±10.0	± 7.8		± 14.2			± 125.6	± 168.7

[a]These two amino acids were not separated on the chromatogram.

[b]Methionine was not measured because there was not a definite peak on this chromatogram.

In comparison with these studies on persons with subclinical vitamin B_6 deficiency, the subjects using oral contraceptives in the present study have a normal excretion of cystathionine. Thus, subclinical vitamin B_6 deficiency, if it exists in the experimental subjects in this study, is not indicated by the excretion of cystathionine before and after a methionine loading dose.

Cysteine Sulfinic Acid Plus Phosphoserine Excretion

Cysteine sulfinic acid and phosphoserine were measured together because they were not separated on the chromatogram. There was no significant difference between the two groups in the excretion of these substances either before or after the methionine load test. The excretion of these substances by both groups increased slightly after the methionine loading dose. These results compare with those of the control subjects studied by Krishnaswamy (1972), who were normal, nonpregnant women apparently free of vitamin B_6 deficiency.

Methionine Excretion

It was impossible to measure methionine in the basal urine because there was no defined peak on the chromatogram. After the methionine loading dose, however, there was a measurable peak.

Following the test dose, there was not much variation in urinary methionine, either between groups or among individuals in each group, indicating that oral contraceptive drugs do not influence the excretion of this amino acid. These levels are in the range of values for normal and vitamin B_6-deficient subjects used in the Krishnaswamy (1972, 1974) studies. Park and Linkswiler (1970) and Shin and Linkswiler (1974) reported values for urinary methionine before and after a methionine loading in the predepletion period that were higher than those in the present study. The fact that the subjects studied by Linkswiler and her associates received 2.5 g of L-methionine daily in addition to that in the diet may account for the higher levels of urinary methionine in their reports. In the present study, the amount of methionine in the subjects' diets was not calculated because complete data on the amino acid content of foods are unavailable in the Nutrient Data Bank.

Homocysteine Excretion

A trace of homocysteine was detected in the urine of three control subjects only after methionine loading. In contrast, traces of homocysteine were found in the basal urine of three oral contraceptive users. After methionine loading, this metabolite was found in the urine of four out of five experimental subjects, two of whom excreted measurable quantities.

These results are not in accord with other studies using a methionine loading dose. Homocysteine was only detected in those studies after two to three weeks of dietary vitamin B_6 deficiency and then only in response to a methionine loading dose. In contrast, cystathionine excretion had increased many times over the normal range at this time (Park and Linkswiler, 1970; Shin and Linkswiler, 1974). Krishnaswamy (1972, 1974) detected no homocysteine in the urine of men and pregnant women who were deficient in vitamin B_6. According to Finkelstein (1974), free homocysteine is absent from serum and tissue extracts of normal persons because this metabolite is rapidly converted to cystathionine or remethylated to methionine.

In the present study, the detection of homocysteine in the urine of several subjects, who excreted normal amounts of cystathionine, may be due to a difference in analytical technique. Cystathionine was at one time not found in the urine of normal persons (Hope, 1947), but it is now routinely found in small amounts (Park and Linkswiler, 1970). The finding of traces of homocysteine in the urine of control subjects after the methionine loading dose suggests that this may be true, in part. However, since the experimental subjects excreted homocysteine in the basal urine and the control subjects did not, the possibility exists that oral contraceptives may affect cystathionine synthase, the pyridoxal phosphate-dependent enzyme catalyzing formation of cystathionine from homocysteine and serine (Figure 2).

Human subjects who lack this enzyme have homocysteinuria (Finkelstein, 1974). Since estrogen causes a redistribution of pyridoxal phosphate among the pyridoxal phosphate-dependent enzymes in rat liver (Mason et al., 1969; Brin, 1971), this hormone may cause cystathionine synthase to give up its cofactor more readily in individuals using oral contraceptives than in those not using these drugs.

An abnormality in either of the two enzymes catalyzing remethylation of homocysteine to methionine (Figure 2) can also cause homocysteinuria. This is accompanied by a decrease in plasma methionine due to decreased biosynthesis of methionine from homocysteine (Finkelstein, 1974).

Low plasma levels of methionine have been reported in pregnant women, suggesting a hormonal-mediated change in methionine metabolism (Zinneman et al., 1967). This may also be a possible explanation for the low plasma methionine values found by Aly et al. (1971) in oral contraceptive users.

Taurine Excretion

The mean value for taurine excreted by the control subjects was 10 times greater than that excreted by the experimental subjects (Table III). In spite of this difference the mean values for the two groups are within the normal range (Jacobson and Smith, 1968). The wide difference in means between the two groups is partly accounted

for by the extremely high taurine excretion by control subject A and the low excretion by experimental subjects Q[3] and P. Taken individually, these values are outside the normal range of 140 to 2650 μmoles per 24 hours reported in other studies (Jacobson and Smith, 1968).

The methionine loading test, on the other hand, had little effect on the individual or group excretion of taurine. The mean taurine excretion, in μmoles per 24 hours, by the control subjects was 1668.8 before and 1692.1 after the methionine loading dose. Similarly, the mean taurine excretion by the experimental subjects was 157.9 and 177.6, respectively.

There was no correlation between the subjects' urinary taurine and their dietary intake of vitamin B_6 or protein, or their transaminase activity levels (reported in Tables IV and V).

A hormonal influence on taurine excretion is suggested by research reporting a decreased taurine excretion during pregnancy in spite of the general aminoaciduria (Armstrong and Yates, 1964; Zinneman et al., 1967; Armstrong, 1973). Armstrong (1973) reports that taurine excretion by oral contraceptive users is lower than normal. On the other hand, Zinneman et al. (1967) report that taurine

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[3] The basal urinary excretion of all amino acids tested as well as creatinine and total α-amino nitrogen by subject Q was much lower on day 1 than on day 2, suggesting an irregularity in the first day's specimen. Regardless of this, the taurine excretion for day 2 was well below the 140 μmoles quoted as the lower limit of the normal range.

excretion was increased by men given progesterone or an estrogen-progesterone combination. In women, estrogen plus cortisol or estrogen alone produced no significant changes in urinary taurine.

Erythrocyte Transaminases

The basal activity and percent stimulation by the *in vitro* addition of pyridoxal phosphate of EGOT and EGPT are reported in Tables IV and V, respectively. Data are expressed as μg pyruvate per mg hemoglobin and mg pyruvate per ml red blood cell (RBC) per hour.

There are no differences between the mean values for the control and experimental subjects in basal activity and percent *in vitro* stimulation of EGOT. These values compare favorably with some studies measuring EGOT activity in normal healthy subjects (Sauberlich et al., 1972) and in oral contraceptive users (Brown *et al.*, 1975). Other studies have reported basal activity to be higher in oral contraceptive users than in non-users, but not the percent *in vitro* stimulation (Rose *et al.*, 1973; Miller *et al.*, 1975). Due to variation that can occur because of different methods of handling and analysis (Sauberlich *et al.*, 1972), a comparison with normal values established in studies done in the same laboratory is the most valid. Miller *et al.* (1975), using the same method and laboratory, studied EGOT activity in untreated control women. The mean basal activity for these women was 6.57 mg pyruvate per ml RBC per hour. In the present study, the

Table IV. EGOT activity in subjects (data expressed two ways).

Subject	µg Pyruvate / mg Hb/hr		mg Pyruvate / ml RBC/hr		Percent stimulation[b]
	Basal	+PALPO[a]	Basal	+PALPO[a]	
			Control		
A	40.0	52.8	12.2	16.1	32.0
B	45.9	63.6	12.9	17.8	38.5
C	30.5	51.1	9.3	15.7	67.9
D	32.3	54.0	10.4	17.4	67.3
Mean	37.2	55.4	11.2	16.7	51.4
St. Dev.	± 7.13	± 5.6	± 2.7	± 1.0	±18.9
			Experimental		
K	31.9	54.4	10.2	17.3	70.4
N	25.0	41.6	8.0	13.3	66.3
S	38.4	53.8	11.1	15.5	40.3
P	30.9	46.5	9.7	14.6	50.5
Q	31.8	44.4	9.9	13.8	39.4
Mean	31.6	48.1	9.8	14.9	53.4
St. Dev.	± 4.2	± 5.7	± 1.28	± 2.5	±14.4

[a] 100 µg Pyridoxal phosphate (PALPO) was added to the assay medium.

[b] $\text{Percent stimulation} = \frac{\text{activity with PALPO added - basal activity}}{\text{basal activity}} \times 100$

Table V. EGPT activity in subjects (data expressed two ways).

Subject	μg Pyruvate / mg Hb/hr		mg Pyruvate / ml RBC/hr		Percent stimulation[b]
	Basal	+PALPO[a]	Basal	+PALPO	
			Control		
A	2.3	2.5	0.72	0.77	7.1
B	2.4	2.6	0.69	0.75	9.6
C	1.1	1.2	0.35	0.40	14.1
D	1.5	1.6	0.49	0.54	9.5
Mean	1.8	2.0	0.56	0.61	10.1
St. Dev.	±0.67	±0.67	±0.18	±0.18	± 2.93
			Experimental		
K	1.1	1.3	0.39	0.44	11.8
N	0.41	0.51	0.14	0.18	24.8
S	0.75	0.93	0.23	0.29	23.8
P	0.73	0.85	0.24	0.28	14.3
Q	1.1	1.2	0.34	0.37	9.0
Mean	0.83	0.96	0.27	0.31	16.7
St. Dev.	±0.31	±0.32	±0.10	±0.10	± 7.2

[a] 100 μg Pyridoxal phosphate (PALPO) was added to the assay medium.

[b] $\text{Percent stimulation} = \frac{\text{activity with PALPO added - basal activity}}{\text{basal activity}} \times 100$

mean basal activity was 11.19 for the control and 9.76 mg pyruvate per ml RBC per hour for the experimental group. In the Miller et al. (1975) study, the percent in vitro stimulation with added pyridoxal phosphate was 89 percent for the control subjects, while in the present study, the mean values were 51 percent for the control and 53 percent for the experimental subjects. Therefore, the subjects in the present study had basal activity levels in the high normal range and percent stimulation after in vitro addition of pyridoxal phosphate in the low normal range, showing that this enzyme was apparently not deficient in vitamin B_6 in either the control or experimental group. According to Sauberlich et al. (1972), a percent in vitro stimulation of up to 100 percent is within the normal range.

There was more variation in EGPT activity among individuals within each group than there was in EGOT activity. The mean basal activity for control subjects in the present study is higher than that obtained in other studies done in the same laboratory (Woodring and Storvick, 1970; Miller et al., 1975). This is mainly due to the high values for control subjects A and B which are above the normal range in these studies. These high EGPT values are not explained by the vitamin B_6 intake of these subjects (Table II), since other subjects had similar intakes of vitamin B_6 without having an unusually high EGPT activity. However, the three days of dietary intake obtained from the subjects may not be representative of their long-term

vitamin B_6 intake, which has a greater influence on this enzyme than short-term intake (Sauberlich et al., 1972). All other values for the basal activity of EGPT lie within the normal range found by Woodring and Storvick (1970) and Miller et al. (1975) except for experimental subject N, whose basal activity level is below the normal range in these studies. The percent of in vitro stimulation for subject N is very close to the upper limit for normal values ($\leq$ 25 percent) proposed by Sauberlich et al. (1972). These values for subject N cannot be explained by her dietary intake of vitamin B_6, which was not as low as that of the other subjects who had a higher EGPT activity and a lower percent in vitro stimulation (Table V). These three exceptional values explain the difference between the mean basal levels of EGPT of 1.8 μg pyruvate per mg hemoglobin for the control group and 0.83 for the experimental group. However, it should be noted that both of these mean values are within or above the normal range reported by other researchers (Woodring and Storvick, 1970; Dobernz et al., 1971; Rose et al., 1973; Brown et al., 1975; Miller et al., 1975).

The difference in the mean percent in vitro stimulation between control and experimental groups is not statistically significant because of the variation in individual values within each group. The means for both groups fall within the normal range reported (Doberenz et al., 1971; Sauberlich et al., 1972; Rose et al., 1973; Miller et al., 1975).

These results for EGPT basal activity and percent in vitro stimulation agree with other studies that have found EGPT activity to be normal in oral contraceptive users (Aly et al., 1971; Brown et al., 1975; Miller et al., 1975). Other studies have reported oral contraceptive users had lower basal EGPT activity (Doebernz et al., 1971) or a higher percent in vitro stimulation (Doberenz et al., 1971; Rose et al., 1973).

The fact that the experimental group had mean values lower in basal EGPT activity and somewhat higher in percent in vitro stimulation suggests that the oral contraceptives may affect the activity of these enzymes, or simply that there is a great deal of individual variation in the activity of EGPT. The small sample size used here limits the conclusions that can be made.

Excretion of Additional Free Amino Acids

Additional free acidic and neutral amino acids that were analyzed in the urine samples besides methionine metabolites of the trans-sulfuration pathway are: threonine, serine, asparagine, glutamic acid, glycine, alanine, valine, leucine, tryosine and phenylalanine. The mean value and range of urinary excretion for each of these amino acids plus the methionine metabolites on day 1 (before methionine) are reported in Table VI. The mean value of each amino acid is lower for the experimental than for the control group. Individual values are

Table VI. Mean of 13 amino acids excreted (μmoles/24 hr) on day 1 (no methionine).

	Subjects	
	Control	Experimental
Cysteine sulfinic acid + phosphoserine[a]	54.9 ± 13.9[b] (38.6 - 71.0)[c]	46.3 ± 10.0 (32.9 - 55.7)
Taurine	1668.8 ± 1630.5 (257.6 - 3760.5)	157.9 ± 125.6 (31.3 - 354.6)
Threonine	206.1 ± 105.9 (128.0 - 357.8)	135.5 ± 39.8 (91.9 - 193.6)
Serine	539.8 ± 346.4 (282.6 - 1044.4)	266.6 ± 80.9 (172.0 - 359.1)
Asparagine	1094.1 ± 709.8 (488.4 - 2100.7)	597.5 ± 154.5 (388.9 - 765.0)
Glutamic acid	32.1 ± 12.4 (19.9 - 47.7)	23.7 ± 8.8 (15.3 - 37.7)
Glycine	3887.5 ± 2528.7 (2095.7 - 7634.1)	1036.7 ± 182.0 (714.4 - 1137.5)
Alanine	394.6 ± 177.3 (277.0 - 658.6)	238.7 ± 69.1 (185.2 - 357.1)
Valine	56.6 ± 17.8 (39.2 - 79.8)	26.6 ± 14.1 (13.6 - 48.0)
Cystathionine	69.6 ± 14.2 (50.3 - 81.3)	53.0 ± 16.1 (28.4 - 68.2)
Leucine	57.3 ± 11.5 (44.2 - 69.7)	44.2 ± 13.9 (22.8 - 58.4)
Tyrosine	98.8 ± 38.0 (75.1 - 155.5)	63.4 ± 21.4 (33.8 - 82.9)
Phenylalanine	70.7 ± 10.9 (63.1 - 86.8)	49.0 ± 16.9 (30.3 - 71.6)

[a] These two amino acids were not separated on the chromatogram.

[b] Standard deviation

[c] Range

reported in Appendix Tables II and III.

The sum of the urinary excretion of these 13 amino acids for each subject is shown in Figure 4. The control subjects excreted greater total amounts of these 13 amino acids than did the experimental ones. The mean total excretion by the control group was 8.243 mmoles per 24 hours as compared with 2.842 mmoles for the group of oral contraceptive users. In spite of the individual variation within each group, the difference between these two means is statistically significant ($p < 0.01$), using an unpaired Student's "t" test.[4]

Except for the reports by Armstrong (1973) and Armstrong and Yates (1964) that taurine excretion was decreased during pregnancy and the postpartum period as well as during oral contraceptive use, there is no report in the literature of a decrease in urinary excretion of amino acids due to the hormonal changes in these conditions.

Total α-Amino Nitrogen Excretion

In view of the results obtained by measurement of the 13 free acidic and neutral amino acids, total α-amino nitrogen was measured in all of the urine samples. These values are expressed three ways in columns II, III and IV of Table VII. The α-amino nitrogen excretion

[4]Since the sample size is so small, an assumption of normal distribution was made in order to use this statistical analysis. Therefore, these data show a trend, but cannot be considered conclusive because of the small sample size.

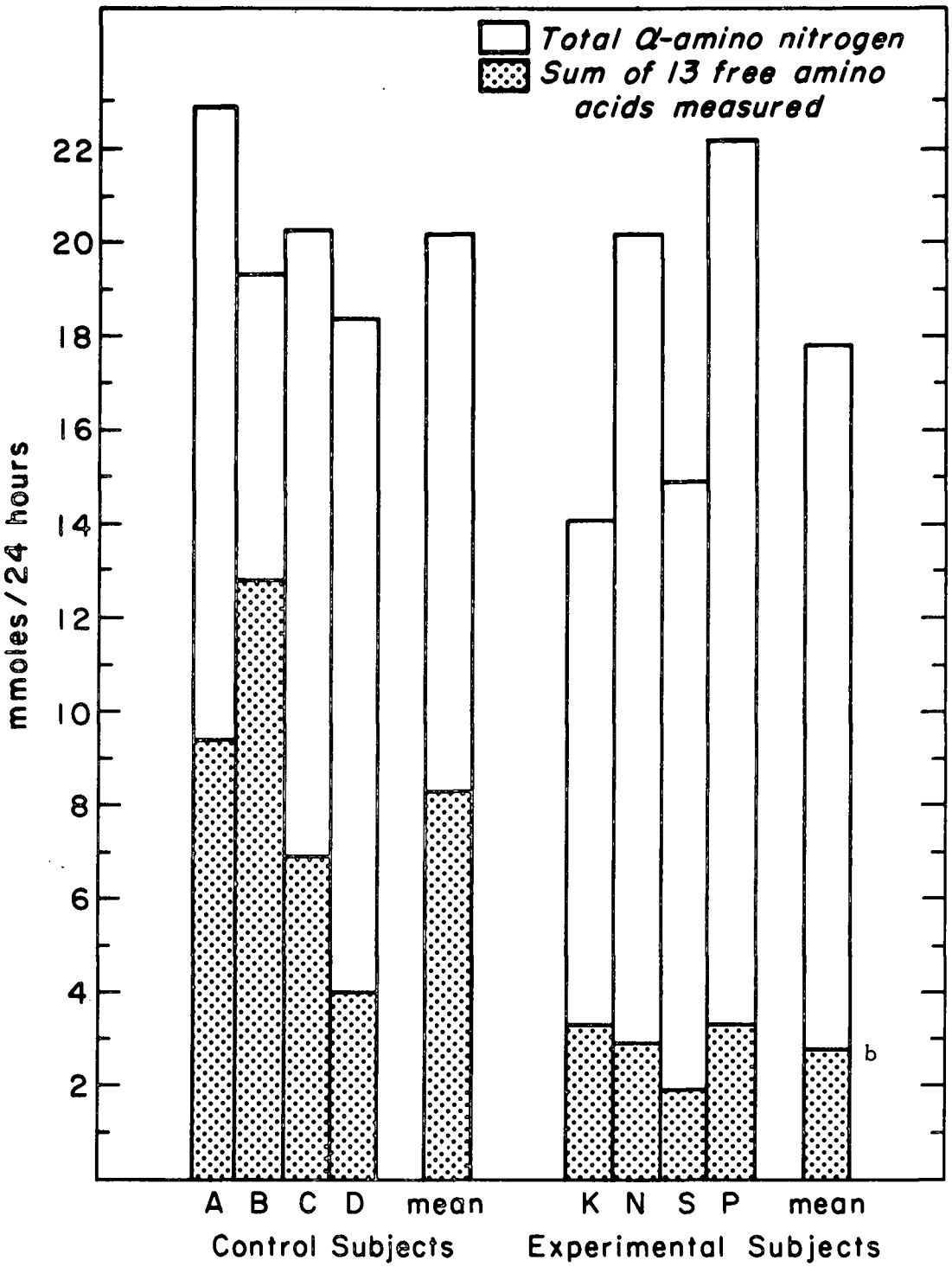

Figure 4. Total α-amino nitrogen and 13 free acidic and neutral amino acids excreted on day 1.[a]

[a]Day when basal urinary excretion was measured.

[b]The data for subject Q is not included because of an irregularity in the first day's urine sample.

Table VII. Creatinine, α-amino nitrogen (expressed three ways) and the sum of 13 free amino acids measured in pre and post methionine urine of subjects.

Subject	Day	Creatinine (mg/24 hr)	Total α-amino nitrogen (mmoles/24 hr)	Total α-amino nitrogen (mg 24 hr)	Total α-amino nitrogen (mg/mg creatinine)	Sum of 13 free amino acids (mmoles/24 hr)	% of Total α-amino nitrogen excreted as 13 free AA
		I	II	III	IV	V	VI
				Controls			
A	1	1192	22.9	320.8	0.269	9.4	41
	2	1095	19.1	267.8	0.245	6.5	34
B	1	897	19.3	269.8	0.301	12.8	66
	2	1094	24.7	345.6	0.316	7.2	29
C	1	1484	20.3	284.8	0.192	6.9	34
	2	1532	21.0	293.8	0.192	7.6	36
D	1	1284	18.5	258.9	0.241	4.0	22
	2	1330	20.3	284.4	0.214	4.2	21
Mean	1	-	20.3	283.6	0.251	8.3	41
	2	-	21.3	297.9	0.242	6.5	30
				Experimentals			
K	1	902	14.1	197.6	0.219	3.3	23
	2	883	15.6	217.8	0.247	3.9	25
N	1	1305	20.2	283.3	0.217	2.9	14
	2	1308	20.7	289.6	0.221	3.5	17

(Continued on next page)

Table VII. (Continued)

Subject	Day	Creatinine (mg/24 hr)	Total α-amino nitrogen (mmoles/24 hr)	Total α-amino nitrogen (mg) 24 hr)	Total α-amino nitrogen (mg/mg creatinine)	Sum of 13 free amino acids (mmoles/24 hr)	% of Total α-amino nitrogen excreted as 13 free AA
S	1	1260	22.4	314.0	0.166	3.3	15
	2	1135	19.4	270.9	0.213	3.3	17
P	1	1170	14.9	208.7	0.268	1.9	13
	2	1196	17.3	242.3	0.227	2.2	13
Q	1	853	13.8	192.6	0.226	2.3	20
	2	1147	20.5	287.0	0.250	4.7	23
Mean	1		17.9[b]	230.9[b]	0.219	2.9[b]	16
	2		18.7	261.5	0.227	3.5	19

[a]Percent calculated by: $\frac{\text{Column V}}{\text{Column II}} \times 100$.

[b]Values for day 1 for subject Q are not included in the mean values.

by the subjects was high compared with the range of 5.2 to 18.5 mmoles per 24 hours reported by Lortenz and Flatter (1974), who used the same method to measure urinary α-amino nitrogen excretion by 74 healthy persons between the ages of 30 and 73 years. The fact that the persons in the present study are younger than those in that group and are all female may have a bearing on this difference. Thompson and Abdulnabi (1950) report that women have a higher excretion of urinary α-amino nitrogen than men. In this present study, there is no significant difference between the control and experimental groups in this measurement, whether expressed as mmoles α-amino nitrogen per 24 hours or mg α-amino nitrogen per mg of creatinine. In column VI of Table VII and in Figure 4 the percentage of the total α-amino nitrogen excreted as the 13 free amino acids measured by ion exchange chromatography is given. The percent of α-amino nitrogen excreted as the 13 free amino acids is lower in the experimental than in the control group, even though the total α-amino nitrogen excreted is similar for both groups.

The difference between the groups in excretion of these 13 free amino acids may be due to the fact that all of the free amino acids found in urine were not measured. However, a few amino acids that are normally present in the urine make up a large percentage of the total free amino acid excretion. Listed in descending order of average excretion they are: glycine, taurine, histidine,

L-methylhistidine, glutamine, serine, 3-methylhistidine, alanine and α-aminoisobutyric acid. These reportedly make up 85 percent of the total free amino acids that are excreted in the urine (Soupart, 1962). Since the 13 amino acids measured include glycine, taurine, alanine and serine, it seems improbable that the basic amino acids, which were not measured in this study, could make up the difference between total α-amino nitrogen measured and the sum of acidic and neutral amino acids.

The p-benzoquinone method used for determining total α-amino nitrogen in this study produces a color reaction with amines, peptides and proteins as well as with free amino acids (Lorentz and Flatter, 1974). Studies on urinary amino acids in which urine samples were analyzed before and after acid hydrolysis have shown that one-half to three-fourths of all urinary amino acids are in the "bound" form, possibly in a conjugated form or in a peptide (Woodson et al., 1948; Echardt and Davidson, 1949; Stein, 1953; Goodwin, 1968). The explanation of the much larger amount of total α-amino nitrogen found in this study than would be expected from the sum of the 13 free amino acids apparently lies in the fact that some "bound" as well as "free" amino acids were measured by this method.

It is possible that oral contraceptive users may excrete more of the amino acids in a "bound" form than in "free" form, thus suggesting an explanation why the analysis of individual free amino acids shows a

difference between the control and experimental groups while the total α-amino nitrogen analysis shows no difference between the two groups.

There have been few studies reporting the effect of oral contraceptives on the urinary excretion of amino acids. However, their effect on the plasma levels of amino acids has been studied more thoroughly. Oral contraceptives decrease certain plasma amino acids (Zinneman et al., 1967; Seal and Doe, 1969; Aly et al., 1971) as well as total α-amino nitrogen (Landau and Lugibihl, 1961; Craft et al., 1970; Craft and Peters, 1971). It has been suggested that this lowering of plasma amino acids may be due to greater utilization or increased urinary excretion of the amino acids (Aly et al., 1971). The latter is suggested by the reports of an aminoaciduria occurring during pregnancy (Wallraff, Brodie and Borden, 1950; Zinneman et al., 1967). Administration of cortisol to men produces a similar aminoaciduria which suggests that increased adrenocortical activity during pregnancy is the cause of this condition (Zinneman et al., 1967). Seal and Doe (1969) report that estrogen administration to women produced an increase in non-protein bound cortisol and an increased urinary excretion of histidine and threonine, but the effect was not the same as the general aminoaciduria of pregnancy. Administration of progesterone or estrogen alone or in combination to men has little effect on urinary amino acid exretion (Zinneman

et al., 1967). Landau and Lugibihl (1961) report that progesterone administered to men produced a lowering of plasma α-amino nitrogen without an increase in urinary amino acid excretion. The results of the present study showing no significant difference between oral contraceptive users and control subjects in total urinary α-amino nitrogen excretion are in agreement with these studies. Metabolic balance studies reporting an increase in lean body mass produced by steroid hormones used for contraceptive purposes also support the thesis that the lowering of plasma amino acids is due to an increased tissue utilization rather than increased urinary excretion (Lecocq, Bradley and Goldzieher, 1967).

Conclusion

The two biochemical tests used to determine vitamin B_6 status in this study did not show evidence of subclinical vitamin B_6 deficiency in the five oral contraceptive users who were studied. Normal amounts of cystathionine were excreted by the oral contraceptive users both before and after the methionine load test. This metabolite is greatly elevated in the urine of persons who are deficient in vitamin B_6 (Park and Linkswiler, 1972; Shin and Linkswiler, 1974). The activities of EGPT and EGOT of these women were normal at the basal level and after stimulation with pyridoxal phosphate added in vitro.

Vitamin B_6-deficient persons have lower basal activity and higher percent stimulation than was found in this study.

Researchers who have used the tryptophan load test to determine the vitamin B_6 status in oral contraceptive users report that 70 to 80 percent of the women taking this drug excrete abnormal amounts of tryptophan metabolites after a loading dose of this amino acid, suggesting that these women may have a vitamin B_6 deficiency (Price et al., 1967; Brown et al., 1969, Rose, 1969; Lubby et al., 1971; Miller et al., 1974). However, it has been demonstrated that estrogen induces tryptophan oxygenase as well as producing changes in the activity of other pyridoxal phosphate-dependent enzymes, including some involved in the tryptophan-kynurenine pathway (Mason et al., 1969; Brin, 1971). Thus, the abnormal tryptophan metabolism observed in women using oral contraceptives may be caused by the channeling of more tryptophan through this pathway rather than a subclinical vitamin B_6 deficiency.

The dietary factor is an important one and may influence the results obtained from biochemical tests in oral contraceptive users. From the dietary calculations done in this study, it is apparent that even persons having a diet that is more than adequate in most nutrients may have a marginal vitamin B_6 intake unless their protein intake is exceptionally high and they include whole grains in their diet. The use of refined grains has brought about this problem

because vitamin B_6 is not included in the nutrients added to the "enriched" refined grain. The studies conducted by Brown et al. (1975) show that oral contraceptive users do not become deficient at a faster rate in vitamin B_6 than control subjects when both groups are receiving the same vitamin B_6 intake.

The results of the present study do show evidence of differences between control and experimental subjects that, although they do not indicate vitamin B_6 deficiency, may be caused by the hormones in the oral contraceptives. The lower urinary excretion of taurine in oral contraceptive users as well as the detection of homocysteine in the urine of these subjects without increased cystathionine excretion are changes that could be hormone-mediated. The fact that the experimental group excreted less of the total α-amino nitrogen in the form of the free amino acids measured than the control subjects suggests that these drugs may cause a higher proportion of amino acids to be excreted in the "bound" rather than the "free" form. The significance of these findings is not known. Further study with a larger sample size than was possible in this study is needed.

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APPENDIX

Appendix Table 1. Mean Dietary Intake[a, b] of Subjects.

Subject	Energy (kcal)	Protein (g)	Fat (g)	CHO (g)	Calcium (%)[c]	Iron (%)[c]	Vitamin A (%)[c]	Thiamine (%)[c]	Ribo-flavin (%)[c]	Niacin (%)[c]	Vitamin C (%)[c]	B_6 (%)[c]
						Control						
A	1518.0	73.1	60.6	172.2	101.9	53.5	167.7	124.6	133.9	131.9	499.5	95
B	1722.0	79.4	61.0	216.4	94.9	80.6	437.5	124.5	203.6	137.8	360.2	80
C	3222.0	124.9	138.9	382.6	350.1	113.9	177.8	186.9	390.2	321.4	721.0	90
D	1644.0	61.7	62.8	207.7	97.0	70.8	275.0	134.8	130.7	140.7	1021.7	50
						Experimental						
K	2452.0	91.4	116.6	270.9	193.5	61.7	185.4	124.1	162.0	80.5	470.1	80
N	1846.0	73.0	75.1	213.6	126.3	60.3	430.0	107.9	165.4	100.7	686.8	65
S	3137.0	124.2	139.7	348.4	167.7	101.7	132.7	198.6	161.2	188.2	589.0	120
P	2366.0	70.7	76.0	326.8	116.0	64.7	110.8	122.9	142.7	134.7	800.3	65
Q	1630.0	54.9	59.1	228.4	84.1	45.0	76.1	139.1	85.8	92.8	811.1	50

[a] Average of three-day dietary intakes.

[b] Calculated from nutrient data bank (tape no. 3610) on file at OSU Computer Center.

[c] Percent of Recommended Daily Dietary Allowances (National Research Council, 1974).

Appendix Table 2. Acidic and Neutral Amino Acids Excreted in Urine (μ m per 24 hours) by Control Subjects before and after Receiving a 3-g Oral Dose of L-methionine.

	A		B		C		D		Average	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Cysteine sulfinic[a] acid plus phosphoserine	6.02	61.9	38.6	44.2	71.0	86.8	49.7	65.2	54.9 ± 13.9[b]	64.5 ± 17.5
Taurine	3760.5	3384.6	498.6	919.8	2158.5	2190.2	257.6	273.7	1668.8 ±1630.5	1692.1 ±1380.0
Threonine	199.5	123.2	357.8	210.3	128.0	151.4	139.1	132.1	206.1 ±105.9	154.3 ± 39.2
Serine	484.2	282.6	1044.4	635.8	342.2	452.0	288.4	265.5	539.8 ±346.4	490.0 ±173.1
Asparagine	1053.4	566.5	2100.7	1278.4	734.0	814.2	488.0	545.3	1094.1 ±709.8	801.1 ±341.0
Glutamic acid	24.9	trace	47.7	6.6	19.5	28.9	36.0	25.6	32.1 ± 12.4	20.4 ± 12.0
Glycine	3022.0	1456.2	7634.1	3811.2	2797.5	3142.1	2095.7	2356.9	3887.5 ±2528.7	2696.6 ±1015.1
Alanine	325.8	232.5	658.6	369.0	277.0	370.3	317.1	262.3	394.6 ±177.3	308.5 ± 71.6
Valine	79.8	108.3	46.9	41.6	60.5	68.7	39.2	37.7	56.6 ± 17.8	64.1 ± 32.5
Cystathionine	81.3	95.8	67.6	80.5	79.2	86.0	50.2	88.6	69.6 ± 14.2	87.7 ± 6.4
Methionine	-[c]	111.3	-	96.8	-	105.9	-	87.6	-	100.4 ± 10.4
Leucine	69.7	72.0	63.7	69.2	51.5	55.2	44.2	55.5	57.3 ± 11.5	63.0 ± 8.9
Tyrosine	75.1	61.1	155.5	114.8	79.6	100.6	85.0	78.1	98.8 ± 38.0	88.7 ± 23.1

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Appendix Table 2. (Continued)

	A		B		C		D		Average	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Phenylalanine	67.3	52.4	86.8	74.2	63.1	85.5	65.5	59.7	70.7 ± 10.9	68.0 ± 14.8
Homocysteine	0	0	0	trace	0	trace	0	trace	-	-
Total	9394.0	6497.0	12801.0	7156.0	6862.0	7638.0	3956.0	4246.0	8256.0	6509.0

[a]These two amino acids were not separated on the chromatogram.

[b]Standard deviation

[c]Methionine was not measured because there was no definite peak on this chromatogram.

Appendix Table 3. Acidic and Neutral Amino Acids Excreted in Urine (μ m per 24 hours) by Experimental Subjects before and after Receiving a 3-g Oral Dose of Methionine.

	K		N		P		S		Q		Average	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Cysteine sulfinic[a] acid + phosphoserine	51.8	53.9	40.9	68.2	55.7	60.8	55.1	66.3	32.9	50.1	47.3 ± 10.0[b]	59.9 ± 7.8
Taurine	354.6	474.5	141.5	117.9	73.4	76.3	188.7	145.8	31.3	73.5	157.9 ±125.6	177.6 ±168.7
Threonine	164.1	181.4	126.1	156.3	91.9	94.3	193.6	173.3	101.6	182.0	135.5 ± 39.8	157.5 ± 36.8
Serine	308.7	322.4	301.9	346.7	172.0	190.2	359.1	358.2	191.4	335.3	266.6 ± 80.9	310.6 ± 68.6
Asparagine	721.0	871.8	607.6	658.2	388.9	418.3	765.0	756.1	505.2	862.5	597.5 ±154.5	713.4 ±186.6
Glutamic acid	21.0	15.0	26.2	40.0	15.3	15.7	37.7	30.4	18.1	36.5	23.7 ± 8.8	27.5 ± 11.7
Glycine	1137.5	1319.1	1140.0	1608.1	714.4	940.9	1075.2	1185.2	116.3	2535.8	1036.7 ±182.0	1517.8 ±617.0
Alanine	240.1	341.4	206.3	308.4	185.2	165.8	357.4	323.1	204.8	360.1	238.7 ± 69.1	299.8 ± 77.4
Valine	48.0	52.2	23.2	17.9	13.6	36.3	32.4	31.1	15.7	31.8	26.6 ± 14.1	33.8 ± 12.3
Cystathionine	68.2	72.5	57.9	63.9	45.9	68.9	64.5	60.6	28.4	61.9	53.0 ± 16.1	65.6 ± 5.0
Methionine	-[c]	100.8	53.2	99.8	-	107.9	47.5	114.6	-	77.0	-	100.0 ± 14.2
Leucine	50.5	63.5	50.7	41.7	38.8	47.2	58.4	43.4	22.8	43.5	44.2 ± 13.9	47.9 ± 9.0
Tyrosine	68.0	52.0	82.7	56.4	49.7	50.7	82.9	55.9	33.8	63.7	63.4 ± 21.4	55.7 ± 5.1

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Appendix Table 3. (Continued)

	K		N		P		S		Q		Average	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Phenylalanine	71.6	58.1	50.8	60.0	34.8	35.6	57.7	40.6	30.3	49.5	49.0 ± 16.9	48.8 ± 10.6
Homocysteine	trace	trace	trace	9.2	0	15.0	trace	trace	0	0	-	-
Total	3305.0	3879.0	2856.0	3504.0	3328.0	3270.0	1880.0	2201.0	2333.0	4686.0	2842.0[d]	3516.0

[a]These two amino acids were not separated on the chromatogram.

[b]Standard deviation.

[c]Methionine was not measured because there was no definite peak on the chromatogram.

[d]Data for subject Q are not included in this average total for day 1.