

EFFECTS OF SELENIUM AND VITAMIN B-6 ON GROWTH OF CHEMICALLY-
INDUCED TRANSPLANTED TUMORS IN BALB/c INBRED MICE

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(ABSTRACT)

Male weanling inbred mice were inoculated with fibrosarcoma cells (hindquarter) originally produced by 2-methylcholanthrene. Before inoculation, mice were randomly divided into three groups of 24 and one of 12 (control). After a one week acclimation period, each group was fed a diet containing either suboptimal vitamin B-6, 0.5 mg/kg diet; adequate, 7.0 mg/kg diet; or excess, 100 mg/kg diet. Controls were fed the adequate vitamin B-6 diet. Twenty-four hours after tumor cell inoculation, a series of sodium selenite injections (0.5 μ g/.10 mL) were given to half of each treatment group and all controls. Mice were sacrificed two wk after tumor inoculation. Tumors were excised and weighed. Selenium-treated mice had significantly smaller tumors as compared to untreated mice regardless of vitamin B-6 treatment. The smallest tumors were found in the selenium-treated group maintained on

adequate B-6, while the largest tumors were developed by mice on the excess B-6 diet without selenium treatments. All groups had similar blood selenium levels as measured by gas chromatography. Tumor selenium levels, analyzed by atomic absorption, were significantly higher for untreated groups than selenium-treated groups (larger tumor size). The excess and adequate vitamin B-6 selenium-treated groups had significantly lower tumor selenium levels than the adequate vitamin B-6 untreated group. Plasma pyridoxal phosphate (concentrations) determined radiometrically and tumor vitamin B-6 levels determined microbiologically, related directly to dietary treatments. Sodium selenite injections and adequate vitamin B-6 diets reduced the size of fibrosarcomas in BALB/c inbred mice.

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INTRODUCTION

For three decades scientists have examined the role of selenium as a cancer therapeutic agent in humans and laboratory animals. As early as 1956 it was reported that selenocystine was effective in reducing leukocyte count and spleen size of patients suffering from acute leukemia. The dose of selenium given, 50-200 mg per day, caused nausea, vomiting, and diarrhea. Although the dosage had toxic side effects, renal and hepatic function were normal and the side effects were no worse than those experienced with other chemotherapeutic agents (1).

Ingestion of large quantities of selenium has been shown to be carcinogenic, while epidemiological studies have indicated that low selenium levels in soil and plant forage are linked to an increased risk of certain cancers. These studies found blood selenium levels ranging from 25.6 $\mu\text{L/dL}$ in high selenium forage areas (Rapid City, SD) to 15.7 $\mu\text{L/dL}$ in low selenium areas (Lima, OH) of the United States. High selenium forage areas were found to be inversely associated with cancers of the lung, rectum, colon, esophagus, bladder, and pancreas, while a positive association was found between forage selenium levels and Hodgkin's disease, leukemia, and cancers of the liver and stomach (2).

Despite these claims, some researchers believe that widespread interstate transport of selenium-rich meat and grain reduces the accuracy of location-based epidemiological data. One must also consider exposure of populations in these areas to chemicals, pollutants, and other results of urbanization. These factors combined with lifestyle may affect blood selenium levels and, consequently, cancer statistics (2).

In order to more closely examine the relationship between selenium and cancer, researchers have compared the correlation between blood selenium levels of patients with various carcinomas and the frequency and behavior of their tumors. Overall, low serum selenium concentrations were associated with multiple primary tumors, increased frequency of distant metastases, multiple recurrences, and decreased survival times. Patients with serum selenium values at or exceeding the mean value of all cancer patients had tumors with a lower frequency of recurrences, less distant metastases, fewer primary neoplasms, and more confined tumors (3). Later observations, however, indicated that reticuloendothelial tumors did not follow the same patterns (4).

In a similar study, a striking decrease in blood selenium concentration was found in every carcinoma

which affected some part of the digestive tract (except the rectum) when compared to healthy control subjects. Cancers in organs which do not come in contact with selenium directly showed no difference in mortality. The lowest blood selenium concentrations were found in patients with cancers of the colon, stomach, liver, and pancreas (5).

It is difficult to determine the ramifications of low blood selenium levels with regard to cancer since it is not clear whether the cancer causes the low blood selenium or whether the low selenium status precedes the condition. The long latency period of most cancers also makes investigation difficult. Chemopreventive studies involving selenium supplementation are nearly impossible to conduct since the Food and Drug Administration and the National Cancer Institute have limited human experimental use of selenium to 200 µg per day (6).

The above stated difficulties in human studies have led researchers to study the relationship between selenium and cancer more vigorously in laboratory animals. To date, research has shown that selenium has an inhibitory effect on chemically-induced epidermal, mammary, and hepatic tumor growth (7,8,9). The inhibition of tumor size extends to transplantable tumor models as well as virally-induced models. There is a

large body of information supporting the claim that selenium is effective against promotion and proliferation of tumors in laboratory animals (9). Detailed information will be given in the following section to further explain the connection between selenium and cancer.

Although researchers agree that selenium is effective against tumor growth, there is no consensus as to how the mineral achieves this end. Some researchers believe that exposure to high concentrations of selenium inhibit DNA synthesis such that cells are blocked in the s-G2 phase of the cell cycle (10). There is some information suggesting involvement of the immune system. Immunity is suppressed under antioxidant-deficient conditions and selenium has been shown to be immunostimulatory (11,12). Some research indicates that selenium is involved in accelerated detoxification of chemicals thereby disrupting carcinogen metabolism (13). Any one or a combination of the above theories may prove correct, yet more intense study is needed to determine the exact mechanism by which the mineral exerts its antitumorigenic effect.

The effect of vitamins as they relate to tumors has been studied in some depth. Research has shown that forms of vitamins A, C, and E have some effective-

ness against tumor development. Other vitamins have also been examined in relation to various types of tumors. Vitamin B-6 has been studied to determine its antitumorigenic effectiveness. Vitamin B-6 is required for normal growth of all animals. Pyridoxal phosphate (PLP) is the coenzyme of many enzymes, thus making it an important growth factor. Studies have established that absence of vitamin B-6 from the diet significantly inhibits growth of Morris hepatoma (14,15,16,17). Restriction of B-6 has been shown to, reduce tumor incidence in mice and rats. Sarcoma-bearing, mice, fed a vitamin B-6 deficient diet, showed above a 50% tumor growth inhibition (18). Research presented by Tryfiates et al (14). examined findings on vitamin B-6 and cancer. The facts presented indicate that vitamin B-6 deficiency in laboratory animals, at the time of tumor cell injection, reduces the size and incidence of tumors. There are many theories as to the efficacy of vitamin B-6 against tumor growth (19). Alterations in enzyme function and protein expression are currently being examined in relation to vitamin B-6 and cancer. Alterations in protein expression due to B-6 deficiency has led researchers to propose a type of regulatory control of protein expression by the vitamin. Vitamin B-6 may also alter gene expression and enzyme induction. Regardless of the reason, in some cases, re-

striction of vitamin B-6 inhibits tumor growth.

Although we have some information about the effectiveness of selenium and vitamin B-6 individually on tumor growth, no information exists as to how they may perform together. Since both nutrients affect protein synthesis and expression, it is necessary to determine their additive effect on tumor growth. This study was designed to examine the effectiveness of selenium injections and dietary levels of vitamin B-6 on the growth of chemically-induced transplantable fibrosarcomas in BALB/c mice. The vitamin B-6 and selenium status of the animals, and the tumor content of these two nutrients were also determined.

REVIEW OF LITERATURE

Attempts to study the relationship between single nutrients and carcinogenesis have provided researchers with mixed, and sometimes contradictory information. To date, investigation regarding the anticarcinogenicity of selenium has yielded important information. Low serum selenium levels are associated with numerous human cancers while selenium supplementation in laboratory animals appears to reduce growth rates of certain types of tumors, hence, the level of selenium is important (1,3,5,7,8). Similarly, low vitamin B-6 status, primarily plasma PLP concentration, is associated with many human cancers. Although vitamin B-6 supplementation reduces recurrences of bladder cancer, research indicates that vitamin B-6 deficiency may reduce tumor growth rates in laboratory animals (14). However, vitamin B-6 inadequacy was observed in most, if not all, individuals having oral cavity, throat, lung, prostate, and bladder malignancies (20,21). The following body of literature may provide some evidence with regard to the efficacy of selenium and vitamin B-6 manipulations and carcinogenesis.

The Efficacy of Selenium as an Antitumorigenic Agent

Selenium seems to have an antitumorigenic role as indicated by studies involving both humans and labora-

tory animals. Human studies have contributed to the pool of knowledge mainly through examination of epidemiological cancer data as it relates to blood and tissue selenium levels. These studies, however, often carry with them a great deal of interference from confounding variables. For this reason researchers have concentrated the bulk of their research on laboratory animals. The use of laboratory animals is ideal in that one has the ability to control variables needed to discover the role of selenium as an antitumorigenic agent.

From the body of data compiled relative to the antitumorigenic effects of selenium, some definite observations have been made. Selenium is known to be effective in the initiation of both chemically- and virally-induced tumors during both the proliferative and promotional phases of development (22,23). The efficacy of selenium, however, is known to depend on the following factors: type of tumor cell used in experimentation, form of selenium used in supplementation, mode and dosage of selenium administration, time and duration of supplementation, and control of interference by other variables. The following text will attempt to explain the relationship between these factors and the antitumorigenicity of selenium.

Inhibition of Chemical Carcinogenesis in Laboratory Animals by Selenium

Inhibition of chemical carcinogenesis in laboratory animals has been explored. A detailed research summary, compiled by Ip (9), indicates that selenium has an inhibitory effect on preneoplastic lesion development and tumorigenesis in rat livers induced by 3-methyl-4-dimethyl-aminoazobenzene, 2-acetylaminofluorene, diethylnitrosamine, and aflatoxins. Similar inhibition was also noted in rat and mouse mammary glands exposed to 7,12-dimethylbenz[a]anthracene (DMBA). Mouse mammary gland tumorigenesis was inhibited when exposed to estrone and progesterone, while rat mammary gland tumorigenesis was inhibited by N-methyl-N-nitrosurea. Inhibition of colon tumorigenesis was noted among rats exposed to dimethylhydrazine, azoxymethane, methylazoxymethanol, and bis(2-oxopropyl)nitrosamine. Skin lesion development was inhibited in mice exposed to benzopyrene, DMBA, and 3-methylcholanthrene. Most of the organ system models presented used inorganic selenium supplements (0.5 to 6 ppm) in drinking water or diet to test for antitumorigenic effects. Ip surmised that since direct carcinogens were used, the primary action of selenium is probably not mediated through interference with carcinogen metabolism. It was also noted that the inhibitory

effect of selenium is nonspecific with respect to organ site carcinogenesis.

Inhibition of Virally-Induced and Transplantable Tumors by Selenium

The effectiveness of selenium against virally-induced and transplantable tumors have also been examined. A detailed summary (23) has presented much of what is known about selenium inhibition of these types of tumor models. Supplementation of 2 mg/mL of selenite in the drinking water of female virgin C3H/st mice, lowered virally-induced, spontaneous mammary tumor development to 10% as compared to 82% of unsupplemented controls. Selenium supplementation, given for 15 mo, led to tumors which grew more slowly, thus increasing the life span of the treated mice by 1 to 2 mo over unsupplemented controls. The same research summary also reported that when BALB/cfC3H mice, containing murine mammary tumor virus (23), were given selenium at 2 and 6 ppm in drinking water, the incidence of tumor development fell to 50% and 80%, respectively, over unsupplemented controls.

A study performed by Schrauzer et al. (24) examined the ability of selenium to lower the incidence of virally-induced tumorigenesis at both the proliferation and promotion phases of tumor development. It was noted, however, that selenium was protective only

during supplementation periods. Incidence of virally-induced adenocarcinoma dropped to 27% in mice fed a torula yeast diet supplemented with 1 $\mu\text{g/g}$ Se/g as selenium yeast. When these same mice reached 13.8 mo of age they were switched to a basal diet containing only 0.15 μg selenium. Rapid onset of mammary tumors was seen in 69% of these mice as compared to 77% in mice fed only the basal diet throughout their post-weanling life span. Mice receiving the basal diet for 13.8 mo which were then given the higher selenium diet, reached a significantly lower (46%) incidence of tumors when compared to control (77%) mice. The researchers concluded that the antitumorigenic effects of selenium were dependent on time and duration of supplementation and that selenium may interfere with replication or transformation of viruses.

A considerable body of evidence now shows that selenium is effective in the growth inhibition of the following transplanted tumor cell models: Ehrlich ascites (25,26), MT-W9B rat mammary tumors (27), canine mammary carcinoma cells (28), L1210 leukemia cells (29), and malignant mouse lymphoid cells (30).

Transplantable tumor models provide an excellent opportunity to study the antitumorigenic effects of selenium due to the ability to manipulate variables and

treatment schedules. Selenium supplementation can be given at any stage of tumor development thus providing important information concerning the ideal time to begin supplementing the mineral. Transplantable tumor models also provide an opportunity to study the best form of selenium to use in supplementation as well as the ideal administration route, and the most effective concentration and duration of supplementation.

Research has indicated that selenium is not chemopreventive below 1.5 ppm (9), while dosage above this level provides a proportional degree of tumor inhibition. Supplementation of selenium at 2.5 ppm has been found to be a non-toxic dose level in that it does not affect the growth rate of rats. At 5 ppm, a commonly effective chemopreventive dose, selenium has been shown to produce a 10% suppression in weight gain. This suppression, however, can not be explained by food consumption data (9).

Selenium supplementation, primarily in the form of sodium selenite, has been shown to be effective in tumor growth inhibition when given at a dose level of 1 $\mu\text{g/g}$ of body weight (31). As the dosage of selenium is increased to 2 mg/g of body weight, symptoms of toxicity are noted in the form of irritability and reduced intestinal tissue mass (25). While 5 ppm of selenium is effective in tumor growth inhibition, decreased

weight gain, liver damage, and death have occasionally been noted as toxic side effects (22). Excess selenium supplementation has also been shown to be carcinogenic in laboratory animals (32).

Administration of selenium supplementation is often provided in food and water of laboratory animals. These administrative routes, however convenient, present problems in actual consumption measurements. For this reason researchers have turned to intraperitoneal injections as a more accurate means of selenium dose administration.

A comprehensive study performed by Poirier and Milner (26) examined both form and mode of selenium administration in order to determine the most effective means of achieving tumor growth inhibition in mice. When sodium selenite was given by intraperitoneal injection (2 μ g given 6 times in 9 days) in one group of mice, and gastric gavage was used in another, several outcomes were noted. Intraperitoneal injection increased survival times of Ehrlich ascites tumor burdened mice by 170% as compared to 20% in mice given selenium by gastric gavage. Survival times were measured against a control group given no selenium treatments. A slight decrease in weight gain was noted in the mice given intraperitoneal selenium injections, due

to reduced ascitic fluid accumulation.

Another portion of the same study examined the effects of four different forms of selenium supplementation on Ehrlich ascites tumor propagation in mice. The forms used for comparison, sodium selenite, sodium selenide, dimethyl selenide and seleno-DL-cystine, were given by intraperitoneal injection in 5 μ g quantities on days 0, 1, 3 and 5 after tumor cell inoculation. Tumor development was 70%, 80%, and 90%, respectively, in mice given the last three forms of selenium listed while no tumors were noted in the group treated with sodium selenite. Similar success was noted by Greeder et al (25).

Overall conclusions drawn from this study (26) include the following: continuous exposure to selenium may be needed to produce the antitumorigenic effects, and sodium selenite, when given by intraperitoneal injection, is the most effective form against development of Ehrlich ascites tumors in mice. In addition, the researchers suggested that one of the compounds formed in the normal process of selenium detoxification may be responsible for the antitumorigenic properties of selenium.

Discussion of the role of selenium as an antitumorigenic agent would not be complete without some mention of confounding variables which may affect the

outcome of experimentation. Aside from environmental factors, growth rates of tumors are known to be affected by vitamins A, C, E, and B-6, and by dietary fat intakes (9,14,33).

Theories Proposed to Explain the Anticarcinogenicity of Selenium

As early as 1943 researchers (32) began to investigate the relationship between selenium and carcinogenesis. Originally the antitumorigenic effects of selenium were thought to be linked to the mineral's function as an antioxidant. With the knowledge that defense against cell-damaging free radicals is achieved by scavengers such as glutathione peroxidase (EC 1.11.1.9), a selenoenzyme, it appeared likely that selenium achieved its antitumorigenic effects in this way. In response to this knowledge, a study was initiated to compare the efficacy of selenium and vitamin E to determine their relative and combined inhibitions of lipid peroxidation (9). The results of the study indicated a failure of high vitamin E supplementation, 1 g α -tocopherol acetate (9), to alter enhanced mammary tumor development caused by selenium deprivation in female Sprague-Dawley rats exposed to DMBA. The researchers also found an absence of a protective effect by vitamin E when selenium was provided in

adequate dietary quantities (0.1 ppm).

Some evidence exists indicating that increased dietary selenium levels inhibit mammary tumorigenesis by decreasing lipid peroxidation and cell damage, which are favorable to neoplastic growth. This idea is supported by the ability of increased glutathione levels to reduce peroxidation levels in cells. A comprehensive review of similar experimentation supports this claim (34). The exact mechanism remains unclear, but decreased lipid peroxidation and changes in DNA and protein synthesis are thought to contribute to the antitumorigenic effects of selenium.

Research efforts continue in the pursuit of an explanation regarding antitumorigenic effects of selenium. Among the theories currently being examined, inhibition of DNA synthesis resulting in reduced rates of tumorigenesis, is one of the more scientifically supported. Research has indicated that exposure to high concentrations of selenium is known to block DNA synthesis in the S-G₂ phase of the cell cycle (10). Other evidence indicates that selenium protects rat liver DNA against strand breakage induced by 2-acetylaminofluorene exposure (35). In a study performed by Ip (9), high levels of selenium were shown to markedly suppress DNA synthesis in DMBA-treated mammary explants. The rat mammary explant cultures exposed to 5

$\times 10^{-5}$ M of selenium in medium, had a low rate of proliferation and a low tumor development potential when transplanted to recipient rats. Only 2 of 25 rats receiving transplants exposed to the high selenium concentrations developed tumors while 12 of 25 control rats, given no selenium treatment, developed tumors.

In addition to these studies, selenium compounds have been examined in relation to inhibition of protein synthesis. Since protein synthesis is directly related to tumor cell growth, attempts were made to examine the effects of sodium selenite in an incubation mixture (cell-free) consisting of free polyribosomes and a 150,000 x g supernatant from rat liver fortified with cofactors. The results of the study showed a complete loss of amino acid incorporation at nmol quantities of sodium selenite per mL of incubation mixture. The inhibition of amino acid incorporation, however, was only apparent if a thiol such as glutathione (GSH) was present in the incubation mixture. In a more purified system, no inhibition was produced by selenite. It was also noted that when GSH reacts with selenite, oxidized glutathione (GSSG) and selenodiglutathione (GSSeSG) are formed. The resulting GSSeSG formed was shown to inhibit ribosome-dependent GTPase activity of elonga-

tion factor 2 (EF-2). In a follow up experiment using an intact cell system containing P815 mastocytoma (36) and L1210 leukemic cells, GSSeSG was again found to inhibit amino acid incorporation into proteins. The authors of this study could not identify the mechanism by which inhibition of amino acid incorporation took place. An indication was given, however, that there was a possibility that selenium decreased the ability of cells to proliferate and that inhibition of protein synthesis may have been involved. (36) Early speculation that selenium promoted its primary antitumorigenic response through interference with carcinogen metabolism has been all but ruled out. The reasoning behind this statement stems from observations that selenium is effective in the inhibition of tumors induced by a wide range of carcinogenic substances including: aromatic amines, aflatoxins, nitrosamines, polycyclic hydrocarbons, and methylnitrosourea (9). Interference with carcinogen metabolism, cannot, however, be ruled out as a contributing factor in the antitumorigenicity of selenium since spontaneous metabolism of these compounds in a normal environment is usually not measured.

Examination of selenium as an immunostimulatory agent is raising new and important questions concerning tumorigenesis and immune system response. To date, it is known that a modest increase in dietary selenium

alone, or in combination with vitamin E, appears to enhance immune responsiveness to vaccine antigens in animals. This is thought to occur due to increased primary response in immunoglobulins (12).

A more recent study examined the effects of sodium selenite on phagocytic functions of human neutrophilic granulocytes from donors having low activities of peroxidase. In the study, human granulocytes were exposed to 100 or 200 ng Se/mL of sodium selenite (in vitro) for 60 min. The phagocytosis of fluorescein-labeled yeast particles and the intracellular killing of Staphylococci were studied in comparison to unsupplemented control cells. Results showed increased phagocytic and bactericidal activity were significant at physiological doses (100 to 200 ng Se/mL). There is a strong indication that supplementation of selenium might enhance phagocytic and bactericidal functions of human granulocytes, thereby improving the host's defense against disease (11).

It is not yet clear which mechanism or combination of mechanisms is responsible for the antitumorigenic effects of selenium. Much experimentation is still needed to answer this question. Although researchers cannot agree on the mechanism, they can verify results. Discovery of the antitumorigenic mechanisms of selenium

is critical if its potential is ever to be maximized in use against cancer.

Effects of Vitamin B-6 on Carcinogenesis

Vitamin B-6 status has been studied in an attempt to determine relationships between immune responses, recurrence rates, and severity of different types of human cancers (20,21). There have also been several attempts to identify a link between vitamin B-6 deficiency and growth rates of tumors in both humans and animals. Research in laboratory animals has led to speculation that vitamin B-6 may play a role in enzyme induction, and alterations in protein synthesis and expression (14,37,38).

To date, human studies regarding vitamin B-6 and cancer have centered on status measurement in the diseased state. Researchers have observed low plasma PLP levels and/or abnormalities of tryptophan metabolism in patients with Hodgkin's disease and cancers of the bladder, breast, throat, oral cavity, and lung (37,38). It is not known, however, whether vitamin B-6 status was suboptimal at the initiation phase of the disease, or whether deficiency actually precipitated the cancers.

A study by Byar et al. (20) indicated that metabolites of tryptophan may cause bladder cancer in labora-

tory animals. This information led the researchers to investigate abnormalities of tryptophan metabolism in humans with bladder cancer. Based on suggestions that vitamin B-6 may correct that abnormality and prevent recurrences of superficial bladder cancers, researchers compared vitamin B-6 supplementation, topical thiotepa, a drug commonly used in treating the disease, and a placebo to determine the efficacy of each on bladder cancer recurrence.

In this study, 121 patients with Stage I bladder cancer were randomly assigned to one of three treatment groups. One group received oral vitamin B-6 supplements (25 mg/day), while a second group was given thiotepa, 60 mg in 60 mL of water, instilled in the bladder for 2 h once a wk for 4 wk and then once a mo. The third group received a placebo. Average follow-up was 31 mo, although some patients were followed for longer periods of time. Overall, the study identified a 46.9% recurrence rate for the vitamin B-6 treatment group, a 47.4% rate for the thiotepa treatment group, and a 60.4% recurrence rate in the placebo group. There was no significant difference noted in bladder cancer recurrence rates between the vitamin B-6 and thiotepa treatment groups, while both had significantly lower rates when compared to the placebo group. Although both treatments demonstrated some effectiveness

against bladder cancer recurrences, researchers failed to measure vitamin B-6 status indices; therefore, no definite conclusions could be drawn regarding the protective role of vitamin B-6 in human bladder cancer.

Potera et al. (21) examined plasma PLP concentrations in patients with early breast cancer, locally recurrent disease, and systemic metastases. In addition urinary 4-pyridoxic acid, the major excretory form of vitamin B-6, was measured. Results of the study indicated that the patients with recurrent disease and systemic metastases had significantly lower PLP levels than 36 healthy controls. Normal PLP levels, however, were noted in the patients with early breast cancers. Urinary 4-pyridoxic acid excretion was shown to be normal in human breast cancer patients. Researchers concluded that low plasma PLP concentrations could have been caused by low dietary intakes of vitamin B-6. Other explanations for the low status were decreased net PLP synthesis or increased tissue binding of PLP in cancer patients. No factual support was given to either supposition.

A recent review article (39) concerning vitamin B-6 and cancer discussed whether or not abnormal tryptophan metabolism is indicative of vitamin B-6 deficiency. It is now suspected that the abnormal metabo-

lism of tryptophan may be linked to enzymes such as indoleamine dioxygenase (a recently described enzyme) which degrade 5 tryptophan to formylkynurenine and kynurenine by a mechanism which is different from that of liver tryptophan dioxygenase (EC 1.13.11.11). These researchers suggested that an abnormal tryptophan metabolism may be a marker for an immunological system activated against a specific cancer and might be independent of vitamin B-6 nutritional effects. Vitamin B-6 may instead be responsible for immune-linked hormone receptor regulation (estrogen and progestogen) which may induce elevated levels of alkaline phosphatase (EC 3.1.3.1) in turn decreasing PLP levels in certain types of cancers. Further clarification of mechanisms and study of enzyme induction is needed before further conclusions can be drawn with regard to vitamin B-6 and carcinogenicity.

Role of Vitamin B-6 in Chemoprevention

Vitamin B-6 has been shown to impair the growth of various transplanted tumors in laboratory animals when omitted from the diet or provided in suboptimal quantities (37). Tumor growth has also been demonstrated to be logarithmically linearly dependent on concentrations of vitamin B-6 in the diets of mice and rats (14). Growth rates of a variety of transplantable Morris hepatomas have been inhibited when vitamin

B-6 availability was restricted (14,15,16,17). The following text will describe the effects of vitamin B-6 on growth inhibition of a variety of tumor models in laboratory animals. Proposed mechanisms by which vitamin B-6 is thought to affect tumor growth will also be discussed.

The effects of vitamin B-6 deficiency on the growth rates of Morris hepatoma lines (#7777, 5123A, 7316B, and 7800) have been investigated based on the suggestion that the vitamin is needed for tumor growth (17). In this study, Buffalo strain weanling rats were maintained on a vitamin B-6 deficient diet for 21 days. After a 3 wk period, the rats were inoculated in the hind legs with tumor cells and divided into four treatment groups. One group was maintained on a deficient diet, a second group was changed to the supplemented diet (70 mg vitamin B-6/kg diet) and was pair fed to the first group and a third group was changed to the supplemented diet provided ad libitum. The control group received vitamin B-6 supplementation from the beginning of the study and received tumors on day 21. Results of the study indicated that significantly lower tumor weights were found in the rats fed a vitamin B-6 deficient diet from the outset of the study. The same observation was made in all four tumor cell lines

tested. Treatment groups receiving vitamin B-6 supplementation, whether ad libitum or restricted (pair fed), had significantly heavier tumors than the deficient group. The study also noted that pyridoxine HCl supplementation reduced host liver enzymes (the specific activity of liver and hepatoma tyrosine aminotransferase, TAT (EC 2.6.1.5). Researchers speculated that reduced tumor growth in the vitamin B-6 deficient rats may have been due to inhibited liver tyrosine aminotransferase induction in the absence of its cofactor. The exact mechanism is not known.

Tryfiates et al. later examined induction of tyrosine aminotransferase in hepatoma-bearing rats with respect to normal and cofactor (PLP) depleted animals (16). The investigators found that in vitamin B-6 deficient mice, the liver was depleted of cofactor (1.47 $\mu\text{g/g}$); whereas, the tumors had accumulated large quantities of cofactor (6.90 $\mu\text{g/g}$). This information suggests that since tumor growth was significantly inhibited by vitamin B-6 deficiency, the need for high concentrations of PLP by the tumor may identify the coenzyme as the growth limiting factor. Similarly, coenzyme activity was examined regarding serine dehydratase (EC 4.2.1.13), a PLP requiring enzyme, in this study Tryfiates (40) found that serine dehydratase activity depended greatly on dietary vitamin B-6 lev-

els. He also proposed that the in vitro activity levels are regulated by the presence or absence of vitamin B-6.

Findings by Tryfiates and coworkers have been supported by Thanassi et al. (41). Additional information provided from the study of Morris hepatomas indicates that metabolism of vitamin B-6 is different in these tumors than in host or control livers. These investigators suggested that the tumor is incapable of complete synthesis of PLP from unphosphorylated forms of vitamin B-6. Without PLP, tumor growth rates are inhibited.

Investigators strongly suspect that involvement of one or more vitamin B-6 coenzyme functions is responsible for its antitumorigenic effects. Vitamin B-6 has been shown to exercise a type of control over protein expression (38). In order to determine the relationship between vitamin B-6 coenzyme functions and antitumorigenicity, Tryfiates (38) examined the effects of a vitamin B-6 deficient diet on serum proteins of Morris hepatoma-bearing rats. Upon examination of sera by polyacrylamide gel electrophoresis (PAGE), the researcher noted absence of one post-albumin band from sera of vitamin B-6 depleted animals while two additional haptoglobins were found. A synergistic positive

effect between tumor presence and vitamin B-6 depletion was found regarding expression of serum proteins.

The relationship between vitamin B-6 and protein expression is not yet clearly understood, but there is some indication that the vitamin's coenzyme form (PLP) binds to and inactivates enzymes such as DNA and RNA polymerases (EC 2.7.7.7.7 and 2.7.7.6 respectively). Tryfiates feels that this inactivation could lead to abnormal protein expression or translational protein modification (38).

Involvement of vitamin B-6 in both humoral and cell-mediated immunity has become the subject of some study. To date, research has indicated that vitamin B-6 deficiency alters normal B lymphocyte and T lymphocyte proliferative responses when stimulated in vitro by mutagens and antigens. Vitamin B-6 has also been shown to reduce human antibody response to vaccines (12). Dietary vitamin B-6 deficiency has been shown to lead to impairment of DNA and protein synthesis. Both B and T lymphocyte and immunoglobulin production abnormalities are thought to be related to these DNA and protein synthesis impairments. Research investigating these claims has been presented by Gebhard et al. (42), who demonstrated what enhanced immune response in mice corresponded to increased dietary vitamin B-6 intakes. In this study BALB/c mice were maintained on either low

or high vitamin B-6 diets (4 mg and 62 mg/kg diet, respectively). Four weeks after dietary treatments began, 40 animals were injected subcutaneously with human malignant melanoma (MZI-HPB) cells. After 4, 8 and 10 wk several animals were sacrificed and blood and spleen samples were obtained. There were no significant or notable differences between tumor sizes of either group when compared to control (non-injected) mice. The high vitamin B-6 diet did, however, precipitate significantly increased oxygen radical production in the blood of both control and tumor-burdened mice. Researchers speculated that dietary intake of vitamin B-6 did not modify tumor growth due to an absence of T lymphocyte-dependent immune mechanisms. A similar study conducted by Gridley et al. (19) suggested that high dietary intake of vitamin B-6 suppressed herpes simplex virus type 2-transformed (H238) cell-induced tumors, either by immune enhancement or PLP growth regulation. In this study, male BALB/cByJ mice were provided diets containing either 0.2, 1.2, 7.7, or 74.3 mg vitamin B-6/kg diet (ad libitum). Thus, the diets ranged from suboptimal (0.2 mg/kg diet) to high (74.3 mg/kg diet) in vitamin B-6 concentration. Results of the study showed overall lower tumor volumes in animals fed 7.7 and 74.3 mg vitamin B-6/kg diet as compared to

the control group (1.2 mg vitamin B-6/kg diet). Mice provided the vitamin B-6 deficient diets, however, had the lowest tumor volume and no incidence of metastases. The study also indicated that blood and spleen lymphoproliferative response to stimulation by phytohemagglutinin or concanavalin A were generally higher in the mice supplemented with 7.7 mg/kg diet and 74.3 mg/kg diet mg when compared to both the control and deficient groups. Researchers found it tempting to speculate that high vitamin B-6 intakes modified tumor growth via an effect on the cell-mediated branch of the immune system.

Role of Selenium and Vitamin B-6 on Tumorigenesis

The mechanisms by which selenium and vitamin B-6 modify tumorigenesis are still a subject of much speculation. It appears possible, however, that immune system enhancement as well as, DNA, RNA, and protein synthesis may all be involved. To date, there is no available information regarding the concomitant effects of selenium and vitamin B-6 on tumorigenesis. The purpose of the following study was to identify the effects of these nutrients on chemically-induced transplantable fibrosarcomas in BALB/c mice. Status levels of selenium and vitamin B-6 in blood were determined in addition to tumor concentrations of these two nutrients.

PROCEDURES

Animal Care and Diet

All procedures were approved by the Virginia Polytechnic Institute and State University Animal Care Committee. Eighty-four BALB/c male weanling inbred mice (Dominion Laboratories, Inc., Dublin, VA) were received in good health and were inspected by a veterinarian to verify wellness. Initial weights were determined to be between 12 and 13 g. The mice were housed in single suspended galvanized cages exposed to 23°C, 50% humidity, and a 12 h light/dark cycle. Ceramic food dishes were acid washed and double rinsed in distilled deionized water to reduce selenium residue. Distilled deionized water was provided ad libitum in plastic bottles with silicone stoppers. Cages and feeding equipment were washed weekly (dishes 3 times weekly) to reduce the risk of infection among the mice.

The mice were maintained on a nutritionally adequate diet ad libitum during a one wk acclimation period. Composition of the diet is described in Table 1. The powdered diet was commercially prepared (ICN Biomedicals, Cleveland, OH) according to specifications. The diet was initially prepared in loose powder form without vitamin B-6 (7 mg/kg of vitamin B-6 was added to the acclimation diet to provide adequate

TABLE 1

DIET COMPOSITION

INGREDIENT	PERCENTAGE
Sucrose	35.0
Cornstarch	30.0
Casein (vitamin-free)	20.0
Fiber (Alphacel)	5.0
Corn oil (tocopherol-stripped)	5.0
AIN Mineral Mix ²	3.5
AIN Vitamin Mix ² (pyridoxine HCl ³ was omitted and vitamin E was added at 50 IU/kg vitamin mix)	1.0
L-Methionine	0.3
Choline bitartrate	0.2

¹Powdered diet was prepared by ICN Biomedicals Inc., Cleveland, OH.

²Composition given in Table 2.

³Pyridoxine HCl was added in desired quantities.

nutrition). Selenium was added in nutritionally adequate quantities (0.1 mg/kg diet). Corn oil was stripped of tocopherol to control antioxidant interference which masks the effects of selenium. Vitamin-free casein was used to further control vitamin levels. Vitamin B-6 was added to the diet in the form of pyridoxine HCl in desired quantities. Specific contents of the mineral and vitamin mixtures used in preparing the diets are listed in Table 2.

After the one wk acclimation period the mice were randomly divided into three treatment groups of 24 animals and a control group of 12. The first of the three treatment groups was provided a diet containing excess vitamin B-6 (100 mg/kg diet); and the third group was provided a suboptimal vitamin B-6 diet (0.5 mg/kg diet). The control group was maintained on the adequate vitamin B-6 diet. All diets were provided ad libitum throughout the study. Food consumption and animal weights were obtained every three days to monitor health status of the mice.

Two wk after dietary vitamin B-6 manipulations began, tumor cell inoculations were initiated. A general outline of the animal treatment schedule is presented in Table 3. Tumor cell preparation and inoculation is described in the following section.

TABLE 2

AIN-76 MINERAL MIX

Manganese	54.0 mg/kg diet
Selenium	0.1 mg/kg diet
Zinc	30.0 mg/kg diet
Chromium	2.0 mg/kg diet
Copper	6.0 mg/kg diet
Iodine	0.2 mg/kg diet
Iron	35.0 mg/kg diet
Calcium	0.52 % mix
Chloride	0.16 % mix
Magnesium	0.05 % mix
Phosphorous	0.40 % mix
Potassium	0.36 % mix
Sodium	0.10 % mix

AIN-76 VITAMIN MIX¹

Vitamin A	4000 IU/kg diet
Vitamin D	1000 IU/kg diet
Vitamin E	50 IU/kg diet
Vitamin K	0.05 IU/kg diet
Vitamin B-12	0.01 mg/kg diet
Biotin	0.20 mg/kg diet
Choline	1000 mg/kg diet
Folacin	2.0 mg/kg diet
Niacin	30.0 mg/kg diet
Calcium Pantothenate	16.0 mg/kg diet
Riboflavin	6.0 mg/kg diet
Thiamin	6.0 mg/kg diet

¹Vitamin E and B-6 contents differ from AIN mix.

TABLE 3

ANIMAL TREATMENT SCHEDULE

Day 7	End of one wk acclimation period
Day 8	Beginning of vitamin B-6 manipulation
Day 21	Tumor cell inoculation
Day 22	Beginning of selenium injections (continued 5 times over 10 days)
Day 32	Day of sacrifice

Preparation of Tumor Cells for Inoculation

Three BALB/c mice were given right hindquarter injections of fibrosarcoma cells, chemically-induced by 2-methylcholanthrene, in a procedure developed by Elgert and Farrar (43,44). All reagents used in this procedure are listed in Appendix A, whereas, chemicals and vendors are listed in Appendix B. The mice were sacrificed by spinal dislocation three wk after tumor cell inoculation. The three animals were placed in a lamina flow hood while the tumors were excised to reduce contamination of cell matter. The tumor cell matter was removed from the hindquarter with a sterile scalpel and was then forced through a fine metal sieve into a mortar containing isotonic RPMI media (cell stabilizer). The resulting cell matter was centrifuged for 5 min at 500 x g in a Jouan GR411 centrifuge at 23°C. The supernatant was poured off and the precipitant was vortexed to resuspend it in fresh RPMI media. Next, 25 μ L of the cell suspension was added to 375 μ L isotonic saline and 100 μ L of trypan blue stain. The 500 μ L cell suspension was then pipetted into a hemocytometer which was placed under a Bausch and Lomb microscope on 2X power. Viable fibrosarcoma cells were counted on the hemocytometer to determine the ultimate live cell concentration needed to produce a tumor in the host mice. The cell dilution was calculated from

the hemocytometer viable cell count to produce a suspension containing 0.5 million tumor cells in 0.10 μ L RPMI media.

Prior to tumor cell inoculation, the hindquarter of each mouse was rinsed with absolute ethanol to prevent infection. Next, each mouse was placed in a restraint to prevent movement during inoculation. The single cell suspension containing the tumor cells was then injected in 0.01 μ L quantities into the right hindquarter of each of 72 BALB/c mice using a 25 gauge tuberculin needle. After the inoculations, the mice were returned to their cages and observed for adverse reactions. None were noted.

Selenium Treatments

Selenium treatments were begun 24 h after tumor cell inoculation. Sodium selenite was administered via intraperitoneal injection of 0.5 μ g in 0.10 mL isotonic Krebs Ringer Phosphate buffer. Selenium injections were given to half of each vitamin B-6 treatment group and all controls using a 25 gauge tuberculin needle. These treatments continued every other day for a ten day period (five times). Intraperitoneal injections were given with the help of a lab assistant who grasped the mouse by the nape of the neck and exposed the abdomen upward. The peritoneum was first rinsed with

absolute ethanol, then pinched and lifted while the needle was gently slipped into the peritoneal cavity. After the contents of the needle were expelled, the mouse was placed back into the cage and observed for signs of infection or distress. None were noted.

Animal Sacrifice Procedures and Plasma and Tumor
Sample Pretreatment

Two wk after the mice had been inoculated with tumor cells, sacrifice was completed in a random manner. The animals were sacrificed by carbon dioxide suffocation in a glass tank. The procedure required approximately 30 sec, after which the mice were secured on a dissection tray. The chest and abdomen were soaked with absolute ethanol before a midline incision was made with sterile scissors to expose the cardiac cavity. After separation of the ribs by retraction, the pericardium was removed and the heart was exposed. A 25 gauge tuberculin needle was inserted to draw the blood by cardiac puncture.

The blood was immediately transferred to 10 mL blood collection tubes treated with sodium EDTA to prevent clotting. The blood samples were placed in ice and covered with aluminum foil to protect them from light. In a dark lab, the blood samples were decanted into conical plastic tubes and centrifuged for 20 min at 2,000 x g at 5° C. The top layer of plasma was

drawn off by Pasteur pipette immediately after centrifugation and was placed in polypropylene tubes which were stored frozen (-20°C) for later analysis.

Tumor Excision

After blood was drawn, the mice were placed in labeled plastic bags and stored on dry ice. The tumors were excised in a semifrozen state in order to protect the vitamin B-6 content. The tumor-burdened leg was removed above the tumor using sterile scissors. The foot was also removed below the tumor. The tumor isolated portion of the leg was stripped of fur and rinsed with distilled deionized water. The alternate leg was removed and stripped in the same manner as the tumor-burdened leg for weight adjustment. The tumor leg portion was weighed as was the alternate leg portion. The latter weight was subtracted from the former in order to obtain an accurate tumor tissue weight. The tissues was immediately frozen at -20°C in labeled plastic bags for later analysis.

It should be noted that blood and tumor samples were paired randomly within treatment groups in order to produce large enough sample sizes for analysis. Half of each paired plasma and tumor sample was used for selenium analysis and half was used for vitamin B-6 analysis.

Selenium Determination in Mouse Blood by Gas Chromatography

Selenium determination in mouse blood was performed using gas chromatography in a method described by McCarthy et al. (45). Chemicals and vendors are listed in Appendix C.

A. Blood sample preparation

Seven mL glass weighing vials were prepared with 50 μ L of whole blood and 1.5 mL of concentrated nitric acid containing 30% magnesium nitrate. The vials were transferred onto a heating block under an acid fume hood. The samples were heated for 1 h (approximately 115°C) followed by 1 h at 125°C. Next, the samples were placed on a hot plate on which the temperature was gradually increased over 1.5 h to 450 °C until no liquid remained in the vials. When the nitrous oxide fumes ceased, the samples were removed from the fume hood. During this time the nitric acid and magnesium nitrate was used to raise the oxidation state of selenium to selenium VI. Digestion of the blood samples was completed in a muffle furnace set at 500°C for 1 h. After the samples cooled, 1.5 mL of concentrated HCl was added to the ash in the bottom of each vial. The capped vials were then placed on a heating block at 90°C for 15 min. The addition of HCl reduced selenium to selenium IV. Next, 2.5 mL of the following solution

was added to the cooled contents of each vial: 1% hydroxylamine sulfate, 1% EDTA, 15% urea (w/v) in water. Addition of these reagents helped remove interference during the gas chromatography peak analysis. The samples were allowed to stand for 10 min after which 200 μ L of complexing solution was added.

B. Complexing solution

Complexing solution, 4-nitro-o-phenylenediamine (4-NPD), was converted to the chloride salt by adding 2 M hot HCl. This solution was filtered and chilled resulting in crystals which were washed with concentrated HCl. Complexing solution was prepared by dissolving the chloride salt in 1 N HCl (0.5 w/v).

The complexing solution was mixed into the vial contents and the vials were placed on a heating block at 45°C for 30 min. Next, 1 mL of toluene was added and the contents of the vial were vortexed for 5 sec. A portion of the toluene layer was pipetted into teflon capped vials for analysis. Four microliters of this solution were used for gas chromatography injection.

C. Instrument conditions

Selenium analysis was performed on the Varian GC 3700. Injection temperature and column temperature were 250°C and 230°C respectively. The electron capture detector temperature was 320°C and the gas flow

was 30 mL/min.

Calculations of selenium in blood samples were based on a standard curve developed from a range of 50 ppb to 800 ppb selenium. The stock standard selenium solution contained 0.10 ppm in 5% nitric acid. A typical standard curve is presented in Appendix D.

Assessment of Selenium in Fibrosarcomas by Atomic Absorption

Assessment of selenium in fibrosarcomas was performed using a modification of a procedure described by Brodie (46). Chemicals and vendors are listed in Appendix E.

A. Tumor sample preparation

Tumor samples were weighed in tared 40 mL Pyrex beakers and placed under an acid fume hood. Each sample was diluted to a volume of 25 mL with nitric and perchloric acids (4:1). Two acid blanks were also prepared for comparison. The contents of the beakers were then wet ashed on heating units at 200°C until approximately 2 mL of sample digest remained in each beaker. The digested material was allowed to cool, after which each sample was brought to 5 mL with distilled deionized water. The contents of the beakers were then decanted into glass conical Kimax tubes (40 mL) containing 5 mL of 20% concentrated HCl. This step reduced selenium VI to selenium IV.

Each tube was brought to a volume of 25 mL with distilled deionized water. Next, the tubes were capped and allowed to stand under an acid fume hood for 1 h. After the elapsed hour, the tubes were inverted twice to mix the contents for analysis.

B. Preparation of selenium standards

Working selenium standard (1 ppm) was prepared by diluting 0.1 mL of 1,000 ppm stock to 100 mL with distilled deionized water. Standards containing 0, 2.5, 5.0, and 10 ppb were prepared by diluting 0, 0.25, 0.5, and 1.0 mL of working standard (1 ppb) to 100 mL, including 20% concentrated HCl.

C. Selenium analysis

Ten mL of diluted sample digest was mixed with 10 mL of distilled deionized water in the reaction vessel of the atomic absorption spectrophotometer by a magnetic stirring bar. The carrier gas (N_2) was allowed to flow for 30 sec during which time the oxygen present was displaced from the quartz tube by nitrogen. Zero was calibrated using the zero selenium standard. A selenium standard curve was prepared for comparison against the tumor samples. A typical curve is represented in Appendix F. All samples and standards were prepared in duplicate for greater accuracy. Calculations of tumor selenium concentrations were based on values obtained from the standard curve according to

atomic absorbance. Data is presented in ppb per gram and per whole tumor.

D. Instrument conditions

Se lamp, 9 mA lamp current, 1.0 nm slit, double-beam, background-196 nm wavelength, peak height mode with 10 sec observation time. Flame:air:acetylene (6:5:2, v/v); N₂ at PSI 20. Equipment: Varian AA-475 Series.

Radiometric Determination of Pyridoxal Phosphate (PLP) in Mouse Plasma

The analysis of plasma PLP was accomplished using the method of Chabner and Livingston (47) as modified by Reinken (48) and Fries et al. (49). Reagents used are listed in Appendix G. Chemicals and vendors are listed in Appendix H.

A. General procedures

In order to protect vitamin B-6 from light and heat destruction, the samples, standards, and reagents were kept cold and were protected from light. Red lamps were used as an alternative light source. The use of radioactive materials prompted protection by latex gloves.

B. Purification of tyrosine decarboxylase apoenzyme

Streptococcus faecalis (ATCC 8043) acetone powder (1.0 to 1.5 g) was ground with 5 g of alumina for 15

min in a chilled mortar. The contents were extracted with 20 mL of cold 0.01 M phosphate buffer (pH 7) and centrifuged for 20 min at 5°C (17,000 x g). The resulting supernatant was measured and added to solid ammonium sulfate at a concentration of 390 mg/mL supernatant (concentration established by Chabner and Livingston). The mixture was dissolved. This suspension was centrifuged again in the same manner listed above. The resulting supernatant was again measured and brought to 85% enzyme saturation by adding 220 mg/mL of ammonium sulfate, stirring mechanically for a 1 h period. The solution was centrifuged again, after which the supernatant was poured off and discarded. The precipitant was dissolved in 2 mL of enzyme buffer solution. The final enzyme solution was frozen in 100 μ L quantities in polypropylene tubes for later use in the radioassay of plasma PLP.

C. Blood sample preparation

Immediately preceding analysis, the frozen plasma samples were thawed in a cold water bath for approximately 25 min. When the plasma was completely fluid, 225 μ L were pipetted into a plastic centrifuge tube with 25 mL of cold 10% trichloroacetic acid (TCA). The contents were vortexed and centrifuged in the same manner stated above. The supernatant was decanted into glass corex tubes with 225 μ L of (20°C) freon amine.

This solution was vortexed and centrifuged for 20 min at 2000 x g (5°C). After centrifugation, the top phase was extracted by Pasteur pipette. This phase was used for radioassay of PLP.

D. Analysis of plasma PLP

Ehrlenmeyer flasks (25 mL) were placed in a rack surrounded by ice. The flasks were set up to contain two blanks, four vitamin B-6 standards, and three plasma samples. The standards were later used to develop a calibration curve for vitamin B-6. Quantities for 40, 100, 200, and 400 ng B-6/20 μ L were placed in the standard flasks and were prepared in triplicate. Plasma samples were also prepared in triplicate.

The first phase of analysis involved adding 10 μ L of cold tyrosine decarboxylase apoenzyme to the back of each flask and either the sample or the standard to the inside front of the flask to prevent mixing until the buffer was added. The contents were then washed into the flask with 1.5 mL of 0.1 M sodium acetate buffer. The flasks were swirled to mix. Next, the flask stoppers were prepared by placing a folded strip of Whatman #42 filter paper (5.1 cm x 0.6 cm) in a suspended plastic center well which was attached to each stopper. The filter paper was then soaked with 200 μ L of cold hyamine hydroxide and the stoppers were

placed in each flask. The stoppers were secured by folding the sides down over the rim to create an air-tight environment within the flask.

In the next phase of the assay, the flasks were placed in a 37°C water bath to initiate holoenzyme formation (15 min). Next, the rack was transferred to an ice bath for 10 min to slow the reaction. After the ice bath, 250 µL of L-tyrosine-1-¹⁴C solution was injected through each stopper along the side of each flask. The flasks were transferred back to the 37°C water bath for 15 min to allow the enzyme-substrate reaction (tyrosine to tyramine) to take place. The flasks were removed from the bath and injected with 0.1 mL of cold 10% TCA to halt the reaction. The flasks were then returned to the 37°C water bath for 1 h to be sure all the radioactivity evolved from the solution collected on the filter paper.

At the end of the hour, the stoppers were removed from the flasks and each center well was clipped off and placed in separate scintillation vials with 5 mL of Turner scintillation medium. The vials were capped tightly, wiped down with fabric softener, and arranged in a Beckman scintillation counter. Each vial was counted for 2 min on Channel 3 set for beta particle emission. The readings for the plasma samples were compared to those of the standards to determine PLP

concentrations. A representative standard PLP calibration curve can be found in Appendix I. PLP data, expressed in ng/mL and nmol/L of plasma, were determined by comparison to the standard calibration curve.

Microbiological Assay of Vitamin B-6 in Tumors

Microbiological assay of tumor vitamin B-6 was performed using a modification of the A.O.A.C. (Association of Official Analytical Chemists) procedure (50). Reagents used in the procedures are listed in Appendix J. Chemicals and vendors are listed in Appendix K.

A. Assay inoculum

Saccharomyces uvarum (ATCC 9080) was aseptically inoculated on Difco bacto YM agar slants which were incubated for 20 h in a 30°C water bath. After the incubation period, assay inoculum was prepared. Ten mL of pyridoxine Y media was steamed in a 50 mL screw top tube for 10 min.

B. Tumor slurry preparation

Tumors were weighed (approximately 1.5 g) and placed in 20 mL glass beakers with 10 mL of distilled deionized water and one drop of antifoam A. The samples were then homogenized for 30 sec with a Polytronic Blender. The samples were then diluted to 200 mL with 0.055 N HCl. These preparations were autoclaved for 5

h at 121°C at 15 PSI. After cooling, the pH was adjusted to 4.5 with 6 N KOH and glacial acetic acid. The samples were then brought to 250 mL with distilled deionized water. Each sample was then filtered through two combined sheets of #42 Whatman filter paper. The clear sample extracts were stored on ice in dark bottles for immediate analysis.

C. Preparation of vitamin B-6 standards

Vitamin B-6 standards were prepared so that 0.025, 0.5, 1.0 and 2.0 mL contained 0.5, 1.0, 2.0, and 4.0 ng of vitamin B-6 ethanol and distilled deionized water.

D. Preparation and analysis of vitamin B-6 standards and tumor samples

Tumor sample extracts and vitamin B-6 standards were added to micro-clean glass tubes submerged in an ice bath.

The tumor extract was measured in quantities of 0.025, 0.50, and 1.0 mL to best find the range of effectiveness when compared to the standards. Each sample was duplicated to provide for a more accurate comparison. When all the tubes were prepared with tumor extract or standard, the contents were brought to a volume of 5 mL with distilled deionized water and to 10 mL with pyridoxine Y media. Each tube was then inoculated with *S. uvarum* inoculum in 20 μ L quantities. All tubes were incubated for 20 h at 30°C, afterwhich they were

steamed for 5 min to halt bacterial growth. The tubes were vortexed to resuspend the *S. uvarum* cells. The contents of all tubes were transferred into spectrophotometer tubes (Bausch & Lomb) and transmittance was measured on a Bausch and Lomb Spectronic 20 at 550 nm transmittance. A calibration curve developed from the vitamin B-6 standard transmittances was used to determine the concentration of the vitamin in the tumor extracts. A sample standard B-6 curve is presented in Appendix L. Data are represented as nmol and nanograms per gram and per whole tumor.

Statistical Analysis

Statistical significance was determined by ANOVA and Duncan's multiple range test (51). Pearson correlations were used to identify interrelationships between variables and treatments. Groups were assigned according to vitamin B-6 and selenium treatments. Data were presented as group means ($\bar{X}$) accompanied by standard errors of the means (SEM).

RESULTS AND DISCUSSION

Animal Weight and Food Consumption Measurements

Animal weights between groups did not differ significantly at any time during the study (Table 4). The adequate vitamin B-6 control group, not injected with tumors, gained the most weight during the study. These animals remained in obvious good health and exhibited no changes in behavior. The smallest net weight gain was noted in the suboptimal vitamin B-6 selenium-treated group. Food consumption patterns coincided with overall total weight gain of these groups during most of the consumption measurement periods (Table 5). In order to clearly evaluate the effects of selenium and vitamin B-6 manipulations on food consumption and animal weights, each treatment group must be discussed separately. The following sections will discuss the separate effects of selenium and vitamin B-6 with regard to growth rates and eating patterns. Observations of side effects and health of the animals were also noted. Individual data regarding animal weight and food consumption are presented in Appendices M and N.

A. Effects of selenium on animal weight and food consumption

Intraperitoneal injection of 5 ppm of sodium

TABLE 4

ANIMAL WEIGHTS

Vitamin B-6 group	Weight Periods							Total gain
	1	2	3	4	5	6	7	
Excess								
Selenium-treated	17.8±0.2	19.2±0.3	19.3±0.4	19.8±0.3 ^g	19.9±0.4	19.8±0.4	20.2±0.5	7.64±0.4
Untreated	17.4±0.3	18.6±0.3	18.6±0.4	19.1±0.4	19.0±0.4	19.1±0.4	19.5±0.4	6.98±0.4
Adequate								
Selenium-treated	17.3±0.2	18.3±0.3	19.0±0.4	19.3±0.3	19.9±0.4	19.8±0.4	19.3±0.5	6.69±0.4
Untreated	17.5±0.2	18.5±0.3	19.1±0.5	19.5±0.4	19.9±0.4	19.7±0.3	19.1±0.6	6.33±0.2
Selenium-treated, no tumor	17.5±0.2	18.1±0.2	18.7±0.2	19.4±0.3	19.6±0.3	19.7±0.2	20.4±0.3	7.72±0.2
Suboptimal								
Selenium-treated	17.7±0.2	18.6±0.3	18.9±0.3	19.0±0.2	18.8±0.2	19.3±0.4	18.9±0.5	5.94±0.3
Untreated	17.9±0.2	19.2±0.4	19.5±0.3	19.3±0.2	19.5±0.3	20.2±0.4	19.4±0.4	6.45±0.3

 $\bar{X}$ =SEM.

TABLE 5

DAILY FOOD CONSUMPTION OF MICE¹
ALL GROUPS

Vitamin B-6 Group	Period 1 ²	Period 2 ³	Period 3 ³	Period 4 ³	Period 5 ³
g					
Excess					
Selenium-treated	3.7±1.0 ⁴	4.2±0.1 ⁴	4.0±0.1 ⁷	3.5±0.1 ⁵	2.4±0.3 ⁶
Untreated	3.8±0.2 ⁴	4.1±0.2 ⁷	4.0±0.1 ⁷	3.5±0.1 ⁵	2.7±0.4 ⁶
Adequate					
Selenium-treated	3.1±0.1 ⁵	3.7±0.1 ⁹	3.7±0.1 ⁸	3.5±0.1 ⁵	4.4±0.5 ⁵
Untreated	3.1±1.2 ⁵	4.0±0.1 ⁹	3.9±0.1 ⁷	3.6±0.1 ⁵	4.0±0.4 ⁵
Selenium-treated, no tumor	3.6±0.0 ⁴	3.9±0.1 ⁹	4.1±0.0 ⁴	4.2±0.1 ⁴	4.7±0.2 ⁷
Suboptimal					
Selenium-treated	3.5±0.1 ⁷	3.5±0.1 ⁸	3.3±0.1 ⁶	3.1±0.2 ⁵	5.9±0.2 ⁴
Untreated	3.7±0.1 ⁴	3.4±0.2 ⁶	3.4±0.2 ⁶	3.2±0.2 ⁵	5.9±0.3 ⁴

¹ $\bar{X} \pm \text{SEM}$, n = 12.

²Mean food consumption for the first 11 days on experimental diets.

³Periods were of three days duration.

⁴Significantly higher at $P < 0.01$.

⁵Significantly different at $P < 0.01$.

⁶Significantly lower at $P < 0.01$.

⁷Homogeneous subgroups of ⁴ and ⁵.

⁸Homogeneous subgroups of ⁵ and ⁶.

⁹Homogeneous subgroups ⁴, ⁵ and ⁶.

selenite (0.5 $\mu\text{g}/0.10\text{ mL}$) produced no toxic side effects and had no significant effects on body weight or food consumption measurements as compared to those of unsupplemented groups (Table 6).

Selenium supplementation at 5 ppm has been shown to produce toxic side effects in some animal studies while others noted no symptoms. At 5 ppm, toxic side effects have been noted in the form of reduced weight gain, liver damage, and death. (22) Selenium supplements given at 2 $\mu\text{g}/\text{g}$ body weight produced symptoms of increased irritability and decreased intestinal tissue mass in mice without tumors (25). Tumor-burdened rats given 5 ppm dietary selenium produced a 10% suppression in weight gain which was directly related to decreased food consumption (9). No symptoms were noted at 2.5 ppm selenium.

Results of our study are in agreement with those found by Poirier and Milner (26) who reported no significant difference in food consumption in Swiss ICR tumor-burdened mice given 2.5 or 5 ppm selenium supplemented diets. The same study reported no difference in body weights (10 days post-weaning) in mice given 2 μg of sodium selenite by gastric gavage or intraperitoneal injection when compared to unsupplemented controls. Overall weight gain, however, was significantly decreased in mice given 5 ppm sodium selenite via intra-

TABLE 6

DAILY FOOD CONSUMPTION OF MICE¹
BY SELENIUM GROUP

Treatment group	Period 1 ⁴	Period 2 ⁵	Period 3 ⁵	Period 4 ⁵	Period 5 ⁵
	g				
Selenium-treated ²	3.5±0.0	3.8±0.1	3.8±0.1	3.6±0.1	4.3±0.2
Untreated ³	3.6±0.1	3.8±0.1	3.7±0.1	3.4±0.1	4.2±0.3

¹ $\bar{x}$ = SEM.

²N = 48.

³N = 36.

⁴Mean food consumption for the first 11 days on experimental diets.

⁵Periods were of three day duration.

peritoneal injection due to decreased ascitic fluid accumulation. No toxic side effects were noted in mice given 5 ppm selenium supplements regardless of administration route.

B. Effects of vitamin B-6 on animal weight and food consumption

Effects of vitamin B-6 manipulations on animal weight and food consumption patterns are presented in Tables 4 & 7. Animal weights among groups, as previously stated, were not significantly different although some trends were noted. The excess and adequate vitamin B-6 groups (untreated) had lower total body weight gains than their selenium-treated counterparts. The mice in the suboptimal vitamin B-6 groups, however, did not follow this trend. The untreated suboptimal vitamin B-6 group had slightly higher total weight gains when compared to their selenium-treated counterparts, perhaps because tumor weights (Table 8) of this group were slightly higher. Food consumption patterns of the suboptimal vitamin B-6 groups were not significantly different from each other after tumor inoculation or during selenium treatment periods 3, 4 and 5, thus ruling out treatment variables as the cause of the total weight gain differences. Prior to food consumption period 1 there were two initial consumption measurement periods which accounted for the seven day

TABLE 7

DAILY FOOD CONSUMPTION OF MICE¹
BY VITAMIN B-6 GROUP

Vitamin B-6 group	Period 1 ⁴	Period 2 ⁵	Period 3 ⁵	Period 4 ⁵	Period 5 ⁵
	g				
Excess ²	3.7±0.1 ⁶	4.1±0.1 ⁶	4.0±0.1 ⁶	3.5±0.1 ⁶	2.6±0.2 ⁸
Adequate ³	3.3±0.1 ²	3.9±0.1 ⁶	3.9±0.1 ⁶	3.8±0.1 ⁶	4.3±0.2 ⁷
Suboptimal ²	3.6±0.1 ⁶	3.5±0.1 ⁸	3.4±0.1 ⁸	3.1±0.1 ⁸	6.0±0.2 ⁶

¹ $\bar{X}$ = SEM.

²N = 24.

³N = 36.

⁴Mice fed experimental diet for 11 days before tumor inoculation.

⁵Periods were of three days duration.

⁶Significantly higher at P<0.01.

⁷Significantly different at P<0.01.

⁸Significantly lower at P<0.01.

TABLE 8

TUMOR WEIGHTS BY GROUP¹

Vitamin B-6 group	g
Excess	
Selenium-treated	2.05±0.13 ⁴
Untreated	2.48±0.15 ²
Adequate	
Selenium-treated	1.79±0.20 ³
Untreated	2.27±0.23 ⁴
Suboptimal	
Selenium-treated	2.05±0.14 ⁴
Untreated	2.30±0.21 ⁴

¹ $\bar{X} \pm \text{SEM}$, n = 12.²Significantly larger at P<0.01.³Significantly smaller at P<0.01.⁴Homogeneous subgroups.

acclimation period. There were no significant explainable differences in food consumption patterns although some differences were noted. These were associated with acclimation differences since no dietary vitamin B-6 manipulations were initiated until the onset of period 1. Periods 1 and 2 encompass the first fourteen days of the vitamin B-6 manipulations. Both the excess and suboptimal groups consumed significantly less food as compared to the adequate vitamin B-6 groups for the first 11 days of the vitamin B-6 manipulation periods. This trend changed during period 2 in that the suboptimal vitamin B-6 group consumed significantly less food than both the excess and adequate vitamin B-6 groups. This trend continued during food consumption periods 2, 3, and 4 of the study. A study similar to the current one using BALB/c tumor-burdened mice found nearly the same animal weight and food consumption patterns (19). In this study mice were provided diets (ad libitum) containing vitamin B-6 in concentrations ranging from 0.2 mg/kg diet (suboptimal) to 74.3 mg/kg diet (excess). Food consumption ranged from 2.8 g to 3.1 g per day, respectively. It should be noted that although the food consumption patterns were similar to those found in the present study, animals in the present study consumed more food. It is also interest-

ing to note that their animals weighed approximately 23 to 32 g at the end of the study while those in the present study ranged from approximately 19 to 20 g. There is no obvious reason for this difference except that strains of mice may vary as inbreeding occurs in mice populations. In a second study involving BALB/c tumor-burdened mice, researchers provided either 4 mg vitamin B-6/kg diet or 62 mg vitamin B-6/kg diet for a 10 wk period. This study found no significant differences in food consumption or animal weights at these dietary vitamin B-6 levels. It was the feeling of these authors that these levels were neither suboptimal or excessive enough to produce significant results.

Vitamin B-6 deficient diets have been shown to result in lower body weights when compared to supplemented controls (19,26). Tryfiates et al. found vitamin B-6 to be a growth limiting factor (15). Rats maintained on vitamin B-6 deficient diets failed to grow and often died prematurely. If the rats were given vitamin B-6 after periods of deficiency, they began to grow normally again. Results of the current study do not generally agree with the findings reported by others (8,9,15) regarding growth inhibition. Mice in the present study, however, were provided diets containing suboptimal levels of vitamin B-6 which were not low enough to inhibit growth, whereas those in other

studies were fed diets deficient in the vitamin. There was, however, some weight loss noted among the adequate and suboptimal vitamin B-6 tumor-burdened mice at the conclusion of the study. These findings could only be explained concerning the suboptimal vitamin B-6 groups, since these mice consumed significantly less food for a large part of the study (periods 2, 3 and 4). The same was not true of the adequate vitamin B-6 groups.

The eating behavior of mice in all three vitamin B-6 treatment groups was altered during the final food consumption period. The suboptimal vitamin B-6 treatment groups, which previously had in general eaten significantly less food than the other groups, now had the highest food consumption of all groups. These mice also displayed unusual behavior patterns including hyperirritability, viciousness toward handlers and frequent periods of voracious food consumption, during which time they ran into and out of the food containers. In addition, the suboptimal vitamin B-6 group developed alopecia, dermatitis of the face, and hindend tremors. All of these symptoms and behaviors are well documented side effects of vitamin B-6 deficiency in laboratory animals (52). The slight suppression in weight gain in this group is also considered to be a

result of increased physical activity and slightly decreased food consumption during the aforementioned periods of the study.

The final period of food consumption measurement also identified the excess vitamin B-6 treatment groups as consuming significantly less food than all other groups. Mice in these groups were among the first to show signs of necrotic tissue on the tumor-burdened legs. These animals also became less physically active, appeared disinterested in their environment, and were noted to consume smaller quantities of food with much less frequency than previously noted. Similar findings were detailed by Driskell et al. (53) in a study which examined food consumption patterns and activity levels of rats provided 100 and 200 times the recommended levels of dietary vitamin B-6. Although the decrease in food consumption and activity levels of the excess vitamin B-6 groups may have been side effects of oversupplementation, these changes may also have been related to the worsening physical condition of these animals due to increasing tumor size.

The adequate vitamin B-6 groups continued to eat normally throughout the study and were noted to be in better general health than the other two vitamin B-6 treatment groups. Smaller tumor sizes of these groups may have contributed to these observations.

Tumor Weights

Tumors were developed in 100% of the inoculated mice and were palpable within three days. Tumor sizes of mice in all treatment groups, represented as grams of tumor, are presented in Table 8. Individual data are presented in Appendix O. Treatment groups can be identified by group number in Appendix P. Tumor sizes of the excess vitamin B-6 group, not treated with selenium, were significantly larger ($P < 0.05$) than those of the adequate vitamin B-6 selenium-treated group. All other treatment groups had statistically similar tumor weights. Optimal tumor growth inhibition can be achieved when vitamin B-6 is given in adequate quantities in combination with selenium supplementation.

When tumor weights are considered without regard to vitamin B-6 manipulations, all selenium-treated groups combined had significantly smaller tumors ($P < 0.05$) than the untreated groups (Table 9). The effect of selenium supplementation on tumor growth inhibition noted in this study is well supported in the literature (3,7,8,9,23,30). Selenium supplementation of 5 ppm, regardless of administration route, significantly inhibited tumor growth in both chemically- and virally-induced tumors in laboratory animals. Some variation may be due to the manner in which the tumors were

TABLE 9

TUMOR WEIGHTS¹
BY SELENIUM GROUPS

Treatment Group	g
Selenium-treated ²	1.97 ± .09 ⁴
Untreated ³	2.35 ± .11

¹ $\bar{X} \pm \text{SEM.}$ ² $N = 46.$ ³ $N = 36.$ ⁴Significantly smaller at $P < 0.05.$

excised.

When the mice were subdivided by vitamin B-6 treatment, their tumor sizes were statistically similar (excess, 2.27 ± 0.11 ; adequate 2.03 ± 0.15 ; and suboptimal, 2.19 ± 0.13 g). Excess vitamin B-6 supplementation, produced larger tumors when no protective selenium treatments were given. This was expected and is supported in the literature. Numerous studies by Tryfiates et al. (14,15,16,17) demonstrated that high vitamin B-6 (70 mg vitamin B-6/kg diet) supplemented diets produced significantly larger tumors in rats when compared to those fed an otherwise optimal diet lacking the vitamin.

There is some evidence, that high vitamin B-6 supplementation may not alter tumor growth rates in mice. A recent study by Gebhard et al. (42), reported no significant differences in tumor (transplanted MZI-HPB) incidence or volume in BALB/c mice provided diets containing either 4 mg or 6 mg vitamin B-6/kg diet. The researchers speculated that vitamin B-6 intake did not modify tumor development due to a lack of T lymphocyte-dependent immune mechanisms. A previous study by Gebhard et al. suggested that high levels of vitamin B-6 may have suppressed tumor development in BALB/c mice through immune enhancement. Subsequent literature

lends support to this claim (19).

The majority of information regarding vitamin B-6 in laboratory animals indicates that diets lacking vitamin B-6 are the most effective in tumor growth inhibition (14,15,16,17). The findings of the present study contradict this observation in that the suboptimal vitamin B-6 group developed larger tumors than the adequate vitamin B-6 group. There is no support for this occurrence in the literature, but the author surmised that extremes at either end of the vitamin B-6 spectrum may be stressful to the mice, and may have affected their immune defense against the tumors. This idea is supported theoretically in that suboptimal levels of vitamin B-6 may lead to impaired immune response and that previously controlled neoplastic cells may proceed to tumors. At the high vitamin B-6 end of the spectrum, immune responses may be enhanced but the vitamin may also stimulate the formation of carcinogenic compounds such as polycyclic hydrocarbons, leading to increased rates of tumorigenesis.

The actual decrease in tumor growth in the adequate vitamin B-6 selenium-treated group may have been primarily the result of selenium's protective effects. It seems likely, however, that adequate dietary vitamin B-6 provided an optimal environment in which to enhance the antitumorigenicity of selenium.

Levels of Selenium in Whole Blood and Tumors

Selenium absorption in rats has been demonstrated to be 90% of the test dose regardless of the dose, compound, or mode of administration (54). Selenium is rapidly absorbed through the gastrointestinal tract, and during acute dose administration; peak tissue concentration is usually achieved within 1 to 2 h. Levander (6) has identified skeletal muscle and liver as the largest tissue pools of selenium in rats, while levels in blood seemingly reflect large body pools of the mineral during long term intake. Erythrocyte and plasma selenium levels were found to be good indicators of muscle and liver selenium content in rats fed constant amounts of selenium in the diet. Muscle selenium concentrations appeared to be among the most stable during a depletion-repletion-depletion cycle, making them ideal for use in the determination of long term status (6).

Poirier and Milner (26) found that large quantities of selenium are cleared from tumor-burdened mice over a 24 h period following administration of 20 μ g of radiolabeled sodium selenite. This was evidenced by significant increases in urinary selenium concentrations and decreasing lung and liver concentrations. These researchers also noted no significant changes in

circulatory selenium over the 24 h test period; however, significant quantities of the radiolabeled selenium were retained by the host tumor (26).

A. Levels of selenium in whole blood

Blood selenium levels, represented in ppb, are presented in Table 10. Information provided in Appendices P and Q is needed to decode individual data given in Appendix R. Overall, there were no significant differences in whole blood selenium levels between treatment groups although all selenium-treated groups had higher levels when compared to unsupplemented groups. The adequate vitamin B-6, selenium-treated group, given no tumor, had the highest blood selenium level of all the treatment groups.

Our findings in the present study regarding blood selenium levels are consistent with those found in the literature. A study by Jacobs (55) determined blood levels of selenium in Sprague-Dawley mice with 1,2-dimethyl-hydrazine (DMH)-induced colon carcinogenesis in animals provided with 4 ppm selenium ad libitum in drinking water. Results of the study showed that selenium-treated rats had higher blood levels of selenium (492 ppb; 0.492 $\mu\text{g/mL}$) as compared to unsupplemented rats having 311 ppb (0.311 $\mu\text{g/mL}$) of whole blood selenium. In addition, selenium-treated rats in the study (no tumor) had the highest blood selenium concen-

TABLE 10

BLOOD SELENIUM LEVELS¹

Vitamin B-6 group	Blood selenium
	ppb
Excess	
Selenium-treated	385 ± 17
Untreated	356 ± 8
Adequate	
Selenium-treated	371 ± 14
Untreated	358 ± 10
Adequate	
Selenium-treated, No tumor	404 ± 13
Suboptimal	
Selenium-treated	374 ± 23
Untreated	361 ± 16

¹ $\bar{x}$ SEM, n = 12.

trations at 551 ppb (0.551 $\mu\text{g/mL}$) of all groups. These trends are in agreement with findings in the current study with the exception that blood selenium levels in the present study were higher for the selenium-treated groups and lower for the untreated groups. It is the supposition of these authors that this may be accounted for by the differences in tumor models and lengths of time over which selenium treatments were given. Selenium supplementation in her study began 5 wk prior to DMH exposure while that in the current study began one day after tumor cell inoculation.

Information regarding blood selenium levels in tumor-burdened mice and rats have largely gone unreported due to the primary focus on tumor size, frequency, and effects of selenium treatment on survival time. Studies reporting blood selenium levels have, however, indicated that a range of 300 to 330 ppb is typical of laboratory rats maintained on diets containing adequate quantities of the mineral (54).

Trends regarding whole blood selenium levels in humans are consistent with those found in laboratory animals in that levels are often significantly lower in patients with cancer as compared to controls. Blood selenium levels of healthy adults living in the United States are known to range from 15.7 to 25.6 $\mu\text{g/dL}$ in low and high selenium forage areas, respectively μg .

Two recent studies involving human cancers have found significantly lower blood selenium levels in diseased patients as compared to controls. Saito et al. (56) found blood selenium levels in patients with stomach cancer to be 19.3 ± 0.8 $\mu\text{g/dL}$ while healthy controls maintained levels of 22.0 ± 0.8 $\mu\text{g/dL}$. Similar results were found in American women with breast cancer. Healthy controls maintained blood selenium levels of 18.3 ng/dL while women with breast cancer had significantly lower levels at 16.4 ng/dL . Researchers noted that both groups had a mean dietary selenium intake of 144 $\mu\text{g/day}$ thus ruling out dietary intake as a contributing factor (57).

Although research regarding blood selenium levels in tumor-burdened animals is scant, levels found in the literature are consistent with those found in this study. Blood selenium levels of tumor-burdened animals were higher in selenium supplemented animals than in the unsupplemented.

B. Tumor selenium levels

Tumor selenium levels represented in ng per whole tumor and per gram of tumor are presented in Table 11. Individual data are presented in Appendix R. Overall results show that regardless of representation (per whole tumor or per gram of tumor), selenium levels were

TABLE 11

TUMOR SELENIUM LEVELS¹
BY GROUP

Vitamin B-6 group	Total tumor selenium	Selenium per gram of tumor
	ng	ng/g
Excess		
Selenium-treated	411 ± 40 ³	200 ± 13 ³
Untreated	533 ± 34 ⁴	225 ± 14 ⁴
Adequate		
Selenium-treated	443 ± 44 ³	260 ± 20 ⁴
Untreated	684 ± 85 ²	285 ± 8 ²
Suboptimal		
Selenium-treated	514 ± 33 ⁴	249 ± 19 ⁴
Untreated	580 ± 70 ⁴	241 ± 13 ⁴

¹ $\bar{x} \pm \text{SEM}$, n = 12.

²Significantly higher at P<0.01

³Significantly lower at P<0.01.

⁴Homogeneous subgroups.

significantly higher in the adequate vitamin B-6 group not treated with selenium as compared to the excess vitamin B-6 selenium treated group. All other groups were homogeneous subgroups. Vitamin B-6 intake had no effect on these values. Selenium did not appear to concentrate in the tumors in any discriminate pattern, nor was there any relationship to selenium treatment. This may be explained, in part, by the nature of the tumor, a fibrosarcoma, which encompassed primarily muscle mass in the animals' hindquarters as previously stated. Muscle tissue is slow to respond to shifts in selenium administration during depletion and repletion cycles (6). This factor, combined with the limited course of selenium exposure could help explain such unremarkable tumor selenium results.

When tumor selenium levels were considered regardless of vitamin B-6 treatments, (Table 12), the combined mean levels were significantly lower for the selenium-treated groups as compared to the untreated groups. This was only true when total tumor selenium levels were considered and may be easily explained by the varied tumor weights. The selenium-treated groups had significantly smaller tumor weights resulting in the difference. Results expressed per gram of tumor provide a more accurate representation of the data due to the variability in tumor size.

TABLE 12

TUMOR SELENIUM LEVELS¹
BY SELENIUM GROUP

Treatment group	Total tumor selenium	Selenium per gram of tumor
	ng	ng/g
Untreated ²	600 ± 39	250 ± 11
Selenium-treated ³	457 ± 24 ⁴	236 ± 11

¹ $\bar{X} \pm \text{SEM}$.²N = 36.³N = 48.⁴significantly lower at $P < 0.01$.

It is difficult to compare the results of the present study to those found in the literature concerning tumor selenium levels because the focus of the study was centered on tumor measurements. Selenium levels in muscle of rats fed adequate diets have been reported to be 400 ppb (0.40 $\mu\text{g/mL}$) which are substantially higher than values found in the current study (55). These data were obtained after 6 wk on dietary supplements containing 5.5 ppm of selenite, a dosage very similar to that in the present study. The discrepancy may have been to the limited course of selenium supplementation used in the present study. Since levels of selenium are more stable in muscle it appears likely that tumor concentrations would not have been susceptible to short term shifts.

In a study by Jacobs (55), described previously, Sprague-Dawley rats exposed to DMH developed tumors of the colon which contained a mean of 412 ppb (0.412 $\mu\text{g/g}$) of selenium without selenium supplementation. These values are in agreement with the findings of Poirier and Milner who demonstrated greater selenium retention in tumors as compared to blood (26). McConnell et al. (58) found differences in tissue concentrations of selenium depending on histological state of particular organs. Abnormal pancreatic tissues of rats

contained a mean of 980 ppm (0.98 μ /g) while normal tissues contained 630 ppm (0.63 μ g/g) of selenium. Selenium stores in normal and diseased livers of rats were, however, widely varied regardless of pathological state. Authors of the study suggested the possibility that enzyme activity or preferential sequestering of selenium by tumor cells may have caused the variability.

Blood levels of selenium in the mice in the present study appeared to better reflect short term shift in status while muscle tumor levels remained stable. Short term selenium supplementation may have affected tumor growth through overall growth inhibition but the stable pools in skeletal muscle were probably not susceptible to the limited course of treatments used in this study.

Plasma PLP Levels and Tumor Total Vitamin B-6 Levels

Vitamin B-6 occurs naturally as pyridoxol, pyridoxal, and pyridoxamine while foods may also contain pyridoxamine-5-phosphate. The forms found in tissues are interconvertible yet PLP and pyridoxamine-5-phosphate are the metabolically active forms. Vitamin B-6 is readily soluble in water and is absorbed primarily in the jejunum and ileum. PLP is bound primarily to albumin and hemoglobin in blood while the vitamin is widely distributed in tissues as PLP. PLP bound to

glycogen phosphorylase of muscle constitutes the largest pool of vitamin B-6 in the body (52). Plasma PLP levels were quantified to determine status of the nutrient. The following sections describe the findings of the present study regarding vitamin B-6.

A. Plasma PLP levels

Plasma PLP levels, determined radiometrically, are represented in ng/mL and nmol/L in Tables 13 and 14. Individual data presented in Appendix R require the detail given in Appendices P and Q for decoding. Plasma PLP levels of all groups were representative of vitamin B-6 levels provided in the diets. The suboptimal vitamin B-6 groups had significantly lower plasma PLP levels as compared to the excess and adequate vitamin B-6 groups when results were considered without regard to selenium treatments (Table 14). These levels were in agreement with those cited in the literature (16). Reynolds and Morris have reported PLP concentrations in the plasma of both tumor-burdened and non-tumor-burdened Buffalo rats fed a vitamin B-6 sufficient diet: those concentrations ranged from 34 to 140 ng/mL (59).

The presence of Morris hepatoma 7777 has been shown to have no significant effect on plasma PLP levels in female Buffalo rats fed vitamin B-6 suffi-

TABLE 13

PLASMA PLP LEVELS BY GROUP¹

Vitamin B-6 group	PLP	PLP
	ng/mL	nmol/L
Excess		
Selenium-treated	148 ± 31 ²	598 ± 125
Untreated	137 ± 13 ²	554 ± 53
Adequate		
Selenium-treated	128 ± 14 ²	518 ± 57
Untreated	103 ± 14 ⁴	417 ± 57
Adequate		
Selenium-treated	130 ± 27 ²	526 ± 109
No tumor		
Suboptimal		
Selenium-treated	64 ± 9 ³	259 ± 36
Untreated	52 ± 7 ³	214 ± 28

¹ $\bar{x} \pm \text{SEM}$.²Significantly higher at $P < 0.05$.³Significantly lower at $P < 0.05$.⁴Homogeneous subgroups.

TABLE 14

PLASMA PLP LEVELS BY¹
VITAMIN B-6 GROUP

Vitamin B-6 group	PLP	
	ng/mL	nmol/L
Excess ²	143 ± 22	576 ± 89
Adequate ³	120 ± 18	487 ± 74
Suboptimal ²	58 ± 8 ⁴	235 ± 32

¹ $\bar{x} \pm \text{SEM}$.²N = 24.³N = 36.⁴Significantly lower at $P < 0.01$.

cient diets (41). Plasma PLP concentrations in this study ranged from 40 to 50 ng/mL. A second group of rats maintained on vitamin B-6 deficient diets had significantly lower plasma PLP levels (below 10 ng/mL) than the vitamin B-6 sufficient groups irrespective of tumor presence. A profound decline in plasma PLP levels resulting from maintenance on vitamin B-6 deficient diets was expected and is supported in the literature.

Plasma PLP levels in the current study were 148 ± 31 ng/mL in the excess vitamin B-6 selenium treated group and 52 ± 7 ng/mL in the suboptimal vitamin B-6 group, not treated with selenium (Table 13). These values were dose responsive and fell within close range of those previously cited in this section. A major difference between the current study and those reported in the literature, is that the present researcher provided a suboptimal vitamin B-6 diet which was not low enough in vitamin content to bring about plasma PLP levels such as those promoted by deficiency. The excess vitamin B-6 diet used in the present study, did, however, exceed the plasma PLP ranges found in the literature (41). This was expected allowing for the dose-dependent nature of plasma PLP. It is interesting to note that all selenium- treated mice had higher plasma PLP levels than their untreated counterparts.

No literature to explain or support this occurrence was found but the present researcher speculated that selenium may possibly protect and/or spare vitamin B-6. This is not unlikely considering selenium's function as an antioxidant.

B. Tumor vitamin B-6 levels

Tumor vitamin B-6 levels, determined microbiologically, are presented in Tables 15 and 16. These are expressed per gram of whole tumor and per whole tumor. Individual data require the information in Appendices P and Q for decoding. Tumor vitamin B-6 levels followed the same dose-dependent trend seen in plasma PLP. Tumor levels increased in proportion to the concentration of vitamin B-6 provided in the diets. Both excess vitamin B-6 groups retained significantly more of the vitamin as compared to the suboptimal vitamin B-6 group, not treated with selenium (Table 15). Tumor vitamin B-6 levels were significantly higher in the excess vitamin B-6 groups compared to the adequate and suboptimal vitamin B-6 groups when results were considered without regard to selenium (Table 16). There is very little published tumor data expressed as total vitamin B-6, yet there is some literature which provides muscle concentrations. The muscular nature of the tumor make these values the most obvious choice for

TABLE 15

TUMOR VITAMIN B-6 LEVELS BY GROUP¹

Vitamin B-6 group	Tumor Vitamin B-6		
	ng/tumor	nmol/tumor	nmol/g
Excess			
Selenium-treated	200 ± 34 ²	1.19 ± .20	0.58 ± 0.10
Untreated	201 ± 42 ²	1.19 ± .25	0.48 ± 0.10
Adequate			
Selenium-treated	138 ± 14 ⁴	0.82 ± .08	0.46 ± 0.04
Untreated	130 ± 11 ⁴	0.77 ± .05	0.34 ± 0.02
Suboptimal			
Selenium-treated	111 ± 14 ⁴	0.66 ± .08	0.32 ± 0.04
Untreated	78 ± 11 ³	0.46 ± .07	0.20 ± 0.03

¹ $\bar{X} \pm \text{SEM}$, n = 12.²Significantly higher at P<0.01.³Significantly lower at P<0.01.⁴Homogeneous subgroups.

TABLE 16

TUMOR VITAMIN B-6 LEVELS BY VITAMIN B-6 GROUP¹

Vitamin B-6 group	Tumor vitamin B-6	
	ng/tumor	nmol/tumor
Excess	201 ± 38 ²	1.19 ± .23
Adequate	134 ± 11	0.80 ± .07
Suboptimal	95 ± 13	0.56 ± .08

¹ $\bar{x} \pm \text{SEM}$, n = 24.

²Significantly higher at $P < 0.01$.

total vitamin B-6 level comparisons. A study by Thiele and Brin (60) sought to determine rat tissue levels of vitamin B-6 vitamers and total vitamin B-6 using microbiological assay and chromatography. Total vitamin B-6 levels were determined after the rats were maintained on either vitamin B-6 deficient or sufficient diets for 32 days. Results of the study demonstrated significantly lower levels of total vitamin B-6 (mean = $6.07 \pm .71$ nmol/g) in the deficient rat muscle compared to those found mice on the sufficient diet (mean = 22.5 ± 2.60 nmol/g). Although these levels are much higher than those found in the tumors in the current study, the same dose-dependent trends were evident. Total tumor vitamin B-6 levels recovered from the tumors in the present study ranged from 0.20 ± 0.03 nmol/g in the vitamin B-6 deficient group to 0.58 ± 0.10 nmol/g in the excess vitamin B-6 group. The large discrepancy noted in comparison to other studies may be the result of differences between species of laboratory animals or it may reflect lack of vitamin B-6 uptake into the tumor as total vitamin B-6.

Plasma PLP levels followed the same general pattern as total vitamin B-6 levels in the current study in that they reflect the dose of supplementation. Rats carrying Morris hepatoma 7777 exposed to either deficient or sufficient diets for several wk were found to

have markedly significant effects with respect to dietary treatment (41) in that tumor PLP levels in the deficient group fell to 0.5 μg of PLP/g of tissue while the group on the sufficient diet maintained levels between 2.2 and 2.7 μg /g of tissue. These findings are in agreement with Reynolds and Morris (57) and Thanassi et al. (41,61), who also identified dose dependency as a factor influencing tumor vitamin B-6 levels.

Correlations

Owing to the dose dependent nature of vitamin B-6, both plasma PLP and tumor vitamin B-6 levels corresponded to the quantities of dietary vitamin B-6 given during the course of the study. There was a positive correlation between plasma PLP and tumor vitamin B-6 levels (Table 17). This was expected considering the relationship between vitamin B-6 intakes and plasma PLP and tumor levels. No correlation was found between blood and tumor selenium levels. Tumor vitamin B-6 levels also showed no correlation to tumor selenium levels.

TABLE 17

CORRELATIONS

Variables	Pearson r	P
Plasma PLP:tumor vitamin ¹ B-6	.56	<0.0001
Blood selenium:tumor selenium	-.30	<0.08
Tumor vitamin B-6:tumor selenium	-.23	<0.18

¹Significant correlation found.

SUMMARY AND CONCLUSIONS

Growth of fibrosarcomas in BALB/c inbred mice was significantly inhibited by a series of intraperitoneal selenium injections coupled with adequate dietary vitamin B-6. Selenium-treated mice developed significantly smaller tumors than their untreated counterparts. Excess and suboptimal levels of dietary vitamin B-6 appeared to enhance tumor growth.

Animal weights were not significantly affected by either selenium treatments or dietary vitamin B-6 manipulations. The smallest total weight gain was noted in the suboptimal vitamin B-6, selenium-treated group. Food consumption patterns coincided with overall net gain during a large part of the study. Selenium treatments had no significant effect on either animal weights or food consumption patterns. The suboptimal vitamin B-6 groups consumed significantly less food during most of the study, yet at the final measurement period, these animals were eating significantly more food than the excess or adequate vitamin B-6 groups. The final consumption measurement period identified significantly lower food consumption in the excess vitamin B-6 groups. These groups were the first to develop necrotic tissue at the tumor site and appear ill as a result of treatments. Both the excess and the

suboptimal vitamin B-6 groups exhibited unusual behavior as the effects of deficiency and oversupplementation began to take hold. The adequate vitamin B-6 groups maintained the most stable body weights, food consumption, and behavior patterns.

Blood selenium levels among groups were not significantly different with regard to vitamin B-6 groups, although all of the selenium-treated groups had higher levels than the untreated groups. Blood selenium levels recovered from mice in the current study closely matched levels found in laboratory animals by other researchers. Selenium in tumors did not appear to concentrate in any discriminate pattern with regard to treatment group. Selenium levels were significantly higher in the adequate vitamin B-6 group, not treated with selenium, as compared to the excess vitamin B-6 selenium-treated group. Treatment with selenium had no significant effect on tumor selenium levels.

Plasma PLP levels were representative of vitamin B-6 provided in the diets. The suboptimal vitamin B-6 groups had significantly lower plasma PLP levels compared to the excess and adequate vitamin B-6 groups. Tumor vitamin levels followed the same dose-dependent trend noted in plasma PLP. Tumor vitamin B-6 levels increased in proportion to levels provided in the diets. The excess vitamin B-6 groups retained signifi-

cantly more of the vitamin than the suboptimal vitamin B-6 group, not treated with selenium.

There was a positive correlation between plasma PLP and tumor vitamin B-6 levels. Vitamin B-6 retention in plasma and tumors appeared to be dose dependent. As vitamin B-6 intake increased, plasma PLP levels and tumor vitamin B-6 levels increased as well.

Adequate vitamin B-6 diets and intraperitoneal injections of selenium had the concomitant effect of significantly inhibiting growth of fibrosarcomas in BALB/c mice. Vitamin B-6 status was dependent on dietary intakes and was reflected in plasma PLP and tumor levels. Selenium status reflected in whole blood levels was slightly improved by selenium supplementation, while tumor selenium levels did not appear to reflect intake or blood levels.

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APPENDIX A

Reagent Preparation for Tumor Cell Inoculation

Krebs Ringer Phosphate Buffer (KRP)

Dissolve 0.935 mg of 0.016 M NaCl, 12.06 mg of 0.085 M sodium phosphate, 0.373 mg of 0.005 M potassium chloride and 7.64 mg of 0.031 M magnesium sulfate in 980 mL of distilled deionized water. Stir until clear. Adjust the pH to 7.4 with 2 N HCl and 2 N NaOH. Bring to volume of 1000 mL with distilled deionized water.

Sodium selenite solution

Dissolve 5 μ g of sodium selenite in 1 mL of KRP buffer. Yield is equal to 5 ppm sodium selenite in 0.10 mL of solution.

Saline

Dissolve 0.9 g NaCl in 100 mL of distilled deionized water. Stir thoroughly until solution is clear.

APPENDIX B

Animal Care and Diet

Chemicals and Vendors

Fisher Scientific Company (Raleigh, NC)

Sodium Phosphate Dibasic

Hydrochloric Acid

Sodium Hydroxide

Sodium Chloride

Magnesium Sulfate

Potassium Chloride

Sigma Chemical Company (St. Louis, MO)

RPMI 1640 Medium 8755

Pyridoxine HCl P9755

U.S. Industrial Chemical Company (Newark, NJ)

Absolute Ethanol

ICN Biomedicals Company (Cleveland, OH)

Mouse Chow

Fisher Scientific Company (Fair Lawn, NJ)

Sodium Selenite

American Optical (Buffalo, NY)

Brightline Hemocytometer

APPENDIX B (Cont'd)

Dominion Laboratories (Dublin, VA)

BALB/c Male Weanling Mice

APPENDIX C

Selenium Analysis by Gas Chromatography

Chemicals and Vendors

Aldrich Chemical Company, Inc. (Milwaukee, WI)

Hydroxylamine Sulfate, 99%

4-Nitro-o-Phenylenediamine, 98%

American Burdick and Jackson (Muskegon, MI)

Toluene, glass distilled

Baker Instra-Analyzed, JT Baker Chemical Company
(Phillipsburg, NJ)

Urea, 15%

Nitric Acid

Hydrochloric Acid

Fisher Scientific Company (Fair Lawn, NJ)

Selenium AA Standard Grade, 1,000 ppb + 1%

Mallinckrodt, Inc. (Paris, KY)

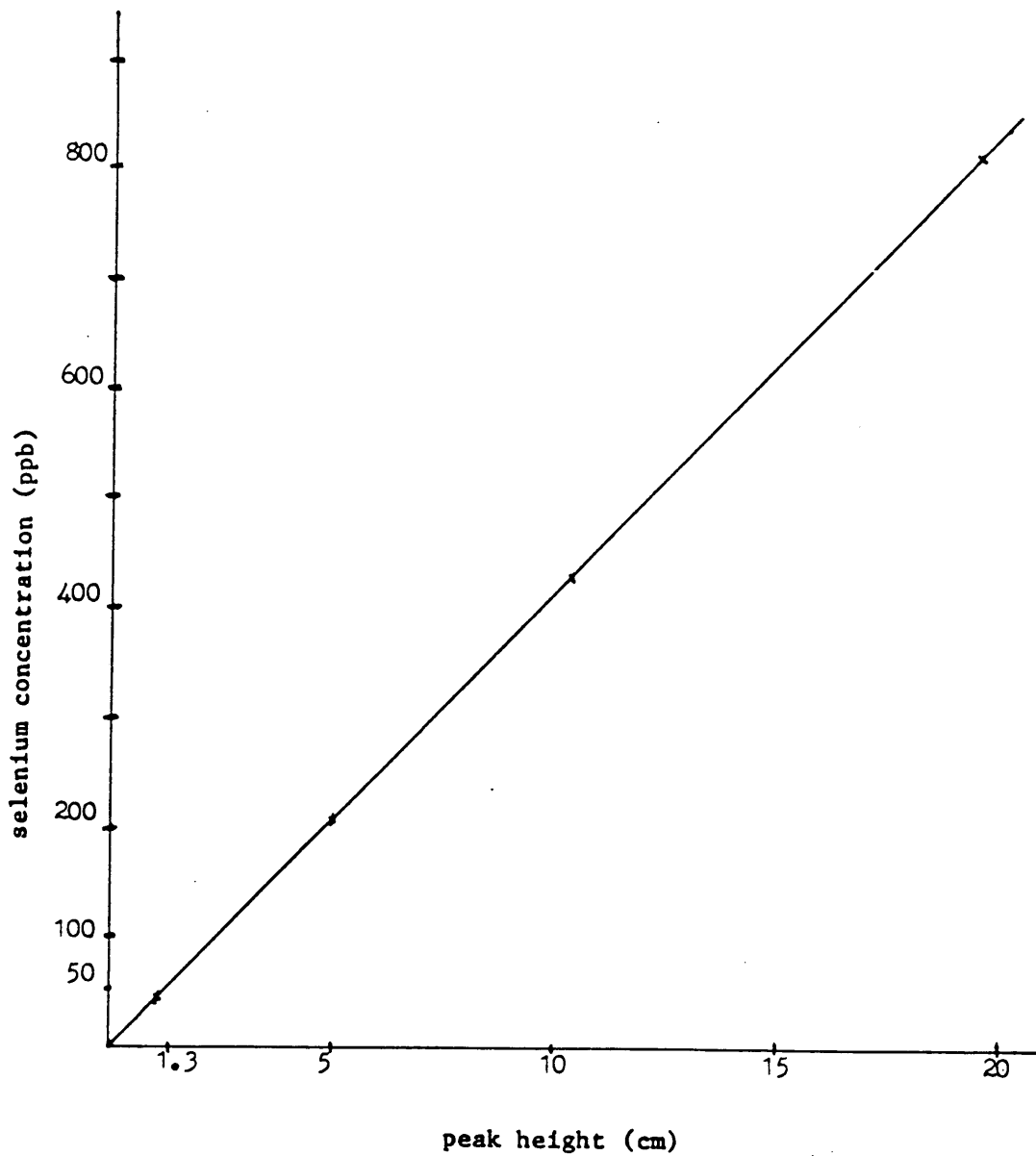
Magnesium Nitrate, 30%

Mallinckrodt, Inc. (St. Louis, MO)

EDTA Disodium Salt

APPENDIX D

Standard selenium calibration curve for gas chromatography of blood



APPENDIX E

Selenium Analysis by Atomic Absorption

Chemicals and Vendors

Baker Instra-Analyzed, J.T. Baker Chemical Company
(Phillipsburg, NJ)

Hydrochloric Acid

Nitric Acid

Perchloric acid, 70%

Fisher Scientific Company (Fair Lawn, NJ)

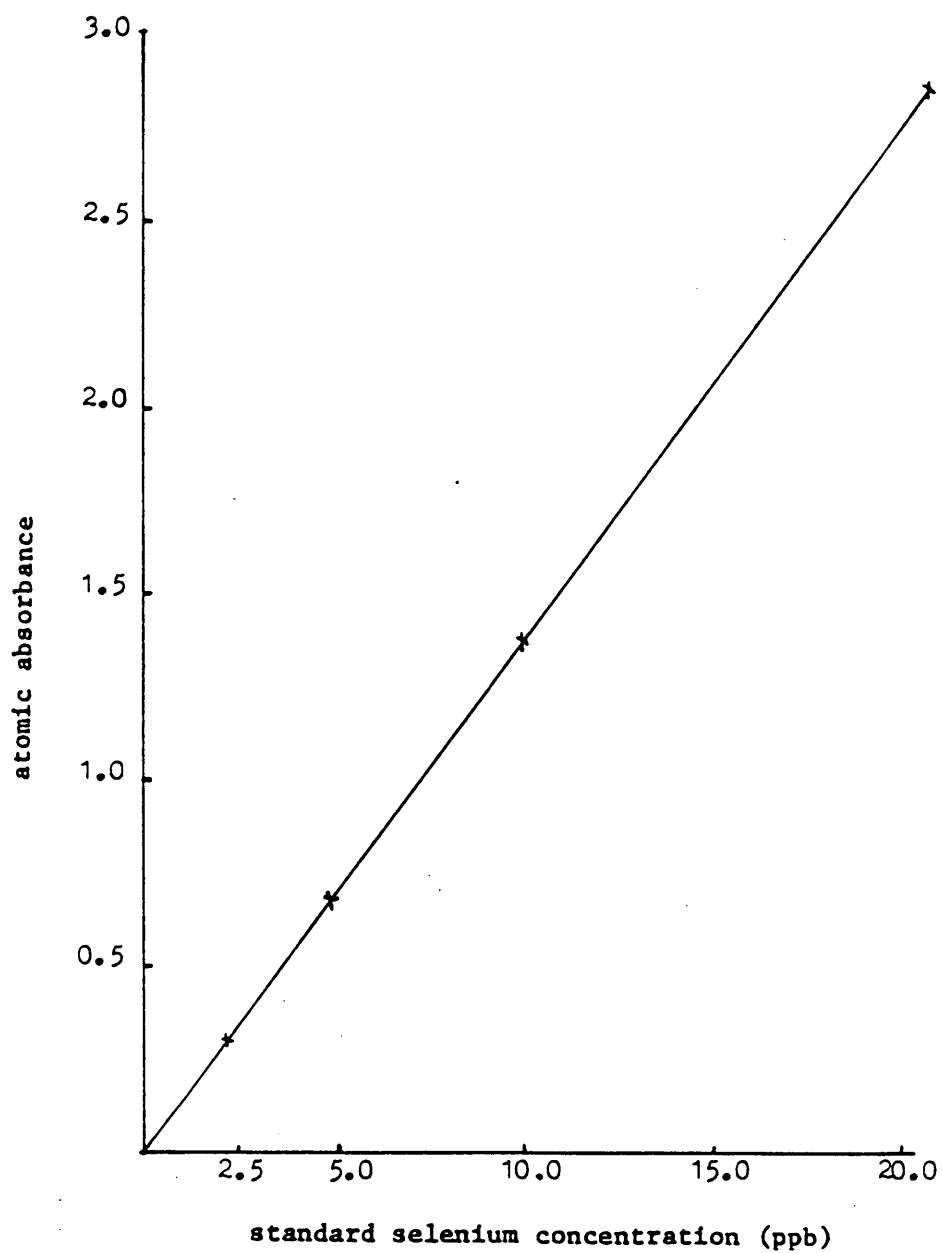
Selenium AA Standard Grade + ppb + 1%

Morton Thiokol Inc. (Danvers, MA)

Sodium Tetrahydride Borate, 98%

APPENDIX F

Standard calibration curve for atomic absorbance of selenium



APPENDIX G

Reagent Preparation for Plasma PLP Radioassay

L-tyrosine-1-¹⁴C

1.25 μ moles L-Tyr-1-¹⁴C, sp. act. 8 μ Ci/mmmole in 0.25 of 0.1 M Na acetate, pH 5.5.

L-tyrosine-1-¹⁴C stock solution

Weigh out 0.283 g tyrosine and add it to 12.5 μ Ci Tyr-1-¹⁴C (125 μ L). Adjust to 0.1 N by adding 5 mL of 1 N HCl. Bring to a volume of 50 mL with distilled deionized water. Store at 5° C in a tightly capped glass bottle within a protective plastic box. Label carefully with radioactive information.

L-tyrosine-1-¹⁴C

Add 0.8 mL stock solution to a 5 mL volumetric flask. Add 0.16 mL of 0.45 N NaOH. Bring to volume (5 mL) with 0.166 M sodium acetate buffer. Prepare fresh daily.

Pyridoxal phosphate standard solution (20 ng/mL)

0.01g pyridoxal phosphate is dissolved in distilled deionized water, brought to 1 L volume. Stir to dissolve. Take 0.2 mL of this solution and dilute to 100 mL with 0.01 M sodium acetate with 0.005 M EDTA. Store in a brown glass bottle at 5°C. Make fresh weekly.

Sodium acetate buffer solution

Prepare 0.2 M sodium acetate buffer by dissolving 16.4 g in 1 L of distilled deionized water. Pour 800 mL of this solution in a 1 L volumetric flask. Adjust the pH to 5.5 with glacial acetic acid (approximately 12 mL/L). Adjust until volume reaches 1 L.

0.166 M sodium acetate solution (50 mL)

Measure 41.5 mL 0.2 M sodium acetate buffer solution into a 50 mL volumetric flask. Bring to 50 mL volume with distilled deionized water.

APPENDIX G (Cont'd)

0.1 M sodium acetate solution

Measure 250 mL of 0.2 M sodium acetate buffer solution and bring to a 500 mL volume with distilled deionized water.

0.01 M sodium acetate buffer with 0.005 M EDTA (pH 5.5)

Weigh out 0.3905 g EDTA and 0.8203 g of sodium acetate. Dissolve in distilled deionized water (800 mL). Adjust to pH of 5.5 with 1 N NaOH. Bring to 1 L with distilled deionized water.

Trichloroacetic acid (10% w/v)

Dissolve 10 g trichloroacetic acid in distilled deionized water. Bring to a volume of 100 mL.

Freon amine solution

Add 0.93 mL Tri-n-octylamine (95%) to a 5 mL volumetric flask. Bring the contents to a volume of 5 mL with freon. Make fresh for each assay.

Turner's scintillation medium

Combine 3 g PPO and 0.1 POPOP. Bring to volume with 300 mL absolute methanol and 700 mL toluene.

Enzyme buffer solution

Combine 0.1 M sodium acetate (pH, 5.5), 24% glycerol (v/v), 10^{-3} M L-tyrosine, and 2×10^{-3} M mercaptoethanol.

Phosphate buffer solution (0.01 M, pH 7)

Add 80.4 mL of K_2HPO_4 solution (1.16g K_2HPO_4 /dL distilled deionized water) to 19.6 mL of KH_2PO_4 solution (0.908 g KH_2PO_4 /dL distilled deionized water). Dilute 15.0 mL of the above combined solutions to 100 mL with distilled deionized water.

APPENDIX H

Plasma PLP Radioassay

Chemicals and Vendors

Fisher Scientific Company (Raleigh, NC)

Acetic Acid, Glacial, 99.7%

Ammonium Sulfate, ACS

2,5-diphenyloxazole (PPO), scintillation grade

Ethylenediamine Tetraacetic Acid (EDTA), ACS

Glycerol, 95%

Hydrochloric Acid

Absolute Methanol

2,2-p-phenylenebis 5-phenyloxazole (POPOP)

Potassium Hydroxide, ACS

Potassium Phosphate, dibasic, ACS

Potassium Phosphate, monobasic, ACS

Sodium Hydroxide, 50%

Toluene, scintanalyzed

Trichloroacetic Acid (TCA), 100%

Matheson Chemical Company (East Rutherford, NJ)

1,1,2-trichlorotrifluorethane, Freon-113

New England Nuclear (Boston, MA)

Hyamine Hydroxide

L-tyrosine-1-¹⁴C

APPENDIX H (Cont'd)

Sigma Chemical Company (St. Louis, MO)

Alumina

2-mercaptoethanol

Pyridoxal-5'-phosphate

Sodium Acetate, 99+%

Tri-n-octylamine, 95%

L-tyrosine

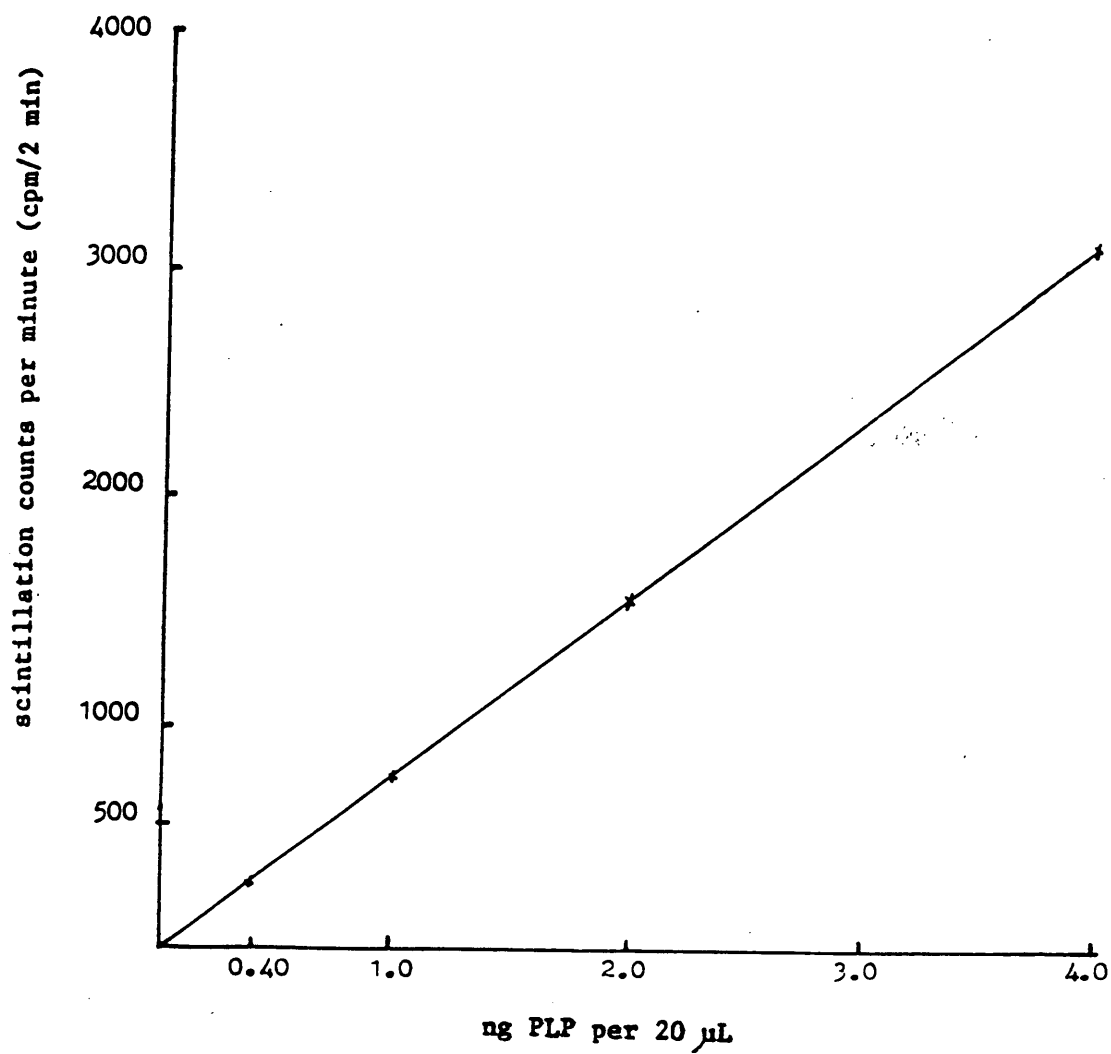
L-tyrosine Decarboxylase Apoenzyme

U.S. Industrial Chemical Company (Newark, NJ)

Absolute Ethanol

APPENDIX I

Standard calibration curve for radioassay of PLP



APPENDIX J

Reagent Preparation for Total Vitamin B-6

Microbiological Assay

YM agar

Add 41 g/L Bacto YM agar in quantities of 5 mL in each 10 mL screw top test tube. Boil in a shaking water bath. Autoclave for 15 min at 121°C (15 PSI). Cool in a slanted position at room temperature. Store at 5°C.

Pyridoxine Y media for inoculum

Dissolve 0.54 g pyridoxine Y media in 20 mL of distilled deionized water. Make fresh for each assay.

Pyridoxine Y media for sample assay

For 75 tubes, dissolve 19.9 g pyridoxine Y media in 375 mL of distilled deionized water. Stir thoroughly to dissolve. Make fresh for each assay.

Vitamin B-6 stock standard

Dissolve 25 mg of pyridoxine HCl in 250 mL 25% ETOH. Store in a dark bottle at 5°C.

Vitamin B-6 working standard

Dilute 1.0 mL of stock standard to 225 mL distilled deionized water. This dilution produces 0.5, 1.0, 2.0, and 4.0 ng/tube with 250 μ L, 500 μ L, 1 mL, and 2 mL standard.

Saline

Dissolve 0.9 g NaCl in 100 mL of distilled deionized water. Pour 10 mL into two test tubes and autoclave for 15 min at 121°C (15 PSI). Store at 5°C.

0.055 N HCl

Dilute 4.6 mL of concentrated HCl to 1 L in distilled deionized water.

6 N KOH

Bring 138 g KOH to 500 mL with distilled deionized water. Stir thoroughly to dissolve. Store at 5°C.

APPENDIX K

Total Vitamin B-6 Microbiological Assay

Chemicals and Vendors

American Type Culture Collection (Rockville, MD)

Saccharomyces uvarum, 9080

Difco Laboratories (Detroit, MI)

Bacto YM Agar, 07-2-01

Pyridoxine Y Medium, 0951-15-2

Fisher Scientific Company (Raleigh, NC)

NaCl, ACS

HCl, ACS

Glacial Acetic Acid, ACS

KOH, ACS

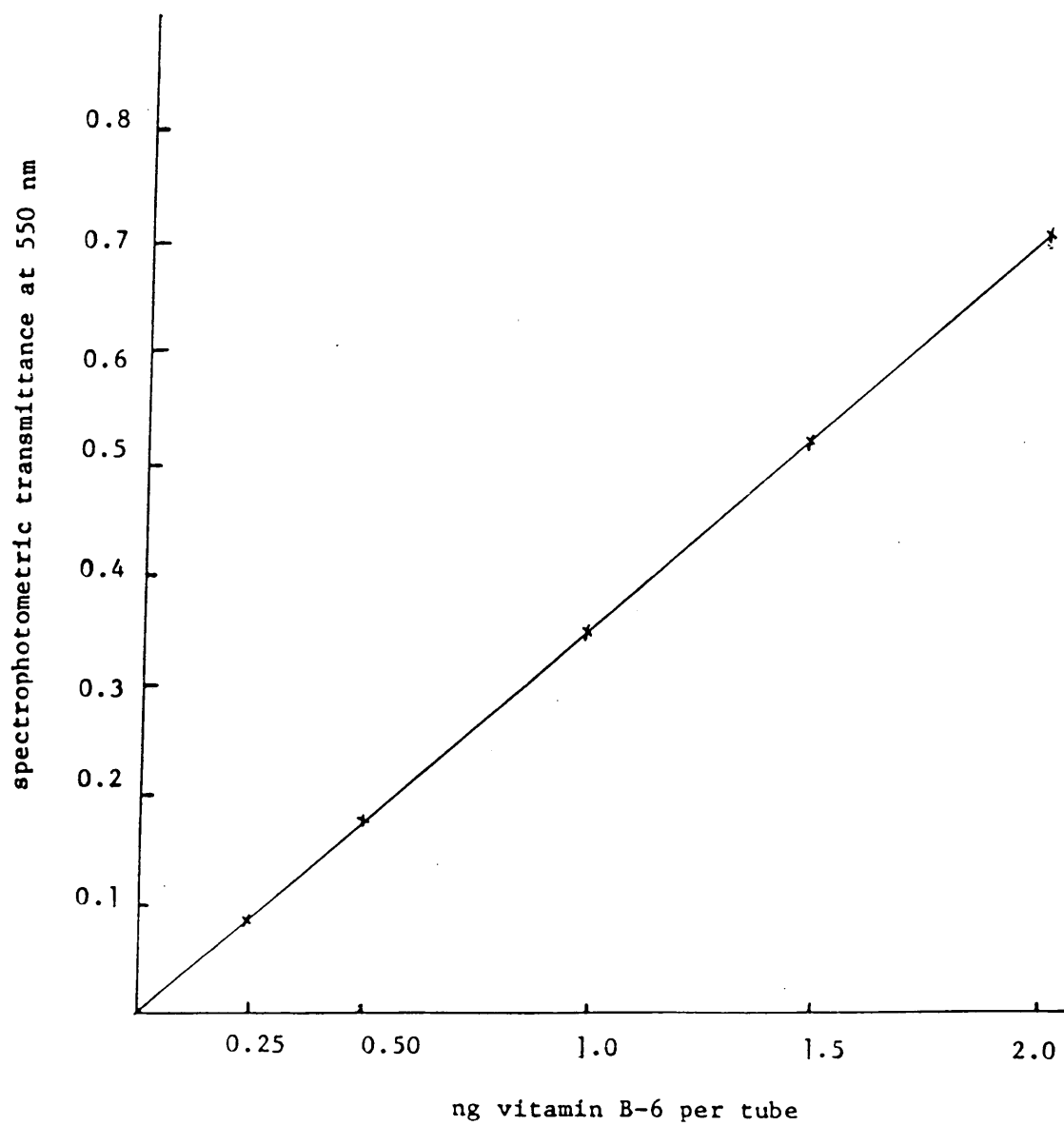
Sigma Chemical Company (St. Louis, MO)

Pyridoxine HCl, P 9755

Antifoam A Emulsion

APPENDIX L

Vitamin B-6 calibration curve for microbiological assay



APPENDIX M

Animal Weight Measurements (g)

Group #	01	02	02	1	2	3	4	5	6	7
1	13.00	14.00	15.50	17.50	18.75	18.75	19.50	20.00	20.00	20.00
1	13.00	15.50	16.50	18.50	19.00	19.00	20.00	20.00	20.00	19.75
1	12.50	13.75	15.00	18.00	19.50	18.50	19.00	20.00	19.50	20.00
1	12.75	14.25	15.50	17.50	19.00	19.00	19.50	20.00	19.50	19.50
1	12.75	13.50	15.00	18.00	18.25	18.50	19.00	19.00	19.00	20.00
1	12.50	14.00	15.00	17.00	17.75	17.25	17.50	17.75	18.00	17.00
1	12.00	13.25	14.50	17.50	19.50	19.75	20.00	20.00	19.00	19.50
1	12.50	13.50	14.50	17.00	18.50	19.00	20.25	20.50	19.00	20.00
1	12.00	13.00	13.50	17.50	21.00	21.75	22.00	23.50	23.50	23.00
1	12.50	14.25	15.00	18.50	19.50	20.00	21.00	18.75	19.25	19.75
1	12.50	13.75	15.00	18.00	18.75	18.75	18.75	18.75	19.25	19.75
1	12.50	13.75	14.50	19.00	20.50	21.25	21.00	21.00	21.50	23.50
2	12.00	14.00	15.50	17.50	18.50	18.75	19.50	20.00	20.00	19.50
2	13.50	14.50	15.00	18.00	18.00	17.00	18.00	18.50	19.00	19.75
2	12.00	13.00	14.00	16.00	17.50	17.75	18.00	18.50	18.50	19.00
2	13.00	14.00	14.50	16.50	19.00	17.25	18.00	18.00	18.00	18.00
2	12.00	13.00	14.50	17.00	18.00	18.00	18.00	18.75	18.00	18.00
2	12.50	13.50	14.00	16.50	17.00	16.75	17.50	18.00	17.00	17.50
2	12.00	13.25	14.50	17.00	18.00	19.00	20.00	20.00	19.00	19.50
2	12.50	13.50	14.50	18.00	20.50	20.50	21.00	21.50	20.00	19.50
2	12.50	13.25	14.50	16.50	18.00	18.75	19.50	20.00	20.00	21.00
2	12.50	13.00	14.50	18.00	19.50	19.25	19.00	19.00	19.00	19.50
2	12.00	13.00	14.00	17.00	18.00	18.25	18.75	18.00	19.00	19.00
2	13.25	14.00	15.50	20.50	21.00	22.25	22.00	22.00	22.00	23.25
3	13.75	14.00	14.00	18.50	19.50	19.50	19.50	19.00	19.75	19.00
3	12.00	13.50	15.00	17.50	18.00	18.00	18.00	18.00	18.00	17.75
3	12.50	13.50	14.00	17.00	17.50	18.00	18.00	18.00	18.25	17.00
3	13.00	14.00	15.00	17.50	18.50	19.25	19.00	18.25	18.00	17.75
3	13.00	14.00	15.00	17.00	17.50	18.00	18.25	18.50	18.50	18.50
3	12.50	13.50	14.00	17.00	18.00	18.50	19.00	19.00	19.75	18.50
3	13.50	14.50	15.50	18.50	20.50	21.00	20.00	20.00	21.50	21.00
3	12.00	13.75	14.50	18.00	20.00	20.00	20.00	20.00	21.50	20.50
3	13.00	13.75	14.50	18.50	19.00	19.00	19.50	19.50	19.75	18.00
3	13.00	14.00	15.00	18.50	18.75	18.75	19.00	19.50	19.75	23.00
3	13.00	13.75	14.00	17.00	17.50	18.00	19.00	18.00	18.25	17.75
3	14.00	13.00	15.00	17.50	18.00	18.50	18.00	17.75	18.25	17.75
4	12.75	13.00	14.50	18.00	19.00	19.75	20.00	20.00	21.00	19.25
4	12.75	14.00	15.00	18.00	20.00	20.75	20.50	21.00	21.00	19.75
4	13.75	14.00	14.50	18.50	20.00	20.00	19.00	20.00	21.00	19.00
4	12.00	13.00	13.50	17.50	20.00	19.75	19.00	18.50	19.25	18.75
4	14.50	14.25	14.00	18.50	19.50	19.75	20.00	20.50	21.00	21.50
4	13.00	14.00	14.50	17.50	19.00	19.00	19.50	20.00	20.50	20.50
4	13.50	14.00	14.50	17.50	18.25	18.75	18.50	18.00	19.00	17.00
4	12.50	14.00	15.00	18.50	20.00	20.50	20.00	20.00	20.50	19.00
4	12.75	13.00	13.50	18.00	21.00	21.00	20.00	21.00	22.00	22.00
4	12.50	13.50	14.50	17.00	19.00	18.75	19.00	19.50	20.00	19.75
4	12.50	13.25	14.00	16.50	17.00	17.00	17.50	17.50	17.00	16.75
4	13.00	14.25	15.50	19.00	19.00	19.00	18.50	20.00	19.75	19.75
5	12.00	13.00	14.00	17.50	21.00	21.50	21.00	22.00	22.50	22.00
5	12.50	13.75	15.00	18.50	19.00	19.50	19.50	20.25	21.00	21.00
5	12.50	13.25	14.00	16.50	18.50	18.00	18.00	18.00	18.00	17.00

01 = initial weights.

02 = weight measurements during acclimation.

1-7 = 3 day measurement periods.

APPENDIX M Cont'd.

Animal Weight Measurements (g)

Group #	01	02	02	1	2	3	4	5	6	7
5	14.00	13.00	15.00	17.00	17.50	19.00	19.00	19.00	18.75	18.00
5	12.00	13.00	14.00	17.00	18.50	19.00	19.25	19.50	19.50	19.50
5	12.00	13.00	14.00	17.50	18.00	19.50	19.75	20.00	20.00	19.00
5	13.00	14.00	14.50	18.50	19.50	20.00	21.00	22.00	21.75	20.00
5	12.00	12.50	13.00	16.50	17.00	17.00	17.50	18.00	17.50	20.00
5	13.50	14.00	14.50	17.50	18.00	18.00	18.50	19.75	19.50	21.00
5	13.00	14.00	14.50	17.50	18.50	20.00	20.00	20.00	19.50	18.00
5	12.25	11.50	10.50	15.50	16.50	17.00	18.00	19.00	19.25	19.00
5	13.00	13.50	14.00	17.50	18.00	19.50	20.00	21.50	20.75	17.00
6	13.50	14.00	14.50	17.00	18.50	18.50	19.00	19.50	19.50	18.00
6	13.00	14.00	15.00	18.00	19.00	20.00	20.00	20.00	20.00	18.00
6	12.50	13.50	14.50	17.50	20.00	21.00	21.00	21.00	21.00	20.00
6	12.25	13.00	13.50	16.50	17.50	18.00	18.00	17.50	18.00	16.75
6	12.50	14.00	15.00	18.50	21.00	21.50	21.75	22.00	21.00	20.50
6	12.00	13.50	14.50	17.00	19.00	19.00	19.50	19.50	19.00	17.50
6	13.00	13.00	13.00	17.00	17.00	17.25	18.00	19.00	18.75	21.00
6	12.00	12.50	13.50	17.00	18.00	19.00	19.50	20.00	19.00	18.00
6	13.50	14.00	14.00	17.50	17.00	17.00	18.00	18.50	18.20	20.00
6	12.50	13.00	14.50	17.50	19.00	21.00	21.00	21.50	21.00	23.50
6	13.50	14.00	15.00	18.00	17.50	17.00	18.50	20.00	20.50	17.00
6	13.00	14.00	14.50	18.00	18.50	20.00	20.00	20.00	20.75	19.00
7	13.25	14.25	15.50	17.50	18.75	19.00	19.50	20.00	20.00	20.50
7	12.75	14.00	15.50	18.00	17.50	19.00	19.00	19.50	20.00	21.00
7	13.00	14.00	15.50	18.50	18.00	19.00	19.00	19.50	20.00	21.00
7	13.50	14.25	15.00	17.50	18.00	18.25	20.00	20.50	21.00	22.55
7	12.75	13.25	14.50	17.00	18.00	18.50	19.50	19.50	19.50	20.00
7	12.50	13.00	14.00	16.00	17.50	18.00	19.00	19.50	19.00	19.50
7	12.25	13.50	15.50	18.50	20.00	20.00	20.75	20.75	20.00	20.75
7	12.50	13.50	15.00	17.50	17.50	18.00	18.00	18.00	18.50	18.50
7	13.75	14.00	15.00	18.00	18.00	18.00	18.00	18.50	19.25	19.75
7	12.00	13.00	14.00	17.00	18.00	18.50	19.00	20.00	19.00	20.00
7	12.00	13.00	14.00	16.50	17.50	18.00	18.00	18.50	19.00	19.50
7	12.50	13.50	14.00	17.50	19.00	19.75	20.00	20.75	21.00	22.00

01 = initial weights.

02 = weight measurements during acclimation.

1-7 = 3 day measurement periods.

APPENDIX N

Food Consumption Measurements (g)

		Periods								
Group #	01	01	(	1	)	2	3	4	5	
1	13.5	13.0	13.5	16.5	9.0	10.0	10.0	10.0	18.0	
1	15.0	14.0	14.0	14.5	11.5	12.0	11.0	12.5	17.0	
1	11.0	12.0	13.0	16.0	8.0	12.5	11.0	8.0	15.0	
1	13.0	13.5	14.0	15.0	8.0	8.0	8.0	8.0	17.0	
1	13.5	14.0	15.0	16.0	10.5	10.0	9.5	8.5	20.0	
1	12.0	13.0	14.0	15.0	12.0	10.0	10.0	10.0	17.0	
1	10.0	11.0	12.0	13.0	11.0	12.0	11.0	10.0	20.0	
1	9.0	12.0	12.0	12.0	9.0	9.0	9.5	10.0	19.0	
1	10.0	11.0	10.5	10.0	8.0	11.0	10.0	7.0	18.0	
1	8.5	9.5	15.0	15.5	13.0	8.5	8.5	8.0	19.0	
1	7.0	10.0	13.5	17.0	12.0	12.0	12.0	12.0	16.0	
1	8.0	13.0	14.0	14.0	10.0	11.0	9.5	8.5	16.0	
2	12.5	13.0	14.0	16.0	13.0	10.0	11.0	11.5	20.0	
2	13.5	14.0	14.0	17.0	10.0	8.0	8.0	8.0	14.0	
2	12.0	13.0	15.5	16.5	10.5	11.0	11.5	12.0	17.0	
2	11.0	12.0	15.0	16.0	9.0	9.0	11.0	12.0	23.0	
2	13.5	14.0	15.0	17.0	16.0	11.0	10.5	10.5	16.0	
2	13.5	14.0	15.0	16.0	10.0	10.0	9.0	8.0	15.5	
2	9.5	12.5	14.0	15.0	12.5	8.0	8.0	8.5	20.0	
2	9.0	13.0	13.0	15.0	12.0	13.0	12.0	7.0	17.0	
2	9.0	12.0	13.0	14.0	11.0	10.0	11.0	7.0	18.0	
2	10.0	12.0	13.0	13.0	12.0	12.0	11.0	9.0	14.0	
2	10.0	13.0	13.5	14.0	11.0	13.0	12.0	12.0	21.0	
2	10.0	12.0	13.5	13.0	14.0	8.0	8.0	8.0	18.0	
3	13.5	13.0	13.0	12.0	11.5	11.0	11.0	11.0	12.0	
3	9.0	9.5	11.0	12.0	11.5	9.0	10.0	11.0	8.0	
3	15.0	12.0	13.0	11.5	12.0	12.5	11.0	9.5	4.0	
3	10.5	11.0	11.0	12.0	14.0	12.5	12.0	13.0	13.0	
3	11.5	12.5	11.5	12.0	11.0	10.0	10.5	11.0	18.0	
3	10.0	11.0	11.0	12.0	12.0	10.0	10.0	10.0	12.0	
3	9.0	9.0	9.5	10.0	11.0	11.0	11.0	10.0	13.0	
3	10.5	11.5	11.0	13.0	8.0	13.0	12.0	11.0	13.0	
3	8.0	8.5	9.5	10.0	12.0	12.5	11.0	10.5	12.0	
3	10.0	9.0	11.0	12.0	11.0	9.5	11.0	10.0	9.0	
3	7.0	8.0	9.5	12.5	11.0	11.0	11.0	11.0	22.0	
3	10.0	10.5	12.0	13.0	12.0	12.0	12.0	8.0	21.0	
4	12.5	12.0	11.0	11.0	11.0	10.0	10.5	11.0	5.0	
4	13.0	12.0	11.5	11.0	10.0	12.0	11.0	9.0	12.0	
4	16.0	15.0	13.0	13.0	15.0	12.0	11.0	10.0	11.0	
4	14.0	13.5	12.0	11.0	12.0	15.0	14.0	13.0	8.0	
4	16.0	15.0	13.0	14.0	17.0	12.5	13.0	11.0	17.0	
4	11.0	11.0	12.0	13.0	10.0	13.0	11.0	10.0	10.0	
4	12.0	12.5	13.0	13.0	10.0	12.0	12.0	12.0	11.0	
4	11.0	11.0	12.0	10.5	12.5	10.0	11.5	9.0	13.0	
4	12.0	11.5	11.5	11.5	11.0	11.0	11.0	12.0	12.0	
4	7.0	9.0	9.0	10.0	13.0	11.5	12.0	12.0	9.0	
4	9.5	10.0	10.5	10.5	7.0	12.5	11.0	10.0	14.0	
4	8.0	9.0	11.0	12.0	12.0	12.0	11.0	11.0	21.0	
5	9.0	13.0	14.5	15.0	10.0	10.0	13.0	14.0	14.0	

01 = consumption measurement periods during acclimation.

1 = first 11 days of vitamin B-6 manipulations.
All other measurement periods include 3 days.

APPENDIX N Cont'd.

Food Consumption Measurements (g)

		Periods								
Group #	01	01	(	1	)	2	3	4	5	
5	10.0	12.0	13.5	15.0	10.0	10.0	12.0	13.5	12.0	
5	9.0	13.5	14.0	15.0	12.0	12.0	12.5	11.0	14.0	
5	8.0	13.0	12.5	13.0	14.0	12.0	12.5	13.5	15.0	
5	9.5	10.0	12.5	13.0	14.5	15.0	12.0	11.0	15.0	
5	6.5	11.0	15.0	16.5	10.0	12.0	12.0	12.5	18.0	
5	8.5	13.5	15.5	16.5	8.5	10.0	11.5	12.5	15.5	
5	9.0	13.0	15.0	16.0	12.0	12.0	12.0	12.0	10.0	
5	9.0	14.0	14.5	16.0	9.0	13.5	12.5	10.5	14.0	
5	10.0	12.5	14.0	13.0	10.0	12.0	12.5	11.5	14.5	
5	6.0	13.0	14.5	16.0	10.0	10.0	13.0	15.5	12.0	
5	7.0	13.5	14.0	14.5	10.0	11.0	12.5	12.5	15.0	
6	16.0	15.0	16.0	16.0	8.0	13.0	12.0	9.0	10.0	
6	12.0	12.5	13.0	13.0	9.0	14.0	13.5	9.0	9.0	
6	12.0	12.5	14.0	17.5	13.0	14.0	13.0	11.0	3.0	
6	10.5	11.5	13.0	14.5	9.0	14.0	12.0	11.0	4.0	
6	11.0	12.0	14.0	16.0	9.5	14.0	13.0	11.0	8.0	
6	13.0	14.0	16.0	19.0	11.0	14.0	13.5	11.0	13.0	
6	8.0	13.0	13.0	17.5	10.0	12.0	11.5	10.0	7.0	
6	9.0	14.5	15.0	13.0	10.0	8.0	9.0	9.5	6.0	
6	11.0	12.0	14.0	16.0	9.0	12.0	12.0	11.5	7.0	
6	12.0	13.0	14.5	15.5	11.0	11.0	11.0	11.5	8.0	
6	11.0	13.0	15.0	16.6	8.5	12.0	12.0	12.0	6.5	
6	8.0	15.0	16.0	18.0	11.0	12.5	12.5	11.0	6.0	
7	12.0	12.0	13.0	14.0	8.0	12.0	12.0	8.0	9.0	
7	14.0	13.5	16.0	20.0	7.0	8.5	9.5	11.0	9.0	
7	20.0	14.5	15.0	20.0	13.0	13.0	12.0	10.0	9.0	
7	16.0	12.5	14.5	22.0	7.0	13.0	11.0	10.0	12.0	
7	9.0	13.0	13.0	16.0	11.0	11.0	11.5	10.0	17.0	
7	14.0	14.5	13.5	17.0	10.0	15.0	14.0	10.0	13.0	
7	8.0	12.5	16.0	17.5	8.5	11.0	11.0	11.0	6.0	
7	8.0	14.5	15.0	16.0	10.0	12.0	12.5	11.0	6.0	
7	12.0	13.0	14.0	22.0	20.0	18.0	13.0	9.5	5.0	
7	8.0	13.0	12.5	10.0	8.0	12.0	12.5	11.0	4.5	
7	9.0	13.5	14.0	15.5	11.0	11.0	11.5	12.0	4.0	
7	15.0	15.0	15.0	17.0	11.0	11.0	12.0	14.0	4.0	

01 = consumption measurement periods during acclimation.

1 = first 11 days of vitamin B-6 manipulations.
All other measurement periods include 3 days.

APPENDIX O
TUMOR WEIGHTS (g)

Sample	<u>Group 1</u> g	Sample	<u>Group 2</u> g
1	1.9484	1	1.9100
2	1.3363	2	1.3711
3	1.6754	3	3.3652
4	4.5570	4	1.4463
5	2.3322	5	2.4796
6	1.7346	6	1.5464
7	2.2602	7	3.0400
8	2.3017	8	2.4294
9	2.8553	9	2.8443
10	2.2382	10	2.9000
11	2.9283	11	1.4911
12	1.7328	12	2.7428

Sample	<u>Group 3</u> g	Sample	<u>Group 4</u> g
1	1.4915	1	1.8313
2	2.2704	2	2.4799
3	1.9561	3	2.1855
4	1.6736	4	1.7480
5	1.9503	5	2.8296
6	1.2293	6	2.8685
7	2.5747	7	2.2453
8	2.7015	8	2.3620
9	2.3424	9	3.1660
10	1.8457	10	3.4325
11	1.8457	11	2.5741
12	2.1094	12	2.0405

APPENDIX O CONT'D

TUMOR WEIGHTS (g)

Sample	<u>Group 5</u> g	Sample	<u>Group 6</u> g
1	1.7716	1	1.6950
2	1.2969	2	1.4248
3	1.0946	3	1.5021
4	2.1226	4	1.5021
5	0.7776	5	2.4528
6	2.7566	6	3.9637
7	2.0541	7	2.7428
8	1.5296	8	2.9700
9	2.2984	9	2.2991
10	2.8665	10	1.6500
11	1.8086	11	1.8365
12	1.1247	12	3.1441

APPENDIX P

Key to Data Sets in Appendices O + R

<u>Group #</u>	<u>Group Treatment</u>
1	Suboptimal vitamin B-6, selenium-treated
2	Suboptimal vitamin B-6, no selenium
3	Excess vitamin B-6, selenium-treated
4	Excess vitamin B-6, no selenium
5	Adequate vitamin B-6, selenium-treated
6	Adequate vitamin B-6, no selenium
7	Adequate vitamin B-6, selenium-treated no tumor

APPENDIX Q

Key to Headings in Appendices O + R

REP = treatment group
PLP = plasma pyridoxal phosphate, ng/mL
SEGT = selenium per gram of tumor, ppb
TTSE = whole tumor selenium, ppb
BLSE = blood selenium, ppb
BSIX = vitamin B-6 per gram of tumor, ng/g
TSIX = whole tumor vitamin B-6, ng/tumor

APPENDIX R

Individual Data

REP	PLP	SEGT	TTSE	BLSE	BSIX	TSIX
1	88.9	224.5	627.65	339	45.5	127.15
1	58.8	325.9	535.20	449	101.8	167.45
1	55.6	278.4	566.10	312	36.6	74.40
1	37.8	215.7	502.70	341	38.2	89.00
1	78.3	245.7	397.05	434	73.4	118.50
1	.	201.5	459.60	367	38.8	88.45
2	30.0	260.2	711.55	399	15.5	37.30
2	59.4	232.6	381.60	408	33.4	54.80
2	73.4	194.8	392.10	373	40.4	82.15
2	61.0	222.9	591.55	341	46.5	100.80
2	32.2	243.6	586.10	314	29.8	81.50
2	55.5	290.0	818.20	328	38.6	108.85
3	215.0	207.6	390.75	396	143.3	269.40
3	206.1	236.6	495.35	397	142.9	324.40
3	165.5	164.3	261.15	341	132.2	210.00
3	65.0	192.7	349.65	454	70.8	128.45
3	86.7	233.5	530.15	373	52.4	118.95
3	.	167.1	440.85	346	55.4	145.95
4	131.7	192.8	549.40	348	123.5	351.35
4	120.0	191.2	440.50	336	104.3	239.90
4	145.2	273.4	481.50	383	117.2	252.60
4	92.2	243.5	668.75	337	32.4	88.60
4	144.4	245.2	482.15	373	94.9	186.60
4	191.1	202.6	581.50	357	30.1	86.40
5	141.1	228.1	350.00	347	61.4	93.95
5	118.8	215.4	380.60	332	96.6	170.45
5	.	259.5	523.00	428	49.5	99.75
5	77.8	312.7	502.95	357	85.9	138.30
5	146.6	326.4	584.90	366	97.3	174.15
5	153.3	217.7	319.25	395	101.7	149.15
6	125.5	308.9	481.80	386	74.5	146.75
6	121.1	264.2	751.20	361	40.7	115.55
6	122.2	297.1	949.30	351	41.2	131.45
6	122.2	262.8	399.10	344	92.3	139.85
6	88.8	279.4	695.70	384	60.3	150.15
6	38.3	294.5	831.45	322	49.9	98.30
7	170.0	.	.	424	.	.
7	65.5	.	.	375	.	.
7	85.5	.	.	454	.	.
7	222.0	.	.	372	.	.
7	166.7	.	.	391	.	.
7	69.4	.	.	406	.	.

**The vita has been removed from
the scanned document**