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Altered Adrenocortical Metabolism in Vitamin B_6 Deficient Rats

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ALTERED ADRENOCORTICAL METABOLISM IN VITAMIN B_6 DEFICIENT RATS

By

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ABSTRACT

ALTERED ADRENOCORTICAL METABOLISM IN VITAMIN B_6 DEFICIENT RATS

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Previous investigations suggest that a defect in adrenocortical metabolism exists in vitamin B_6-deficient rats. The symptoms are consistent with decreased glucocorticoid production, with increased mineralocorticoids. This study was undertaken to test the hypothesis that 11β-hydroxylation is decreased in the adrenal glands of vitamin B_6-deficient rats. Two strains of rats which are known to differ in susceptibility to B_6-deficiency were used.

Sprague-Dawley (CD) or Wistar (WIS) male rats were fed a semi-purified diet lacking vitamin B_6 or one with pyridoxine (30 mg/kg). At the end of 3 or 10 weeks the in vitro steroidogenic capacity of the adrenal glands was assayed. The capsule and inner cortex of the adrenals were incubated separately in Krebs-Ringer bicarbonate buffer containing glucose (200 mg/dl) and progesterone (51 uM). The steroids released into the medium were determined by high-pressure liquid chromatography.

11-Deoxycorticosterone (DOC) production from progesterone was increased in tissue from the inner zones of CD and WIS rats consuming the vitamin B_6-deficient diet for 10 weeks, but not 3 weeks. The

synthesis of corticosterone and 18-OH-DOC by the inner zones of WIS deficient rats was decreased only at 3 weeks. DOC production in the capsules from deficient animals was not different than that of control. Capsular corticosterone and 18-OH-DOC syntheses were decreased at 3 weeks in deficient WIS rats, but there was no effect of the deficient diet in tissue from CD rats After 10 weeks, corticosterone synthesis was decreased in the capsules from both strains, but 18-OH-DOC production was not. Aldosterone production was markedly depressed in the capsules from both strains at 3 and 10 weeks.

These results demonstrate that 11β- and 18-hydroxylases are decreased in vitamin B_6-deficient rats, resulting in enhanced mineralocorticoid production (DOC) but decreased glucocorticoid (corticosterone). The effect on WIS rats is greater than CD rats.

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INTRODUCTION

Pyridoxine was isolated and identified as a required nutrient for rats in the 1930s and 40s. Among the earliest symptoms resulting from deficiency of this vitamin include histological changes in the adrenal gland indicative of altered function. During this same period of time, comprehensive studies of the adrenal gland were undertaken. Although numerous investigators studied the relationship between vitamin B_6 deficiency and the adrenals, knowledge of the structure and functions of the steroids produced by the cortex was inadequate, as was the methodology available to study such a problem. Since then, the major corticosteroids in the rat have been isolated and identified and more is known about the control of adrenal steroidogenesis. The recent increase in availability of high pressure liquid chromatography makes practical the separation and quantification of such compounds produced by the adrenal glands of the rat. The objective of this work will be to review the literature regarding this problem, and test the hypothesis that steroid synthesis is abnormal in the adrenal glands of vitamin B_6 deficient rats, specifically that the activity of 11 -hydroxylase is depressed.

VITAMIN B_6

Discovery and Isolation

The isolation and identification of the individual components of the B-complex vitamins in the early part of this century represents one of the most exciting periods in nutrition research. The initial impetus to such work was the recognition that the human diseases

beri-beri and pellegra were due to dietary inadequacies rather than infectious agents or toxicities. Numerous laboratories around the world became engaged in intense competition to discover the specific nutrients. The isolation and purification of vitamin B_6 was accomplished in 1938 by four independent labs (Lepkovsky, 1979). The observation that the various test organisms had different growth responses to the various forms of the vitamin B_6 (Snell & Rannefeld, 1945) led to the development of assays for the individual compounds in natural materials. The fact that they were interchangeable in animal nutrition (Snell & Rannefeld, 1945) provided the first clues regarding the metabolism of vitamin B_6.

Pharmacokinetics

The structures and official nomenclature of the vitamin B_6 compounds are shown in Figure 1. "Vitamin B_6" is the recommended term for all of the substances derived from 3-hydroxy-2-methylpyridine which "exhibit the biological activity of pyridoxine in rats" (Mayes et al., 1974). In animals each of the B_6 vitamers is readily converted to the biologically active forms and thus are equally potent in supporting growth. Figure 1 shows the known pathways of metabolism and interconversion, as well as the oxidation to the biologically inactive metabolite 4'-pyridoxic acid (PA). The numbers refer to the enzymes which catalyze the designated reaction(s).

The unphosphorylated forms are more commonly found in foods. They are absorbed in the intestine by passive diffusion, as shown by kinetic analyses of transport behavior (Yamada & Tsuji, 1980). Although there is some intracellular accumulation evident at low doses, this is

Figure 1. Pathways of metabolism and interconversion of B_6 vitamers. The numbers refer to the enzymes which catalyze the designated reaction(s). Pyridoxal kinase (#1) is a cytosolic enzyme which phosphorylates pyridoxal (PL), pyridoxine (PN), and pyridoxamine (PM) (Snell & Haskell, 1970). Dephosphorylation (#2) is accomplished by a various phosphatases, including alkaline phosphatase (Lumeng & Li, 1980). Pyridoxine oxidase (#3) catalyzes the conversion of PN to PL, but will not utilize pyridoxine phosphate (PNP). Pyridoxamine phosphate (PMP) oxidase (#4) is a cytosolic enzyme which catalyzes the oxidation of the amine and alcohol forms to the aldehydes, pyridoxal phosphate (PLP) and PL (McCoy & Columbini, 1972). PM and PMP may also be converted to the aldehyde forms by the actions of transaminases (#5, #6). The inactivation of the vitamers by oxidation of PL to 4'-pyridoxic acid (PA) is catalyzed by aldehyde oxidase (#7) or aldehyde dehydrogenase (#8) (McCormick & Merrill, 1980).

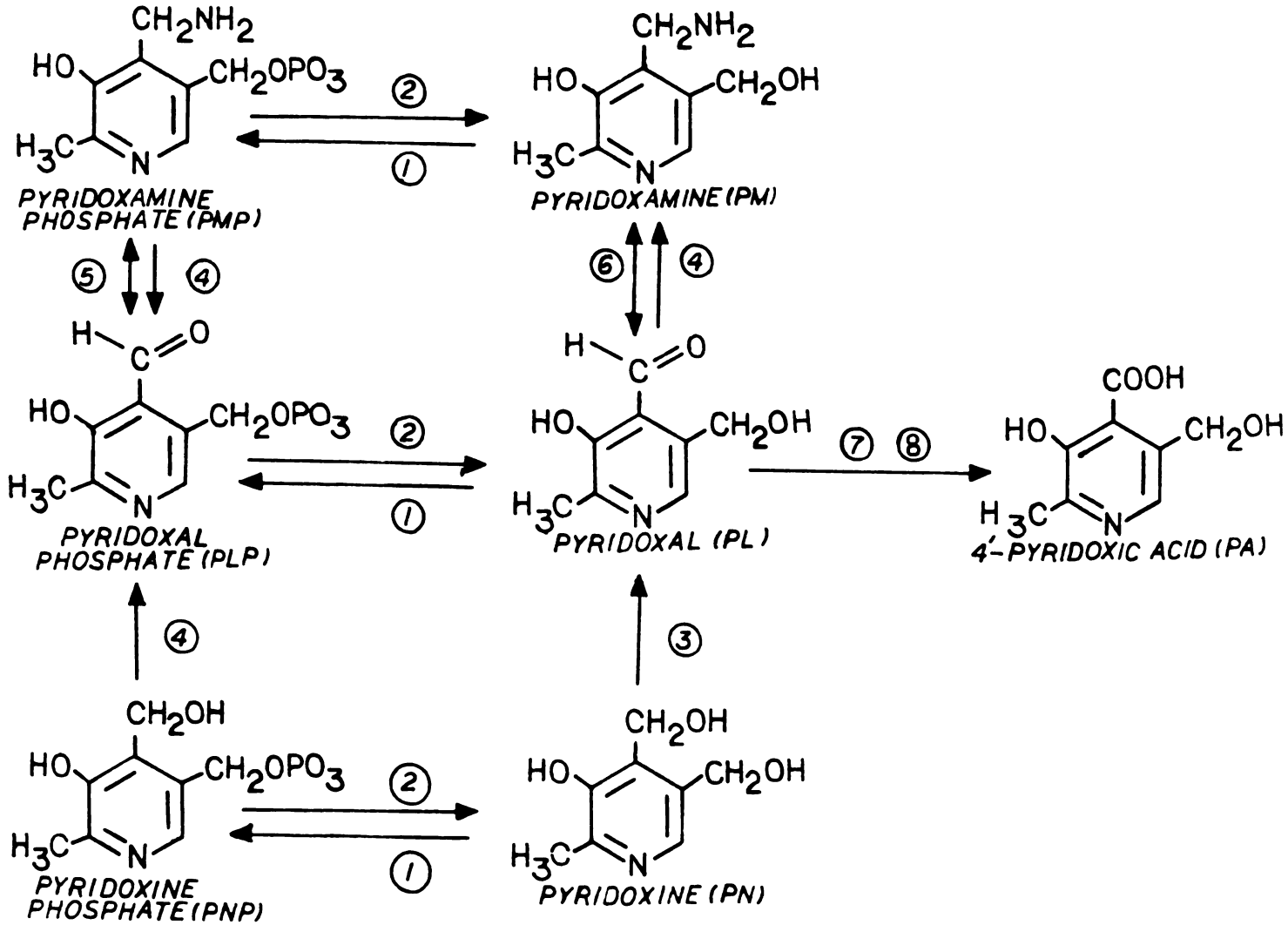

PYRIDOXAMINE PHOSPHATE (PMP)
PYRIDOXAMINE (PM)
PYRIDOXAL PHOSPHATE (PLP)
PYRIDOXAL (PL)
4'-PYRIDOXIC ACID (PA)
PYRIDOXINE PHOSPHATE (PNP)
PYRIDOXINE (PN)

Figure 1

thought to be due to rapid conversion to the phosphorylated forms which do not readily cross cell membranes (Snell & Haskell, 1970). Pyridoxal phosphate (PLP) in the intestinal lumen is hydrolyzed and transported as pyridoxal (PL), although the intact PLP is slowly transported (Mehansho et al., 1979). The site of absorption is the small intestine, with little or no absorption occurring in the stomach or colon. Most of an orally administered dose is absorbed in the jejunum, although the ileum has the capacity (McCoy & Columbini, 1972).

Once in the portal circulation, the vitamers go to the liver where they are rapidly phosphorylated. The role of the liver as the primary source of plasma PLP was established by Lumeng and coworkers (1974). Most tissues can produce PLP from the other vitamers, but only the liver contributes to plasma PLP; the B_6 in excess of hepatic need is released into the circulation as PL, PLP, or PA (Lumeng et al., 1980). PL exists largely in free form with little binding to plasma proteins, whereas PLP is secreted by the liver as a complex with albumin. One function of albumin binding is to prevent hydrolysis of plasma PLP. Following ingestion of PN supplements by human volunteers, plasma PLP, PL, and PA increase 6-12 times the basal concentrations, while the concentration of the other vitamers do not change significantly (Lumeng et al., 1980).

The majority of PLP is hydrolyzed to facilitate transport across cell membranes, although the intact molecule can be transported into the red blood cell (Lumeng et al., 1974; Suzue & Tachibana, 1970). PLP and pyridoxamine phosphate (PMP) are the major intracellular forms in tissues, with little pyridoxine phosphate (PNP) and less than 10% of the non-phosphorylated forms (Lumeng & Li, 1980). The equilibrium

ratio of PMP to PLP varies among tissues and depends on the nature of the binding proteins, the majority of which appear to be enzymes. Lumeng & Li (1980) found the ratio of PMP to PLP in liver, muscle, and brain to be 1.0, 0.2, and 1.9, respectively. The distribution of vitamers may be genetically determined, as the brain and liver PMP/PLP ratios from two strains of mice were significantly different (Lyon et al., 1962).

The concentrations of PLP are very closely regulated in the liver and brain; with increasing B_6 in the diet there is a maximum of PLP in the tissues of rats (Lumeng & Li, 1980). In the liver, such regulation is accomplished by hydrolysis of excess PLP. In contrast, PLP concentrations in blood and muscle do not become saturated during supplementation because of the considerable binding capacity of red blood cells, plasma albumin, and muscle phosphorylase (Black et al., 1977; Lumeng & Li, 1980). At normal intakes the ratio of the concentration of PLP in red blood cells to that in plasma is 1:1, but with increased intakes ratios of 13 to 50 have been obtained (Shane, 1978). This is due to the large capacity for PLP binding to hemoglobin. Based on such observations, skeletal muscle and erythrocytes have been considered storage depots for vitamin B_6 (Lumeng & Li, 1980). However, the PLP associated with muscle phosphorylase is not readily accessible during vitamin depletion, and would therefore not appear to be an effcient reservoir (Black et al., 1978). In contrast, Brin and Thiele (1967) found muscle and kidney to be more severely depleted of total B_6 during deficiency than liver, heart, or brain.

Vitamin B_6 is catabolized to PA which is readily excreted by

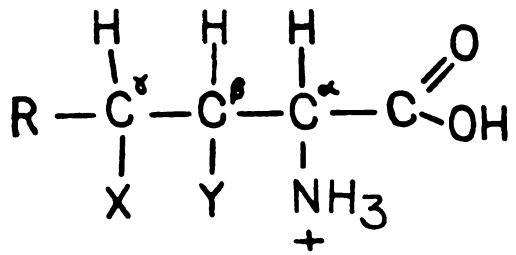
the kidneys. PA represents 28-41% of the urinary B_6 substances for humans fed a normal diet (Stanulovic, 1980). In rats, PA represents only 10% of the B_6 compounds in urine and the rest is PL and PN. In the isolated perfused kidney of the rat, PL and PN are both filtered, and it appears that there is net secretion of PN but net reabsorption of PL (Hamm et al., 1980). PLP is filtered at the glomerulus; this probably involves dephosphorylation and rephosphorylation. However because of the small amount of PLP found free in the plasma, urinary excretion of this compound is negligible. No evidence exists for the fecal excretion of any B_6 compound (Stanulovic, 1980).

Biochemical Roles of B_6 Vitamers

The ultimate active forms of B_6 at the cellular level are the phosphorylated derivatives, PLP and PMP. Most biological reactions involving these molecules occur at the aldehyde or amino functional groups, although evidence exists for the catalytic role of the phosphate in glycogen phosphorylase (Takagi et al., 1982). In addition, the phosphate group functions to retain the molecule within the cell and likely has a steric role in some enzymes (Schnackerz & Feldman, 1980).

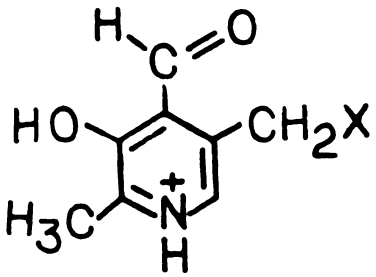
The basis of chemical reactivity of the B_6 compounds is the ability of the amine and aldehyde derivatives (PL, PM, PLP, PMP) to form Schiff bases with other molecules, notably amino acids and amino acyl residues in proteins (Figure 2). The resulting structure, a Schiff base attached to the aromatic pyridine ring, is a highly conjugated system which facilitates the breaking and forming of bonds

Figure 2. Formation of a Schiff base between pyridoxal (PL) or pyridoxal phosphate (PLP) and an amino acid.

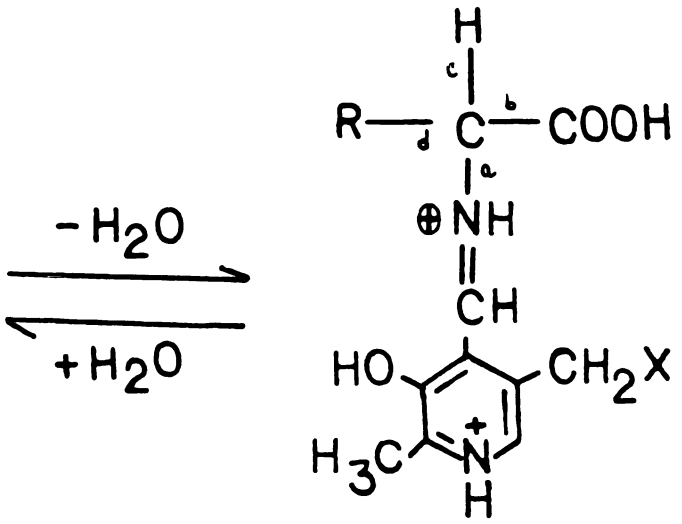


AMINO ACID

+



PL OR PLP



SCHIFF BASE

Figure 2

with amino or keto acids (Walsh, 1979). Furthermore, the nitrogen at position 1 can be reversibly protonated, allowing for the flow of electrons in and out of the ring (Barker, 1971).

Following the formation of the Schiff base with the amino group of an amino acid, a number of reactions are possible (Figure 2). Under appropriate conditions the B_6 vitamers can perform the same reactions in vitro as in vivo ; however a mixture of products results (Barker, 1971; Walsh, 1979). In contrast, when associated with an enzyme the reaction is directed toward the formation of specific product(s) (Dunathan, 1971; Walsh, 1979).

Transaminases are ubiquitous in mammalian tissues and catalyze transfer of the amino group from an amino acid to a receptor α-keto acid. These enzymes are necessary for utilization of glucogenic amino acids and the synthesis of non-essential amino acids. Transamination occurs as a two-step process (Walsh, 1979). PLP bound to the enzyme forms a Schiff base with the substrate amino acid and bond a (Figure 2) is broken. The complex is thus resolved into PMP-enzyme and the product keto acid. A second Schiff base is formed with the PMP and the recipient keto acid. When this bond is broken, PLP is regenerated and the new amino acid released.

Decarboxylation involves cleavage of bond b, with release of CO_2 and an amine. Many biologically active amines are formed by the action of PLP-dependent decarboxylases (Holtz & Palm, 1964). Glutamic acid decarboxylase catalyzes the synthesis of gamma-aminobutyric acid (GABA), and histamine is formed by histidine decarboxylase. Aromatic amino acid decarboxylase will form the neurotransmittor dopamine from the precursor dihydroxyphenylalanine

(DOPA), and serotonin is derived from the decarboxylation of 5-hydroxytryptophan. Additionally, synthesis of the heme precursor δ-aminolevulonic acid (ALA) is catalyzed by ALA synthetase, a decarboxylase.

Serine hydroxymethylase (serine aldolase) is an unusual enzyme which requires both PLP and an additional cofactor, tetrahydrofolate (THF). This enzyme catalyzes the C -C cleavage of serine to glycine with the ultimate formation of N^5, N^{10}-methylene THF, a compound important in the synthesis of purines and pyrimidines (Walsh, 1979). Kyneurinase, an enzyme involved in tryptophan metabolism, is an example of β -replacement. Cystathionase is found in the pathway of methionine metabolism, and represents α-elimination.

Numerous xenobiotics and therapeutic agents interfere with the biological actions of vitamin B_6. A chemical may form a stable Schiff base with the PLP molecule, thus making the coenzyme unavailable for association with an enzyme and/or reaction with the substrate. Furthermore, the urinary excretion of this complex may result in depletion of PLP from the body. Penicillamine is thought to act by this mechanism (DeJesus Sevigny et al., 1966). Some compounds are inactive analogs of the coenzymatic forms of vitamin B_6. They can associate with B_6-requiring enzymes, but are unable to support the catalytic action of the enzyme. The classic B_6 antagonist, 4'-deoxypyridoxine (DOP) may be phosphorylated by PL kinase and subsequently compete with PLP for enzymatic binding sites (Umbreit & Waddell, 1949). The action of enzymes involved in the interconversion of the vitamers may be affected. For example, PL kinase may be inhibited by isonicotinic acid hydrazide and other PL analogs inhibit

PL kinase, preventing formation of the phosphorylated coenzymes (Cornish, 1969; Mizuno et al., 1980) Phosphorylated derivatives of vitamin B_6 also inhibit PNP oxidase (Snell & Haskell, 1970).

Many antimetabolites have significant physiological actions, and have often been used to hasten the development of experimental vitamin B_6 deficiency in animals and humans. However, these compounds may have effects other than on B_6 metabolism. Penicillamine is an effective chelator of several trace elements, and deleterious effects on copper status may be more significant than effects on B_6 nutriture (Heddle et al., 1963; Takeda et al., 1980). The rate of depletion of PLP from several tissues with isoniazid treatment (DeJesus Sevigny et al., 1966) or DOP (Stoerk, 1950a) may not duplicate that of vitamin depletion alone. Although antimetabolites may be useful for certain studies, the syndromes produced by administration of these agents may not be valid models of vitamin B_6 deficiency (Coburn & Mahuren, 1976).

The activity of most vitamin B_6-dependent enzymes is responsive to the level of B_6 nutriture, as well as other dietary and hormonal factors. Several mechanisms are possible to explain the loss of activity which occurs during vitamin deficiency. Enzymes which bind the coenzyme loosely or those which contain subunits that depend on PLP for association may lose PLP during depletion. In such cases, the enzyme may be reactivated by adding exogenous PLP <u>in vitro</u> (Greengard, 1964). In contrast, the activity of alanine aminotransferase was lower in deficient animals but could not be stimulated by PLP (Lee et al., 1977). This was determined to be due to synthesis of defective apoenzyme in the absence of the coenzyme.

The concentration of the apoenzyme may change as the result of vitamin B_6 deficiency. The rate of degradation of several B_6-dependent enzymes appears to be correlated with the affinity for the coenzyme and it has been suggested that for such enzymes the coenzyme protects the apoenzyme against degradation (Anonymous, 1978). However, Lee et al. (1977) have argued that the degradation rate and the affinity for coenzyme reflect a common structural feature of the protein rather than being causally related.

Katunuma (1973) has described proteases which degrade several PLP-dependent enzymes and act by rendering the enzymes susceptible to attack by the non-specific proteolytic enzymes in the cell. The presence of the coenzyme will protect the protein against degradation by the PLP enzyme degrading proteases. These proteases do not degrade hepatic tyrosine aminotransferase (Katunuma, 1973); however, inactivation of this enzyme occurs in homogenates from the liver of vitamin B_6-replete rats (Reynolds & Thompson, 1974), but not from similar preparations from deficient rats (Reynolds, 1978; Sloger et al., 1978). This may explain the observation of Hunter and Harper (1977) that basal and glucocorticoid-induced tyrosine aminotransferase (TAT) was higher in deficient animals. The synthesis and degradation of vitamin B_6 dependent enzymes such as TAT may be controlled by separate mechanisms which are individually responsive to dietary and hormonal factors. Thus, in order to examine the regulation of enzymatic activity, studies must be conducted in which dietary (vitamin deficient vs. replete) and hormonal factors (adrenalectomized, sham-operated) are considered in concert.

Recent evidence suggests that PLP may have a role in the actions

of steroid hormones in the cell. Cidlowski and Thanassi (1981) have proposed that PLP facilitates the recycling of steroid receptors. PLP would both increase the activation of the steroid/receptor complex in the cytosol (Sekula et al., 1982) as well as hasten its dissociation from the nuclear DNA (Cake et al., 1978). In this manner the availability of the receptor for the next steroid molecule would be increased. Such actions of PLP on steroid receptors have been observed with the glucocorticoid receptor (DiSorbo et al., 1980), the prostate androgen receptor (Hipakka & Liao, 1980; Mulder et al., 1980), and the uterine progesterone receptor (Chen et al., 1981).

Deficiency Symptoms

Within two to three weeks after the introduction of a vitamin B_6 deficient diet, the food intake of weanling rats decreases compared to the intake of rats fed a control diet. The deficient animals grow at a slower rate and the body weight reaches a plateau, usually after 6-7 weeks. The weight gain per 100 g food ingested is less in vitamin B_6 deficient rats than that of animals pair-fed a control diet (Sure & Easterling, 1949).

The first symptom by which vitamin B_6 deficiency was distinguished from that of other B complex vitamins was a form of dermatitis termed acrodynia. The skin in the affected areas is scaly, often with a bloody exudate. In rats, the lesion involves the snout, paws, ears, and tail. In extreme cases, the tail curls rigidly into a flat spiral which does not straighten out under anesthesia (Stoerk, 1950a). Microscopically the tissues exhibit hyperkeratinosis, intercellular edema, congested capillaries, atrophy of hair follicles

and sebaceous glands, and infiltration with inflammatory cells (Antopol & Unna, 1942). Although alopecia has been noted in some cases, the condition does not become severe. Humans also develop dermatitis around the mouth and eyes during experimental B_6 deficiency (Vilter et al., 1953).

Vitamin B_6 has several important roles in the functioning of the nervous system. Pyridoxal phosphate is a coenzyme for enzymes which catalyze the synthesis of biogenic amines such as serotonin, GABA, catecholamines, dopamine, and taurine (Holtz & Palm, 1964; Ebadi, 1978). Convulsions occur in deficient animals (Loo, 1980) and were observed in human infants fed a vitamin B_6 deficient formula (Coursin, 1955). The synthesis of sphingomyelin requires vitamin B_6 (Loo, 1980) and the lipid composition of the brain is altered during deficiency of the vitamin (Loo, 1980). Depletion of B_6 resulted in decreased synthesis of cholesterol, cerebrosides, long chain fatty acids, and myelin (Loo, 1980). Peripheral neuritis has been reported in adult humans consuming a vitamin B_6 deficient diet plus an antimetabolite (Vilter, et al., 1953).

Vitamin B_6 deficiency has deleterious effects on the immune system. The lymphatic tissues of deficient monkeys and rats atrophy, with decreased density of small lymphocytes in the thymus and an increase in the number of abnormal cells (Robson & Schwarz, 1980). Impaired cellular immunity is also manifested as the inability of lymph cells *in vitro* to respond to genetically dissimilar cells (Robson & Schwarz, 1980). The effects of B_6 deficiency on the immune system are not mediated by the adrenal cortex or inanition (Agnew & Cook, 1949), but are likely to be due to impaired synthesis of nucleic acids

(Robson & Schwartz, 1980).

Pyridoxal phosphate is involved at several points in the metabolism of tryptophan (Henderson & Hulse, 1978). The conversion of tryptophan (TRP) to tryptamine or 5-hydroxytryptophan to serotonin require PLP-dependent decarboxylases. The most quantitatively important pathway, however, is that from TRP to xanthurenic acid or N'-methylnicotinamide. Following administration of a tryptophan load, a larger amount of kynurenine is formed from TRP. Kynurenine accumulates in the cytosol and migrates into the mitochondria. In the mitocondria, kynurenine is hydroxylated, because the affinity of the hydroxylase for kynurenine is 100 times that of the transaminase. Further metabolism of 3-hydroxykynurenine by PLP-dependent enzymes in the cytosol is depressed in the vitamin deficient state, whereas that in the mitochondria is affected to a lesser degree, resulting in preferential production of xanthurenic acid.

Vitamin B_6 also participates in the metabolism of sulfur-containing amino acids (Sturman, 1978). Increasing the amount of methionine has been noted to aggravate the growth depressing effects of a vitamin B_6 deficient diet (DeBey et al., 1952). PLP-requiring enzymes occur in the biosynthetic pathway for taurine, which is required for growth and functioning of the brain. In addition, the synthesis of the polyamines putrescine, spermidine, and spermine require vitamin B_6 in ornithine decarboxylase; the role of PLP in S-adenosyl methionine decarboxylase is not certain (Pegg, 1977).

Vitamin B_6 is involved in several pathways of amino acid metabolism, and the effects of deprivation on intermediary metabolism have been studied extensively. The symptoms of vitamin B_6

deficiency are aggravated when a high-protein diet is used, and the requirement for the vitamin appears to increase with the protein content of the diet (Canham et al., 1969; Holtz & Palm, 1964). However, Williams (1964) observed that increased pyridoxine improves growth when rats are fed low protein diets which are also limited in an amino acid.

It has been suggested that the observed effects of B_6 deficiency on protein synthesis, as measured by amino acid incorporation, represent alterations in amino acid precursor pools (Okada & Suzuki, 1974). Deficiency of vitamin B_6 alters the utilization and the cellular uptake of amino acids which would affect availibility (Anon., 1979). The net effect of vitamin B_6 deficiency on protein metabolism would depend on several factors, which include: changes in enzyme activities accompanying increased protein and/or decreased B_6 intake, the amino acid composition of the protein, the rates of individual B_6-dependent reactions, and endocrinological sequelae of changes in protein and B_6 nutriture (Williams, 1964).

A role of vitamin B_6 in lipid metabolism has long been suspected; however, reports in the literature are conflicting and inconclusive. A decrease in the proportion of arachadonic acid in the lipids from tissues of deficient rats has been reported (Mueller, 1964). Arteriosclerotic lesions were observed in the arteries of monkeys (Greenberg, 1964). Studies on cholesterol metabolism have proven to be equally contradictory, with reports of hypercholesteremia, no change, and hypocholesteremia in deficient animals (Mueller, 1964). It is difficult to compare the results of

studies, due to differences in the fat and protein composition of the experimental diets (Mueller, 1964).

Sabo and coworkers (1971) concluded that although B_6 deficient animals have less body fat, the ability of liver and adipose tissue to synthesize lipid may be greater than that of controls if glucose is available. They also noted that insulin administration increased the activity of glucose-6-phosphate dehydrogenase to a larger extent in deficient animals (50%) than in similarly treated contols (13%). Further studies by this group indicate that changes in lipid and glucose metabolism in adipose tissue from deficient rats may be secondary to increased transport of glucose into the cells in vitro (Ribaya & Gershoff, 1977).

Many of the effects of B_6 deficiency on intermediary metabolism have been ascribed to actions on endocrine systems. Insulin administration can prevent several of the symptoms of B_6 deficiency, including changes in fatty acid composition (Gershoff, 1968), decreased appetite and impaired lipid storage (Beaton et al., 1956). Insulin activity was found to be reduced in the plasma and pancreatic tissue from deficient rats. Additionally, Singh (1980) observed diminished amylase content in acinar cells of the pancreas, as well as impaired nucleic acid and protein metabolism. It is possible that the effects on insulin production result from similar depression of pancreatic metabolism.

Early studies indicated that pituitary function is affected in vitamin B_6 deficiency. Administration of growth hormone (GH) to deficient animals resulted in a growth response which was not observed in control animals (Huber & Gershoff, 1965). Deficient rats had low

pituitary and serum GH levels compared to ad libitum (Huber & Gershoff, 1965) but not pair-fed controls (Rose, 1978). The vitamin B_6 content of the pituitary was found to be high compared to other organs (Huber & Gershoff, 1965), suggesting a possible role of the vitamin in the functioning of the gland. Beare and coworkers (1953a) observed that administration of GH aggravated the effects of the deficiency (severity of acrodynia, increased BUN, decreased thymus weight, increased adrenal weight). Administration of the GH to pyridoxine-sufficient rats also increased the adrenal weight and decreased thymus weight.

The follicle-stimulating activity (FSH) in the pituitary of vitamin B_6-deficient rats was greatly increased compared to controls, whereas interstitial cell stimulating hormone (ICSH; lutenizing hormone) and prolactin were only slightly affected (Wooten et al., 1955). However, the amount of FSH necessary to stimulate follicular growth was much higher in deficient rats (Wooten, 1958). The authors suggested that the release of FSH was impaired in vitamin B_6 deficient rats.

Increased kidney weight and kidney/body weight ratios have been reported in vitamin B_6-deficient rats (Olsen & Martindale, 1954; Agnew, 1955). Agnew (1949, 1951) observed hematuria in hooded Lister rats maintained on a B_6 deficient-diet for longer than 11 weeks. Microscopic examination of the kidneys from these animals revealed fibrosis and areas of calcification at a much greater frequency than found in rats fed a control diet. Albino (Wistar) rats did not develop hematuria or fibrosis, thereby demonstrating a marked strain difference in response to the deficient diet. Lesions were observed in the kidneys of rats fed a vitamin B_6 deficient diet for 33 weeks

(Reynolds & Slaughter, 1980). These consisted of focal necrosis, inflammation, and regenerative hyperplasia in the tubules; however, data were not available comparing the various nephron segments.

Elevated levels of protein in the urine of deficient male rats were reported by Linkswiler and coworkers (1952). The proteinuria was aggravated by increasing the percentage of protein in the diet, and still further by increasing the amount of methionine. This was not a non-specific result of a dietary deficiency inasmuch as rats fed a low-protein diet exhibited no change in amount of protein excreted, and pantothenic acid-deficient rats excreted less protein. Davis and Sloop (1965) reported decreased renal concentrating ability in rats maintained on a vitamin B_6-deficient diet for five months. This defect was apparent only when both the deficient rats and pair-fed controls were given urea in the drinking water, a manipulation which increased the maximum osmolality of the urine in both groups. No histological abnormalities were found in any area of the kidney in this study.

Blood urea nitrogen (BUN) in vitamin B_6 deficient animals has been reported by several groups to be increased (Lyon et al., 1958; DiPaolo et al., 1974; Hawkins et al., 1946). The increase was found to be strain- and age- dependent for mice (Lyon et al., 1958). In young mice, which are usually more susceptible to B_6 deficiency than adults, no consistent difference in BUN could be found during six weeks on the experimental diets. Furthermore the rise in BUN of adult animals was transient, and exogenously administered urea was cleared from the blood as rapidly as in animals with no increase in BUN (Beaton et al., 1953).

Olsen and Martindale (1954) reported that the sodium space of the B_6 deficient rats was elevated (26.4% ± 0.8) but not significantly different from control animals (23.8% ± 0.8). Hsu and Kawin (1962) demonstrated that plasma volume was higher in deficient rats (3.24 ± .08 ml/100 gm BW), though again not significantly different from controls (2.80 ± .04 ml/100 gm BW). Hsu et al. (1958) postulated that the elevated serum sodium concentration in B_6 deficient rats which they observed was responsible for the hypertension noted by others. Based on their observation of enhanced KCl preference in deficient rats, Stewart and Bhagavan (1982) further suggested that B_6 deficiency is associated with hyperaldosteronism.

An association between deficiency of B-complex vitamins and increased blood pressure was first noted in 1942 by Calder. Subsequent studies indicated that vitamin B_6 deficiency in rats produces an elevation in blood pressure. Olsen and Martindale (1952) noted a rise in systolic pressure which reached a maximum (15 mm Hg) after four months on the deficient diet. Inclusion of DOP in the diet elicited no further increase, and supplementation of the diet with PL resulted in a rapid increase in weight and a concomitant fall (20 mm Hg) in blood pressure. Further studies showed that dietary DOP could produce increases in the blood pressure of control rats (Olsen & Martindale, 1954).

The blood pressure of rats was found to increase during the first seven weeks on a vitamin B_6 deficient diet, but decreased slightly between the 7th and 14th weeks (DeLorme et al., 1975). Plasma renin acitvity and angiotensin concentration in vitamin B_6-deprived animals were twice that of the controls. The measurements were taken

in the eighth week of the study, several weeks after the blood pressure of the deficient animals had reached a plateau. At this time the animals were significantly affected by the vitamin depletion; the authors noted cessation of growth with the appearance of acrodynia by five to six weeks. At an advanced stage of deficiency it is possible that the effects on renin activity and angiotensin II could be the result of a debilitated state; for example, Brown and Pike (1960) reported hypoproteinemia in vitamin B_6 deficient rats. It would be difficult to conclude from the data presented that the increased plasma renin or angiotensin determined at 8 weeks contributed to the elevation of blood pressure observed at three weeks.

ADRENAL GLANDS

Corticosteroid Synthesis and Metabolism

In most mammals, the adrenal glands consist of a cortex of steroid-secreting cells surrounding a medulla containing cells which produce catecholamines. The cortex is further stratified into three histologically distinct layers (Wheater et al., 1979). The zona glomerulosa, in which the cells are arranged in clumps, lies immediately beneath the capsule of the gland. The zona reticularis is adjacent to the medulla, and the cells of this cortical zone are small and arranged in an irregular network. Interposed between the zona glomerulosa and zona reticularis is the broadest layer of the cortex, the zona fasciculata, comprised of columns of cells separated by strands of connective tissue. The blood supply to the adrenals is from three arteries which ramify within the cortex (Wheater et al.,

1979). Branches of these arteries also descend directly into the medulla and form a network around the chromaffin cells. All of the capillaries and venules of the cortex and medulla drain into the central vein of the medulla; therefore, blood flow in the adrenal is from the outer regions to the inner ones.

Three major classes of steroids are synthesized in the adrenal cortex: glucocorticoids, mineralocorticoids, and androgens. The glucocorticoids largely affect intermediary metabolism and have an important role in response of the animal to stress. Mineralocorticoids modulate salt and water homeostasis. The adrenal androgens supplement sex steroids produced by the gonads, and are a major source of androgens in females. The pathways of adrenal cortical steroidogenesis are shown in Figure 3. Cholesterol is the common precursor for all corticosteroids and is stored as esters within lipid droplets in the cells of the cortex. Although adrenal cells have the capacity to synthesize cholesterol, plasma is the primary source of cholesterol for steroidogenesis (Boyd et al., 1983). The synthesis of pregnenolone involves removal of the side chain of cholesterol following hydroxylation at C_{21} and C_{22} (Sandor et al., 1976; Boyd et al., 1983). These reactions are catalyzed by an enzyme complex which contains a hemoprotein, cytochrome P450, and a flavoprotein (NADPH-adrenodoxin reductase). Additionally the non-heme iron-sulfur protein, adrenodoxin, is required (Omura et al., 1966). The availability of cholesterol to the cytochrome P450 which catalyzes the side-chain cleavage (cytochrome $P450_{scc}$) is the rate-limiting step in corticosteroid synthesis.

The transformation of pregnenolone to progesterone occurs on the

Figure 3. Pathways of steroidogenesis in the adrenal cortex. Enzymes in the pathways of glucocorticoid and mineralocorticoid production are designated by numbers.

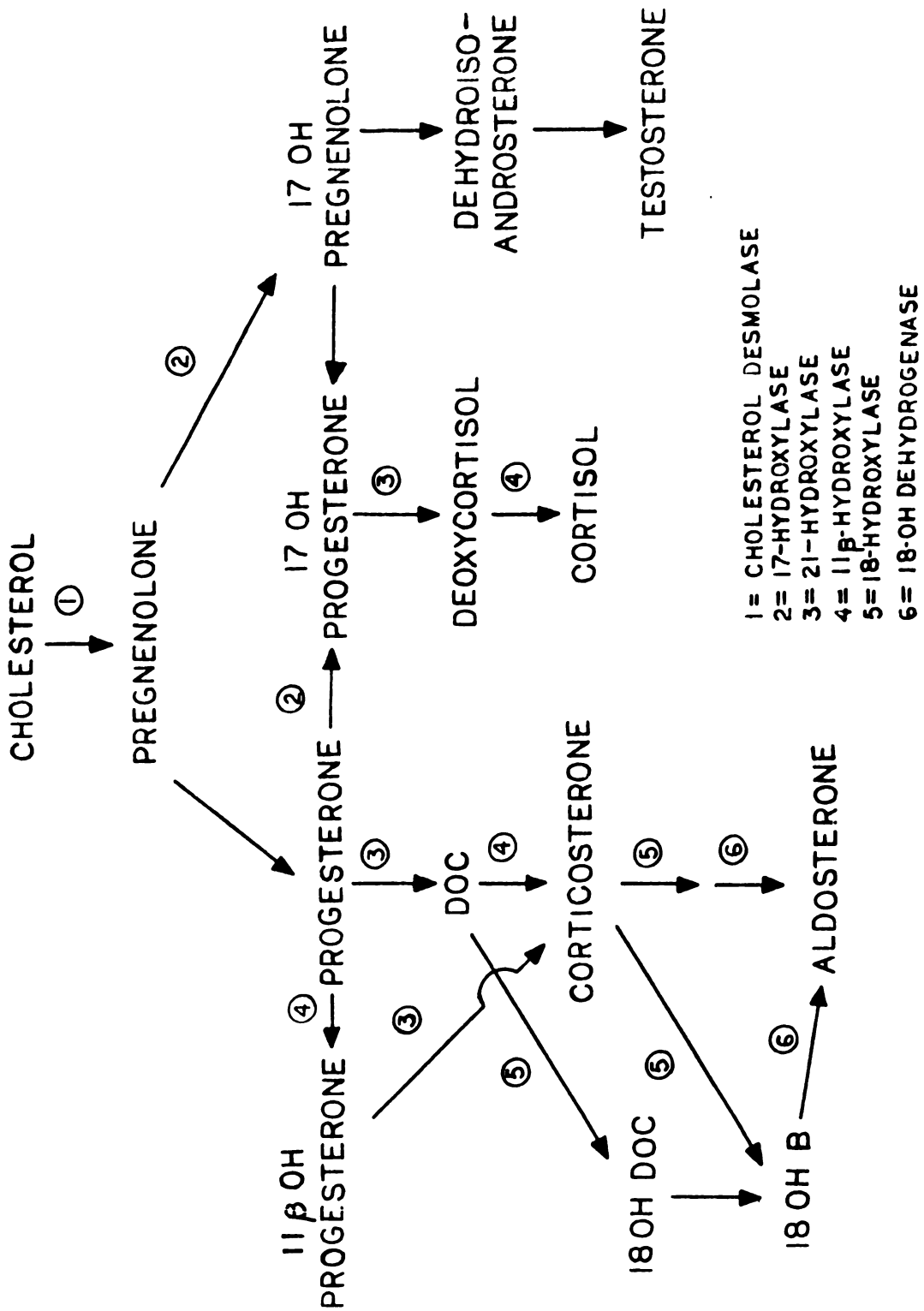

CHOLESTEROL
①
PREGNENOLONE
②
17 OH PREGNENOLONE
DEHYDROISO-ANDROSTERONE
TESTOSTERONE
②
17 OH PROGESTERONE
③
DEOXYCORTISOL
④
CORTISOL
②
PROGESTERONE
③
DOC
④
CORTICOSTERONE
⑤
⑥
ALDOSTERONE
④
11β OH PROGESTERONE
③
⑤
18OH DOC
⑤
⑥
18 OH B
1= CHOLESTEROL DESMOLASE
2= 17-HYDROXYLASE
3= 21-HYDROXYLASE
4= 11β-HYDROXYLASE
5=18-HYDROXYLASE
6= 18-OH DEHYDROGENASE

Figure 3

endoplasmic reticulum in the cytoplasm of the cells and is believed to involve two enzymes (Sandor et al., 1976). Progesterone may be further metabolized on the endoplasmic reticulum by hydroxylation at C_{21} or C_{17}. These reactions are catalyzed by two cytochrome P450-dependent enzymes which differ from the mitochondrial hydroxylases in the lack of requirement for adrenodoxin (Sandor et al., 1976).

The formation of the glucocorticoids corticosterone (B) and cortisol from the 21-hydroxylated products occurs in the mitochondria. This reaction is catalyzed by 11β-hydroxylase, one of the most intensely studied enzymes of the adrenal cortex. Similar to the cholesterol side-chain cleavage enzymes, 11β-hydroxylase is located on the inner membrane of the mitochondria and requires a cytochrome P450 (Omura & Sato, 1964), adrenodoxin (Nakamura & Otsuka, 1966), and NADPH-adrenodoxin reductase (Omura et al., 1966). 11-Deoxycorticosterone (DOC) and B may alternatively be hydroxylated at C_{18} to form 18-hydroxy-11-deoxycorticosterone (18-OH-DOC) and 18-hydroxy-corticosterone (18-OH-B) respectively (Fraser & Lantas, 1978).

The diagram of steroidogenesis in Figure 3 is a composite of known pathways for several species. The activity of 17α-hydroxylase is low in rats, rabbits, and mice, and therefore these species produce B as the major glucocorticoid (Sandor et al., 1976; Vinson & Whitehouse, 1970). For other species, including humans, cortisol is the most important glucocorticoid. Similar differences exist in mineralocorticoid metabolism. Although aldosterone is the most potent mineralocorticoid produced by the adrenals, the adrenal gland of the rat synthesizes greater quantities of DOC (Vinson & Whitehouse, 1970).

In contrast, aldosterone is physiologically more important in humans.

In addition to histological heterogeneity, the zones of the adrenal cortex appear to be distinct in function (production of steroids) and in their response to trophic and regulatory stimuli. The zona fasciculata and zona reticularis are the primary site of glucocorticoid and androgen synthesis, whereas mineralocorticoid production occurs in the zona glomerulosa. However DOC and 18-OH-DOC, which have mineralocorticoid properties, are formed in significant quantities in the inner cortex (Vinson & Whitehouse, 1970). Furthermore aldosterone synthesis from cholesterol proceeds through B in the zona glomerulosa. Thus, the three zones of the adrenal cortex are not strictly distinguished by the type of steroid produced.

The zones do however respond differently to regulatory stimuli. Such stimuli may have acute effects on specific enzymes and/or chronic trophic effects on the cells. The inner regions (fasciculata and reticularis) appear to depend on adrenocorticotropic hormone (corticotrophin; ACTH) for appropriate functioning. The response of the zona glomerulosa to ACTH is much less than that of the inner zones, and this zone does not atrophy following hypophysectomy (Tait et al., 1970), but is affected by factors which accompany changes in electrolyte and fluid balance (Muller et al., 1970; Aguilera et al., 1981). The mechanisms by which regulatory stimuli such as ACTH exert selective effects on corticosteroid metabolism despite common synthetic pathways is unknown. In particular, schemes such as those in Figure 3 imply that some of the enzymes may be involved in the synthesis of several hormones. It is difficult to reconcile this with the specificity of action of the regulatory factors.

Lieberman and coworkers (1984) have developed a model for adrenal steroidogenesis which attempts to account for such discrete actions. The central construct of their model is that the steroids are produced in units termed "hormonads". The hormonads may be enzymes which are membrane-bound and functionally linked, or may be those with affinity for a particular substrate. The enzymes involved would be arranged so that the steroid molecule would be transformed by a concerted series of reactions without leaving the unit. Each hormone would be produced in its own hormonad and the factors which regulate the synthesis of a particular hormone(s) would act at the level of the hormonad, rather than a type of cell or an enzyme.

Although the concept of the hormonad as a functional grouping of enzymes is interesting, the authors do not persuasively rule out the possibility that the functional unit is the cell. That is, single cell types produce distinct hormonal products, and the cells respond individually to regulatory stimuli. Additionally, Lieberman and coworkers review evidence that there are multiple forms of cytochrome $P450_{scc}$, and 11β-, 18-, and 21-hydroxylase, and that the isoenzymes in the zona glomerulosa have different characteristics than those in the inner zones. This would provide another mechanism for the differential effects of ACTH on corticosteroid synthesis.

The primary regulatory factor influencing glucocorticoid synthesis and secretion is ACTH. ACTH is a single-chain peptide hormone of 39 amino acid residues which is synthesized in the adenohypophysis as part of a larger precursor molecule. Release of ACTH is controlled by the hypothalamic corticotropin releasing factor (CRF). CRF is released as the result of excitatory stimuli (such as "stress") on the CRF neurons

in the hypothalamus (Jones et al., 1982) and is delivered via portal vessels directly to the pituitary where it induces the release of ACTH. The principle target cells for ACTH in the adrenal cortex are those of the inner zones. ACTH increases the synthesis of steroids by stimulating the association of cholesterol with cytochrome P450 (Kido & Kimura, 1982) and possibly by increasing 11β -hydroxylation (Lieberman et al., 1984). The actions of ACTH on steroidogenesis are mediated by receptors in the plasma membrane of the cell. At least two sets of receptors have been postulated to be involved; one group which acts via cAMP and the others independently of cAMP (Schwyzer, 1980).

The hypothalamus, pituitary, and adrenal act in concert to control circulating levels of ACTH and glucocorticoids (Jones et al., 1982). The operation of the hypothalamus-pituitary-adrenal (HPA) axis is complex, involving multiple, interrelated feedback mechanisms. The basic elements of regulation of plasma glucoroticoid concentration are a negative feedback controller with a varible set-point (Yates & Urquhart, 1962). In addition, recent evidence indicates that there are multiple sites of control, as well as a pathway that bypasses several of these sites (Keller-Wood & Dallman, 1984). The plasma corticosteroid and ACTH response to a stimulus depends on several factors, including (Keller-Wood & Dallman, 1984):

1. the type and magnitude of the stimulus,
2. the plasma corticosteroid concentrations, both current and in the past few days, and
3. the present sensitivity of the system to both the stimulus and the corticosteroid feedback mechanisms.

Mineralocorticoid synthesis is modulated in response to changes in electrolyte and fluid balance. The major mineralocorticoids are

aldosterone and DOC, although several other steroids may have salt-retaining properties under certain conditions. The renin-angiotensin system is the primary factor regulating mineralocorticoid production in most species. Renin is a proteolytic enzyme produced in the kidneys by cells of the juxtaglomerular apparatus (Oparil & Haber, 1974). The substrate for renin (renin substrate; angiotensinogen) is a protein produced by the liver and is hydrolyzed by renin to the decapeptide angiotensin I. Angiotenin I is metabolized to the octapeptide angiotensin II by converting enzyme, the highest concentration of which is in the lungs.

Angiotensin II (AII) is the agent which stimulates aldosterone secretion; AII will increase aldosterone synthesis in vitro whereas renin will not. This action is believed to occur primarily on a later step of aldosterone synthesis, most likely after B, though some evidence exists for stimulation at an earlier stage (Muller et al., 1970). However, AII does not increase cortisol at doses which induce aldosterone synthesis. Renin release, and hence AII production, is stimulated by a number of factors including decreased renal perfusion pressure, increased sympathetic activity from receptors in renal vessels, and decreased sodium flux across the macula densa of the nephron. AII may not be the principle stimulus to aldosterone synthesis in rat adrenals that it is in most other species (Tait et al., 1970; Vinson & Kenyon, 1976). Potassium and ACTH also stimulate aldosterone synthesis, as does sodium depletion (Muller et al., 1970).

Corticosteroids exist in plasma in several forms, including the native molecule which may be free or bound to plasma proteins, and conjugated or unconjugated metabolites which may also be free or

protein-bound. The unbound form of the steroids is available to the tissues. The plasma proteins to which native corticosteroids bind are transcortin (corticosteroid binding globulin; CBG), sex hormone binding globulin, and albumin. Albumin has the greater concentration of steroid binding sites in plasma, however CBG has a higher affinity for corticoids (Siiteri et al., 1982). Binding to proteins increases the plasma half-life of the steroids by decreasing conversion to inactive metabolites as well as reducing renal excretion. The differing affinities of the steroids for the proteins may thus contribute to differences in metabolic clearance rate among the steroids. Furthermore, Siiteri et al. (1982) have proposed that CBG is important in the intracellular mechanism of action of these steroids.

The major site of degradative metabolism of the corticosteroids is the liver, although extrahepatic sites may be significant for some compounds (Yates & Urquhart, 1962). The inactivation process primarily involves reduction of ring A of the molecule by hepatic Δ^4-steroid hydrogenases which are specific for each steroid (Yates, 1965). Removal of the corticosteroids from plasma by the liver follows first-order kinetics: when the concentration in plasma rises, hepatic extraction increases by a constant proportion. Changes in hepatic Δ^4-steroid hydrogenase activity can be accomplished by various factors, including administration of androgens, estrogens, or thyroid hormones, thyroidectomy, and sodium-depletion (Yates & Urquhart, 1962). However, these enzymes are not induced by their substrate; instead the adrenal secretion rate changes to accommodate alterations in hepatic metabolism. Therefore, although the hepatic degradation of steroids affects the secretion rates of the adrenal cortex, it is not a

determinant of plasma corticosteroid concentrations (Yates, 1965).

BIOLOGICAL EFFECTS OF CORTICOSTEROIDS

The biological actions of steroid hormones result from the interaction of these compounds with cellular genetic material (O'Malley & Schrader, 1976). Initially the hormones diffuse across the plasma membrane and in target cells combine with high affinity receptors in the cytoplasm. The binding of the hormones induces a conformational change in the receptors producing activated receptor/hormone complexes. These complexes are translocated to the nucleus where they bind to chromatin and increase the expression of DNA, including that coding for specific messenger RNA. In order to respond to a corticosteroid, a tissue must have specific receptors for that hormone. The concentration of receptors is not constant and may change during maturation or as a result of the actions of other hormones (Giannopoulos, 1975). Thus, the activities of steroid hormones in the body are modulated by the target tissues, as well as by control of hormone synthesis and release.

The physiological and biochemical actions of glucocorticoids are diverse, and attempts to unify these effects under the auspices of a single comprehensive "function" have met with limited success. In the 1930s, the association of the adrenal cortex with resistance to various noxious conditions (trauma, severe exercise, infections) had been made. Such observations were unified by Seyle in the 1940s, who applied the term "stress" to these stimuli and determined that glucocorticoids were the adrenal agents responsible for the resistance to stress (Seyle, 1946). Recently, Munck and coworkers (1984) have again related

the functions of glucocorticoids to stress; they suggest that increased activity of the adrenal cortex during stress serves to limit the extent of the body's response to stress, rather than facilitate it. The authors summarize evidence that the glucocorticoids antagonize a number of homeostatic mechanisms including the inflammatory response to tissue damage, the immune reaction to infection, fluid loss in response to antidiuretic hormones, insulin during certain metabolic disturbances, and to modulate the production of biologically active peptides, including ACTH and β-endorphin (Munck et al., 1984). Alternatively, the beneficial effects of glucocorticoids during stress have been attributed to maintenance of blood pressure and blood glucose (Yates et al., 1974).

Ingle (1954) suggested that the actions of glucocorticoids were permissive; i.e. they were required to facilitate the normal response to other hormones and were effective at basal concentrations. The permissive actions of hormones may occur through several possible mechanisms (Nelson, 1980). First, glucocorticoids may have a permissive effect on the actions of agents such as glucagon, epinephrine, ACTH, and GH via specific receptor-mediated action. In some cases, glucocorticoids may alter the rate of enzymatic reactions (eg. alkaline phosphatase, Na-K ATPase) by inducing the synthesis of a protein which increases the efficiency of the reaction. For example, the permissive actions of glucocorticoids on the effect of glucagon or epinephrine on the transport of α-aminobutyric acid (AIB) have been noted to be dependent upon the synthesis of protein. Finally, some permissive effects may be due to actions of glucocorticoids on the physical properties of membrane phospholipids.

Glucocorticoid administration increases the concentration of glucose in the plasma (Loeb, 1976; Wilcke & Davis, 1982). The most immediate action is to reduce the uptake of glucose by peripheral tissues, antagonizing the effects of insulin. Such a decrease in glucose transport requires protein synthesis. Subsequent enhancement of hepatic glucoenogenesis contributes to elevated blood glucose. Gluconeogenesis is facilitated by induced synthesis of enzymes involved in amino acid degradation (eg. transaminases, tryptophan dioxygenase). In addition there is increased availability of gluconeogenic precursors due to changes in peripheral protein and fat metabolism as well as increased activation of glycogen synthetase and deactivtion of glycogen phosphorylase (Wilcke & Davis, 1982). Glycogen accretion has been observed in fasting animals receiving glucocorticoids. Synthesis of new protein in peripheral tissues is decreased by glucocorticoid administration, and protein catabolism is enhanced. Lipid metabolism is also altered by glucocorticoids; synthesis of long chain fatty acids is reduced, as well as mobilization of fatty acids from lipid depots. Thus, in several respects, glucocorticoids can be seen to be antagonists of insulin. Tissues from glucocorticoid deficient animals are more sensitive to insulin.

Glucocorticoids also have significant effects on cellular growth and proliferation. Administration of glucocorticoids results in increased liver size due to cellular hypertrophy. There is accumulation of glycogen, protein, water, and mRNA in the cells with concommitant inhibition of hepatic cell proliferation in neonatal and weanling rats (Loeb, 1976; Weiss & Silbermann, 1981). The effect on cell proliferation, as measured by thymidine incorporation into DNA,

varies among tissues. The liver, heart, kidneys, and skeletal muscle were sensitive, whereas the brain, testis, spleen, bone marrow and GI tract were found to be resistant to the inhibition of cellular proliferation by glucocorticoids (Loeb, 1976). Others have noted these effects to be sex- and age-dependent (Takahashi et al., 1982). In tissue culture, the effects of glucocorticoids on various cells of hepatic origin were found to be similar to the results in vivo. Removal of gluccorticoids from the culture medium resulted in immediate DNA synthesis and cell proliferation.

Glucocorticoid excess, as well as deficiency, has significant effects on salt and water balance. Prolonged administration of glucocorticoids is associated with hypertension. This may be due in part to redistribution of body water, with an increase in plasma volume. On the other hand, adrenalectomized animals do not excrete a water load as efficiently as do intact animals, and administration of glucocorticoids will correct this antidiuresis. Potassium excretion is increased following dexamethasone administration (Bia et al., 1982). Dexamethasone had a greater kaliuretic effect than aldosterone; furthermore sodium excretion was not affected. The authors concluded that the effects of dexamethasone on potassium excretion were not due to mineralocorticoid activity. Furthermore, the data indicates that enhanced urine flow contributed to the increased K^+ excretion, and in contrast to earlier studies with glucocorticoids (Garrod et al., 1955), dexamethasone did not increase glomerular filtration rate (GFR).

EFFECTS OF VITAMIN B_6 DEFICIENCY ON THE ADRENAL GLANDS

Histological evidence from several papers suggests increased activity in the adrenal glands from vitamin B_6 deficient rats. During a detailed study of pathological changes in various tissues of B_6 deficient Wistar male rats, Antopol and Unna (1942) observed that the zona fasciculata of the adrenals was wider and the cells were enlarged compared to those from rats receiving adequate vitamin B_6. The reticular zone was narrow and the cells were more compact, as were those of the adrenal medulla. Following administration of pyridoxine to deficient rats the cytoplasm in the fasciculata became clearer and less granular, and the medullary cells were larger and less dense. The adrenals of vitamin B_6 deficient rats did not hemorrhage, as did those of pantothenic acid deficient rats.

Deane and Shaw (1947) studied the time course of histochemical changes in the adrenal cortex of Long-Evans male rats. They observed increased sudanophilic and Schiff reagent staining in the inner zones of the cortex, indicative of increased lipid content and ketosteroids, respectively. By six to ten weeks on the deficient diet, the histochemical behavior of these areas was comparable to that of controls. The mitochondria were normal in appearance at all times, as was the histology of the zona glomerulosa. The adrenals of the B_6 deficient rats were heavier than those from controls, until 6 weeks. In contrast to the B_6 deficient animals, the adrenals of thiamine deficient rats had less lipid in the zona fasciculata and the mitochondria were swollen and irregular. There were no significant changes in the adrenals from riboflavin deficient rats. In food

restricted rats, sudanophillic lipids in the zona fasciculata were normal at the end of one week, markedly increased by three weeks, but decreased by the fourth week. The ketosteroids increased throughout the duration of the experiment. The authors concluded that the abnormalities in thiamin-deficient animals, as well as in the pantothenic acid deficient rats studied earlier (Deane & McKibbin, 1946), resembled the adrenal response to stress which had recently been described by Seyle (1946). In contrast, the changes observed during vitamin B_6 or riboflavin deficiency were not similar to changes found during stress.

The adrenals of weanling male Carsworth rats receiving the vitamin B_6 antagonist DOP concurrently with a vitamin B_6 deficient diet were compared with those of pair-fed rats, as well as rats receiving the deficient diet alone (Stebbins, 1951). Histochemical examination of adrenals from the DOP-treated rats showed decreased lipid and ascorbic acid content in the inner zones of the cortex by two weeks. After 3 weeks, similar results were found in adrenals of the deficient rats not receiving the antagonist. No abnormalities were noted in the adrenals of the pair-fed rats. The adrenal weight/body weight ratio (relative adrenal weight) was increased only in those rats receiving DOP. Administration of pyridoxine to deficient rats normalized the histological appearance of the adrenals, regardless of DOP treatment. The author suggested that the lack of vitamin B_6 reduced the capacity of the adrenal cortex to produce its hormone.

Thus, despite some inconsistencies, the histochemical data suggest that vitamin B_6 deficiency affected the adrenal glands. In view of the inadequacies of the available methodology for studying

adrenal function, evidence for alterations in adrenal function was sought using indirect methods. Two approaches were used: the first involved studying the effects of an excess or deficiency of adrenal hormones on the symptomology of vitamin B_6-deficiency. The second involved examination of the disturbances in intermediary metabolism (fat, carbohydrate, protein) and electrolyte balance in vitamin B_6 deficient animals in order to compare with the effects of adrenal hormone administration or deficiency on the same parameters.

Adrenalectomy prevented the acrodynia of B_6 deficiency whereas administration of ACTH or adrenal cortical extract had no effect on the skin lesions (Beaton et al., 1952; Beare et al., 1953b). Draper and Johnson (1953) noted that intraperitoneal administration of cortisone (2.5 mg/day) had no effect on the length of survival of B_6 deficient Sprague Dawley rats, and the data of Stoerk (1950b) support this observation. However, Ershoff (1951) reported that vitamin B_6-deficient animals had decreased resistance to cold.

Rats maintained on a vitamin B_6-deficient diet for 5 weeks showed an impaired ability to excrete an oral water load compared to the ad-lib control group (Stebbins, 1951). Furthermore, 84% of the deficient animals died by the second day following the experiment and 33% in the ad-lib fed rats, but none in the pair-fed group. Administration of pyridoxine 18 hours prior to the water load corrected the impaired diuresis and reduced the mortality to 67%. Adrenal cortical hormones (DOCA, 2 mg, plus "lipo-extract" of adrenal glands, 0.6 ml) also restored diuresis to control levels, and eliminated mortality. Similarly, Guggenheim (1954) reported that vitamin B_6-deficient rats excreted significantly less urine during the

first 3 hr after administration of a water load (8% body wt), compared to both ad-lib and restricted intake controls. DOCA (1 mg/rat) administered 60 minutes prior to the water load had no influence on the impaired diuresis. In contrast, cortisone (3 mg/rat, 90 min prior to the test) or ACTH (1 mg/rat, 60 min before) restored the diuresis in the deficient animals, but did not affect that of controls. Following a saline load B_6 deficient rats excreted less water, sodium, and chloride than ad-lib or pair-fed control rats (Diamont & Guggenheim, 1957). There was no effect of cortisone in either control group. These results suggest that vitamin B_6 deficient rats are lacking in glucocorticoid.

Diamont and Guggenheim (1957) observed an increase in sodium and chloride concentration, and decreased potassium, in the muscle of deficient animals. Chloride concentrations in plasma were elevated. No effect of pair-feeding was noted in these experiments. In contrast, adrenalectomized rats exhibited reduced sodium and elevated potassium in plasma, with increased potassium and water content of muscle. Administration of cortisone (2.5 mg X 3 days) to control rats decreased muscle water and plasma sodium, and increased plasma potassium. In adrenalectomized rats there was no effect of cortisol other than decreased muscle water. Cortisone treatment of vitamin B_6 deficient rats significantly reduced muscle sodium and chloride, and increased muscle potassium. Plasma sodium and potassium concentrations increased, with a concomitant decrease in chloride. In pair-fed animals, cortisone administration increased plasma sodium and muscle potassium. Hsu and coworkers (1958) observed a significant elevation of serum sodium in vitamin B_6-deficient rats, with no change in

serum potassium. The sodium content of tissues from deficient rats was increased only in muscle. Kidney potassium was greater in the deficient animals than controls, but less in muscle. Administration of pyridoxine corrected the serum sodium of deficient rats but had no effect on the distribution of potassium. The increased sodium content of tissues from deficient rats, with concomitantly decreased potassium is indicative of excess mineralocorticoid production in vitamin B_6 deficient rats.

Stewart and Bhagavan (1982) observed no significant difference in the taste preference for sodium chloride of deficient rats, whereas the deficient animals had a stronger preference for KCl than did control animals. The authors speculated that aldosterone secretion is increased in vitamin B_6-deficient rats and suggested that the zona glomerulosa would hypertrophy. However the histological reports described above indicated that this zone was not affected. The possibility of increased aldosterone synthesis would be consistent with the observation of DeLorme et al. (1975) of increased plasma renin activity and angiotensin concentration in deficient rats.

Despite such provocative data, few studies have been directed toward examining adrenal steroid metabolism in vitamin B_6 deficient animals. Swell et al. (1961) measured corticosteroids produced by adrenals from rats fed a vitamin B_6 deficient diet for 40 days. No significant difference was found in total steroid production from adrenals of deficient animals compared to adrenals of controls. In contrast to other reports, neither the cholesterol nor lipid content of the adrenal glands was altered by B_6 deficiency. Furthermore, the spectrophotometric method used (Eisenstein, 1956) detects all steroids

with an α, β unsaturated ketone structure in ring A, and qualitative differences in the proportion of steroids produced would be undetected. It is also possible that differences in the rate of steroid metabolism would not be detected by the protocol used. The glands were bisected and incubated for two hours in the presence of ACTH, with no added substrate. The incubation period may have been of sufficient length to allow the substrate to be depleted and differences in the rate of production might be obliterated.

RATIONALE

It appears probable that a specific defect in adrenocortical metabolism exists in vitamin B_6-deficient rats, distinct from the possible effects of stress. Based on the increase in relative weight of the glands and the histological evidence of hypertrophy in the inner zones of the adrenal cortex, it is likely that there is an overproduction of trophic factors (ACTH). Although one would expect an increase in the production of B in this case, the available evidence suggests a decrease in glucocorticoid production. First, the disturbances of carbohydrate metabolism in B_6 deficient rats resemble lack of glucocorticoid rather than excess, specifically the tendency toward hypoglycemia and the decrease in hepatic glycogen. Second, humans with Cushing's syndrome --in which glucocorticoid secretion is pathologically increased-- exhibit thinning of the skin and obesity due to excessive fat deposition. In contrast, deficient rats lose body fat and have areas of hyperkeratinization. However, both states do have in common hypertension and edema. Finally, cortisone administration corrected the abnormal electrolyte patterns in

B_6 deficient rats, but had no effect on control animals. These results indicate that excessive corticosterone production is not a major factor in vitamin B_6-deficient animals. A more attractive hypothesis is that of adrenal insufficiency; that is, a defect in production of corticosteroids.

Three observations in early reports provide critical clues as to the nature of the lesion: 1) elevated blood pressure, 2) increase in sodium, and decrease in potassium concentrations in the tissues of deficient rats, and 3) administration of cortisone corrected the abnormal electrolyte distribution. The increased blood pressure and altered electrolytes suggested an excess of mineralocorticoids, and the reversibility by cortisone was consistent with a deficit in glucocorticoids. Such symptoms are similar to the syndromes found in humans known collectively as congenital adrenal hyperplasia (CAH), or the adrenogenital syndromes. The hallmark in these conditions is the lack of glucocorticoid (cortisol in humans) accompanied by hyperplasia of the adrenal cortex, particularly the inner zones. The hyperplasia is believed to be caused by the lack of feedback inhibition of ACTH production by plasma cortisol (New et al., 1981). The specific symptoms of the various forms of CAH depends upon where in the biosynthetic pathways cortisol production is interrupted (Finkelstein & Shaeffer, 1979).

Diminished 21-hydroxylation is the most frequent anomaly found. Usually enough enzyme activity remains so sufficient aldosterone and cortisol are produced. However, when this is not the case, significant amounts of salt may be lost in the urine. In both instances, the relative block at the level of 21-hydroxylase (enzyme #3 in Figure 3)

would cause a shunting of substrate into 17α-hydroxylase (enzyme #2) resulting in androgen excess. Inasmuch as this occurs during the maturation of the individual, the result is virilization of female children and precocious puberty in males.

The syndrome which would most closely approximate that of vitamin B_6-deficient rats is 11β-hydroxylase deficiency. Animals with a deficit in hydroxylation at this point would show increased levels of DOC and consequently hypertension, which has been observed in B_6 deficient rats. Although the enzyme blockage may not be complete, decreased secretion of glucocorticoid will be found if the activity of 11β-hydoxylase is affected. Rats do not have significant basal 17-hydroxylase activity, and may not exhibit increased androgen production (Laplante et al., 1964).

Changes which occur in the plasma level of a corticosteroid may represent effects on the controlling mechanism(s) or alterations in adrenocortical steroidogenic capacity. Furthermore, measurement of adrenal steroid metabolism in vitro is therefore the most direct method to assess the functioning of the adrenal glands and is therefore used in the present study.

Strain differences in response to vitamin B_6 depletion have been observed by others. Agnew (1949) reported that Wistar-derived rats on a vitamin B_6 deficient diet developed acrodynia much earlier than hooded Listar rats. In contrast, the deficient Listar rats developed hematuria but the Wistar-derived did not. Sprague-Dawley rats were found to be more resistant to the development of vitamin B_6 deficiency with respect to appearance of acrodynia, weight gain, and survival time (Cerecedo, 1946). The distribution of

B_6 vitamers among tissues was found to differ significantly between two strains of mice (Lyon et al., 1962) which may affect the rate of appearance of the deficiency. Similarly, strain differences in adrenocortical metabolism have also been reported. Brownie et al. (1973) found no differences in plasma B of quiescent male Wistar and Holtzman rats. However, following 10 minutes of ether stress the response of the Wistar rats was less than that of Holtzman. The adrenal content of cytochrome P450 was also less in Wistar rats than Holtzman, both in the stressed and non-stressed state. Differences in adrenocortical metabolism and vitamin B_6 metabolism may affect the results of studies designed to determine the effects of B_6 deficiency on adrenal metabolism.

This study was designed in order to test the hypothesis that adrenocortical synthesis of steroid hormones is altered in vitamin B_6 deficient rats; specifically, that the activity of 11β-hydroxylase is reduced. The use of an <u>in vitro</u> system will allow estimation of adrenal steroidogenic capacity in the absence of extra-adrenal factors. Additionally, the capsule and inner cortex may be separated and assessed individually. The two strains of rats used in the study (Wistar and Sprague-Dawley) are known to differ in susceptibility to vitamin B_6 deficiency.

METHODS

Animals and Diets

Sprague-Dawley (CD) or Wistar (WIS) male rats weighing 50-75 g were obtained from Charles River Breeding Laboratories, Inc., Kingston, NY. The animals were housed in stainless steel wire-bottom cages in rooms with a 12 hour light/dark cycle. The rats were fed a semi-purified control diet (Tables 1, 2, and 3) following arrival, with distilled water to drink. After 5-7 days half of the rats of each strain were switched to the vitamin B_6-deficient diet and the rest remained on the control diet (C), resulting in four treatment groups: WIS/C, WIS/D, CD/C, and CD/D.

At the end of 3 weeks (Experiment 1) or 10 weeks (Experiment 2) on the experimental diets, the rats were anesthetized with pentobarbital sodium (50 mg/kg BW) and exsanguinated through the abdominal aorta. The adrenal glands were excised and placed in cold saline. The liver, kidneys, and heart were also removed, trimmed, and weighed on an analytical balance.

Adrenal Steroidogenesis

The adrenals were decapsulated by slitting the capsule and extruding the inner gland with gentle pressure. The inner sections of each adrenal were quartered with a razor blade, and the cut in the capsule completed to produce two hemispheres. Capsular and inner adrenal samples were thereafter handled separately. This method of separating the zones of the rat adrenal results in 10-20% contamination of the zona glomerulosa with outer fasciculata (Giroud et al., 1956).

The tissue was preincubated in a 10 ml Erlenmeyer flask containing

Table 1

Composition of Rat Diet

	Recommended level	Provided by control diet
Casein, Vitamin-free[a] (90% protein)	20.0	22.0
Sucrose[b]	50.0	25.0
Cornstarch[b]	15.0	31.0
Cellulose[b]	5.0	7.2
Corn Oil[a]	5.0	7.0
Choline chloride[a]	0.2	0.2
DL-Methionine[c]	0.3	0.8
Vitamin Mix	d	0.7
Mineral mix, Bernhardt-Tomarelli[a]	e	4.0
Mineral mix, Supplemental	e	2.0

[a]United State Biochemicals
[b]Teklad, Madison, WI
[c]Sigma Chemical Co., St. Louis, MO
[d]See Table 2.
[e]See Table 3.

Table 2

Vitamin mix for B_6 study

Ingredient	Recommended Level	Provided by Control Diet
	(mg/100 gm diet)	
pyridoxine HCl	.7[a,b]	3.0
vitamin B12	.001[b]	.02
nicotinic acid	1.5[a]	4.0
menadione	1.0[b]	4.0
calcium pantothenate	0.8[a]	2.4
m-inositol	10.0[c]	10.0
thiamine HCl	0.6[b]	1.5
riboflavin	0.6[b]	1.5
biotin	.02[b]	.03
folacin	0.2[b]	0.5
p-aminobenzoic acid	1.1[c]	1.0
retinyl palmitate	1200 U[a]	3600 U
∝-tocopherol	5 U[b]	10 U
cholecalciferol	100 U[a,b]	250 U

[a] Nutritional Requirements of Laboratory Animals, National Academy of Sciences, 1972.

[b] Report of the American Institute of Nutrition Ad Hoc Committee on Standards for Nutritional Studies. J. Nutr. 107:1340-1348, 1977.

[c] Yeh & Leveille, 1969.

Table 3

Mineral Mixes for B_6 study

A. Composition of Supplemental Mineral Mix

	(%)
glucose	24.855
Na_2SO_4	40.0
NaCl	35.0
$CrK(SO_4)\ 12H_2O$	0.1441
Na_2SeO_3 (anhyd.)	0.000657

B. Minerals Provided by Mineral Mixes

	NAS Recommendation[a]	Bernhardt-Tomarelli[b]	Supplemental Mix[c]	Total in diet
		(mg/100 g diet)		
Ca	600	900	0	900
Cl	25	74	423	497
Na	50	76	535	611
K	500	268	0	268
P	500	746	0	746
S	---	50	181	231
Mn	5	4.6	0	4.6
Mg	50	60	0	60
Fe	3.5	3.7	0	3.7
Zn	1.2	1.7	0	1.7
Cu	0.5	0.65	0	0.65
Cr	0.24	0	0.30	0.30
I	0.015	0.022	0	0.022
Se	0.004	0	0.006	0.006

[a] Recommended levels are for pregnancy/lactation or growth.
[b] Added at 4% of the diet.
[c] Added at 2% of the diet.

3 ml Krebs-Ringer bicarbonate buffer with 10 mM glucose. The buffer was bubbled with 95% O_2/CO_2 until the pH was 7.4. The flask was flushed with the gas, closed with a rubber stopper, and incubated at 37° in a Dubnoff metabolic shaker at 100 rpm. After 15 minutes, this medium was aspirated with a pasteur pipette and discarded, leaving the tissue in the flask. Fresh buffer (3 ml) was added and the reaction started by the addition of progesterone (Sigma Chemical Co., St. Louis) in 10 ul of dimethyl sulfoxide, for a final concentration of 51 uM progesterone. The vessel was gassed, stoppered, and incubated for 1 hr under the same conditions as above.

At the end of this period the medium was removed with a pasteur pipette and placed in a 15 ml screw-cap test tube on ice. A known amount of 11-deoxycortisol (5 μg/10 ul dimethyl sulfoxide) was added to the media as an internal standard for chromatography, and the samples frozen for later analysis.

The tissue was blotted, weighed, and homogenized in 3 ml of Reagent A used for the protein assay. The homogenate was allowed to digest for 1-2 days in this solution. Following neutralization with 1 N HCl, the samples were diluted with distilled water to 10 ml for capsules and 25 ml for the inner cortical samples. Aliquots were taken for determination of protein by the method of Lowry et al. (1951).

Sample Extraction

The screw cap test tubes used during the extraction were fitted with teflon liners resistant to the solvents used, and all glassware which came in contact with the steroids was sialinized in order to mask the reactive groups in glass. This was accomplished by soaking the

glassware in the sialinizing reagent (40 ml dimethyldichlorosilane/1 gal hexanes) for at least 20 minutes. The glass was rinsed twice with hexanes, twice with methanol, and allowed to dry. These procedures were done under a fume hood.

Chromatography grade methylene chloride (2 ml) was added to the thawed media, and the caps secured. The samples were shaken vigorously for 1 minute and centrifuged briefly to facilitate separation of the liquid phases. The organic layer was removed with a pasteur pipette and placed in a 30 ml screw cap test tube. The media were further extracted wih 3 ml, then 5 ml methylene chloride and the organic fractions for each sample pooled. The exhausted incubation media were discarded. Five ml of dilute sodium hydroxide (.05 N) were added to the methylene chloride containing the extracted steroids, the test tubes capped and shaken for 1 minute. The phases separated cleanly in a few minutes and the upper aqueous layer was removed by aspiration. The sample was similarly washed with 10 ml distilled water.

The methylene chloride extract for each sample was percolated through a column of anhydrous sodium sulfate in order to remove the water. The columns were constructed from sialinized 9 inch pasteur pipettes filled two-thirds with Na_2SO_4, with a small plug of sialinized glass wool for a bed support. The sample was added to the top, collected into a test tube, and the methylene chloride evaporated under a stream of nitrogen in a 37^{o} water bath. The test tube was sealed with Parafilm^{TM}, and stored dessicated in the freezer until analysis of the steroids.

Chromatography

The steroids in the samples were separated and quantified by high pressure liquid chromatography (HPLC), using a modification of the method of Gallant et al. (1978). The HPLC system included a Waters System Controller (Waters Corp., Milford, MA), Data Module™ (chart recorder, integrator) M6000-A pump, M4500 pump, Model 440 UV detector set at 254 nm with .02 AUFS, and either a Model U6k injector, or Waters automatic injector (WISP). An isocratic mobile phase was used, comprised of 40% antioxidant-free tetrahydrofuran (THF; Burdick & Jackson, Muskegon, MI) and 60% high quality water (Milli-Q Reagent Water System, Continental Water Systems Corp., El Paso, TX).

The steel column (15 cm X 4.6 mm) contained a reverse phase 5μ packing (Ultrasphere ODS, Beckman Co., Berkeley, CA), which was reported to optimize the chromatographic behavior of the 18-hydroxylated steroids (O'Hare & Nice, 1981). The efficiency of the column when new was determined to be 67,560 plates/m for 18-OH-DOC, providing 10,000 theoretical plates in the column. This allowed separation of the steroids under investigation within 18 minutes at a solvent flow rate of 1 ml/min. The number of plates was calculated as $5.55(t_{1/2}/w_{1/2})$, where $t_{1/2}$ is the peak retention time and $w_{1/2}$ is the peak width at half-height.

The samples were reconstituted in 400-600 ul of the mobile phase, filtered using a microfilter apparatus using a nitrocellulose membrane with a pore size of 0.2 μ (Anspech, Ann Arbor, MI), and 15-30 μl was injected onto the column. Identification and quantification of these compounds was accomplished by comparing elution patterns of the samples with those of known amounts of pure standards (Steraloids Inc., Wilton,

NH). The standards were prepared by dissolving aldosterone (2.52 mg), 18-OH-DOC (1.13 mg), corticosterone (2.80 mg), 11-deoxycortisol (2.78 mg), and DOC (2.24 mg) in 100 ml of HPLC grade methanol. Aliquots of this solution (1.0, 0.8, 0.5, 0.3, 0.1 ml) were placed in small sialinized vials. The methanol was evaporated at 37^{o} under a stream of nitrogen, and the vials closed with teflon-lined caps. The standards were stored dessicated in the freezer and reconstituted with 3.0 ml mobile phase (40% THF) when needed. This procedure conveniently provided a stable storage form for reference quantities of the steroids.

For every batch of samples, one vial of each stock concentration was reconstituted, allowing calculation of linear regression statistics for the five compounds. Aliquots of these were run several times during the day in order to monitor the chromatographic conditions (retention times, peak height) which were sensitive to factors such as changes of solvent composition due to evaporation and variation in room temperature. The concentrations of steroids in the samples were calculated from the regression statistics (slope, intercept) of the standard curves. Correction was made for recovery of the internal standard in the samples (generally 85-105%).

In the absence of progesterone, the steroids recovered in the medium were below the detection limits of the method used. Preliminary studies indicated that production of B, 18-OH-DOC, and DOC was linear with progesterone concentration (400 nM, 2 μM, 10 μM, and 51 μM) for at least one hour. Aldosterone production was detectable only with 51 μM progesterone in the medium, therefore 51 μM was used in the experiments.

Statistical Analyses

Data were subjected to a factorial analysis of variance. Differences between the means were tested by Duncan's multiple range test, using 0.05 as the criterion of significance (Steel & Torrie, 1960).

RESULTS

Body Weight

The mean body weights of all groups were not significantly different at week 0, as shown in Table 4 for the 3 week study (Experiment 1). WIS rats consuming the deficient ration for one week were lighter than WIS controls, while there was no difference in the CD rats at that time. After two weeks on the diet, and for all subsequent time points (Tables 4 and 5), vitamin B_6 deficient rats of both strains were significantly lighter than the controls. The deficient animals gained until the sixth week, but lost weight thereafter. Although CD control rats were heavier than WIS, this difference was not significant at any point.

Organ Weights

The kidneys, liver, adrenals, and heart of the deficient WIS rats killed during the third week weighed significantly less than the organs from the WIS control animals (Table 6). The heart and liver of deficient CD rats were smaller than control, but not when expressed as a percentage of body weight (relative weight). The relative weight of the liver from deficient WIS rats was significantly smaller than control. Relative adrenal and kidney weights were larger in both the WIS and CD deficient rats.

Following 10 weeks on the diets, the heart and liver of deficient CD rats were still smaller than that of controls. All of the organs of the deficient rats were heavier when factored by body weight (Table 7). However, the absolute weights were different only for the kidneys of the deficient WIS rats, which were significantly enlarged.

Table 4

Effect of Vitamin B_6 Deficiency on Body Weight

	Time on Diet (weeks)			
	0	1	2	3
	Body Weight (grams)			
WISTAR				
Control	73±2[a]	108±4*	153±7*	206±9*
Deficient	71±2	93±7*	117±7*	142±12*
CD				
Control	81±2	111±6	155±8*	205±13*
Deficient	82±2	112±2	127±4*	142± 4*

[a]Values are expressed as the mean ± SEM of 9 determinations.

*Significantly different from group of the same strain consuming the control diet ($P<0.05$).

Table 5

Effect of Vitamin B_6 Deficiency on Body Weight

	Time on Diet (weeks)				
	2	4	6	8	10
	Body Weight (grams)				
WISTAR					
Control	187±6[a]	203±11*	236±11*	258±6*	278±7*
Deficient	150±8*	175± 7*	172± 7*	165±11*	160±13*
CD					
Control	186±4*	211±6*	242±6*	245±16*	289±8*
Deficient	159±5*	165±6*	166±5*	161± 6*	153±6*

[a]Values represent the mean ± SEM of 4 or 5 animals.

*Significantly different from the group of the same strain consuming the control diet ($P<0.05$).

Table 6

Effects of Consuming Vitamin B_6 Deficient Diet for 3 Weeks on Organ Weights of Rats

	WISTAR		CD	
	Control	Deficient	Control	Deficient
Adrenal wt.$_3$(mg)	44.8±1.8[a]	37.6±1.0*	37.8±1.3	33.5±1.6*
AW/BW (X 10^3)	23.1±1.0	27.0±1.2	19.5±0.7+	25.5±0.9*
Kidney wt. (gm)	2.43±.10	2.14±.06*	2.28±.08+	2.13±.10*
KW/BW (X 10)	1.25±.07	1.51±.06*	1.18±.04	1.63±.07*
Heart wt. (gm)	.770±.023	.556±.027*	.770±.038	.513±.020*
HW/BW (X 10)	.396±.012	.388±.015	.395±.007	.387±.010
Liver wt. (gm)	10.95±.55	6.83±.36*	9.50±.36+	6.62±.44*
LW/BW (X 10)	5.62±.24	4.78±.18*	4.94±.10	5.00±.26

[a]Values represent the means ± SEM of 6 to 9 animals.

*Significantly different from control group of same strain ($P<0.05$).

+Significantly different from Wistar group consuming the same diet ($P<0.05$).

Table 7

Effect of Consuming a Vitamin B6 Deficient Diet for 10 Weeks on Organ Weights of Rats

	WISTAR		CD	
	Control	Deficient	Control	Deficient
Adrenal wt.$_3$(mg)	48.4±2.7[a]	45.6±2.9*	45.7±3.5	42.2±2.7*
AW/BW (X 10^3)	17.4±0.9	29.5±3.4*	15.9±.13	27.7±2.6*
Kidney wt. (gm)	2.24±.08	3.23±.12*+	2.38±.09	2.58±.20*+
KW/BW (X 10)	.809±.024	2.05±.137*+	.825±.022	1.70±.18*+
Heart wt. (gm)	.800±.018	.803±.030*	.921±.062	.662±.059*+
HW/BW (X 10)	.296±.010	.513±.043*+	.319±.020	.436±.048*+
Liver wt. (gm)	9.37±.29	9.10±.83*	9.14±.42	7.01±.84*+
LW/BW (X 10)	3.37±.14	5.73±.47*+	3.16±.09	4.62±.56*+

[a]Values represent the mean ± SEM of four determinations.
*Significantly different from control group of same strain ($P<0.05$).
+Significantly different from Wistar group consuming the same diet ($P<0.05$).

Protein Content of the Adrenal Glands

There was no effect of diet on the protein content of the inner section (inner cortex plus medulla) of the adrenal gland for either strain at 3 or 10 weeks (Table 8). The capsular portion of the adrenal glands from WIS deficient rats contained less protein than control whereas that of CD deficient was not affected. The concentration of protein was higher in the inner part of the adrenal gland from CD deficient animals; the protein concentration in the CD control tissue was significantly less than that of WIS control. No differences were noted in the protein concentration of the capsular gland from either strain. After 10 weeks on either diet, no differences were observed in the protein content or concentration of the capsule or inner adrenal gland from WIS or CD groups.

Adrenal Steroidogenesis

Estimation of total metabolism of added progesterone was made by summing the net production rates for the individual metabolites (aldosterone, 18-OH-DOC, B, and DOC). As illustrated in Figure 4, the production of steroids from progesterone was decreased in WIS rats consuming a vitamin B_6 deficient ration for 3 weeks. In contrast, after 10 weeks on the diet the net formation of the steroids of interest was enhanced. A significant reduction of steroid production was noted in the tissue from deficient CD rats at three weeks, but no changes were noted at 10 weeks. In the capsular tissue from deficient WIS, steroid formation was reduced both at 3 and 10 weeks (Figure 5). Production of steroids by the capsules of deficient CD animals was

Table 8

Protein Content of Adrenal Glands

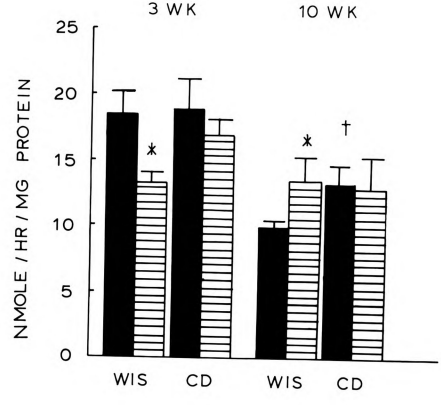
		INNER (mg protein)	CAPSULE (mg protein)	INNER (mg protein/gm tissue)	CAPSULE (mg protein/gm tissue)
		3 WEEKS			
WISTAR:	Control	2.69±.57[a]	0.72±.10*	85.6±6.1	84.9±5.4
	Deficient	2.12±.17	0.55±.04*	85.0±4.4	68.5±5.1
CD:	Control	2.09±.12	0.51±.05+	61.8±5.7+*	75.3±4.4
	Deficient	1.75±.25	0.54±.12	80.4±8.3*	76.4±17.2
		10 WEEKS			
WISTAR:	Control	3.95±.20	0.72±.09	119.6±6.8	61.1±6.3
	Deficient	3.59±.29	0.67±.14	125.6±5.8	58.3±21.0
CD:	Control	3.27±.33	0.76±.13	113.8±9.3	73.5±4.5
	Deficient	4.11±.25	0.55±.14	132.8±3.8	70.5±18.0

[a] Values are expressed as mean + SEM for four to six animals.

* Significantly different from corresponding control group of the same strain ($P<0.05$).

+ Significantly different from WIS group of the same diet and week ($P<0.05$).

Figure 4. Effect of diet and strain on formation of steroids from progesterone by quartered decapsulated (inner) adrenal glands. Wistar (WIS) or Sprague-Dawley (CD) rats were maintained on a vitamin B_6 deficient diet (shaded bars) or a control diet containing 30 mg/kg pyridoxine (solid bars) for 3 weeks (Experiment 1) or 10 weeks (Experiment 2). Net production of steroids of interest (sum of DOC, B, 18-OH-DOC, and aldosterone) is expressed as umole steroids/hr/mg protein (top) or as the total amount formed (bottom). Data represented are the mean $\pm$ SEM of four determinations. Asterisks indicate a significant difference from corresponding control group of the same strain, and daggers indicate significant difference from corresponding WIS group consuming the same diet ($P<0.05$).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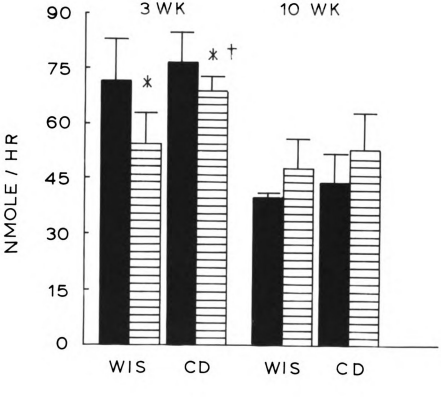
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WIS
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WIS
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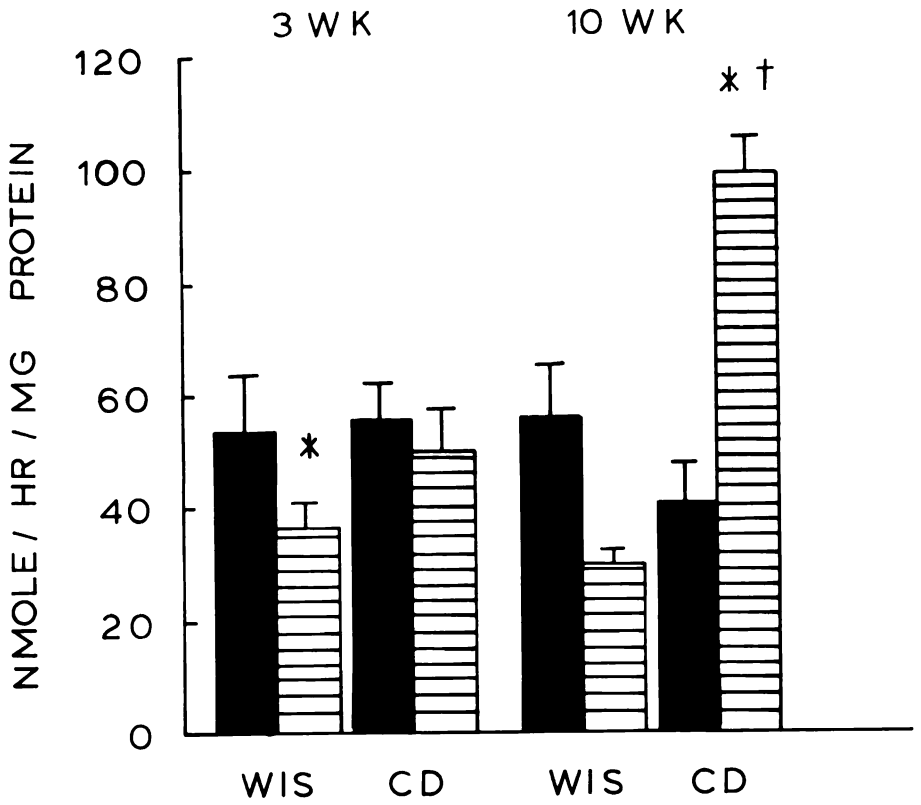
Figure 5. Effect of diet and strain on formation of steroids from progesterone by capsular portion of adrenal glands. Wistar (WIS) or Sprague-Dawley (CD) rats were maintained on a vitamin B_6 deficient diet (shaded bars) or a control diet containing 30 mg/kg pyridoxine (solid bars) for 3 weeks (Experiment 1) or 10 weeks (Experiment 2). Net production of steroids of interest (sum of DOC, B, 18-OH-DOC, and aldosterone) is expressed as umole steroids/hr/mg protein (top) or as the total amount formed (bottom). Data represented are the mean $\pm$ SEM of four determinations. Asterisks indicate a significant difference from corresponding control group of the same strain, and daggers indicate significant difference from corresponding WIS group consuming the same diet ($P<0.05$).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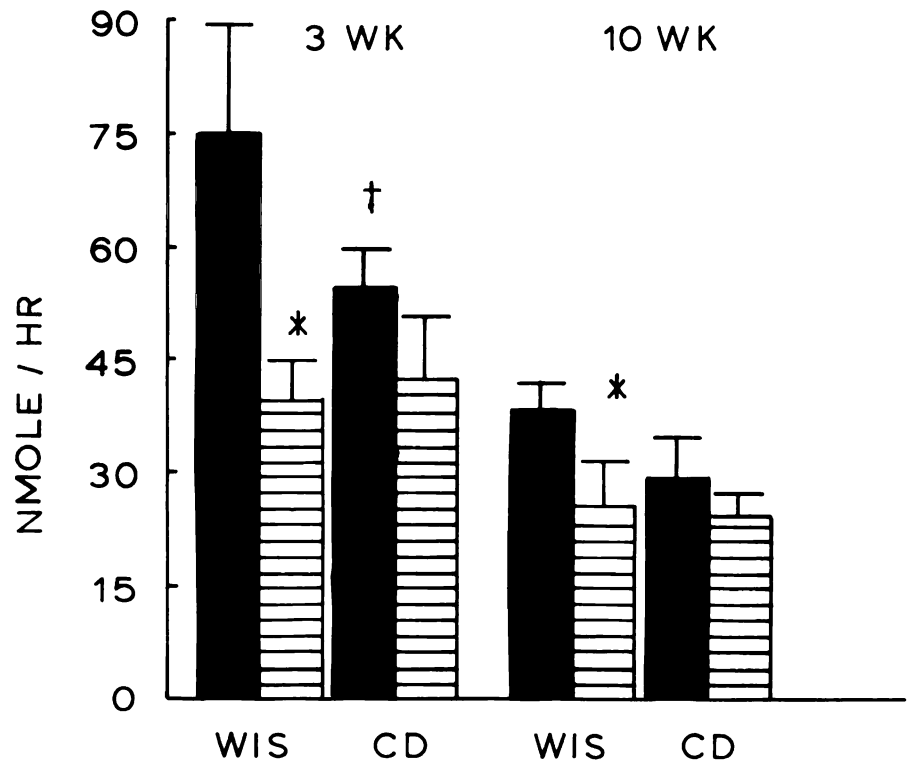
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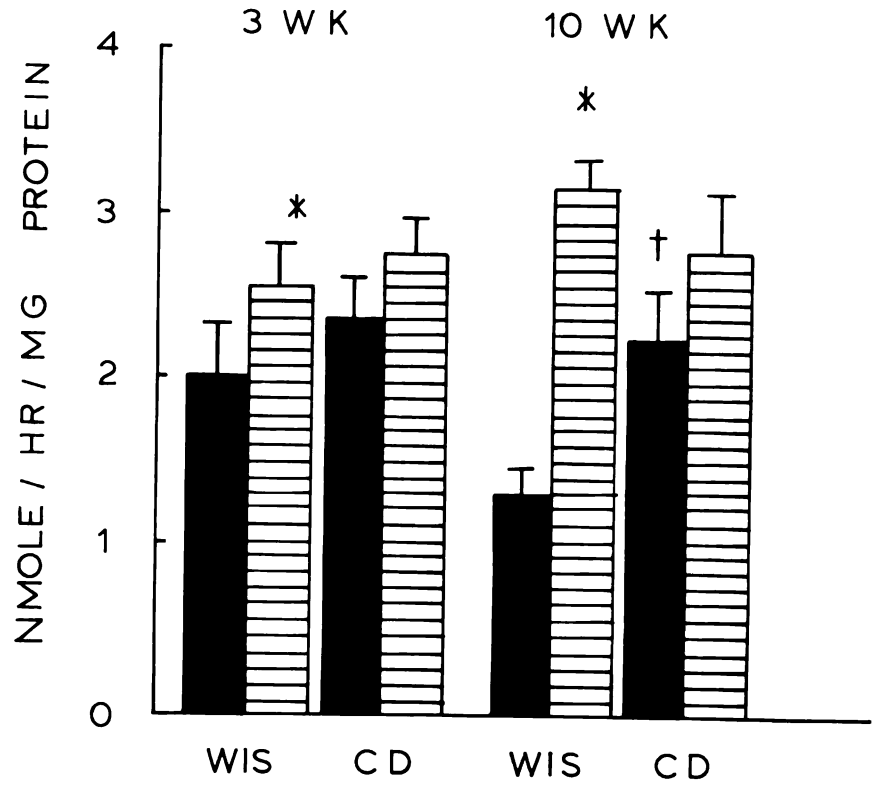
markedly elevated at 10 weeks when based on protein content, but no other significant differences were detected.

Net formation of DOC by the inner adrenal glands was significantly greater in deficient WIS rats compared to WIS controls following 3 or 10 weeks on the experimental diets (Figure 6, top). The slight increase noted in the deficient CD group was not statistically significant. At three weeks, total production of DOC by tissue from deficient animals (Figure 6, bottom) was not different from controls for either strain. However, the total production of DOC was significantly greater in the deficient groups of both strains after 10 weeks. In contrast, capsular production of DOC was elevated only in tissue from deficient CD rats at 10 weeks (Figure 7).

The net formation of B was significantly depressed in tissue from the inner adrenal glands of WIS deficient rats at 3 weeks (Figure 8), whereas that of CD deficient was not altered. Although at 10 weeks the CD groups had a higher rate of production than comparable WIS groups, there was no effect of vitamin B_6 deficiency on B production for either strain. Capsular tissue from deficient WIS rats also exhibited a significant decrease in the net formation of B at 3 weeks (Figure 9). Additionally, total B production by capsules from the deficient groups of both strains was less than that of control groups (Figure 9, bottom). The production of 18-OH-DOC by inner adrenal sections of deficient animals was different only for WIS rats at 3 weeks (Figure 10). No significant differences due to diet were observed at 10 weeks. Capsular synthesis of 18-OH-DOC was depressed in WIS rats at 3 and 10 weeks (Figure 11), but for CD rats the reduction was significant at 10

Figure 6. Effect of diet and strain on formation of 11-deoxycorticosterone (DOC) from progesterone by quartered decapsulated (inner) adrenal glands. Wistar (WIS) or Sprague-Dawley (CD) rats were maintained on a vitamin B_6 deficient diet (shaded bars) or a control diet containing 30 mg/kg pyridoxine (solid bars) for 3 weeks (Experiment 1) or 10 weeks (Experiment 2). Net production is expressed as umole DOC/hr/mg protein (top) or as the total amount formed (bottom). Data represented are the mean $\pm$ SEM of four determinations. Asterisks indicate a significant difference from corresponding control group of the same strain, and daggers indicate significant difference from corresponding WIS group consuming the same diet ($P<0.05$).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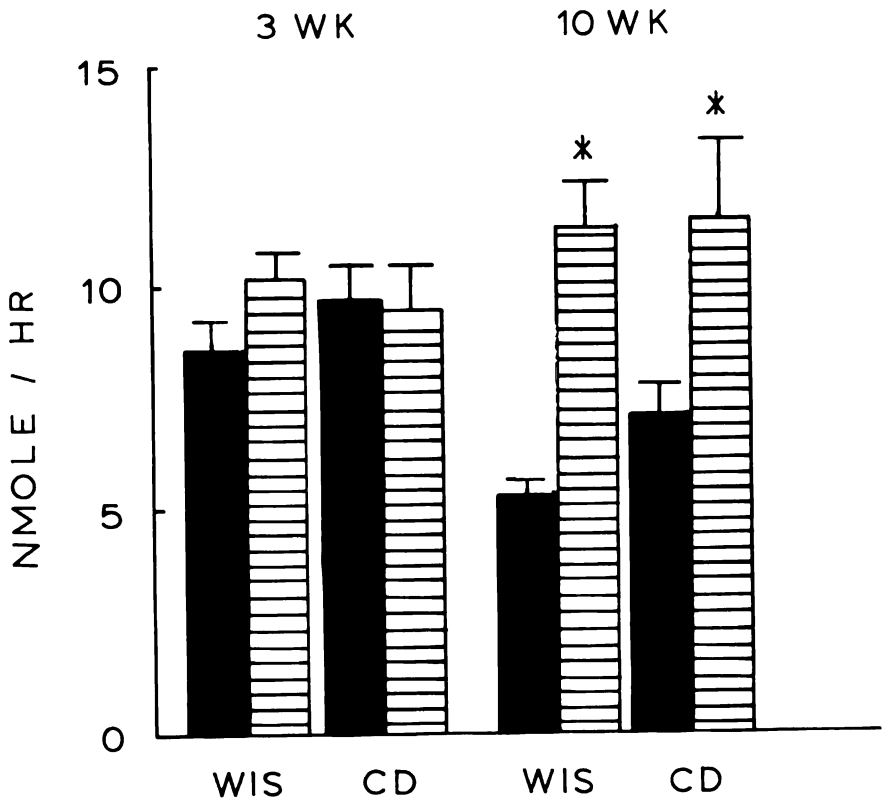
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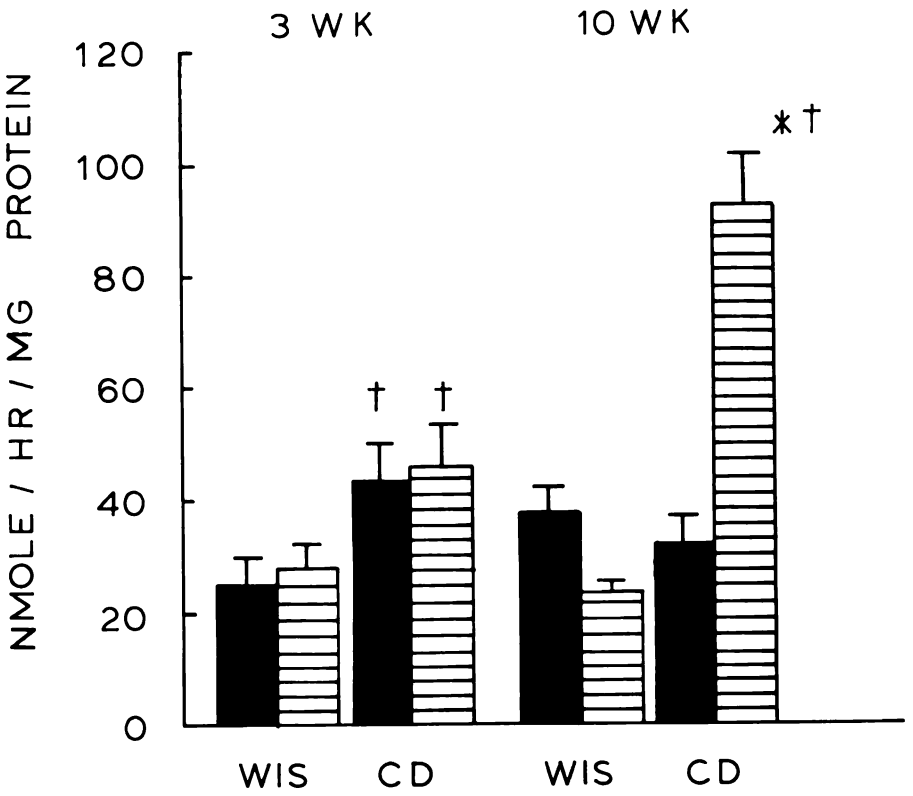
Figure 7. Effect of diet and strain on formation of 11-deoxycorticosterone (DOC) from progesterone by capsular portion of adrenal glands. Wistar (WIS) or Sprague-Dawley (CD) rats were maintained on a vitamin B_6 deficient diet (shaded bars) or a control diet containing 30 mg/kg pyridoxine (solid bars) for 3 weeks (Experiment 1) or 10 weeks (Experiment 2). Net production is expressed as umole DOC/hr/mg protein (top) or as the total amount formed (bottom). Data represented are the mean $\pm$ SEM of four determinations. Asterisks indicate a significant difference from corresponding control group of the same strain, and daggers indicate significant difference from corresponding WIS group consuming the same diet ($P<0.05$).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10 WK
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WIS
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WIS
CD

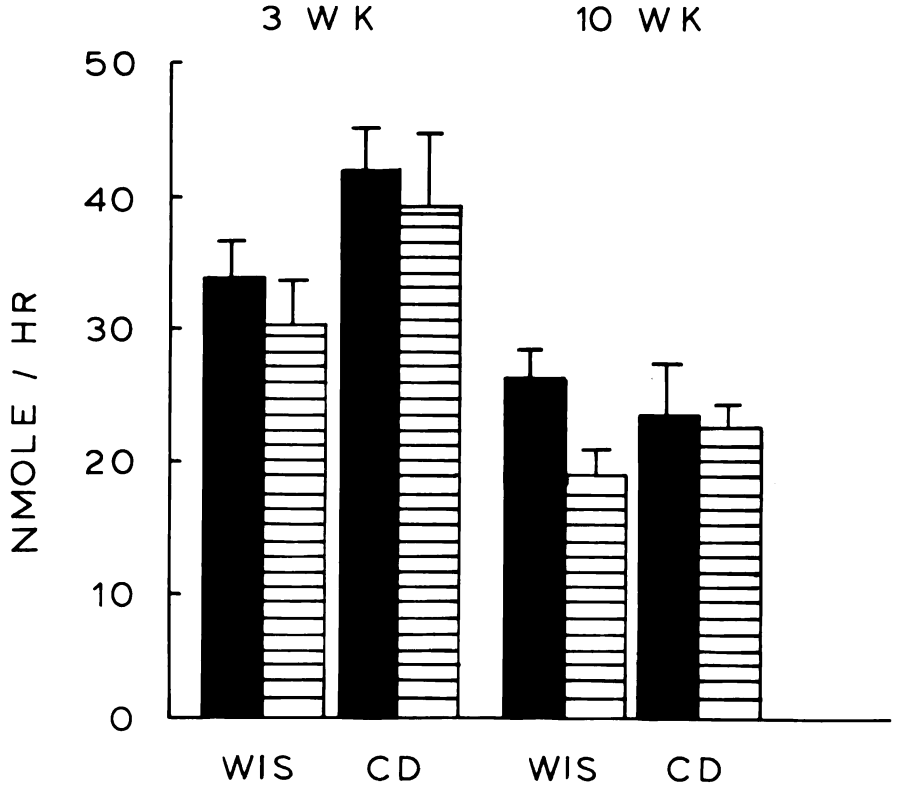

3 WK
10 WK
NMOLE / HR
50
40
30
20
10
0
WIS
CD
WIS
CD

Figure 8. Effect of diet and strain on formation of corticosterone (B) from progesterone by quartered decapsulated (inner) adrenal glands. Wistar (WIS) or Sprague-Dawley (CD) rats were maintained on a vitamin B_6 deficient diet (shaded bars) or a control diet containing 30 mg/kg pyridoxine (solid bars) for 3 weeks (Experiment 1) or 10 weeks (Experiment 2). Net production is expressed as umole B/hr/mg protein (top) or as the total amount formed (bottom). Data represented are the mean $\pm$ SEM of four determinations. Asterisks indicate a significant difference from corresponding control group of the same strain, and daggers indicate significant difference from corresponding WIS group consuming the same diet ($P<0.05$).

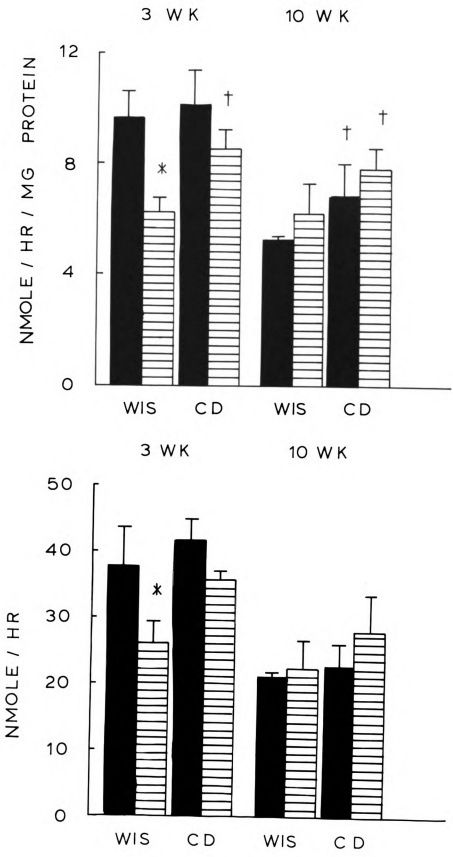
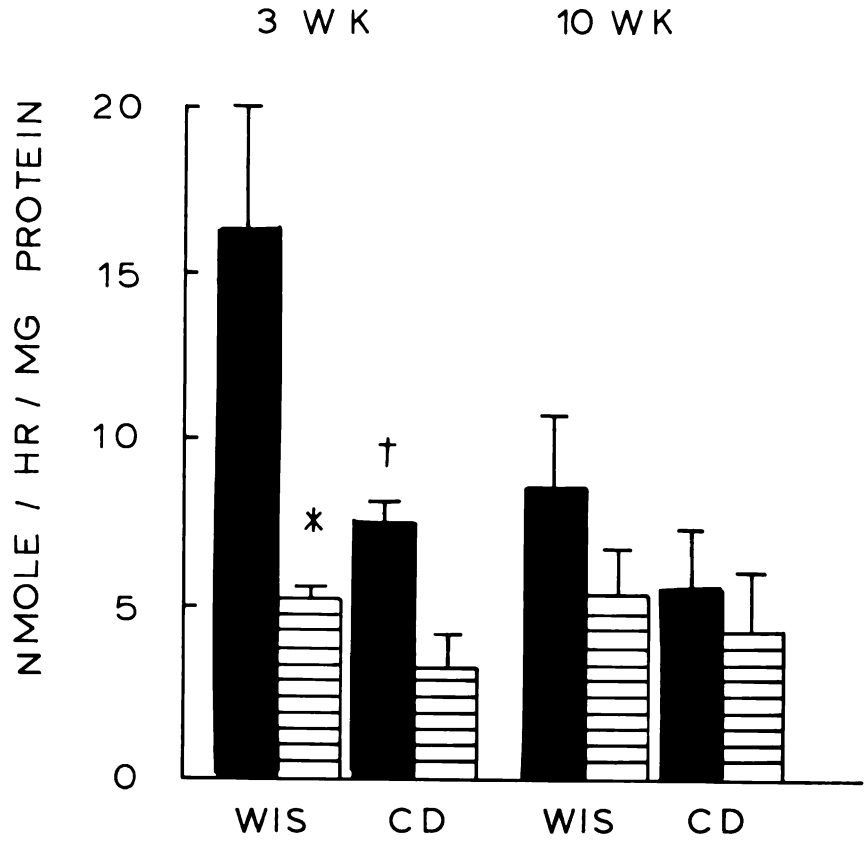

3 WK
10 WK
NMOLE / HR / MG PROTEIN
12
8
4
0
*
†
†
†
WIS
CD
WIS
CD
3 WK
10 WK
NMOLE / HR
50
40
30
20
10
0
*
WIS
CD
WIS
CD

Figure 9. Effect of diet and strain on formation of corticosterone (B) from progesterone by capsular portion of adrenal glands. Wistar (WIS) or Sprague-Dawley (CD) rats were maintained on a vitamin B_6 deficient diet (shaded bars) or a control diet containing 30 mg/kg pyridoxine (solid bars) for 3 weeks (Experiment 1) or 10 weeks (Experiment 2). Net production is expressed as umole B/hr/mg protein (top) or as the total amount formed (bottom). Data represented are the mean $\pm$ SEM of four determinations. Asterisks indicate a significant difference from corresponding control group of the same strain, and daggers indicate significant difference from corresponding WIS group consuming the same diet ($P<0.05$).


3 WK
10 WK
NMOLE / HR / MG PROTEIN
20
15
10
5
0
*
†
WIS
CD
WIS
CD

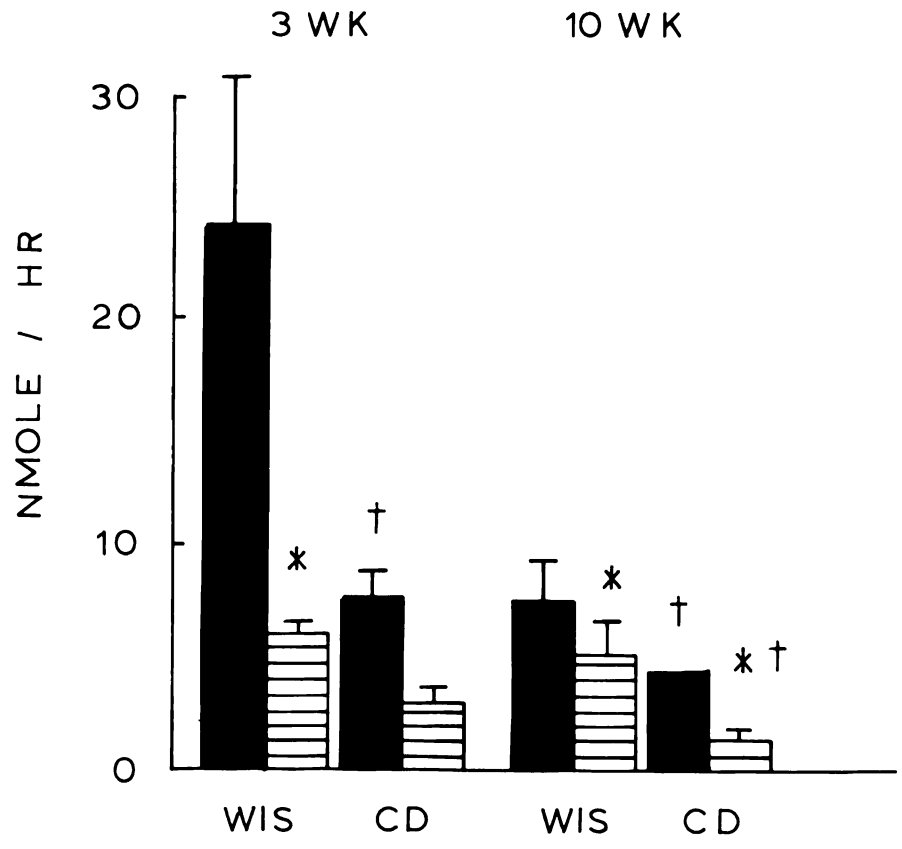
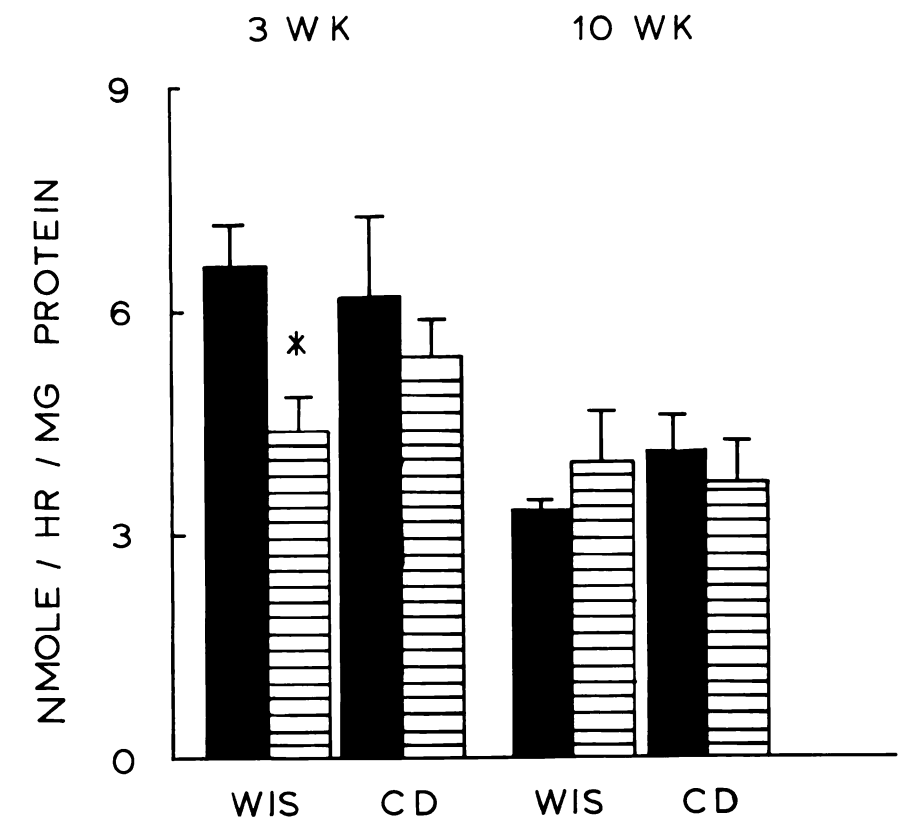

3 WK
10 WK
NMOLE / HR
30
20
10
0
*
†
*
†
*
†
*†
WIS
CD
WIS
CD

Figure 10. Effect of diet and strain on formation of 18-hydroxy-11-deoxycorticosterone (18-OH-DOC) from progesterone by quartered decapsulated (inner) adrenal glands. Wistar (WIS) or Sprague-Dawley (CD) rats were maintained on a vitamin B_6 deficient diet (shaded bars) or a control diet containing 30 mg/kg pyridoxine (solid bars) for 3 weeks (Experiment 1) or 10 weeks (Experiment 2). Net production is expressed as umole 18-OH-DOC/hr/mg protein (top) or as the total amount formed (bottom). Data represented are the mean $\pm$ SEM of four determinations. Asterisks indicate a significant difference from corresponding control group of the same strain, and daggers indicate significant difference from corresponding WIS group consuming the same diet ($P<0.05$).


3 WK
10 WK
9
6
3
0
NMOLE / HR / MG PROTEIN
*
WIS
CD
WIS
CD

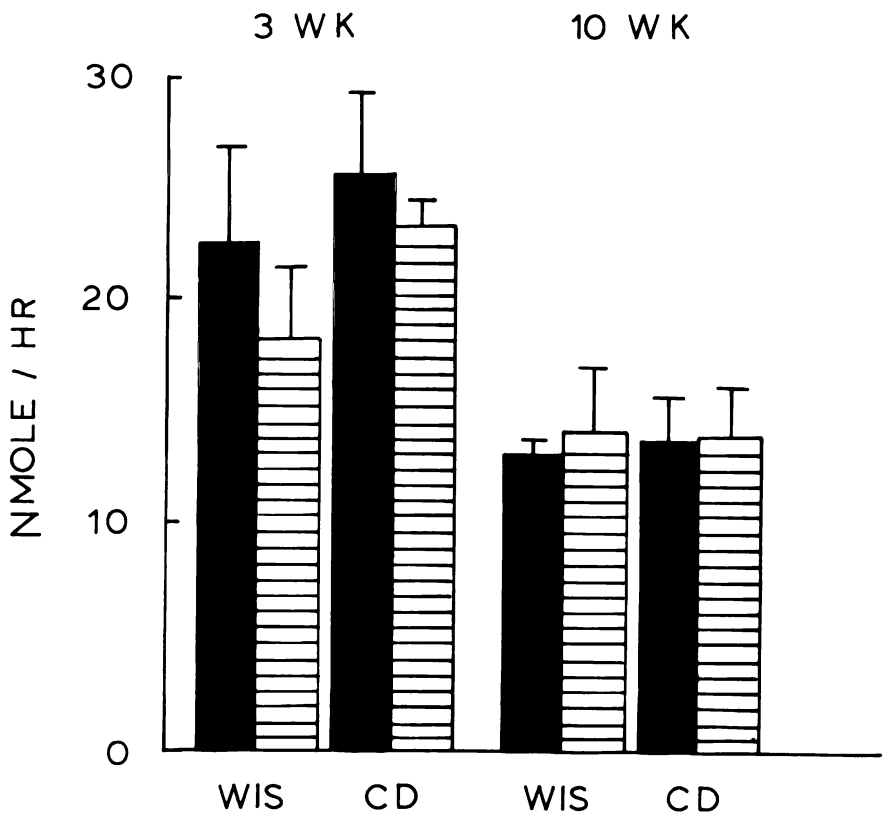

3 WK
10 WK
30
20
10
0
NMOLE / HR
WIS
CD
WIS
CD

Figure 11. Effect of diet and strain on formation of 18-hydroxy-11-deoxycorticosterone (18-OH-DOC) from progesterone by capsular portion of adrenal glands. Wistar (WIS) or Sprague-Dawley (CD) rats were maintained on a vitamin B_6 deficient diet (shaded bars) or a control diet containing 30 mg/kg pyridoxine (solid bars) for 3 weeks (Experiment 1) or 10 weeks (Experiment 2). Net production is expressed as umole 18-OH-DOC/hr/mg protein (top) or as the total amount formed (bottom). Data represented are the mean $\pm$ SEM of four determinations. Asterisks indicate a significant difference from corresponding control group of the same strain, and daggers indicate significant difference from corresponding WIS group consuming the same diet ($P<0.05$).

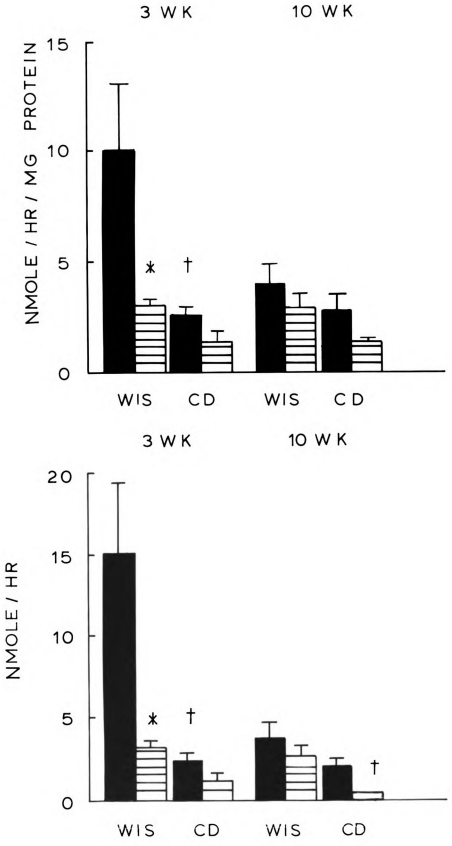
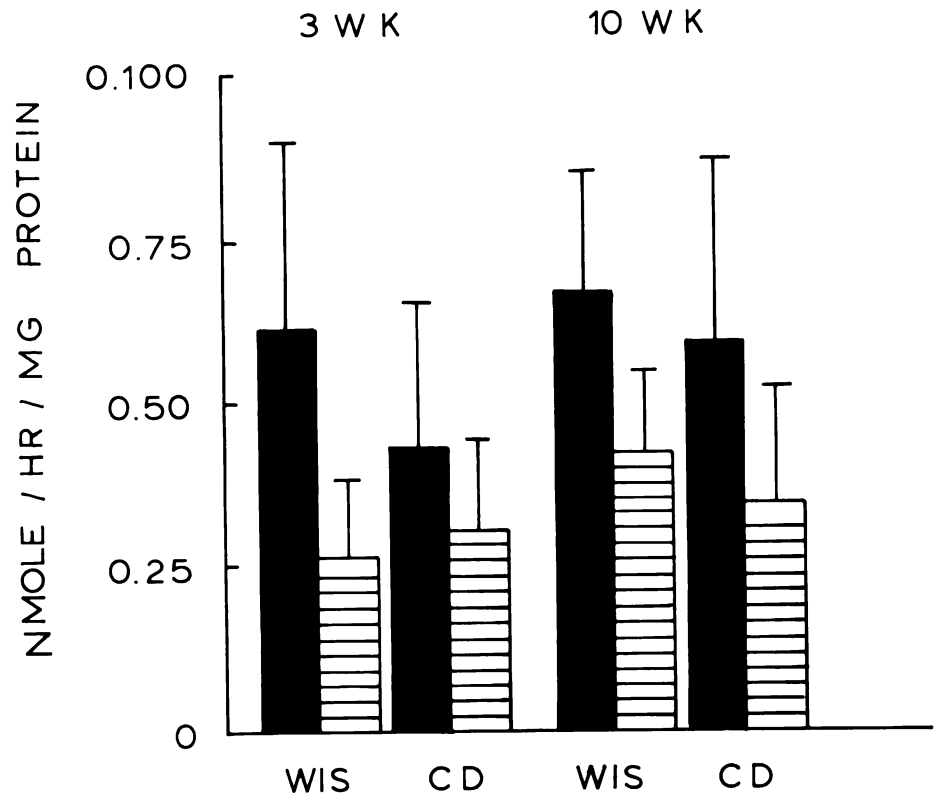
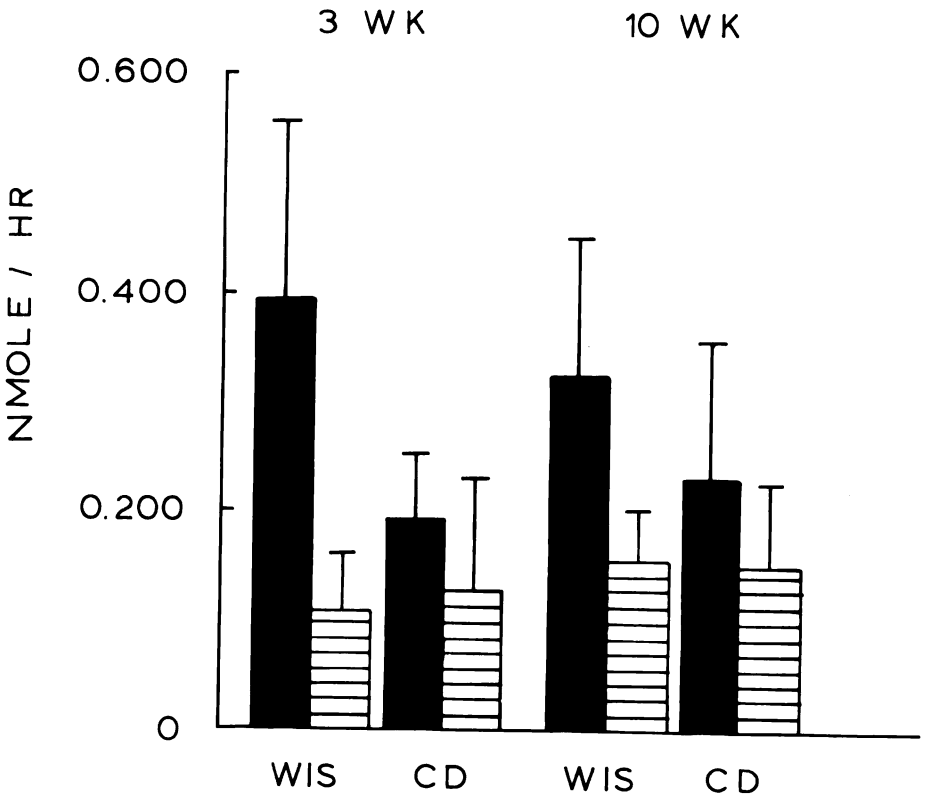

3 WK
10 WK
NMOLE / HR / MG PROTEIN
15
10
5
0
*
†
WIS
CD
WIS
CD
3 WK
10 WK
NMOLE / HR
20
15
10
5
0
*
†
†
WIS
CD
WIS
CD

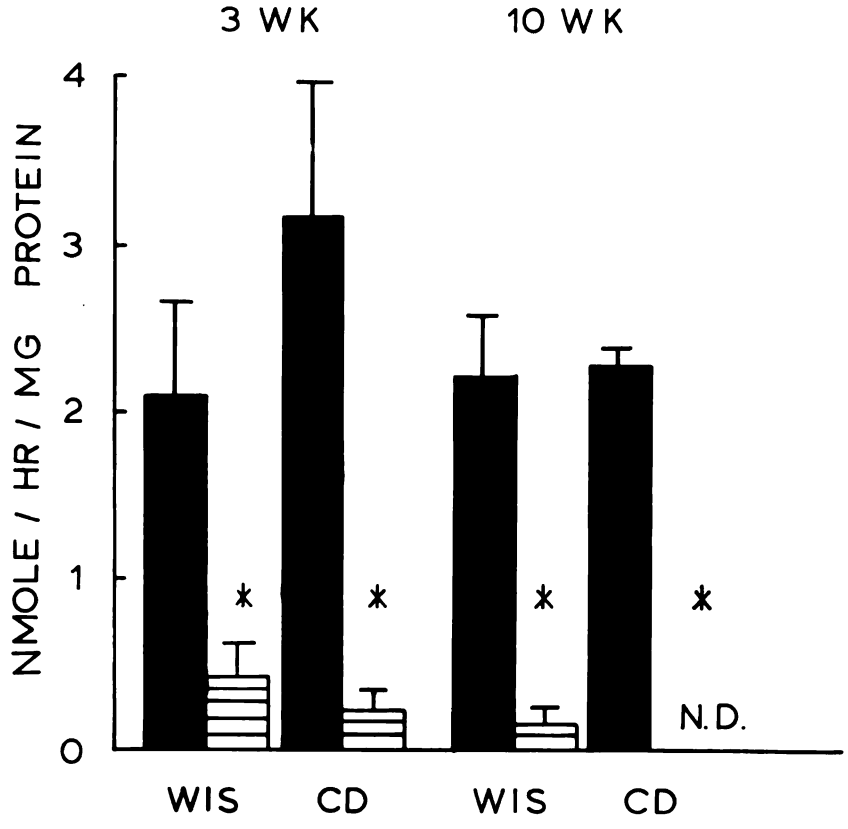
Figure 12. Effect of diet and strain on formation of aldosterone from progesterone by quartered decapsulated (inner) adrenal glands. Wistar (WIS) or Sprague-Dawley (CD) rats were maintained on a vitamin B_6 deficient diet (shaded bars) or a control diet containing 30 mg/kg pyridoxine (solid bars) for 3 weeks (Experiment 1) or 10 weeks (Experiment 2). Net production is expressed as umole aldosterone/hr/mg protein (top) or as the total amount formed (bottom). Data represented are the mean $\pm$ SEM of four determinations. Asterisks indicate a significant difference from corresponding control group of the same strain, and daggers indicate significant difference from corresponding WIS group consuming the same diet ($P<0.05$).

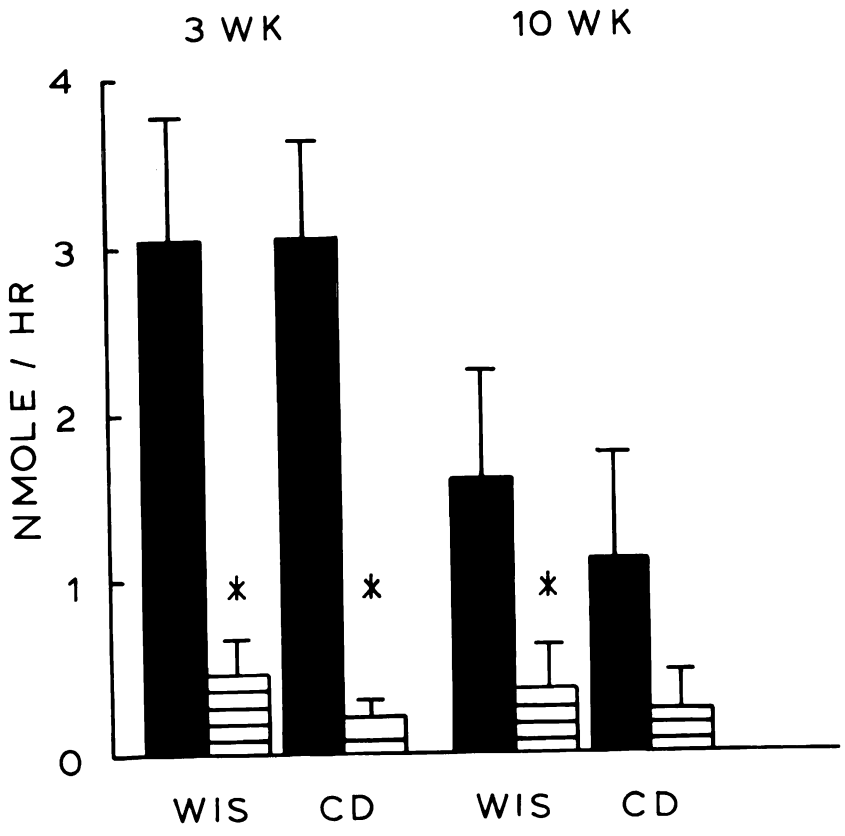

3 W K
10 W K
0.100
0.75
0.50
0.25
0
NMOLE / HR / MG PROTEIN
WIS
CD
WIS
CD


3 W K
10 W K
0.600
0.400
0.200
0
NMOLE / HR
WIS
CD
WIS
CD

weeks only when factored by protein content. Although aldosterone production by tissue from the inner adrenal glands was depressed in all deficient groups (Figure 12), the differences were not statistically significant. Net formation of aldosterone by capsular tissue of deficient rats was significantly reduced for both strains at 3 and 10 weeks (Figure 13).

Figure 13. Effect of diet and strain on formation of aldosterone from progesterone by capsular portion of adrenal glands. Wistar (WIS) or Sprague-Dawley (CD) rats were maintained on a vitamin B_6 deficient diet (shaded bars) or a control diet containing 30 mg/kg pyridoxine (solid bars) for 3 (Experiment 1) or 10 weeks (Experiment 2). Net production is expressed as umole aldosterone/hr/mg protein (top) or as the total amount formed (bottom). Data represented are the mean $\pm$ SEM of four determinations. Asterisks indicate a significant difference from corresponding control group of the same strain, and daggers indicate significant difference from corresponding WIS group consuming the same diet ($P<0.05$).


3 WK
10 WK
NMOLE / HR / MG PROTEIN
4
3
2
1
0
*
*
*
*
N.D.
WIS
CD
WIS
CD


3 WK
10 WK
NMOLE / HR
4
3
2
1
0
*
*
*
*
WIS
CD
WIS
CD

DISCUSSION

Previous investigators (Swell et al., 1961; Eisenstein, 1959) were unable to demonstrate changes in adrenal metabolism in vitamin B_6-deficient rats, despite histological and physiological evidence which suggested that the functioning of the adrenal glands was impaired. Eisenstein (1959) measured steroid production by the adrenals of rats maintained on a vitamin B_6 deficient diet for two months as well as those receiving the control diet ad-libitum or pair-fed to the deficient animals. The rate of steroid production for the deficient animals (76.1±5.8 μg/100 mg adrenal weight) was higher than ad libitum (62.6 ±2.0 μg/100 mg) controls, though the difference was not significant, and was similar to that of the pair-fed controls (77.8 ±3.6 μg/100 mg). The relative adrenal weight of the pair-fed rats was significantly greater than from those fed ad libitum, and that of the B_6 deficient group was greater than either control. Swell et al. (1961) observed no differences in the secretion of corticosteroids by adrenal glands from control rats or those maintained on a vitamin B_6 deficient diet for 40 days.

Swell and coworkers (1961) and Eisenstein (1959) concluded that vitamin B_6 deficiency did not alter the functioning of the adrenal glands. However the results of the present study show significant differences in adrenocortical metabolism of the deficient rats. Following three weeks on the vitamin B_6-deficient diet, the production of total corticosteroids by both capsular (Figure 5) and decapsulated (Figure 4) adrenal glands from WIS rats was significantly less than that of control animals. The reduction in synthesis of these

compounds by tissue from deficient animals was not as pronounced in CD rats. At the end of 10 weeks, steroid production remained depressed in the capsular tissue from deficient WIS rats (Figure 5), but was elevated in the decapsulated glands from the same animals (Figure 4). For the CD rats there was no effect of diet on total production of steroids during the incubation period (Figures 4 and 5, bottom), although when factored for protein content there was a marked stimulation in the capsule.

The previous papers (Swell et al., 1961; Eisenstein, 1959) used the same method to assess in vitro adrenal steroid secretion (Eisenstein, 1956), and several differences in experimental protocol exist which may influence the outcome. The previous investigators used bisected adrenal glands, containing both the capsular and inner adrenal tissue, whereas these tissues were incubated separately in the present study. The presence of the zona glomerulosa modifies steroidogenesis in the inner zones (Muller et al., 1970; Vinson & Whitehouse, 1976), and may alter the effects of vitamin B_6 deficiency on the enzymes of the inner zones.. The incubation medium used is identical in all three studies (Krebs-Ringer solution with 200 mg/dl glucose) and contains ACTH (1 unit/2 ml) in the previous studies, but progesterone (51 μM) in the present one. The incubation time was two hours for the method of Eisenstein (1956) and one hour herein. It is possible that the endogenous substrate (adrenal cholesterol) would be totally metabolized to corticosteroids during the longer incubation period of the previous papers. Therefore differences between control and deficient tissue in the rate of production of steroids may have been obscurred. The use of a high concentration of exogenous substrate

(progesterone) in the present study should result in maximal stimulation of the enzymes and unmask any modest impairment of activity.

Alternatively, the method of detection of the corticosteroids may influence the results. In the present study, the metabolism of progesterone is estimated by summing the quantity of DOC, 18-OH-DOC, B, and aldosterone produced. Other steroids produced in minor amounts would not be included in this calculation. The method of Eisenstein (1956) utilizes the UV spectrum of the mixture to determine all steroids which contain the α-, β-ketone group in ring A. The total amount of steroid produced by control animals was comparable for both methods: 80.1 µg/100 mg adrenal weight/2 hr (Eisenstein, 1956) and 48 µg/100 mg inner adrenal/1 hr, calculated from the data for WIS control in Figure 4. It is possible that vitamin B_6 deficiency may cause alterations in the synthesis of unknown steroids which contribute to the differences in results observed among the studies.

An advantage to the use of HPLC is that the individual corticosteroids can be identified and quantified. The vitamin B_6 deficient diet had little effect on the synthesis of DOC by adrenal tissue from either strain in Experiment 1 (Figures 6 and 7). In contrast, the appearance of B and 18-OH-DOC in the media was reduced in WIS deficient rats. No significant effect is apparent in B or 18-OH-DOC production in CD rats. Thus the hydroxylation of DOC is diminished in WIS but not CD rats after three weeks on the vitamin B_6 deficient diet. The appearance of DOC in the media is markedly increased in the inner cortex from deficient rats of both strains fed the deficient diet for 10 weeks (Figure 6), whereas B and 18-OH-DOC are

equivalent to control in most cases. Although the synthesis of aldosterone by the decapsulated glands from deficient animals was consistently decreased (Figure 12), the differences are not significant. The production of aldosterone by this tissue was very low and probably represents contamination of inner cortical tissue with zona glomerulosa cells. The data from the capsular tissue (Figure 13) does show markedly diminished aldosterone recovery from the media of all deficient groups compared to the respective controls.

The total amount of steroids (nM/hr) synthesized from progesterone by the capsules from WIS control rats (Figure 4) was similar to that from the inner gland (Figure 5) despite the larger mass of the latter. The specific activity of production (nM/hr/mg protein) by capsular tissue was more than 200% the rate of the inner tissue. This is in contrast to reports by other investigators, who found that steroid production (DOC, 18-OH-DOC, and B) was greater in the inner cortex (Vinson & Whitehouse, 1976). In the present study, DOC production by adrenal tissue from WIS control rats was much greater in the capsule (Figure 7) than the inner zone (Figure 6). Aldosterone synthesis represented a small fraction of the total steroid production from either tissue, and was greater in the capsule (Figure 13) than the inner zone (Figure 12), consistent with previous reports (Bankiewicz et al., 1968). The production of B and 18-OH-DOC was higher in the inner zone (Figures 8 and 10) than the capsule (Figures 9 and 11), although not when factored by protein concentration.

The apparently higher metabolic capacity of the capsular tissue in these experiments may be due to the method of isolating the tissue. The medulla was not removed when the inner glands were quartered, and

the steroid-synthesizing cells of the zona fasciculata and reticularis were thereby diluted. In the tissue preparations used, the zona glomerulosa cells were attached as a narrow layer to the capsule so it is likely that a greater proportion of the zona glomerulosa cells were adequately exposed to substrates during the incubation period.

The synthesis of steroids from progesterone by the adrenal inner zones (Figure 4) and capsules (Figure 5) was less in Experiment 2 (10 weeks) than in Experiment 1 (3 weeks), and is particularly striking for capsular B (Figure 9) and 18-OH-DOC production (Figure 11). This may represent developmental effects occuring in the rats between the 3 week study and the 10 week study, or may be due to variation between the batches of rats used. Alternatively, the rats sacrificed at 10 weeks may have lower adrenocortical activity because they were adapted to the experimental conditions (housing, handling) for a longer period of time. Therefore, for each experiment the results for vitamin B_6 deficient animals were compared with similarly treated controls.

The response of adrenal tissue from vitamin B_6 deficient CD rats was smaller in magnitude than that of WIS rats in the early stage of depletion (3 weeks). Following 10 weeks, the response of the inner glands of the two strains to the diets were comparable. Steroid production by the capsules, however, were markedly different. Production of total steroids per mg protein was elevated in CD deficient rats, but depressed in WIS. This could be accounted for by the tremendous increase in the production of DOC (Figure 7), since B and 18-OH-DOC were decreased. Thus, the effects of vitamin B_6 deficiency on adrenocortical metabolism vary with both the strain of rat and the severity of the deficiency.

The use of *in vitro* methods to assess adrenal function is based on the assumption that the recovery of steroids in the medium reflects the *in vivo* capacity of the glands. This assumption was examined by Vinson and Rankin (1965). Recalculation of their data indicates that the percent distribution of steroids produced by the *in vitro* incubation of rat adrenals with no added substrate was reasonably close to that found in the adrenal venous effluent of rats (DOC, 16.3%; 18-OH-DOC, 39%; B, 47.7%; aldosterone, 0.4%), although the distribution *in vitro* with exogenous progesterone as the substrate differed considerably (DOC, 36.7%; 18-OH-DOC, 21.0%; B, 41.4%; and no detectable aldosterone). In Table 9 the synthesis of corticosteroids by control and vitamin B_6 deficient rats illustrated in Figures 4 through 13 is summarized as percentage of total steroids measured in the incubation medium. For the inner cortex of WIS control rats DOC represented 12% of the steroids determined, 18-OH-DOC was 31.5%, B was 52.4%, and 0.5% was aldosterone. These results are similar to those found in the study cited above. Although the *in vitro* steroid production can be used to approximate enzyme activity, the application of such data *in vivo* may be limited.

Decreased food intake often accompanies deficiency of an essential nutrient, and itself has profound direct and indirect effects on several endocrine glands. The plasma concentrations of various hormones are controlled simultaneously by acute and chronic mechanisms. Consequently, when the endocrine status of an individual changes as the result of a nutritional deficiency it may be difficult to distinguish appropriate endocrine responses necessary for survival from those due to pathological interference in hormone metabolism.

For example, the decreased plasma T_4 which occurs during protein-calorie malnutrition (PCM) is the result of both impaired synthesis of the hormone, as well as reduced plasma half-life subsequent to decreased binding to plasma proteins (Graham & Blizzard, 1973; Inglebleek & Malvaux, 1980). Insulin secretion in decreased in malnutrition, and it has been difficult to determine whether the changes represent adaptation to the nutritional state or are pathologic in nature (Lunn et al., 1973). Growth hormone is significantly increased in malnourished children, and Parra et al. (1973) have proposed that this is a homeostatic action of the hypothalamus which serves to maintain plasma free fatty acids in order to protect the brain. These examples illustrate that the changes in endocrine systems during malnutrition may represent appropriate hormonal adaptation to the nutritional insult. Alternatively, there may be pathological effects on the glands. Such effects may represent specific actions on the functioning of the tissue, or may be due to deterioration of control mechanisms.

It is difficult to determine the extent of adrenal involvement in nutritional deprivation. Adrenocortical hormones are involved in control of intermediary metabolism as well as salt and water balance, and are stimulated during periods of stress. During the development of a nutritional deficiency the homeostatic mechanisms of the body are under pressure to maintain normalcy and -in the extreme case- survival.

Thus, during periods of malnutrition, the body can be considered to be under "general metabolic stress" (Hastings & Van, 1981). The demand for adaptation is considered to be the basis for stress (Vander, 1981).

The more extreme the malnutrition (i.e. the further from the "zone of homeostasis"), the greater is the stress sustained by the body (Krehl, 1956).

The response of the hypothalamic-pituitary-adrenal system to stress is hypertrophy, with increased production of glucocorticoids (Seyle, 1976). Leonard (1973) has argued that many of the symptoms observed in patients with kwashiorkor resemble those in individuals suffering from overproduction of cortisol by the adrenal glands (Cushing's syndrome). It has been reported that rats receiving cortisone develop features characteristic of marasmus- decreased body weight, muscle loss, but normal plasma albumin (Anonymous, 1977). The adrenal glands of rats starved for seven days were enlarged compared to ad-lib fed controls (Mulinos et al., 1942). In contrast, the glands of rats subjected to chronic inanition (50% of normal food intake for 3 months) weighed less, but the adrenal weight/body weight ratio was larger than that of the controls.

The synthesis of plasma proteins which bind cortisol is reduced in PCM, resulting in a greater fraction of free cortisol, so even when total plasma cortisol is within the normal range the free, physiologically-active steroid is more available to the tissues (Leonard, 1973). Hepatic catabolism of corticosteroids to inactive metabolites is also impaired and the plasma half-life increased (Smith et al., 1975). The urinary excretion of corticosteroid metabolites are reduced although urinary cortisol is increased. The plasma concentration of glucocorticoids may therefore be normal or slightly elevated despite reduced activity of the adrenal glands.

In contrast, Beas and coworkers (1973) have suggested that

patients with marasus have adrenal insufficiency. They base this hypothesis on several observations: 1) marasmic children do not conserve sodium adequately, 2) these children exhibit impaired water diuresis in response to a water load; this is alleviated by cortisone treatment, and 3) following ACTH administration, the plasma cortisol concentration and urinary excretion of corticosteroid metabolites is less when compared to fed children. Such observations would be consistent with decreased capacity for steroidogenesis in the adrenal cortex rather than interference with the control system. However, the plasma concentrations of aldosterone were normal in marasmic children and the secretion rates were elevated (Migeon et al., 1973). In patients with kwashiorkor plasma aldosterone concentrations were greater than well-fed controls but the secretion rates were normal.

Other investigators have utilized in vitro methods to assess adrenal function in animals deprived of various nutrients. Hayashida and Portman (1959) examined adrenocortical metabolism in rats deficient in essential fatty acids (EFA). After 15 weeks on the experimental diet, the adrenal weight of deficient animals was less than normal, though larger when factored by body weight. Production of corticosteroids by quartered adrenal glands was significantly reduced in EFA-deficient rats, as determined by the method of Eisenstein (1956). Vitamin A also appears to be involved in the functioning of the adrenal glands. Animals depleted of vitamin A have less fat, cholesterol, and glycogen in the body than control animals; this has been ascribed to "chemical adrenalectomy" in vitamin A deficient animals (Johnson & Wolf, 1960). These authors incubated quartered adrenal glands of rats for 1 hour in the presence of ACTH and TPN

(NAD), and observed that the production of B by tissue from deficient rats was 50% that of control. B production by adrenal quarters from pair-fed, starved, or vitamin E-deficient rats was not different from that of animals fed ad libitum, indicating that the observed effects of vitamin A deficiency were not due to inanition.

Further investigations showed that in pig adrenal tissue, conversion of ^{14C}-cholesterol to progesterone, DOC, and B was reduced while the production of 17-OH-DOC was markedly increased. In the adrenal glands from mildly deficient rats, the production of B from labelled cholesterol was decreased to a much greater extent than that of DOC or progesterone. However in severely depleted rats, the recovery of ^{14}C as progesterone was significantly depressed. Administration of vitamin A to deficient animals four hours before killing restored glucocorticoid production, as did addition of vitamin A to the incubation media from deficient animals. The results of these studies suggest that adrenocortical hydroxylations are impaired in vitamin A deficiency, and that the formation of B may be more severely affected in pigs. Increased DOC recovery was not found in vitamin A deficient rats, in contrast to that observed for vitamin B_6 deficiency. It is likely that different mechanisms are responsible for the altered adrenal metabolism of vitamin B_6 and vitamin A deficiencies.

From such studies, it is apparent that the behavior of the adrenal glands during food restriction and malnutrition is not solely a reflection of increased adrenocortical activity due to stress. The functional capacity of the adrenals appear to be maintained in protein-calorie malnutrition, although the production of

glucocorticoids is decreased secondary to changes in distribution and inactivation of the hormones. Therefore, during the development of a nutritional deficiency specific effects on corticosteroid metabolism may occur in addition to the postulated effects of general metabolic stress.

In the present study, it is apparent that hydroxylation of steroid hormones in adrenal tissue from vitamin B_6-deficient animals is significantly depressed. This abnormality is distinct from any possible effects of inanition or stress on the adrenal glands. The 11β - and 18-hydroxylations of DOC are reduced in B_6 deficiency, inasmuch as during the third week there is decreased synthesis of the products of these reactions but no simultaneous change in DOC recovery. Conversely in week 10 of depletion, when B synthesis is normal, DOC accumulates in the media. These results indicate that 11β- and 18-hydroxylase activities are decreased in vitamin B_6 deficient rats.

Speculations

The data presented indicate that adrenocortical biosynthesis of steroid hormones is impaired in vitamin B_6 deficient rats, but do not confirm a role of B_6 in the normal functioning of the adrenal cortex. The adverse effects of the deficiency may be due to indirect actions. For example, abnormal metabolic products which accumulate in the plasma or cells (eg. xanthurenic acid, cystathionine) may inhibit the hydroxylases. As indicated for the case of vitamin A, vitamin B_6 deficiency may reduce steroid hydroxylation by affecting the

redox state of the cell or by decreasing the supply of other cofactors for steroid production. However, Gomikawa & Okada (1980) found no difference in the NAD/NADH ratios of the liver from deficient and control rats.

The synthesis of a component of the steroid hydroxylase may be adversely affected in B_6 deficiency. The concentration of hepatic cytochrome P450 is depressed in vitamin B_6 deficient rats and the V_{MAX} for ethyl morphine demethylase and aniline hydroxylase were also depressed (Wade & Evans, 1977). The decreased cytochrome P450 is not surprising inasmuch as PLP is a cofactor for ALAS, a requisite enzyme in heme synthesis (Holtz & Palm, 1964). Alternatively, the synthesis of the hydroxylase hemoprotein may be depressed because of non-specific effects of the deficient state on protein or amino acid metabolism. Protein deficiency decreases hepatic drug-metabolism by impairing the interaction of the components of cytochome P450-dependent enzymes (Nerurkar et al., 1978). PLP is known to be an allosteric effector for several multiunit enzymes, and lowered PLP availability may adversely affect steroid hydroxylations by reducing the efficiency of association of the components.

It is difficult to ascertain the nature of the effects on other enzymes in the corticosteroid biosynthetic pathways. For example, the hydroxylation of progesterone may be impaired in depleted animals and partially obscure the effect on 11β-hydroxylase. Aldosterone recovery is diminished in the capsule from deficient animals; however it should be noted that synthesis of B and 18-OH-DOC are also depressed, and the reduction of aldosterone synthesis may be due to decreased rate of

production of precursors. It is possible that the 11-β and 18-hydroxylases are preferentially diminished in vitamin B_6 deficient animals. The activities of mitochondrial hydroxylases from the adrenals of hypophysectomized rats decay at the same rate as mitochondrial cytochrome P450 (Purvis et al., 1973). In contrast, microsomal hydroxylase activity appears to decay much slower than the associated P450 content. Such observations might explain differing effects of vitamin B_6 deficiency on the various steroid hydroxylases.

PLP has been found to alter the binding of oxygen to hemoglobin (Maeda et al., 1976, 1979). During storage of blood at low temperature 2,3-diphosphoglycerate (2,3-DPG), the endogenous allosteric effector of hemoglobin, is lost. This results in increased oxygen affinity and decreased functional properties of the blood. Unlike exogenous 2,3-DPG, PLP can enter red blood cells and restore the oxygen transport capacity. Covalent attachment of PLP to cytochrome c decreases the ability of cytochrome c to interact with both cytochrome oxidase and cytochrome c reductase (Avirim & Schejter, 1973; Atanasov et al., 1980). These two examples suggest that PLP can directly affect certain reactions -and possibly steroid hydroxylations- by altering oxygen binding and/or electron transfer.

Therefore, though B_6 vitamers are not known to act in a catalytic capacity in hydroxylating enzymes, there are several mechanisms possible for the observed effects of vitamin B_6 deficiency on adrenal steroidogenesis. Kinetic studies using isolated organelles (microsomes, mitochondria) would be desirable in order to

determine the specificity of the effect on the steroid hydroxylases. Additionally, by using cell fractions from deficient and control animals it should be possible to determine whether an activator is decreased in the deficient state or whether an inhibitor is involved.

The use of *in vitro* methods to assess adrenal function is based on the assumption that the recovery of steroids in the medium reflects the *in vivo* capacity of the glands. This assumption was examined by Vinson and Rankin (1965). Recalculation of their data indicates that the percent distribution of steroids produced by the *in vitro* incubation of rat adrenals with no added substrate was reasonably close to that found in the adrenal venous effluent of rats (DOC, 16.3%; 18-OH-DOC, 39%; B, 47.7%; aldosterone, 0.4%), although the distribution *in vitro* with exogenous progesterone as the substrate differed considerably (DOC, 36.7%; 18-OH-DOC, 21.0%; B, 41.4%; and no detectable aldosterone). In Table 9 the synthesis of corticosteroids by control and vitamin B_6 deficient rats illustrated in Figures 4 through 13 is summarized as percentage of total steroids measured in the incubation medium. For the inner cortex of WIS control rats DOC represented 12% of the steroids determined, 18-OH-DOC was 31.5%, B was 52.4%, and 0.5% was aldosterone. These results are similar to those found in the study cited above. Although the *in vitro* steroid production can be used to approximate enzyme activity, the application of such data *in vivo* may be limited.

Investigations with intact animals would permit assessment of the significance of the impaired adrenal function. Plasma glucocorticoid concentration is an important regulatory factor for ACTH synthesis and

Table 9

Summary of Data from Figures 4-13

	WIS		CD		WIS		CD	
	C	D	C	D	C	D	C	D
	(3 weeks)				(10 weeks)			
INNER:								
DOC	12.0[a]	18.7	12.6	13.7	13.4	23.7	16.2	21.6
18-OH-DOC	31.5	33.6	33.1	33.8	33.4	29.8	31.4	26.8
B	52.4	47.5	54.0	51.8	52.3	46.3	51.7	52.2
ALDOSTERONE	0.5	0.2	0.2	0.2	0.8	0.3	0.5	0.3
CAPSULE:								
DOC	44.0	75.0	75.9	90.6	67.7	70.6	77.9	92.6
18-OH-DOC	19.9	8.1	4.4	2.6	9.5	9.3	5.9	1.1
B	31.9	14.7	14.0	6.3	19.6	18.7	12.8	5.2
ALDOSTERONE	4.0	1.1	5.6	0.4	4.2	1.5	3.4	0.9

[a]Data are expressed as percentage (%) of steroid measured in the incubation medium.

release, with decreased glucocorticoid secretion resulting in ACTH release. In vitamin B_6 deficient animals the diminished B synthesis resulting from decreased 11β-hydroxylation would stimulate ACTH release enhancing the conversion of cholesterol to pregnenolone and progesterone. However, with reduced activity of 11β- and 18-hydroxylases, DOC secretion would increase, as found in the present study (Figure 6). Thus the measurement of plasma and urinary corticosteroids would be desireable.

In human 11β-hydroxylase deficiency, the increased production of DOC contributes to the development of hypertension. The blood pressure of vitamin B_6 deficient rats has been reported to be elevated (DeLorme et al., 1975; Olsen & Martindale, 1954), and it is possible that increased DOC may be involved in the etiology of this hypertension. The production of DOC was increased by approximately 20-50% in the present study, whereas that of aldosterone in the capsule decreased 64-90%. Aldosterone is 30 times more potent than DOC (Frieden & Lipner, 1971) and the total mineralocorticoid activity from adrenal glands of B_6 deficient rats is thus decreased. This suggests that the adrenal glands are unlikely to be involved in the hypertension found in vitamin B_6 deficient rats.

Vitamin B_6 nutriture in humans is often suboptimal due to inadequate intake and/or increased requirements. Several segments of the population are at risk with respect to intake. At least one-third of the elderly population ingested less than the recommended level of B_6 (Driskell, 1978). Alcoholics may be undernourished with respect to vitamin B_6 due to both an inadequate intake and interference

with the hepatic metabolism of the vitamin (Li, 1978). Patients with uremia or liver disease (cirrhosis, hepatitis) show increased metabolic clearance of PLP, resulting in subnormal plasma concentrations of the vitamin despite an otherwise normal intake (Spannuth et al., 1978).

The requirement for B_6 is influenced by dietary and physiological factors. Higher levels of protein in the diet increases the need for the vitamin. However, many high protein foods are not good sources of B_6, and consumption of a diet based on these foods may result in marginal intakes (Brown, 1972). The need for B_6 increases greatly during pregnancy due to the requirements of the fetus (Cleary et al., 1975). Patients with hypertensive disorders of pregnancy were found to have lower plasma B_6 concentrations than normotensive women (Brophy & Siiteri, 1975). Many drugs (isoniazid, penicillamine, imipramine) and xenobiotics to which humans may be exposed (CS_2, decaborane) interfere with the utilization of the vitamin (Brown, 1972). Fever and infection may also increase the requirement temporarily.

Altered corticosteroid synthesis is one of the earliest symptoms of vitamin B_6 deficiency appearing in the rat, preceding the more classical external hallmarks, suggesting that the activity of these enzymes is among the most labile during depletion. The observed impairment of steroid hydroxylases in the adrenals from deficient rats is not absolute and it is possible that the control systems for maintaining hormone secretion are adequate in the basal state. For example, plasma B and DOC concentration may be affected only following stimulation, and the ability of the adrenal glands to respond to stress

may therefore be compromised. This may be an important consequence for humans with marginal intakes of the vitamin and/or other contributing dietary and physiological factors.

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