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ADRENOCORTICAL FUNCTION AND CHOLESTEROL IN PANTOTHENIC ACID DEFICIENCY

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AN ABSTRACT

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ABSTRACT

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IN PANTOTHENIC ACID DEFICIENCY

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To evaluate adrenocortical function in pantothenic acid (PA) deficiency, weanling Sprague-Dawley rats were fed a PA-deficient or control diet. After 11 weeks, the rats received an i.v. injection of ACTH (2.2 units/kg body weight) 20 minutes before blood and tissue collection. Serum corticosterone concentrations after ACTH injection in the PA-deficient rats were significantly lower than in controls ($p<.01$) although basal concentrations of serum corticosterone at weeks 6, 9, and 11 were not statistically different between groups. Serum cholesterol concentrations at weeks 9 and 11 were significantly lower in the PA-deficient rats than in controls ($p<.005$). At week 11, adrenal and liver cholesterol concentrations in the PA-deficient rats were also significantly less than in controls ($p<.005$). Since cholesterol is the precursor of corticosterone, the decreased concentration of serum and tissue cholesterol in the PA-deficient rats may explain the observed adrenocortical insufficiency in response to ACTH.

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INTRODUCTION

Pantothenic acid is an essential B-complex vitamin which functions at the cellular level as a component of both coenzyme A (CoA) and acyl carrier protein (ACP). CoA is a cofactor required in many acyl group transfer reactions including the activation, transport, and oxidation of fatty acids, the synthesis of cholesterol from acetate, and the oxidation of pyruvate, ketone bodies and amino acids. Acyl carrier protein, a functional arm of the fatty acid synthase complex, is necessary for fatty acid synthesis.

The signs of pantothenic acid deficiency are broad and presumably related to the function of CoA or acyl carrier protein. Clinical and biochemical signs of an experimental pantothenic acid deficiency also suggest that adrenal function is compromised in the deficiency. Animals and/or humans deficient in pantothenic acid show weakness, fatigue, anorexia, hyperpigmentation, increased insulin sensitivity, abnormal glucose tolerance, reduced gluconeogenesis, and impaired eosinopenic response to ACTH (Hodges et al, 1959; Srinivasan and Belevady, 1976; Song, 1990). Most of these signs are also commonly seen in the well-defined Addison's disease caused by adrenal insufficiency and subsequent

glucocorticoid deficiency (Cullen et al, 1980).

One frequently reported result of feeding rats a pantothenic acid deficient diet is hemorrhage, necrosis and depletion of lipid material from the adrenal cortex (Deane & McKibbin, 1946; Daft & Sebrell, 1939; Nelson, 1939). The regions of the adrenal cortex affected by the deficiency were the fasciculata and reticularis which are mainly responsible for the production and secretion of glucocorticoids. Furthermore, reduced plasma glucocorticoid concentrations in rats fed a pantothenic acid deficient diet compared to those fed a supplemented diet has been reported by several investigators (Eisenstein, 1957; Longwell et al, 1956; Goodman, 1960; Pietrzik et al, 1974). These authors, however, do not agree on whether basal or ACTH-stimulated glucocorticoid concentrations, or both, are affected by the deficiency. Also, possible mechanisms responsible for the decreased glucocorticoid output and adrenal necrosis in pantothenic acid deficiency have not been investigated.

Glucocorticoid deficiency can be caused by many factors including autoimmune damage to the adrenal cortex, adrenal destruction due to malignancy, infection or vascular disease, and decreased concentrations of ACTH due to pituitary or hypothalamic lesions (Cullen et al, 1980). It has also been shown that drug-induced hypocholesterolemia can result in decreased in vitro adrenal production of glucocorticoids (Szabo et al, 1984). Since CoA is required

for the synthesis of cholesterol from acetyl-CoA, pantothenic acid deficiency leading to reduced tissue CoA concentration could possibly impair cholesterol synthesis, thereby reducing the amount of cholesterol available for glucocorticoid synthesis.

The clinical and biochemical signs of a pantothenic acid deficiency which relate to glucocorticoid deficiency as well as the evidence of adrenal damage in rats fed a pantothenic acid deficient diet suggest that adrenal endocrine function may be impaired in the deficiency. A complete evaluation of pantothenic acid and CoA concentrations in blood and tissues along with an evaluation of adrenal endocrine function in rats fed a pantothenic acid deficient diet has not yet been performed. A mechanism for the adrenal damage and impaired function is also not known. The hypothesis of this study was that pantothenic acid deficiency decreases the ability of the adrenal gland to produce glucocorticoids by lowering tissue CoA concentration and adrenal and plasma cholesterol concentrations, thereby limiting steroid hormone synthesis. To test the hypothesis, the specific objectives of this study were:

1) To measure serum glucocorticoid concentration at different stages of pantothenic acid deficiency in rats,
2) To determine, in pantothenic acid deficient rats, the ability of the adrenals to increase serum

glucocorticoid concentration in response to ACTH injection,

3) To measure serum, adrenal, and liver cholesterol in pantothenic acid deficient rats,

4) To relate pantothenic acid status of the rats with glucocorticoid and cholesterol concentrations of serum or tissues.

REVIEW OF LITERATURE

MORPHOLOGY OF THE ADRENAL GLAND

The adrenal gland can be separated into at least two functionally and structurally distinct glands. The medulla makes up the center of the adrenal gland and secretes mainly epinephrine and norepinephrine. The cortex, which surrounds the medulla, is composed of three zones: the glomerulosa, the fasciculata, and the reticularis. These zones can be distinguished by structural location, cell type and arrangement, vascular pattern, and steroid hormones produced.

The glomerulosa, the outer-most layer of the adrenal cortex, normally makes up about 5% of total cortical thickness. The cells are small, form clusters, and are low in lipid content. The adrenal artery enters the glomerulosa and becomes highly branched in this zone. Mineralocorticoids are the main secretory product of the glomerulosa.

The fasciculata, the middle layer, occupies about 70% of a normal cortex. The cells are large and abundant in lipid material. The outer portion of the fasciculata has a higher lipid content and a lower mitochondrion content than

the inner portion of the fasciculata. This suggests that the outer fasciculata functions more as a storage site of lipid precursors of steroid hormone synthesis and the inner fasciculata functions more as the site of energy-requiring production of the steroid hormones. In the fasciculata, the branched blood vessels become straighter and run toward the center of the gland. Glucocorticoids are the main secretory product of the fasciculata.

The inner-most 25% of a normal cortex is the reticularis. Low lipid content, numerous mitochondria, and lipofuscin are characteristic of the reticularis. The blood vessels of the reticularis become thicker and empty into the medullary sinuses which drain into the central adrenal vein. Androgens are the main secretory product of the reticularis although some glucocorticoids are produced here.

The main regulator of steroid hormone production and secretion from the glomerulosa is the renin-angiotensin system with minor stimulation coming from adrenocorticotrophic hormone (ACTH). The fasciculata and reticularis, however, are controlled predominantly by ACTH. ACTH is secreted from the pituitary in response to corticotropin-releasing factor (CRF), a peptide hormone secreted from the hypothalamus in response to neural stimulation. A rise in blood glucocorticoid concentration exhibits a negative feedback effect on ACTH production at both the pituitary and hypothalamic level. ACTH stimulates

cortical growth, via cell hypertrophy and hyperplasia, and the release of adrenocortical hormones. ACTH also induces cellular changes in the cortex reflecting increased synthesis and metabolism such as depletion of lipid stores and the changes in the morphology of fasciculata cells to resemble those of the reticularis. Physiological stress can also cause these adrenocortical changes through increased CRF and ACTH secretion (Idelman, 1978).

HISTOLOGICAL CHANGES OF THE ADRENAL GLAND IN PANTOTHENIC ACID DEFICIENCY

The interest in the relationship between the adrenal gland and pantothenic acid nutrition began with the observation of a characteristic adrenal lesion in rats fed a pantothenic acid deficient diet. The lesion was variably accompanied by necrosis, hemorrhage, and lipid depletion (Daft and Sebrell, 1939; Nelson, 1939; Deane and McKibbin, 1946).

Deane and McKibben (1946) conducted an anatomical and histological examination of the adrenal cortex in rats fed a pantothenic acid diet. One month old rats were fed a purified pantothenic acid deficient or control (10 mg pantothenic acid/kg diet) diet for six weeks. Two to 3 rats from each group were killed weekly and adrenals removed and sectioned. The sections were either stained with Sudan IV, Sudan black B, the Schiff reagent, and 2-4 dinitrophenyl-

hydrazine for lipid determination; left unstained and observed with polarizing and fluorescence microscopes; or stained with hematoxylin for mitochondria determination.

For the first two weeks of the study, the weight of the adrenal cortices of the rats fed the deficient diet made up a smaller proportion of the gland than those of the control rats. Beginning with the fourth week, the cortical weight of the deficient group represented a greater proportion of the total gland than in controls. The widths of the adrenal cortices correspond to the weight: the width of the cortices of the rats fed the deficient diet for 1 week was less than that of controls and that of the rats fed the deficient diet for 6 weeks was greater than that of controls.

A gradual loss of lipid material from the adrenal cortex was seen in the pantothenic acid deficient group but not in the controls. Lipid droplets had disappeared from the reticularis after the first week of the study, from the inner fasciculata after four weeks, and from the entire fasciculata after six weeks in the pantothenic acid deficient group. Also, after rats were fed the deficient diet for one week, the reticularis and inner fasciculata were exhausted of ketosteroid material as determined by birefringence under a polarizing microscope. Necrosis of the adrenal cortex was seen in both the reticularis and fasciculata after two weeks in the deficient group. In contrast, the glomerulosa was not affected by the

deficiency.

Since ACTH stimulates adrenocortical secretion of glucocorticoids and increases adrenocortical size, the results of this study can be explained by changes in ACTH secretion throughout the deficiency. The initial decrease in relative cortex size could have been the result of an acute rise in ACTH leading to the secretion of glucocorticoids and the depletion of stored lipid material but not yet resulting in adrenocortical enlargement. If the depletion of lipid material continues and results in decreased production of glucocorticoids from cholesterol, ACTH concentration will remain high, resulting in adrenal enlargement and possibly cell damage and necrosis.

Schultz et al (1952) further studied the changes in adrenal histology in a pantothenic acid deficiency using young rats weaned for 3 weeks from dams fed a pantothenic acid deficient diet after littering. The young rats were then weaned to a pantothenic acid deficient diet. Control rats were fed a complete diet (pantothenic acid content data not reported) after being weaned from mothers on a complete diet. Rats from both groups received an intraperitoneal injection of ACTH (4 mg/day) or cortisone (2 mg/day) or were left untreated. The length of feeding and the lengths of the ACTH and cortisone treatments were not reported. Cholesterol was determined from visual inspection of photomicrographs of adrenal slices fixed in formalin,

imbedded in paraffin and stained with hematoxylin and phloxine B.

The adrenals of the deficient rats treated with ACTH were enlarged (adrenal weight: 57.1 mg, 0.159% body weight), showed hemorrhage, had severe and widespread necrosis, and had extremely low cholesterol content of the cortices compared with untreated controls (adrenal weight: 22.2 mg, 0.033% body weight) and untreated deficient rats (adrenal weight: 34.7 mg, 0.089% body weight). The adrenals of the control rats treated with ACTH were slightly enlarged (29.1 mg, 0.044% body weight), had excess storage of cholesterol compared with untreated controls, and showed no hemorrhage or necrosis of the cortex.

The adrenals of the deficient rats treated with cortisone were slightly smaller than normal (7.7 mg, 0.032 % body weight) and free from morphological abnormalities. These findings support the hypothesis that a low glucocorticoid concentration in pantothenic acid deficiency results in the characteristic adrenal damage since replacement with cortisol lessened the adrenal damage and greater stimulation with ACTH aggravated the damage. However, the lengths of the various treatments necessary to obtain these results were not reported in this study. Also, serum corticosterone and ACTH concentration were not monitored in order to determine basal concentrations.

ADRENOCORTICAL HORMONE PRODUCTION

The adrenal cortex synthesizes steroid hormones such as corticosterone from cholesterol (Figure 1). Cholesterol used for steroidogenesis in the adrenal is derived from uptake of serum lipoproteins and from endogenous synthesis. Uptake of plasma cholesterol esters, in the form of LDL in the human or HDL in the rat, is through receptor-mediated endocytosis by the adrenal cortex. ACTH then stimulates the conversion of the LDL or HDL cholesterol esters to free cholesterol in the cytosol. Cholesterol is synthesized endogenously in cortical cells from acetate units (Figure 2) and the rate-limiting step, mediated by HMG-CoA reductase, is the conversion of HMG-CoA to mevalonate. The synthesized cholesterol is added to the adrenal cholesterol pool available for steroid hormone production. The proportion of cholesterol for steroidogenesis coming from plasma lipoproteins versus de novo synthesis varies among species. In the human and rat, about 80% of cholesterol used for steroidogenesis is derived from plasma lipoproteins (Sabine, 1977).

When needed, cholesterol is transported to mitochondria where ACTH stimulates the transport of cholesterol from the outer to the inner mitochondrial membrane. This is thought to be the rate-limiting step in steroid hormone synthesis. The first enzymatic reaction in steroidogenesis is the conversion of cholesterol to pregnenolone. This reaction,

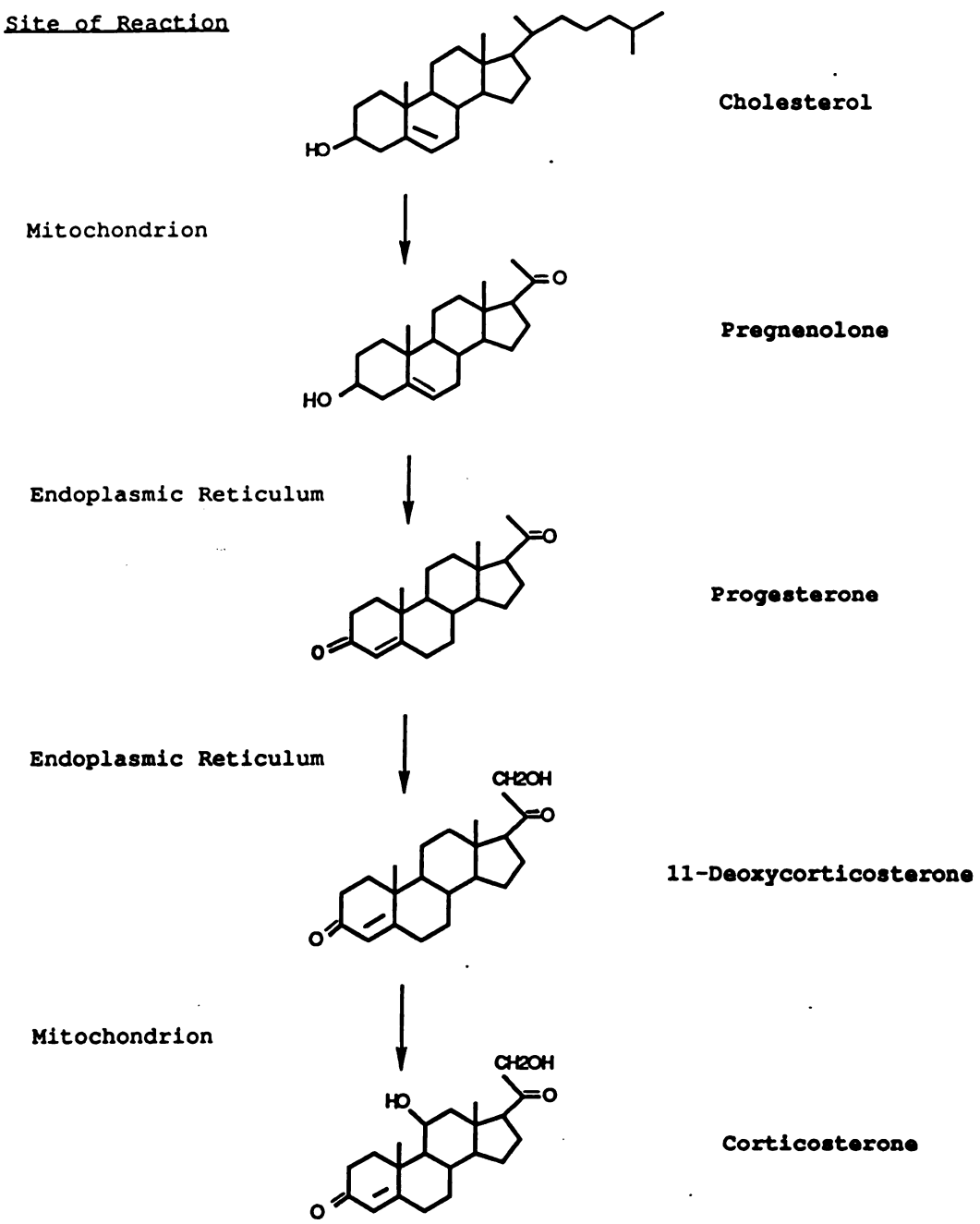

FIGURE 1 Synthesis of Corticosterone from Cholesterol

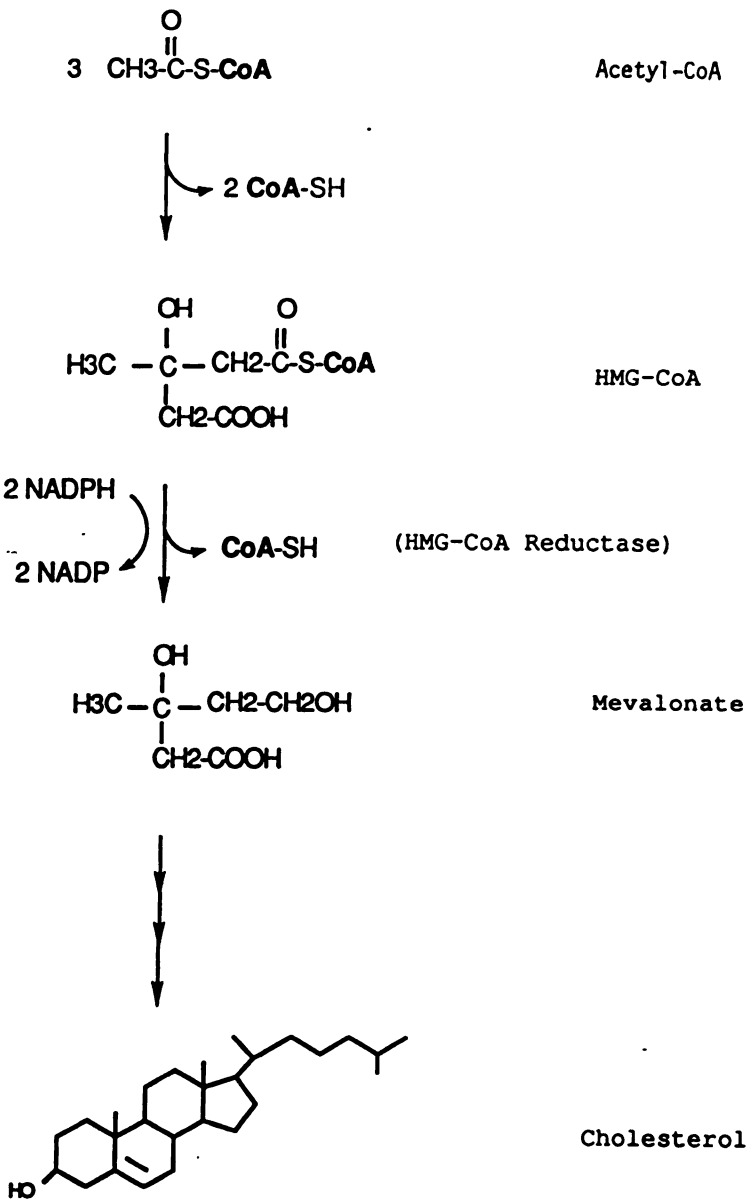

FIGURE 2 Synthesis of Cholesterol from Acetyl-CoA

mediated by $CytP_{450}SCC$, is stimulated by ACTH and involves the cleavage of a 6 carbon unit. Pregnenolone can be dehydrogenated to progesterone which can be subsequently hydroxylated to produce cortisol, the main glucocorticoid in man, corticosterone, the main glucocorticoid in the rat, or aldosterone, the main adrenal mineralocorticoid. Alternatively, production of 17-hydroxypregnenolone from pregnenolone leads to the formation of the adrenal androgens. The first step in the production of corticosterone occurs in the mitochondria. The conversion of pregnenolone to progesterone occurs in the smooth endoplasmic reticulum and further conversion to corticosterone is in the mitochondria.

EVALUATION OF ADRENOCORTICAL ENDOCRINE FUNCTION

Many clinical and biochemical methods are used to diagnose insufficiency or overactivity of the adrenal gland. The clinical signs of primary adrenocortical insufficiency (insufficiency not due to a defect in ACTH or CRF response to stress) include weakness, fatigue, weight loss, loss of appetite, nausea, and pigmentation of skin (Cullen et al, 1980). These clinical signs develop progressively in almost all cases but none are unique to this disease.

Biochemical tests are much more specific than the clinical signs in detecting adrenocortical insufficiency but the proper techniques must be used to obtain valid results.

Basal concentrations of corticosteroids (cortisol in human, corticosterone in rat) in blood and urine, basal blood concentrations of ACTH, and ACTH-stimulation tests are the most commonly used tests to evaluate adrenocortical function. Stress introduced to the subject, time of day of sample collection, and type of anesthesia used must all be considered when performing adrenal function tests.

Any disturbance in the environment can stimulate the hypothalamic-pituitary-adrenal axis of the subject. Immediate blood collection from undisturbed animals is necessary to obtain basal blood values of corticosteroids and ACTH. Rats and humans exhibit a diurnal variation of corticosteroid secretion and most likely exhibit a similar variation in ACTH secretion. When rats are fed throughout the night, plasma concentrations of ACTH and corticosterone peak late in the light phase while plasma concentrations of both hormones reach a low point between the end of the dark phase and the middle of the light phase (Depaolo and Masoro, 1989). In humans, the peak plasma cortisol concentration occurs near the beginning of the light phase while the minimum plasma concentration of corticosterone occurs near the beginning of the dark phase (Depaolo and Masoro, 1989). Feeding schedules and the time of day for blood collection must be consistent for valid comparisons between treatment groups. Barbiturate anesthesia has been reported to have the least stimulatory effect on the hypothalamic-pituitary-

adrenal axis, while ether activates the system within 5-10 minutes.

In adrenal insufficiency, low or normal plasma corticosteroid concentration is reported and the diurnal variation of corticosterone may or may not be present. However, adequate increases in plasma glucocorticoid concentration in response to stress is consistently absent in all cases of adrenocortical insufficiency. Urinary excretion of corticosteroid metabolites may not always be reduced in the insufficiency. Plasma ACTH concentration however, is consistently increased in adrenal insufficiency although the diurnal variation of ACTH is still intact (Cullen, 1980).

Using a data base of 20 patients diagnosed with primary adrenocortical insufficiency between 1987 and 1989 and their laboratory findings, Snow et al (1992) determined which test or combination of tests were most valid and reliable for the diagnosis. In those with primary adrenocortical insufficiency, basal, morning plasma cortisol concentrations were in the normal range in 3 of 20 patients. Two of 8 also had basal, evening plasma cortisol concentrations in the normal range. All 20 patients had plasma ACTH concentrations above the normal range, indicating the absence of negative feedback control from glucocorticoids. Eight patients underwent an ACTH-stimulation test and none were able to properly increase

plasma cortisol concentration (data for dose, duration, and cortisol concentration not reported). Two of 6 patients with primary adrenocortical insufficiency had urinary free cortisol within the normal range and 1 patient out of 4 had urinary 17-ketosteroids within the normal range. These results indicate that basal plasma and urinary corticosteroid concentrations alone cannot detect adrenocortical insufficiency. ACTH-stimulated plasma corticosteroids and basal plasma ACTH concentration are more specific for diagnosing the insufficiency as no patient had normal values for either of these tests.

ADRENAL STEROIDOGENESIS IN DIETARY PANTOTHENIC ACID DEFICIENCY

Adrenal damage seen in pantothenic acid deficiency involves mainly the zona fasciculata of the cortex which secretes glucocorticoids. Adrenal function in pantothenic acid deficiency has been evaluated by serum corticosterone concentration, the predominant glucocorticoid in the rat. Several authors (Longwell et al, 1956; Eisenstein, 1957; Goodman, 1969; Pietrzik et al, 1974; Remer and Pietrzik, 1989) have reported evidence of decreased plasma glucocorticoid concentrations or glucocorticoid secretion in rats fed a diet deficient in pantothenic acid and/or supplemented with a pantothenic acid antagonist.

Eisenstein (1957) fed weanling rats (35-40 g) a

pantothenic acid deficient diet ad libitum, a control diet (10 mg pantothenic acid/kg diet) ad libitum, or a control diet pair-fed to the deficient group for 5 weeks. The adrenals were then removed, placed in 2 mL of Krebs-Ringer-phosphate solution containing 1 unit of ACTH and 200 mg/dL glucose and incubated for 2 hours at 37° C. The steroids secreted into the medium were quantified using either the spectrophotometric method, which detects steroids with an unsaturated ketone on ring A (i.e., progesterone, 17-alpha-hydroxyprogesterone, 11-deoxycorticosterone, corticosterone, dehydrocorticosterone, deoxycortisol, cortisol, aldosterone), or the more specific Porter-Silber method, which detects steroids having a 17,21-dihydroxy-20-ketone side chain (i.e., corticosterone, dehydrocorticosterone, cortisol, cortisone).

The mean adrenal weight of the deficient group expressed relative to body weight, was much higher than that of either control group (PA-: 38 ± 1.9 mg/100 g body weight; PA+, ad lib: 15.7 ± 2.4 mg/100 g body weight; PA+, pair-fed: 22.8 ± 1 mg/100 g body weight). The adrenals of the deficient group also secreted less cortical hormone than did pair-fed controls: 22-39% depending on the method of determination. The researchers did not report serum glucocorticoid concentrations or serum or tissue pantothenic acid concentrations in the rats.

Longwell et al (1956) fed 150 g rats a pantothenic acid

deficient diet for 24 days, adding the pantothenic acid antagonist, omega-methyl pantothenic acid (0.5 g/100 g diet) to the diet for the last 14 days of feeding. A second group of rats was pair-fed a pantothenic acid supplemented diet (6.6 mg/100 g diet) without an antagonist and a third group was pair-fed a pantothenic acid supplemented diet (11.6 mg/100 g diet) containing the antagonist (0.5 g/100 g diet). Adrenal vein blood was then collected and values for both pantothenic acid supplemented groups were averaged and reported as one control value.

The deficient group had a lower output of adrenal corticosterone than either control group (PA-: 5.4 ug/mL vs control: 8.9 ug/ml or 80 ug/adrenal/kg/hr vs 136 ug/adrenal/kg/hr). The authors did not report the type of anesthesia used for the cannulation of the adrenal vein or the time of day for blood collection. Standard deviations and statistical analyses were also not reported.

Goodman (1960) conducted several studies on pantothenic acid deficiency and adrenal function. The author also reported that the output of corticosterone into the adrenal vein was decreased in rats fed a pantothenic acid deficient diet for 7 weeks. The effect of a pantothenic acid or ACTH dose on corticosterone concentration in rats fed a pantothenic acid deficient diet plus omega-methyl pantothenic acid was also examined. Rats (50-60 g) were fed a pantothenic acid deficient diet plus antagonist (0.75

g/100 g diet) or pair-fed the deficient diet without antagonist for 11 days. On the 11th day, one-half of each group (n=6) underwent adrenal vein cannulation for collection of the blood sample while the other half (n=7) received a subcutaneous injection of 65 mg pantothenic acid and underwent the same cannulation 24 hours later.

The rats fed the deficient diet plus antagonist and injected with pantothenic acid had a corticosterone output that was greater than that of the rats in this group that did not receive a pantothenic acid injection (0.24 ± 0.05 vs 0.15 ± 0.03 ug/min/adrenal, $p<.01$). The rats fed the pantothenic acid deficient diet without antagonist showed no differences in corticosterone output between those injected with pantothenic acid and those not injected (0.28 ± 0.04 vs 0.27 ± 0.04 ug/min/adrenal). These values were also not different from the group fed the antagonist and injected with pantothenic acid (0.24 ± 0.05 ug/min/adrenal).

These data support the assumption that the decrease in corticosterone concentration in rats fed a pantothenic acid deficient diet plus antagonist was caused by pantothenic acid deficiency because corticosterone output increased after replacement with pantothenic acid. The effect of the antagonist could be reversed by pantothenic acid replacement but since data on corticosterone output by rats on a complete diet was not reported, it cannot be concluded whether any effect of the deficient diet alone on

corticosterone concentrations could be reversed by the replacement.

Using the same diets and duration of feeding as above, Goodman examined the effects of ACTH on rats treated with a pantothenic acid antagonist. Rats fed the experimental diets for 11 days received one of two ACTH treatments: 1) daily subcutaneous injections of ACTH (8 units/day) throughout the 11 days plus a subcutaneous injection of ACTH (2 units/rat) 20 minutes before adrenal cannulation or 2) daily subcutaneous injections of ACTH (4 units/day) beginning five days before cannulation plus a subcutaneous injection of ACTH (4 units) 20 minutes before cannulation. Results from the two types of ACTH treatments were averaged together and expressed as one group.

The group fed the pantothenic acid deficient diet plus antagonist and treated with ACTH had a corticosterone output that was significantly higher than the group fed the same diet but not treated with ACTH (0.37 ± 0.11 vs 0.12 ± 0.06 ug/min/adrenal, $p<.01$). The corticosterone output of the group fed the deficient diet plus antagonist and the group fed the deficient diet without antagonist did not differ after ACTH treatment (0.37 ± 0.11 vs 0.36 ± 0.08 ug/min/adrenal). Of the rats not treated with ACTH, those fed the deficient diet plus antagonist had a lower corticosterone output than did those fed the deficient diet without antagonist (0.12 ± 0.06 vs 0.30 ± 0.2). Again, the

effect of a pantothenic acid deficient diet without antagonist on corticosterone output cannot be determined from this study.

Pietrzik et al (1974) investigated plasma corticosterone concentrations in pantothenic acid deficiency. Rats weighing 130 g were fed a pantothenic acid deficient or supplemented (15 mg/kg diet) control diet either with or without omega-methyl pantothenic acid (500 mg/kg diet) for 8 weeks. In the morning on days 1 and 28, blood was collected retrobulbarly three hours after intramuscular injection of 1 unit of ACTH.

No differences in circulating corticosterone concentration were seen between any of the groups on days 1 and 28. Blood was again collected on day 56 after Nembutal anesthesia and without prior ACTH injection. All groups fed the pantothenic acid supplemented diet had higher corticosterone concentration than the groups fed a pantothenic acid deficient diet or the deficient diet plus antagonist (approximate data from bar graph; PA+: 100 ug/dL, PA-: 60 ug/dL, PA- + antagonist: 10 ug/dL). The authors also showed that although only those rats fed the deficient diet with antagonist exhibited necrosis of the adrenal, the rats fed the deficient diet without antagonist also had decreased plasma corticosterone concentration. This demonstrates that the reduced corticosterone output is not dependent on and may precede adrenal damage if there is no

toxic effect of the antagonist itself on the adrenal cortex.

More recently, Remer and Pietrzik (1989) have studied plasma corticosterone concentrations in weanling rats fed a pantothenic acid deficient diet ad libitum, a control diet (50 mg pantothenic acid/kg diet) ad libitum, or a control diet and pair-weighted to the deficient group. At approximately 3, 5, 7, 9, 11, and 13 weeks of the study, blood was collected by orbital sinus puncture under light ether anesthesia at 8:00 am. Corticosterone was determined by a fluorometric method. At each time point, the plasma corticosterone concentration of the pair-weighted control group was significantly higher than both the pantothenic acid deficient group and the ad libitum control group. The physiological stress of decreased weight gain in the pair-weighted group probably contributed to the observed elevation in corticosterone. Also, food was given to the pair-weighted group at 11 am each day which would lead to a "pre-feeding" corticosterone peak at the time of blood collection at 8:00 am. Therefore, a comparison of a single plasma corticosterone value between groups would be invalidated.

Remer and Pietrzik (1989) also determined plasma corticosterone concentrations 30 minutes after an intramuscular injection of ACTH (2.5 units/rat) at weeks 3, 5, 7, and 12. There were no differences in plasma corticosterone concentrations among the three groups at any

time point studied, indicating that each group was able to increase plasma corticosterone concentration normally in response to the ACTH.

The authors also studied the circadian rhythm of plasma corticosterone concentration in another set of rats fed the same diets as above for 7 weeks. Every 3 hours over a 24-hour period, 6 rats from each group were taken for a one time blood collection from the orbital sinus under light ether anesthesia. The mean corticosterone concentration at each time point was plotted over the 24 hours to obtain the circadian rhythm. The researchers reported an interruption in the normal circadian rhythm of corticosterone (D'Agostino et al, 1982) in the pantothenic acid deficient and pair-weighted groups. The peak corticosterone concentration seen at the beginning of the dark cycle (9:00 pm) was amplified in the pantothenic acid deficient rats (approximately 50 ug/dL for the deficient group vs 30 ug/dL for the two control groups). This may indicate that there is an increased stimulation of the adrenal by ACTH in the deficient group which results in a higher evening corticosterone peak although ACTH was not measured by the researchers. The pair-weighted group showed a second peak in plasma corticosterone at the beginning of the light cycle which was not seen in the other two groups, possibly due to the altered feeding schedule of the pair-weighted group.

In summary, researchers have either reported a

reduction or no change in plasma corticosterone concentration or corticosterone secretion in response to the intake of a pantothenic acid deficient diet. Of the three studies which determined corticosterone concentration or output in rats without prior ACTH treatment, Longwell et al (1956) and Pietrzik et al (1974) found either a reduced rate of output or a reduced plasma concentration of corticosterone in rats fed a pantothenic acid deficient diet with or without added antagonist compared to pair-fed controls. Surprisingly, Pietrzik et al (1974) used larger rats (150 g) and found an effect of the deficiency on non-stimulated corticosterone concentration after 8 weeks of feeding. In another study, Remer and Pietrzik (1989) used weanling rats fed the deficient diet for 13 weeks and could not duplicate these results.

The studies that employed ACTH to stimulate adrenal steroidogenesis did not agree on the ability of pantothenic acid deficient rats to produce corticosteroids in response to the ACTH. Longwell (1956) reported that adrenals removed from pantothenic acid deficient rats and incubated with ACTH secreted less cortical hormone than did adrenals from control animals. In Goodman's study (1960), rats fed a pantothenic acid deficient diet with antagonist were able to increase corticosterone output after ACTH treatment to match that of rats on a deficient diet without antagonist. Pietrzik et al (1974) showed that after 4 weeks on a

pantothenic acid deficient diet, plasma corticosterone concentration after ACTH treatment did not differ from pair-fed controls. Also, Remer and Pietrzik (1989) found that the plasma corticosterone concentration seen after an ACTH dose were similar in pantothenic acid deficient and control groups. From these studies, there is actually more evidence of a decreased basal corticosterone concentration than an ACTH-stimulated corticosterone concentration in pantothenic acid deficiency.

These studies on adrenal function in pantothenic acid deficiency did not determine pantothenic acid concentration in the rats. Only Remer and Pietrzik (1989) attempted to do so by determining urinary excretion of pantothenic acid in the rats. The validity of these data are minimized because of the high concentration of pantothenic acid supplementation of controls (50 mg/kg diet) which would cause the excretion of pantothenic acid in these animals to be invariably higher than deficient animals. It is important to assess both blood and tissue pantothenic acid throughout the experimental period to confirm depletion of the vitamin and identify the relationship between this depletion and the onset of adrenal alterations. It is also desirable to measure specific forms of pantothenic acid, such as CoA, to determine if a reduction in this form could be responsible for the alterations seen in the deficiency.

The vitamin antagonist, omega-methyl pantothenic acid,

along with a pantothenic acid deficient diet, produced a more rapid effect of the deficiency on the adrenal gland than a pantothenic acid deficient diet alone. However, the effect of the antagonist on adrenal function independent of pantothenic acid metabolism is not known so benefits of its use in future studies are not warranted.

DEPENDENCE OF ADRENOCORTICAL STEROIDOGENESIS ON CHOLESTEROL

Because cholesterol is the precursor to steroid hormone synthesis, the effect of a reduction in plasma lipoproteins and/or adrenal cholesterol on the steroidogenic ability of adrenocortical cells has been examined. Szabo et al (1984) studied the structure and endocrine function of adrenocortical cells isolated from adult rats treated with or without 4-aminopyrazolo(3,4-d)pyrimidine (4-APP), a known hypocholesterolemic drug. Treated rats were injected intraperitoneally with 2 mg 4-APP/100 g body weight for two consecutive days while untreated control rats were injected with vehicle only. Rats were killed by decapitation 24 hours after the last injection, adrenals removed, and cell suspensions prepared. ACTH was added to cell suspensions in 4 concentrations (2×10^{-11} - 5.1×10^{-9} M) and the experiment was completed in triplicate. Lipid content of cryostat sections of freshly removed adrenals was determined by a polarizing microscope and corticosterone concentration of the incubation media was determined by a fluorometric

method.

The fasciculata region of the adrenal sections of rats treated with 4-APP was depleted of lipids compared to those of untreated controls. The fasciculata cells of the 4-APP-treated rats also secreted significantly less corticosterone into the media after in vitro ACTH treatment compared to controls at each concentration of ACTH. The mean reduction in corticosterone concentration of the media for all ACTH concentrations and all runs was 51%. Glomerulosa cells isolated from rats treated with 4-APP showed almost no differences in lipid content or steroidogenic response to ACTH when compared with untreated controls. The authors did not report the cholesterol concentration of plasma or isolated cells to verify the extent of cholesterol depletion due to 4-APP treatment. Therefore, the plasma cholesterol concentration which will impair adrenocortical steroidogenesis is not known.

Toth et al (1990) tried to determine the role of de novo cholesterol synthesis in the endocrine function of adrenal glomerulosa and fasciculata cells. Adrenal cells isolated from adult rats fed a complete diet were treated with or without the cholesterol synthesis inhibitor, BM 15.766 (4-[2-[1-(4-chlorocinnamyl)piperazin-4-yl]ethyl]-benzoic acid) at a concentration of 2×10^{-7} to 2×10^{-5} M. When used, ACTH was added to the cell suspension in a concentration of 1×10^{-9} M along with BM 15.766 treatment.

Cells were incubated for 2 hours. The corticosterone concentration of the incubation media was determined by fluorometry.

Upon addition of ACTH, the corticosterone production of the fasciculata cells treated with 2 x 10^{-5} M BM 15.766 was significantly decreased to 75% of that of untreated fasciculata cells. The corticosterone production of glomerulosa cells was significantly decreased to about 70% of cells not treated with BM 15.766. Fasciculata cells treated with BM 15.766 and not stimulated by ACTH showed no change in corticosterone production compared to cells not treated with BM 15.766. Glomerulosa cells, however, showed a reduction in corticosterone production in the absence of ACTH to 80% of untreated controls. The authors did not quantitate adrenal cholesterol concentration to substantiate the proposed relationship between adrenal de novo cholesterol synthesis and steroidogenesis.

CHOLESTEROL IN PANTOTHENIC ACID DEFICIENCY

Perry et al (1953) studied adrenal function in pantothenic acid deficiency by measuring adrenal cholesterol and ascorbic acid concentration which are normally reduced upon ACTH stimulation. Weanling rats were fed a pantothenic acid deficient diet or pair-fed the same diet plus a daily dose of 100 ug pantothenic acid for 18 to 90 days. Rats were at killed after 18, 30, 50, and 90 days of feeding and

adrenals quickly removed for analysis.

The adrenal cholesterol concentration of the deficient group was significantly less than that of pair-fed controls after 30, 50, and 90 days of feeding (control: 42.5, 56.1, 47.6 vs deficient: 24.2, 42.0, 21.5 mg/g ww, $p<.05$). Conversely, plasma cholesterol concentration was slightly higher in the deficient group than the control group on days 30, 50, and 90 (control: 94.7, 82.3, 96.3 vs deficient: 99.6, 96.0, 105.1 mg/dL, not significant). In the same study, rats fed a diet deficient in all B-complex vitamins except pantothenic acid for 50 days showed no difference in adrenal cholesterol concentration compared to controls.

Carter and Hockaday (1962) analyzed liver and adrenal lipids as well as ketone body formation in pantothenic acid deficiency. Rats weighing 55-60 g were fed a pantothenic acid deficient diet that was either low-fat (5% cod liver oil by weight) or high-fat (19% margarine and 5% cod liver oil by weight). One-half of the rats in each of these dietary groups received a daily dose of 240 ug pantothenic acid and was pair-fed to the rats not receiving pantothenic acid. After 80 days of feeding, rats were fasted for 17 hours, decapitated, and their livers and adrenals removed and immediately frozen. Liver and adrenal lipids were extracted and cholesterol determined by the Liebermann-Burchard reaction.

Rats on the low-fat, pantothenic acid deficient diet

had adrenals with reduced total cholesterol concentration compared to pair-fed controls on the low-fat diet (10 ± 1 vs 87 ± 6 mg/g ww, $p<.05$). A difference in adrenal cholesterol concentration between pantothenic acid deficient and controls was also evident in the high-fat diet group (32 ± 7 vs 64 ± 10 g/100 g ww, $p<.05$). Liver cholesterol was also decreased in the low-fat, pantothenic acid deficient group compared to pair-fed controls but not to the extent seen in the adrenal (10 ± 1 vs 13 ± 1 mg/g ww, $p<.05$). A significant difference in liver cholesterol was not seen between the pantothenic acid deficient and control groups fed the high-fat diet.

Remer and Pietrzik (1989) have shown no difference in adrenal cholesterol concentration between two groups of rats fed a pantothenic acid deficient or an ad libitum control diet for 9 weeks (13.66 ± 1.14 vs 13.05 ± 1.08 mg/g ww). The rats fed a control diet but pair-weighted to the deficient group had adrenal cholesterol concentrations that were significantly higher than that of the two other groups (35.81 ± 1.24 mg/g ww, $p<.001$ compared to both groups). Plasma cholesterol concentration was decreased in both the pantothenic acid deficient and the pair-weighted control group compared to ad libitum controls (deficient: 74.5 ± 8.3 and control, pair-weight: 71.9 ± 3.1 vs control, ad lib: 121.2 ± 6.3 mg/dl), suggesting that decreases in food intake may be the cause of the decrease in plasma cholesterol.

In each of the studies on adrenal cholesterol, pantothenic acid deficient animals exhibited lower adrenal cholesterol concentration than pair-fed or pair-weighted controls. Since the depletion of adrenal cholesterol in rats on a pantothenic acid deficient diet was seen when compared to pair-fed controls, decreased food intake cannot completely account for the effect of the deficiency on adrenal cholesterol. The response of plasma cholesterol in pantothenic acid deficiency was not consistent in the two studies in which it was evaluated. The mechanism of how dietary pantothenic acid deficiency leads to decreased adrenal and plasma cholesterol independent of underfeeding and stress has not yet been studied.

TISSUE COA CONCENTRATION IN PANTOTHENIC ACID DEFICIENCY

The regulation of CoA synthesis and degradation in vivo have not yet been completely defined. The first and rate-limiting step of CoA synthesis is catalyzed by pantothenate kinase forming 4'-phosphopantothenic acid (4'-PPA) from pantothenic acid. The pantothenate kinase activity has been shown to be inhibited in vitro by CoA, 4'-PPA, dephospho-CoA, and acetyl-, propionyl-, and malonyl-CoA. The degree of inhibition by the CoA derivatives differs among the tissues studied. For example, the K_i for CoA was shown to be much lower in the rat heart (0.2 uM) than in either the kidney (72 uM) or liver (47 uM). Since the content of CoA

in myocardial cells is sufficiently high to inhibit the pantothenate kinase of the rat heart, some other regulatory factor, such as carnitine, is thought to mediate CoA synthesis in vivo (Tahiliani and Beinlich, 1991).

The response of CoA in pantothenic acid deficiency also differs among studies. Srinivasan and Belevady (1976) fed weanling male rats a pantothenic acid deficient diet or a control diet pair-fed to the deficient group for six weeks. The group fed the deficient diet had a 43% reduction in liver total pantothenic acid (40.2 ± 3.4 vs 74.5 ± 7.1 ug/g) and total CoA (53.2 ± 7.8 vs 93.9 ± 11.7 units/g) compared with pair-fed controls (Srinivasan and Belevady, 1976).

Reibel et al (1982) fed 250-300 g rats a pantothenic acid deficient or control (50 mg pantothenic acid/kg diet) diet for only four weeks. A 72-93% decrease in the free pantothenic acid concentration of the tissues of the pantothenic acid deficient group compared to ad libitum controls was found. The tissues included the liver (PA-: 7 ± 1 vs PA+: 26 ± 7 nmol/g dry tissue) and adrenal (PA-: 4 ± 1 vs PA+: 28 ± 4 nmol/g dry tissue). The total CoA concentration of these tissues, however, was not reduced.

Although the reduction in pantothenic acid concentration of these tissues appears greater than what was seen by Srinivasan and Belavady (1976), the two sets of data are not comparable because Reibel et al (1982) determined free pantothenic acid in dried tissue while Srinivasan and

Belevady (1976) determined total pantothenic acid in wet tissue. Tissue free pantothenic is normally reduced first, indicating that a reduction seen here is not as critical as a reduction in tissue total pantothenic acid which encompasses CoA. The fact that the rats in Reibel's study were not weanling and the deficient diet was fed for only four weeks may also explain the maintenance of CoA concentration in the tissues in pantothenic acid deficiency.

Reibel et al (1982) also demonstrated that tissue CoA concentration may be under hormonal and metabolic control. Rats fed either a pantothenic acid deficient or supplemented diet showed an increase in liver pantothenic acid after fasting and an increase in liver pantothenic acid and CoA after both fasting and alloxan-induced diabetes. In myocardial tissue, diabetes caused a decrease in pantothenic acid in control rats and an increase in CoA in both pantothenic acid deficient and control rats. It is apparent from these studies that tissue CoA concentration is not solely dependent on tissue pantothenic acid concentration and any hormonal regulation of CoA concentration is tissue specific.

ADRENAL FUNCTION IN OTHER B-COMPLEX DEFICIENCIES

Pyridoxine is the only B-complex vitamin, other than pantothenic acid, for which there is evidence for altered adrenal histology and function in the deficiency. The

adrenal changes seen in pyridoxine deficiency include adrenal enlargement, loss of lipid material and cholesterol from the fasciculata, and disorganization of the mitochondrial membrane and structure. Reduction of food intake to match that of the pyridoxine deficient rats resulted in no significant changes in the adrenal cortex (Datsis, 1991). The mechanism responsible for these alterations has not been explored but pyridoxine has been linked with the function of the glucocorticoid receptor. Bender et al (1989) showed that isolated hepatocytes from rats deficient in pyridoxine accumulated more dexamethasone than did hepatocytes from replete animals. A review by Allgood et al (1990) described the alterations in structure and function of the glucocorticoid receptor induced by pyridoxine. When working with whole cells, pyridoxine supplementation produced an alteration in the isoelectric point of the receptor, changed the steroid and DNA binding capacities of the receptor, and the pyridoxine deficiency increased the responsiveness of the receptor to the steroid hormone. A direct connection has not been made between the role of pyridoxine in glucocorticoid receptor regulation and the altered adrenal histology seen in the deficiency.

SUMMARY

Presently, one set of data suggests that adrenal production of glucocorticoids is decreased in a pantothenic

acid deficiency, and another set of data indicates that adrenal and blood concentrations of cholesterol may be decreased in the deficiency. Also, there is evidence that a hypocholesterolemic state can result in a decreased output of glucocorticoids in response to ACTH. The decrease in adrenal and blood cholesterol concentrations may be responsible for the inadequate adrenal function seen in pantothenic acid deficiency. Studies incorporating an evaluation of adrenal function, pantothenic acid status, and cholesterol concentration are needed to test this hypothesis.

MATERIALS AND METHODS

MATERIALS

D-pantothenic acid (hemicalcium salt), alkaline phosphatase (EC 3.1.3.1), rabbit serum albumin, CoA-SH (sodium salt), ATP, Triton X-100, bovine serum albumin, bovine gamma globulin, acyl-CoA synthetase (EC 6.2.1.3), ACTH (1-39), and the enzymatic total cholesterol kit were purchased from Sigma Chemical Company (St. Louis, MO). D-[1-C^{14}]-pantothenic acid (sodium salt, 40-60 mCi/mmol), [1-C^{14}]-palmitate (40-60 mCi/mmol), and [1,2,6,7-H^3]-corticosterone (80-105 mCi/mmol) were purchased from New England Nuclear (Boston, MA). Dithiothreitol was from Boehringer Manheim Biochemicals (Indianapolis, IN), Soluene was from Packard Instruments Company (Downer's Grove, IL), Safety Solve was from Research Products International (Indianapolis, IN), EDTA was from JT Baker Chemical Company (Phillipsburg, NJ), and the corticosterone antibody was from Endocrine Sciences (Calabasas Hills, CA).

RESEARCH DESIGN

Thirty-two weanling (21-day old) Sprague-Dawley male rats (Charles River Laboratories, Portage, MI) were received

with an initial body weight of 49.4 ± 5.1 g. The rats were cared for according to the Michigan State animal care guidelines. The rats were housed individually in suspended stainless steel cages to limit coprophagy and to measure individual food intake. The rats were maintained on a 12-hour light/dark cycle with lights on at 7:00 am. Temperature (20-22° C) and humidity (68-70%) were controlled. The rats were randomly divided into two groups (n = 16 per group). One group was fed, ad libitum, the AIN 76 semi-purified diet deficient in pantothenic acid (ICN Biochemicals, Irvine, CA; Appendix A) and the other group was fed, ad libitum, the same diet supplemented with pantothenic acid (12 mg/kg diet). The total pantothenic acid content of both diets was measured by radioimmunoassay (Wyse et al, 1979). The deficient diet contained 0.3 mg/kg diet while the supplemented diet contained 12.3 mg/kg diet. Rats were weighed weekly for the first six weeks of the study. Beginning with week seven, rats from the deficient group were weighed every two to three days in agreement with laboratory animal care regulations that animal weight loss does not occur. Food was given approximately 2 times per week and food intake was calculated weekly.

Blood samples were collected at weeks 6, 9, and 11. At each week, rats were moved to the room where blood was drawn and left undisturbed for two hours prior to blood collection. At 5:00 pm, the rats were anesthetized with

ether and, within ten minutes, a maximum of one mL of blood was collected from the tail artery. Two days after the blood collection at week 11, all rats were anesthetized with ether and injected intravenously with 2.2 units ACTH (25 ug)/kg body weight. Twenty minutes later, blood was collected by heart puncture after anesthesia in CO_2. Adrenals and liver were removed, weighed, frozen in dry ice, and stored at -70° C.

Throughout the study, blood was left to clot at room temperature for approximately one hour after collection and then centrifuged at 3500 x g for 15 minutes. Two to four aliquots of serum were frozen at -20° C, and one aliquot was thawed once for each analysis. Free pantothenic acid, corticosterone, and total cholesterol were determined in the serum samples. Total pantothenic acid and cholesterol were measured in adrenal and liver tissue. The CoA concentration of the liver was also determined.

SUPPLEMENTARY STUDIES

Diurnal Variation of Corticosterone:

Rats possess a classical diurnal rhythm of serum corticosterone concentration with the peak occurring at the beginning of the dark cycle and the nadir occurring at the beginning of the light cycle (D'Agostino et al, 1982; Jasper and Engeland, 1991). The objective of the first supplementary study was to confirm that the peak and nadir

of serum corticosterone of the pantothenic acid deficient group are the same as those of the control group. This must occur for a valid comparison of serum corticosterone concentration between groups.

To determine the diurnal variation in serum corticosterone, seven rats were selected from each group at week 6 of the study and moved to a separate room at 3:00 pm where blood was collected. Two hours later, blood was collected from the tail artery of each rat within 10 minutes after ether anesthesia. The remaining eight rats in each group underwent the same procedure the following day at 7:00 am. The two collections provided an evening and morning blood sample which were used for serum corticosterone determination.

ACTH Dose:

Since the weight of the two treatment groups were significantly different after the first week of the study, the second supplementary study was carried out to determine what type of ACTH dose will equally elevate serum corticosterone in rats of differing weights. One ACTH dose given relative to body weight (2.2 units ACTH/kg body weight) and one given uniformly for all rats were employed (0.7 units/rat). Two groups of rats fed commercial rat chow and with differing mean body weights (185 ± 48 vs 300 ± 4.7, n=3 per group) were removed from their cages at 3:00 pm.

The rats were injected intravenously with the first dose of ACTH (2.2 units/kg body weight) and 20 minutes later blood was collected from the tail artery. Forty-eight hours later, the same rats were injected with the second dose of ACTH (0.7 units/rat) and blood was collected as before. The second dose of ACTH is the approximate dose that the larger group received with the first dose. Serum corticosterone was determined in all blood samples.

ANALYTICAL METHODS

Determination of Pantothenic Acid:

A) Rat Diet Analysis

Two grams of each diet, in triplicate, were combined with an enzyme mixture containing 30 units alkaline phosphatase, 40 units pantetheinase, 0.4 mL phosphate buffered saline, and distilled water to make up the final volume to 2 mL. The test tubes containing the diet/enzyme mixture were covered and incubated at 37° C for at least four hours in a shaking water bath. The contents of each tube were then filtered into 100 mL volumetric flasks using Whatman #1 paper. The filters were rinsed and the volume of the filtrate was adjusted to 100 mL with distilled water. This final solution was used directly in the radioimmunoassay for pantothenic acid. The pantothenic acid content of the enzyme solution alone was also determined and later subtracted from the pantothenic acid concentrations of

the samples.

B) Serum Analysis

Serum is known to contain only free pantothenic acid. Serum was first deproteinated by adding equimolar concentrations of saturated $Ba(OH)_2$ followed by 10% $ZnSO_4$ with a combined volume of two times the volume of serum used (i.e., 150 uL serum + 140 uL $Ba(OH)_2$ + 160 uL $ZnSO_4$). The mixture was vortexed and centrifuged at 5000 x g for 10 minutes. Normally, 50 uL of the resulting supernatant was used for the radioimmunoassay.

C) Tissue Analysis

Total pantothenic acid concentration of liver and adrenal tissues was determined. Both adrenal glands (30-90 mg) from each rat or portions of liver after the organ was manually minced (0.8-1 g) were homogenized in cold distilled water (1:20 w:v dilution for adrenals and 1:4 w:v dilution for liver) using a Brinkman Polytron at a speed of four for 30 seconds. To release all bound forms of pantothenic acid, 1.5 units of alkaline phosphatase and 3 units of pantetheinase in enough Tris-HCl buffer to make the volume to 50 ul was added to 100 uL of the tissue homogenate. The mixture was then incubated for at least four hours at 37° C. Saturated $Ba(OH)_2$ followed by 10 % $ZnSO_4$ were added as before, contents mixed and centrifuged at 5000 x g for 10 minutes. Normally, 50 uL of the resulting supernatant was used for the radioimmunoassay.

D) Pantothenic Acid Radioimmunoassay

Pantothenic acid was determined using the radioimmunoassay as described by Wyse et al (1979). Fifty uL of the deproteinated supernatant was pipetted into 5 mL polypropylene tubes and combined with 250 uL of an antisera mixture containing a 1:20 final dilution of rabbit pantothenic acid antisera, 6000-8000 dpm of C^{14}-labelled pantothenic acid, and enough 1.5% rabbit serum albumin in phosphate buffered saline to make up the volume. The mixture was vortexed and left on a shaker for 15 minutes to allow equilibration of the pantothenic acid-antibody complex. Three-hundred uL of saturated $(NH_4)_2SO_4$ was added to each tube and the contents were vortexed and centrifuged under refrigeration at 8500 x g for 15 minutes. The resulting supernatant, containing pantothenic acid not bound to the antibody was siphoned off and discarded. The pellet was resuspended with 500 uL of 50% $(NH_4)_2SO_4$, vortexed and centrifuged at 8500 x g for 15 minutes. The resulting supernatant was again siphoned off and discarded. The pellet was solubilized by adding 300 uL Soluene tissue solubilizer and incubating at 60° C for 30 minutes. The contents were vortexed, combined with 3 mL Safety-Solve scintillation cocktail and measured for radioactivity (dpm) using a Packard 4430 scintillation counter. Fifty uL of standards containing 0, 0.001, 0.005, 0.01, 0.05, 0.1, 0.2, 0.35, 0.6, and 1 nmol pantothenic acid were concurrently

assayed with the serum samples.

The resulting dpm readings represent the amount of radiolabeled pantothenic acid bound to the antibody. Since the amount of labeled pantothenic acid added to each sample is constant, the variable amount of unlabeled pantothenic acid in the sample competes with the radiolabeled pantothenic acid for the antibody binding sites. Therefore, a greater amount of radiolabeled binding in the protein precipitate indicates a lower amount of unlabeled pantothenic acid present in the sample. A standard curve was prepared using a log scale of pantothenic acid concentration of the standards versus the dpm readings of the standards. The pantothenic acid concentration of the samples was then determined from the regression equation of the standard curve.

According to Wyse et al (1979), the cross-reactivity of the antibody with compounds structurally similar to pantothenic acid should be negligible at concentrations present in biological systems.

Determination of Coenzyme A:

A) Tissue Preparation

Liver tissue was prepared following a slight modification of the method of Knights and Drew (1988). Minced liver tissue (0.1-0.2 g) was homogenized in a 1:7 dilution (w:v) with 8% $HClO_4$ using a Brinkman Polytron at a

speed of 4 for approximately 30 seconds. The homogenate was centrifuged at 1500 x g for 10 minutes and the resulting supernatant, containing acid-soluble CoA, was removed and saved. Then, 6% $HClO_4$ was added to the pellet in the same volume as 8% $HClO_4$. The contents were vortexed to resuspend the pellet and centrifuged at 1500 x g. The supernatant was removed and added to the previously obtained supernatant. The pH of the combined supernatants was adjusted to 6-7 using 3N KOH. The contents were again centrifuged at 1500 x g for 10 minutes to precipitate potassium perchlorate. One mL of supernatant was removed and 10 uL of 0.2 M dithiothreitol (DTT) was added for a final concentration in the supernatant of 2 mM. DTT was added to keep the functional sulfhydryl group of CoASH in its reduced form. This supernatant was used directly in the CoA radioisotopic assay (Knights and Drew, 1988).

B) CoA Radioisotopic Assay

Free CoA was determined according to the method described by Knights and Drew (1988). The assay relies on the acyl-CoA synthetase reaction which activates palmitic acid to palmitoyl-CoA. The assay conditions were set up so that the amount of free CoA present in the sample limits the reaction. C^{14}-palmitic acid is the substrate and the quantity of C^{14}-palmitoyl-CoA formed determines the amount of CoA present in the sample.

Forty uL of eight standards containing 0-250 pmol CoA

or 40 uL of the tissue samples prepared as above were combined with 200 uL of a reaction mixture containing the necessary cofactors for the reaction: $MgCl_2$ (6.2 mM), EDTA (2mM), ATP (2.5 mM), DTT (1 mM), Triton X-100 (.05%) and approximately 400,000 dpm C^{14}-palmitic acid (20 mCi/mmol) in Tris-HCl (150 mM, pH 8.4). The reaction mixture plus standards or samples were equilibrated in a water bath at 37° C for one minute. Acyl CoA synthetase (0.034 units) was then added to each tube and the reaction was allowed to occur at 37° C in a shaking water bath for 10 minutes. The reaction was terminated by adding 3.25 mL of methanol/chloroform/heptane (1.41:1.25:1). The contents were mixed briefly and 1.05 mL of sodium acetate (0.1 M, pH 4) was added to each tube to ease the separation of solvent layers. The tubes were tightly sealed, placed on a mechanical shaker for 20 minutes, and centrifuged at 500 x g for 10 minutes to clearly separate the solvent layers. One mL of the upper heptane layer was transferred to 20 mL scintillation vials, combined with 10 mL Safety-Solve scintillation fluid and measured for radioactivity in a Packard 4430 scintillation counter.

A standard curve was formed using the concentrations of the CoASH standards versus their dpm readings which was linear up to 250 pmol, the highest concentration of standard used. The concentration of each sample was determined from the regression equation of the standard curve.

According to Knights and Drew (1988), no significant formation of [C^{14}]palmitoyl-CoA occurred in the presence of acetyl-CoA, dephospho-CoA, or CoA-ss-CoA.

Determination of Corticosterone:

A) Serum Preparation

Serum was prepared according to the method accompanying the corticosterone antibody from Endocrine Sciences (Calabasas Hills, CA). Ten uL of serum was mixed with 90 uL of 0.25% (w:v) bovine serum albumin (BSA) in borate buffer (.05 M, pH 8) and heated at 60° C for 30 minutes. Four-hundred uL of 100% ethanol was then added and the tubes were vortexed three times at five minute intervals. After centrifugation for 10 minutes at 3000 x g, 50 uL of the supernatant containing unbound corticosterone was transferred to 1.5 mL microcentrifuge tubes and dried in a Savant refrigerated centrifuge evaporator. Immediately following complete evaporation, 50 uL of 0.25% BSA in borate buffer was used to reconstitute the dried material. Fifty uL aliquots of corticosterone standard containing 0-20 ng corticosterone/mL methanol were also evaporated and reconstituted as were the serum samples. This mixture was used directly in the corticosterone radioimmunoassay.

B) Corticosterone Radioimmunoassay

Serum corticosterone was determined using a radioimmunoassay described in the literature accompanying

the corticosterone antibody from Endocrine Sciences (Calabasas Hills, CA). An antisera mixture was prepared so that a 200 uL aliquot contained 4000-8000 dpm H^3-corticosterone, a 1:40 dilution of 10% BSA, a 1:40 dilution of bovine gamma globulin, and borate buffer (.05 M, pH 8) to make up the volume. Two-hundred uL of this mixture was added to two tubes containing a blank standard in order to determine non-specific binding of the tritiated corticosterone to substances other than the antibody. Non-specific binding was less than 5% in all assays used for final data. Corticosterone antibody was then added to the remaining antisera mixture to obtain a final dilution of 1:40. Two hundred uL of this antisera mixture was then added to all remaining standards and samples. Tubes were vortexed and incubated at 37° C for 45 minutes with gentle shaking. The samples were then incubated at room temperature for at least two hours. The fraction of antibody-bound corticosterone was separated from free corticosterone by precipitation using 250 uL of saturated $(NH_4)_2SO_4$. Tubes were vortexed, centrifuged at 3000 x g for 10 minutes and 400 uL of the supernatant containing unbound corticosterone was transferred to 20 mL scintillation vials. Five mL Safety Solve scintillation cocktail was added and the radioactivity of the samples was determined in a Packard 4430 liquid scintillation counter.

The dpm readings from the standards and samples

represent the fraction of radiolabeled corticosterone which was not bound to antibody. A higher dpm reading indicates a higher amount of unlabeled corticosterone in the standard or sample because the unlabeled corticosterone competitively binds to the antibody, resulting in more radiolabeled corticosterone unbound to antibody. A standard curve was formed using a log scale of the concentrations of the standards versus their dpm readings. The corticosterone concentration of the samples was then determined by the regression equation of the standard curve.

According to the literature accompanying the antibody, the only substance which has a greater than 1% cross-reaction with the corticosterone antibody is deoxycorticosterone at 4%. It is also stated that deoxycorticosterone is substantially separated from corticosterone by solvent partitioning.

Determination of Cholesterol:

A) Tissue Preparation

Adrenals were homogenized with a 20-fold volume (w:v) of distilled water and liver was homogenized with a 4-fold volume of distilled water using a Brinkman Polytron at a speed of 4 for approximately 30 seconds. The Folch method (Folch et al, 1957) was used for the extraction of lipid material from the tissue homogenate. First, the volumes of methanol and chloroform used were determined so that the

final proportion of water(homogenate):methanol:chloroform was 3:4:8. Methanol was added to 190-250 uL of the homogenate and the contents vortexed for one minute. Chloroform was then added to the mixture and vortexed for two minutes. Approximately 80 uL of 4.2% KCl was added to each tube to ease the separation of the two solvent layers. The contents were centrifuged at 500-800 x g and the lower, chloroform, layer was quantitatively removed to a clean, disposable test tube. The chloroform was evaporated under a stream of nitrogen and the dried lipid extract was redissolved in 100 uL of isopropanol for use in the subsequent cholesterol determination.

B) Determination of Total Cholesterol

Ten uL of serum or 10 uL of isopropanol tissue extract was assayed using a kit for the quantitative, enzymatic determination of total cholesterol (Sigma Chemical Company, St. Louis, MO). The assay involved the enzymatic hydrolysis of cholesterol esters to free cholesterol by cholesterol esterase, the oxidation of free cholesterol to cholest-4-en-3-one and H_2O_2 by cholesterol oxidase, and the formation of quinoneimine dye from the hydrogen peroxide, 4-aminoantipyrine and p-hydroxybenzenesulfonate by peroxidase. The intensity of the color produced is directly proportional to the concentration of cholesterol in the sample and the standard is linear up to 600 mg/dL. Within 30 minutes after combining the enzyme reagent with the serum or tissue

samples, the absorbance of the standards and samples were read at 500 nm in a Bausch and Lomb spectronic 21 spectrophotometer.

Statistical Analysis:

Student's t-test was used to compare rat weight, diet intake, serum pantothenic acid, corticosterone, and cholesterol as well as tissue pantothenic acid, CoA, and cholesterol between groups. Homogeneity of variances between groups was confirmed using the f-test. When variances were not equal, the proper corrections in the statistical analysis were made using the Minitab statistical program. A paired t-test was used to compare serum pantothenic acid, corticosterone, and cholesterol within a group before and after ACTH treatment.

RESULTS

SUPPLEMENTARY STUDIES

Diurnal Variation of Corticosterone:

In both the control and pantothenic acid deficient groups, the mean corticosterone concentration in the evening sample was 3-4 ug/dL (25-45%) higher than in the morning sample although the differences were not statistically significant due to a large variation within each group (Table 1). In both groups, the observed pattern of serum corticosterone concentration agrees with the known diurnal variation in rats (D'Agostino, 1982). At both time points, the serum corticosterone concentration of the pantothenic acid deficient group was only slightly higher than that of the control group and statistical differences were not seen (Table 1). It was concluded that any comparisons of serum corticosterone concentration between groups would not be affected by the time of day of blood collection. Blood samples were collected from all rats in the evening throughout the remainder of the study to contrast the normally high serum concentration of corticosterone at this time with any low concentration seen in the deficient rats.

TABLE 1

Evening and morning serum corticosterone concentrations in rats fed a pantothenic acid (PA) deficient or control diet[1]

Diet	Corticosterone (ug/dL)	
	Evening Sample	Morning Sample
Control	11.6 ± 8.2 (n=7)	7.9 ± 9.9 (n=6)
PA-deficient	15.0 ± 6.4 (n=7)	12.2 ± 8.0 (n=4)

[1]Rats were fed a pantothenic acid deficient (0.3 mg PA/kg diet) or control (12.3 mg PA/kg diet) diet for 6 weeks. An evening blood sample was collected between 5-7 pm and a morning blood sample was collected between 9-11 am. Data are mean ± sd. Student's t-test showed no significant differences between groups.

ACTH Dose:

Serum corticosterone concentration in both weight groups of rats under both types of ACTH stimulation were above the normal reported range for Sprague-Dawley rats (1-23 ug/dL, Depaolo and Masoro, 1989), indicating that the ACTH doses were effective in stimulating corticosterone release. When the dose of ACTH was given relative to body weight (2.2 units/kg body weight), the serum corticosterone concentrations of the two weight groups were comparable even though the smaller group received a lower total dose (Table 2, $p=.59$). When the rats in both groups were given one uniform dose of ACTH (0.7 units/rat), the serum corticosterone concentration of the smaller group was significantly greater than that of the larger group (Table 2, $p<.005$). This uniform dose was approximately equal to the dose received by the larger group and greater than the total dose received by the smaller group during the first ACTH dose of 2.2 units/kg body weight.

These data indicate that an ACTH dose given relative to body weight produces similar serum corticosterone concentrations among rats with differing body weights. Thus, the pantothenic acid deficient rats in our study, which had a significantly lower body weight than controls at the time of ACTH injection, were expected to have the same serum corticosterone concentration after an ACTH dose given according to body weight if adrenal endocrine function

TABLE 2

Serum corticosterone concentrations after ACTH injection in rats with differing body weights[1]

		Corticosterone (ug/dL)	
	Weight (g)	ACTH Dose 1 (2.2 units/kg bw)	ACTH Dose 2 (0.7 units/rat)
Group A	185 ± 48	62.7 ± 12.3	165.4 ± 14.3[a,b]
Group B	300 ± 4.7	56.1 ± 15.5	42.1 ± 11.0

[1]Rats first received an i.v. injection of ACTH Dose 1 and blood was collected 20 minutes later from the tail artery. Forty-eight hours later, the same rats received an i.v. injection of ACTH Dose 2 and blood was collected in the same manner. Data are mean ± sd, n = 3 per group.

[a]Paired t-test for Group A showed that serum corticosterone at ACTH dose 2 is higher than that at ACTH dose 1 ($p<.005$).

[b]Student's t-test showed that at ACTH dose 2, the serum corticosterone concentration of Group A is higher than that of Group B ($p<.005$).

was intact. Also, in the smaller group, the increase in total ACTH dose from 0.4 units/rat during the per weight dose (2.2 units /kg body weight X .185 kg body weight) to 0.7 units/rat during the uniform dose was paralleled by a significant increase in corticosterone concentration (42.1 - 165.4 ug/dL, Table 2, $p<.005$), indicating that the first ACTH dose was sub-maximal in stimulating corticosterone release.

BODY WEIGHT AND DIET INTAKE

The mean and standard deviation of the weekly body weights of rats fed a pantothenic acid deficient or control diet for 11 weeks is shown in Figure 3. After one week of the study, the rats fed a pantothenic acid deficient diet weighed less than controls (89.1 ± 8.4 vs 99.5 ± 8.6 g, $p<.005$). The rats fed the deficient diet gained weight throughout the study although the rate of growth was much slower than that of the control rats. After 11 weeks of the study, the deficient rats weighed less than 50% of the controls (193.7 ± 34.1 vs 462.2 ± 46.3 g, $p<.0005$).

The mean and standard deviation of the weekly diet intakes of both groups is shown in Figure 4. The intake of the control rats increased throughout the study from 70 g/week/rat at week 1 to 160 g/week/rat at week 11 while that of the deficient rats remained constant throughout the study at 60-70 g/week/rat. Decreased food intake accompanying a

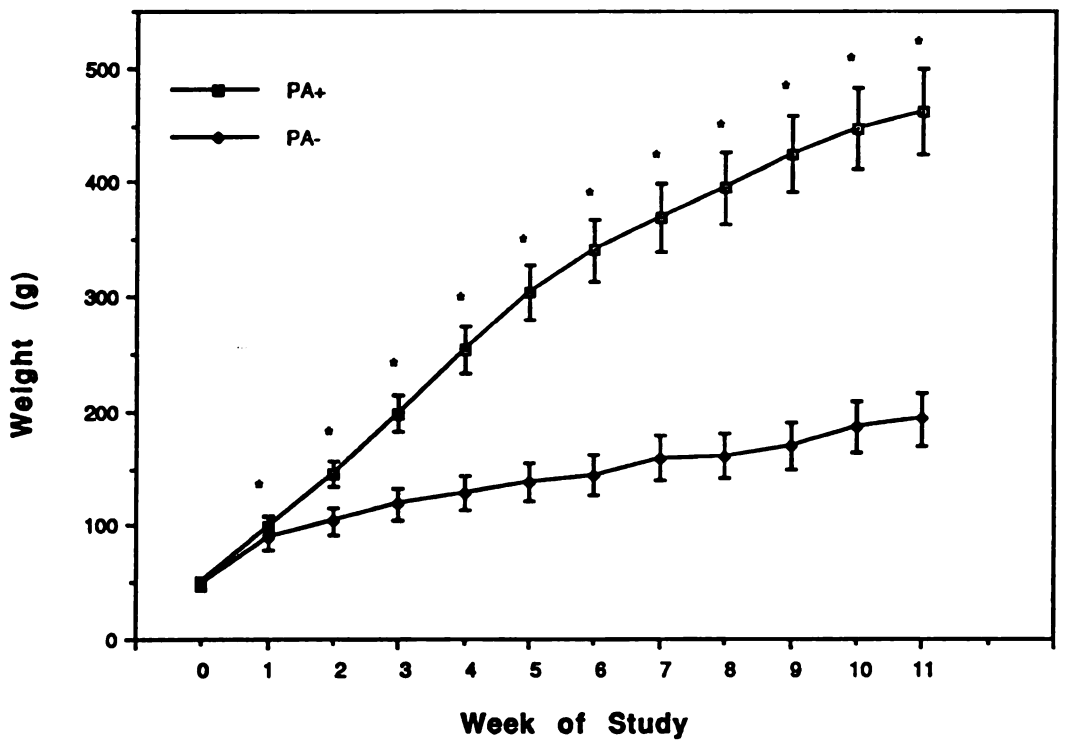

FIGURE 3 Weight of Pantothenic Acid Deficient and Control Rats

Weights of rats maintained on either a pantothenic acid deficient or control (12.3 mg pantothenic acid/kg diet) diet for 11 weeks were determined weekly. Data are expressed as mean ± sd, n=8-15 per group. *Student's t-test showed the weight of the pantothenic acid deficient rats was lower than that of controls ($p<.005$).

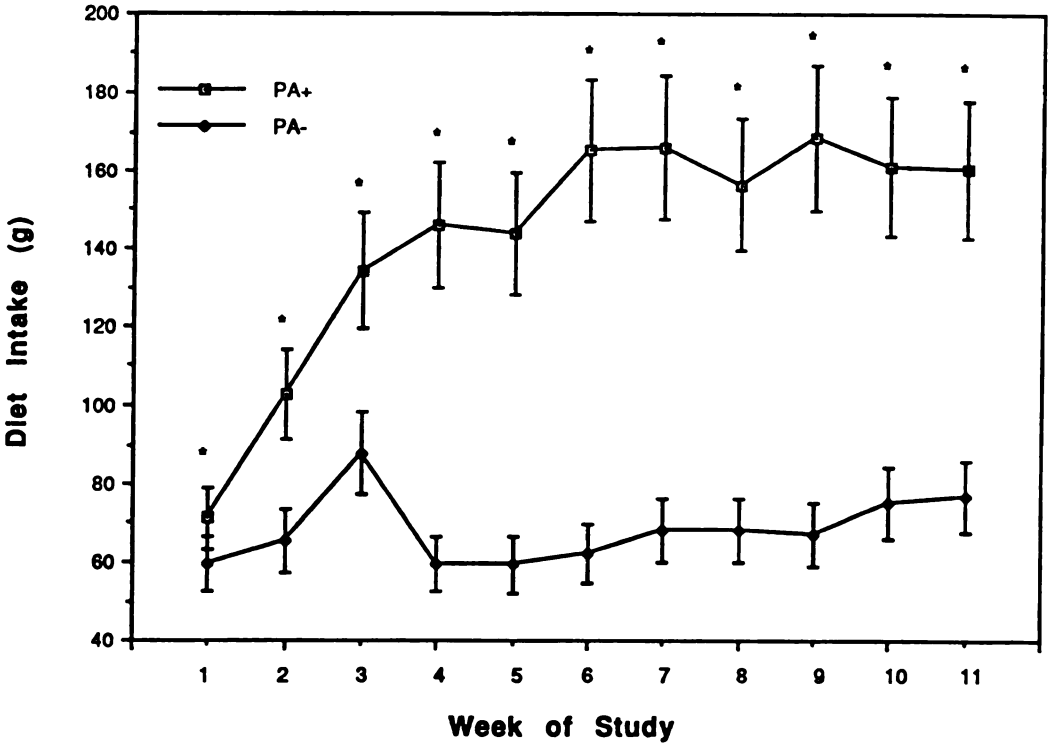

FIGURE 4 Diet Intake of Pantothenic Acid Deficient and Control Rats

Diet intake of rats on a pantothenic acid deficient or control (12.3 mg pantothenic acid/kg diet) diet was determined weekly. Data are expressed as mean ± sd, n = 8-15 per group. *Student's t-test showed the diet intake of the pantothenic acid deficient rats was lower than that of controls ($p<.001$).

pantothenic acid deficiency has been reported previously (Remer and Pietrzik, 1989; Srinivasan and Belavady, 1976) and has been repetitively seen in our laboratory. However, the decrease in food intake accounts for only part of the decreased weight gain of the deficient group based on reports that rats given a control diet and pair-fed to the deficient rats consistently weigh more than the deficient rats (Perry et al, 1953; Eisenstein, 1957; Carter and Hockaday, 1962; Srinivasan and Belevady, 1976).

Other signs of the pantothenic acid deficiency appeared by the sixth week of the study such as hair loss, pigmentation of the fur coat from white to yellowish-brown and porphyrin coated whiskers.

ORGAN WEIGHT

Adrenals were removed and weighed immediately after sacrifice which occurred 20 minutes after ACTH injection at week 11 of the study. Although the pantothenic acid deficient rats weighed less than 50% of controls at this time, the adrenals of the deficient rats weighed slightly more than those of the controls (Table 3, $p=.51$). When adrenal weight was expressed as a percentage of body weight, the adrenals of the pantothenic acid deficient rats weighed significantly more than those of the control rats (Table 3, $p<.005$).

Unlike adrenal weight, the mean liver weight of the

TABLE 3

Adrenal and liver weights of rats fed a pantothenic acid (PA) deficient or control diet[1]

Diet	Adrenal Weight		Liver Weight	
	(mg)	(% body weight)	(g)	(% body weight)
Control	59.3±8	.013±.0015	18.2±2.5	4.03±.23
PA-	64.4±20	.035±.0145[a]	8.6±1.7[a]	4.49±.95

[1]Rats were fed the experimental diets for 11 weeks and received an i.v. injection of ACTH (2.2 units/kg bw) 20 minutes before sacrifice. Tissues were immediately removed and weighed. Data are mean ± sd, n = 7-12 per group.
[a]Student's t-test showed the organ weight of the PA-deficient group is significantly different from that of control group ($p<.005$).

pantothenic acid deficient rats was significantly less than that of the control group ($p<.0005$). The liver weight expressed as a percentage of body weight, however, was not different from that of the control group (Table 3, $p=.23$).

SERUM PANTOTHENIC ACID AND CORTICOSTERONE

Concentrations at weeks 6, 9 and 11 without prior ACTH injection:

As shown in Table 4, serum free pantothenic acid concentration was significantly lower in the rats fed a pantothenic acid deficient diet than in the rats fed a control diet ($p<.005$ at week 6 and 9, $p=.059$ at week 11). At week 11, the pantothenic acid deficient group included a smaller number of rats than at week 6 or 9 due to loss of rats during the course of the experiment. Also, serum pantothenic acid concentration of the deficient rats at week 11 exhibited a larger variability than at week 9.

A difference in serum corticosterone concentration was not seen between groups at week 6 or 9 of the study (Table 4). At week 11, the mean serum corticosterone concentration of the deficient group was lower than that of the control group but even with the small sample size of the deficient group ($n=3$), the significance level of this difference was $p=.17$. The three rats in the deficient group in which serum corticosterone concentration was determined at week 11 showed a 3.8, 12.0, and 22.5 ug/dL decrease in serum

TABLE 4

Serum free pantothenic acid (PA) and corticosterone concentrations in rats fed a PA-deficient or control diet[1]

	Control	PA-deficient
Pantothenic Acid (nmol/mL)		
Week 6	.322 ± .105 (n=11)	.167 ± .093[a] (n=12)
9	.459 ± .144 (n=14)	.221 ± .042[a] (n=8)
11	.302 ± .042 (n=11)	.156 ± .087[b] (n=7)
11 + ACTH	.345 ± .209 (n=14)	.303 ± .208 (n=8)
Corticosterone (ug/dL)		
Week 6	11.6 ± 8.2 (n=7)	15.0 ± 6.4 (n=7)
9	14.5 ± 4.6 (n=15)	18.0 ± 6.3 (n=12)
11	11.1 ± 4.8 (n=7)	6.5 ± 2.2 (n=3)
11 + ACTH	26.8 ± 5.9[c] (n=11)	17.6 ± 6.5[c,d] (n=7)

[1]Rats were fed the experimental diets for 11 weeks and received an i.v. injection of ACTH (2.2 units/kg bw) 20 minutes prior to blood collection. Data are mean ± sd.

[a]Student's t-test showed serum PA of the deficient group to be lower than that of the control group ($p<.001$).

[b]Student's t-test showed the significance of the difference in serum PA at week 11 at $p=.059$.

[c]Paired t-test using rats with values before and after ACTH (control: n=5, PA-: n=2) showed the serum corticosterone concentration after ACTH treatment to be higher than that prior to ACTH treatment ($p<.05$).

[d]Student's t-test showed the serum corticosterone concentration of the PA deficient group to be lower than that of the control group ($p<.01$).

corticosterone from week 9. It appears that the adrenals of rats on a pantothenic acid deficient diet for 11 weeks may be insufficient in producing and secreting corticosterone.

Response to ACTH:

At week 11 of the study, serum pantothenic acid and corticosterone were measured in samples taken twenty minutes after an intravenous ACTH dose (2.2 units ACTH/kg body weight). The mean serum pantothenic acid concentration in the control group did not change with ACTH injection while that of the deficient group doubled to that of controls (Table 4). The increase seen in the deficient group was not, however, statistically significant ($p=.35$) because the ACTH injection resulted a large variability in the serum pantothenic acid values. This rise in serum pantothenic acid in the deficient group in response to the ACTH dose was not expected and has not been reported previously.

ACTH injection resulted in a significant increase in serum corticosterone concentration in both the control and deficient group compared to that seen prior to ACTH treatment (Table 4, $p<.05$). The serum corticosterone concentration after ACTH injection in the deficient group was, however, significantly less than that of the control group (Table 4, $p<.01$). Although ACTH was administered to each group to achieve a similar blood concentration of ACTH (approximately .04 units/mL blood), the deficient rats only increased serum corticosterone concentration to be similar

to their non-stimulated concentrations at weeks 6 and 9.

Figure 5 shows the percent increase in serum pantothenic acid and corticosterone after ACTH injection. Data from only those rats with values both before and after ACTH at week 11 were used. The increase in serum pantothenic acid after ACTH is greater in the deficient group then the control group (40% vs 23%) while the increase in corticosterone in the deficient group is less than that of the control group (105% vs 180%).

SERUM CHOLESTEROL

Rats in the control group maintained fairly constant concentrations of serum total cholesterol throughout the study (100-116 mg/dL). In the pantothenic acid deficient group, serum total cholesterol decreased from 100 mg/dL at week 6 to 72 mg/dL at week 11 and remained low at 79 mg/dL after ACTH injection (Table 5). At weeks 9, 11, and 11 after ACTH, serum cholesterol was lower in the pantothenic acid deficient group than in the control group (Table 5).

TOTAL PANTOTHENIC ACID AND TOTAL CHOLESTEROL CONCENTRATION OF ADRENALS

The total pantothenic acid concentration of the adrenals of the pantothenic acid deficient rats was reduced significantly (66%) from controls (Table 6, $p<.05$). Adrenal CoA concentration was not assessed due to a limited amount

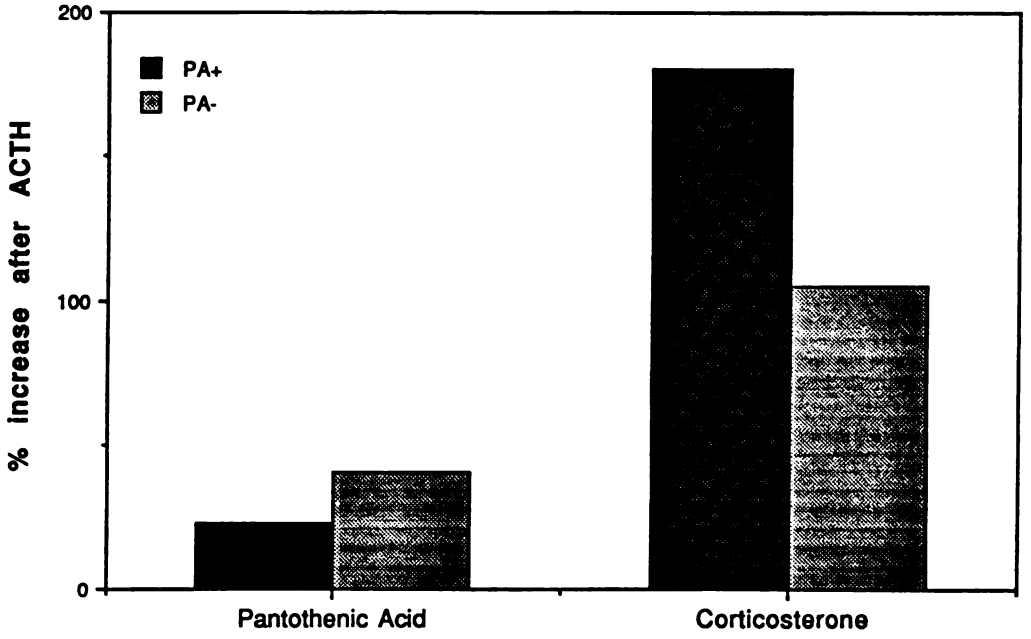

FIGURE 5 Percent Increase in Serum Pantothenic Acid and Corticosterone in Response to ACTH Injection

Rats were fed a pantothenic acid deficient or control (12.3 mg pantothenic acid/kg) diet for 11 weeks and blood was collected from the tail artery at 3:00 pm. Forty-eight hours later, rats received an i.v. injection of ACTH (2.2 units/kg body weight) and blood was collected 20 minutes later from the tail artery. Percentages are derived from only those rats with both before and after ACTH values.

TABLE 5

Serum total cholesterol concentration in rats fed a pantothenic acid (PA) deficient or control diet[1]

Week of Study	Cholesterol (mg/dL)	
	Control	PA-deficient
6	115 ± 24 (n=13)	100 ± 25 (n=13)
9	116 ± 26 (n=15)	85 ± 14[a] (n=10)
11	100 ± 22 (n=13)	72 ± 29[a] (n=7)
11 + ACTH	115 ± 26 (n=11)	79 ± 12[a] (n=8)

[1]Rats were fed the experimental diets for 11 weeks and received an i.v. injection of ACTH (2.2 units/kg bw) 20 minutes prior to blood collection. Data are mean ± sd.

[a]Student's t-test showed the serum cholesterol concentration of the PA deficient group to be lower than that of the control group ($p<.05$).

TABLE 6

Adrenal total pantothenic acid (PA) and total cholesterol concentrations of rats fed a control or PA-deficient diet[1]

Diet	Adrenal PA	Adrenal Cholesterol
	nmol/g ww	mg/g ww
Control	29.4 ± 10.4 (n=4)	15.5 ± 2.8 (n=10)
PA-deficient	10.1 ± 2.4[a] (n=4)	5.8 ± 1.7[b] (n=6)

[1]Rats were fed the experimental diets for 11 weeks and received an i.v. injection of ACTH (2.2 units/ kg bw) 20 minutes prior to sacrifice. Adrenals were immediately removed and frozen at -70° C. Data are mean ± sd.

[a]Student's t-test showed the adrenal PA content of the PA-deficient group to be lower than that of the control group ($p<.05$).

[b]Student's t-test showed the adrenal cholesterol content of the PA-deficient group to be lower than that of the control group ($p<.001$).

of adrenal tissue available. When one assumes that about 20% of total pantothenic acid of tissues is in its free form and that about 70% of the tissue weight is water, the free pantothenic acid in the adrenal available for CoA synthesis is less than 3 uM. This estimated concentration of free pantothenic acid in the adrenals is below the K_m for pantothenate kinase (.011-.018 mM) which is the first and rate-limiting step in CoA biosynthesis. Hence, the depleted adrenal pantothenic acid in _the deficient group is expected to have led to the decreased biosynthesis of CoA in the adrenal.

The total cholesterol concentration of the adrenals of the pantothenic acid deficient rats was also significantly reduced (63%) from controls (Table 6, $p<.001$). The adrenals in this study may have responded to the ACTH injection with some adrenal cholesterol depletion. Since both groups were injected with ACTH based on body weight to produce similar blood concentration of ACTH, the depletion of cholesterol is not expected to differ between groups. These data, along with the reported decrease in serum and liver cholesterol concentration seen in the pantothenic acid deficient group, suggest that the adrenals of the deficient group exhibited lower cholesterol concentration prior to the injection of ACTH as well.

TOTAL PANTOTHENIC ACID, CoA, AND TOTAL CHOLESTEROL CONCENTRATION OF LIVER

The total pantothenic acid concentration of the livers of the deficient rats was reduced 66% from controls as was adrenal total pantothenic acid (Table 6 and 7, $p<.05$). Liver free CoA concentration was also significantly lower in the pantothenic acid deficient group than in the control group (Table 7, $p<.005$).

Liver cholesterol was significantly, but only slightly, lower in the pantothenic acid deficient group than in controls (Table 7, $p<.005$). Liver cholesterol concentration of the pantothenic acid deficient group, however, was decreased only 9% from controls in contrast to the 63% decrease seen in the adrenals of the pantothenic acid deficient group (Table 7). The concurrent reduction in pantothenic acid, CoA, and cholesterol concentrations in the liver may suggest that liver cholesterol synthesis is limited due to the low CoA concentration in this tissue.

TABLE 7

Liver total pantothenic acid (PA), free CoA, and total cholesterol concentration of rats fed a control or PA-deficient diet[1]

Diet	Liver PA	Liver CoA	Liver Cholesterol
	nmol/g ww	nmol/g ww	mg/g ww
Control	329 ± 31	52.5 ± 12.7	1.78 ± .14
PA-deficient	111 ± 49[a]	37.1 ± 5.3[b]	1.62 ± .08[b]

[1]Rats were fed the experimental diets for 11 weeks and received an i.v. injection of ACTH (2.2 units/ kg bw) 20 minutes prior to sacrifice. Livers were immediately removed and frozen at -70° C. Data are mean ± sd, n = 8-14 per group.
[a]Student's t-test showed the liver PA concentration of the PA-deficient group to be lower than that of controls ($p<.0001$).
[b]Student's t-test showed the liver CoA and cholesterol concentration of the PA-deficient group to be lower than that of the control group ($p<.005$).

RESULTS SUMMARY

1. Rats fed a pantothenic acid deficient diet for 11 weeks are unable to increase circulating corticosterone concentration in response to ACTH treatment to that seen in controls.
2. Serum total cholesterol levels of rats fed a pantothenic acid deficient diet for 9 and 11 weeks is significantly lower than that of controls.
3. Adrenal total cholesterol concentration is reduced 63% from controls after 11 weeks on a pantothenic acid deficient diet.
4. Liver total cholesterol concentration is decreased 9% in rats fed a pantothenic acid deficient diet for 11 weeks compared to controls.
5. Adrenal total pantothenic acid concentration is reduced 66% from controls after 11 weeks on a pantothenic acid deficient diet.
6. Liver total pantothenic acid and CoA concentration is reduced 66% from controls after 11 weeks on a pantothenic acid deficient diet.
7. The adrenal weight, expressed as a percentage of body weight, of rats fed a pantothenic acid deficient diet is significantly higher than rats fed a control diet.

DISCUSSION

Rats fed a pantothenic acid deficient diet for 11 weeks had a reduced concentration of serum free pantothenic acid at weeks 6, 9 and 11 as well as reductions in adrenal and liver total pantothenic acid concentration compared to controls. Significant reductions in plasma total pantothenic acid after 3 weeks of feeding a deficient diet to weanling rats (Bates, 1988) and serum free pantothenic acid after 4 weeks of feeding a deficient diet to 250-300 g rats (Reibel et al, 1982) have also been reported.

The rise in serum free pantothenic acid in response to i.v. injection of ACTH in our deficient group has not been reported previously and was not expected. It has been reported that a rise in serum free pantothenic acid occurs after 48 hours of fasting or alloxan-induced diabetes in both control and pantothenic acid deficient rats (Reibel et al, 1982). Also, it was reported that fasting and diabetes in the deficient group resulted in no change in tissue total CoA concentration and an increase in tissue free pantothenic acid (kidney, gastrocnemius, testes, diaphragm, adrenal). The data suggest that the hormonal and metabolic regulation under fasting and diabetes results in the mobilization of a

yet unknown store of pantothenic acid for acquisition by serum and tissues. ACTH results in increased glucocorticoids as does fasting and diabetes. In this way, ACTH may stimulate the mobilization of the pantothenic acid store and increase serum pantothenic acid.

After 11 weeks of the study, a 66% decrease in both liver and adrenal total pantothenic acid was observed ($p<.05$). Reibel et al (1982) have shown an 85% reduction in adrenal and a 73% reduction in liver free pantothenic acid in rats on a pantothenic acid deficient diet for 4 weeks.

In this study, adrenal function of rats, as determined by serum corticosterone concentration after ACTH injection, was decreased after 11 weeks on the pantothenic acid deficient diet. An impaired ability to increase serum glucocorticoid concentration in response to ACTH has a negative effect on several physiological processes. These include the regulatory response to stress in which glucocorticoids suppress the primary defense mechanisms such as inflammation, increased insulin secretion, and the immune reaction (Munck et al, 1984). Also, exercise endurance is decreased in rats which do not have the ability to increase glucocorticoids in response to ACTH (Sellers et al, 1988). Indeed, signs of adrenocortical insufficiency are seen in Addison's disease where basal concentrations of glucocorticoids may not be altered while the response to ACTH treatment is reduced (Cullen et al, 1980; Snow et al,

1992).

The non-stimulated serum corticosterone concentration of our pantothenic acid deficient rats were not statistically different from controls at weeks 6, 9, or 11 of the study. When considering the decreased weight gain of the pantothenic acid deficient rats as a chronic stress to the rats, a basal serum corticosterone concentration of the deficient rats that is not higher than ad libitum controls may be meaningful. Remer and Pietrzik (1989) reported that the mean plasma corticosterone concentration over 24 hours was higher in rats pair-weighted to the deficient group compared to both the deficient and ad libitum control groups. That the pantothenic acid deficient group did not exhibit the same increase in mean corticosterone concentration over 24 hours as did the pair-weighted controls may indicate impaired adrenal function in the deficient group.

The disparity in adrenal weight, expressed relative to body weight, between the groups fed a pantothenic acid deficient or control diet can be explained in terms of adrenal function. Sayers et al (1944) have shown that 3, 6, and 9 hours after intraperitoneal injection with ACTH (50 mg/kg body weight), mean adrenal weights of normal, adult, fasted rats remained unchanged. Therefore, the intravenous ACTH (25 ug/kg body weight) injection 20 minutes prior to adrenal removal should not explain the difference in adrenal

weights between the two groups.

Chronic ACTH stimulation, however, results in adrenal cell hypertrophy and proliferation as well as increased adrenal blood volume. These effects together contribute to the increase in adrenal weight seen under chronic ACTH stimulation. If the adrenal gland cannot secrete adequate amounts of corticosterone, chronic stimulation by ACTH could result because of decreased negative feedback by serum corticosterone on ACTH secretion from the pituitary and CRF secretion from the hypothalamus. Therefore, impaired adrenal function could result in low serum corticosterone concentration, elevated serum ACTH concentration, and an enlarged adrenal gland relative to body weight. It has been consistently reported that the adrenal weight relative to body weight of pantothenic acid deficient rats is higher than rats pair-fed to the deficient group (Deane and McKibben, 1946; Perry et al, 1953; Eisenstein, 1957; Carter and Hockaday, 1962; Remer and Pietrzik, 1989). The pair-fed group, having chronic stress from decreased weight gain but normal adrenal function, would exert negative feedback by corticosterone on ACTH secretion, thereby lowering ACTH concentration and sparing the adrenal from the effects of chronic ACTH elevation.

The decrease in adrenal cholesterol concentration seen in the pantothenic acid deficient rats could be explained by increased removal of adrenal cholesterol for adrenocortical

hormone synthesis, decreased acquisition of adrenal cholesterol from blood, or decreased de novo synthesis of cholesterol. ACTH stimulates both the conversion of adrenal cholesterol esters to free cholesterol and the subsequent conversion of free cholesterol to pregnenolone, the first step in steroid hormone synthesis. A sudden increase in ACTH concentration in the blood will result in a reduction of adrenal cholesterol within 3 hours with low adrenal cholesterol levels being sustained for at least 9 hours (Sayers et al, 1944). If, in a pantothenic acid deficiency, the adrenal is not able to maintain adequate circulating corticosterone concentration, then blood ACTH concentration would remain high. This chronic ACTH stimulation would result in a permanent reduction of adrenal cholesterol.

Alternatively, a decrease in the uptake or de novo synthesis of cholesterol by the adrenal may occur in pantothenic acid deficiency. CoA is required as an acetate carrier for HMG-CoA reductase, the enzyme which catalyzes the rate limiting step in the conversion of acetyl-CoA to cholesterol. If the activity of this enzyme is compromised because of insufficient amounts of CoA, cholesterol synthesis will be decreased, reducing the cholesterol available for steroid synthesis. Also, the decrease in serum cholesterol seen in the deficient animals could have impaired the uptake of serum cholesterol by the adrenal. It is known that rats obtain up to 80% of the cholesterol used

for steroidogenesis from plasma lipoproteins (Szabo et al, 1984). It has been shown that by reducing plasma cholesterol with hypocholesterolemic drugs, the output of glucocorticoids in response to ACTH by isolated adrenal cells is decreased (Szabo et al, 1984; Toth et al, 1990). Thus, the combination of decreased adrenal and plasma cholesterol seen in pantothenic acid deficient rats could conceivably result in a decreased steroidogenic response of the adrenocortical cells to ACTH, a condition which was seen in our rats.

The effect of pantothenic acid deficiency was also studied in liver, a tissue important in cholesterol synthesis and regulation. Like the adrenal, the total pantothenic acid concentration of the livers of the deficient rats was decreased 66% from controls. Liver CoA concentration of the deficient rats were also decreased 30% from control levels. Unlike the drastic decrease seen in the adrenal, liver cholesterol decreased only 9% from controls. It may be that since the adrenal gland is most active during stress, such as that seen during the deficiency, it is this organ that is uniquely damaged in pantothenic acid deficiency. Therefore, the depletion of adrenal cholesterol seen in pantothenic acid deficiency may be a combined result of a decreased ability to synthesize cholesterol and an increased removal due to stress whereas the decrease in liver cholesterol may be the result of

decreased cholesterol synthesis alone.

We have concluded from this study that, in pantothenic acid deficiency, the ability of the rat adrenal gland to normally elevate serum corticosterone after ACTH injection is reduced. Also, this reduction in serum corticosterone may be caused by decreased adrenal, liver, and serum cholesterol concentrations. These conclusions are supported by the knowledge that pantothenic acid is required for the synthesis of cholesterol and this vitamin or its metabolite, CoA, were shown to be decreased in serum, adrenals, and liver of the rats fed a pantothenic acid deficient diet.

LIMITATIONS

Certain limitations were unavoidable in our attempt to determine adrenal function and cholesterol levels in pantothenic acid deficiency:

1. It cannot be claimed that the rats in this study were completely free from stress when the blood samples were collected for determination of corticosterone concentration at weeks 6, 9, and 11 of the study. The hypothalamic-pituitary-adrenal axis is known to respond immediately to small changes in the environment, resulting in increased serum concentrations of corticosterone. Our interpretation of the serum corticosterone concentrations in the rats of our study, however, are considered to be valid because 1) a similar diurnal variation of serum corticosterone was detected in both groups of rats, 2) basal serum corticosterone concentrations of the control rats were consistent at 6, 9, and 11 weeks of the study, and 3) the main difference in adrenal function between groups was seen after administration of ACTH, which should have resulted in consistent increases in glucocorticoids.

2. Without having a pair-fed or pair-weighted group in

our study, the effect of decreased weight gain on cholesterol and corticosterone concentrations in the deficient rats could not be assessed. Undernutrition and/or anorexia similar to that seen in pantothenic acid deficiency have been reported to increase in serum corticosterone concentration (Pugliese, 1990), increase adrenal cholesterol (Remer et al, 1989), and not change adrenal weight relative to body size (Remer et al, 1989). Our data indicate an opposite response of each of these measures in our deficient group, i.e, we found basal serum corticosterone concentrations in the deficient group to be similar to controls, adrenal cholesterol concentration to be decreased in the deficient group compared to controls, and adrenal weight, relative to body size, to be increased in the deficient group compared to controls. Therefore, our findings are considered to be due to the vitamin deficiency itself rather than due to decreased weight gain.

3. Although pantothenic acid concentration was determined in the serum, liver, and adrenals of the rats in our study, the active form of the vitamin, CoA, was not determined in the adrenal due to the limited amount of tissue available for several assays. Decreased adrenal CoA concentration was important in supporting our hypothesis that low adrenal CoA impaired adrenal cholesterol synthesis and subsequent corticosterone synthesis. The regulation and

distribution of CoA is not fully understood but considering the length of our study and that liver CoA and serum and adrenal PA was decreased in the deficient rats, it is a reasonable assumption that adrenal CoA was decreased as well.

FUTURE RESEARCH

We have reported pantothenic acid deficiency, decreased concentrations of cholesterol in serum and tissues, and insufficient adrenocortical response to ACTH occurring together. Future research should focus on determining causative relationships between these factors:

1. The effect of pantothenic acid deficiency on cholesterol synthesis should be studied using adrenal tissue from pantothenic acid deficient rats. The cholesterol synthesis can be determined by incubating adrenal slices with [C^{14}]acetate followed by determination of the conversion of [C^{14}]acetate into cholesterol. If cholesterol synthesis is decreased in the adrenal slices from deficient rats, pantothenic acid would be added to the adrenal slices prior to the determination of cholesterol synthesis. These data would indicate whether the concentration of adrenal pantothenic acid in the deficient rats was responsible for the impaired cholesterol synthesis.

2. A similar study can be performed which evaluates the reliance of adrenal steroidogenesis on adrenal pantothenic acid and CoA. First, steroidogenesis would be determined in

adrenal slices from pantothenic acid deficient rats. If steroidogenesis is decreased compared to controls, pantothenic acid or CoA would be added to the system and steroidogenesis again determined. An increase in steroidogenic ability in this case would indicate that the low concentration of pantothenic acid and CoA in the deficient rats resulted in the decreased steroidogenesis.

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APPENDIX

APPENDIX A: Composition of Pantothenic Acid Deficient Diet

Pantothenic acid deficient diet, modified composition

Ingredient	Amount
Vitamin Free Casein	18.0%
DL-Methionine	0.3%
Sucrose	62.7%
Alphacel, Non-Nutritive Bulk	5.0%
Cottonseed Oil	10.0%
Calcium Carbonate	6.250 gm/kg
Manganese Sulfate-H20	0.180 gm/kg
Zinc Carbonate	0.050 gm/kg
Cupric Sulfate-5H2O	0.025 gm/kg
Chromium Potassium Sulfate	0.022 gm/kg
Sodium Fluoride	0.005 gm/kg
Potassium Iodide	0.001 gm/kg
Sodium Selenite	0.001 gm/kg
Salt Mixture No.2, U.S.P. XIII	4.0 %
Calcium Biphosphate	13.58%
Calcium Lactate	32.69%
Ferric Citrate (16-17% Fe)	2.96%
Magnesium Sulfate	13.70%
Potassium Phosphate Dibasic	23.99%
Sodium Biphosphate	8.73%
Sodium Chloride	4.35%

AIN Vitamin Mixture 76

Ingredient	per kg of mixture
Thiamine Hydrochloride	600.0 mg
Riboflavin	600.0 mg
Pyridoxine Hydrochloride	700.0 mg
Nicotinic Acid	3.0 gm
Folic Acid	200.0 mg
D-Biotin	20.0 mg
Cyanocobalamin (Vitamin B-12)	1.0 mg
Retinyl Palmitate (Vitamin A) (250,000 IU/gm)	1.6 gm
DL-alpha-Tocopherol Acetate (250 IU/gm)	20.0 gm
Cholecalciferol (Vitamin D3) (4000,000 IU/gm)	250.0 mg
Menaquinone (Vitamin K)	5.0 mg
Sucrose, finely powdered	to make 1000.0 g