# THE ROLE OF NUTRITIONAL FACTORS IN THE FORMATION OF CATECHOLAMINES

by

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THESIS

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## GENERAL INTRODUCTION

The work to be presented in this thesis forms part of a larger program of research carried out in this laboratory on mechanisms of homeostasis. The fact that the catecholamines play a part in these mechanisms is undisputed, yet much remains to be understood regarding their role. The more specific object of the present studies was to obtain information with respect to the part played by nutritional factors in the biosynthesis of adrenaline and noradrenaline and also to investigate the possibility that more than one pathway for their synthesis exists. Dietary deficiencies of riboflavin, pyridoxine and thiamine in young rats were investigated. In addition, phenylalanine-tyrosine deficient diets supplemented with putative precursors were employed. The results of these studies are presented in the pages to follow.

# HISTORICAL

## Pathways of catecholamine biosynthesis

Blaschko (1) in 1939 proposed a mechanism for the biosynthesis of adrenaline and noradrenaline. This scheme involves the following reaction sequence:

Phenylalanine ⟶ Tyrosine ⟶ DOPA ⟶ DOPAmine

Noradrenaline ⟶ Adrenaline

Much work has been done which tends to establish the correctness of this pathway in the animal body. There is no proof, however, that this is the only pathway of catecholamine biosynthesis.

The postulation of this scheme depended upon the assumption that DOPA decarboxylase is involved in the synthesis of the catecholamines. The position of DOPA in the reaction sequence was indicated by the fact that neither tyrosine nor N-methyl-DOPA was decarboxylated by the enzyme. This meant that the decarboxylation could only take place after the introduction of the second hydroxyl group on to tyrosine and before N-methylation occurred. Evidence for the validity of this scheme has been furnished by isotopic studies both in the intact animal and with isolated organs.

Gurin and Deluva (2) in 1947 administered phenylalanine labelled with both tritium and radioactive carbon to rats and were able to obtain radioactive adrenaline

from the adrenals. Udenfriend, Cooper, Clark and Baer (3) administered $C^{14}$-labelled phenylalanine and tyrosine intraperitoneally to rats and rabbits and again obtained radioactive adrenaline. Van Arman (4), using rats whose adrenals had been depleted of adrenaline by insulin, administered various possible precursors and determined the state of recovery of the catecholamines after a period of six hours. Of the substances tested, DOPA brought about a significant recovery. Udenfriend and Wyngaarden (5) found the three substances, phenylalanine, tyrosine, and DOPA, when given to intact rats, served as precursors for adrenal adrenaline and noradrenaline. Tyramine and phenylethylamine were found to be inactive. They also noted that the turnover rates of adrenaline and noradrenaline in the adrenals were very low, both having half lives of about one week. Goodall and Kirshner (6), employing beef adrenal slices, found labelled hydroxytyramine, noradrenaline and adrenaline were formed from tyrosine and from DOPA. Hydroxytyramine also gave rise to noradrenaline and adrenaline. Although no evidence for the participation of dihydroxyphenylserine in the reaction sequence from tyrosine and DOPA was obtained, its participation could not be excluded. However, in a subsequent publication, Kirshner (7), by manipulating the conditions of the experiment, was again able to obtain data which only supported the reaction sequence: DOPA to DOPAmine to noradrenaline, and concluded that dihydroxyphenylserine was not an intermediate.

The Blaschko scheme for the biosynthesis of the catecholamines, as outlined above, appears to be well established, and probably represents the major route of synthesis; however, evidence has appeared from time to time which indicates that this sequence may not be the only means whereby adrenaline and noradrenaline may be synthesized in the body. Logical schemes can be postulated for the inclusion of members of the phenylserine series and for meta-tyrosine with, at least, some experimental support.

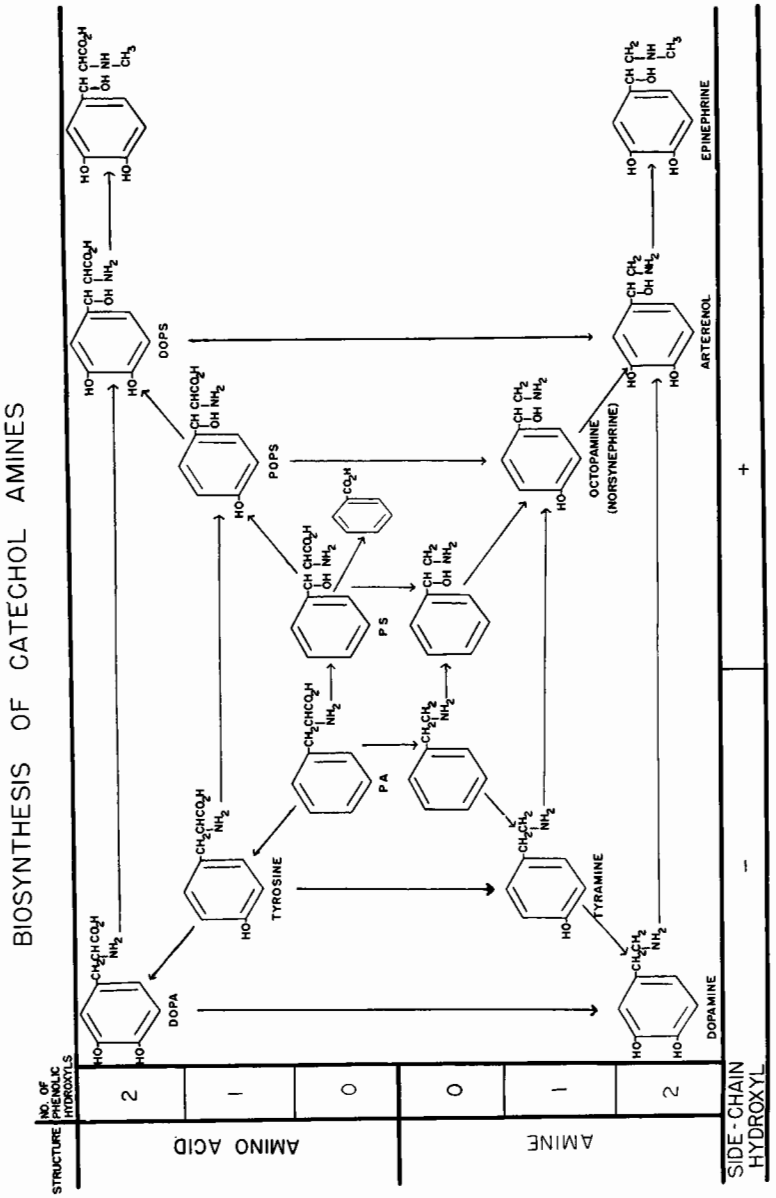
DL-erythro-3, 4-dihydroxyphenylserine has been shown to be decarboxylated by enzymes from both hog kidney and rat liver (8). The product of this reaction is noradrenaline.

Mitoma et al. (9) have studied the uptake and metabolism of the ortho- and meta- forms of tyrosine by brain tissue. Both these forms were found to be taken up by rat and rabbit brain and to be decarboxylated to the corresponding amines.

There is thus some experimental basis for postulating other routes of catecholamine synthesis than that proposed by Blaschko. More will be said about these substances in the sections devoted to the phenylserines and meta-tyrosine.

The various possible routes for adrenaline and noradrenaline synthesis have been assembled in Figure 1.

Fig. 1. Various possible routes for adrenaline and noradrenaline synthesis.




See legend on page 6.

Fig. 1. The starting point for the various postulated routes of adrenaline synthesis, phenylalanine (PA), is to be found in the center of the figure. All the compounds in the top half are amino acids and in the lower half are amines. On the left side, the substances have no side chain hydroxyl group whereas on the right side, they are present. In the center, no phenolic hydroxyl groups occur but they increase in number as one proceeds toward the periphery. The writer is indebted to Dr. T.L. Sourkes for permission to use this figure.

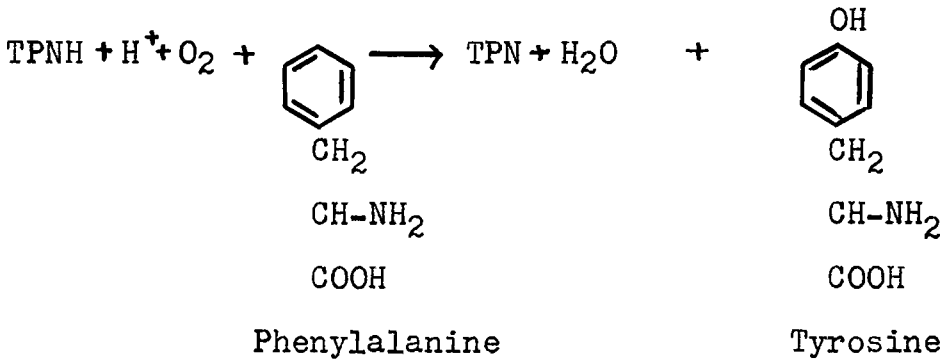
## Phenylalanine Oxidation

The first reaction in the currently accepted pathway for the formation of noradrenaline and adrenaline is the ring oxidation of phenylalanine to form tyrosine. In 1913 Embden and Baldes (10) and again in 1922, Dakin (11), suggested that phenylalanine was converted to tyrosine in the body and that this was probably an important step in phenylalanine metabolism. During the course of investigations concerning the dietary requirements for amino acids carried out in Rose's laboratory, Womack and Rose (12) demonstrated that phenylalanine could replace tyrosine in the diets of young rats. This suggested that phenylalanine is converted to tyrosine *in vivo*. However, it appears that the reverse reaction, from tyrosine to phenylalanine, does not take place *in vivo* since young rats will grow on a tyrosine-containing diet only if phenylalanine is also included (12,13).

It was also found that the D(+) enantiomorph of phenylalanine is about as effective for growth promotion as the naturally occurring L(-) form (14). Moss and Schoenheimer (15) found, by administering deuterium-labelled phenylalanine to both growing and adult rats, that tyrosine was in fact formed from phenylalanine *in vivo*. Bernheim and Bernheim (16), working with liver slices, observed the formation of an hydroxyphenol after incubation with L-phenylalanine. The reaction product was not chemically identified, but was presumed to be tyrosine.

Using both surviving liver slices and liver extracts, Udenfriend and Cooper (17) found L-phenylalanine to be converted to tyrosine and determined by isotopic means that the tyrosine was actually formed from the added phenylalanine. A soluble enzyme was prepared from rat liver which required a pyridine nucleotide and oxygen in order for the reaction to take place. The crude preparation could use both diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN). However, DPN appeared to be the more effective of the two after the crude preparation had been dialysed. Mitoma (18), in studying the coenzyme requirement, came to the conclusion that the actual requirement of the enzyme was for the reduced form of DPN, that is, DPNH. Oxidized DPN was catalytically effective only when sufficient additional substances such as aldehydes or alcohols were present to ensure its reduction. When Kauf-

mann (19), on the other hand, studied this system, he found that TPNH was the more active coenzyme and postulated the following reaction:




Phenylalanine Tyrosine

Mitoma and Leeper (20) fractionated rat liver homogenate into two portions, each of which contained principles active toward the oxidation of phenylalanine, but each fraction by itself was inactive. Activity was only demonstrable when the two fractions were incubated together with L-phenylalanine, DPN (or DPNH) and certain aldehydes or alcohols. Fraction I precipitated between 33% and 45% saturation with ammonium sulfate and Fraction II between 45% and 55%. It should be noted that although the whole animal can use D-phenylalanine equally as well as the L-form for growth, the liver enzyme is specific for the L-form. Fraction I was extremely labile and was confined to the liver of the tissues investigated. Fraction II was more stable and was found in kidney and heart as well (18).

Udenfriend and Cooper (17) studied the effects of certain substances upon the reaction rate of the enzyme

found in the unfractionated preparations and observed marked inhibition with azide and cyanide, indicating interference with a heavy metal system. Arsenite produced moderate inhibition, but arsenate, fluoride and phenylserine did not bring about inhibition. That the metal involved is probably iron is indicated by the work of Mitoma (18) who found that ferrous ions appeared to be an integral part of the enzyme system, and that of Kaufmann (19) who observed that ferrous ions sometimes stimulate enzymatic activity. However, the enzyme was inhibited by cupric ions.

A lag period in the oxidation of phenylalanine to tyrosine has led Kaufmann to postulate that the function of TPNH is to reduce another cofactor which is the actual hydroxylating agent (21, 22).

The presence of this cofactor has been demonstrated in the boiled extracts of livers from rat, sheep, rabbit and beef and from beef adrenal glands (23). The nature of the cofactor has not yet been determined although the following have been found not to be active: ascorbic acid, glutathione, thiamine, vitamin $B_{12}$, cysteine, adrenaline, pyridoxal phosphate, $FeCl_2$, DOPA, riboflavin monophosphate, thiamine monophosphate, folic acid, acetylcholine, choline, lipoic acid, coenzyme A, N-methylnicotinamide, DPN, thyroxine, triiodothyronine, DPNH, biotin, methylthioadenosine, adenosine-2-phosphate, -3-phosphate, and -5-phosphate, adenosine, inosine, uridine, and cytidine.

Kenny, et al.(24) colorimetrically measured the amount of tyrosine formed from phenylalanine under standard conditions and found that foetal livers of rats, pigs and rabbits lacked Mitoma's Fraction I. Rat foetal livers were found to be unable to convert phenylalanine to tyrosine, those from rats less than twenty-four hours old virtually could not, however, livers from animals four to twelve days old were capable of carrying out this reaction. Thus the enzyme appears not to be active until sometime after birth.

Evidence has recently accumulated to show that in the condition known as oligophrenia phenylpyruvica the enzyme catalysing phenylalanine oxidation to tyrosine is at least markedly diminished in activity if not totally absent. The deficiency in the human disease appears to be associated with the active principle found in Mitoma's Fraction I. ( 26-28 ).

Fellman and Devlin (25) have found free phenylalanine in the adrenal glands of beef, rabbit, guinea pig, rat, monkey and human.

Tyrosine Oxidation

Very little is known about the oxidation of tyrosine to DOPA in the synthesis of the catecholamines. Most of the investigations concerning this oxidation have been carried out in connection with the metabolic pathway for the formation of melanin. It seems unlikely that the same enzyme is involved in the two processes. The tyrosinase of

pigmented tissues carries out, in effect, a double reaction. The first of these is the oxidation of tyrosine to DOPA, but the second follows immediately, the oxidation of DOPA to DOPA quinone. Indeed, tyrosinase has to be primed by the second reaction before it is capable of carrying out the former (29, 30). On the other hand, DOPA takes a different course during the synthesis of the catecholamines in which it is decarboxylated to DOPAmine. Thus the mechanics of the reaction whereby tyrosine is hydroxylated to form DOPA in, for example, the adrenals, appears to preclude the participation of the tyrosinase of pigmented tissues.

DOPA has been observed in brain as well as possibly its amine (31) by Montagu.

Dihydroxyphenylalanine Decarboxylation

The formation of DOPAmine (hydroxytyramine, oxytyramine) by the decarboxylation of dihydroxyphenylalanine (DOPA) has been the subject of a number of studies. Holtz, Heise and Lüdtke (32) gave the name DOPA decarboxylase to the enzyme they found in mammalian tissues which was capable of carrying out this reaction. Blaschko (1) investigated the properties of L-DOPA decarboxylase and observed that while the enzyme was active toward L-DOPA, it had no activity toward D-DOPA, DL-N-methyl DOPA, L-tyrosine, DL-N-methyl tyrosine or L-phenylalanine. However, both L-2, 5-DOPA

and L-meta-tyrosine were decarboxylated by DOPA decarboxylase in guinea pig kidney and rat liver extracts (33). 2,3-DOPA is also decarboxylated by guinea pig kidney extract (34).

Sourkes et al. (35) showed that pig and guinea pig kidney enzyme decarboxylated 2,5-, 3,5- and 2,4-DOPA, but at slower rates than the naturally occurring 3,4-DOPA. 2,6-DOPA was also shown to be decarboxylated by the guinea pig kidney enzyme (36), however, again at a slower rate. On the other hand, the decarboxylation rate of 2,3-DOPA was faster than that of the natural isomer.

DOPA decarboxylase is dependent upon pyridoxine for activity (37). Blaschko et al. (38) found that when rats are deprived of pyridoxine, the ability of their livers to decarboxylate DOPA is diminished.

DOPA decarboxylase has been shown to be present in the ox adrenal medulla by Langemann (39,40).

Holtz, Credner and Koepp (41) reported finding DOPAmine in the urine of guinea pigs, rabbits, cats and man after either the oral or parenteral administration of DOPA. The DOPAmine existed partly in the free state and partly in a bound inactive form. It has also been found in normal human urine by Holtz et al. (42) and by von Euler, Hamberg and Hellner (43). The latter authors state that DOPAmine occurs in the urine largely in the free state and estimated the daily output at between 0.1 and 0.2 mg. DOPAmine has also been found in the adrenal medullae of sheep, ox and

cow by Shepherd and West (44), but these authors were unable to find it in the medullae of adult pig, dog, cat or man.

Carlsson et al. (45) have found DOPAmine in rabbit brain to an extent of about 0.4 µg/g. This is approximately equal to the noradrenaline concentration. The injection of DOPA increased the concentration to about 2.0 µg/g. in less than an hour.

Weil-Malherbe and Bone (46) have observed the presence of DOPAmine in normal human urine.

The Dopamine to Noradrenaline Reaction

Little or nothing is known regarding the actual mechanism of the insertion of the side chain hydroxyl on to dopamine to form noradrenaline. However, the reaction has been shown to take place by a number of studies. Holtz and Kroneberg (47) noted that after incubation of dopamine with beef, pig, or guinea pig adrenals there was an increased hypertensive activity in the extract. Hagen (48) has found radioactive noradrenaline after incubation of chick adrenals with labelled dopamine in the presence of ATP, DPN, TPN, FAD, nicotinamide, methionine and alpha-ketoglutarate. Neri et al. (49) found dopamine to give rise to a noradrenaline-like material after incubation with beef adrenal acetone powder. Noradrenaline was not identified, but its presence was indicated by rat colon assay. ATP with either DPN or TPN was required for the reaction to

take place. Ellman (50) has also observed the conversion of DOPAmine to noradrenaline by beef adrenals. Rosenfeld et al. (51) perfused intact calf adrenals with labelled substrates. Tyrosine and DOPAmine were demonstrated to give rise to noradrenaline; DOPAmine and noradrenaline both gave rise to adrenaline provided a methyl donor was added to the perfusion medium. Methionine was the most effective of the donors employed.

It is difficult to study the biosynthesis of the catecholamines in man due to the small amounts to be found in blood and the difficulty in getting fresh tissue. However Sjoerdsma, Leeper and Udenfriend (52) have obtained evidence with pheochromocytoma slices that both DOPA and DOPAmine are precursors for noradrenaline. The turnover in this tissue was found to be much higher than that in animals.

Noradrenaline Methylation

In 1949 Bülbring (53) found that suspensions of ground dogs' and cats' adrenals were capable of converting noradrenaline to adrenaline. Adenosine triphosphate (ATP) was required for this methylation to take place. Keller et al. (54) found labelled adrenaline in the adrenals of rats which had received in the diet methionine bearing $C^{14}$-labelled methyl groups. These results were consistent with those of Borsook and Dubnoff (55) who had shown that ATP and methionine were required for the methylation of guanidoacetic acid. Cantoni (56) later reported evidence

which suggested that methionine must be activated by ATP before transfer of the methyl group to an acceptor can occur. The active intermediate in the methylation process was identified as S-adenosylmethionine (57). Masuoka (58) and his coworkers found $C^{14}$-labelled adrenaline in the adrenal glands of rats which had been administered $\alpha$-$C^{14}$-noradrenaline intraperitoneally. In 1957, Kirschner and Goodall (59) were able to obtain a system from the supernatant of bovine adrenal medullae after centrifugation at 20,000 times gravity which could methylate noradrenaline to form adrenaline. Either methionine and ATP on the one hand, or S-adenosylmethionine on the other, could be used for methylation. In either case the methyl group of the methionine was tagged and was found on the adrenaline after incubation. S-adenosylmethionine was, however, some twenty to thirty times more active than the combination ATP and methionine. Reduced glutathione and magnesium ions were required. The enzyme could be precipitated with ammonium sulfate, and the greatest activity was found in the fraction precipitating between 35 and 50% saturation.

The ATP concentration of the adrenals has been shown to be high (60).

Pyridoxine Deficiency

The clinical signs of Vitamin $B_6$ deficiency in the rat have been described by Olsen and Martindale (61) as follows: fulminating diarrhea, scaly nose, paws and tail,

and, finally, edematous paws and convulsions. The blood pressure is also higher in deficient rats than in normal controls. There is an increase in the size of the liver and the $B_6$ content is less than in normal controls. Broken cell preparations from $B_6$ deficient livers consume oxygen at a reduced rate (62).

Studies carried out by Beaton et al. (63) indicate that the time of onset of the deficiency varies with the component studied. For example, differences in carcass total and crude fatty acids between deficient and control young rats become evident within a week while significant differences in nitrogen metabolism do not become apparent for at least four weeks. Liver transaminase activity of the deprived rats does not decrease with time, nor does it increase as in the control animals. In this connection, Hope (64) has shown that taurine remains at a normal level in brain during advanced $B_6$ deficiency at a time when liver taurine has disappeared. This is believed due to the persistence in brain of the enzyme which forms taurine from cysteine sulfinic acid while that of liver has disappeared.

Certain neurological features also appear in pyridoxine deficiency. Davenport and Davenport(65) have shown that in mild deficiency there is a lowered electroshock threshold as compared with controls. Pyridoxine raises the threshold in the deficient animals, but not in the

controls. The effect is noted within five hours after pyridoxine injection in mild deficiency and is somewhat slower in more severe deficiency states. Since glutamic acid tends to ameliorate the $B_6$ deficiency effects, the authors suggest that the maintenance of a normal transaminase activity is necessary for a normal electroshock threshold.

In addition to the lowering of the electroshock threshold, a convulsive syndrome has been associated with vitamin $B_6$ deficiency. In 1938 Chick et al. noted that young pigs on a synthetic diet lacking in $B_6$ developed epileptiform fits (66). The same phenomena have also been seen in dogs (67), chicks (68), rats (69), and humans (70). The fits can be prevented or cured by the administration of pyridoxine (71,72).

Pyridoxine forms coenzymes which are active in transamination reactions and in decarboxylations (73). DOPA decarboxylase has itself been shown by Green et al. (37) to be dependent upon vitamin $B_6$ for activity and Blaschko (38) has observed a diminished DOPA decarboxylase activity in the livers of pyridoxine deficient rats.

Vitamin $B_1$ Deficiency

The effect of thiamine deficiency on the catecholamine content of rat heart, adrenals and liver has been studied by Goodall (74). The control rats were fed an unspecified diet designated as "normal" by the author while the experimental animals were placed on a synthetic

diet deficient in thiamine. The control rats were killed at about eight weeks of age, those on the deficient diet when severe symptoms of beri beri were manifest. The experimental animals were placed on the synthetic diet at about the age of three weeks and required from three to five weeks for the deficiency symptoms to appear. Therefore, they too were killed at about eight weeks of age.

It was found that both the adrenaline and noradrenaline in the heart were higher in the deficient rats, the adrenaline by about 100% and the noradrenaline by about 50%. On the other hand in the adrenals, both catecholamines were lower in the thiamine deficient animals. The adrenaline fell from 1550 µg/g to 740 µg/g whereas the noradrenaline fell only from 200 to 180 µg/g which was not significant. In the liver the noradrenaline concentration doubled while that of the adrenaline fell from 0.002 µg/g to an amount undetectable by biological assay.

It has been known for a long time that there are marked changes in the heart in beri beri. In 1930, Drury, Harris and Maudsley (75) observed that the adrenals were enlarged in thiamine deficiency and in 1939 Sarfy (76) found that the adrenaline content was diminished. Raab and Supplee (77) had previously shown that the adrenaline content of the thiamine deficient rat heart was elevated.

Insulin and Reserpine

Insulin has been found to bring about the release of adrenaline from the adrenal glands (73). Reserpine causes

the release of both adrenaline and noradrenaline from the adrenals and also causes a discharge of noradrenaline from the hypothalamus (79). Shore et al. (80) estimating noradrenaline fluorimetrically, confirmed the release of noradrenaline from the brain stem of the cat by reserpine, and observed that normal values returned only after a number of days. Carlsson and Hillarp (81) found that the catecholamines have disappeared from the adrenal medullae of rabbits eighteen hours after the injection of 5 mg. reserpine per Kg. body weight. Muscholl and Vogt (82) have shown reserpine to cause losses of up to 80% of the noradrenaline content of the right superior cervical ganglion. When the loss was 75% or greater, stimulation of the pre- or post-ganglionic fibres caused no or very weak effects on the eyelid or pupil of rabbits, probably by causing loss of the transmitter. Practically complete depletion of the catecholamines from the rabbit heart has been brought about by reserpine (83,84). After administration of 5 mg./Kg., only 10% or less of the control amounts of noradrenaline are found in rat heart (85). In rabbits, 5 mg./Kg. causes almost complete disappearance of catecholamines from the brain in one hour, the heart in three hours, and the adrenals in sixteen hours (86).

Serotonin (88) and 3-hydroxytyramine (45) release from brain can also be brought about by reserpine.

Phenylserine and the Hydroxyphenylserines

Phenylserine has been implicated in the formation

of adrenaline and noradrenaline since 1919 when Rosenmund and Dornsaft (89) proposed it as a possible precursor of noradrenaline. Phenylserine has been shown to participate in several reactions in the body. As early as 1909, Dakin (90) found that when this compound is administered to cats it is excreted in the form of hippuric acid. It has also been shown to act as an inhibitor of phenylalanine (91), the degree of antagonism varying with the phenylserine isomer (92). One would expect that the metabolic reactions would also depend upon the isomeric form. This has been found to be the case for the cleavage of beta-phenylserine to benzaldehyde and glycine (93), the L-erythro isomer being most rapidly split.

Armstrong and Lewis (94) found that when phenylserine is administered to rats in diets deficient in phenylalanine and tyrosine, it does not support growth, and may indeed be somewhat toxic.

Van Arman (4) carried out experiments in which the adrenaline stores of rats were partially depleted by insulin and investigated the efficacy of presumed precursors to restore the catecholamine content of the adrenals. Phenylserine was found not to be effective.

Several studies have been carried out with the dihydroxy derivative of phenylserine. Blaschko, Burn and Langemann (95) incubated dihydroxyphenylserine anaerobically with minced guinea pig kidney and found that carbon dioxide

was given off and at the same time noradrenaline was formed. Only the laevoisomer was produced. N-Methyl-dihydroxyphenylserine was not decarboxylated. In 1955, Hartman et al. ( 8 ) carried out studies concerning the decarboxylation of the stereoisomers of dihydroxyphenyl-serine, m-hydroxyphenylserine, and p-hydroxyphenylserine. Using hog kidney enzyme, erythro-dihydroxyphenylserine is decarboxylated at an appreciable rate, though it is slower than is the decarboxylation of DOPA. The isomeric threo-dihydroxyphenylserine is also decarboxylated by this enzyme, but at a very slow rate. The erythro form of m-hydroxyphenylserine is also slowly decarboxylated. However, both forms of dihydroxyphenylserine were found to be decarboxylated at the same rate by whole liver homogenate. In the same paper, noradrenaline was reported in the urine of rats which had been injected with both threo- and erythro-dihydroxyphenylserine. The natural biologically active optical isomer of noradrenaline was found after administration of the threo form while that arising from erythro-dihydroxyphenylserine was the relatively inactive optical antipode.

Metatyrosine

Blaschko (96) has found m-tyrosine to be decarboxylated by both guinea pig kidney and rat liver DOPA decarboxylase and bacterial tyrosine decarboxylase. The mammalian enzyme carries out the reaction with m-tyrosine

at a rate only slightly less than that of DOPA. The rate using the bacterial enzyme was about one-third that with the mammalian enzyme. The mammalian enzyme can also decarboxylate o-tyrosine, but not the naturally occurring p-tyrosine. On the other hand, the bacterial enzyme was unable to decarboxylate o-tyrosine. Since only one half the added DL-amino acid could be accounted for in terms of the carbon dioxide produced, it was concluded that only the L-form was decarboxylated.

In 1957, Mitoma et al. ( 9 ) have studied the ability of brain tissue to decarboxylate the three isomeric forms of tyrosine. Rat brain homogenates were observed to decarboxylate m-tyrosine. When m-tyrosine was injected intraperitoneally into rats or rabbits, the corresponding amine was also found in the brain.

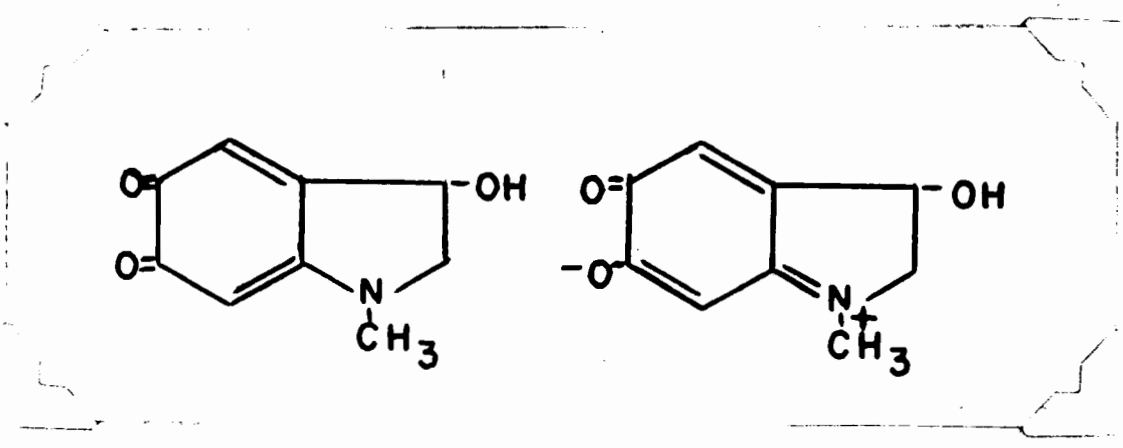
Some of the metabolites of m-tyrosine have been found both in normal human urine and in that of phenylketonurics. The presence of m-hydroxyphenylacetic acid in normal urine was detected by Boscott and Bickle (97) but it was not found in phenylketonurics. Armstrong, Shaw and Wall (98) found m-hydroxyphenylhydracrylic acid in normal urine and reported amounts up to 150 mg. per day were excreted by phenylketonurics. The same authors also reported that m-hydroxyhippuric acid occurs normally.

# METHODS

## Introduction

In the investigations to be reported in this thesis, adrenaline and noradrenaline were measured by adaptations of the method of Sourkes and Drujan (99). This method depends upon the oxidation of adrenaline and noradrenaline to their corresponding chromes followed by conversion of the chromes by alkaline rearrangement to their lutins. Adrenochrome may be formally described as N-methyl-2,3-dihydro-3-hydroxyindole-5, 6-quinone, the structure assigned to it by Green and Richter (100) (who also proposed the name adrenochrome), although it may not actually possess the true o-quinone structure. Harley-Mason (101) has produced evidence that it can also be formulated as a zwitterion p-quinoneimine. The two structures are given below in Fig. 2.

Figure 2

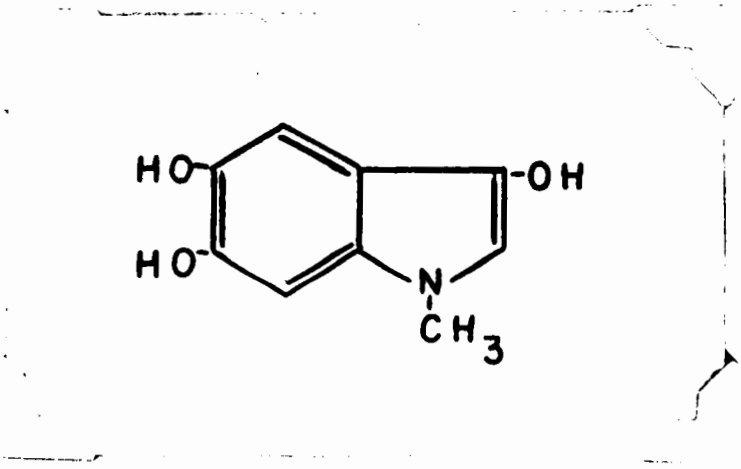



The structural formulas proposed for adrenochrome; that of Green and Richter is to the left, Harley-Mason's is to the right.

Adrenochrome is a red colored compound which has usually been described as unstable, giving rise to melanin-like products both in solution and, somewhat more slowly, in the dry state. Heacock, Nerenberg and Payza (102) have recently published a method by which a more stable product has been obtained.

Adrenolutin is N-methyl-3,5,6-trihydroxyindole (103,104); the structural formula is given below in Fig. 3. It is a yellow compound with a green fluorescence.

Figure 3




The structural formula of adrenolutin.

Sobotka (105) has proposed the term "aminochromes" for those compounds which are related to adrenochrome. Noradrenaline also undergoes chrome and lutin formation under the same conditions as adrenaline. Iodo derivatives are formed when iodine serves as the oxidant (106).

A comprehensive review of the chemical methods that have been used for the estimation of the catecholamines has been given by Sourkes(99). In summary, the steps of

such procedures are as follows. The catecholamines are first adsorbed on either alumina gel or suitably prepared aluminum oxide. After removal of the supernatant following adsorption the alumina is washed, and elution is accomplished with dilute acetic acid. Estimation of the catecholamine content can be made by oxidation of these substances to the chromes; if the concentration is sufficiently high, absorbance can be measured. Usually, however, rearrangement of the chromes by alkali to their lutins is carried out, a procedure which allows for greater sensitivity of measurement. Although the lutins are fairly unstable, the stability can be increased sufficiently by addition of ascorbic acid to the solution to allow fluorescent estimation. This is the basis of the Euler-Floding method (107), which is based in turn upon Lund's work (108). An alternative procedure has been devised by Weil-Malherbe and Bone (109) who condense the catechol chromes with ethylene diamine under alkaline conditions. This again yields products which can be measured fluorometrically.

Animals Used and Methods of Sacrifice.

The animals used in these experiments were young, male, albino rats of the Sprague-Dawley strain weighing between 80 and 100 g. when received from the breeder. The animals were placed on various dietary

regimens to be described in the appropriate experimental sections until time for sacrifice. They were killed by decapitation and the carcass was drained of blood as completely as possible. Immediately after each animal was sacrificed, the organs desired for study were removed, weighed, wrapped in parafilm and placed in the deep-freeze at about -20°C until trichloroacetic acid (TCA) extracts were prepared.

Preparation of TCA Extracts.

Adrenals. Both of the adrenals removed from one animal were homogenized in 2.0 or 3.0 ml. of 5% TCA. The "Virtis 45" homogenizer fitted with the micro assembly was found convenient for this purpose, and homogenization was carried out for one minute at the highest speed, or until the tissue was completely ground as determined visually. The blades and flask were washed with 2.0 ml. of 5% TCA and the washings were added to the homogenate. The flask was washed twice more with like portions of TCA and, again the washings were combined with the ground suspension. The whole was then made to a total volume of 50.0 ml. with 10% TCA.

Brain. The brains were homogenized with the "Virtis 45" fitted with the macro assembly. Homogenization was carried out in 10 ml. of 5% TCA and the blades and flask were washed with 5 ml. of 10% TCA which was added to the homogenate. The total volume of added fluid was 15 ml.

Liver, Spleen and Heart. These were homogenized with 10 ml. of 5% TCA, and washings were effected with two 5 ml. portions of 10% TCA which were combined with the homogenate. The total volume of added fluid here was 20 ml.

All tissue extracts except those from the adrenals were centrifuged to remove the proteins before preparation of the eluates. After removal of the supernatant, the precipitate was washed with 3.0 ml. of 10% TCA, recentrifuged, and the washings added to the original supernatant. In the case of brain, the entire supernatant was used for adsorption of the catecholamines. The volumes of the liver, spleen and heart supernatants were accurately measured and then divided into two equal portions for adsorption. Because the amount of protein found in the TCA extracts of the adrenals was small, 10 ml. of the uncentrifuged extract was used for adsorption.

The volumes chosen for the adsorption procedure were the resultant of two considerations. In the first place, the volumes had to be kept low. Ten ml. of supernatant was found to be about optimum and 15 ml. maximum to allow for thorough mixing of the alumina. On the other hand, sufficient quantity of the TCA extract had to be employed to ensure sufficient of the catecholamines for analysis. For these reasons the volume of the brain

extract was kept to 15 ml. in order to be able to use the entire extract for adsorption. This was made necessary due to the small quantities of catecholamines found in brain. The extracts of the other organs could safely be divided, permitting duplicate runs.

Adsorption and Preparation of Eluates.

A measured portion of the TCA extract was put into 25 x 150 mm. pyrex test tubes. To this was added 2.5 ml. of 10% versene and 0.5 g. "non-alkaline" aluminum oxide (Woelm) suitable for chromatography. One drop of 1% alcoholic solution of phenolphthalein was then placed in each tube and the addition of alkali commenced. Speed is essential at this point since the catecholamines are extremely labile to air oxidation in an alkaline medium. The samples were made alkaline to the first permanent, light pink color of the indicator by adding first 20% NaOH, and then as the end-point was approached, lesser concentrations of the alkali. Excess alkalinity must be avoided. The test tubes were fitted with rubber stoppers immediately after each had been made alkaline. They were then placed in a mechanical shaker and thoroughly shaken for 15 minutes. After about three minutes, the shaker was stopped for a few seconds to ensure that all samples had remained alkaline. If the phenolphthalein color had disappeared from any of the samples, 5% NaOH was added to restore the original faint pink, and shaking was continued. At the end of 15 minutes,

when shaking was completed, the samples were unstoppered five at a time and the supernatant removed by suction through a fine bore pipette. The entire time taken for this step, including the shaking time should not exceed 20 minutes. The alumina was then washed with 2.5 ml. of 0.2 M sodium acetate, the shaking period at this step was 10 minutes. After removal of the sodium acetate in the same fashion as above, 2.5 ml. of glass distilled water were added and again the samples were shaken for 10 minutes. The wash water was then removed and 3.5 ml. of 0.5 N acetic acid added to the alumina for elution of the catecholamines. After shaking for 15 minutes, the alumina was allowed to settle and the acid eluate was decanted into suitable small centrifuge tubes for storage if the procedure was to be interrupted at this point. These tubes were centrifuged to settle the alumina before aliquots were removed for analysis.

At the same time as the experimental samples were put through this procedure, tubes were also prepared for adrenaline and noradrenaline recovery. Seven of these tubes were employed for each run. The first was a blank containing only TCA of the same concentration and volume as was found in the experimental samples. The remaining six tubes contained varying amounts of adrenaline and noradrenaline, also in a TCA medium. Convenient amounts of

these substances when adrenals were being run were found to be 1.0, 2.0 and 3.0 µg. noradrenaline, each in a total TCA volume equal to that of the experimental samples and 0.5, 1.0 and 1.5 µg. of adrenaline. When other tissues were being analyzed these amounts were halved in order that the readings for the recovery samples might be in the approximate range of the experimental samples.

Fluorometric Analysis

Fluorometric analysis was carried out at two pH's in order to determine both the adrenaline and noradrenaline content of the samples. At pH 6.0 both of these catecholamines are oxidized to their corresponding chromes, while at pH 3.0 only the adrenaline is found to be oxidized. The detailed procedure was as follows. In doing analyses on the eluates prepared from adrenals,0.2 and 0.4 ml. of the eluate were added to 15 x 150 mm. test tubes and for those of other tissues, 0.4 and 0.8 ml. were added to similar tubes. The volume in each tube was then made to 1.0 ml. with glass distilled water. After this 2.0 ml. buffer were added, either at pH 6.0 or 3.0, depending on the analysis. After mixing, the contents of the tubes were oxidized with 0.5 ml. of 0.009 N iodine solution for exactly three minutes, the reaction being stopped at the end of this time by the addition of 0.5 ml. 0.01 N sodium thiosulfate. Lutin formation was brought about by adding 1.0 ml. of 18% NaOH containing 2 mg. per ml.

ascorbic acid. The samples were then allowed to stand for 10 minutes before readings were commenced. This procedure was followed since it had been found that the fluorescence immediately produced after the addition of the alkali fell during 10 minutes to stable values which were proportional to the concentration of the catecholamine. The iodine and thiosulfate solutions were prepared by dilution from normal stock solutions twice a week; the alkaline ascorbic acid solution was prepared immediately before use by dissolving the ascorbic acid in sufficient glass distilled water to make one tenth the final volume of the solution, and then diluting to final volume with 20% NaOH. Standard curves were carried through the fluorometric procedure at the same time as the eluates were being run. Convenient amounts of noradrenaline to employ for this purpose were 0.10, 0.20 and 0.30 µg.; however, due to the greater fluorescence of adrenaline, the amounts of this substance were reduced to 0.05, 0.10 and 0.15 µg. A reagent blank, from which the catecholamine was omitted, was also carried through the procedure.

During the first experiments readings were carried out with the Farrand Model A fluorimeter using Corning filters 5113 and 3389 to select the 436 mµ line of the mercury vapor lamp for the exciting wave-length, and a Corning 3486 filter in the secondary position. The experiments in the later portion of the work were read on

the Aminco-Bowman Spectrophotofluorometer using the wavelength settings which gave maximum excitation and fluorescence. These were 420 mμ for the exciting light and 510 mμ for the emission on our instrument, using the widest slit widths. A yellow Corning filter (No. 3385) was inserted between the secondary grating and the phototube to reduce the amount of scattered light from the curette.

Calculations

All galvanometer readings were corrected for the value of the reagent blank. An average calibration factor (F) was calculated from the standard curve and defined as the reading of one μg. of the catecholamine. Because both adrenaline and noradrenaline contribute to the fluorescence when the procedure is run through at pH 6.0 and because adrenaline fluoresces at different intensities when oxidized at the two pH's, it was necessary to obtain three calibration factors: one for noradrenaline at pH 6.0, one for adrenaline at pH 6.0 and one for adrenaline at pH 3.0. These have been given the symbols $F_6n$, $F_6a$ and $F_3a$, respectively.

Losses during extraction occur, necessitating the application of a suitable correction. This is obtained by determining the percentage recovery of noradrenaline and adrenaline from the recovery samples described above. Pn and Pa are defined as the per cent recovery of noradrenaline and adrenaline, respectively.

In carrying out the calculations of the catecholamine content of the samples, the readings obtained for the various aliquots were first increased appropriately to values corresponding to the whole eluate and designated $M_3$ or $M_6$, depending upon the pH at which the oxidation was carried out.

The calculations for the noradrenaline content of the "unknown" samples were carried out by use of a formula derived as follows.

Let: $M_6$ = Observed readings for sample at pH 6.0 increased appropriately to value corresponding to the whole eluate.

$F_6n$ = Calibration factor for noradrenaline at pH 6.0

$N$ = µg. noradrenaline

$Pn$ = Per cent recovery for noradrenaline

$F_6a$ = Calibration factor for adrenaline at pH 6.0

$A$ = µg. adrenaline in eluate

$Pa$ = Per cent recovery for adrenaline

Then: $$M_6 = F_6n \cdot N \cdot \frac{Pn}{100} + F_6a \cdot A \cdot \frac{Pa}{100} \qquad (1)$$

$$F_6n \cdot N \cdot \frac{Pn}{100} = M_6 - \frac{F_6a \cdot A \cdot Pa}{100} \qquad (2)$$

$$N = \frac{100\ M_6}{F_6n\ Pn} - \frac{F_6a \cdot A \cdot Pa}{100} \cdot \frac{100}{F_6n\ Pn} \qquad (3)$$

$$N = \frac{100}{Pn} \cdot \frac{M_6}{F_6n} - \frac{F_6a}{F_6n} \cdot A \cdot \frac{Pa}{Pn} \qquad (4)$$

This equation can be simplified to:

$$N = \frac{1}{Pn \cdot F_6 n} \quad (100\ M_6 - F_6 a \cdot Pa \cdot A)\ ; \quad (5)$$

however, since $M_6$ and A are the variable terms, all others being constant, a more convenient simplification was as follows:

$$N = k_1 M_6 - k_2 A \qquad (6)$$

where $k_1 = \frac{100}{Pn F_6 n}$ and $k_2 = \frac{F_6 a}{F_6 n} \cdot \frac{Pa}{Pn}$

The formula for calculating adrenaline was as follows:

$$A = \frac{M_3}{F_3 a} \cdot \frac{100}{Pa}$$

Where: A = µg. adrenaline

$M_3$ = observed reading for sample at pH 3.0 increased appropriately to value corresponding to the whole eluate

$F_3 a$ = Calibration factor for adrenaline at pH 3.0

Pa = Per cent recovery for adrenaline.

To convert the values obtained by the use of these formulas to the quantity of catecholamine found in the whole organ, the result was multiplied by 5, 2 or 1 depending upon the proportion of the TCA extract taken for analysis.

In carrying out these analyses, duplicate eluates of each TCA extract were made where possible. These were

usually made in different runs for two reasons. First, the time during which the samples are alkaline is critical and should be as short as possible. Therefore, in order to reduce the number of samples run at any one time, only one aliquot from each of the TCA extracts was taken for any given run. Second, the recoveries may vary somewhat from run to run, and it was felt that by making two runs with the same TCA extract, the actual error of the final result would be minimized.

The method for determining the catecholamines in urine was essentially the same as that for tissues. Twenty-four hour specimens were collected in acid (5 cc of 6 N HCl per 2.5 l. collection bottle) with a few drops of toluene. Ten ml. of the twenty-four hour samples were measured into pyrex test tubes, 25 X 150 mm., and carried through the procedures for adsorption and elution just as with the tissue samples. For fluorimetry, 0.4 ml. and 0.8 ml. portions of the eluate were used.

Table I below gives a measure of the standard error of the differences between duplicates in the determinations of adrenaline and noradrenaline carried out on the various organs of the rat and on human urine.

Table I

Standard Error of Differences
Between Duplicates

Organ or Body Fluid

| | No. of Obs. NA | No. of Obs. A | Standard Error NA | Standard Error A |
|---|---|---|---|---|
| Liver | 24 | 24 | 0.16 | 0.03 μg. |
| Heart | 10 | 10 | 0.03 | 0.02 μg. |
| Spleen | 10 | 10 | 0.07 | 0.04 μg. |
| Adrenals | 30 | 30 | 1.44 | 1.73 μg. |
| Urine | 20 | 20 | 0.64 | 0.50 μg./100 ml. |

Diets Used in Experiments

Various types of diets were employed in the experiments to be reported later. Some of these were prepared diets obtained commercially while others were prepared in the laboratory. In one instance Purina fox checkers served as the diet.

When compounded in the laboratory, the basal diets were prepared omitting the vitamin mixture until just prior to use. This was done because choline was not included in the vitamin mixture due to its hygroscopic properties until the mixture was required. After preparation, the diets were stored in the refrigerator and the amounts required for feeding were removed as needed. The composition of the various diets is given in the following tables.

| | Diet No. 1 | | % |
|---|---|---|---|
| Basal Diet: | Alcohol extracted casein | 200 g. | 20 |
| | Crisco | 130 | 13 |
| | Powdered sucrose | 520 | 52 |
| | GBI salt mixture | 40 | 4 |
| | Cod liver oil | 50 | 5 |
| | Granulated agar or cellulose | 20 | 2 |
| | Vitamin mixture | 40 | 4 |
| | Total | 1,000 g. | |

| | | |
|---|---|---|
| Vitamin Mixture: | Thiamine | 150 mg. |
| | Riboflavin | 150 |
| | Biotin | 6 |
| | Calcium pantothenate | 400 |
| | Nicotinamide | 1,000 |
| | Pyridoxine | 150 |
| | Menadione | 20 |
| | Folic acid | 30 |
| | Inositol | 10,000 |
| | Para-aminobenzoic acid | 2,000 |
| | Choline | 4,000 |
| | Vitamin $B_{12}$ (in mannitol) | 5 |
| | Powdered sucrose to make 200 g. | |

The above vitamin mixture is the complete mixture. In the vitamin deficiency experiments, the appropriate vitamin, or vitamins, were omitted from the mixture and were added separately to the control diets. This diet was one which had been in use in this laboratory for a number of years.

Diet No. 2

Diet No. 2 consisted of the pyridoxine deficient diet of Nutritional Biochemicals Company, Inc. fortified with 5 mg./Kg. pyridoxine to render it complete. Five mg. of the vitamin was chosen on the basis of a report by Brown and Sturtevant (110).

Diet No. 3

The diets used in the amino acid deficiency experiments were based on the phenylalanine-tyrosine deficient diet of Armstrong and Lewis (94). This diet as prepared by the directions of Armstrong and Lewis was found to contain too much fat to be palatable. Therefore it was slightly modified. The two modifications used in our laboratory follow.

Modification A.

| | | | % |
|---|---|---|---|
| Basal Diet. | Amino acid mixture | 1,000 g. | 17.4 |
| | Powdered sucrose | 2,700 | 47.0 |
| | Crisco | 1,300 | 22.6 |
| | USP Salt mixture | 200 | 3.5 |
| | Cod liver oil | 250 | 4.3 |
| | Cellulose | 100 | 1.7 |
| | Vitamin mixture | 200 | 3.5 |
| | Total | 5,750 g. | 100.0 |

Amino Acid Mixture

| | |
|---|---|
| Glycine | 30 g. |
| DL-Alanine | 25 |
| DL-Valine | 110 |
| L-Leucine | 110 |
| DL-Isoleucine | 90 |
| L-Cystine | 10 |
| DL-Methionine | 40 |
| DL-Threonine | 70 |
| DL-Aspartic acid | 50 |
| L-Glutamic acid | 100 |
| DL-Tryptophan | 45 |
| L-Arginine-HCL | 45 |
| L-Lysine-HCL | 125 |
| L-Histidine-HCL-$H_2O$ | 50 |
| Sodium bicarbonate | 100 |
| Total | 1,000 g. |

Vitamin Mixture. This was the same as that used in Diet No. 1.

Modification B.

| | | | % |
|---|---|---|---|
| Basal Diet. | Amino acid mixture | 1,000 g. | 20 |
| | Powdered sucrose | 2,600 | 52 |
| | Crisco | 650 | 13 |
| | USP XIV Salt mixture | 200 | 4 |
| | Cod liver oil | 250 | 5 |
| | Cellulose | 100 | 2 |
| | Vitamin mixture | 200 | 4 |
| | Total | 5,000 g. | 100 |

Amino acid mixture. This remained unchanged from Modification A above.

Vitamin mixture. This was the same as that used in Diet No. 1.

In both modifications of the Armstrong-Lewis diet, Crisco was used for the "hydrogenated vegetable oil". In Modification A, the sucrose content was raised from 43% to 47%; in Modification B, only half the recommended weight of Crisco was used and the difference in weight was made up with sucrose. Cellulose was substituted for agar.

Diet No. 4

This diet consisted of fox checkers manufactured by The Ralston Purina Company of Canada. It was the normal laboratory ration.

## EXPERIMENTAL RESULTS

### Experiment No. 1, Preliminary Experiment

The object of the first experiment was to investigate the adrenal content of adrenaline and noradrenaline in rats comparable to those which would be used later on. It was in the nature of a control experiment to determine how the catecholamines would vary with age, body size and adrenal weight. The animals used in this experiment were male, albino rats of the Sprague-Dawley strain and weighed between 94 g. and 119 g. when first placed on the experimental diet. Thirty-two rats, after receipt from the breeder were caged, given fox checkers and allowed two days to become acclimatized to the new surroundings. After 48 hours, they were weighed and assorted into groups of four on a weight basis. This was accomplished by first arranging the weights on a descending basis and cutting the list into four equal parts. The first animal in each part comprised the first group, the second animal in each part comprised the second group, etc. In this fashion, groups of nearly comparable weight were obtained. Of the eight groups formed, seven were chosen for the experimental procedure and one was left as a spare. They were then placed on Diet No. 2, and the first group to be sacrificed were killed. The order of sacrifice of groups was chosen by the method of random numbers. The remaining group after the first seven were chosen was left as the spare group. Groups were killed at

weekly intervals until the last one when there was a two week interval. Thus animals were maintained over a period of seven weeks.

The results of this experiment are given in the tables below.

In Table II the amounts of adrenaline, noradrenaline and the sum of the two (designated "Total") found in the pair of adrenals from each rat are presented. In Table III these results are expressed in terms of 100 mg. of adrenal tissue and in Table IV in terms of 100 g. of body weight of the animal.

Table II

Adrenaline and Noradrenaline in Rat Adrenals

µg. CA* per pair of Adrenals

| Time | Animal No. | NA | A | Total |
|---|---|---|---|---|
| 0 Week | 16 | 3.24 | 6.96 | 10.20 |
| | 24 | 3.77 | 8.20 | 11.97 |
| | 8 | 4.17 | 8.03 | 12.20 |
| | 32 | 4.31 | 9.57 | 13.88 |
| 1st. Week | 9 | 4.00 | 8.38 | 12.38 |
| | 17 | 6.53 | 11.38 | 17.91 |
| | 1 | 5.33 | 13.38 | 18.71 |
| | 25 | 4.48 | 9.30 | 13.78 |
| 2nd. Week | 11 | 5.58 | 12.13 | 17.71 |
| | 19 | 5.20 | 14.43 | 19.63 |
| | 3 | 5.78 | 14.48 | 20.26 |
| | 27 | 4.03 | 11.85 | 15.88 |
| 3rd. Week | 18 | 4.33 | 8.60 | 12.93 |
| | 26 | 4.68 | 11.78 | 16.46 |
| | 2 | 4.30 | 9.18 | 13.48 |
| | 10 | 2.83 | 8.20 | 11.03 |
| 4th. Week | 4 | 3.60 | 14.54 | 18.14 |
| | 12 | 3.08 | 15.84 | 18.92 |
| | 20 | 5.33 | 17.38 | 22.71 |
| | 28 | 5.35 | 14.12 | 19.47 |
| 5th. Week | 6 | 7.52 | 16.68 | 24.20 |
| | 14 | 6.31 | 19.52 | 25.83 |
| | 22 | 8.00 | 19.14 | 27.14 |
| | 30 | 5.05 | 13.90 | 18.95 |
| 7th. Week | 5 | 13.23 | 23.75 | 36.98 |
| | 21 | 7.08 | 17.08 | 24.16 |
| | 13 | 6.88 | 16.55 | 23.43 |
| | 29 | 7.78 | 17.68 | 25.46 |

* The following abbreviations are used throughout the tables: CA, Catecholamines; NA, noradrenaline; A, adrenaline.

The values for adrenaline (A), noradrenaline (NA) and the sum of the two (designated "Total") given in this table represent the total amounts found in the combined adrenals from one rat.

Table III

Adrenaline and Noradrenaline in Rat Adrenals Expressed on a Constant Adrenal Weight Basis

µg. CA per 100 mg. Adrenal Tissue

| Time | Animal No. | NA | A | Total |
|---|---|---|---|---|
| 0 Week | 16 | 14.7 | 31.6 | 46.4 |
| | 24 | 16.5 | 36.0 | 52.5 |
| | 8 | 17.9 | 34.5 | 52.4 |
| | 32 | 23.4 | 52.0 | 75.4 |
| 1st. Week | 9 | 16.4 | 34.3 | 50.7 |
| | 17 | 23.5 | 40.9 | 64.4 |
| | 1 | 17.1 | 42.9 | 60.0 |
| | 25 | 19.6 | 40.8 | 60.4 |
| 2nd. Week | 11 | 24.7 | 53.7 | 78.4 |
| | 19 | 29.2 | 81.1 | 110.3 |
| | 3 | 20.1 | 50.3 | 70.4 |
| | 27 | 14.5 | 42.6 | 57.1 |
| 3rd. Week | 18 | 18.0 | 35.8 | 53.9 |
| | 26 | 18.4 | 46.4 | 64.8 |
| | 2 | 17.9 | 38.3 | 56.2 |
| | 10 | 12.3 | 35.7 | 48.0 |
| 4th. Week | 4 | 11.3 | 45.4 | 56.7 |
| | 12 | 11.8 | 60.9 | 72.8 |
| | 20 | 17.0 | 55.4 | 72.3 |
| | 28 | 19.2 | 50.8 | 70.0 |
| 5th. Week | 6 | 22.9 | 50.9 | 73.8 |
| | 14 | 20.2 | 62.6 | 82.8 |
| | 22 | 23.8 | 57.0 | 80.8 |
| | 30 | 19.4 | 53.5 | 72.9 |
| 7th. Week | 5 | 43.2 | 77.6 | 120.8 |
| | 21 | 21.9 | 52.7 | 74.6 |
| | 13 | 24.6 | 59.1 | 83.7 |
| | 29 | 32.4 | 73.7 | 106.1 |

The values reported in this table are those taken from Table II and expressed in terms of 100 mg. of adrenal tissue.

Table IV

Adrenaline and Noradrenaline in Rat Adrenals Expressed on a Constant Body Weight Basis

µg. CA per 100 g. Body Weight

| Time | Animal No. | NA | A | Total |
|---|---|---|---|---|
| 0 Week | 16 | 3.15 | 6.76 | 9.91 |
| | 24 | 3.81 | 8.28 | 12.09 |
| | 8 | 3.79 | 7.30 | 11.09 |
| | 32 | 4.59 | 10.18 | 14.77 |
| 1st. Week | 9 | 3.28 | 6.87 | 10.15 |
| | 17 | 6.40 | 11.16 | 17.56 |
| | 1 | 4.52 | 11.34 | 15.86 |
| | 25 | 4.67 | 9.69 | 14.36 |
| 2nd. Week | 11 | 3.38 | 7.35 | 10.73 |
| | 19 | 3.61 | 10.02 | 13.63 |
| | 3 | 4.86 | 12.17 | 17.03 |
| | 27 | 3.70 | 10.87 | 14.57 |
| 3rd. Week | 18 | 2.58 | 5.12 | 7.70 |
| | 26 | 2.59 | 6.51 | 9.10 |
| | 2 | 3.61 | 7.71 | 11.32 |
| | 10 | 2.16 | 6.26 | 8.42 |
| 4th. Week | 4 | 1.53 | 6.19 | 7.72 |
| | 12 | 1.57 | 8.08 | 9.65 |
| | 20 | 4.10 | 13.37 | 17.47 |
| | 28 | 4.31 | 11.39 | 15.70 |
| 5th. Week | 6 | 3.02 | 6.70 | 9.72 |
| | 14 | 2.76 | 8.52 | 11.28 |
| | 22 | 5.52 | 13.20 | 18.72 |
| | 30 | 3.88 | 10.69 | 14.57 |
| 7th. Week | 5 | 4.38 | 7.86 | 12.24 |
| | 21 | 2.69 | 6.49 | 9.18 |
| | 13 | 3.91 | 9.40 | 13.31 |
| | 29 | 5.68 | 12.91 | 18.59 |

The values reported in this table are those taken from Table II and expressed in terms of 100 g. body weight of the animal.

The average weekly values from each of the methods of presentation given in the foregoing tables are presented in Table V.

Table V
Table of Average Values
µg. Catecholamine
Week

| | | 0 | 1 | 2 | 3 | 4 | 5 | 7 |
|---|---|---|---|---|---|---|---|---|
| per pr. Adr. | NA | 3.88 | 5.09 | 5.15 | 4.03 | 4.34 | 6.73 | 8.75 |
| | A | 8.19 | 10.61 | 13.23 | 9.44 | 15.47 | 17.31 | 18.77 |
| | Total | 11.07 | 15.70 | 18.38 | 13.47 | 19.81 | 24.04 | 27.52 |
| per 100 mg. Adr. | NA | 18.1 | 19.2 | 22.1 | 1.67 | 14.8 | 21.3 | 30.5 |
| | A | 38.5 | 39.7 | 56.9 | 39.1 | 53.1 | 56.0 | 65.8 |
| | Total | 56.6 | 58.9 | 79.0 | 55.8 | 67.9 | 77.3 | 96.3 |
| per 100 g. Body Wt. | NA | 3.82 | 4.72 | 3.89 | 2.74 | 2.88 | 3.80 | 4.17 |
| | A | 8.13 | 9.77 | 10.10 | 6.40 | 9.76 | 9.78 | 9.17 |
| | Total | 11.95 | 14.49 | 13.99 | 9.18 | 12.64 | 13.58 | 13.34 |

The average weekly values taken from Tables II, III, and IV. Each figure represents the average of four animals.

From the first section of Table V, it can be seen that the adrenal content of the catecholamines rises as the rats become older. Both the adrenaline and noradrenaline contents are more than doubled after seven weeks over the values found at the start of the experiment. A similar increase is also noted when the results are expressed on a constant adrenal weight basis, although it is not quite of the same magnitude. On the other hand, when the results are expressed on a body weight basis, the values for both amines remain at about the same level of magnitude throughout the experimental period. It therefore appears that the quantities of adrenaline and noradrenaline to be found in the adrenal glands depends more on the size of the animal than on the size of the adrenals.

These findings can be more readily seen in Figure 4 in which the total CA values reported in Table V are plotted as a graph.

Figure 4

Total CA in terms of three bases expressed as a function of time

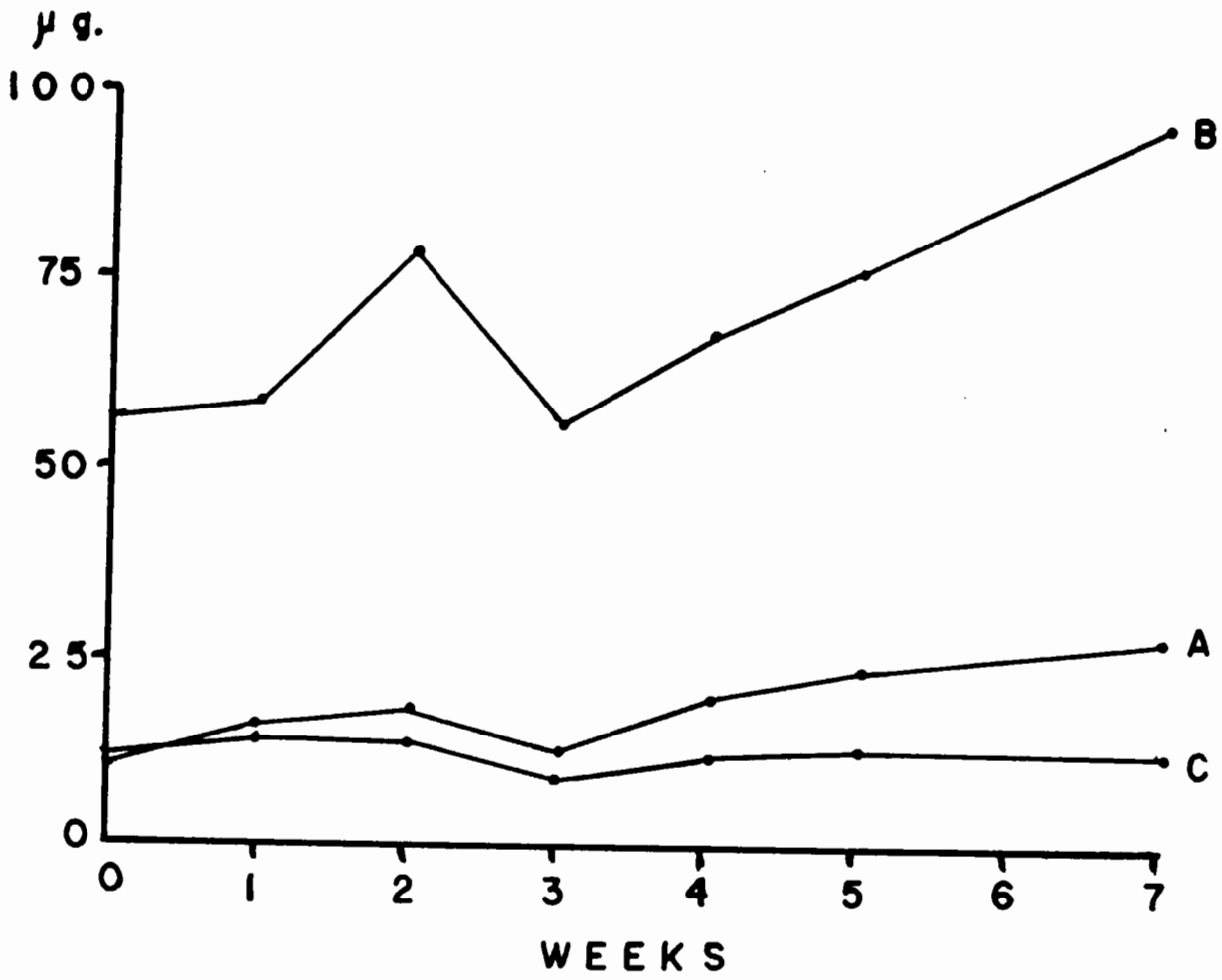



Fig. 4. The total values of Table V expressed in terms of total content for each pair of adrenals (Curve A), total content per 100 mg. of adrenal tissue (Curve B) and total adrenal content per 100 g. body weight (Curve C) are plotted as a function of time.

## Vitamin Deficiency Experiments

### Experiment No. 2. The Effect of Riboflavin and Pyridoxine Deficiencies on the Catecholamine contents of Adrenal Glands, Liver, Brain, Heart and Spleen of Young Rats.

This experiment was designed to investigate the effects of riboflavin and pyridoxine deficiencies on the catecholamine contents of various rat organs. The organs chosen for study were the adrenals, brain, liver, spleen and heart. It appeared to be of interest to study riboflavin and pyridoxine deficiencies in view of the following considerations. First, Udenfriend and Cooper (17), among others, have shown that the pyridine nucleotides are involved in the hydroxylation of phenylalanine to form tyrosine. Kaufmann (19) has postulated a reaction mechanism for this oxidation which involves the formation of water. Since flavoproteins have been associated with the pyridine nucleotides in the classical hydrogen transport system, in which water is formed, we thought it would be of interest to investigate whether riboflavin deficiency would modify the amounts of catecholamines synthesized by the animal. Although tyrosine was supplied in the protein components of the diet, making it unlikely that a riboflavin deficiency at this step would result in altered catecholamine synthesis, there are two other hydroxylation steps in adrenaline and noradrenaline biosynthesis which might be affected.

In the second place, Green, Leloir and Nocito (37) have demonstrated that DOPA decarboxylase depends

upon pyridoxine for activity. One would expect, therefore, that a deficiency of vitamin $B_6$ would interfere with the decarboxylation of DOPA and thus impede the biosynthesis of these amines. Indeed, Blaschko (38) observed diminished DOPA decarboxylase activity in livers of pyridoxine deficient rats. In a later preliminary report not yet amplified (112), however, he found a normal concentration of adrenaline and noradrenaline in the adrenals of such deficient animals. But if the adrenals of both normal and $B_6$ deficient rats had been previously depleted of adrenaline by insulin, then the deficient adrenals contained much less than the control after a period of recovery. With these considerations in mind, the following experiment was carried out.

Twenty-eight rats weighing between 90 g. and 100 g. were divided into seven groups of four on a descending weight basis. The members of each group were then randomly distributed amongst four diets: one member received the complete No. 1 diet, the other three members of each group received the same diet modified by omission of riboflavin in one instance, pyridoxine in another, and the final diet lacked both of these vitamins in order to investigate a possible interaction between the two deficiencies. The animals were sacrificed one group at a time over periods ranging from 19 to 54 days.

The results of this experiment are presented in the tables to follow.

Table VI

Brain

Adrenaline (A), noradrenaline (NA) and total catecholamine content (Total) of brains from rats on riboflavin and pyridoxine deficient diets. Results expressed as µg. per whole organ.

| | Days on Diet | Diet: Complete | $-B_2$ | $-B_6$ | $-B_2B_6$ |
|---|---|---|---|---|---|
| NA | 19 | 1.61 | 1.24 | 0.79 | 1.94 |
| | 31 | 0.48 | 0.38 | 0.59 | 0.36 |
| | 32 | 1.21 | 1.27 | 1.47 | 1.12 |
| | 38 | 1.04 | 1.08 | 1.19 | 1.07 |
| | 39 * | 0.89 | 0.75 | 0.89 | 0.82 |
| | 46 | 0.96 | 0.95 | 0.95 | 0.67 |
| | 54 | 1.09 | 1.11 | 1.05 | 0.81 |
| A | 19 | 0.35 | 0.06 | 0.13 | 0.18 |
| | 31 | 0.19 | 0.11 | 0.18 | 0.13 |
| | 32 | 0.37 | 0.48 | 0.43 | 0.33 |
| | 38 | 0.33 | 0.34 | 0.28 | 0.29 |
| | 39 * | 0.15 | 0.10 | 0.15 | 0.15 |
| | 46 | 0.09 | 0.13 | 0.08 | 0.10 |
| | 54 | 0.10 | 0.13 | 0.13 | 0.03 |
| Total | 19 | 1.96 | 1.30 | 0.92 | 2.12 |
| | 31 | 0.67 | 0.49 | 0.77 | 0.49 |
| | 32 | 1.58 | 1.75 | 1.90 | 1.45 |
| | 38 | 1.37 | 1.42 | 1.47 | 1.36 |
| | 39 | 1.04 | 0.85 | 1.04 | 0.97 |
| | 46 | 1.05 | 1.08 | 1.03 | 0.77 |
| | 54 | 1.19 | 1.24 | 1.18 | 0.84 |
| NA | Sum | 7.28 | 6.78 | 6.93 | 6.79 |
| | Mean | 1.04 | 0.97 | 0.99 | 0.97 |
| A | Sum | 1.58 | 1.35 | 1.38 | 1.21 |
| | Mean | 0.23 | 0.19 | 0.20 | 0.17 |
| Total | Sum | 8.86 | 8.13 | 8.31 | 8.00 |
| | Mean | 1.27 | 1.16 | 1.19 | 1.14 |

* The control animal for this group died during the course of the experiment. The values reported for this animal were estimated by the formula for missing data (111).

Table VII

Spleen

Adrenaline, noradrenaline and total catecholamine contents of spleens from rats on riboflavin and pyridoxine deficient diets. Results expressed as µg. per whole organ.

| | | Diet | | | |
|---|---|---|---|---|---|
| | Days on Diet | Complete | $-B_2$ | $-B_6$ | $-B_2B_6$ |
| NA | 19 | 1.49 | 0.70 | 0.56 | 1.35 |
| | 31 | 0.32 | 0.34 | 0.31 | 0.29 |
| | 32 | 0.90 | 0.94 | 1.13 | 0.77 |
| | 38 | 0.97 | 1.02 | 1.00 | 1.02 |
| | 39 * | 0.43 | 0.39 | 0.45 | 0.37 |
| | 46 | 0.55 | 0.45 | 0.35 | 0.50 |
| | 54 | 0.53 | 0.52 | 0.49 | 0.74 |
| A | 19 | 0.45 | 0.02 | 0.22 | 0.16 |
| | 31 | 0.25 | 0.22 | 0.42 | 0.16 |
| | 32 | 0.45 | 0.33 | 0.25 | 0.43 |
| | 38 | 0.35 | 0.39 | 0.31 | 0.28 |
| | 39 * | 0.13 | 0.06 | 0.13 | 0.10 |
| | 46 | 0.09 | 0.03 | 0.00 | 0.00 |
| | 54 | 0.04 | 0.00 | 0.00 | 0.04 |
| Total | 19 | 1.94 | 0.72 | 0.78 | 1.51 |
| | 31 | 0.57 | 0.56 | 0.73 | 0.45 |
| | 32 | 1.35 | 1.27 | 1.38 | 1.20 |
| | 38 | 1.32 | 1.41 | 1.31 | 1.30 |
| | 39 | 0.56 | 0.45 | 0.58 | 0.47 |
| | 46 | 0.64 | 0.48 | 0.35 | 0.50 |
| | 54 | 0.57 | 0.52 | 0.49 | 0.78 |
| NA | Sum | 5.19 | 4.36 | 4.29 | 5.04 |
| | Mean | 0.74 | 0.62 | 0.61 | 0.72 |
| A | Sum | 1.76 | 1.05 | 1.33 | 1.17 |
| | Mean | 0.25 | 0.15 | 0.19 | 0.17 |
| Total | Sum | 6.95 | 5.41 | 5.62 | 6.21 |
| | Mean | 0.99 | 0.77 | 0.80 | 0.89 |

* See note under Table VI, p. 49

Table VIII

Heart

Adrenaline, noradrenaline and total catecholamine contents of hearts from rats on riboflavin and pyridoxine deficient diets. Results expressed as µg. per whole organ.

| | | Diet | | | |
|---|---|---|---|---|---|
| | Days on Diet | Complete | $-B_2$ | $-B_6$ | $-B_2B_6$ |
| NA | 19 | 2.36 | 1.07 | 1.76 | 1.71 |
| | 32 | 1.16 | 1.30 | 1.00 | 0.97 |
| | 38 | 1.18 | 1.03 | 1.06 | 1.15 |
| A | 19 | 0.57 | 0.11 | 0.86 | 0.22 |
| | 32 | 0.64 | 0.38 | 0.46 | 0.29 |
| | 38 | 0.36 | 0.39 | 0.30 | 0.35 |
| Total | 19 | 2.93 | 1.18 | 2.62 | 1.93 |
| | 32 | 1.80 | 1.68 | 1.46 | 1.26 |
| | 38 | 1.54 | 1.42 | 1.36 | 1.50 |
| NA | Sum | 4.70 | 3.40 | 3.82 | 3.83 |
| | Mean | 1.57 | 1.13 | 1.27 | 1.28 |
| A | Sum | 1.57 | 0.88 | 1.62 | 0.86 |
| | Mean | 0.52 | 0.29 | 0.54 | 0.29 |
| Total | Sum | 6.27 | 4.28 | 5.44 | 4.69 |
| | Mean | 2.09 | 1.42 | 1.81 | 1.57 |

Table IX

Liver

Adrenaline, noradrenaline and total catecholamine contents of livers from rats on riboflavin and pyridoxine deficient diets. Results expressed as µg. per whole organ.

| | Days on Diet | Diet: Complete | -$B_2$ | -$B_6$ | -$B_2B_6$ |
|---|---|---|---|---|---|
| NA | 19 | 11.67 | 5.67 | 5.27 | 7.59 |
| | 31 | 5.11 | 1.16 | 3.83 | 2.10 |
| | 32 | 6.59 | 3.54 | 8.67 | 2.19 |
| | 38 | 4.07 | 2.80 | 4.48 | 1.74 |
| | 39 * | 8.97 | 4.16 | 7.17 | 4.37 |
| | 46 | 6.60 | 2.36 | 3.89 | 1.71 |
| | 54 | 8.07 | 2.18 | 7.75 | 0.63 |
| A | 19 | 3.66 | 1.17 | 3.00 | 1.85 |
| | 31 | 4.03 | 1.05 | 3.19 | 1.31 |
| | 32 | 4.00 | 1.55 | 3.74 | 0.95 |
| | 38 | 1.74 | 0.87 | 1.33 | 0.69 |
| | 39 * | 3.06 | 0.83 | 2.63 | 0.92 |
| | 46 | 1.47 | 0.71 | 0.85 | 0.41 |
| | 54 | 2.83 | 1.13 | 3.35 | 0.33 |
| Total | 19 | 15.33 | 6.84 | 8.27 | 9.44 |
| | 31 | 9.14 | 2.21 | 7.02 | 3.41 |
| | 32 | 10.59 | 5.09 | 12.41 | 3.14 |
| | 38 | 5.81 | 3.67 | 5.81 | 2.43 |
| | 39 | 12.03 | 4.99 | 9.80 | 5.29 |
| | 46 | 8.07 | 3.07 | 4.74 | 2.12 |
| | 54 | 10.90 | 3.31 | 11.10 | 0.96 |
| NA | Sum | 51.08 | 21.87 | 41.06 | 20.33 |
| | Mean | 7.30 | 3.13 | 5.87 | 2.90 |
| A | Sum | 20.79 | 7.31 | 18.09 | 6.46 |
| | Mean | 2.97 | 1.04 | 2.58 | 0.92 |
| Total | Sum | 71.87 | 29.18 | 59.15 | 26.79 |
| | Mean | 10.27 | 4.17 | 8.45 | 3.82 |

* See note under Table VI

Table X

Adrenals

Adrenaline, noradrenaline and total catecholamine contents of adrenals from rats on riboflavin and pyridoxine deficient diets. Results expressed as µg. per whole organ.

| | Days on Diet | Diet: Complete | $-B_2$ | $-B_6$ | $-B_2B_6$ |
|---|---|---|---|---|---|
| NA | 19 | 30.86 | 9.24 | 24.67 | 11.87 |
| | 31 | 4.78 | 6.04 | 3.03 | 0.84 |
| | 32 | 5.87 | 6.25 | 4.46 | 6.34 |
| | 38 | 7.14 | 4.54 | 9.96 | 7.98 |
| | 39 * | 6.60 | 6.67 | 5.54 | 7.01 |
| | 46 | 8.82 | 4.60 | 8.47 | 8.28 |
| | 54 | 2.95 | 4.14 | 6.29 | 6.15 |
| A | 19 | 26.23 | 18.96 | 19.42 | 10.82 |
| | 31 | 17.73 | 17.73 | 16.48 | 14.12 |
| | 32 | 18.20 | 9.76 | 7.30 | 10.35 |
| | 38 | 13.78 | 11.86 | 23.06 | 21.10 |
| | 39 * | 15.29 | 15.74 | 13.28 | 10.25 |
| | 46 | 25.66 | 19.06 | 24.27 | 18.36 |
| | 54 | 21.95 | 23.11 | 26.31 | 16.61 |
| Total | 19 | 57.09 | 28.20 | 44.09 | 22.69 |
| | 31 | 22.51 | 23.77 | 19.51 | 14.96 |
| | 32 | 24.07 | 16.01 | 11.76 | 16.69 |
| | 38 | 20.92 | 16.40 | 33.02 | 29.08 |
| | 39 | 21.89 | 22.41 | 18.82 | 17.26 |
| | 46 | 34.48 | 23.66 | 32.74 | 26.64 |
| | 54 | 24.90 | 27.25 | 32.60 | 22.76 |
| NA | Sum | 67.02 | 41.48 | 62.42 | 48.47 |
| | Mean | 9.57 | 5.93 | 8.92 | 6.92 |
| A | Sum | 138.84 | 116.22 | 130.12 | 101.61 |
| | Mean | 19.83 | 16.60 | 18.59 | 14.52 |
| Total | Sum | 205.86 | 157.70 | 192.54 | 150.08 |
| | Mean | 29.40 | 22.53 | 27.51 | 21.44 |

* See note under Table VI, p. 49

Table XI

Mean Catecholamine Contents
(Whole Organ)

Results expressed as µg. noradrenaline (NA) and adrenaline (A) per whole organ or pair of adrenals. Each figure represents the mean of 3 rats (hearts) or 7 rats (all other organs).

| Diet | | Complete | $-B_2$ | $-B_6$ | $-B_2B_6$ |
|---|---|---|---|---|---|
| Adrenals | NA | 9.57 | 5.93 | 8.92 | 6.92 |
| | A | 19.83 | 16.60 | 18.59 | 14.52 |
| | Total | 29.40 | 22.53 | 27.51 | 21.44 |
| Brain | NA | 1.04 | 0.97 | 0.99 | 0.97 |
| | A | 0.23 | 0.19 | 0.20 | 0.17 |
| | Total | 1.27 | 1.16 | 1.19 | 1.14 |
| Liver | NA | 7.30 | 3.13 | 5.87 | 2.90 |
| | A | 2.97 | 1.04 | 2.58 | 0.92 |
| | Total | 10.27 | 4.17 | 8.45 | 3.82 |
| Heart | NA | 1.57 | 1.13 | 1.27 | 1.28 |
| | A | 0.52 | 0.29 | 0.54 | 0.29 |
| | Total | 2.09 | 1.42 | 1.81 | 1.57 |
| Spleen | NA | 0.74 | 0.62 | 0.61 | 0.72 |
| | A | 0.25 | 0.15 | 0.19 | 0.17 |
| | Total | 0.99 | 0.77 | 0.80 | 0.89 |

Table XII

Organ Weights

Wet weights of organs from rats on riboflavin and pyridoxine deficient diets. Adrenal weights in milligrams, all others in grams.

| | Days on Diet | Diet: Complete | $-B_2$ | $-B_6$ | $-B_2B_6$ |
|---|---|---|---|---|---|
| Brain | 19 | 1.63 | 1.52 | 1.74 | 1.63 |
| | 31 | 1.63 | 1.49 | 1.66 | 1.53 |
| | 32 | 1.80 | 1.75 | 1.72 | 1.51 |
| | 38 | 1.90 | 1.60 | 1.90 | 1.47 |
| | 39 * | 1.51 | 1.53 | 1.65 | 1.28 |
| | 46 | 1.74 | 1.50 | 1.73 | 1.61 |
| | 54 | 1.55 | 1.70 | 1.70 | 1.65 |
| | Sum | 11.76 | 11.09 | 12.10 | 10.68 |
| | Mean | 1.68 | 1.58 | 1.73 | 1.53 |
| Liver | 19 | 7.21 | 4.00 | 5.60 | 3.70 |
| | 31 | 11.40 | 5.26 | 5.95 | 3.96 |
| | 32 | 12.16 | 7.42 | 7.64 | 4.75 |
| | 38 | 11.70 | 5.33 | 8.60 | 4.37 |
| | 39 * | 12.35 | 5.53 | 7.35 | 5.40 |
| | 46 | 10.69 | 4.76 | 7.08 | 2.61 |
| | 54 | 13.25 | 5.76 | 7.87 | 6.31 |
| | Sum | 78.70 | 38.06 | 50.09 | 31.10 |
| | Mean | 11.25 | 5.44 | 7.16 | 4.44 |
| Spleen | 19 | 0.87 | 0.24 | 0.45 | 0.18 |
| | 31 | 0.59 | 0.35 | 0.37 | 0.25 |
| | 32 | 0.85 | 0.42 | 0.43 | 0.40 |
| | 38 | 0.39 | 0.33 | 0.33 | 0.35 |
| | 39 * | 0.65 | 0.32 | 0.47 | 0.29 |
| | 46 | 0.55 | 0.17 | 0.37 | 0.16 |
| | 54 | 0.58 | 0.26 | 0.31 | 0.30 |
| | Sum | 4.48 | 2.09 | 2.73 | 1.93 |
| | Mean | 0.64 | 0.30 | 0.39 | 0.28 |
| Adrenals (Pair) | 19 | 32.1 | 31.0 | 28.1 | 23.8 |
| | 31 | 26.0 | 35.0 | 21.0 | 33.3 |
| | 32 | 35.4 | 26.2 | 30.0 | 31.4 |
| | 38 | 32.1 | 25.2 | 30.0 | 31.6 |
| | 39 * | 29.8 | 32.4 | 33.0 | 40.0 |
| | 46 | 30.6 | 35.6 | 29.0 | 24.6 |
| | 54 | 25.6 | 28.4 | 46.8 | 40.8 |
| | Sum | 211.6 | 213.8 | 217.9 | 225.5 |
| | Mean | 30.2 | 30.5 | 31.1 | 32.2 |
| Heart | 19 | 0.80 | 0.51 | 0.50 | 0.48 |
| | 32 | 1.04 | 0.55 | 0.58 | 0.60 |
| | 38 | 0.97 | 0.51 | 0.81 | 0.38 |
| | Sum | 2.81 | 1.57 | 1.89 | 1.46 |
| | Mean | 0.94 | 0.52 | 0.63 | 0.49 |

* See note under Table VI

Before considering the results as expressed on a constant organ weight basis, we might first consider the whole organ contents of adrenaline and noradrenaline. As seen from Table XI, the various deficiencies had little effect on either noradrenaline or adrenaline in brain. The various dietary group means were approximately 1.0 µg. for noradrenaline and 0.20 µg. for adrenaline. Although there was some depression of the total catecholamines in the vitamin deficient animals, in no instance was it of appreciable maghitude nor was it statistically significant.* The spleens also showed a non-significant decrease of both catecholamines in the deficient animals. In the case of hearts, all the deficient animals showed somewhat lowered noradrenaline contents, however there was a fairly uniform decrease among the various deficiency categories. Adrenaline, on the other hand, was diminished only in the $B_2$-deficient and the $B_2B_6$-deficient groups. Since $B_6$-deficiency did not cause any decrease in the adrenaline of the heart as compared with the controls, the decrease in the doubly deficient group was probably due only to lack of riboflavin.

The noradrenaline and adrenaline levels of liver were not affected by $B_6$-deficiency to any significant extent; however $B_2$-deficiency markedly reduced the amounts of both catecholamines in this organ. When the two $B_2$-deficient groups ($-B_2$ and $-B_2B_6$) were summed and compared

* See Appendix for Statistical tables.

with the sum of the remaining two groups (Complete and -$B_6$), both of which were supplied with dietary riboflavin, there was seen to be a highly significant decrease in both the noradrenaline and adrenaline contents, significant at the 99% level of confidence.

Pyridoxine deficiency had no apparent effect on the levels of either noradrenaline or adrenaline in the adrenals as judged from the mean values. However, when the figures for the $B_6$-deficient animals are calculated as a per cent of the animals on the complete diet and plotted as a function of time (Fig. 6), it can be seen that there was actually a decrease in the earlier portion of the experimental period. After about 5-6 weeks on the diets, the adrenal catecholamine content of the deficient animals reaches, or even surpasses, that of the animals on the complete diet. This change as a function of time appeared to be specific for pyridoxine since it did not occur in the riboflavin-deficient rats.

The results of the whole organ contents are presented graphically in Figures 4 through 6.

Figure 4

Adrenaline and Noradrenaline Contents of Rat Brain and Liver

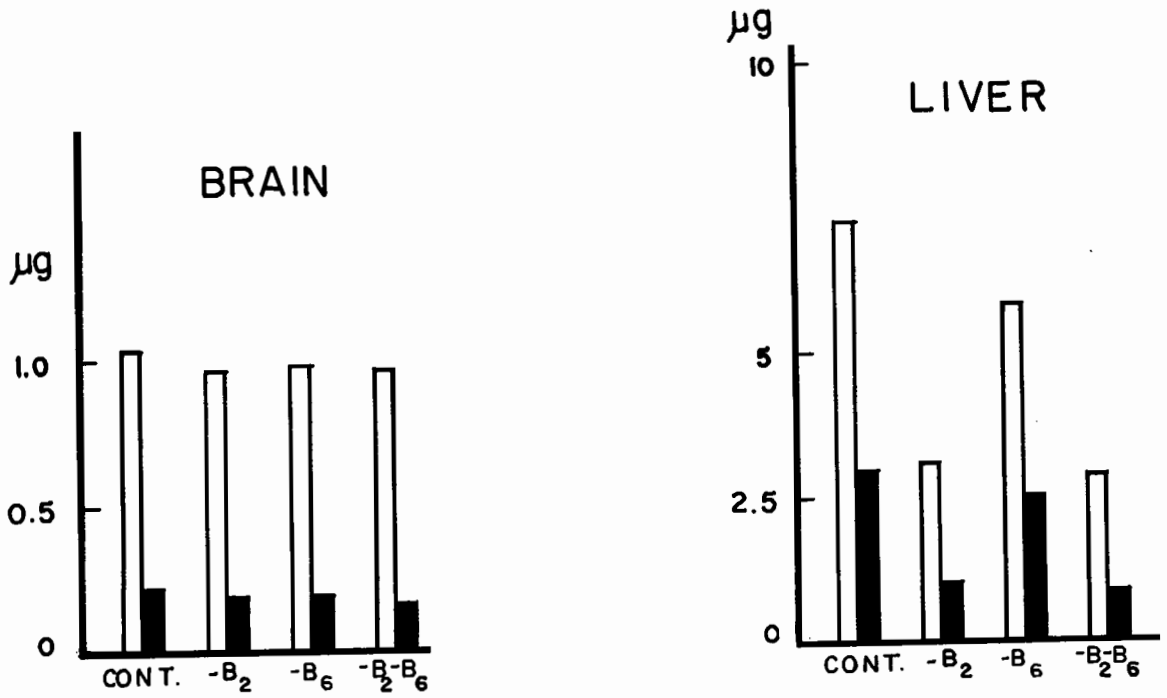



Fig. 4. The open bars represent the mean organ content of noradrenaline of seven animals, the solid, of adrenaline. The control diet (Cont.) was fully supplemented, the others deficient as indicated.

Figure 5

Adrenaline and Noradrenaline Contents of Rat Spleen and Adrenals

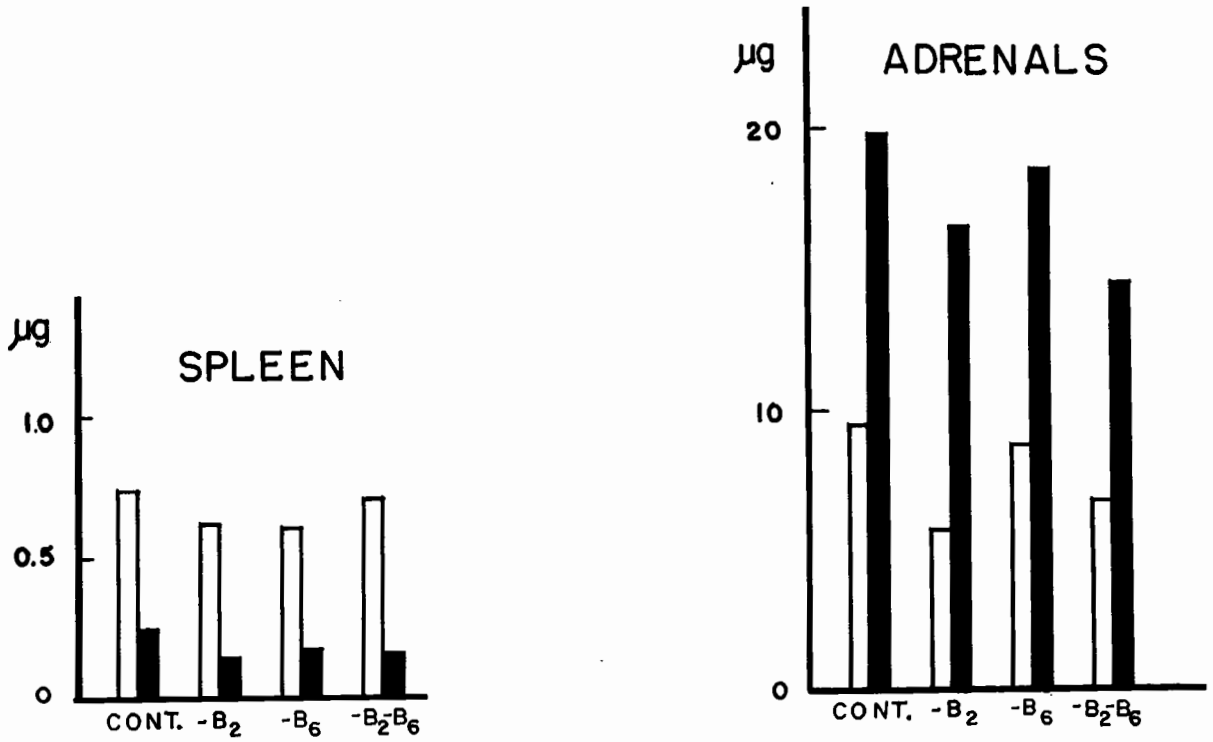



Fig. 5. The open bars represent the mean organ content of noradrenaline of seven animals, the solid, of adrenaline. The control diet (Cont.) was fully supplemented, the others deficient as indicated.

Figure 6

Adrenaline and Noradrenaline of Pyridoxine-Deficient Rat Adrenals

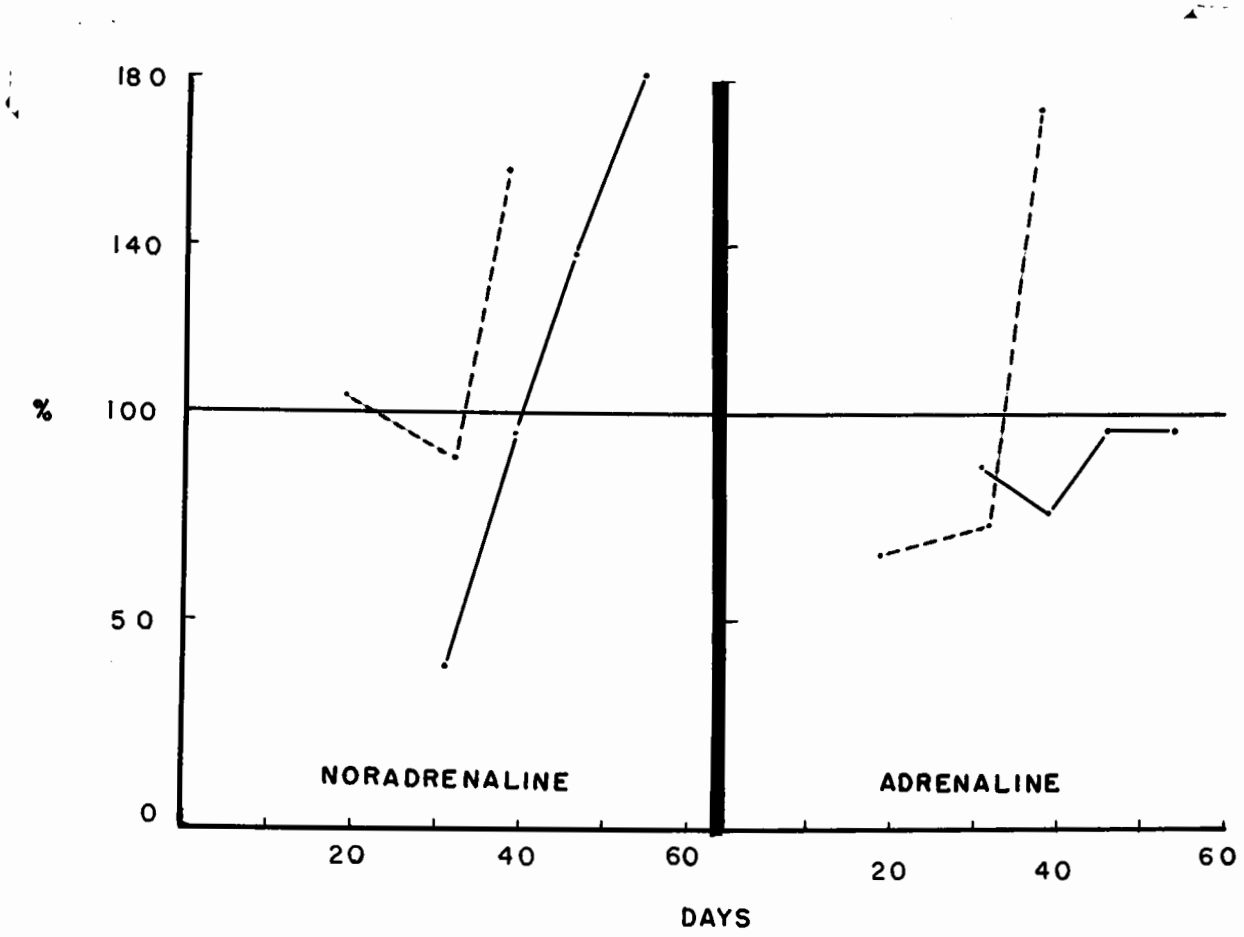



Fig. 6. The adrenaline and noradrenaline values for the pyridoxine-deficient animals are expressed as a percentage of the controls on $B_6$ supplemented diets and plotted as a function of time. The control values, set arbitrarily at 100%, are represented by the horizontal line across the center of the figure. The dotted lines represent the animals sacrificed on days 19, 32 and 38 which were actually carried out as a duplicate experiment at a later time than those represented by the solid lines. Each point is the mean adrenal content of two animals, one on each of the $B_6$-deficient diets ($-B_6$ and $-B_2B_6$). The control figures, set at 100%, are the mean values of the animals on the complete and $-B_2$ diets.

The results of this experiment are also presented in terms of constant organ weight in tables XIII through XVIII. Brain, spleen, heart and liver contents are expressed as µg. per gram whereas those of the adrenals are given as micrograms per milligram.

Table XIII

Brain

Adrenaline, noradrenaline and total catecholamine contents of brains from rats on riboflavin and pyridoxine deficient diets.

µg. per g. tissue

| | Days on Diet | Diet: Complete | -$B_2$ | -$B_6$ | -$B_2B_6$ |
|---|---|---|---|---|---|
| NA | 19 | 0.99 | 0.82 | 0.45 | 1.19 |
| | 31 | 0.29 | 0.25 | 0.36 | 0.24 |
| | 32 | 0.67 | 0.73 | 0.84 | 0.74 |
| | 38 | 0.55 | 0.67 | 0.63 | 0.72 |
| | 39 ✱ | 0.59 | 0.49 | 0.54 | 0.64 |
| | 46 | 0.55 | 0.63 | 0.55 | 0.42 |
| | 54 | 0.70 | 0.65 | 0.62 | 0.49 |
| Total | | 4.34 | 4.24 | 3.99 | 4.44 |
| Mean | | 0.62 | 0.61 | 0.57 | 0.63 |
| A | 19 | 0.22 | 0.04 | 0.07 | 0.11 |
| | 31 | 0.12 | 0.07 | 0.11 | 0.09 |
| | 32 | 0.20 | 0.27 | 0.25 | 0.22 |
| | 38 | 0.17 | 0.21 | 0.15 | 0.20 |
| | 39 ✱ | 0.10 | 0.07 | 0.09 | 0.12 |
| | 46 | 0.05 | 0.09 | 0.05 | 0.06 |
| | 54 | 0.06 | 0.08 | 0.08 | 0.02 |
| Total | | 0.92 | 0.83 | 0.80 | 0.82 |
| Mean | | 0.13 | 0.12 | 0.11 | 0.12 |
| Total CA | 19 | 1.21 | 0.86 | 0.52 | 1.30 |
| | 31 | 0.41 | 0.32 | 0.47 | 0.33 |
| | 32 | 0.87 | 1.00 | 1.09 | 0.96 |
| | 38 | 0.72 | 0.88 | 0.78 | 0.92 |
| | 39 ✱ | 0.69 | 0.56 | 0.63 | 0.76 |
| | 46 | 0.60 | 0.72 | 0.60 | 0.48 |
| | 54 | 0.76 | 0.73 | 0.70 | 0.51 |
| Total | | 5.26 | 5.07 | 4.79 | 5.26 |
| Mean | | 0.75 | 0.73 | 0.68 | 0.75 |

✱ The control animal of the group sacrificed at this time died during the course of the experiment. The values for this animal were estimated using the formula for missing data (111).

Table XIV

Spleen

Adrenaline, noradrenaline and total catecholamine contents of spleen from rats on riboflavin and pyridoxine deficient diets.

µg. per g. tissue

| | Days on Diet | Diet: Complete | $-B_2$ | $-B_6$ | $-B_2B_6$ |
|---|---|---|---|---|---|
| NA | 19 | 1.71 | 2.90 | 1.25 | 7.49 |
| | 31 | 0.54 | 0.97 | 0.84 | 1.16 |
| | 32 | 1.06 | 2.23 | 2.62 | 1.93 |
| | 38 | 2.49 | 3.09 | 3.04 | 2.91 |
| | 39 * | 0.66 | 1.22 | 0.96 | 1.28 |
| | 46 | 1.00 | 2.65 | 0.95 | 3.13 |
| | 54 | 0.91 | 2.00 | 1.58 | 2.46 |
| Total | | 8.37 | 15.06 | 11.24 | 20.36 |
| Mean | | 1.20 | 2.15 | 1.61 | 2.91 |
| A | 19 | 0.51 | 0.08 | 0.50 | 0.88 |
| | 31 | 0.42 | 0.63 | 1.13 | 0.64 |
| | 32 | 0.53 | 0.77 | 0.57 | 1.08 |
| | 38 | 0.89 | 1.18 | 0.93 | 0.79 |
| | 39 * | 0.20 | 0.19 | 0.28 | 0.35 |
| | 46 | 0.16 | 0.18 | 0.00 | 0.00 |
| | 54 | 0.07 | 0.00 | 0.00 | 0.13 |
| Total | | 2.78 | 3.03 | 3.41 | 3.87 |
| Mean | | 0.40 | 0.25 | 0.49 | 0.55 |
| Total CA | 19 | 2.22 | 2.98 | 1.75 | 8.37 |
| | 31 | 0.96 | 1.60 | 1.97 | 1.80 |
| | 32 | 1.59 | 3.00 | 3.19 | 3.01 |
| | 38 | 3.38 | 4.27 | 3.97 | 3.70 |
| | 39 * | 0.86 | 1.41 | 1.24 | 1.63 |
| | 46 | 1.16 | 2.83 | 0.95 | 3.13 |
| | 54 | 0.98 | 2.00 | 1.58 | 2.59 |
| Total | | 11.15 | 18.09 | 14.65 | 24.23 |
| Mean | | 1.59 | 2.58 | 2.09 | 3.46 |

* See note under Table XIII, p. 62

Table XV

Heart

Adrenaline, noradrenaline and total catecholamine contents of hearts from rats on riboflavin and pyridoxine deficient diets.

µg. per g. heart

| | Days on Diet | Diet: Complete | -$B_2$ | -$B_6$ | -$B_2B_6$ |
|---|---|---|---|---|---|
| NA | 19 | 2.95 | 2.10 | 3.51 | 3.56 |
| | 32 | 1.11 | 0.24 | 1.72 | 1.61 |
| | 38 | 1.21 | 2.01 | 1.31 | 3.02 |
| Total | | 5.27 | 4.35 | 6.54 | 8.19 |
| Mean | | 1.76 | 1.45 | 2.18 | 2.73 |
| A | 19 | 0.71 | 0.22 | 1.72 | 0.46 |
| | 32 | 0.62 | 0.69 | 0.80 | 0.49 |
| | 38 | 0.38 | 0.76 | 0.37 | 0.91 |
| Total | | 1.71 | 1.67 | 2.89 | 1.86 |
| Mean | | 0.57 | 0.56 | 0.96 | 0.62 |
| Total CA | 19 | 3.66 | 2.32 | 5.23 | 4.02 |
| | 32 | 1.73 | 0.93 | 2.52 | 2.10 |
| | 38 | 1.59 | 2.77 | 1.68 | 3.93 |
| Total | | 6.98 | 6.02 | 9.43 | 10.05 |
| Mean | | 2.33 | 2.01 | 3.14 | 3.35 |

Table XVI

Liver

Adrenaline, noradrenaline and total catecholamine contents of liver from rats on riboflavin and pyridoxine deficient diets.

µg. per g. tissue

| | Days on Diet | Diet: Complete | $-B_2$ | $-B_6$ | $-B_2B_6$ |
|---|---|---|---|---|---|
| NA | 19 | 1.62 | 1.42 | 0.94 | 2.05 |
| | 31 | 0.45 | 0.22 | 0.64 | 0.53 |
| | 32 | 0.54 | 0.48 | 1.14 | 0.46 |
| | 38 | 0.35 | 0.53 | 0.52 | 0.40 |
| | 39 ✱ | 0.73 | 0.75 | 0.98 | 0.81 |
| | 46 | 0.62 | 0.50 | 0.55 | 0.65 |
| | 54 | 0.61 | 0.38 | 0.98 | 0.10 |
| Total | | 4.92 | 4.28 | 5.75 | 5.00 |
| Mean | | 0.70 | 0.61 | 0.82 | 0.71 |
| A | 19 | 0.51 | 0.29 | 0.54 | 0.50 |
| | 31 | 0.35 | 0.20 | 0.54 | 0.33 |
| | 32 | 0.33 | 0.21 | 0.49 | 0.20 |
| | 38 | 0.15 | 0.16 | 0.16 | 0.16 |
| | 39 ✱ | 0.25 | 0.15 | 0.36 | 0.17 |
| | 46 | 0.14 | 0.15 | 0.12 | 0.16 |
| | 54 | 0.21 | 0.20 | 0.43 | 0.05 |
| Total | | 1.94 | 1.36 | 2.64 | 1.57 |
| Mean | | 0.28 | 0.20 | 0.38 | 0.22 |
| Total CA | 19 | 2.13 | 1.71 | 1.48 | 2.55 |
| | 31 | 0.80 | 0.42 | 1.18 | 0.86 |
| | 32 | 0.87 | 0.69 | 1.63 | 0.66 |
| | 38 | 0.50 | 0.69 | 0.68 | 0.56 |
| | 39 ✱ | 0.98 | 0.90 | 1.34 | 0.98 |
| | 46 | 0.76 | 0.65 | 0.67 | 0.81 |
| | 54 | 0.82 | 0.58 | 1.41 | 0.15 |
| Total | | 6.86 | 5.64 | 8.39 | 6.57 |
| Mean | | 0.98 | 0.81 | 1.20 | 0.93 |

✱ See note under Table XIII, p. 62

Table XVII

Adrenals

Adrenaline, noradrenaline and total catecholamine contents of adrenals from rats on riboflavin and pyridoxine deficient diets.

µg. per mg. Adrenals

| | Days on Diet | Diet: Complete | $-B_2$ | $-B_6$ | $-B_2B_6$ |
|---|---|---|---|---|---|
| NA | 19 | 0.96 | 0.30 | 0.88 | 0.50 |
| | 31 | 0.18 | 0.17 | 0.14 | 0.03 |
| | 32 | 0.17 | 0.24 | 0.15 | 0.20 |
| | 38 | 0.22 | 0.18 | 0.33 | 0.25 |
| | 39 ✱ | 0.22 | 0.21 | 0.17 | 0.18 |
| | 46 | 0.29 | 0.13 | 0.29 | 0.34 |
| | 54 | 0.12 | 0.15 | 0.13 | 0.15 |
| Total | | 2.16 | 1.38 | 2.09 | 1.65 |
| Mean | | 0.31 | 0.20 | 0.30 | 0.24 |
| A | 19 | 0.82 | 0.61 | 0.69 | 0.46 |
| | 31 | 0.68 | 0.51 | 0.78 | 0.42 |
| | 32 | 0.51 | 0.38 | 0.24 | 0.33 |
| | 38 | 0.43 | 0.47 | 0.77 | 0.67 |
| | 39 ✱ | 0.51 | 0.49 | 0.40 | 0.26 |
| | 46 | 0.84 | 0.54 | 0.84 | 0.75 |
| | 54 | 0.86 | 0.81 | 0.56 | 0.41 |
| Total | | 4.65 | 3.81 | 4.28 | 3.30 |
| Mean | | 0.66 | 0.55 | 0.61 | 0.47 |
| Total CA | 19 | 1.78 | 0.91 | 1.57 | 0.96 |
| | 31 | 0.86 | 0.68 | 0.92 | 0.45 |
| | 32 | 0.68 | 0.62 | 0.39 | 0.53 |
| | 38 | 0.65 | 0.65 | 1.10 | 0.92 |
| | 39 ✱ | 0.73 | 0.70 | 0.57 | 0.44 |
| | 46 | 1.13 | 0.67 | 1.13 | 1.09 |
| | 54 | 0.98 | 0.96 | 0.69 | 0.56 |
| Total | | 6.81 | 5.19 | 6.37 | 4.95 |
| Mean | | 0.97 | 0.74 | 0.91 | 0.71 |

✱ See note under Table XIII, p. 62

Table XVIII

Mean Catecholamine Contents

(Constant Organ Weight)

Results expressed as ug. noradrenaline (NA) and adrenaline (A) per mg. wet weight adrenals and per g. wet weight all other organs. Each figure represents the mean of 3 rats (hearts) or 7 rats (all other organs).

| Diet | | Complete | $-B_2$ | $-B_6$ | $-B_2B_6$ |
|---|---|---|---|---|---|
| Adrenals | NA | 0.31 | 0.20 | 0.30 | 0.24 |
| | A | 0.66 | 0.55 | 0.61 | 0.47 |
| | Total | 0.97 | 0.75 | 0.91 | 0.71 |
| Brain | NA | 0.62 | 0.61 | 0.48 | 0.63 |
| | A | 0.13 | 0.12 | 0.11 | 0.12 |
| | Total | 0.75 | 0.73 | 0.59 | 0.75 |
| Liver | NA | 0.70 | 0.61 | 0.82 | 0.71 |
| | A | 0.28 | 0.20 | 0.38 | 0.22 |
| | Total | 0.98 | 0.81 | 1.20 | 0.93 |
| Heart | NA | 1.76 | 1.45 | 2.18 | 2.73 |
| | A | 0.57 | 0.56 | 0.96 | 0.62 |
| | Total | 2.33 | 2.01 | 3.14 | 3.35 |
| Spleen | NA | 1.20 | 2.15 | 1.61 | 2.91 |
| | A | 0.40 | 0.25 | 0.49 | 0.55 |
| | Total | 1.60 | 2.40 | 2.10 | 3.46 |

The results presented in terms of constant organ weight do not appreciably alter the conclusions drawn from a consideration of the whole organ contents. In the case of brain, the small decrease in catecholamine content observed in the deficiency states in Table VI are not apparent when the results are expressed on a per gram basis, the values being relatively consistent in each of the four dietary categories. On the other hand, the gram contents of the catecholamines in spleen markedly increase in the deficiency states as compared with the vitamin supplemented controls. The greatest increase is seen in the double deficiency state and the second largest in riboflavin deficiency. $B_6$-deficiency displays the smallest increase over the controls. A glance at Table XII reveals that the mean weights of the spleens and the increase in catecholamine contents vary inversely. Therefore the apparent increases in the concentrations of adrenaline and noradrenaline in the spleen are reflections of the weight changes accompanying vitamin deficiency, but the amounts found are independent of the deficiency itself. The total amount of splenic catecholamines in the animals of this experiment appears to be regulated by a mechanism which is quantitatively relatively constant despite variations in organ size, body weight (Table XIX) or pyridoxine or riboflavin deficiency. The values for heart also appear to reflections of the weight changes.

In liver, the two groups lacking riboflavin have less of each of the catecholamines than do their corresponding groups supplied with vitamin $B_2$, that is, $-B_2$ compared with the Complete group and $-B_2B_6$ compared with $-B_6$. This is in spite of the fact that each of the $B_2$-deficient groups possess much smaller livers than do their corresponding control groups. Therefore, it appears that riboflavin deficiency causes a very real decrease in liver ability to synthesize adrenaline and noradrenaline.

The decrease in catecholamine contents of the adrenals brought about by riboflavin deficiency as observed in the whole organ is also apparent when the results are expressed in terms of constant organ weight. And, again, there is little reduction of these amines in the $B_6$-deficient group as compared with those supplemented with the vitamin. There is probably little justification for expressing the adrenal results on a constant organ weight basis as the adrenals are, in effect, a double organ. The cortex differs histologically from the medulla and serves different functions. It is reasonable to assume that the two may not react in the same fashion to the stress imposed by vitamin deficiencies.

Table XIX gives a summary of the body weights of the animals in the various dietary categories at the time of sacrifice, and Table XX presents the mean organ content of adrenaline and noradrenaline per 100 g. body weight. The amounts of the catecholamines found in the

various organs appear to be independent of the body weight of the animal. Making this assumption, the results are consistent with those presented in Table XI.

From Table XIX it can be seen that the various deficiency diets had a pronounced effect on the terminal body weight of the animal. Riboflavin deficiency had a greater effect in weight reduction than did pyridoxine deficiency, although in this deficiency the weight reduction also was marked. When the average change in body weight per day was treated for significance by analysis of variance, it was found that all groups were significantly different from each other at greater than the 99% level of confidence.

Table XIX

Body Weights (in Grams) at time of Sacrifice

A

| Days on Diet | Diet: Complete | -$B_2$ | -$B_6$ | -$B_2B_6$ |
|---|---|---|---|---|
| 19 | 175 | 101 | 150 | 95 |
| 31 | 293 | 124 | 160 | 116 |
| 32 | 288 | 152 | 183 | 118 |
| 38 | 282 | 131 | 195 | 123 |
| 39 * | 334 | 128 | 184 | 144 |
| 46 | 310 | 96 | 201 | 102 |
| 54 | 385 | 166 | 212 | 148 |
| Sum | 2067 | 898 | 1285 | 846 |
| Mean | 295 | 128 | 184 | 121 |

* See note under Table XIII, p. 62

B Analysis of variance for average body weight changes per day of rats in Vitamin $B_2$, $B_6$ deficiency experiment.

| Sources of Variation | D.F. | Mean Square | F | Significance |
|---|---|---|---|---|
| Total | 31 | - | - | - |
| Between diets | 3 | 38.162140 | 150.52 | <1% |
| Between -$B_2$, +$B_2$ | 1 | 78.882392 | 311.14 | <1% |
| Between -$B_6$, +$B_6$ | 1 | 19.709142 | 77.74 | <1% |
| Interaction | 1 | 15.876887 | 62.62 | <1% |
| Between replicates | 7 | 0.471084 | 1.86 | >5% |
| Remainder | 21 | 0.253531 | (1) | |

In the analysis of body weight changes one more replicate of four animals was included which were omitted from the reports of analytical values. Hence, the degrees of freedom are 31 for total and 7 for between replicates.

Table XX

Mean Catecholamine Contents
(Constant Body Weight)

Results expressed as µg. noradrenaline (NA) and adrenaline (A) per 100 g. body weight. Each figure represents the mean of 3 rats (hearts) or 7 rats (all other organs).

| Diet | | Complete | $-B_2$ | $-B_6$ | $-B_2B_6$ |
|---|---|---|---|---|---|
| Adrenals | NA | 4.20 | 4.87 | 5.15 | 6.07 |
| | A | 7.26 | 13.52 | 10.11 | 12.27 |
| | Total | 11.46 | 18.39 | 15.26 | 18.34 |
| Brain | NA | 0.39 | 0.78 | 0.54 | 0.85 |
| | A | 0.09 | 0.15 | 0.11 | 0.15 |
| | Total | 0.48 | 0.93 | 0.65 | 1.00 |
| Liver | NA | 2.72 | 2.57 | 3.21 | 2.60 |
| | A | 1.09 | 0.82 | 1.45 | 8.16 |
| | Total | 3.81 | 3.39 | 4.66 | 10.76 |
| Heart | NA | 0.72 | 0.90 | 0.75 | 1.18 |
| | A | 0.23 | 0.22 | 0.32 | 0.25 |
| | Total | 0.95 | 1.12 | 1.07 | 1.43 |
| Spleen | NA | 0.29 | 0.49 | 0.33 | 0.63 |
| | A | 0.10 | 0.11 | 0.11 | 0.14 |
| | Total | 0.39 | 0.60 | 0.44 | 0.77 |

## Experiment No. 3. The effect of Thiamine Deficiency on the Adrenaline and Noradrenaline Content of Rat Adrenal Glands.

The effect of thiamine-deficiency upon the catecholamine contents of adrenals has not been extensively investigated. Goodall (74) has obtained some evidence that thiamine deficiency reduces the adrenaline content of the rat adrenal, although little effect was observed on the noradrenaline content. In this work, however, the control animals were fed an unspecified diet described as "normal" by the author whereas the experimental animals were fed a synthetic diet; thus the results were of limited value.

Enzymes making use of thiamine as cofactor are not known to occur in any of the reactions by which the catecholamines are synthesized. However, thiamine deficiency produces marked physiological changes in the organism which might very well be reflected in modified amounts of these amines in the organs of the body. It was of interest, therefore, to investigate the problem of thiamine deficiency in relation to catecholamine biosynthesis in the adrenals.

The adrenal glands used in this experiment were obtained from animals prepared for another experimental procedure for which $B_1$-deficient animals were required. The writer is deeply indebted to Miss Edith Townsend of this laboratory for making the adrenals available for the present study. Because homogeneous groups of both the

control and the vitamin deficient rats were also tryptophan deficient it was possible to determine at the same time whether this amino acid influences catecholamine biosynthesis. It has been shown by Scott (113) that the histological appearance of the adrenal cortex is affected by tryptophan deficiency; he was, however, unable to detect any change in the appearance of the medulla.

The study to be reported in this section was actually carried out in the form of three experiments. In the first two of these only the total catecholamines at pH 6.0 were measured. The results of these two are expressed as total catecholamine in terms of their noradrenaline equivalent in micrograms.

In the three experiments of this section (designated Experiments A, B and C) the rats were all fed a basal diet similar to Diet No. 1 (p. 36). In Experiment C, the diet was Diet No. 1 itself, however in A and B, it was modified by replacing the alcohol-extracted casein with acid hydrolysed casein in order to render the diet free of tryptophan. Vitamin $B_1$ was omitted from the mixture as well. In the first two experiments twelve male albino rats of the Sprague-Dawley strain were randomly distributed into four groups. The first group were given the basal diet without further supplement; the second received additional thiamine; the third, a supplement of tryptophan;

and the fourth received both thiamine and tryptophan as supplements. The animals of Experiment A were sacrificed over periods of time ranging from 11 to 13 days, and in Experiment B from 13 to 28 days.

The results of Experiments A and B are presented in Tables XXI and XXII.

Table XXI

Effect of Vitamin $B_1$ Deficiency on Adrenal Catecholamine Content

Experiment A

µg. Noradrenaline equivalent per pair adrenals

| Diet Supplement: | | None | $B_1$ | Trypt | $B_1$ + Trypt |
|---|---|---|---|---|---|
| Days on Diet: | 13 | 20.3 | 20.1 | 15.8 | 19.1 |
| | 12 | 14.7 | 19.6 | 16.4 | 11.8 |
| | 11 | 11.5 | 16.1 | 12.6 | 18.7 |
| | Mean: | 15.5 | 18.6 | 14.9 | 16.5 |
| Adrenal Wt. (mg.) | 13 | 12.8 | 14.8 | 13.6 | 19.6 |
| | 12 | 12.7 | 14.8 | 16.8 | 16.0 |
| | 11 | 14.5 | 15.6 | 20.3 | 18.2 |
| | Mean: | 13.3 | 15.1 | 16.9 | 17.9 |
| Body Wt. (g.) | 13 | 98 | 89 | 139 | 158 |
| | 12 | 100 | 97 | 120 | 140 |
| | 11 | 93 | 92 | 147 | 147 |
| | Mean: | 97 | 93 | 135 | 148 |

Table XXII

Effect of Vitamin $B_1$ Deficiency on Adrenal Catecholamine Content

Experiment B

µg. Noradrenaline equivalent per pair adrenals

| Diet Supplement: | | None | $B_1$ | Trypt | $B_1$ + Trypt |
|---|---|---|---|---|---|
| Days on Diet | 24 | 25.4 | 29.9 | 31.5 | 24.1 |
| | 13 | 11.7 | 16.2 | 13.8 | 11.3 |
| | 28 | 29.9 | 26.8 | 28.6 | 28.2 |
| | Mean: | 22.3 | 24.3 | 24.6 | 21.2 |
| Adrenal Wt. (mg.) | 24 | 17.2 | 18.3 | 35.0 | 24.1 |
| | 13 | 13.2 | 10.7 | 18.0 | 15.6 |
| | 28 | 19.3 | 21.4 | 25.8 | 24.3 |
| | Mean: | 16.6 | 16.8 | 26.3 | 21.3 |
| Body Wt. (g.) | 24 | 58 | 58 | 102 | 153 |
| | 13 | 85 | 68 | 110 | 112 |
| | 28 | 63 | 61 | 114 | 166 |
| | Mean: | 69 | 62 | 109 | 144 |

An inspection of the above tables reveals that six of the twelve rats in each experiment were on diets devoid of thiamine and the remaining six were on diets containing $B_1$. Likewise, the animals were split six and six with regard to tryptophan-free and -supplemented diets. Table XXIII summarizes the results of these two experiments with respect to both thiamine and tryptophan.

Table XXIII

Effect of Thiamine and of Tryptophan on Adrenal Catecholamine Contents

| Experiment: | A | | B | |
|---|---|---|---|---|
| No. of rats making up the mean | 6 | 6 | 6 | 6 |
| | $-B_1$ | $B_1$ | $-B_1$ | $B_1$ |
| | 15.2 | 17.6 | 23.5 | 22.8 |
| | -Trypt | Trypt | -Trypt | Trypt |
| | 17.1 | 15.7 | 23.3 | 22.9 |

The results of Experiment C are presented in Table XXIV. Ten animals in all were used, five on thiamine-deficient diet and the remainder on diets supplemented with the vitamin. Both adrenaline and noradrenaline were estimated. The animals were sacrificed after 21 and 23 days on the diets.

Table XXIV

Effect of Thiamine Deficiency on the Adrenaline and Noradrenaline Content of Rat Adrenals

µg. per pair adrenals

| Days on Diet | $-B_1$ NA | $-B_1$ A | $-B_1$ Total | $B_1$ NA | $B_1$ A | $B_1$ Total |
|---|---|---|---|---|---|---|
| 21 | 4.87 | 12.97 | 17.84 | 4.69 | 14.39 | 19.08 |
| 21 | 6.24 | 18.18 | 24.42 | 6.56 | 17.25 | 23.81 |
| 23 | 5.03 | 13.75 | 18.78 | 7.50 | 16.80 | 24.30 |
| 23 | 4.15 | 12.73 | 16.88 | 6.25 | 17.23 | 23.48 |
| 23 | 5.73 | 15.40 | 21.12 | 8.05 | 14.78 | 22.83 |
| Mean | 5.20 | 14.61 | 19.81 | 6.61 | 16.09 | 22.70 |

Adrenal Weights (mg.)

| Days on Diet | $-B_1$ | $B_1$ |
|---|---|---|
| 21 | 19.8 | 30.0 |
| 21 | 25.6 | 30.4 |
| 23 | 30.8 | 28.6 |
| 23 | 28.0 | 29.4 |
| 23 | 31.6 | 32.0 |

Overall Body Weight Change (g.)

| | | |
|---|---|---|
| 21 | 4 | 74 |
| 21 | 1 | 93 |
| 23 | 1 | 88 |
| 23 | -11 | 90 |
| 23 | -6 | 95 |

The results of Experiments A and B indicate that tryptophan deficiency does not influence the amounts of adrenal catecholamines.

The degree of tryptophan deficiency can be observed in Tables XXI and XXII (Experiments A and B). When the mean body weights in columns $B_1$ and $B_1$ + Trypt are compared, there is seen to be a marked loss of weight in the tryptophan deficient animals. The same thing is observed on comparison of the mean weights in columns None and Trypt. Loss of body weight due to thiamine deficiency becomes apparent only in the presence of tryptophan (columns Trypt and $B_1$ + Trypt, and columns None and $B_1$).

Thiamine and tryptophan deficiencies both tended to decrease the adrenal weight as observed in Tables XXI and XXII.

In all three experiments thiamine deficiency likewise had no effect on adrenal catecholamine content, As seen by the overall changes in body weight of the two groups of Experiment C, the animals on the $B_1$-deficient diet suffered marked deficiency although the neurological symptoms of beri beri did not appear. The present experiment which employed adequate controls answers the question posed by that of Goodall.

Experiment No. 4. Amino Acid Deficiency Experiments.

The experiments carried out under this heading were designed to investigate the effect of dietary deficiency of natural precursors on the catecholamine content of rat organs. A second objective was to determine if some of the precursor analogues, as phenylserine and m-tyrosine, would support catecholamine biosynthesis in the phenylalanine-tyrosine deficient rat and thus provide evidence for an alternate route of synthesis.

The animals used were male, albino rats of the Sprague-Dawley strain and weighed initially between 90 and 100 grams. They were fed Diets 3A and 3B which were modelled after Armstrong and Lewis (94), and which were devoid of phenylalanine and tyrosine. The first two experiments of this series (Experiments A and B) are from the unpublished results of Dr. T.L. Sourkes to whom the writer is deeply grateful for permission to use them to introduce his own studies. In Experiments A and B, total catecholamines were measured; in the later three (Experiments C, D and E) both noradrenaline and adrenaline were estimated. The results of all five of these experiments are reported in Tables XXV through XXXV.

Experiments A and B. No pretreatment was administered to the rats of Experiment A, however those of Experiment B all received convulsive doses of insulin in order to deplete the adrenals of adrenaline before being placed on

their respective diets. The animals were brought out of the insulin convulsions by injections of glucose.

In each experiment nine rats were divided into three groups on a descending weight basis. The members of each group were then randomly distributed among three diets: Diet 3B for the control animals, Diet 3B plus 2% phenylalanine and Diet 3B plus 2% tyrosine for the other two groups. The animals remained on their respective diets for 2-3 weeks before being sacrificed. The mean values of the results of these two experiments are presented in Table XXV.

Table XXV

Effect of Phenylalanine and Tyrosine Deficiency on the catecholamine content of rat adrenals.

| Experiment: | A | B |
|---|---|---|
| Pretreatment: | None | Insulin |
| Time on Diet: | 2-3 Weeks | 2-3 Weeks |
| Supplement to Basal Diet | µg. CA / pair Adrenals | |
| None | 14.6 * | 11.4 |
| Phenylalanine | 13.7 | 13.8 |
| Tyrosine | 14.9 | 14.2 |

* Each figure represents a mean of 3 rats.

Experiment C. Both noradrenaline and adrenaline estimations were made on adrenals, spleen and liver in this experiment. Fifteen rats of the same type and weight

range as in the previous experiments were used. The animals were all pretreated by being placed on Diet 3A for a period of two days; following this they were fasted overnight to diminish the stores of liver glycogen. After the overnight fast, all the rats were given convulsive doses of insulin. The insulin dosage was based on the report of Burn, Hutcheon and Parker (78) and amounted to 0.4 unit of insulin per rat, since the animals were of fairly uniform weight, or 4 units per Kilogram. A few rats that became comatose but did not convulse at this dose level were given an additional 0.16 to 0.32 unit. After one or two severe convulsions, the animals were brought out of this state by injecting 4.0 ml. of 5.4% glucose (isotonic solution), and orally administering 20% glucose when they were alert enough to receive it. After all animals had recovered from the convulsive condition, they were divided into three groups of five on a descending weight basis and the members of each group were then randomly distributed among five diets. The control animals of each group received Diet 3A. The remaining four groups received the same basal diet, but supplemented with one of the following: 1.5% DL-phenylalanine, 1.5% L-tyrosine, 1.5% L-DOPA, or 2% DL-threo-B-phenylserine. Two per cent sucrose was added to the control diet and 0.5% sucrose was added to all the rest except the phenylserine supplemented one in order to make all the diets uniform.

DOPA was added to the basal diet daily just before feeding time in order to minimize loss due to oxidation. Ordinarily no obvious conversion of DOPA to melanin occurred, however, on one or two occasions when an animal had urinated into his food box, the food remaining next day was tinged with dark purple. In all such instances the food box was thoroughly cleaned and fresh food added.

All the animals were sacrificed by decapitation seven days after insulin treatment. This length of time was chosen because the half-life of the adrenal contents has been reported to be of the order of one week (114 ).

The results of Experiment C are given in Tables XXVI through XXIX.

Table XXVI

Adrenals

Adrenaline and Noradrenaline contents of Adrenals of rats on Phenylalanine-Tyrosine Deficient Diets.

Insulin Pretreatment

µg. NA per pair of Adrenals

| Supplement: | None | Pa* | Tyr | DOPA | Ph.Ser. |
|---|---|---|---|---|---|
| | 0.89 | 3.03 | 2.43 | 4.19 | 1.34 |
| | 1.86 | 2.41 | 5.09 | 3.32 | 2.71 |
| | 7.29 | 3.71 | 5.72 | 1.80 | 7.44 |
| Sum: | 10.04 | 9.15 | 13.24 | 9.31 | 11.49 |
| Mean: | 3.35 | 3.05 | 4.41 | 3.10 | 3.83 |

µg. A per pair of Adrenals

| Supplement: | None | Pa | Tyr | DOPA | Ph.Ser. |
|---|---|---|---|---|---|
| | 6.87 | 13.33 | 14.26 | 19.73 | 12.26 |
| | 9.57 | 9.06 | 16.79 | 10.10 | 9.70 |
| | 14.46 | 8.84 | 9.40 | 6.78 | 8.17 |
| Sum: | 30.90 | 31.23 | 40.45 | 36.61 | 30.13 |
| Mean: | 10.30 | 10.41 | 13.48 | 12.20 | 10.04 |

µg. Total per pair of Adrenals

| Supplement: | None | Pa | Tyr | DOPA | Ph.Ser. |
|---|---|---|---|---|---|
| | 7.76 | 16.36 | 16.69 | 23.92 | 13.60 |
| | 11.33 | 11.47 | 21.88 | 13.42 | 12.41 |
| | 21.75 | 12.55 | 15.12 | 8.58 | 15.61 |
| Sum: | 40.94 | 40.38 | 53.69 | 45.92 | 41.62 |
| Mean: | 13.65 | 13.46 | 17.90 | 15.31 | 13.87 |

* Abbreviations: Pa, phenylalanine; Tyr, tyrosine; DOPA, dihydroxyphenylalanine; Ph.Ser, phenylserine.

Table XXVII

Liver

Adrenaline and Noradrenaline contents of Livers of Rats on Phenylalanine-Tyrosine Deficient Diets.

Insulin Pretreatment

µg. NA per whole Liver

| Supplement: | None | Pa | Tyr | DOPA | Ph.Ser. |
|---|---|---|---|---|---|
| | 1.25 | 1.08 | 0.98 | 1.11 | 1.07 |
| | 1.02 | 1.61 | 1.54 | 1.22 | 1.08 |
| | 1.32 | 1.58 | 1.39 | 1.01 | 1.67 |
| Sum: | 3.59 | 4.27 | 3.91 | 3.34 | 3.82 |
| Mean: | 1.20 | 1.42 | 1.30 | 1.11 | 1.27 |

µg. A per whole Liver

| Supplement: | None | Pa | Tyr | DOPA | Ph.Ser. |
|---|---|---|---|---|---|
| | 0.35 | 0.39 | 0.29 | 0.29 | 0.31 |
| | 0.24 | 0.41 | 0.33 | 0.30 | 0.28 |
| | 0.26 | 0.36 | 0.30 | 0.20 | 0.38 |
| Sum: | 0.85 | 1.16 | 0.92 | 0.79 | 0.97 |
| Mean: | 0.28 | 0.39 | 0.31 | 0.26 | 0.32 |

µg. Total per whole Liver

| Supplement: | None | Pa | Tyr | DOPA | Ph.Ser. |
|---|---|---|---|---|---|
| | 1.60 | 1.47 | 1.27 | 1.40 | 1.38 |
| | 1.26 | 2.02 | 1.87 | 1.52 | 1.36 |
| | 1.58 | 1.94 | 1.69 | 1.21 | 2.05 |
| Sum: | 4.44 | 5.43 | 4.83 | 4.13 | 4.79 |
| Mean: | 1.48 | 1.81 | 1.61 | 1.37 | 1.59 |

Table XXVIII

Spleen

Adrenaline and Noradrenaline contents of Spleens of Rats on Phenylalanine-Tyrosine Deficient Diets.

µg. NA per whole Spleen

| Supplement: | None | Pa | Tyr | DOPA | Ph.Ser. |
|---|---|---|---|---|---|
| | 0.43 | 0.58 | 0.37 | 0.42 | 0.51 |
| | 0.68 | 0.74 | 0.76 | 0.61 | 0.35 |
| | 0.77 | 0.87 | 0.60 | 0.67 | 0.58 |
| Sum: | 1.88 | 2.19 | 1.73 | 1.70 | 1.74 |
| Mean: | 0.63 | 0.73 | 0.58 | 0.57 | 0.58 |

µg. A per whole Spleen

| Supplement: | None | Pa | Tyr | DOPA | Ph.Ser. |
|---|---|---|---|---|---|
| | 0.23 | 0.26 | 0.24 | 0.24 | 0.26 |
| | 0.22 | 0.25 | 0.21 | 0.27 | 0.26 |
| | 0.28 | 0.22 | 0.23 | 0.22 | 0.26 |
| Sum: | 0.73 | 0.73 | 0.68 | 0.73 | 0.78 |
| Mean: | 0.24 | 0.24 | 0.23 | 0.24 | 0.26 |

µg. Total per whole Spleen

| Supplement: | None | Pa | Tyr | DOPA | Ph.Ser. |
|---|---|---|---|---|---|
| | 0.66 | 0.84 | 0.61 | 0.66 | 0.77 |
| | 0.90 | 0.99 | 0.97 | 0.88 | 0.91 |
| | 1.05 | 1.09 | 0.83 | 0.89 | 0.84 |
| Sum: | 2.61 | 2.92 | 2.41 | 2.43 | 2.52 |
| Mean: | 0.87 | 0.97 | 0.80 | 0.81 | 0.84 |

Table XXIX

Organ Weights

Organ Weights of Rats on Phenylalanine-Tyrosine Deficient Diets.

Insulin Pretreatment

Adrenals (mg.)

| Supplement: | None | Pa | Tyr | DOPA | Ph.Ser. |
|---|---|---|---|---|---|
| | 31.2 | 28.8 | 22.8 | 20.2 | 19.6 |
| | 26.0 | 31.0 | 28.6 | 29.6 | 26.3 |
| | 29.2 | 21.4 | 30.2 | 21.6 | 27.8 |
| Sum: | 86.4 | 81.2 | 81.6 | 71.4 | 73.7 |
| Mean: | 28.8 | 27.1 | 27.2 | 23.8 | 24.6 |

Liver (g.)

| Supplement: | None | Pa | Tyr | DOPA | Ph.Ser. |
|---|---|---|---|---|---|
| | 3.40 | 6.56 | 2.91 | 3.42 | 3.13 |
| | 3.00 | 6.11 | 2.91 | 3.04 | 3.03 |
| | 3.17 | 4.55 | 2.53 | 2.59 | 2.45 |
| Sum: | 9.57 | 17.22 | 8.35 | 9.05 | 8.61 |
| Mean: | 3.19 | 5.74 | 2.78 | 3.02 | 2.87 |

Spleen (g.)

| Supplement: | None | Pa | Tyr | DOPA | Ph.Ser. |
|---|---|---|---|---|---|
| | 0.26 | 0.36 | 0.30 | 0.37 | 0.23 |
| | 0.23 | 0.35 | 0.17 | 0.21 | 0.22 |
| | 0.30 | 0.37 | 0.27 | 0.28 | 0.27 |
| Sum: | 0.79 | 1.08 | 0.74 | 0.86 | 0.72 |
| Mean: | 0.26 | 0.36 | 0.25 | 0.29 | 0.24 |

Experiment D. Fifteen male, albino rats of the Sprague-Dawley strain, weighing between 90 g. and 100 g. were placed on Diet 3B overnight. Next morning twelve of these animals received 5 mg. per Kg. reserpine (Serpasil, Ciba) via the tail vein and the remaining three received a like volume of the solvent in which the reserpine was dissolved (Serpasil Placebo, Ciba). This dose level is based on a report by Carlsson et al. (45). After injection, the animals were placed on the control diet lacking in phenylalanine and tyrosine. After 24 hours the twelve reserpinized animals were divided into three groups of four on a descending weight basis and the members of each group were randomly distributed among four diets: one remained on the control diet, the other rats received Diet 3B plus supplements of 1.5% DL-phenylalanine, 1.5% L-tyrosine and 2% DL-threo-B-phenylserine. The placebo animals also received the control diet. In order to render the diets uniform, 2% sucrose was added to Diet 3B for the controls and 0.5% sugar added to the phenylalanine and tyrosine diets.

The animals receiving reserpine became comatose, there was marked ptosis, and generally they had diarrhea for one or two days following the injection. Frequently there was some bleeding about the nose. The animals receiving the placebo injection showed none of these signs and remained alert. The animals were largely recovered

after 24 to 48 hours. Because the reserpinized animals were comatose and ate very little during the first 24 hours there was a delay of a day before commencing feeding the supplemented diets. All animals were sacrificed eleven days after reserpine injections and ten days after being placed on their supplemented diets.

The results of this experiment are found in Tables XXX through XXXI.

Table XXX

Adrenals

Adrenaline and Noradrenaline contents of Adrenals of Rats on Phenylalanine-Tyrosine Deficient Diets.

Reserpine Pretreatment

µg. NA per pair of adrenals

| PC* | RC | PA | Tyr | Ph.Ser. |
|---|---|---|---|---|
| 7.83 | 8.77 | 7.68 | 10.14 | 4.86 |
| 4.08 | 6.27 | 8.34 | 7.71 | 6.17 |
| 5.41 | 4.64 | 6.22 | 7.18 | 4.80 |
| Sum:17.32 | 19.68 | 22.24 | 25.03 | 15.83 |
| Mean: 5.77 | 6.56 | 7.41 | 8.34 | 5.28 |

µg. A per pair of adrenals

| PC | RC | PA | Tyr | Ph.Ser. |
|---|---|---|---|---|
| 9.29 | 11.41 | 9.72 | 11.73 | 11.76 |
| 4.87 | 8.81 | 9.03 | 12.86 | 8.39 |
| 6.61 | 8.27 | 7.88 | 11.80 | 8.85 |
| Sum:20.77 | 28.49 | 26.63 | 36.39 | 29.00 |
| Mean: 6.92 | 9.50 | 8.88 | 12.13 | 9.67 |

µg. Total per pair of adrenals

| PC | RC | PA | Tyr | Ph.Ser. |
|---|---|---|---|---|
| 17.12 | 20.18 | 17.40 | 21.87 | 16.62 |
| 8.95 | 15.08 | 17.37 | 20.57 | 19.56 |
| 12.02 | 12.91 | 14.10 | 18.98 | 13.65 |
| Sum:38.09 | 48.17 | 48.87 | 61.42 | 44.83 |
| Mean:12.69 | 16.06 | 16.29 | 20.47 | 14.95 |

* Abbreviations: PC, placebo control; RC, reserpine control; PA, phenylalanine; Tyr, tyrosine; Ph.Ser., phenylserine.

Table XXXI

Brain

Adrenaline and Noradrenaline contents of Brains of Rats on Phenylalanine-Tyrosine Deficient Diets.

Reserpine Pretreatment

µg. NA per whole brain

| | PC | RC | PA | Tyr | Ph.Ser. |
|---|---|---|---|---|---|
| | 0.81 | 0.33 | 0.39 | 0.46 | 0.42 |
| | 0.89 | 0.35 | 0.47 | 0.67 | 0.29 |
| | 1.00 | 0.66 | 0.81 | 0.66 | 0.31 |
| Sum: | 2.70 | 1.34 | 1.67 | 1.79 | 1.02 |
| Mean: | 0.90 | 0.45 | 0.56 | 0.60 | 0;34 |

µg. NA per whole brain

| | PC | RC | PA | Tyr | Ph.Ser. |
|---|---|---|---|---|---|
| | 0.04 | 0.06 | 0.05 | 0.10 | 0.07 |
| | 0.10 | 0.05 | 0.01 | 0.04 | 0.10 |
| | 0.05 | 0.08 | 0.06 | 0.07 | 0.02 |
| Sum: | 0.19 | 0.19 | 0.12 | 0.21 | 0.19 |
| Mean: | 0.06 | 0.06 | 0.04 | 0.07 | 0.06 |

µg. Total per whole brain

| | PC | RC | PA | Tyr | Ph.Ser. |
|---|---|---|---|---|---|
| | 0.85 | 0.39 | 0.44 | 0.56 | 0.49 |
| | 0.99 | 0.40 | 0.48 | 0.71 | 0.39 |
| | 1.05 | 0.74 | 0.87 | 0.73 | 0.33 |
| Sum: | 2.89 | 1.53 | 1.79 | 2.00 | 1.21 |
| Mean: | 0.96 | 0.51 | 0.60 | 0.67 | 0.40 |

Experiment E. Fifteen rats of the usual sex and strain, weighing between 90 and 100 grams were placed overnight on Diet 3B. The following morning twelve of these animals were given reserpine (Serpasil, Ciba) at a dose level of 5 mg. per Kg. by tail vein. The remaining three were given a similar injection of the reserpine solvent (Serpasil Placebo, Ciba). All animals continued on the same diet for three days and were given a second injection of reserpine or placebo at the end of this time. After the second injection, the usual randomization procedure was carried out and the animals were placed on the experimental diets. In this experiment all the rats receiving the placebo injection, as well as one member randomly chosen from each of the remaining groups, received the control diet (Diet 3B plus 1.5% sucrose). Other supplements to the basal diet were 1.5% DL-tyrosine, 1.5% DL-DOPA and 1.5% DL-m-tyrosine. All animals were sacrificed six days after the last injection of reserpine.

The results of this experiment are presented in Tables XXXII and XXXIII.

Table XXXII

Adrenals

Adrenaline and Noradrenaline contents of Adrenals of Rats on Phenylalanine-Tyrosine Deficient Diets.

Reserpine Pretreatment

µg. NA per pair of adrenals

| PC* | RC | Tyr | DOPA | m-Tyr. |
|---|---|---|---|---|
| 3.06 | 4.50 | 3.49 | 2.48 | 2.35 |
| 4.15 | 3.98 | 4.11 | 1.43 | 2.80 |
| 2.99 | 4.76 | 5.35 | 5.13 | 2.56 |
| Sum:10.20 | 13.24 | 12.95 | 9.04 | 7.71 |
| Mean: 3.40 | 4.41 | 4.32 | 3.01 | 2.57 |

µg. A per pair of adrenals

| PC | RC | Tyr | DOPA | m-Tyr. |
|---|---|---|---|---|
| 6.08 | 8.88 | 7.90 | 6.56 | 4.98 |
| 6.06 | 7.45 | 9.74 | 3.18 | 6.13 |
| 6.66 | 9.46 | 9.42 | 6.47 | 5.79 |
| Sum:18.80 | 25.79 | 27.06 | 16.21 | 16.90 |
| Mean: 6.27 | 8.60 | 9.02 | 5.40 | 5.63 |

µg. Total per pair of adrenals

| PC | RC | Tyr | DOPA | m-Tyr. |
|---|---|---|---|---|
| 9.14 | 13.38 | 11.39 | 9.04 | 7.33 |
| 10.21 | 11.43 | 13.85 | 4.61 | 8.93 |
| 9.65 | 14.22 | 14.77 | 11.60 | 8.35 |
| Sum:29.00 | 39.03 | 40.01 | 25.25 | 24.61 |
| Mean: 9.67 | 13.01 | 13.34 | 8.41 | 8.20 |

* Abbreviations: PC, placebo control; RC, reserpine control; Tyr. tyrosine; DOPA, dihydroxyphenylalanine; m-Tyr, m-tyrosine.

Table XXXIII

Brain

Adrenaline and Noradrenaline contents of Brains of Rats on Phenylalanine-Tyrosine Deficient Diets.

Reserpine Pretreatment

µg. NA per whole brain

| | PC | RC | Tyr | DOPA | m-Tyr. |
|---|---|---|---|---|---|
| | 0.62 | 0.21 | 0.20 | 0.19 | 0.10 |
| | 0.66 | 0.21 | 0.31 | 0.13 | 0.14 |
| | 0.77 | 0.27 | 0.32 | 0.15 | 0.22 |
| Sum: | 2.05 | 0.69 | 0.83 | 0.47 | 0.46 |
| Mean: | 0.68 | 0.23 | 0.28 | 0.18 | 0.15 |

µg. A per whole brain

| | PC | RC | Tyr | DOPA | m-Tyr. |
|---|---|---|---|---|---|
| | 0.02 | 0.01 | 0;01 | 0.00 | 0.01 |
| | 0.02 | 0.01 | 0.01 | 0.00 | 0.01 |
| | 0.03 | 0.02 | 0.01 | 0.01 | 0.01 |
| Sum: | 0.07 | 0.04 | 0.03 | 0.01 | 0.03 |
| Mean: | 0.02 | 0.01 | 0.01 | 0.003 | 0.01 |

µg. Total per whole brain

| | PC | RC | Tyr | DOPA | m-Tyr. |
|---|---|---|---|---|---|
| | 0.64 | 0.22 | 0.21 | 0.19 | 0.11 |
| | 0.68 | 0.22 | 0.32 | 0.13 | 0.15 |
| | 0.80 | 0.29 | 0.33 | 0.16 | 0.23 |
| Sum: | 2.12 | 0.73 | 0.86 | 0.48 | 0.49 |
| Mean: | 0.70 | 0.24 | 0.29 | 0.16 | 0.16 |

Summary: Catecholamine content of Adrenals of Rats on Diets Deficient in Phenylalanine and Tyrosine.

| Experiment | A | B | C | | | D | | | E | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Pretreatment | None | Insulin | Insulin | | | Reserpine | | | Reserpine | | |
| Length of time on diet | 2-3 Weeks | 2-3 Weeks | 7 days after insulin | | | 11 days after reserpine | | | 5 days after reserpine | | |
| Item measured, µg. / pair of adrenals | Total CA | Total CA | NA | A | Total | NA | A | Total | NA | A | Total |
| Supplement to basal Diet | | | | | | | | | | | |
| None | 14.6 | 11.4 | 3.35 | 10.30 | 13.65 | 6.56 | 9.50 | 16.06 | 4.41 | 8.60 | 13.01 |
| Phenylalanine | 13.7 | 13.8 | 3.05 | 10.41 | 13.46 | 7.41 | 8.88 | 16.29 | | | |
| Tyrosine | 14.9 | 14.2 | 4.41 | 13.48 | 17.89 | 8.34 | 12.13 | 20.47 | 4.32 | 9.02 | 13.34 |
| DOPA | | | 3.10 | 12.21 | 15.31 | | | | 3.01 | 5.40 | 8.41 |
| Phenylserine | | | 3.83 | 10.04 | 13.87 | 5.28 | 9.67 | 14.95 | | | |
| m-Tyrosine | | | | | | | | | 2.57 | 5.63 | 8.20 |

Each figure represents a mean of three rats.

Table XXXIV

Summary: Catecholamine content of Organs of Rats on Diets Deficient in Phenylalanine and Tyrosine.

| Experiment | C | | | D | | | E | | |
|---|---|---|---|---|---|---|---|---|---|
| Pretreatment | Insulin | | | Reserpine | | | Reserpine | | |
| Length of time on diet | 7 days after insulin | | | 11 days after reserpine | | | 5 days after reserpine | | |
| Organ | Liver | | | Spleen | | | Brain | | |
| Item measured, µg. / whole organ<br>Supplement to basal diet | NA | A | Total | NA | A | Total | NA | A | Total |
| None | 1.20 | 0.28 | 1.48 | 0.63 | 0.24 | 0.87 | 0.45 | 0.06 | 0.51 |
| Phenylalanine | 1.42 | 0.39 | 1.81 | 0.73 | 0.21 | 0.97 | 0.56 | 0.04 | 0.60 |
| Tyrosine | 1.30 | 0.31 | 1.61 | 0.58 | 0.23 | 0.81 | 0.60 | 0.07 | 0.67 |
| DOPA | 1.11 | 0.26 | 1.37 | 0.57 | 0.24 | 0.81 | - | - | - |
| Phenylserine | 1.27 | 0.32 | 1.59 | 0.58 | 0.26 | 0.84 | 0.34 | 0.06 | 0.40 |
| m-Tyrosine | - | - | - | - | - | - | - | - | - |

| Experiment | E | | |
|---|---|---|---|
| Pretreatment | Reserpine | | |
| Length of time on diet | 5 days after reserpine | | |
| Organ | Brain | | |
| Item measured, µg. / whole organ<br>Supplement to basal diet | NA | A | Total |
| None | 0.23 | 0.01 | 0.24 |
| Phenylalanine | - | - | - |
| Tyrosine | 0.28 | 0.01 | 0.29 |
| DOPA | 0.18 | 0.01 | 0.19 |
| Phenylserine | - | - | - |
| m-Tyrosine | 0.15 | 0.01 | 0.16 |

Each figure represents a mean of three rats.

Table XXXV

Table XXXVI

Organ Weights of Experiment C

Adrenals (mg.)

| | None | PA | Tyr | DOPA | Ph. Ser. |
|---|---|---|---|---|---|
| | 31.2 | 28.8 | 22.8 | 20.2 | 19.6 |
| | 26.0 | 31.0 | 28.6 | 29.6 | 26.3 |
| | 29.2 | 21.4 | 30.2 | 21.6 | 27.8 |
| Sum: | 86.4 | 81.2 | 81.6 | 71.4 | 73.7 |
| Mean: | 28.8 | 27.1 | 27.2 | 23.8 | 24.6 |

Liver (g.)

| | None | PA | Tyr | DOPA | Ph.Ser. |
|---|---|---|---|---|---|
| | 3.40 | 6.56 | 2.91 | 3.42 | 3.13 |
| | 3.00 | 6.11 | 2.91 | 3.04 | 3.03 |
| | 3.17 | 4.55 | 2.53 | 2.59 | 2.45 |
| Sum: | 9.57 | 17.22 | 6.35 | 9.05 | 8.61 |
| Mean: | 3.19 | 5.74 | 2.12 | 3.02 | 2.87 |

Spleen (g.)

| | None | PA | Tyr | DOPA | Ph.Ser. |
|---|---|---|---|---|---|
| | 0.26 | 0.36 | 0.30 | 0.37 | 0.23 |
| | 0.23 | 0.35 | 0.17 | 0.21 | 0.22 |
| | 0.30 | 0;37 | 0.27 | 0.28 | 0.27 |
| Sum: | 0.79 | 1.08 | 0.74 | 0.86 | 0.72 |
| Mean: | 0.26 | 0.36 | 0.25 | 0.29 | 0.24 |

Table XXXVII

Organ Weights of Experiment D

Adrenals (mg.)

| | PC | RC | PA | Tyr | Ph.Ser. |
|---|---|---|---|---|---|
| | 20.2 | 22.1 | 19.1 | 21.9 | 20.2 |
| | 16.1 | 21.6 | 18.2 | 22.1 | 24.6 |
| | 18.1 | 17.0 | 20.2 | 21.0 | 18.9 |
| Sum: | 54.4 | 60.7 | 57.5 | 65.0 | 63.7 |
| Mean: | 18.1 | 20.2 | 19.2 | 21.7 | 21.2 |

Brain (g.)

| | PC | RC | PA | Tyr | Ph.Ser. |
|---|---|---|---|---|---|
| | 1.49 | 1.55 | 1.40 | 1.51 | 1.60 |
| | 1.55 | 1.14 | 1.60 | 1.55 | 1.40 |
| | 1.55 | 1.50 | 1.60 | 1.52 | 1.45 |
| Sum: | 4.59 | 4.19 | 4.60 | 4.58 | 4.45 |
| Mean: | 1.53 | 1.40 | 1.53 | 1.53 | 1.48 |

Table XXXVIII

Organ Weights of Experiment E

Adrenals (mg.)

| | PC | RC | Tyr | DOPA | m-Tyr |
|---|---|---|---|---|---|
| | 17.8 | 22.1 | 27.0 | 25.1 | 24.0 |
| | 20.8 | 24.0 | 22.6 | 47.0 | 27.6 |
| | 20.4 | 22.3 | 26.1 | 25.0 | 23.8 |
| Sum: | 59.0 | 68.4 | 75.7 | 97.1 | 75.4 |
| Mean: | 19.7 | 22.8 | 25.2 | 32.4 | 25.1 |

Brain (g.)

| | PC | RC | Tyr | DOPA | m-Tyr |
|---|---|---|---|---|---|
| | 1.55 | 1.60 | 1.35 | 1.45 | 1.55 |
| | 1.60 | 1.45 | 1.55 | 1.50 | 1.52 |
| | 1.55 | 1.52 | 1.49 | 1.45 | 1.65 |
| Sum: | 4.70 | 4.57 | 4.39 | 4.40 | 4.72 |
| Mean: | 1.57 | 1.52 | 1.46 | 1.47 | 1.57 |

The results of the amino acid deficiency experiments are summarized in Tables XXXIV and XXXV. From Table XXXIV it is apparent that insulin tended to lower the adrenal catecholamine content of those animals not receiving dietary supplements. This is most apparent when Experiments A and B are compared. These two experiments were actually replicates of one another, carried out in parallel, the first without and the second with insulin pretreatment. Also, the rats treated with insulin gave mean values on the three diets which are in the expected sequence. Supplementing the diets with either phenylalanine or tyrosine increased the catecholamine content of the adrenals when compared with the controls, and, moreover, tyrosine supplementation brought about the greater increase. This was to be expected since tyrosine is a later intermediate in the catecholamine biosynthesis route than is phenylalanine.*

In the subsequent experiments in which the depleting agent was either insulin or reserpine, tyrosine supplementation again led to increased adrenal catecholamines. The effect of phenylalanine was not as consistent. This, however, is not necessarily surprising in view of the fact that the oxidation of phenylalanine is known to be rate limiting in several biosynthetic pathways.

The effect of supplementing the diet with DOPA was variable. In Experiment C, there was an increased

* See addendum, pg. 100 a

ADDENDUM

(Refer back to p.100)

The mean adrenal CA content of all the animals on diets supplemented with the known precursors of adrenaline are summarized in the table below. Only the total catecholamine contents are considered. When the data were analysed for statistical significance, the increase due to tyrosine was found to be significant at the 95% level of confidence. Phenylalanine did not bring about a significant increase, nor did DOPA.

Effects of Amino Acid Supplements to Phenylalanine-Tyrosine Deficient Diet.

| | Supplement | | | |
|---|---|---|---|---|
| | None | PA | Tyr. | DOPA |
| No. of Rats making up the mean | 15 | 12 | 15 | 6 |
| Mean adrenal content of total catecholamines, micrograms | 13.8 | 14.3 | 16.2 | 11.9 |

amount of catecholamines in the adrenals of animals receiving DOPA over those of the controls. On the other hand, the values were not as high as in the animals receiving tyrosine. DOPA is known to give rise to adrenaline both *in vivo* and *in vitro*. In Experiment E, DOPA appeared to be actually inhibitory toward the formation of the catecholamines. There is the possibility that spontaneous oxidation of DOPA occurred in the diet, which might account for these results. This was minimized by adding DOPA to the diets only in the amounts required for daily feeding.

Phenylserine (Experiments C and D) and *m*-tyrosine (Experiment E) were used to test for alternate pathways of catecholamine biosynthesis. Phenylserine had very little effect on the adrenal catecholamine content, in either the insulin or the reserpine pretreated rats, although the mean noradrenaline content tended to be higher than in the controls after insulin pretreatment and lower after reserpine. *m*-Tyrosine appeared to somewhat inhibit both noradrenaline and adrenaline formation in the one experiment in which it was tested using reserpinized rats.

The catecholamine contents of livers, spleens and brains were examined in Experiments C, D and E. In liver, phenylalanine and tyrosine supplementation of the diet tended to raise both the noradrenaline and adrenaline levels as compared with the controls. Phenylserine may have

increased the levels slightly and DOPA slightly decreased them.

Neither phenylalanine, tyrosine, DOPA nor phenylserine appeared to affect the catecholamine contents of the spleen.

Brain noradrenaline contents were somewhat elevated by phenylalanine and tyrosine but not by DOPA, phenylserine or m-tyrosine.

Experiment No. 5. The Influence of Methionine and Nicotinic Acid on the Proportion of Adrenaline Excreted in Urine.

Some preliminary experiments have been carried out employing methionine and nicotinic acid in an attempt to influence the methylation of noradrenaline to form adrenaline. Nicotinic acid is known to be excreted in the urine as its methyl derivative (115 ). Altschul et al. (116 ) have also shown that administration of nicotinic acid lowers the concentration of blood cholesterol, presumably by depletion of the methyl group pool. On the other hand, it has long been known that methionine is a very active methyl group donor. Therefore it appeared possible that administration of these two substances might influence the methylation of noradrenaline and that such influence might be reflected in an altered ratio of noradrenaline to adrenaline.

Three experiments were carried out. In two of these DL-methionine was administered to three healthy,

adult, male subjects and in the third, nicotinic acid was given. Twenty-four hour urine specimens were collected and analysed for noradrenaline and adrenaline.

Experiment A. In this experiment twenty-four hour specimens of urine were collected from each of three healthy, adult, male subjects on two days prior to the test day and two after administration of 5 g. DL-methionine. A placebo consisting of 5 g. DL-alanine was given on the control days and all amino acids were taken with fruit juice. The two amino acids were Merck and Company products. The results are presented in Table XXXIX, and are expressed as the per cent of total catecholamines excreted as adrenaline.

Table XXIX

DL-methionine, 5 g.

Percent of Adrenaline in Urinary Catecholamines.

| Subject: | | W | S | L | Mean |
|---|---|---|---|---|---|
| Day | Treatment | | | | |
| 1 | Control | 23.6 | 27.2 | 27.2 | 26.0 |
| 2 | Control | 27.2 | 24.2 | 23.2 | 24.9 |
| 3 | Test | 21.4 | 21.4 | 28.1 | 23.6 |
| 4 | Control | 20.2 | 24.7 | 25.1 | 23.3 |
| 5 | Control | 25.6 | 22.7 | 29.4 | 25.9 |
| Average of: | | | | | |
| | Control Days | 24.2 | 24.7 | 26.2 | 25.0 |
| | Test Days | 21.4 | 21.4 | 28.1 | 23.6 |

The results of this experiment show no very striking changes produced as a result of methionine administration. Although the mean percentage of adrenaline

excreted on the test day is lower than on the control days, this was true of only two subjects out of the three taking the amino acid. The lowered adrenaline percentage was not in accordance with the predicted result. No ready explanation presents itself. There was a possibility, however, that the dose level of methionine was too low, consequently the amount of methionine administered did not raise the body's methionine pool sufficiently to influence the methylation of noradrenaline. Therefore a second experiment was carried out in which the dose level was raised four-fold.

Experiment B. In this experiment control urines were collected on a day prior to administration of 20 g. of DL-methionine and on the day following. Placeboes of DL-alanine were administered on the control days. Three subjects, adult males, again participated in the experiment. The amino acids were taken with fruit juice. The results are given in Table XL, expressed as the percentage of total catecholamines excreted as adrenaline.

Table XL

DL-methionine, 20 g.

Percent of Adrenaline in Urinary Catecholamines.

| Subject: | | W | S | L | Mean |
|---|---|---|---|---|---|
| Day | Treatment | | | | |
| 1 | Control | 17.8 | 20.6 | 18.0 | 18.8 |
| 4 | Test | 19.9 | 19.9 | 21.4 | 20.4 |
| 5 | Control | 17.6 | 16.0 | 19.2 | 17.6 |
| Average of: | | | | | |
| | Control Days | 17.7 | 18.3 | 18.6 | 18.2 |
| | Test Days | 19.9 | 19.9 | 21.4 | 20.4 |

The values for the test day are higher than the mean values of the control days, which is in line with the predicted results. However, the differences that occur are not of great magnitude.

Experiment C. In this experiment doses of nicotinic acid ranging from 2.2 to 4.5 grams were administered orally to three adult, male subjects over a period of two test days. The dose level depended upon the severity of the flush produced. Urine was also collected on each of two control days, one prior to taking the drug and the day following administration. On the control days placeboes consisting of 5 g. DL-alanine were given. The results of this experiment, expressed as the per cent of total catecholamines excreted as adrenaline, are given in Table XLI.

Table XLI

Nicotinic acid

Percent of Adrenaline in Urinary Catecholamines.

| Day | Subject: / Treatment | W | S | L | Mean |
|---|---|---|---|---|---|
| 1 | Control | 28.6 | 33.4 | 38.9 | 33.6 |
| 3 | Test | 33.6 | 33.7 | 41.9 | 36.4 |
| 4 | Test | 36.1 | 35.5 | 33.0 | 34.9 |
| 5 | Control | 35.6 | 28.7 | 32.7 | 32.3 |
| Average of: | | | | | |
| | Control Days | 32.1 | 31.0 | 35.8 | 33.0 |
| | Test Days | 34.9 | 34.6 | 37.5 | 35.7 |

The results of this experiment show that there is a slightly higher proportion of adrenaline excreted on the test days than on the control. This was contrary to the predicted results, and may reflect an effect of the

intense vasodilation in reducing the activity of the sympathetic post-ganglionic fibers which release nor-adrenaline, thus bringing about the increased adrenaline percentage.

## DISCUSSION

The data for the catecholamine contents of the pairs of adrenals presented in Experiment No. 1, Tables II and V, show that with the exception of the third week on the diet, there was a steady increase in organ content with time. Both adrenaline and noradrenaline showed this increase. When the results were expressed on a constant organ weight basis, the same general tendency toward an increase was apparent. However, expressing the catecholamine content as a function of body weight produced a much more uniform result and little or no increased amounts of the amines at the end of seven weeks on the diet. During this time the body weights of the animals were more than doubled. It appears that the amounts of catecholamines to be found in the adrenals depend more on body size, or perhaps age, than on the size of the organs. This is not surprising in the case of the adrenals since these are, in effect, double organs. The medulla and cortex are of different embryonic origins and serve different functions. It seems, therefore, that whole organ comparisons are valid as long as the rats are within the same, or nearly the same, age group and body size. Van Arman (4) has also come to the conclusion that whole organ comparisons are valid from a different set of considerations. In his study the adrenals were of remarkably uniform weight, however, the animals were all adult females and were of fairly uniform

body weight as well. Butterworth and Mann (118) found that there was a closer relationship between the catecholamine contents of the two adrenal glands from a single cat when expressed on a per gland basis rather than as per 100 mg. of gland.

Further evidence that whole organ comparisons are valid is found in Experiment No. 2. The spleens had reasonably constant amounts of both adrenaline and noradrenaline, as seen from Table VII, pg. 50; however, in Table XIV, pg. 63 it can be seen that in the deficient animals where the organ weight is reduced, expressing the amounts in terms of concentration per gram of tissue gives a misleading impression of increased quantities. One possible explanation for this is that if the spleen and other organs hold catecholamines in granules as has been shown by Blaschko et al. (119) to be the case for the adrenals, a reduction in cytoplasmic volume without a concomitant reduction in granule number would account for these findings.

Pyridoxine deficiency brought about an interesting effect. We might first consider the mean whole organ contents of the $B_6$-deficient group as compared with the controls, that is, the two groups which were supplied with riboflavin. Following this, we will consider the effect of pyridoxine deficiency in the presence of an associated $B_2$ deficiency.

The first portion of the discussion will center about Table XI, p. 54 and Figures 4, 5 and 6, pp. 58, 59

and 60, respectively. In brain, spleen and heart, $B_6$ deficiency in the presence of riboflavin supplementation caused no apparent reduction in either adrenaline or noradrenaline contents. The same thing holds true for the adrenals. In the liver, there was a slight reduction in catecholamine content, but still not significantly so. That these animals were actually deficient as compared with the controls can be seen from a comparison of their body weights in Table XIX, p. 71. The average weight of the $B_6$-deficient group at the time of sacrifice was 184 grams whereas that of the controls was 295 grams. The mean adrenal wet weights, despite the marked difference in body weight, did not differ, nor did those of brain. On the other hand, liver, spleen, and heart wet weights were less in the pyridoxine deficient animals than in the controls (Table XII, p. 55).

One explanation for the lack of a real reduction in the catecholamine contents of the various organs in $B_6$ deficiency is that pyridoxine is firmly bound and, consequently is not readily lost from the organs of the $B_6$-deficient rat. Beaton et al. (63) have shown that the various signs of pyridoxine deficiency do not appear simultaneously, but rather over a protracted period of time indicating a greater affinity between certain tissues and the vitamin than of others. Further evidence that metabolic lesions due to lack of vitamin $B_6$ may be difficult to produce has

been brought forth by Yefimochkina et al. (120). These authors maintained rats on $B_6$-deficient diets for 45, 86 and 98 days and found that there was no difference between the ability of the deficient animals to transaminate $N^{15}$-labelled amino groups from glycine, DL-glutamic acid, or ammonia from that of the controls. They considered that this was due to a high residual activity in the aminopherases of the organs of the $B_6$-deficient animals.

Evidence that the adrenals may not readily lose pyridoxine when rats are placed on $B_6$-deficient diets has been produced by Blaschko and his group. Von Euler (124) quotes Burn (125) as showing that there is no difference in the adrenaline content of the suprarenal glands between rats fed a pyridoxine deficient diet and their normal controls. Blaschko (112) found that both the adrenaline and noradrenaline of adrenals from $B_6$-deficient rats were present in normal concentration. He further states (126) "that without insulin no differences were observed, except in one almost moribund animal in which the amine content was very low".

Firm binding of the vitamin to prevent loss during deficiency may not be the whole story in the case of the adrenals. Figure 6, p. 60 shows the state of affairs when the data for adrenals are plotted on a time basis. In this figure, the mean adrenal content of adrenaline and noradrenaline of the animals on the two $B_6$-deficient diets

are expressed as a percentage of that of the animals on the two $B_6$-supplemented diets for each group sacrificed at a given time. Under these conditions it can be seen that during the early periods on the diets, the deficient animals do actually contain less of the catecholamines than their supplemented controls. After 5 - 6 weeks the deficient animals catch up to the controls and they may finally surpass them. The explanation for this phenomenon is not known, but it appears that there are at least two processes working. One of these tends to lower the catecholamine content of the deficient rats whereas the other tends to retard loss. A reasonable explanation for the loss of adrenaline and noradrenaline during the early part of the experimental period is that loss of pyridoxine reduces the activity of DOPA decarboxylase, and thereby results in a diminished synthesis of these amines. The second process is more difficult to explain, but it could be due to prolonged deficiency causing damage to the splanchnic nerves which results in an impaired release of the catecholamines from the adrenals. Thus, even though synthesis is retarded, inability to discharge the amines would result in their accumulation within the gland. That pyridoxine deficiency does cause impairment of nervous function has been amply demonstrated. Daniel (72), Chick (66, 69, 71), Fouts (67) and Jukes (68) have all reported the appearance of convulsive states associated with $B_6$ deficiency in young animals of various species

including pig, dog, chick and rat. Tower (121) has described a similar condition in man. Davenport and Davenport (65) have shown that in pyridoxine deficiency in the rat there is a reduced electroconvulsive threshold.

The time effect observed in the pyridoxine deficient animals appeared to be specific for $B_6$-deficiency. It did not appear in the riboflavin- nor thiamine-deficient rats.

The discussion so far has been concerned mainly with pyridoxine deficiency in the presence of adequate amounts of the other vitamins. However, in this investigation $B_6$ deficiency superimposed upon a $B_2$ deficiency has also been studied. These results are recorded in Table XI, p. 54 as well, and are found in Columns $\underline{-B_2}$ and $\underline{-B_2B_6}$. The mean noradrenaline content was slightly higher in the adrenals, heart and spleen of the doubly deficient animals, equal in brain, and slightly lower in the liver. The adrenaline content varied independently of the noradrenaline, and was slightly lower in the adrenals, brain, and liver of the $-B_2B_6$ group, equal in heart, and very slightly higher in the spleen. But in no instance was the change of any significance. Thus a concomitant riboflavin deficiency did not modify the $B_6$ deficiency. The mean body weights of these two groups were not greatly different, that of the doubly deficient group being 121 g. while the mean control group weight was 128 g.

Riboflavin deficiency results may be seen in Table XI, p. 54 and in Figures 4 and 5, pp. 58 and 59, respectively. The deficiency of the single vitamin had no effect on the catecholamines of brain when compared with the fully supplemented control, nor did imposition of $B_6$ deficiency on the riboflavin deficient animals bring about any effect. There was no effect of riboflavin deficiency in the spleen. One is tempted to speculate that in brain riboflavin is firmly bound and that loss during deficiency would be minimized thereby, or that it is unnecessary for catecholamine biosynthesis. It has, however, been shown by Efremov et al. (122) that riboflavin deficiency in dogs is followed by changes in conditioned reflex activity, thus implying cerebral loss of the vitamin, and evidence to be discussed below indicates that riboflavin is required for synthesis of adrenaline.

A third possibility is, of course, that the catecholamines are not synthesized in brain, but are transported to it from some other site of synthesis.

The hearts of the animals on the $B_2$-deficient diet contained less adrenaline than did those of the controls. This occurred both in the rats receiving pyridoxine as well as in those that did not.

Riboflavin deficiency reduced the amount of noradrenaline when pyridoxine was supplied in the diet as can be seen by comparing the means of the animals on the control and $-B_2$ diets. However, when the animals were

pyridoxine deficient ( $-B_6$ and $-B_2B_6$), restriction of riboflavin intake did not reduce the noradrenaline content. However, in both categories of rats, whether supplied with $B_6$ or deprived of this vitamin, the total catecholamines were reduced when riboflavin intake was restricted. There was thus an apparent differential effect of $B_2$-deficiency depending on the presence of pyridoxine. This experiment with regard to heart is of a preliminary nature only since the number of rats in the series is small, being a total of twelve. The results are, however, compatible with those of the liver and adrenals.

In the adrenals $B_2$-deficiency caused a reduction in both the adrenaline and the noradrenaline contents of the glands. This reduction took place regardless of the presence of $B_6$ in the diet. The fall in adrenaline was significant at the 95% level of confidence. In the liver as well, riboflavin deficiency brought about marked reductions in both the adrenaline and noradrenaline contents. And, again, pyridoxine deficiency failed to modify the riboflavin deficiency effect. The adrenaline and noradrenaline reductions in liver were both significant at greater than the 99% level of confidence. It thus appears that riboflavin is involved in the synthesis of the catecholamines. There has been published no direct evidence for the involvement of riboflavin as such, or in the form of flavoprotein, in any of the individual reactions leading to the synthesis of the catecholamines.

The work of Udenfriend and Cooper (17) who found a requirement for pyridine nucleotides and oxygen in the oxidation of phenylalanine to tyrosine suggests an involvement of the flavoproteins. Further support for the involvement of riboflavin through the flavoproteins is furnished by Kaufmann's postulated mechanism of phenylalanine oxidation in which water is formed (19). It has been known for some time that the cytochrome system, with which the flavoproteins are known to be associated, is present in the adrenal medulla (123). A fair amount of indirect evidence is available, therefore, which indicates that riboflavin is necessary for catecholamine biosynthesis, and gives credence to the experimental findings reported in this thesis.

There are three logical sites in the Blaschko reaction sequence for adrenaline biosynthesis in which riboflavin might be required. The first is in the oxidation of phenylalanine to tyrosine, the second is in the oxidation of tyrosine to form DOPA, and the third is in the side chain hydroxylation of DOPAmine to form noradrenaline. Inasmuch as the amino acid tyrosine was included in the protein component of the diets fed to our animals, it seems unlikely that a diminished conversion of phenylalanine to tyrosine could explain the reduced amounts of catecholamines found in the livers and adrenals of the $B_2$-deficient animals. It is therefore suggested that either one or both of the remaining two hydroxylations is riboflavin dependent.

The thiamine deficiency results are found in Tables XXIII and XXIV, pp. 77 and 78, respectively. In Table XXIII total catecholamines of the first two experiments are considered, whereas in Table XXIV both noradrenaline and adrenaline are considered. Lack of thiamine did not bring about any modification of the amounts of catecholamines synthesized by the adrenals. None of the known enzymes involved in adrenaline and noradrenaline synthesis requires thiamine as cofactor. However, it was felt that secondary effects possibly resulting from the stress of vitamin deficiency might influence the amounts of catecholamines found in the adrenals. Goodall's study (74) indicated that, at least in extreme thiamine deficiency, there may be a diminution in adrenal catecholamine content. The present studies do not support this view, nor, apparently, does the stress per se which is attendant upon vitamin deficiency bring about any modification.

To sum up these results, riboflavin appears to be required for catecholamine biosynthesis in liver and in the adrenals. Pyridoxine seems to be firmly bound to cellular protein in most of the organs studied so that simple deficiency of this vitamin does not affect adrenaline and noradrenaline synthesis. However, in the adrenals, $B_6$ deficiency may result in a two-fold effect influencing both the biosynthesis and the release of the catecholamines. Thiamine apparently is not directly involved in the formation of these substances.

The results of the experiments involving amino acid supplementation are found in Tables XXXIV to XXXVIII, pp. 95 to 99, respectively. For ready reference, the summary tables (XXXIV and XXXV) may be used to follow the discussion. The basal diet used in these experiments was based on the phenylalanine-tyrosine deficient diet of Armstrong and Lewis (94). Using weanling rats, these authors showed that the unsupplemented diet not only failed to promote growth of the animals, but actually permitted the animals to lose weight. However, supplementation with 0.9% DL-phenylalanine allowed growth to take place. Similar results were obtained in our own experiments; when the diets were supplemented with tyrosine, DOPA, phenylserine, or m-tyrosine the animals failed to grow and in most instances lost weight.

The wet weights of the adrenals were not affected by the various dietary supplements. The animals on the phenylalanine supplemented diet, however, possessed livers that weighed nearly twice as much as those of the controls. Such other supplements as tyrosine, DOPA and phenylserine did not affect the control liver weight. The differences in spleen weight paralleled those of the liver. The weight of the brain was unaffected by any of the supplements.

The effect of the various amino acid supplements on the catecholamine contents of the organs investigated varied with the individual amino acid and with the type of pretreatment the animal received. Phenylalanine

is known to be a precursor of noradrenaline and adrenaline. Gurin and Delluva (2) and Udenfriend et al. (3, 5) have demonstrated by isotopic means the conversion of phenylalanine to adrenaline. Van Arman (4), on the other hand, was unable to find any increase after six hours in the rate of adrenaline regeneration in the adrenals of rats whose stores of adrenaline had been depleted with insulin. This may have been due in part to slow oxidation to tyrosine; the same author was unable to find that tyrosine stimulated the rate of regeneration. In our experiments the effect of phenylalanine in raising the adrenal catecholamine content above that of the controls was variable. In one experiment (Table XXXIV, Experiment B) lasting up to 3 weeks following pretreatment with insulin, the total catecholamine content was increased over that of the control animals. In Experiment D, lasting 11 days after pretreatment with reserpine, the total catecholamines were slightly higher due to an increased noradrenaline content. In other experiments, however, no effect was noted. As for the other organs investigated, phenylalanine supplementation increased the liver content of both amines and brought about small increases in spleen and brain.

In our experiments, there may be an explanation for the lack of a consistent effect of phenylalanine in promoting catecholamine synthesis other than a low rate

of oxidation to tyrosine. Grau and Steele (127), investigating the metabolic fate of labelled phenylalanine fed in the diet to young mice, observed that when the mouse had been previously made phenylalanine deficient, it converted less of the labelled amino acid to liver protein tyrosine and to carbon dioxide than did the control animals fed a diet containing enough phenylalanine for normal growth. The explanation offered for this was that when the phenylalanine supply was insufficient, a greater proportion of the amount absorbed was used for protein synthesis than when the amount supplied was sufficient to satisfy this primary requirement. Although our rats were not deliberately made phenylalanine deficient prior to administration of the supplemented diets, it is possible that in the young, growing rat phenylalanine is predominantly laid down as protein and only insignificant amounts of that administered in the diet is available for catecholamine synthesis. From this point of view, it would be interesting to repeat this experiment with fully mature rats whose growth had ceased. The drawback to using adult rats is that a longer time would probably be required to produce an effect. However, since Rose and Womack (14) have found that maximum growth is obtained when phenylalanine makes up about 0.9% of the diet, our supplement of 1.5% should have been sufficient to supply all of the growth requirements with an excess left over for other biosynthetic purposes.

In the adrenals, tyrosine was consistently more effective in promoting catecholamine biosynthesis than was phenylalanine. This might have been expected since tyrosine is closer to the catecholamine end-product in the biogenetic sense than is phenylalanine, and is consistent with the view that the oxidation of phenylalanine is rate-limiting. On the other hand, tyrosine was not superior to phenylalanine in liver, spleen or brain. Indeed, the liver and spleen contents of the catecholamines were less after tyrosine administration than after phenylalanine. This was surprising in view of the results obtained with adrenals.

Although DOPA is well known to be a precursor of noradrenaline and adrenaline and is an intermediate which occurs farther along the metabolic pathway toward the catecholamines than does tyrosine, it did not support better synthesis of the amines than did tyrosine in any of the organs studied. There are two possible explanations which might account for this. One is that spontaneous air oxidation of the DOPA in the diet occurred thereby reducing the amount available to the animal. This possibility was taken into account in the preparation of the diet. Only the amount of diet to be consumed during a day was supplemented with DOPA, thereby ensuring a fresh supply each day. Moreover, examination of the amount of diet remaining in the food cage from the previous day's supply did not usually reveal any melanin-like material.

The only exception to this occurred when the animal urinated into his food dish.

The other possible explanation is that much of the DOPA might have been either destroyed by the intestinal flora or after being carried to the liver by the hepatic portal vein, was converted to the amine which, in turn was oxidized by amine oxidase, before the DOPA could be released to the general circulation. A method of avoiding these possibilities in future experiments might be to administer the DOPA by injection. Van Arman (4) found 300 mg. of DOPA per rat, administered by forced feeding, effective in restoring adrenal catecholamines after insulin depletion. Toxic symptoms were, however, produced.

Phenylserine has long been known to be metabolized by the body. Dakin (90) in 1909 found that phenylserine administered to cats gave rise to hippuric acid in the urine. Bruns and Fiedler (128) have recently shown that the splitting of L-threo-beta-phenylserine to benzaldehyde and glycine is a reversible reaction. The enzyme catalysing this reaction is present in livers and kidneys of men, rats, mice, guinea pigs, pigs, sheep and cows. In the synthetic reaction both the threo and erythro isomers are formed, possibly by two different enzymes. Although Blaschko (95) and Hartmann et al. (8) have observed noradrenaline formation from dihydroxyphenylserine by kidney and liver enzymes and Schmiterlow (129) has obtained evidence for its formation from the same

precursor *in vivo*, Van Arman (4) showed that phenylserine was unable to restore the catecholamines of insulin-depleted adrenals over a six hour period after administration. He also observed that this compound produced toxic symptoms.

When we fed rats (pretreated with insulin) diets supplemented with phenylserine, little change in the catecholamine contents of the adrenals, livers or spleens could be observed, although there was some increase in the noradrenaline content of the adrenals and both amines were slightly increased in liver. However, when rats were pretreated with reserpine, phenylserine reduced the noradrenaline content of the adrenals to a value considerably less than that of the controls.

*m*-Tyrosine administered to one group of rats pretreated with reserpine depressed the amounts of both adrenaline and noradrenaline in the adrenals as compared with the controls. This was also seen in brain. Thus *m*-tyrosine appeared to act in the same manner as phenylserine under comparable circumstances.

Phenylserine and *m*-tyrosine were administered to our rats in an attempt to determine whether alternate pathways for catecholamine biosynthesis exist. The results obtained with phenylalanine, tyrosine and DOPA supplementation of the phenylalanine-tyrosine deficient diet indicate that this method lacks the required sensitivity. It is not as sensitive as isotopic tracer

techniques, for example. Therefore these attempts must be considered as preliminary measures in searching for suitable procedures to be used in such studies.

The effect of the depleting agent upon the results obtained with the various dietary supplements deserves mention. A comparison of Experiments A and B, Table XXXIV, showed that pretreatment with insulin definitely lowered the catecholamine content of the adrenals of those animals on the basal diet as compared with those of the rats not receiving this agent. Moreover, pretreatment allowed the effects of phenylalanine and tyrosine supplementation to become visible. These two experiments were carried out under the same set of conditions except for pretreatment with insulin. A comparison of the effects of reserpine pretreatment with those of insulin is possible in several instances. The depleting agent did not modify the effect of tyrosine supplementation in the adrenals. On the other hand, the effect of DOPA in insulinized rats was annulled in reserpinized animals. A comparison of Experiments C and E reveals that after insulin DOPA increased the amount of adrenaline found in the adrenals over that found in those of the animals on the unsupplemented diet, whereas after reserpine the amounts of both catecholamines were greatly reduced. Phenylserine tended to raise the adrenal content of noradrenaline after insulin (Experiment C), but this component

was reduced following reserpine (Experiment D). In the case of m-tyrosine, data are available only after reserpine pretreatment, however this compound behaved in the same manner as did phenylserine following reserpine. It is tempting to speculate that the two would behave similarly after insulin as well. Future experiments should explore this possibility.

The results obtained after feeding tyrosine for 5 and 11 days following reserpine differ. After 5 days there is a slight increase in the total catecholamines of the adrenals of the tyrosine-fed rats over those of the rats on the basal diet. This is due to a somewhat increased amount of adrenaline. The noradrenaline values do not significantly differ. After 11 days of the tyrosine-supplemented diet following reserpine, the amounts of both noradrenaline and adrenaline show a much greater increase over those of the controls.

There are three avenues of possible further exploration. One is to determine whether length of time on the diets quantitatively affects the results; the second, to find out whether the type of pretreatment - insulin or reserpine - causes a real difference in the type of result obtained; and the third, to test whether m-tyrosine and phenylserine act similarly following pretreatment with the two depleting agents.

One further aspect of this study remains to be discussed. When one compares the effect of reserpine pre-

treatment in those animals on the deficient diet with those receiving the placebo injection (also on the deficient diet) in Columns PC and RC, Tables XXX through XXXIII, the adrenals of the reserpinized animals are found to contain a higher amount of catecholamines than those of the placebo animals. This is contrary to the expected result. On the other hand, in brain the situation is reversed. Here the noradrenaline content is reduced in the reserpinized animals while the adrenaline levels remain the same, thereby resulting in a diminished total catecholamine content. These results are in agreement with those reported in the literature (79, 130). The explanation for the adrenal findings is not known, but in a further experiment completed too late to be reported in the experimental section, the catecholamine-depleting action of reserpine on the adrenals was observed. The results are found in Table XLII, p. 126.

Table XLII

Reserpine-Placebo Experiment

μg. NA per pair of adrenals

| | SD * | PD | RD | SCh | PCh | RCh |
|---|---|---|---|---|---|---|
| | 3.03 | 4.98 | 2.10 | 4.85 | 4.98 | 4.80 |
| | 4.23 | 3.98 | 3.43 | 7.03 | 4.60 | 4.48 |
| Mean: | 3.63 | 4.48 | 2.77 | 5.94 | 4.79 | 4.64 |

μg. A per pair of adrenals

| | SD | PD | RD | SCh | PCh | RCh |
|---|---|---|---|---|---|---|
| | 8.03 | 11.10 | 5.95 | 11.23 | 10.85 | 17.13 |
| | 10.13 | 10.75 | 9.68 | 12.70 | 12.38 | 16.93 |
| Mean: | 9.08 | 10.93 | 7.82 | 11.97 | 11.62 | 17.03 |

μg. Total per pair of adrenals

| | SD | PD | RD | SCh | PCh | RCh |
|---|---|---|---|---|---|---|
| | 11.06 | 16.08 | 8.05 | 16.08 | 15.83 | 21.93 |
| | 14.36 | 14.73 | 13.11 | 19.73 | 16.98 | 21.41 |
| Mean: | 12.71 | 15.41 | 10.58 | 17.91 | 16.41 | 21.67 |

* S, P and R represent saline, placebo and reserpine, respectively. D represents phenylalanine-tyrosine deficient diet and Ch, Purina fox checkers.

Three pairs of rats were given a phenylalanine-tyrosine deficient diet and three were allowed Purina fox checkers. At the beginning of the experimental period one pair of animals on each diet received reserpine, 5 mg./Kg., one pair a volume of placebo equal to the reserpine solution, and one a like volume of saline. The animals were maintained on the diets for 11 days before being sacrificed.

The adrenals of the reserpinized animals on the phenylalanine-tyrosine deficient diet contained less adrenaline and noradrenaline than did those from animals treated with the placebo, which is in contrast to the results of the first two reserpine experiments. The animals remained on the diets for 11 days. It appears that under certain conditions, depleting the adrenals with reserpine provided a stimulus for speedy recovery of the lost catecholamines whereas under other conditions (e.g. Third Experiment, Table XLII) it did not. It is possible that some factor prior to receiving the animals from the breeder determined the type of response, although in general appearance the animals in all these experiments were indistinguishable.

Finally there remain only the experiments in which attempts were made to alter the adrenaline / noradrenaline ratio in human volunteers by means of nicotinic acid and methionine administration. The results were not clear-cut although, as seen from Table XL, p. 104, there was some indication that at the higher dose level, methionine increased the proportion of adrenaline found in the urine. This would be the expected outcome following the administration of large quantities of a methyl donor. On the other hand, nicotinic acid, by depleting the body of methyl groups would be expected to reduce the proportion of adrenaline. Table XLI, p. 105, shows that this

did not happen. It is possible that the somewhat increased proportion of adrenaline excreted in the urine is due to an effect of the intense vasodilation in reducing the activity of the sympathetic post-ganglionic fibres which release noradrenaline. Further experiments should be carried out over a long period of time using experimental animals to clarify this point. In such experiments not only might the adrenaline / noradrenaline ratio of the urine be determined, but also the effect of the drugs on the proportion in the adrenals and other organs be investigated. In addition, other compounds as choline and guanidoacetic acid might be used to supply or deplete methyl groups, respectively.

## SUMMARY

1. A study has been made of the role of certain nutritional factors in the biological synthesis of adrenaline and noradrenaline. These factors included dietary deficiencies of riboflavin, pyridoxine and thiamine; supplements of phenylalanine, tyrosine, DOPA, phenylserine and m-tyrosine to a phenylalanine-tyrosine deficient diet; and the administration of methionine and nicotinic acid. Young rats were used for the vitamin deficiency and amino acid supplementation experiments; methionine and nicotinic acid were administered to human volunteers.

2. It was found that under our conditions, i.e. measuring catecholamine contents of organs after the intact animal had undergone the experimental procedure, expression of the results in terms of whole organ content led to more meaningful comparisons since the amounts of catecholamines within the organ tended to vary independently of organ or body weight.

3. Riboflavin deficiency was found not to influence the catecholamine content of brain or spleen; however, both the adrenaline and noradrenaline contents of adrenals and livers were significantly reduced. The adrenaline content of heart was reduced by riboflavin deficiency but the noradrenaline content was unaffected. The data indicate that riboflavine is required for catecholamine biosynthesis; and since tyrosine was

supplied in the dietary protein, it is suggested that at least one requirement for this vitamin is in one or both of the hydroxylation steps coming after the formation of tyrosine.

4. Thiamine deficiency did not affect the catecholamine contents of the adrenals.
5. Pyridoxine deficiency did not significantly reduce the catecholamine contents of brain, liver, spleen or heart. The mean adrenaline and noradrenaline contents of a group of 14 $B_6$-deficient rats was not significantly lower than those of the pyridoxine supplemented controls. These animals were sacrificed over periods of time ranging from 19 to 54 days. Analysis of the data on a time basis reveals that during the earlier phases of the experiment the adrenals of the $B_6$-deficient animals contained less catecholamines than did those of the controls. After about 5-6 weeks on the diets, the adrenals of the deficient animals contained as much as, or more, of the catecholamines than did the controls.
6. Pretreatment with insulin was found to lower the catecholamine contents of adrenals in animals on a phenylalanine-tyrosine deficient diet, and permitted the effects of phenylalanine and tyrosine supplementation of such diets to become visible.
7. It was found that a supplement of phenylalanine to the basal diet failed to consistently give rise to higher amounts of the catecholamines in the adrenals, liver

spleen or brain than found in the non-supplemented controls. This is interpreted as further "negative" evidence, obtained by an *in vivo* technique that the conversion of phenylalanine to tyrosine takes place slowly.

8. The data obtained in the amino acid supplementation experiments indicate that the agent used to deplete the animal of catecholamines may modify the results produced by the supplement.
9. Supplementing the basal diet with tyrosine led to increased amounts of the adrenal catecholamines after either insulin or reserpine pretreatment. The average increase after reserpine was less than that after insulin. In an experiment in which the animals were given two injections of reserpine during the pretreatment period, and were sacrificed five days after the second injection, the increase in the amount of catecholamines was only very slight. This might have been due to the more drastic pretreatment or to an insufficient time lapse after the last reserpine injection. Tyrosine led to increased quantities of catecholamines in the brain, however, no increases were noted in either liver or spleen.
10. Although DOPA is a known precursor of the catecholamines, supplementing the basal diet with this amino acid led to an increase in adrenal catecholamines,

due solely to a rise in adrenaline, in only one experiment, and after insulin pretreatment. In a second experiment, after reserpine, DOPA appeared to be inhibitory. DOPA failed to cause any effect in the brain, liver or spleen.

11. Phenylserine and m-tyrosine did not cause a significant increase in the amounts of catecholamines in any of the organs investigated. The tendency toward increased noradrenaline in the adrenals brought about by phenylserine after insulin pretreatment was not seen after reserpine.

12. Demonstration of alternate pathways of catecholamine biosynthesis involving phenylserine or m-tyrosine were not possible dsing the dietary deficiency technique. The results obtained with phenylalanine, tyrosine and DOPA supplements indicate that this method of approach lacks the necessary sensitivity.

13. The oral administration of 20 g. per day of DL-Methionine increased the proportion of adrenaline found in the urine of human volunteers. A dose level of 5 g. per day was found not to affect the proportion nor did nicotinic acid raise the adrenaline / noradrenaline ratio.

## Claims of Original Research or Contributions to Knowledge

1. Riboflavin deficiency was found to reduce the amounts of adrenaline and noradrenaline of rat adrenal glands and liver, but only the adrenaline of heart. No effect was produced on the catecholamine content of brain or spleen.
2. Pyridoxine deficient diets were shown to decrease the catecholamine content of rat adrenal glands as compared with the controls during the earlier part of the experimental period but after 5 - 6 weeks on the diet, the glands from deficient animals contained as much as the controls and later surpassed them.
3. Thiamine deficiency has been found not to affect the adrenaline or noradrenaline content of the adrenals of the rat.
4. In chronic deficiency experiments, significant increases in the catecholamine content of the adrenals was brought about by tyrosine but not by phenylalanine.
5. Phenylserine and m-Tyrosine, when added to deficient diets, did not cause an increased synthesis of catecholamines.
6. The two catecholamine depleting agents, insulin and reserpine, affect differentially catecholamine biosynthesis from DOPA. In respect to phenylserine, the results obtained after insulin and reserpine were analagous to those of DOPA.

# APPENDIX I

The following tables give the analysis of variance for adrenaline and noradrenaline in liver, adrenals and heart of rats on $B_2$- and $B_6$- deficient diets. Analysis of variance for brain and spleen are omitted since it was obvious from the raw data that no statistically significant differences were possible.

Table XLIII

Analysis of Variance for Liver Adrenaline

| Sources of Variation | D.F. | Mean Square | F | Significance |
|---|---|---|---|---|
| Total | 27 | - | - | - |
| Between diets | 3 | 7.696873 | 26.42 | < 1% |
| Between $-B_2, +B_2$ | 1 | 22.518296 | 76.34 | < 1% |
| Between $-B_6, +B_6$ | 1 | 0.450096 | 1.53 | > 5% |
| Interaction | 1 | 0.122226 | 0.41 | > 5% |
| Between replicates | 6 | 1.721955 | 5.84 | < 1% |
| Remainder | 18 | 0.294992 | (1) | |

Analysis of Variance for Liver Noradrenaline

| Sources of Variation | D.F. | Mean Square | F | Significance |
|---|---|---|---|---|
| Total | 27 | - | - | - |
| Between diets | 3 | 32.137477 | 13.47 | < 1% |
| Between $-B_2, +B_2$ | 1 | 89.071571 | 37.33 | < 1% |
| Between $-B_6, +B_6$ | 1 | 4.772648 | 2.00 | > 5% |
| Interaction | 1 | 2.568212 | 1.08 | > 5% |
| Between replicates | 6 | 10.929632 | 4.58 | < 1% |
| Remainder | 18 | 2.385748 | (1) | |

Table XLIV -ii-

Analysis of Variance for Adrenal Adrenaline

| Sources of Variation | D.F. | Mean Square | F | Significance |
|---|---|---|---|---|
| Total | 27 | - | - | - |
| Between diets | 3 | 38.014988 | 2.46 | >5% |
| Between $-B_2$, $+B_2$ | 1 | 93.367096 | 6.04 | <5% |
| Between $-B_6$, $+B_6$ | 1 | 19.438956 | 1.26 | >5% |
| Interaction | 1 | 1.238914 | 0.08 | >5% |
| Between replicates | 6 | 62.547711 | 4.05 | <1% |
| Remainder | 18 | 15.457419 | (1) | |

Analysis of Variance for Adrenal Noradrenaline

| Sources of Variation | D.F. | Mean Square | F | Significance |
|---|---|---|---|---|
| Total | 27 | - | - | - |
| Between diets | 3 | 20.232143 | 1.17 | >5% |
| Between $-B_2$, $+B_2$ | 1 | 55.695028 | 3.22 | >5% |
| Between $-B_6$, $+B_6$ | 1 | 0.204028 | 0.01 | >5% |
| Interaction | 1 | 4.797372 | 0.28 | >5% |
| Between replicates | 6 | 107.270116 | 6.20 | <1% |
| Remainder | 18 | 17.309122 | (1) | |

Table XLV -iii-

Analysis of Variance for Heart Adrenaline

| Sources of Variation | D.F. | Mean Square | F | Significance |
|---|---|---|---|---|
| Total | 11 | - | - | - |
| Between diets | 3 | 0.058564 | 1.43 | >5% |
| Between $-B_2$, $+B_2$ | 1 | 0.175210 | 4.28 | >5% |
| Between $-B_6$, $+B_6$ | 1 | 0.000076 | 0.002 | >5% |
| Interaction | 1 | 0.000407 | 0.01 | >5% |
| Between replicates | 2 | 0.011109 | 0.27 | >5% |
| Remainder | 6 | 0.040930 | (1) | |

Analysis of Variance for Heart Noradrenaline

| Sources of Variation | D.F. | Mean Square | F | Significance |
|---|---|---|---|---|
| Total | 11 | - | - | - |
| Between diets | 3 | 0.099519 | 0.93 | >5% |
| Between $-B_2$, $+B_2$ | 1 | 0.138675 | 1.34 | >5% |
| Between $-B_6$, $+B_6$ | 1 | 0.016875 | 0.16 | >5% |
| Interaction | 1 | 0.143008 | 1.38 | >5% |
| Between replicates | 2 | 0.510475 | 4.93 | >5% |
| Remainder | 6 | 0.103453 | (1) | |

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