# Dopamine Metabolism in the Copper Deficient Rat

By

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# PREFACE

The history of the catecholamines--dopamine, norepinephrine, and epinephrine--is a relatively short one, for although Halle first postulated a scheme in 1906, it was not until 1939 that Blaschko suggested the pathway now accepted as being the most important. It was not until 1946 that Von Euler pointed out that the true adrenergic neuro-transmitter is norepinephrine, not epinephrine, as previously supposed. Since then the work on catecholamines has become enormously complex.

The medical importance of the catecholamines, especially norepinephrine and epinephrine is considerable. As norepinephrine is the adrenergic transmitter and epinephrine is a powerful hormone, this is not unexpected. Applications in this field range from mental and neuro-muscular disorders to heart disease and chronic hyper-tension.

Dopamine is the first, metabolically speaking, of the catecholamines. From it norepinephrine and subsequently epinephrine are synthesized in the body. It is the enzyme Dopamine-$\beta$-hydroxylase that is responsible for the conversion of dopamine to norepinephrine. As dopamine is becoming medically important in its own right, and norepinephrine is already of considerable importance, it is the dopamine to norepinephrine conversion that will be studied in detail in this presentation.

## ABSTRACT

Male Sprague-Dawley rats were fed a copper deficient diet for periods up to three months. The dopamine-β-hydroxylase activity was decreased to as little as 20% of the controls. The activity of dopamine-β-hydroxylase was found to be correlated with the concentrations of copper in the liver and heart. An intraperitoneal injection of disulfiram caused 100% inhibition of the enzyme. The most satisfactory basal diet of several tested in this work consisted of 50% powdered milk and 50% corn-starch, with added vitamins and minerals. In a study of simultaneous deficiencies of copper+iron it was observed that the concentration of iron in the liver rose considerably in copper deficiency, and in iron deficiency the copper level rose. A study of the heart rates of copper-deficient and of control animals in response to stress showed no differences.

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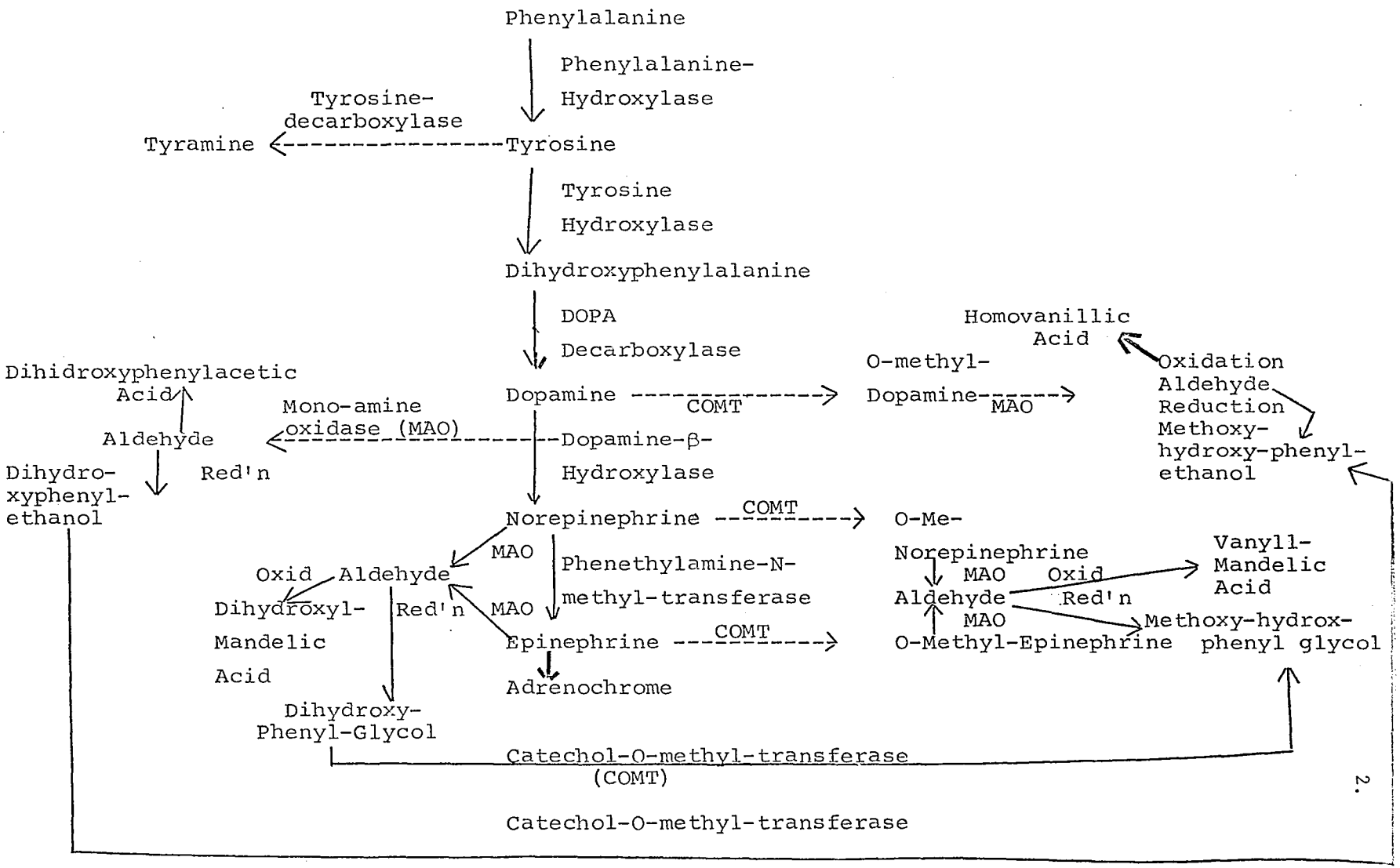
# I. INTRODUCTION

## A. General Biosynthesis and Metabolism of Catecholamines

The general scheme for catecholamine metabolism, as understood today, is indicated in Table I (1-3). Those metabolites appearing in the urine are listed in Table II, the conjugates occurring as sulphates and glucosiduronidates. This scheme took a period of 60 years to develop, and may be said to have been initiated by Spiro (4) in 1902 when he demonstrated the decarboxylation of phenylalanine in extracts of mammalian tissue. In the same year Emerson (5) showed a similar conversion of tyrosine, yielding tyramine. This led Halle, in 1906, to propose the first pathway of catecholamine synthesis (6). This was tyrosine (tyr.)→dihydroxyphenylalanine(DOPA)→dopamine→epinine→ epinephrine. This has subsequently been suggested to be of minor, if any importance.

After this there was a lapse in activity and not until the discovery by Holtz et al. in 1938 of DOPA decarboxylase in the pig kidney (7) was scientific interest really renewed. This formation of dopamine from DOPA has subsequently been confirmed by many others. Langemann found DOPA decarboxylase in the adrenal medulla (8), as has Hagen (9) and Sourkes et al. (10). The discovery by Holtz led Blaschko (11,12) to propose the scheme now

TABLE I: Metabolic Pathway of the Catecholamines



accepted as being the major pathway for the biosynthesis of the catecholamines i.e. tyrosine→DOPA→dopamine→norepinephrine. Each of these steps has been confirmed by others, predominantly by isotope dilution techniques (10, 13-36). Some of the organs in which this pathway occurs are: bovine sympathetic nerves and ganglia, which appear unable to convert tyrosine to DOPA but have all the other necessary enzymes (16); cat brain (25); rat brain (23, 24); guinea pig vas deferens (33); and the adrenal chromaffin tissue of snakes (35). Kaufman and Friedman (30) demonstrated in the bovine adrenal medulla that the only major precursor of norepinephrine is tyrosine.

In 1940, Devine (37) claimed that phenylethylamine could be converted to epinephrine. His claims were confirmed by Udenfriend et al. (38) and Hagen and Welch (9) although some doubt was cast on this by Udenfriend and Wyngaarden (39). Hagen and Welch (9) showed that the amine oxidase of the adrenal medulla readily attacks ring-substituted phenylethylamines but the introduction of an OH into position 2 of the side chain eliminates all activity (see section on Dopamine-β-hydroxylase). Of practical interest, Björling and Waldeck showed that injected dl-norepinephrine reflects the fate of l-norepinephrine except for very short periods of time after injection (31).

Gurin and Delluva (40) were the first to demonstrate phenylalanine as a precursor of epinephrine in vivo. They showed that in the biosynthesis of epinephrine from phenylalanine the side chain and ring remained intact apart from decarboxylation. Udenfriend and Wyngaarden found that phenylalanine, tyrosine and DOPA all could give rise to adrenal norepinephrine and epinephrine; DOPA was the best substrate (39). Goldstein and Musacchio (27) have also confirmed this. Bulbring (41) in 1949, Keller et al. (42) and Masuoka et al. (43) in 1950 demonstrated that the conversion of norepinephrine to epinephrine involves the methyl group from methionine and ATP. Wurtman (44) and Wurtman and Axelrod (45) showed that the enzyme involved in this step is highly localized in the adrenal medulla, and that the control of this step is by the anterior pituitary (ACTH) and the adrenal cortex (glucocorticoids). From experiments on the sympathetic nerves and ganglia of the dog, Goodall and Kirshner (46) concluded that the catecholamine pathway stops at norepinephrine in sympathetic nerves. Iyer et al. (47) showed that the complete pathway of tyrosine to norepinephrine is present in brain in vitro and that DOPA decarboxylase is also responsible for the decarboxylation of 5-hydroxytryptophan to serotonin. Van Arman showed that out of all the amino

acids DOPA alone could replenish rapidly the stores of pressor amines in the adrenals of rats depleted by insulin hypoglycaemia (48) and West (49) demonstrated that this repletion was slower when the rats were $B_6$ deficient. Demis et al. (14) showed the conversion of DOPA to norepinephrine by bovine adrenal homogenates, suggesting dopamine as the likely intermediate.

Goldstein and co-workers (23) studied the metabolism of the catecholamines in the rat and found that dopamine, norepinephrine, 3-0-methyldopamine and 3-0-methyl norepinephrine, dihydroxyphenylacetic acid (DOPAC) and methoxy-hydroxy-phenylethanol (MHPE) were obtained from DOPA, dihydroxyphenylethanol (DHPE) and MHPE resulted from dopamine administration. Goldstein and Musacchio (27) found in rats given mono-amine oxidase (MAO) inhibitors that dopamine was metabolized to norepinephrine, 3-0-methyldopamine and 3-0-methyl norepinephrine while tyramine resulted in norsynephrine (octopamine).

Much interest has been focused on the studies to determine which is the rate-limiting step in catecholamine biosynthesis. The measurement of the rate-limiting step is complicated by the fact that dopamine is an effective substrate for MAO and it also acts both as a precursor and depleting agent for norepinephrine (53).

Udenfriend and Wyngaarden (39) were the first to suggest tyrosine hydroxylase as the slow step in catecholamine synthesis. Since their work in 1956 much evidence has been obtained in support of their statement.

In 1963, Musacchio and Goldstein (26) claimed tyrosine hydroxylase to be the rate-limiting step in MAO-inhibited rats, and the work of Nagatsu et al. (54) on the isolated enzyme supported this hypothesis. Kaufman and Friedman (30) stated that in the adrenal medulla and, to a lesser extent, in the heart, there is evidence to support the conclusion that the slowest step in norepinephrine biosynthesis is the conversion of tyrosine to DOPA. In brain and nerve it is not known what the rate-limiting step is. Levitt et al. (55) concurred with this conclusion that tyrosine hydroxylase is this rate-limiting enzyme. Similarly, Merrills and Offerman (32) agreed as they found that more norepinephrine was obtained from dopamine than from tyrosine. Further crediting tyrosine hydroxylase as the rate-limiting enzyme Glowinski et al. (56) demonstrated that for the formation of catecholamines in rats depleted of their catecholamines by reserpine the synthesis of dopamine from tyrosine was 30% of normal while that of norepinephrine was 45% of normal. Using

measurements in vivo, Udenfriend et al. (57) found that with α-Me-tyrosine the measured inhibition of norepinephrine synthesis from tyrosine was exactly the same as the calculated inhibition of tyrosine hydroxylase. They claimed that this could be true only if tyrosine hydroxylase were the rate-limiting step in the overall synthesis of norepinephrine.

The challenge to this was presented by Sedvall and Kopin (36). They found that stimulated adrenal glands synthesized norepinephrine four times as rapidly as decentralized glands, the stimulation occurring at or before the tyrosine hydroxylase step. They suggested rate-limiting mechanisms other than tyrosine hydroxylase. These included transport, endogenous inhibitors, and the possibility that accumulation of norepinephrine in storage sites may inhibit synthesis. Iyer et al. claimed that tyrosine hydroxylase is not the rate-limiting step in the brain, (47).

One of the other major unresolved problems is whether or not tyramine takes an active part in the synthesis of any of the catecholamines. In 1956, Udenfriend and Wyngaarden (39) claimed that neither norepinephrine nor epinephrine could be formed by tyramine. Goldstein

and Musacchio (26, 27) support this view by stating that tyramine can give rise to norsynephrine, but neither dopamine nor norepinephrine. However, Chidsey and Kaiser (58) claim that the canine heart can convert tyramine to dopamine as well as norsynephrine and Lemberger et al. (59) used a soluble liver fraction plus microsomes to get tyramine converted to dopamine and octopamine to norepinephrine. In 1965, Lemberger and co-workers (60) using female rabbit liver microsomes found tyramine converted to dopamine. They called the enzyme tyramine hydroxylase and found it in the liver of male and female guinea pigs, rats and mice. They suggest this enzyme as a minor pathway of norepinephrine synthesis.

A brief summary of the knowledge to date is found in Table III.

TABLE II

Urinary Metabolites of the Catecholamines

| Metabolite | Unchanged | Conjugated | N-Acetylated |
|---|---|---|---|
| Dopamine | X | X | X |
| Norepinephrine | X | X | X |
| Epinephrine | X | X | X |
| 3-MeO-Epinephrine | X | X | X |
| DOMA | X | | |
| VMA | X | | |
| MHPG | | X | |

TABLE III

| Enzyme | Step | Occurrence in Tissue | References |
|---|---|---|---|
| Phenylalanine Hydroxylase | Phenylalanine → Tyrosine | Rat | 4,9,27,38-40 |
| Tyrosine Hydroxylase | Tyrosine → DOPA | Bovine, canine, sympathetic nerves and granules; cat brain especially hypothalamus; rat heart; bovine and rat adrenals; guinea pig vas deferens; fowl. | 11,12,16,17,24, 26-28,30,32-34, 36,39,47,54-57. |
| DOPA Decarboxylase | DOPA→ dopamine | Bovine, canine, sympathetic nerves and granules; rat brain, adrenal medulla of cow, cat, chicken, pig, rat, rabbit, adrenal chromaffin tissue of snakes; pig kidney; guinea pig vas deferens. | 2,6-12,14,17, 18,23,26,27,29,32-35,39,46-48 |
| Dopamine-β-Hydroxylase | Dopamine → Norepinephrine also the hydrox. of other phenylethylamines. e.g. | Bovine, canine sympathetic nerves and ganglia; brain of cat, cow, dog, rat and sheep; heart of dog, rabbit, rat; Adrenal | 9,11-13,15-27, 29-33,35,39,44-47,53,56,59. |

TABLE III (Continued)

| Enzyme | Step* | Occurrence in Tissue | References |
|---|---|---|---|
| | Tyramine→ Octopamine | chromaffin tissue of rats and snakes; bovine adrenal medulla; spleen, liver and lungs of rat; guinea pig vas deferens. | |
| Phenylethylamine-N-Me-Transferase. (PNMT) | Norepinephrine→ Epinephrine | Rat brain and adrenal medulla; adrenal chromaffin tissue of snakes; cat skin, lungs and salivary glands. | 35,39-45,47, 50,52,60. |
| Catechol-)-Me-Transferase (COMT) | DOPA→O-Me-DOPA<br>Epinine→O-Me-Epinine<br>DA→O-Me-DA<br>NE→O-Me-NE<br>E→O-Me-E<br>DHPG→MHPG<br>DOMA→VMA | Rat brain, liver, spleen and heart. | 1,22,23,45,50. |
| Mono-amine oxidase (MAO) | Epinine→()→DOPAC, DHPE<br>DA→()→DOPAC,DHPE<br>O-Me-DA→()→HVA,MHPE<br>NE→()→DOMA,DHPG | Brain, heart, liver, spleen, blood, lung, adrenals and kidney. | 1-3,9,23,50, 51,53,62. |

TABLE III (Continued)

| Enzyme | Step* | Occurrence in Tissue | References |
|---|---|---|---|
| | O-Me-NE→()→VMA,MHPG<br>E→()→DOMA,DHPG<br>O-Me-E→()→VMA,MHPG | | |
| Tyramine Hydroxylase (same as DA-β-Hydrox.?) | Tyramine→DA | Canine heart, female rabbit liver microsomes; guinea pig,rat and mice livers. | 2,27,38,58-60. |
| ? | Epinephrine →Adrenochrome | Salivary glands, skin and lungs of the cat. | 52 |

* = () denotes aldehyde, which is very labile.

(i) Norepinephrine

a) Uptake and Storage:

The major organ for the uptake and storage of norepinephrine appears to be the adrenal medulla. It was Bennett in 1941 (63) who introduced the concept that the adrenal medullary granules were different from mitochondria, and he suggested that they represented oxidized catecholamines. That the pressor amines occur in these granules was experimentally demonstrated by Blaschko and Welch (64) and Hillarp and Nilson (65). Lever showed that typical mitochondria are present in the chromaffin cells of the rat (66). In 1956 Hagen and Welch (9) showed that these granules were from 0.1 to 0.6 μ in diameter and contained up to 19% of dry weight as pressor amines. It was claimed, by the same authors, that these granules possess a cytochrome system, amine oxidase, and most of the citric acid cycle enzymes. Later, Blaschko et al. (67) demonstrated that the chromaffin granules are the same as cytoplasmic granules and that two types are present, the light granules and the heavy granules. They have different ATP: Catecholamine ratios, and the ATP content of the granules shows an increase when the catecholamine content increases. Blaschko also demonstrated (68) that the light granules

are rich in succinoxidase, fumarase, and amine oxidase (i.e. they are mitochondria) and that the heavier granules are different from mitochondria and contain the catecholamines and ATP. Further differentiation was made by Eade (69) when by sucrose density centrifugation he obtained two different granules, one rich in norepinephrine the other rich in epinephrine. This has recently been confirmed by Biscardi and Donoso (35) who showed that in the adrenal, the epinephrine and norepinephrine containing cells are separated anatomically, the norepinephrine cells being contained in the peripheral chromaffin tissue and the epinephrine cells in the central tissue.

Hillarp (70), in 1959, postulated that the stored amines cannot exist in a free form in the extragranular water, but are bound with adenosine phosphates and probably at least a third compound, which he suggested is a protein. This protein complex is destroyed by the rupture of the granular membrane. Smith et al. (71) found two proteins present in the vesicles that can bind catecholamines in the presence of ATP and $Mg^{++}$. This agrees with the finding of Kirshner et al. (72) that dopamine uptake is stimulated by ATP and $Mg^{++}$. It has been estimated that the number of negative charges present as

adenosine nucleotides is equivalent to the number of charges present as catecholamines (71,73,74).

Kirshner showed that the chromaffin granules of the adrenal medulla (20) will take up catecholamines at 37°, but not at 0°, this uptake being inhibited by EDTA. From this he suggests an energy requiring transport mechanism for catecholamines, saying that they react with some component of the granular membrane. Lever and others (63,66,75) showed that these so-called granules are actually vesicles (i.e. they have a sac-like membrane), the granular appearance being a staining-fixation artifact representing the precipitation of oxidized catecholamines.

It has been shown by several authors (76-81) that the store of norepinephrine in sympathetically innervated tissue cannot be considered a homogeneous entity. Neff et al. (82) proposed an open two-compartment system consisting of a readily available compartment of free norepinephrine in dynamic equilibria with a larger compartment of norepinephrine complexes in the granules. Montanari et al. (83) said that the readily available pool of norepinephrine is approximately 40% of the total pool.

Paton (84) and Kopin et al. (79) postulated that the re-uptake of norepinephrine is believed to play a major role in limiting the physiological action of norepinephrine released from the sympathetic nerve endings. In accordance with this, Merrills and Offerman (32) concluded, using isolated guinea pig atria that the newly formed norepinephrine enters a fraction of the total pool. The accumulation of norepinephrine in the tissue will inhibit formation of new norepinephrine, thus avoiding saturation of the storage sites and enabling the "re-uptake" process to operate. Blakely et al. (85) found that substantial amounts of the transmitter norepinephrine liberated by the sympathetic post-ganglionic nerves are reincorporated into the stores upon which the nerves call during activity. They showed that injected norepinephrine and that liberated from nerves compete for the same uptake site.

Chromaffin granules also appear in other sympathetically innervated tissues and in the brain, but in some cases are not associated with all the dopamine, norepinephrine and epinephrine. For example, Von Euler and Lishajko (86) found a specific kind of norepinephrine granule in the vesicular gland and vas deferens of the bull.

Whole heart, heart slices, heart microsomes and brain slices have all been demonstrated to have the ability to take up and concentrate norepinephrine (29, 86-88) and by these measurements, it has been calculated and experimentally confirmed that in the normal rat about 80% of heart norepinephrine is synthesized there, the rest coming from the circulating pool (86). The norepinephrine that is taken up can be released from slices by electrical stimulation. This binding is not affected by disulfiram (89).

Norepinephrine has been found in the brain of all animals studied (91). Goldstein and Nakajima (92) demonstrated the accumulation of norepinephrine in the hypothalamus, the brain stem, and caudate nucleus in normal rats. Iversen and Glowinski (93) injected norepinephrine into the lateral ventricles where it is taken up and retained in the catecholamine-retaining neurones. They found that the cerebellum had the most rapid turnover and the hypothalamus had the slowest turnover of norepinephrine.

Glowinski and co-workers (56) showed that reserpine (a depletor of brain catecholamines) apparently affects both the uptake and release of norepinephrine in

isolated nerve. However, it is claimed that the reserpine probably only affects the intra-neuronal storage and not the actual uptake of catecholamines. The amount of norepinephrine retained after the reserpine injection is 45% of normal.

Other tissues that contain norepinephrine are: the heart of guinea pig and rabbit, the spleen of the rat, dog and cat, and the kidney of the turtle.

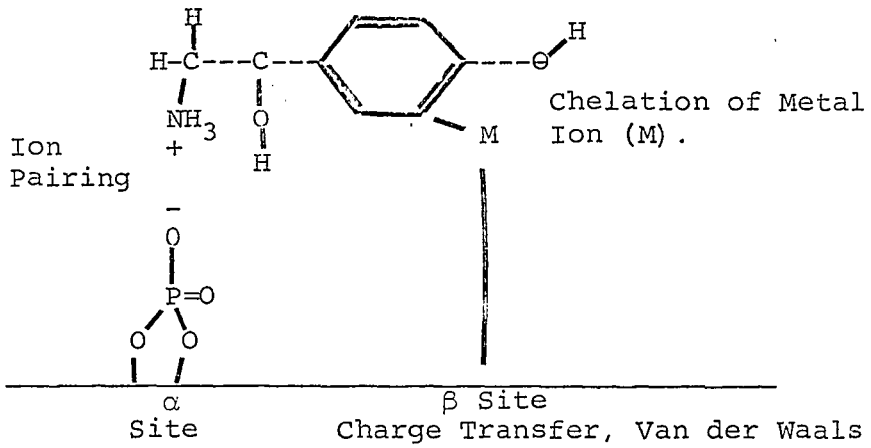
b) Physiological Actions and Importance of Norepinephrine:

It was T.R. Elliot in 1904 (95) who first postulated the chemical nature of the transmitter of the sympathetic nervous system. He claimed that the transmitter was epinephrine and it was not until 1946 that the true sympathetic neurotransmitter, norepinephrine was established by U.S. von Euler (96,97). From this discovery onwards it has been shown that, as the sympathetic neurotransmitter, norepinephrine plays a host of extremely important roles in the body.

Those nerve terminals which have norepinephrine as the transmitter liberated at the end of the postganglionic fibres are called adrenergic nerves (98).

Adrenergic receptor sites are considered to consist of two varieties, the α site and the β site. The reaction of norepinephrine with these sites may take place as in SCHEME A (99, 100).

**Scheme A.** The Adrenergic Receptor Site




i.e. The adrenergic receptor can be pictured as possessing two adjacent sites. Excitor effects are due to ion-pair formation at the α-site and chelation at the β-site binds the transmitter more firmly to the receptor surface. If combination at the α site cannot occur, then only inhibitory effects are seen.

Blood vessels of the skeletal muscles possess sympathetic vasoconstrictor fibres, i.e. norepinephrine results in vasoconstriction (101) and increased blood pressure (102). Stimulation of adrenergic nerve fibres

to the appropriate organ causes the following effects: (norepinephrine is the substance bridging the gap between the nerve ending and the effector cell), increase in heart rate; diastole is shortened and filling may be decreased; there is an increase in systolic and diastolic blood pressure (103); there is little or no change in cardiac output; an increase in the force of the heart and an increase in myocardial oxygen consumption; in small doses norepinephrine increases the depth and frequency of respiration, and causes relaxation of the bronchial musculature; there is a decrease in the amount of the secretion of the glands of the gastro-intestinal tract and respiratory passages, but the salivary and sweat glands are stimulated.

At one time there was speculation on the role of norepinephrine in schizophrenia. In 1958, Cohen et al. (104) found that both norepinephrine and epinephrine are very stable in normal and schizophrenic serum and that no difference could be detected with regard to normal subjects and schizophrenic patients. They showed that due to the stability of the pyrocatechols in plasma and serum it is unlikely that circulating plasma could mediate the development of schizophrenia via the metabolites of

norepinephrine and/or epinephrine. Berthiaume et al. (105) found no major metabolite formed when dl-epinephrine was incubated with schizophrenic serum and were in complete agreement with Cohen et al. (104). This is in direct variance with the finding of Leach and Heath (106) who presented data tending to support the thesis that epinephrine was relatively unstable in plasma or serum obtained from schizophrenic patients. This was used in support of the adrenochrome hypothesis of Hoffer et al. (107, 108).

(ii) Dopamine

a) Uptake and Storage:

Most of the work on the uptake, storage, action and importance of dopamine has been carried out recently. In 1958, Eade (69) claimed that although the chromaffin granules of the adrenal medulla contain little dopamine, most of it is associated with the 'light' granules. Bertler et al. (109) reported that the cow, sheep, and goat have special chromaffin cells that non-ruminants do not possess. These cells were found to correlate with the dopamine concentration in the lungs, liver, splenic nerves and submucosa and mucosa of the intestine. However, no correla-

tion was found between cell distribution and amine content in the spleen or heart.

In the brain, dopamine is highly localized in certain of the basal ganglia as well as in the hypothalamus and thalamus (110, 111). It is the catecholamine present to the largest extent in the brain of rats, guinea pigs, rabbits, dogs, and pigeons, and the second largest in most other animals (91). Fuxe (112) and Carlsson et al. (113) showed that the following areas of the brain have dopaminergic nerve terminals; neostriatum, tuberculum olfactorum, nucleus accumbens, median eminence, nucleus amygdaloideus centralis and the dorsal part of the nucleus interstitialis striae terminalis. In these dopamine neurones, diethyldithiocarbamate does not seem to affect the level of dopamine as it is the end-point of catecholamine metabolism in these tissues (114). It is claimed that by the use of diethyldithiocarbamate separation of the norepinephrine and dopamine nerve terminals can be obtained. Goldstein and Nakajima (94) showed that dopamine accumulates in the caudate nucleus, hypothalamus and brain stem. This accumulation occurs in both disulfiram treated and normal animals.

Kirshner and co-workers (72) found that reserpine inhibited the uptake of dopamine by about 98%, the inhibition lasting about 48 hours. Denervation of the adrenals themselves did not cause a loss of the catecholamine content. ATP and $Mg^{++}$ were found to stimulate the uptake of dopamine and this stimulation was completely inhibited by EDTA. If the granules remained intact, the EDTA only affected the accessibility of the dopamine to the granules and not its conversion to norepinephrine. The explanation is that the reserpine causes a depletion of the catecholamine stores by inhibiting the transport of dopamine into the granules. During the transport process, dopamine is supposedly bound until it reaches the site of action of dopamine-β-hydroxylase (32).

b) Physiological Actions and Importance of Dopamine:

Until recently dopamine was thought of only as the precursor of norepinephrine and epinephrine with no biological activity of its own. However, in the past years dopamine has become potentially important as a neurotransmitter and the methylated metabolites may have a major relationship in mental disease, notably Parkinson's disease and schizophrenia.

It was Harley-Mason (115), in a paper by Osmond and Smythies, who was the first to suggest methylation of the phenol ring in the occurrence of schizophrenia. He suggested 3,4-dimethoxyphenylethylamine (dimethyl dopamine, DIMPEA) as the most likely compound to be involved. Friedhoff and Van Winkle (116) in 1962 claimed to have isolated a compound from the urine of schizophrenics which is not present in normals. They said that this compound was DIMPEA. Takesada (117), Sen and McGeer (113), and Kuehl et al. (119) all claim to have identified DIMPEA in the urine of schizophrenics, but some of them found DIMPEA in the urine of normals, but not to the same extent as in schizophrenics. Friedhoff and Van Winkle (120) infused schizophrenic patients with $C^{14}$ dopamine and claim to have isolated 3,4-dimethoxyphenylacetic acid, the acid metabolite of DIMPEA, from their urine. Kuehl et al. (121) demonstrated this by the administration of DIMPEA itself. The latter also tried to demonstrate DIMPEA in schizophrenics and not in normals, but their differences were not statistically significant. They also failed in an attempt to demonstrate the conversion of dopamine to DIMPEA in vitro. Schweitzer and Friedhoff (122) claimed that DIMPEA does not occur in plants and that when injected into rats it distributes itself in a manner analagous to mescaline

(trimethoxyphenylethylamine). In a very extensive test, Bourdillon et al. (123) investigated 808 individuals and said although DIMPEA was not shown conclusively to be the compound, they did find a "pink spot" upon chromatography of the urine and confirmed its association with schizophrenia, suggesting it as a result rather than as a cause of the illness. They could not find the "pink spot" in normals. Barbeau et al (62) injected DIMPEA into the rat and found an increase in the dopamine of the grey nucleus during the period of clinical akinesia. They claimed that DIMPEA complexes with dopamine at specific dopaminergic nerve endings where dopamine is the end point in catecholamine metabolism (e.g. the striatum and lungs) and where it probably has an independent physiological action.

Wagner and co-workers in 1966 (124) suggested that O-methylation of catecholamines does not occur abnormally in schizophrenia. They also failed to demonstrate the conversion of S-Adenosyl-L-methionine-$C^{14}$ to DIMPEA in experiments in vitro using the liver and brain of both normal and schizophrenic individuals. They also state that DIMPEA and 3,4-dimethoxylphenylacetic acid are not metabolites in the chronic schizophrenic.

Ehringer and Hornykiewicz (125) were the first to report that the concentrations of dopamine and norepinephrine and 5-hydroxytryptamine are significantly lower in the brain of Parkinsonian patients than in normals. Dopamine was found to be especially lower. This was later confirmed by Bernheimer et al. (126) and Hornykiewicz (127). Hornykiewicz et al. (128) demonstrated the virtual absence of dopamine from the caudate nucleus and putamen of patients dying of Parkinson's disease. In 1962, Ernst (129) found that O-methylated derivatives of dopamine, when injected, would cause phenomena of the hypokinetic rigid type. He suggests that in the disease state, this type of phenomenom is created by O-methylation of dopamine in the p-position with or without O-methylation in the 3-position in consequence of a defect in dopamine metabolism through the methyl transferase system. He also states that reserpine produces a Parkinson-like syndrome by depleting the dopamine in the corpus striatum. Several authors (126,130,131) have suggested that the enzymic mechanism catalyzing the formation of dopamine from its precursors is defective in Parkinson's disease.

Sourkes and Poirier (132) reported in 1963 that monkeys in which brain stem lesion have been placed and had subsequently developed postural tremor, had the

concentration of both dopamine and norepinephrine lower in the basal ganglia of the lesioned side than the intact side. The difference was greater for the lenticular than the caudate nucleus. Poirier and Sourkes later demonstrated (133,134), again in monkeys with brain stem lesions, that the animals with a marked unilateral loss of cells in the substantia nigra showed in the corresponding caudate nucleus and putamen much lower dopamine and norepinephrine concentrations than in the striatum of the intact side. They suggested that the pars compacta of the substantia nigra normally exerts through its efferent nervous pathways a direct influence on the catecholamine concentration of the corresponding ipsilateral striatum. These authors in 1966 were the first to demonstrate directly a function of dopamine in the central nervous system (135). They found that the postural tremor and hypokinesia of the limbs after the production of electrolytic lesions in the upper brainstem were associated contralaterally with a lesion of substantia nigra and depletion of striatal dopamine and Serotonin. From this they deduced that the nigro-striatal fibres were dopaminergic and that dopamine has a role in the control of limbs and posture.

In 1966, Farmer (136) reported that dopamine is a sympathomimetic amine with direct and indirect actions. It has a direct action on the heart and nictitating membrane. The vasodepressor action of dopamine is both direct and indirect. The indirect action is due to the ability of dopamine to displace norepinephrine. The release of norepinephrine by dopamine has also been reported by Harrison et al. (137).

## B. Dopamine-β-Hydroxylase

### (i) Historical

Blaschko (12) in 1939 was the first person to postulate the hydroxylation of dopamine to norepinephrine and thus he may be said to be the first to suggest the existence of the enzyme, Dopamine-β-hydroxylase. Later he suggested that it might be the side-chain hydroxylation that is the rate-limiting step in norepinephrine biosynthesis (138). However, more recent evidence (72, 139) does not support this, at least in the adrenal medulla and heart.

In 1956, Hagen demonstrated the presence of the enzyme in the adrenal medulla (15) and Leeper and Udenfriend (140) showed that dopamine is converted to norepinephrine in the rat adrenal in vivo. This was confirmed by Goodall and Kirshner (17) in the following year. It was also demonstrated that Dopamine-β-hydroxylase is contained in the particulate fraction of adrenal homogenates (141). It has since been shown by several authors (19,20,142,143) that it is located in the catecholamine-containing particles of the adrenal medulla. Only two other enzymes are known to occur in these particles, an ATPase and an ADP transphosphorylase (70,144).

The presence of Dopamine-β-hydroxylase in the heart was demonstrated by Booker and co-workers (145) by the conversion of dopamine to norepinephrine, and soon confirmed by others (29,58,146). Potter and Axelrod (147) suggested that the enzyme is associated with the vesicular elements isolated from cardiac tissue, and Musacchio et al. (143) substantiated this. That the enzyme is present in the heart has also been demonstrated using tyramine as substrate (26, 148). The employment of tyramine as substrate was used to show the presence of Dopamine-β-hydroxylase in the salivary gland of the rat in vivo (149) and in adrenal and brain slices (150). This pathway has been suggested as an alternate route for norepinephrine biosynthesis. Dopamine-β-hydroxylase has also been shown to occur in the brain of the rat, dog, sheep and cow (18). It has been suggested that the enzyme does not occur in those granules containing large amounts of dopamine (109, 151, 152).

Dopamine-β-hydroxylase has been implicated in the production of epinephrine from epinine both in vitro (153,154) and in vivo (22). This pathway had already been suggested as having significance in those tissues in which there is a relatively large amount of epinephrine as compared to norepinephrine (155,156).

The enzyme has been isolated and purified by several workers. Levin et al. (19) first purified it in 1960, followed by Goldstein and Contrera (22). In 1965 Goldstein et al. (157) purified the enzyme 87.5 fold, using ammonium sulphate fractionation and precipitation followed by calcium phosphate gel adsorption, elution and chromatography twice on DEAE cellulose.

(ii) Cofactors and Characteristics of Dopamine-β-Hydroxylase

a) Classification:

The formal designation of the enzyme is EC 1.99.1. According to the definition of Mason (158) Dopamine-β-hydroxylase can be classified as a mixed function oxidase, i.e. it requires aerobic oxygen and an external electron donor. The hydroxyl O is derived from atmospheric oxygen. In 1962 Kaufman et al. (159), proved unequivocally that Dopamine-β-hydroxylase is a mixed function oxidase. They showed that in the Dopamine-β-hydroxylase catalyzed reaction of phenylethylamine to phenylethanolamine, the hydroxyl oxygen atom in the synthesized ethanolamine was derived from molecular oxygen and not water.

b) Cofactors:

(1) ATP and $Mg^{++}$:- Neri and co-workers (160), in 1956 were the first to show stimulation of the conversion of dopamine to norepinephrine by ATP. This was confirmed by Kirshner (141,161). However, Levin et al. found that there was only a slight stimulation with the partially purified enzyme (19). Levin and Kaufman have since demonstrated (162) that in the presence of catalase, ATP has no effect and thus it is not intimately involved in the hydroxylation reaction. Goldstein et al. (157) claim that the ATP stimulates Dopamine-β-hydroxylase by affecting the enzyme's stability. The above workers (141,160,161) also demonstrated the ability of oxygen and $Mg^{++}$ to stimulate the dopamine to norepinephrine conversion in adrenal homogenates or acetone powders. Carlsson et al. (142) and Kirshner (20) have since shown that $Mg^{++}$ is required for the uptake of dopamine by adrenal vesicles. The activity of the enzyme does not appear to be necessary for the uptake of the amines, but is necessary for the storage of amines such as tyramine (142,143).

(2) Catalase:- Catalase apparently acts in a manner similar to ATP (157) although as mentioned above, ATP is not active when catalase is present. In 1961,

Levin and Kaufman (162) reported that small amounts of $H_2O_2$ will inactivate the enzyme and that catalase will protect the enzyme from this inactivation. Van der Schoot et al. (163) confirmed that Dopamine-β-hydroxylase reactions are stimulated by added catalase, and that with tyramine as substrate, there is an absolute requirement for catalase. In the purified enzyme preparation, catalase is essential for activity (157).

$Fe^{++}$ can replace catalase in protection of the enzyme from inactivation at low pH, and is superior to catalase below pH 5.5 (157).

(3) Fumarate:- Fumarate also stimulates the enzyme, but not equally for all substrates. There is strong stimulation for dopamine and phenylethylamine (162) and for epinine (153), but there is only slight stimulation for tyramine as substrate (163,164). The consumption or conversion of fumarate to any other compound during the hydroxylation has not been demonstrated (30).

It has been suggested that fumarate may act by inducing an allosteric change in the enzyme molecule, i.e. a change to a conformation of higher activity (165). It is not known if fumarate is active in vivo.

(4) Ascorbate:- Levin and co-workers (19) in 1960 demonstrated that one mole of ascorbate produced one mole of dehydroascorbate for each mole of dopamine converted to norepinephrine. That ascorbic acid is a co-substrate which is stoichiometrically reduced to dehydroascorbic acid was confirmed by Levin and Kaufman (162). This reaction is the first one in which it has been demonstrated that ascorbate is stoichiometrically consumed (30). Dopamine + Ascorbate + $O_2$ → L-norepinephrine + Dehydroascorbate + $H_2O$ (19).

The in vivo function of ascorbate is uncertain. Dependence of the conversion of dopamine to norepinephrine could not be demonstrated in the ascorbate-deficient guinea pig (166). The role in vivo is made more difficult to ascertain by the report of an enzyme that can catalyze the conversion of dehydroascorbate to ascorbate (167).

c) Copper:

It has been shown that Dopamine-β-hydroxylase is a copper protein (157). The evidence for this is as follows:

(1) The enzyme is inhibited by chelating agents (164), especially copper-sensitive ones (see below).

(2) The presence of copper in the purified protein. In the purified protein there is 0.65-1.0 μg. of copper per mg. protein, i.e. 4-7 moles copper per mole protein (21,157). The $Cu^{++}$ content is constant at about 2 moles per mole of enzyme (21).

(3) The ratio of copper to protein increases during purification, the increase in activity being proportional to the increase in copper content (157).

(4) The enzyme is inhibited by copper and peroxides (157). This, along with the protection by catalase from reaction inactivation is common to copper enzymes (168,169).

The copper nature of the enzyme might explain its protection by catalase in a manner analagous to ascorbate oxidase (168). $H_2O_2$ causes a significant inactivation of ascorbate oxidase only when the enzyme is functioning. It is claimed that the $H_2O_2$ reacts with the active site of ascorbate oxidase when the copper is in the cuprous form (170). The proposed role for copper in Dopamine-β-hydroxylase is to accept electrons from ascorbate and transfer them to oxygen (30).

Possible other roles for copper, for which there is no direct evidence, are as follows:

(1) The copper may bind oxygen (171) as it does in hemocyanin (172,173). This does not appear to involve a change in the valency of copper (174).

(2) It may play a role in binding the substrate to the enzyme surface as it does in other enzymes (175,176). The cuprous copper present, may function in this respect. Charge transfer complexes have been observed between enzyme-bound copper and substrate in other cases (176).

It has been suggested by Kaufman and co-workers (159) that the function of the metal is to co-ordinate with the molecule of atmospheric oxygen which contributes the O atom to the newly formed hydroxyl group in the substrate.

d) Kinetic and Physical Characteristics:- Dopamine-β-hydroxylase is a colourless enzyme, which in the purified state (21), converts dopamine to L-norepinephrine (162). In the highly purified state it catalyzes the hydroxylation of approximately 1000 moles of dopamine per minute at 25°C, has an $s_{20,w}$ of 8.9 in 0.1M NaCl + 0.005 M. potassium

phosphate buffer pH 6.8, a specific partial volume (v) of 0.720 and a molecular weight of about 290,000 (21). The ultracentrifugal analysis of a pure preparation by a different group of workers (157) gives a major component with an $s_{20}$ of 5.4 and a minor component with an $s_{20}$ of 12.5

The kinetic measurements vary considerably between whole homogenates and pure preparations (Table IV). These kinetic measurements (in the whole homogenate) may be artificial and inaccurate due to the rather slow uptake and decrease in substrate concentration (32). Another problem is that dopamine is an effective substrate for MAO and is destroyed rapidly. It also is both the precursor and a depleting agent for norepinephrine (24).

(iii) Substrate Specificity

Dopamine-β-hydroxylase catalyzes the hydroxylation of a wide variety of phenylethylamine derivatives to form the corresponding secondary alcohols (150,154,162), i.e. it catalyzes the β-hydroxylation not only of dopamine, but also amines structurally related to dopamine (178,179). That it is a single enzyme that hydroxylates all these

TABLE IV

Summary of Kinetic Information on Dopamine-β-Hydroxylase

| Reference | Substrate | pH Optimum | Km. | Other Information |
|---|---|---|---|---|
| 32 | Dopamine | | Km apparent $=3x10^{-3}M.$ | Isolated guinea pig atria. Vmax= 25 m/kg/hr. |
| 157 | Dopamine | 5.5 | | |
| 22,154 | Dopamine | 6.2 | $5.8x10^{-3}M.$ | |
| 163 | Tyramine | 5,0-5.5 | | |
| 177 | Dopamine | 5.5 | $6x10^{-3}M.$ | |
| 177 | Tyramine | 5.5 | $8x10^{-4}M.$ | |

TABLE V

Substrates of Dopamine-β-Hydroxylase

| Substrate | Product | Relative Activity | References |
|---|---|---|---|
| p-Tyramine | octopamine (norsynephrine) | 100 | 22,27,58, 154,157. |
| m-Tyramine | m-octopamine | 60-80 | 154 |
| o-tyramine | - | 0 | 154 |
| Dopamine | norepinephrine | 85-90 | Table III |
| Epinine | epinephrine | 25 | 22,26,148, 153,154. |
| N-Me-tyramine | N-Me-octopamine | 25 | 154 |
| N,N-diMe-tyramine | - | 0 | 154 |
| α-Me-tyramine | α-Me-octopamine | 60-70 | 154 |
| 3-0-Me-tyramine | 3-0-Me-octopamine | 50-70 | 154 |
| 3-0-Me-dopamine | 3-0-Me-norepinephrine | - | 157 |
| Mescaline | β-OH-Mescaline | 2-5 | 154 |

TABLE V (Continued)

| Substrate | Product | Relative Activity | References |
|---|---|---|---|
| β-phenylethylamine | β-phenylethanolamine | 10-15 | 22,154 |
| 3,4-dimethoxy-phenylethylamine | 3,4-dimethoxyphenylethanol-amine | 2-5 | 154 |
| 3-methoxy-4-OH-phenylethyl-amine | 3-O-Me-norepinephrine | - | 22 |
| δ-phenylpropylamine | δ-phenylpropanolamine anolamine | + | 154 |
| p-OH-δ-phenylpropylamine | p-OH-δ-phenylpropanolamine | ++ | 154 |
| DL-Amphetamine | Norephedrine | - | 22 |
| DL-p-hydroxyamphetamine | p-hydroxynorephedrine | - | 22 |

amines, was shown by Levin and Kaufman (162) and Goldstein and Contrera (22). It has also been demonstrated that any amine that is taken up by the granules and is a substrate for Dopamine-β-hydroxylase will be hydroxylated (143,148,149).

From a comparison of the chemical structure of the substrates (Table V) it is evident that compounds with electron-withdrawing groups in the para-position of the benzene ring, with a weakly basic amino nitrogen on an ethyl side chain, are the best substrates for the enzyme (22). The basic requirements for activity as a substrate of Dopamine-β-hydroxylase are a benzene ring with a 2 or 3 C atom side chain with a terminal amine. Hydroxyl substitution on the ring in the meta- or para-position, or both, increases activity (30).

The importance of this wide range of action of Dopamine-β-hydroxylase may be seen in its action on many of the sympathomimetic drugs such as the amphetamines. It is claimed that at least some of the activity of the parent drug is due to the hydroxylated product (154).

From Table V it is seen that O-methylation of the substrate at the p-OH position abolishes nearly all activity. The m-hydroxy derivatives and the unsubstituted

parent compounds are less active than the p-OH derivatives. The o-OH derivatives have essentially no activity. A methyl group on the side chain or on the terminal nitrogen reduces activity 75-90% and tertiary amines are inactive (163).

(iv) Inhibition of Dopamine-β-Hydroxylase

a) Antabuse (Disulfiram) and Diethyldithiocarbamate:

The first report on the inhibition of Dopamine-β-hydroxylase by Antabuse was by Carlsson and co-workers (180). Goldstein et al. (157,181) reported that Antabuse is the most effective inhibitor of the enzyme in vivo and in vitro. Musacchio et al. stated that Antabuse apparently inhibits the enzyme by complexing the copper (143). However, Udenfriend et al. (184) reported that Antabuse is not a very active inhibitor of norepinephrine biosynthesis in vivo using either DOPA or tyrosine as precursors.

b) Other Metal Chelators:

The first chelating agent shown to be an inhibitor of Dopamine-β-hydroxylase was cyanide (19). Since then many other chelators have been reported to inhibit the enzyme (19,20,164,185). EDTA has been found to completely inhibit

Dopamine-β-hydroxylase in lysed adrenal medullary granules, but ineffective if the granules are intact (20,72). The explanation is that the enzyme is bound to the inner surface of the vesicular membrane or some other site inacessable to EDTA and not capable of reacting with the substrate until the substrate enters the vesicle.

Of these chelators, $CN^-$, diethyldithiocarbamate, 2,9,dimethyl-o-phenanthroline, bathocuproinesulfonate and cuprizone are especially sensitive to copper (186). There is no effect of pre-incubation with enzyme on the inhibition by the chelators (164) and most of them, except for diethyldithiocarbamate, can be reversed by $Fe^{++}$ or $Co^{++}$ (157).

c) Analogue Inhibitors:

The activity of competitive inhibitors is similar to their activity as substrates of Dopamine-β-hydroxylase, as in Table V. If the inhibitor is pre-incubated with the enzyme the inhibition is apparently non-competitive with respect to substrate. If the substrate and inhibitor are added to the enzyme simultaneously the inhibition is competitive or partially competitive (163,177). No competitive inhibitor of Dopamine-β-hydroxylase has been uncovered that lowers the catecholamine content of the tissue solely on the basis of its inhibition of the enzyme (30).

Benzyloxamines and benzylhydrazines have been found to be potent inhibitors of Dopamine-$\beta$-hydroxylase both in vitro (55, 177) and in vivo (188, 189). The inhibitory action does not seem to be due to the benzyloxamine structure as not all benzyloxyamines are active inhibitors. Apparently the benzyloxyamines act by interaction at the substrate site and are relatively specific for Dopamine-$\beta$-hydroxylase. It has been suggested that the benxyloxyamine-dopamine-$\beta$-hydroxylase complex dissociates in vivo as the inhibition resulting from 200 mg/kg intraperitoneal injection lasts only 5 hours (188).

The dopamine to norepinephrine conversion is more strongly inhibited by phenylethylamines and their derivatives than by phenylpropylamines and their derivatives. The primary amines are much more active inhibitors than the secondary amines, and catechol and para-phenolic amines are more potent inhibitors than the corresponding unsubstituted amines (22).

d) Other Types of Inhibition:

It is of some controversy as to whether feedback inhibition is of any importance with respect to Dopamine-$\beta$-hydroxylase. Harrison et al. (24) claimed that it does not appear to affect the enzyme. They found that the same

TABLE VI

Inhibitors of Dopamine-β-hydroxylase

| Inhibitor | Type | Concentration (% Inhib.) | Notes | References |
|---|---|---|---|---|
| Antabuse | Chelator(?) | $10^{-6}$M.(100) | Both in vivo and in vitro. | 157,180,181 |
| Diethyldithio-carbamate | Chelator | $10^{-6}$M.(100) | Both in vivo and in vitro. Breakdown product of Antabuse. Gives a tightly bound yellow complex when added to a pure preparation. | 21,114,157, 181-183 |
| EDTA | Chelator | $10^{-4}$M.(100) | $10^{-5}$ stimulates activity slightly. | 20,72,157 |
| 2,9,dimethyl-o-phenanthroline | Chelator | $5x10^{-6}$M. | | |
| o-phenanthroline<br>Na bathocuproine-sulfonate<br>8-OH quinoline<br>8,2 dipyridyl<br>Cuprizone | Chelators | | | 20,21,157, 164 |
| Colchiceine | Chelator | | Colchicine does not inhibit due to lack of the free OH group. | 164 |

TABLE VI (Continued)

| Inhibitor | | | | |
|---|---|---|---|---|
| CO | | | Dopamine-β-hydroxylase and Phenolase are the only colorless, Cu-containing enzymes, inhibited by CO. | 187 |
| p-OH benzyloxyamine | Analogue | $10^{-5}$M. (98-preincubate 40-simultaneousincubation) | | 163,177 |
| Benzyloxyamines | Analogues | $10^{-5}$M.(60-80) $10^{-3}$M.(100) | $K_i$=6.6x$10^{-6}$M. in vitro. | 163,177 |
| NSD-1055 | ? | | Also inhibits DOPA decarboxylase. | 191 |
| TM-10 | | K 1.75x$10^{-1}$M. | Stimulates at 8x$10^{-2}$M. | 192 |

amounts of norepinephrine were synthesized from administered dopamine when the tissue contained its normal complement of norepinephrine as when it was almost depleted, i.e. the Dopamine-β-hydroxylase level is the same when its product is low as when it is normal. However, Merrills and Offerman (32) found that addition of norepinephrine to an organ bath results in considerable reduction in the rate of Dopamine-β-hydroxylase and they suggest that this is due to end-product inhibition of the enzyme.

It has been reported that Dopamine-β-hydroxylase inhibition is more effective if the animals are kept in the cold (190).

(v) Mechanism of Action

As stated earlier the enzyme is of the mixed oxidase type. The dehydrogenation-hydration type of reaction is unlikely as ascorbate is an essential component of the Dopamine-β-hydroxylase system (19) and this type of reaction would require an electron acceptor. not an electron donor such as ascorbate.

2,6-Dichlorophenolindophenol is highly active as a substitute for ascorbate (30) and in the absence of ascorbate, dopamine can function as a reducing agent in

the following reaction (162):

$$2 \text{ dopamine} + O_2 \rightarrow \text{dopamine-quinone} + H_2O + \text{norepinephrine}$$

This can be coupled to DPNH oxidation (non-enzymatic) (193) as follows:

$$\text{Dopamine-quinone} + \text{DPNH} + H^+ \rightarrow \text{Dopamine} + DPN^+$$

i.e. $$\text{Dopamine} + O_2 + \text{DPNH} + H^+ \rightarrow \text{norepinephrine} + DPN^+ + H_2O$$

Pyrocatechol can function as a reducing agent for substrates without the catechol group, but is only one-tenth as active as ascorbate (162). Friedman and Kaufman (21) reported that on the basis of the pyrocatechol and 2,6-dichlorophenolindophenol reactions, the first step in the Dopamine-β-hydroxylase catalyzed reaction involves an interaction between enzyme, electron donor and oxygen.

$$E + \text{ascorbate} \rightarrow E^= + 2H^+ + \text{dehydroascorbate}$$

This reaction has been shown to occur both aerobically and anaerobically (21). The reduced enzyme has been prepared and shown to be an intermediate in the next reaction:

$$E^= + O_2 + 2H + RH \rightarrow ROH + E + H_2O$$

It has been suggested that copper is the reducing group. After addition of substrate the amount of copper oxidized was found to be equivalent to the amount of pro-

duct formed.

i.e. $E(Cu^{++})_2 + 2 \text{ electrons} \rightarrow E(Cu^{+})_2$

The overall reaction of Dopamine-β-hydroxylase is:

$$E(Cu^{+})_2 + O_2 + 2H^{+} + RH \rightarrow E(Cu^{++})_2 + ROH + H_2O$$

C. Copper and Iron

As the subjects of normal copper and iron metabolism are beyond the scope of this thesis, only the deficiency states will be examined.

(i) Copper

Copper has been shown by many authors to be of vital importance in maintaining a normal, healthy animal. It is present in all tissues (194). Some of the compounds in which copper is normally present are:

Erythrocuprein in erythrocytes (195)
Cerebrocuprein in brain (195)
Hepatocuprein in liver (195)
Tyrosinase (195)
Ceruloplasmin (195)
Spermine oxidase of ox plasma (196)

It has been suggested that the role of ceruloplasmin is to donate copper to the tissues (198).

Schultze (199) was one of the first to show a function of copper in vivo. It was shown that the cytochrome oxidase activity of heart, liver and bone marrow

were greatly decreased in copper deficient rats, and that it was quickly regenerated with the addition of copper to the diet. Gallagher et al. (200) demonstrated that the depletion of cytochrome oxidase activity in copper deficiency led to the loss of succinoxidase activity. They found that in extreme copper deficiency there was a loss of ability of the mitochondria to oxidize substrates. They also found increased isocitric dehydrogenase levels.

There have been several reports on the loss of activity of pig plasma benzylamine oxidase activity in copper deficiency (201,202). This enzyme has 3-4 atoms copper per mole of enzyme, does not act on catecholamines, and is inactive in copper deficiency with its activity being restored by copper sulphate treatment.

Another defect in copper deficient animals is that their aortas appear to contain less elastin than normals and that their elastin contains an abnormally high amount of lysine (203).

It has been reported that injections of tremorine cause a reduction of brain copper 30 minutes after administration. The level returns to normal after 2 hours (204). The same authors reported that LSD and

diethylcysteamine also reduced brain copper. From this they suggested that mobilisation of copper in the brain causes intention tremor. Cessa and Schneider (205) found that the administration of copper sulphate potentiated the toxicity of tremorine and increased the death rate from 50% to 100%.

The route of copper absorption appears to be from the stomach and not from the small intestine as it is for metals such as cadmium and zinc (206).

(ii) Iron

The liver and the spleen are the organs that contain most of the body's accumulated reserves of iron, in the form of two non-heme protein components, ferritin and hemosiderin (207). Even in the most severe cases of iron deficiency some of this non-heme storage iron remains in the liver and spleen (208,209). This type of iron has been termed 'parenchymal iron' by Whipple et al. (210,211), is apparently physiologically different from other storage iron and is essential for the normal function of liver and spleen. One source of this could be the breakdown of hemoglobin and the resultant non-heme iron.

Iron stored either as ferritin or as hemosiderin is equally readily released when required for hemoglobin synthesis (207,212).

Upon attainment of sexual maturity there is a noticeable difference in iron stores in male and female rats. The normal storage iron is much higher in liver and spleen in female rats (207,213-215). Female rats also have a higher level of plasma iron (216). These sex differences are maintained even after severe iron depletion (207). Castration of the female results in decreased iron storage and thus the differences are probably due to hormonal causes (213,217).

Chronic haemorrhagic anemia will result in iron deficiency. Bleeding alone and in combination with an iron low diet are common methods of obtaining experimental iron deficiency (207). A significant reduction in the content of iron in the rat brain is claimed after the administration of tremorine or LSD (218).

Iron absorbed from the gastro-intestinal tract and soluble iron injected in small amounts is stored rapidly and almost entirely in the liver (219).

(iii) Copper-Iron Interrelationship and Other Trace Metals

Owing to the existence of interrelationships between some of the trace elements a simple dietary deficiency in one of these will probably affect the metabolism of at least one other element and this relationship may eventually be of more importance than the deficiency itself. This has been known or at least implied for some time.

In 1932 Elvehjem and Sherman (220) reported that when rats deficient in both iron and copper (i.e. doubly deficient) were fed iron, the iron content of the liver and spleen were increased two to three times over that of the deficient controls. Doubly deficient rats fed iron alone for two weeks followed by copper alone for two weeks regenerated hemoglobin, and simultaneously decreased liver and spleen iron to the double-deficient value. Stein and Lewis (221) found that combined iron and copper deficiency gave rise to hyperchromic, microcytic anemia. The rats responded to copper therapy by an increase in the number of red blood cells without a concomitant increase in the hemoglobin concentration. This has been confirmed by Cartwright et al. (222).

They found injected iron did not prevent the development of anemia in copper deficient swine but the red blood cells were normocytic. The addition of copper to the diet of milk anemic or iron/copper-deficient rats, rabbits or swine, increases the number of reticulocytes. Iron does not do this. The reticulocytosis following copper therapy appears to be persistent whereas that following combined iron and copper therapy is transitory. The anemia in double deficiency develops more rapidly and is more severe than in the iron deficient animal (207). No essential difference has been found between the bone marrow of iron-deficient and copper-deficient rats (223). Both have been found to be hyperplastic.

In doubly deficient animals low hemoglobin and low plasma iron values are found, however, it is claimed that there is adequate storage iron in the liver and spleen (207). On the basis of these observations, Morgan has suggested that the mobilization of storage iron and/or its utilization for haemoglobin synthesis is depressed. Gubler et al. (224) arrived at a similar conclusion.

Cohn and Elvehjem (225) reported that cytochrome a was absent or markedly reduced in heart or liver of milk anemic rats. Copper alone restored the

cytochrome a levels. They also found a slight decrease in cytochromes b and c in the heart and liver of doubly deficient rats. This was reversed only when both iron and copper were added to the diet. Schultze and Kuiken (226) found a decrease in catalase activity in liver, kidney and blood in the iron and copper-deficient rats. This appeared to be more marked in copper deficiency than in iron deficiency; the activity returned to normal when copper was fed.

Bunn and Matrone (227) have corroborated the findings of Elvehjem and Sherman (220) by reporting that in copper-deficient animals the iron content is twice that of the controls. Cox and Harris (228) found that copper can mobilize iron from the liver even when the dietary level of iron is low. Magee and Matrone (229) found that supplemental copper was associated with a reduction in the iron content of the liver, thus supporting the work of Cox and Harris.

The copper-iron relationship is also apparently dependent upon zinc to some extent. In 1958 Swarma Sasty and Sarma (230) reported that the apparent antagonism between zinc and copper is actually a reflection

of zinc on iron metabolism. Magee and Matrone (229) found that zinc directly interferes with the utilization of iron but not with its absorption (231). In the rat, zinc interferes with copper metabolism by decreasing the utilization and increasing the excretion of copper. Zinc apparently has little or no effect on the absorption of copper (231). Gubler et al. (224) suggested that when copper was omitted from the diet and zinc was added to the diet, iron accumulated in the liver because the copper concentration was inadequate for the normal mobilization and utilization of iron.

Bunn and Matrone (227) found that in rats, but not in mice, both cadmium and zinc lowered the liver copper concentration. They also found that cadmium lowers liver iron and zinc increases liver iron. This latter finding is in contradiction to the findings of most authors, i.e. that zinc decreases liver iron (227, 228,232).

D. Antabuse and Diethyldithiocarbamate

(i) Antabuse

Antabuse is one of the many common names for (diethyldithiocarbamyl) disulfide. Some of the other common names are: tetraethylthiuram disulfide; disulfiram; Abstensil; Antietanol and others (233).

The structure of Antabuse is:

$$(C_2H_5)_2\text{-N-}\overset{\overset{S}{\|}}{C}\text{-S-S-}\overset{\overset{S}{\|}}{C}\text{-N-}(C_2H_5)_2$$

One of its major uses is in the treatment of alcoholism. Hald et al. (234) demonstrated that Antabuse causes the accumulation of acetaldehyde and Morimura (235) showed that it inhibits the endogeneous respiration of the liver. Casier and Polet (236) showed that Antabuse is a metal chelator and that it slows down the oxidation of alcohol.

Bradley and Hewer (237) reported that, along with the above metabolic changes, Antabuse gives rise to drowsiness, headaches, memory impairment, decreased libido, gastro-intestinal symptoms, halitosis, skin rashes and peripheral neuropathy. Goldstein and Nakajima (190) found that Antabuse leads to moderate sedation, a loss of balance and increased sensitivity to the cold.

Hassinen (238) claimed that Antabuse inhibits mitochondrial oxidations of NAD linked substrates, but does not affect succinoxidase activity or mitochondrial respiration. The DNP-activated ATPase is inhibited by 60% whereas there is little effect on the Mg-activated ATPase. There is a 50% irreversible inhibition of the ATP-Pi exchange ratio. 2-mercaptoethanol protects the mitochondria if added before the Antabuse.

(ii) Diethyldithiocarbamate

Goldstein and co-workers demonstrated that Antabuse is degraded to diethyldithiocarbamate in vivo (181). Knowing this, the work that has been accomplished on diethyldithiocarbamate can be applied, at least partially, in understanding the effects of Antabuse.

Pfeifer and co-workers (239) found that diethyldithiocarbamate diminished the hypermotility caused by amphetamine or cocaine. This was not observed in the presence of an MAO inhibitor (Nialamide). From this information they conclude that diethyldithiocarbamate may inhibit the hypermotility by its effect in decreasing the norepinephrine content of the brain. They also found that diethyldithiocarbamate increases the sleeping time

in mice, for 50 mg/kg hexobarbitone, from 7 minutes to 40 minutes.

Howell (240,241) reported that diethyldithiocarbamate depresses blood copper levels and that foetal resorption follows its administration to pregnant rabbits. After 7 and 1/2 months of daily intraperitoneal injections of diethyldithiocarbamate rabbits showed signs of incoordination and had nervous degeneration and lesions of the spinal cord. The copper levels of the liver and central nervous system had increased at this time (241).

# II. METHODS

## A. Chronic Animal Experiments

Four long term copper deficiency experiments were carried out.

### (i) Experiment No. 1

Forty-six male Sprague-Dawley albino rats, weighing about 35 grams each initially, were used in this experiment. The cages used were nickel-plated stainless steel metabolism cages and were machine-washed and then rinsed with glass distilled water, twice a week. All rats were fed the copper deficiency diet for 7 days and then were separated into 2 groups (18 control and 28 copper deficient). Rats were sacrificed twice a week, the number on each occasion being noted below. They were fed Crino Powdered Milk obtained from the Cooperative Agricole de Granby and were supplemented daily with the following vitamins, given daily in their drinking water.

Daily supplement per rat:

| | |
|---|---|
| $Fe^{++}$ | 0.4 mg |
| $Mn^{++}$ | 0.05 mg |
| Thiamine HCl | 0.05 mg |

| | | |
|---|---|---|
| Riboflavin-5-$PO_4$ | 0.05 | mg |
| Pyridoxine | 0.01 | mg |
| Nicotinic Acid | 0.1 | mg |
| Calcium Pantothenate | 0.4 | mg |
| p-aminobenzoic Acid | 0.1 | mg |
| Folic Acid | 0.02 | mg |
| Inositol | 5.00 | mg |
| Choline HCl | 20.0 | mg |
| Vitamin $B_{12}$ | 0.0005 | mg |
| Biotin | 0.0005 | mg |

The control group also received a daily supplement of 0.05 mg $Cu^{++}$.

(ii) Experiment No. 2

This experiment consisted of two different groups of rats. The first group were 30 Norwegian Hooded Rats obtained from the Animal Care Centre of McGill University. Initially these rats weighed between 35 and 40 grams and were divided into two groups, one on a copper deficiency diet and the other on the same diet supplemented with copper as in Experiment No. 1. The food supplied to the animals was as follows: Days 1-7 inclusive the rats were given 100% Crino Powdered Milk as in Experiment No. 1.

Owing to a high mortality rate, presumably because of the very high protein content, this diet was changed to Farmer's Wife Evaporated Milk No. 2, purchased from the Cow and Gate Co., Montreal. This was diluted 1:1 with twice glass distilled water. During the first week of the experiment the vitamin and mineral supplement was given orally by dropper, after which it was added directly to the milk from a stock solution. The rats were housed in nickel-plated stainless steel metabolism cages in an isolated room.

In the other part of this experiment the diet and housing conditions were as above. The animals were 62 male albino rats of the Sprague-Dawley strain received from the Animal Care Centre. The initial weight range was between 35 and 40 grams.

The hooded rats were killed by decapitation at 49 and 89 days. The albino rats were sacrificed in a similar manner on the 47th and 91st days.

(iii) Experiment No. 3

Thirty-four male albino rats of the Sprague-Dawley strain were received from the Animal Care Centre and divided into two groups. The first group was fed

a diet of 50% Benson's Original Cornstarch and 50% Crino Powdered Milk. The animals were then subdivided into copper-deficient and control groups, the latter receiving a supplement as in the previous experiments. The second group received a diet of Farmer's Wife Evaporated Milk No. 2 diluted 1:1 with twice glass-distilled water. All rats were fed the deficient diet, and supplements where applicable, were added directly to the liquid milk. The animal housing and cage cleaning were as in the previous experiments.

(iv) Experiment No. 4

Sixty male albino rats of the Sprague-Dawley strain were purchased from the Canadian Animal Breeding Laboratories, Laprairie, Quebec, initial weight being 35-40 grams. The basal diet was 50% Benson's Original Cornstarch and 50% Crino Powdered Milk. The animals were subdivided into four groups:

(a) Control-28 rats with all supplements.

(b) Deficient in both copper and iron, i.e. supplemented in neither, 12 rats.

(c) Copper deficient, 12 rats.

(d) Iron deficient, 28 rats.

A summary of the principle features of these long-term animal experiments will be found in Table VII.

TABLE VII

| Experiment No. | Diet | State | Type of Animal | Day Killed (*) |
|---|---|---|---|---|
| I | Powdered Milk | Control, Cu.Def. | Albino | 7(4,0);14(2,2);21(2,2) 24(1,1);28(1,1);35(1,1); 38(1;1);43(1,1). |
| IIa | Liquid Milk | Control, Cu.Def. | Hooded | 49(5,5);89(3,4). |
| IIb | Liquid Milk | Control, Cu.Def. | Albino | 47(10,10);91(6,6). |
| IIIa | Powdered Milk and Cornstarch | Control, Cu.Def. | Albino | Growth curves only were obtained as the organs removed were lost when the refrigerator broke down and the freezing compartment heated up during the summer vacation. |
| IV | Powdered Milk and Cornstarch | Control, Fe,Cu.Def. Cu Def. Fe Def. | | 25(2,2,2,2);37(2,2,0,2); 66(2,2,2,2);78(1,1,2,2). |

(*) The numbers in parentheses refer to the number of animals sacrificed on the given day, the order being the same as listed under State in the headings.

B. Radioactive Dopamine-β-Hydroxylase Experiments

(i) Injection and Sacrifice

All rats were injected intramuscularly with a standard dose of $^{14}$C-dopamine (New England Nuclear Corporation, Boston, Mass.), 5.9 x $10^6$ dpm, the specific activity of which was 6 mc/mmole. The time interval between injection and sacrifice was exactly one hour, except for the study of time curves. The method of sacrifice was by decapitation.

(ii) Extraction and Chromatography of Catecholamines from Tissues

a) Extraction Procedure:

This varied during the first experiments.

First Procedure:- The heart and spleen were removed from the rat, rinsed in copper-free water and then frozen on dry ice. They were then cut into pieces, placed in 7 ml of absolute ethanol containing 0.01% HCl and 50 mg ascorbic acid, and sonified by a Branson sonifier in a scintillation vial. The sonifying was carried out with the sample immersed in an ice bath in order to minimize heating. The sonicate was then transferred to a polyallomer (Beckman) centrifuge tube. The sonification vial was washed with 2 ml ethanol-HCl which was added to

the centrifuge tubes. The tubes were placed on dry ice for 20 minutes and after cooling they were rotated in a Spinco Model L Ultracentrifuge at 33,000 r.p.m. (98,000 x g) for one hour.

After centrifugation, 0.2 ml of the supernatant was removed for counting. The remainder of the supernatant was then transferred to lyophilization vessels and placed in dry ice-acetone until solid. This was lyophilized, the residue being redissolved in 0.2 ml ethanol-HCl for chromatography.

Second Procedure:- The above procedure was first changed during the control tests as it was noticed that a great deal of radioactivity was lost during lyophilization. This was rectified by removing the lyophilization step, instead drying the sample under a stream of purified nitrogen. The increase in recovered radioactivity was very large.

Third Procedure:- The next alteration was adopted when it was noticed that in spite of the ice bath the material became very hot during sonification, turning a dark olive-brown colour. The sonification time was decreased, but it was soon decided to discontinue the use

of sonification. The alternative procedure introduced was as follows: After being frozen on dry ice the tissue was cut into four pieces and placed in a glass mortar with a small amount of sea sand that had been previously washed with acid and rinsed. The tissue was then ground very fine with a glass pestle with the continuous addition of ethanol-HCl. The suspension was then transferred to a centrifuge tube with washings for centrifugation. There was no noticeable difference in quantitative results, but the colour of the ground tissues remained pink.

b) Chromatography:

The dried samples were redissolved in 0.2 ml ethanol-HCl and then centrifuged in a bench centrifuge for 10 minutes to remove precipitate. 100-200 lambda were then spotted using a nitrogen stream to dry the material on the paper. Each sample was at first spotted on both phosphocellulose-81 ion exchange paper and aminoethylcellulose-81 ion exchange paper (Whatman). In the later experiments the extract was spotted on the PC-81 paper only. (The dimensions of the paper were as follows: a width of 2 cm and a length of 48 cm.) Then the papers were developed in butanol:acetic acid:water

(4:1:1) for 40 hours. They were then dried in a fume hood. The chromatograms were then cut into 1 cm long strips and put into vials containing 10 ml toluene and 0.3% 2,5-diphenyloxazole (PPO) and 0.02% 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (dimethyl POPOP) (Packard Instrument Company, Lagrange, Illinois). These were counted in a Tricarb scintillation spectrometer, model No. 3003, with a gain of 7.5% and window setting of 70-1000.

(iii) Calculations

a) Catecholamines:

In all radioactive experiments, the counts from norepinephrine (NE) and dopamine (D) areas of the chromatograms were used in the calculations.

$$\%NE \quad \frac{\text{cpm NE}}{\text{cmp(NE D)}}$$

$$\%D \quad \frac{\text{cpm D}}{\text{cpm(NE D)}}$$

The ratio NE:D was also used as an index of deficiency, with a ratio 1:1 being considered as normal control value for one hour after injection.

For the deficiency states: decrease in conversion;

$$\% \frac{\%NE(def.)}{\%NE(control)} \times 100\%$$

also

$$\frac{NE:D(def.)}{NE:D(control)}$$

b) Recovery of Radioactivity:

This was measured by the use of an aliquot before and after each step. The cpm were measured in toluene-ethanol scintillator except for the counts on the chromatography paper, which have an efficiency of 2% in the toluene scintillator. If the aliquot has C cpm in a volume of n ml, the total cpm in a sample = $\frac{C}{n}$ x Volume of sample before aliquot is removed.

Thus, % recovery $\frac{\text{Total cpm before a given step}}{\text{Total cpm after a given step}} \times 100\%$

C. Measurement of Metals

(i) Copper

Copper was measured in the liver of all animals, and in the radioactive studies it was often measured in the heart. Studies by G. Gregoriadis (243) show that there is no significant amount of copper remaining in the supernatant after the centrifugation and that copper levels in the precipitate were the same as in the whole heart. As a result, the copper levels in the heart were determined on the precipitate obtained after centrifugation.

a) Wet Ash Method:

This is the method of Eden and Green (244), as used in our laboratory in the first three experiments. A sample of less than 2.5 grams was used. All glassware (as in the radioactive experiments) was treated with chromosulfuric acid and then washed with tap and glass-distilled water. Two glass beads were placed in a digestion tube and the sample was then added. If the sample was tissue, it was cut into small pieces. To this was added 1 ml concentrated $H_2SO_4$ and 3 ml 60% perchloric acid. The sample could be stored in this condition.

Blanks were prepared in duplicate with beads, sulfuric acid and perchloric acid. Digestion was carried out on a Kjeldahl apparatus, manufactured by the Laboratory Construction Company. Heating was commenced with setting No. 4. The sample was agitated while heating and was watched continuously while boiling. If the sample stopped boiling, it was agitated to avoid overheating. The sample became black and then transparent. At this point, the heat was raised to setting No. 5-5$\frac{1}{2}$ until it became white, then yellow. At this point the heat was raised to No. 6. It remained there for 45 minutes and was then raised to No. 7. The perchloric was allowed to evaporate as a white vapour, leaving 1 ml sulfuric acid. This was allowed to cool and it solidified owing to the salts present. The samples were transferred to extraction tubes with 3 x 3 ml twice glass-distilled water.

To this was added 8-10 ml saturated sodium pyrophosphate, making sure that none got on the lip of the tube. Five ml of concentrated ammonia was added to make the sample strongly alkaline. It was then allowed to cool for 30 minutes; 2 ml of diethyldithiocarbamate (Matheson, Coleman and Bell, Toronto) was added while shaking the sample continuously. The colour was then

extracted with 5 ml amyl alcohol by shaking for 1 minute. This was then transferred by Pasteur pipette to clean, dry tubes. A small amount of anhydrous sodium sulphate was added and the clear supernatant was transferred to a Coleman tube and read at 435 m in a Coleman Junior Spectrophotometer.

b) Dry Ash Method:

This method was used for copper determination in the last experiment only. It was developed in our laboratory by G. Gregoriadis. The tissue was weighed and then placed in a Vycor brand 20 ml crucible and mashed slightly with a small glass pestle. The sample was placed on a Lindberg heater (which was turned off) underneath a General Electric Reflection 250 watt infrared lamp, placed about 7 inches above the sample. The crucibles were uncovered but an inverted Petri dish was placed about 1 cm above them so as to allow fumes to escape, but prevent any dust from falling into the crucibles. The infrared lamp was turned on and moved 2 inches closer every 20-30 minutes until the lamp was almost touching the Petri dish. The samples were left like this until they turned completely black. At this

point the heater was turned on to low and left for 30 minutes. The heat was increased every 30 minutes until it was at position 3 and it was left there until no further vapours could be observed escaping. Then the crucibles were removed, their lids placed upon them and then put in a Blue M Lab Heat Muffle Furnace at position 6 ($600^{o}$) and left overnight. After this the samples had turned into a small heap of black ash at the bottom of the crucible. The door of the muffle furnace was left open for 20-30 minutes to allow the samples to cool. They were then placed, with the use of crucible tongs on a sheet of asbestos until the samples had cooled to a degree at which they could be handled. Three ml of 6 N HCl was then added and heated for 15 minutes, but not allowed to boil. This was then transferred, along with 3 x 1 ml twice glass-distilled water rinses to a test tube. To this was added 5 ml of 0.03% dibenzyl-dithiocarbamate ("Arazate", Naugatuck Chem., Elmira, Ontario) in $CCl_4$ and shaken for 30 seconds. The organic (lower) phase was transferred to a clean tube and a small amount of anhydrous sodium sulphate was added. The clear liquid was examined in a Coleman Junior Spectrophotometer at 440 mμ.

It was discovered that high concentrations of iron will interfere with the determination of copper. This is eliminated by the addition of 5.0 ml of saturated Na pyrophosphate to the acid extract. For the samples that had been determined with the interference, a suitable correction was made from a standard curve for iron under the conditions of copper analysis.

All the crucibles that were used for ashing were filled after use with aqua regia and allowed to stand overnight. The acid was then discarded and the crucibles washed several times with glass-distilled water. All the other glassware was first machine washed and then left standing overnight in 6 N HCl. After this they were washed several times with twice glass-distilled water.

(ii) Iron Determination

All iron determinations were performed by H. Birnbaum of this laboratory. The method was essentially that of Gubler et al. (224). The dry ashing was as for copper, except that the maximum temperature reached was 450 C. The material was taken up in HCl to a volume of 10 ml. To 1.5 ml of this was added 0.1 ml of 0.5M $Na_2SO_3$, 0.4 ml of a 0.1% solution of ortho-phenanthroline (Fisher)

and after mixing 3 ml of 3 M sodium acetate (final pH of 5), water was added to a final volume of 5 ml. This was then examined at 510 mμ in a Coleman Junior Spectrophotometer.

a) Calculations:

i) Iron:- Calculated from the following formula:-

micrograms iron/gram wet weight tissue

$$\frac{\text{Optical density at 510 m}\mu \times \text{Iron/O./D./unit}}{\text{Fresh weight of the liver}}$$

ii) Copper:- The calculations for copper are the same as for iron except that the optical density is read at 440 mμ.

iii) The line of best fit was calculated by the method of least squares for the figures on pages 125, 127, 128, 129, 130, 136, 137, 152.

D. Measurement of Catecholamines in Vitro

(i) Tissue Preparations

The frozen tissues were homogenized in 10% TCA, with a glass mortar and pestle (motor driven teflon homogenizer for brain), according to the method of Sourkes and Murphy (245). All homogenization was carried out in an ice bath, the final volume being about 10 ml. The homogenate was transferred to a centrifuge tube and spun down at 15,000 rpm for 30 minutes in an International refigerated centrifuge, Model PR-2. The resulting supernatant was then transferred to a storage tube.

(ii) Absorption and Elution on Alumina

The method used was that of Sourkes and Murphy (245). The supernatant obtained from step (i) was transferred to a 50 ml test tube with 2 mg ascorbic acid in 1 ml water. Further additions consisted of 2.5 ml 10% EDTA, 1 drop of 1% phenolphthalein and 0.5 gm alumina (Woelm non-alkaline alumina, Alupharm Chemicals, New Orleans). The pH was then adjusted approximately to 8.2-8.5 with 5N and 2.5N NaOH using the phenolphthalein end point and agitating constantly.

The tubes were then shaken in a mechanical shaker for 3 minutes, the pH readjusted, and then shaken for a further 10 minutes. After the alumina had settled the supernatant was aspirated and the alumina washed twice with 10 ml glass distilled water (manual shaking). The supernatant was discarded and the catecholamines were eluted with 0.5 ml of 5.0M acetic acid (mechanical shaking, 15 minutes). The tubes were then centrifuged at 2,500 rpm for 15 minutes and the supernatant transferred to a storage flask.

(iii) Procedure for Dopamine

Dopamine was determined by the method of Sourkes and Murphy (245). To 1.0 ml of the eluate was added 2 ml of acetate buffer (1M Na acetate adjusted to pH 6 with 1M acetic acid). The final pH was 5.3. To this was pipetted 0.05 ml of 0.1N iodine solution. The oxidation was stopped after exactly 3 minutes with 0.5 ml of 4.5N NaOH which was 0.2M with respect to Na sulfite (90 ml 5N NaOH plus 10 ml 2M $Na_2SO_3$). The blank was identical except that the alkali contained 2 mg ascorbic acid in addition to the sulfite. Three minutes after the addition of the alkali 1.0 ml of 5N HCl containing

2 mg ascorbic acid was added. The blanks received 1.0 ml 5N HCl. After 45 minutes the fluorescence was read in an Aminco-Bowman spectrophotofluorometer at an activation of 330 mμ and a fluorescence wavelength of 375 mμ.

(iv) Procedure for Epinephrine

To a 1.0 ml aliquot of the eluate was added 2 ml glycine buffer (0.1M glycine adjusted to pH 3.0 with 0.1N HCl). The catechols were oxidized by the addition of 0.05 ml of 0.1N iodine solution. At exactly 3 minutes 0.5 ml of 0.01N Na thiosulfate was added followed by 1.0 ml of 5N NaOH containing 2 mg ascorbic acid. After 45 minutes the samples were read against a reagent blank at an activation wavelength of 410 mμ and a fluorescence wavelength of 510 mμ.

(v) Procedure for Norepinephrine

The procedure used for norepinephrine was that described by Sourkes and Murphy (245). It was exactly the same as the procedure for epinephrine except that the buffer that was used is the pH 6.0 acetate buffer instead of the glycine buffer.

(vi) Calculations

Calculations for dopamine and epinephrine were made from a standard curve for which recovery corrections had been made. The value obtained for norepinephrine was corrected for epinephrine and dopamine on the basis that the relative intensities of the three fluorescence substances, under the reaction conditions for norepinephrine, were 100:60:10.

E. Antabuse Experiments

Antabuse (disulfiram) was obtained from Ayerst, McKenna and Harrison Ltd., Montreal, lot No. 54876. The animals used were male albino (Sprague-Dawley) rats of about 250 grams in weight. All controls were injected intraperitoneally with 1 ml. of 0.9% NaCl. The test animals were injected intraperitoneally with 200 mg/kg of Antabuse suspension in distilled water. The Antabuse was made up by adding 500 mg to 10 ml, sonifying for 10 minutes and adding 1 drop of Triton-X (emulsifying agent, non-ionic surfactant). Two hours later all animals were injected intramuscularly with $^{14}$C-dopamine (New England Nuclear Corporation). Simultaneously with the dopamine, the control group was injected with NaCl and the test group received the second injection of Antabuse.

One rat was killed,

(1) 15 minutes after injection

(2) 45 minutes after injection

(3) 90 minutes after injection

The catecholamines were extracted and chromatographed as in II,B,ii.

F. Heart Rate Experiments

Heart rate determinations were made on copper deficient and control animals. The apparatus used was a Physiograph (E and M, Instrument Co. Inc., Houston, Texas). The electrodes used were safety pins which were fastened through the skin, underneath each forelimb. This did not cause the animal any apparent discomfort. The animal was then placed in a specially designed jacket which effectively prevented the movement of any of the limbs. The animal was grounded through the tail. The rat was then suspended from the ceiling of a small, disconnected refrigerator and the door closed. A light remained on in the container and an air supply led into it. The animal was left for 10 minutes to become accustomed to its environment. At this point the recorder was turned on for 10 minutes in order to register the basal heart rate. The rat was then shocked by means of an electric buzzer which sounded for 5 seconds. The animal was given at least 5 minutes to recover before the buzzer was sounded again.

# III. RESULTS

## A. Growth Curves

### (i) Experiment No. 1.

Forty-six male albino rats were fed a 100% Crino powdered milk diet plus added vitamins. The weight table for this experiment is in Table VIII and the growth curve in Figure 1.

TABLE VIII

Growth Table for Experiment No. 1*

| Day No. | 7 | 12 | 15 | 19 | 34 |
|---|---|---|---|---|---|
| Control | | 57.5 | 66 | 91 | 112 |
| Copper Deficient | 50.3 | 55 | 60.5 | 67.5 | 82 |

* Weight is expressed in grams, average of all rats in each group.

### (ii) Experiment No. 2.

Both groups were fed Crino powdered milk for the first 7 days and Farmer's Wife evaporated milk thereafter.

a) Thirty male Norwegian hooded rats. The growth of these is recorded in Table IX and Fig. No. 2.

TABLE IX

Norwegian Hooded Rats *

| Day No. | 0 | 2 | 4 | 6 | 8 | 10 | 12 | 14 | 16 | 18 | 20 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Control | 38.3 | 39.7 | 46 | 50.9 | 57.3 | 62.5 | 65.2 | 72.5 | 71.1 | 78.6 | 82.9 |
| Copper Deficient | 39.8 | 42 | 47.3 | 52.8 | 56.7 | 64.2 | 69.5 | 77.9 | 74.3 | 80.7 | 85.3 |

| Day No. | 24 | 26 | 28 | 30 | 32 | 34 | 36 | 38 | 40 | 43 |
|---|---|---|---|---|---|---|---|---|---|---|
| Control | 80.2 | 87.5 | 95.4 | 99.9 | 106.4 | 105.9 | 118 | 125.8 | 121.4 | 131.5 |
| Copper Deficient | 94.5 | 96.6 | 100.3 | 105.7 | 114.7 | 113.9 | 126 | 132.5 | 126 | 140 |

* This table represents the average body weight of 2 groups of Norwegian Hooded rats (30 rats initially in each group) over a period of 6 weeks.

Figure No. 1. Growth Curve, Experiment 1

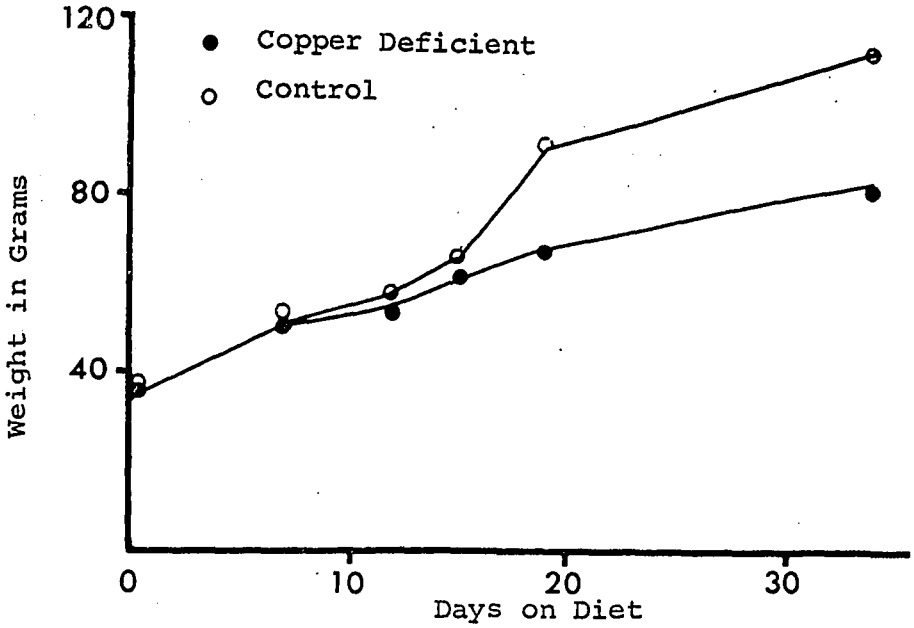



Figure No. 2. Growth Curve, Experiment 2
Hooded Rats

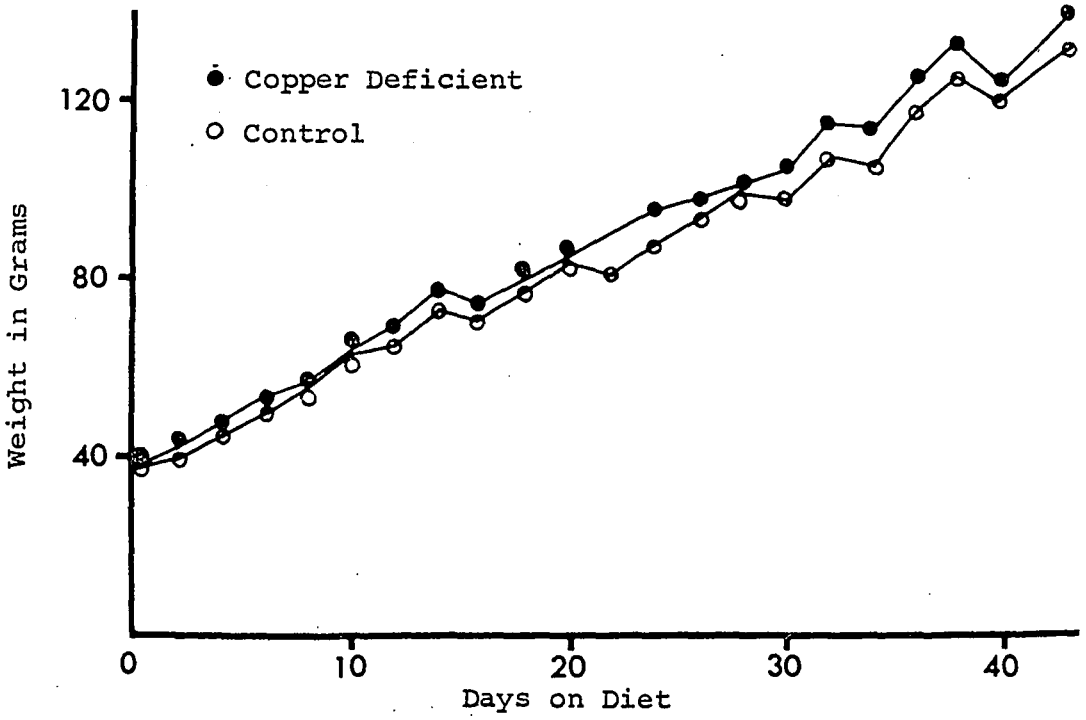
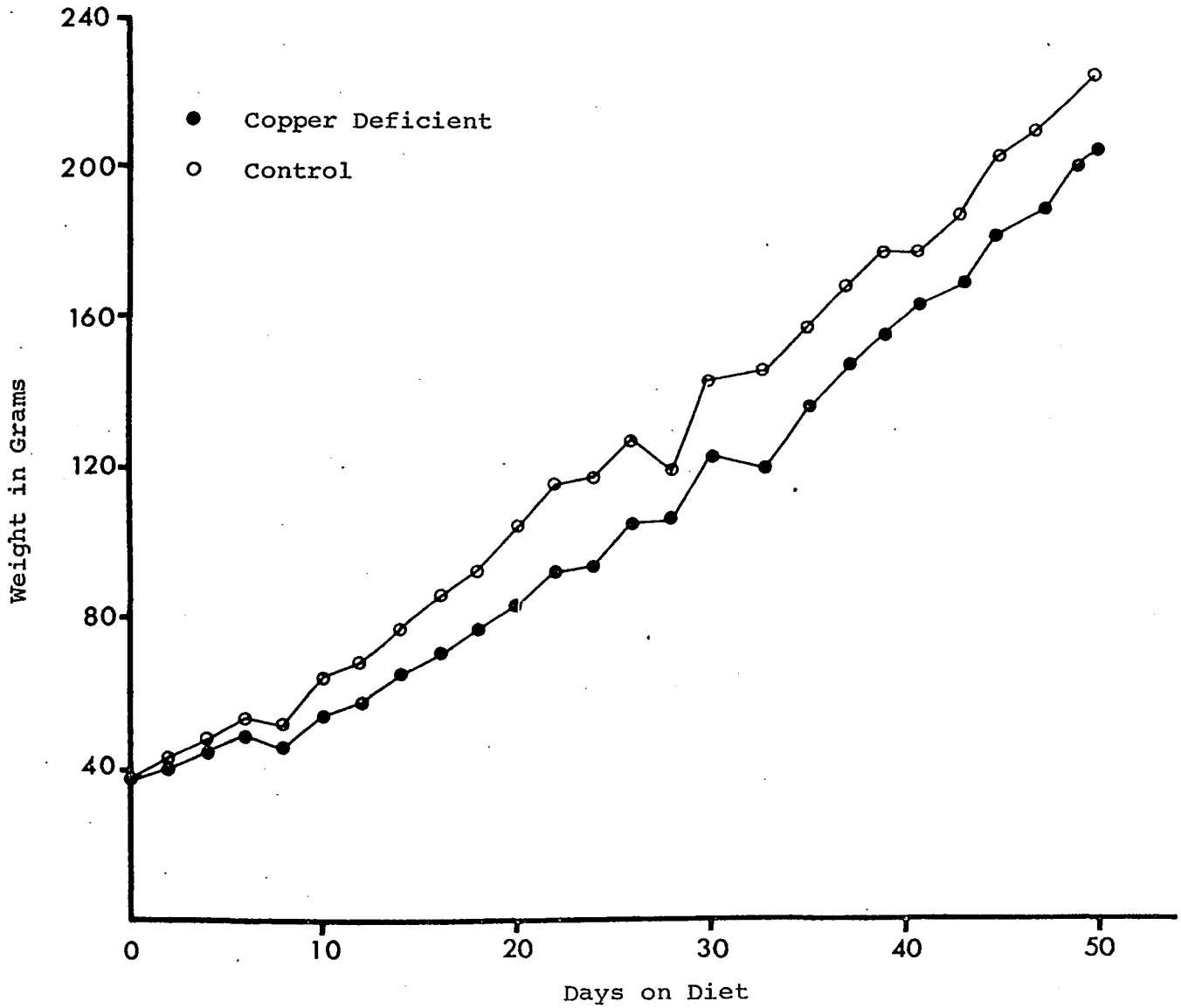


Figure No. 3. Growth Curve, Experiment No. 2.
Albino Rats



b) Sixty-two male albino rats. Table X and Figure No. 3.

(iii) Experiment No. 3

This group contained 36 male albino rats divided into 4 groups: (a) Seven animals fed starch (50%) and dry milk (50%) diet plus added vitamins, with copper added (control).

(b) Seven animals on starch plus powdered milk plus vitamins (copper-deficient).

(c) Ten animals on liquid milk plus vitamins (liquid copper-deficient).

(d) Twelve animals on liquid milk diet plus vitamins with 20 mg diethyldithiocarbamate per day per rat supplied in the milk. (deficient diet with supplemental diethyldithiocarbamate).

The growth results from this group are summarized in Table XI and Figure No. 4.

(iv) Experiment No. 4.

Sixty male albino rats were divided into four groups. The basal diet was 50% cornstarch plus 50% powdered milk with added vitamins.

TABLE X

Albino Rats*

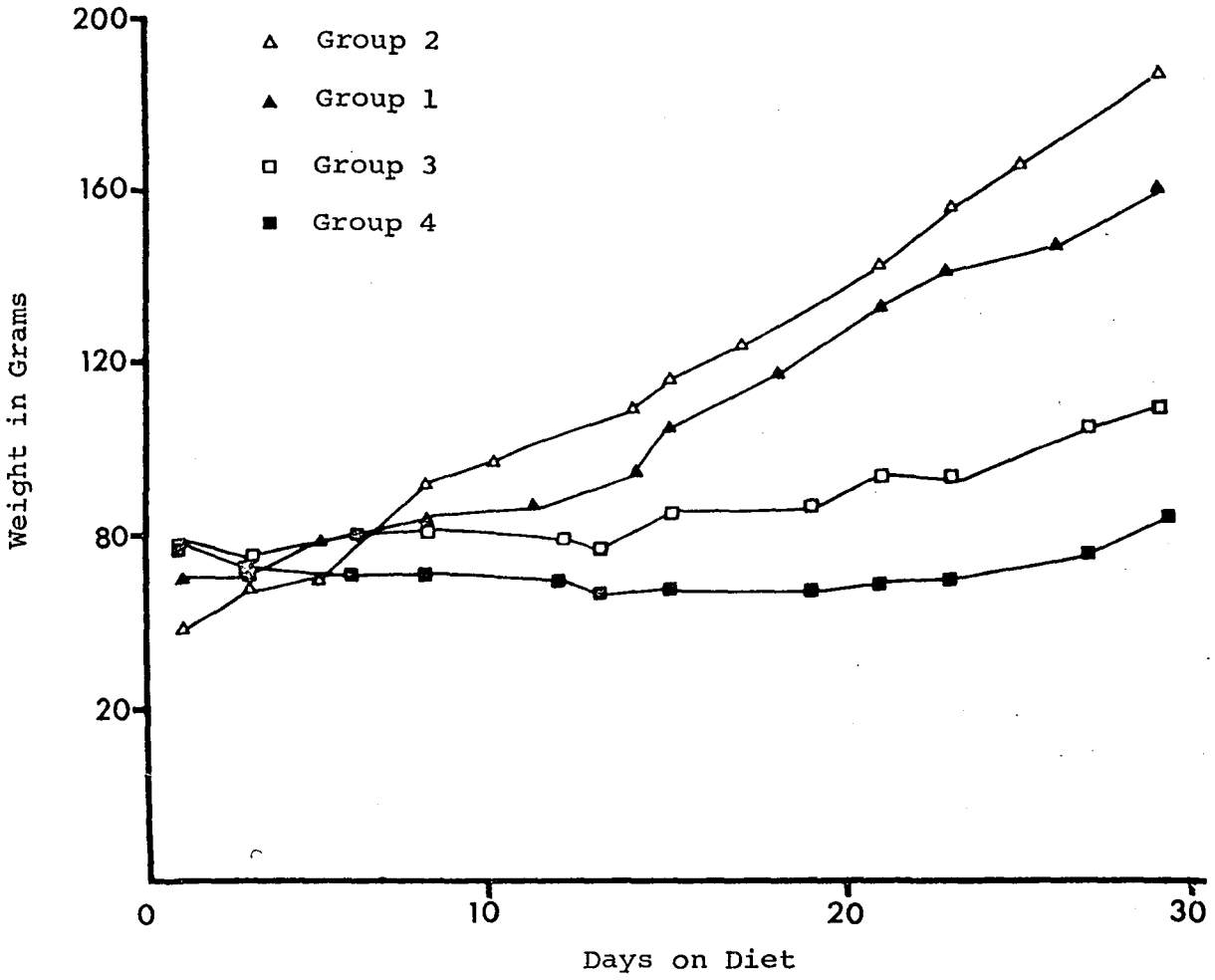
| Day No. | 0 | 2 | 4 | 6 | 8 | 10 | 12 | 14 | 16 | 18 | 20 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Control | 35.6 | 42 | 47.5 | 51.7 | 51.1 | 63.4 | 67.4 | 77.9 | 85.3 | 90.5 | 103.2 |
| Copper Deficient | 36.8 | 39.4 | 43.4 | 48.0 | 46.1 | 52.6 | 56.6 | 63.2 | 69.0 | 76.9 | 80.4 |

| Day No. | 22 | 24 | 26 | 28 | 30 | 33 | 35 | 37 | 39 | 41 | 43 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Control | 114.3 | 118.4 | 126. | 117.9 | 141.5 | 143.8 | 156 | 168.1 | 176.4 | 177.5 | 184 |
| Copper Deficient | 90.1 | 91.5 | 104.5 | .06.8 | 122.4 | 119.9 | 134.6 | 146.1 | 153.3 | 162.3 | .67.9 |

| Day No. | 45 | 47 | 49 | 50 |
|---|---|---|---|---|
| Control | 200.8 | 208.4 | | 222.3 |
| Copper Deficient | 180.3 | 186.9 | 199.8 | 202 |

* This table represents the average weight (in grams) of the two groups of rats in Experiment No. 2. Initially there were 31 animals in each group.

Figure No. 4. Growth Rate, Experiment No. 3.*




* See text, p. 87 for a detailed description of the diets.

TABLE XI *

| Day No. | 1 | 3 | 5 | 6 | 8 | 10 | 11 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|---|---|---|---|
| Group a | 68.9 | 71 | 78.1 | | 83.4 | | 85.4 | | | 94.3 |
| Group b | 57.8 | 66.6 | | | 90.9 | 97.0 | | | | 109.3 |
| Group c | 78.6 | 73.7 | | 79.9 | 80.9 | | | 79.3 | 77.2 | |
| Group d | 78.2 | 70.7 | | 71.2 | 70.1 | | | 68.1 | 65.7 | |

| Day No. | 15 | 17 | 18 | 19 | 25 | 26 | 27 | 29 | 31 |
|---|---|---|---|---|---|---|---|---|---|
| Group a | 103.7 | | 117.4 | | | 146.1 | | 158.9 | |
| Group b | 116.3 | 123.4 | | | 165 | | | 185 | 193.1 |
| Group c | 83.9 | | | 85.9 | | | 104.6 | 109.6 | |
| Group d | 67.9 | | | 67.1 | | | 75 | 83.9 | |

* This table represents the average weights of the following:
Group a = Control, 7 animals;
Group b = Copper deficient, 7 animals;
Group c = Liquid copper deficient, 10 animals;
Group d = Deficient diet, supplemented with diethyldithiocarbamate, 12 animals.

(a) Control group, 18 animals, both copper and iron supplements were added to the diet.

(b) Twelve animals with neither copper nor iron added to the diet (doubly deficient).

(c) Twelve animals with iron added to the basal diet (copper deficient).

(d) Eighteen animals with copper added to the diet (iron deficient).

The weight relationships for these animals are shown in Table XII and Figure No. 5.

(v) Mortality

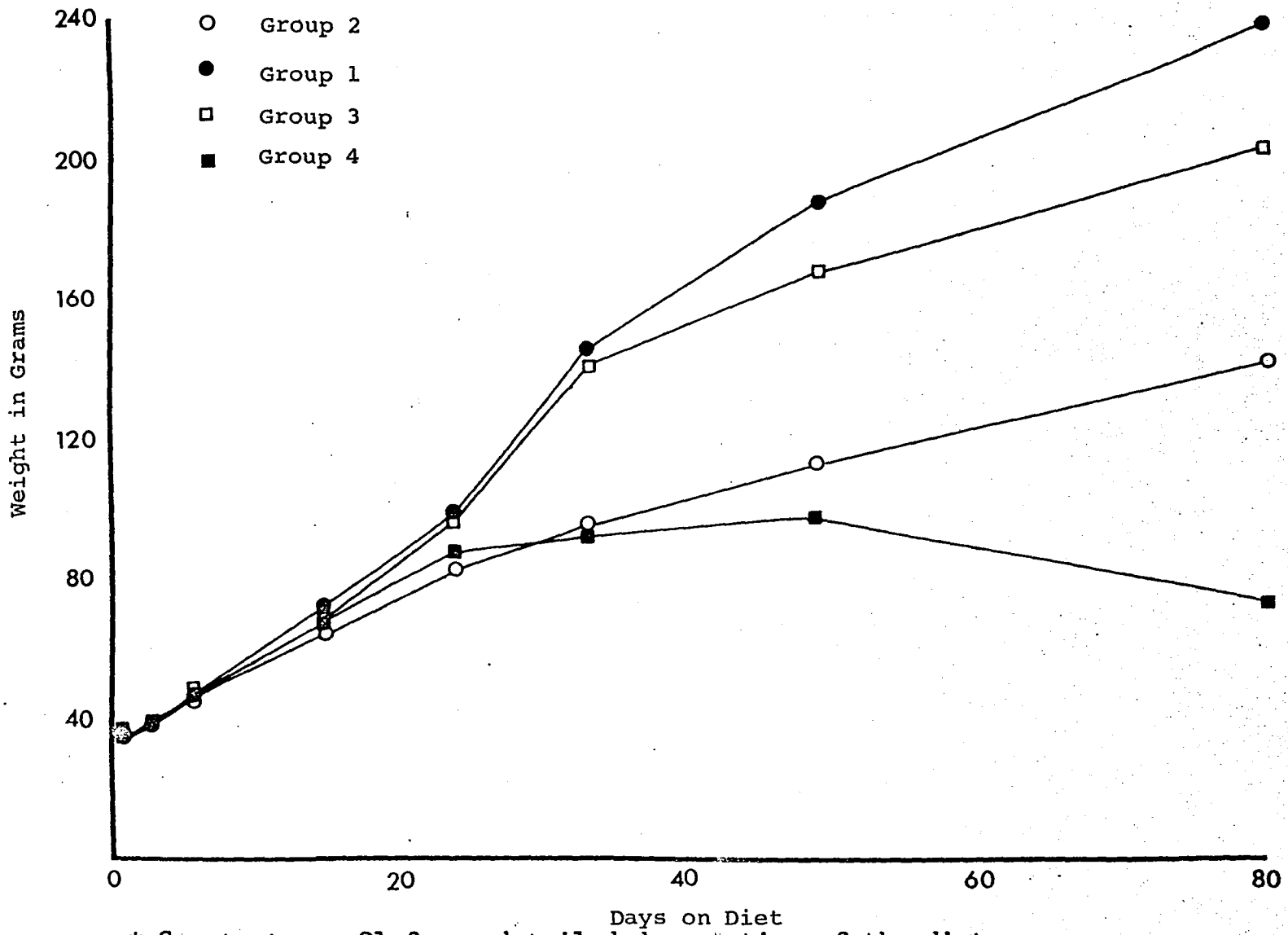
A comparison of the number of deaths and relative percentages under the different diets and experiments are to be found in Table XIII.

From the next Tables and Graphs it is seen that the best basal diet was that consisting of 50% cornstarch and 50% powdered milk with added vitamins. The least satisfactory diet was the one consisting of 100% powdered milk plus vitamins.

The deficiency diet that had the greatest effect on weight gain and mortality was the iron deficient diet. This gave a poorer rate of growth than even the diet deficient in both iron and copper.

This will be considered further in the discussion.

Figure No. 5. Growth Rates for Experiment No. 4.*




* See text, p. 91 for a detailed description of the diets

TABLE XII

Experiment No. 4 *

| Day No. | 1 | 3 | 6 | 15 | 24 | 35 | 49 | 78 |
|---|---|---|---|---|---|---|---|---|
| Group a | 34.3 | 38.1 | 48.3 | 70.9 | 99.4 | 136.6 | 189.9 | 236.5 |
| Group b | 33.5 | 38.6 | 47.5 | 65.1 | 80.4 | 96.0 | 113.9 | 144 |
| Group c | 34.8 | 38.5 | 48.3 | 68.3 | 98.5 | 130.3 | 169.1 | 203.7 |
| Group d | 34.7 | 38.8 | 47.8 | 68.3 | 86.6 | 93.5 | 98.2 | 75.3 |

* This table represents the average weight gain (in grams) of 4 groups of animals in Experiment 4, over an 11-week period:

(a) Control group, initially 18 animals,
(b) Doubly deficient group, initially 12 animals,
(c) Copper deficient group, initially 12 animals,
(d) Iron deficient group, initially 18 animals.

TABLE XIII

Mortality

| Experiment | Diet | Type of Rat | Deaths | % Dead of Original No. of Animals |
|---|---|---|---|---|
| 1 | Powdered milk Control | Albino | 5 | 31.1 |
| 1 | Powdered Copper Deficient | Albino | 5 | 19.2 |
| 2a | Evaporated milk Control | Hooded | 1 | 6.7 |
| 2a | Evaporated Copper Deficient | Hooded | 6 | 40.0 |
| 2b | Evaporated Milk Control | Albino | 11(11)* | 35.5 (35.5)** |
| 2b | Evaporated Copper Deficient | Albino | 12(8)* | 38.7 (25.8)** |
| 3a | Starch + Powdered Milk Control | Albino | 0 | 0 |
| 3b | Starch + Powdered Milk Copper Deficient | Albino | 0 | 0 |
| 3c | Liquid Milk Deficient | Albino | 1 | 10.0 |
| 3d | Liquid Milk Deficient | Albino | 2 | 17 |
| 4a | Starch + Powdered Milk Control | Albino | 0 | 0 |

TABLE XIII (Continued)

| Experiment | Diet | Type of Rat | Deaths | % Dead of Original No. of Animals |
|---|---|---|---|---|
| 4b | Starch + Powdered Milk Doubly Deficient | Albino | 2 | 11.1 |
| 4c | Starch + Powdered Milk Copper Deficient | Albino | 0 | 0 |
| 4d | Starch + Powdered Milk Iron Deficient | Albino | 5 | 27.8 |

* Number of rats that died in the first 7 days when the animals were fed a diet of 100% powdered milk plus vitamin supplement.

** % of rats that died in first days, of the total number of rats that died.

B. Standards, Time Curves and Recovery for Dopamine-β-Hydroxylase in Vivo

Dopamine-$C^{14}$ was injected intramuscularly. At varying lengths of time the animals were sacrificed and the conversion of dopamine to norepinephrine was studied. The purpose of this investigation was to determine the optimum period of time after injection, at which to sacrifice the animal in order to determine labelled catecholamines in the heart. All the rats used in these studies were normal rats fed on Purina Laboratory Chow.

(i) Time Curves

a) 200-250 Gram Albino Rats:

TABLE XIV

Heart

| Rat Weight In grams | Time of Sacrifice (min) | % Norepinephrine | % Dopamine |
|---|---|---|---|
| 252 | 15 | 15 | 85 |
| 220 | 30 | 29 | 71 |
| 245 | 45 | 40 | 60 |
| 202 | 60 | 47 | 53 |
| 250 | 90 | 69 | 31 |

Figure No. 6. Time Curve for Heart, 200-250 Gram Albino Rats

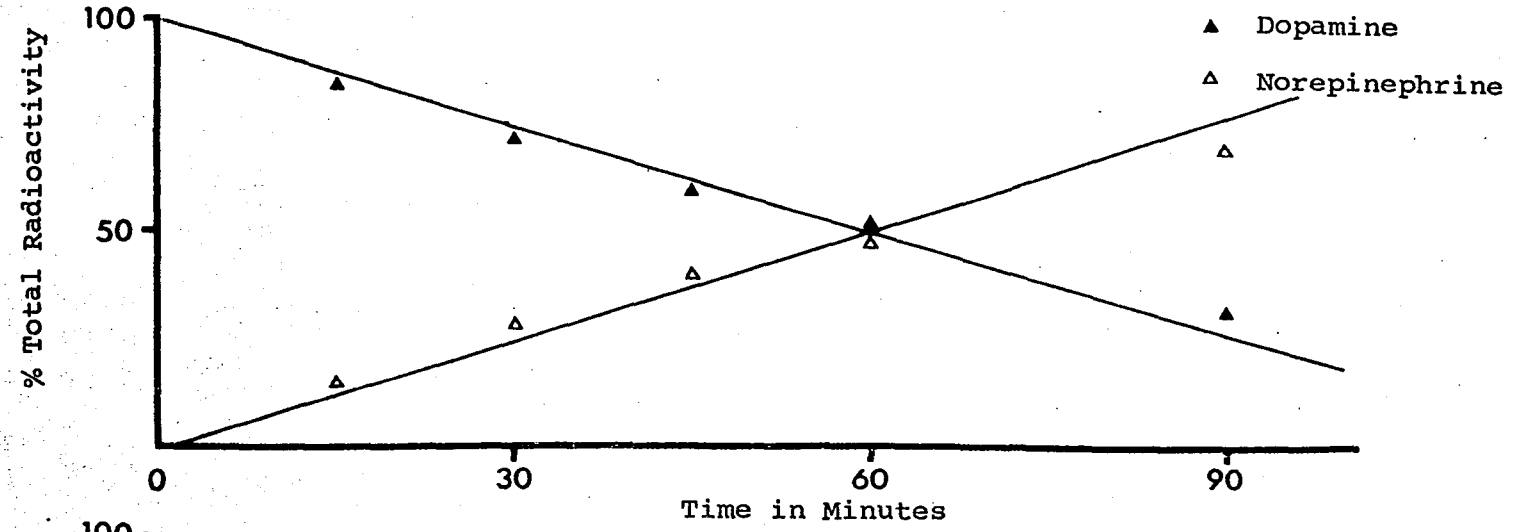
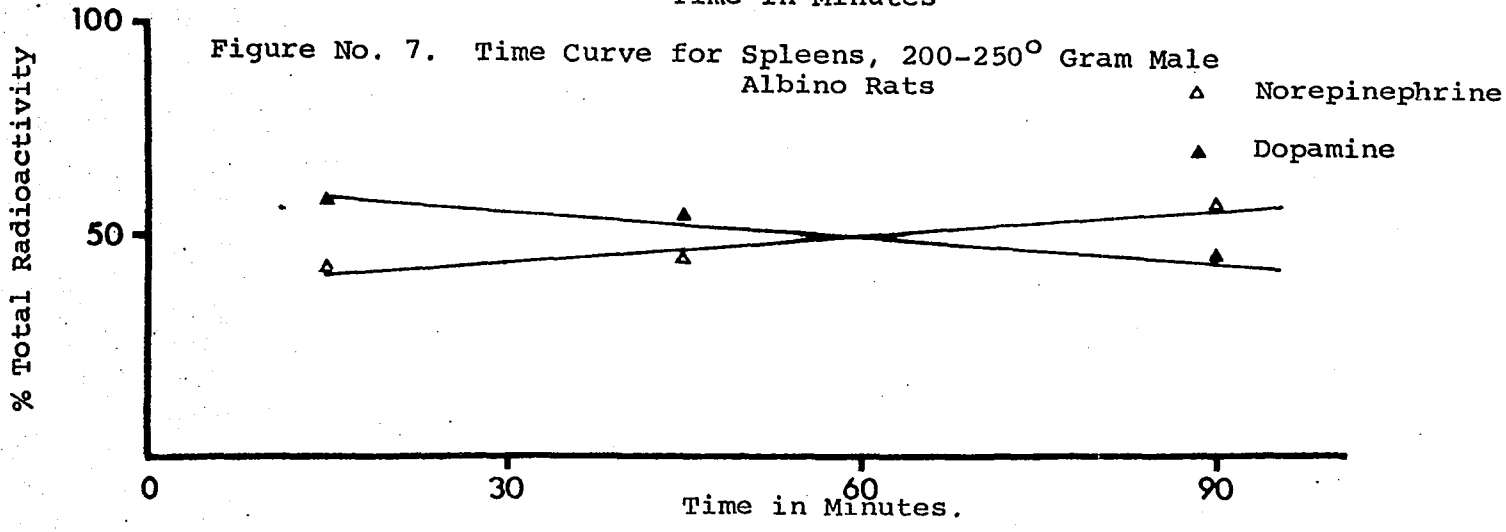



Figure No. 7. Time Curve for Spleens, 200-250° Gram Male Albino Rats



From this Table and Fig. No. 6, it is seen that, for the heart, the 50% conversion point (1:1 ratio of DA:NE) is at one hour. Thus, it was decided to sacrifice all rats one hour after the injection of the dopamine-$C^{14}$.

TABLE XV

Spleen

| Rat Weight in grams | Time of Sacrifice (min)* | %Norepine-phrine | % Dopamine |
|---|---|---|---|
| 250 | 15 | 41 | 59 |
| 242 | 45 | 46 | 54 |
| 242 | 90 | 56 | 44 |

* Expressed as minutes after injection of Dopamine-$C^{14}$.

From Table XV and Figure No. 7 it is seen that for the spleen the 50% conversion occurs at 45-90 min. after injection.

Figure No. 8. Time Curve for Heart, 50 Gram Albino Rats

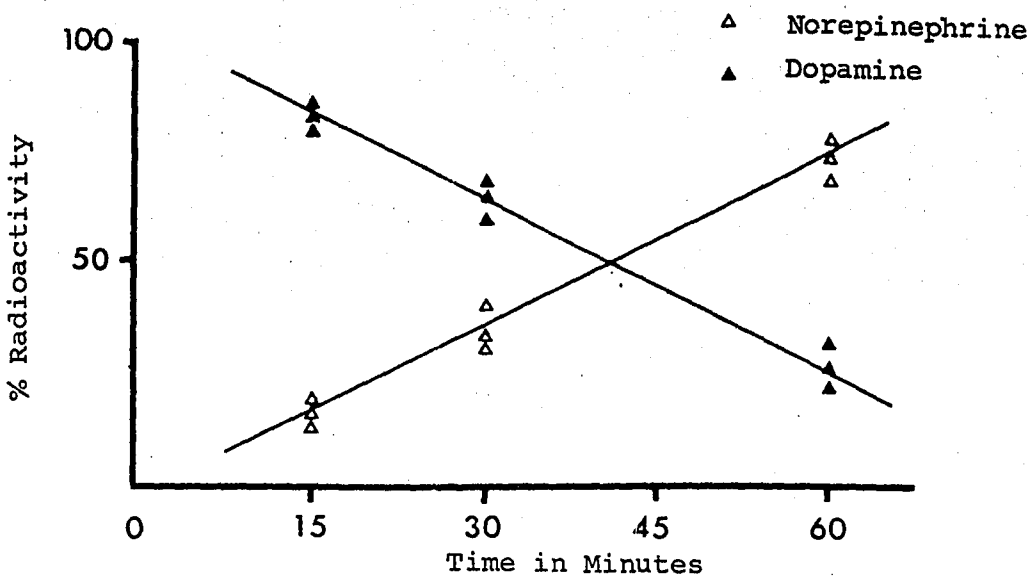
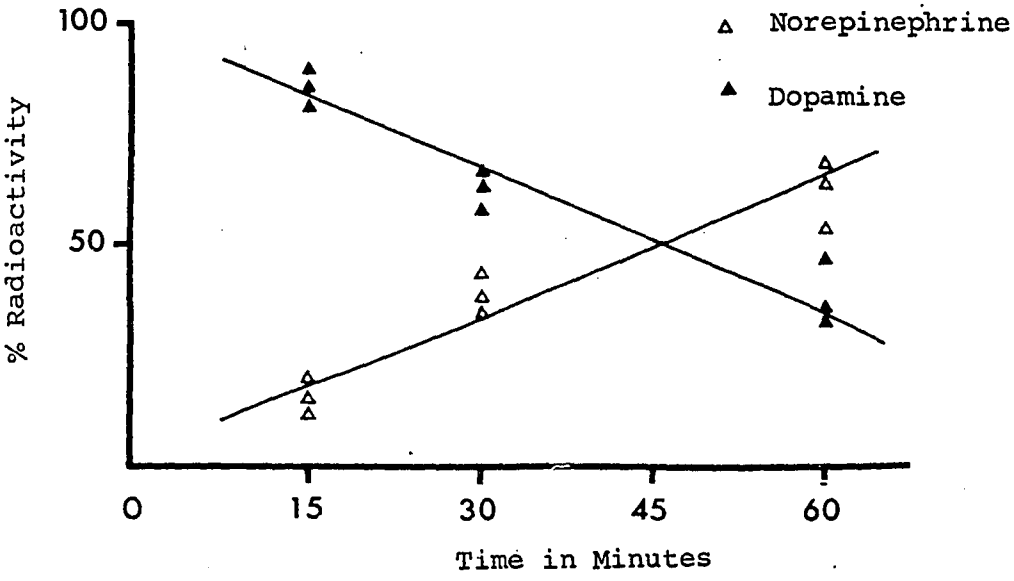



Figure No. 9. Time Curve for Heart, 100 Gram Albino Rats



b) 50 Gram Albino Rats:

TABLE XVI

Heart

| Time of Sacrifice (min)* | % Norepinephrine | % Dopamine |
|---|---|---|
| 15 | 16 | 84 |
| 15 | 14 | 86 |
| 15 | 18 | 82 |
| 30 | 33 | 67 |
| 30 | 42 | 58 |
| 30 | 36 | 64 |
| 60 | 74 | 26 |
| 60 | 78 | 22 |
| 60 | 69 | 31 |

* Footnote Table XV

From this Table and Fig. No. 8, it is seen that in the heart of 50 gram albino rats, the 50% conversion point occurs about 40 minutes after injection.

c) 100 Gram Albino Rats:

Table XVII and Fig. No. 9.

TABLE XVII

Heart

| Time of Sacrifice (min)* | % Norepinephrine | % Dopamine |
|---|---|---|
| 15 | 15 | 85 |
| 15 | 19 | 81 |
| 15 | 11 | 89 |
| 30 | 33 | 67 |
| 30 | 42 | 58 |
| 30 | 36 | 64 |
| 60 | 67 | 33 |
| 60 | 66 | 34 |
| 60 | 52 | 48 |

* Footnote Table XV

The time of 50% conversion of cardiac dopamine-$C^{14}$ to norepinephrine was found to be 46 minutes.

d) 50 Gram Hooded Rats:

Table XVIII and Fig. No. 10.

Figure No. 10. Time Curve for Heart, 50 Gram Hooded Rats

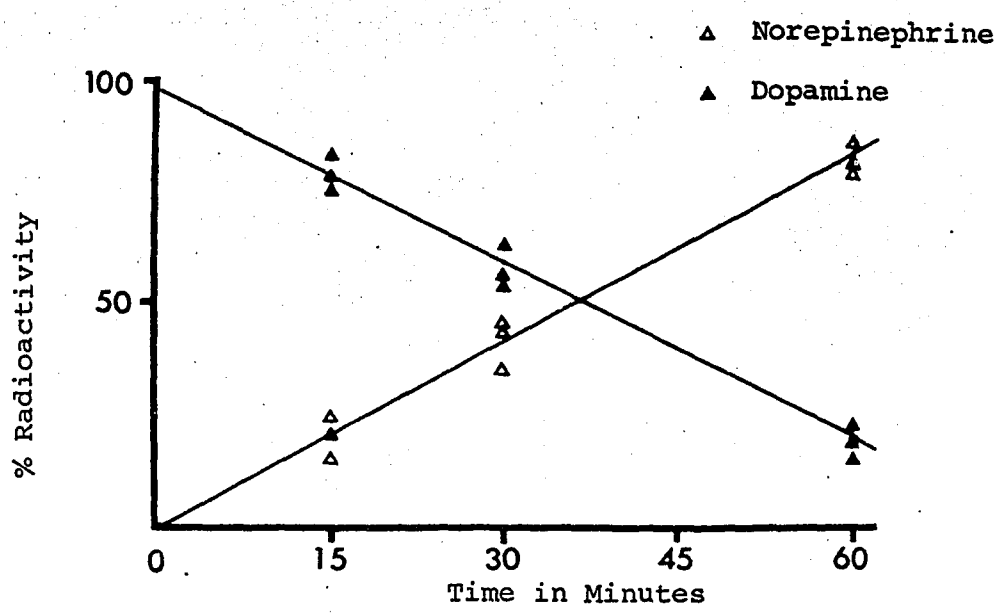



Figure No. 11. Time Curve for Heart, 100 Gram Hooded Rats

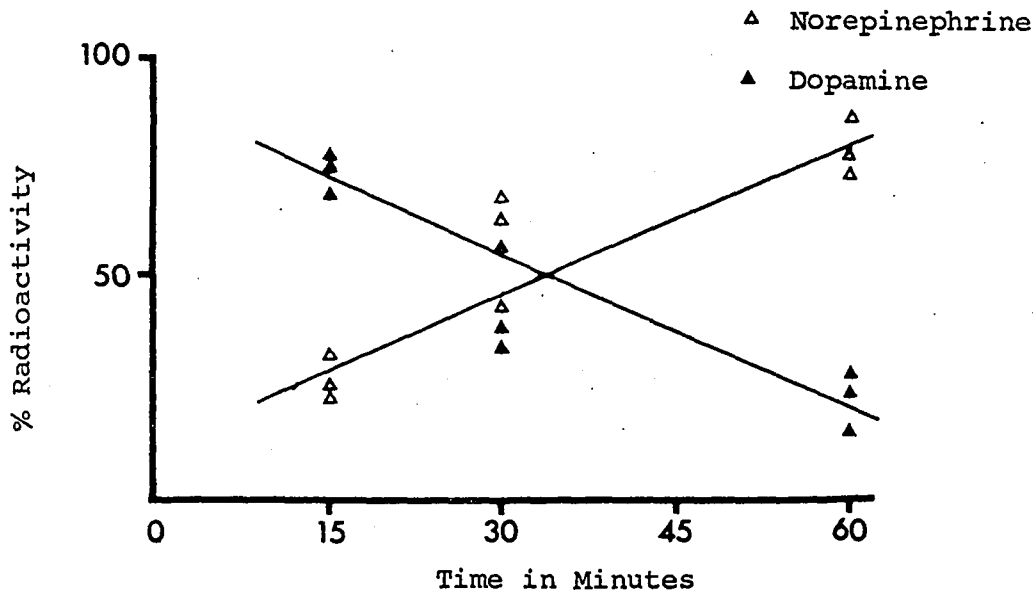


TABLE XVIII

Heart

| Time of Sacrifice (min)* | % Norepinephrine | % Dopamine |
|---|---|---|
| 15 | 21 | 79 |
| 15 | 16 | 84 |
| 15 | 23 | 77 |
| 30 | 37 | 63 |
| 30 | 55 | 45 |
| 30 | 44 | 56 |
| 60 | 84 | 16 |
| 60 | 80 | 20 |
| 60 | 79 | 21 |

* Footnote Table XV

From this Table and Fig. No. 10, it is seen that the 50% conversion point of cardiac $C^{14}$ dopamine was reached 38 minutes after injection.

e) 100 Gram Hooded Rats:

Table XIX and Fig. No. 11.

TABLE XIX

Heart

| Time of Sacrifice (min)* | % Norepinephrine | % Dopamine |
|---|---|---|
| 15 | 31 | 69 |
| 15 | 26 | 74 |
| 15 | 24 | 76 |
| 30 | 62 | 38 |
| 30 | 66 | 34 |
| 30 | 42 | 58 |
| 60 | 72 | 28 |
| 60 | 86 | 14 |
| 60 | 77 | 23 |

* Footnote Table XV

In the heart of 100 gram hooded rats, the 50% conversion point of injection $C^{14}$ dopamine was reached 30 minutes after injection.

f) 250-300 Gram Hooded Rats:

Table XX and Fig. No. 12.

Figure No. 12. Time Curve for Heart, 250-300 Gram Hooded Rats

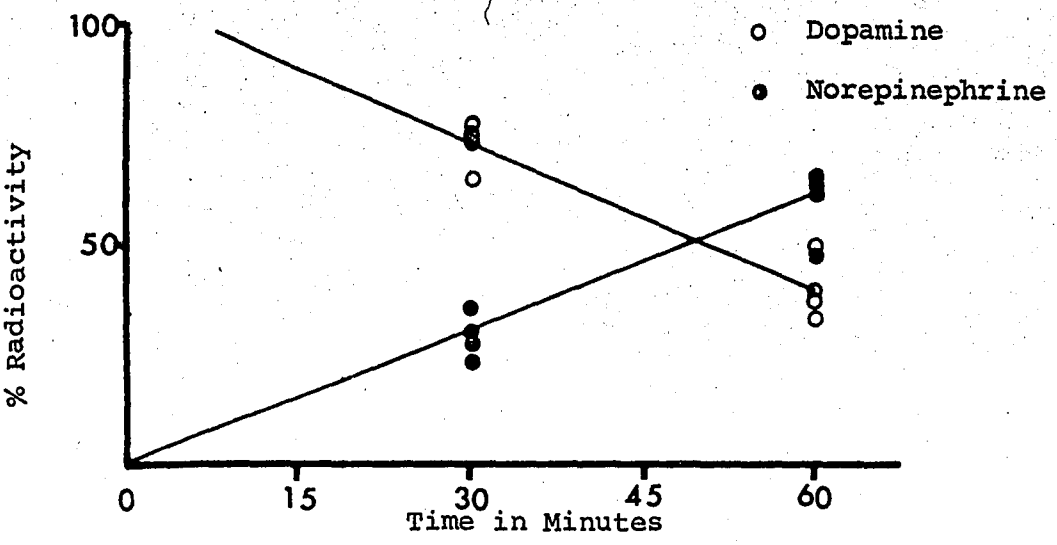



Figure No. 13. Age Effect on Dopamine-β-Hydroxylase Albino Rats

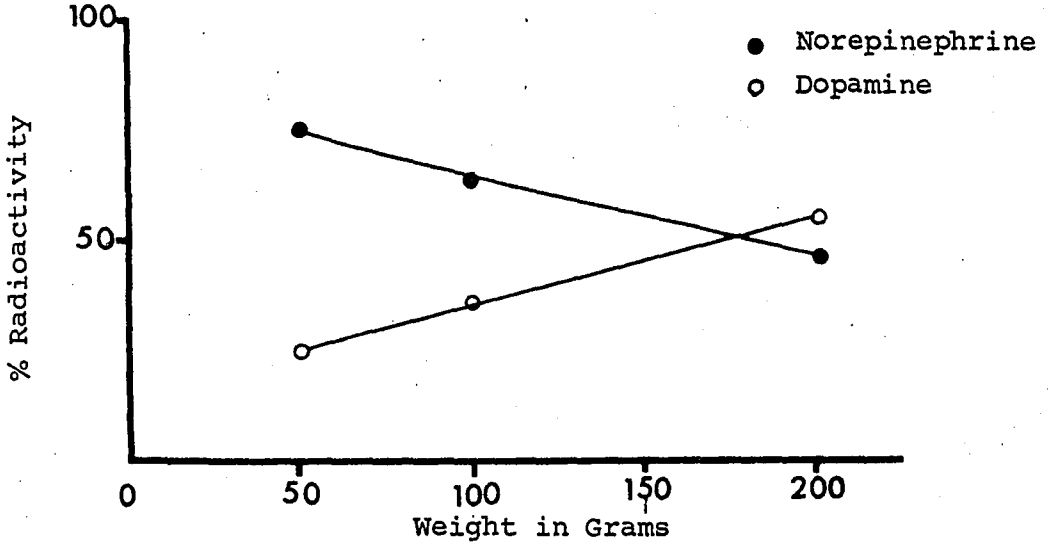


TABLE XX

Heart

| Rat Weight | Time of Sacrifice (Min)* | % Norepinephrine | % Dopamine |
|---|---|---|---|
| 285 | 30 | 28 | 72 |
| 274 | 30 | 23 | 77 |
| 245 | 30 | 36 | 64 |
| 223 | 30 | 26 | 74 |
| 271 | 60 | 62 | 38 |
| 266 | 60 | 61 | 39 |
| 279 | 60 | 65 | 35 |
| 281 | 60 | 61 | 39 |
| 244 | 60 | 49 | 51 |

* Footnote Table XV

In the heart of adult hooded rats, the 50% conversion point was reached 52 minutes after injection.

(ii) Effect of Age on Dopamine-β-Hydroxylase Activity

In this experiment all rats were sacrificed exactly one hour after injection.

a) Albino Rats:

Table XXI and Fig. No. 13.

Figure No. 14 Effect of Age on Dopamine-β-Hydroxylase in Hooded Rats

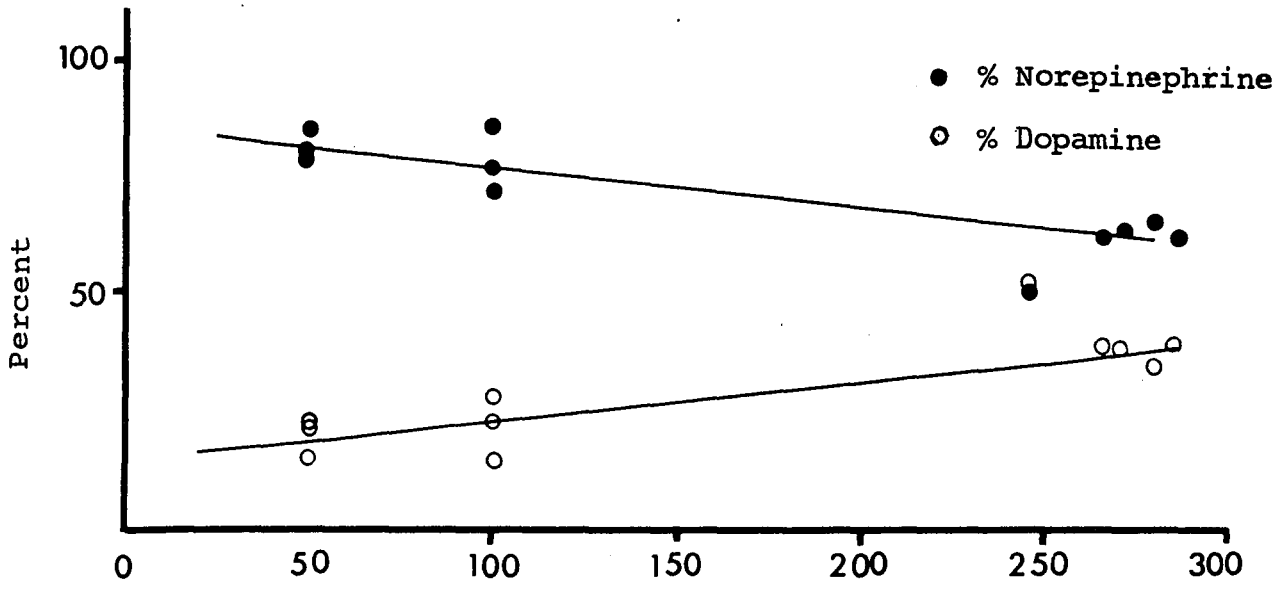


TABLE XXI

Heart*

| Rat Weight in Grams | % Norepinephrine | % Dopamine |
|---|---|---|
| 50 | 74 | 26 |
| 100 | 62 | 38 |
| 200 | 47 | 53 |

* 50 and 100 grams values are the average of three rats from the previous section.

It is seen from this Table and Graph that the activity of Dopamine-β-hydroxylase apparently decreases slightly with age.

b) Hooded Rats:

Table XXII and Fig. No. 14.

TABLE XXII

Heart*

| Rat Weight in Grams | % Norepinephrine | % Dopamine |
|---|---|---|
| 50 | 81 | 19 |
| 100 | 78 | 22 |
| 271 | 62 | 38 |
| 266 | 61 | 39 |
| 279 | 65 | 35 |
| 281 | 61 | 39 |
| 244 | 49 | 51 |

* Footnote Table XXI

The results from these rats agree with the results from the albino rats and seem to indicate that there is no strain difference in the loss of activity of Dopamine-$\beta$-hydroxylase with age.

(iii) Recovery of Added Radioactivity

Three rats were sacrificed, the hearts and spleens removed and frozen on dry ice. The organs were then minced and 10 lambda of solutions of dopamine $C^{14}$ ($2.96 \times 10^5$ dpm) and of norepinephrine $C^{14}$ ($2.22 \times 10^5$ dpm) were added to the tissue. This was then carried throughout the extraction procedure.

TABLE XXIII

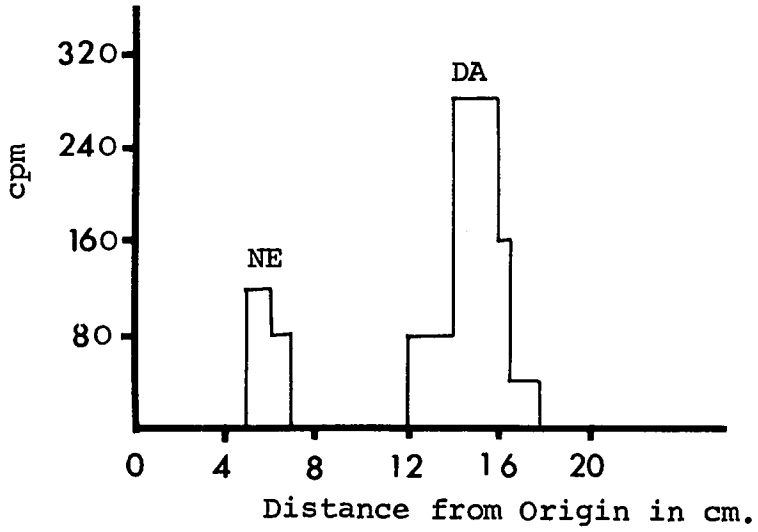
Recovery of Radioactivity

| Rat Weight | % Recovery | |
|---|---|---|
| | Heart | Spleen |
| 195 | 78 | 67 |
| 203 | 77 | 64 |
| 160* | 74±1 | 70 |

* Average of 4 rats

A correction term obtained from this Table was applied to the radioactivities found.

Figure No. 15 Phosphocellulose 81 Ion, Exchange Paper Chromatogram



(iv) Standards

Several standards were run in the butanol:acetic acid:water (4:1:1) system.

TABLE XXIV

Relative $R_f$'s of Catecholamines on PC81 Paper

| Catecholamine | $R_f$ | Distance Travelled in 40 hr.(cm) |
|---|---|---|
| Dopamine | 0.62 | 14-16 |
| Norepinephrine | 0.28 | 6.5-7.5 |
| Normetanephrine | 1.00 | 22-24 |
| Epinephrine | 0.54 | 11-12 |
| Metanephrine | 1.63 | 35-37 |

As can be seen from Table XXIV and Fig. No. 15, a good separation of metabolites was obtained.

After many experiments only the first 25 cm. of the chromatogram were cut to be counted, as most of the radioactivity on the paper consisted of dopamine and norepinephrine.

C. Copper Excess Experiment

These rats were supplied by Mr. G. Gregoriadis of our laboratory. Copper was administered as a $CuSO_4$ solution (500 μg $Cu^{++}$/ 1 saline) or as CuCl suspension (500 μg Cu/ 1 saline). The animals were injected intraperitoneally every day or two days, depending on the health of the animal. All rats were sacrificed 1 hour after injection with dopamine-$C^{14}$.

TABLE XXV

Effect of Excess Copper on Dopamine-$C^{14}$ Conversion by Heart in Vivo

| Rat Weight | | Copper μg/gm Heart | Liver | Total injected Copper | % Norepinephrine | % Dopamine |
|---|---|---|---|---|---|---|
| Control | 475 | 6.6 | 4.16 | | 53 | 47 |
| Control | 490 | 6.6 | 3.83 | | 54 | 46 |
| Control | 350 | 5.3 | 4.09 | | 62 | 38 |
| + $Cu^{+}$ | 355 | 8.7 | 132.1 | 58.2 | 55 | 45 |
| + $Cu^{+}$ | 355 | 7.8 | 95.1 | 56.2 | 65 | 35 |
| + $Cu^{+}$ | 330 | 4.9 | 101.1 | 58.2 | 53 | 47 |
| + $Cu^{+}$ | 295 | 10.1 | 72.5 | 55.4 | 58 | 42 |
| + $Cu^{+}$ | 338 | 9.9 | 231 | 73.6 | 81 | 19 |
| + $Cu^{+}$ | 485 | 6.4 | 90.9 | 73.8 | 55 | 45 |
| + $Cu^{+}$ | 545 | 5.7 | 121 | 75.8 | 54 | 46 |
| + $Cu^{++}$ | 262 | 7.2 | 123.6 | 54.8 | 78 | 22 |
| + $Cu^{++}$ | 434 | 10.2 | 112 | 58.1 | 50 | 50 |
| + $Cu^{++}$ | 530 | 10.4 | 128.7 | 89. | 64 | 36 |

This Table indicates that injected excess copper (cuprous or cupric) has no effect on Dopamine-β-hydroxylase activity, as determined by this method of assay. In two rats apprecially increased conversion was found, but this is not statistically significant.

D. Effect of Antabuse

Albino rats were injected intraperitoneally with 200 mg/kg of a suspension of Antabuse, while the controls were injected with an equal volume of 0.9% NaCl. Two hours later all animals were injected intramuscularly with $C^{14}$-Dopamine. Simultaneously with the dopamine, the control group was injected with NaCl and the test group received a second injection of Antabuse. One rat of each group was sacrificed 15 minutes, 45 minutes, and 90 minutes after injection, dopamine and norepinephrine were extracted from heart and spleen and then chromatographed on phosphocellulose-81 ion exchange paper. The results are in Table XXVI.

From this Table it is seen that Antabuse inhibits Dopamine-β-hydroxylase in vivo, when injected at a concentration of 200 mg/kg.

TABLE XXVI

Dopamine-β-Hydroxylase Inhibition by Antabuse*

| Weight | Group | Time of Sacrifice | % Norepinephrine | | % Dopamine | | NE/DA | |
|---|---|---|---|---|---|---|---|---|
| | | | Heart | Spleen | Heart | Spleen | Heart | Spleen |
| 250 | Antabuse | 15 | 0 | 0 | 100 | 100 | 0:1 | 0:1 |
| 242 | Antabuse | 45 | 0 | 0 | 100 | 100 | 0:1 | 0:1 |
| 242 | Antabuse | 90 | 0 | 0 | 100 | 100 | 0:1 | 0:1 |
| 252 | Control | 15 | 15 | 41 | 85 | 59 | 0.18 | 0.67 |
| 245 | Control | 45 | 40 | 46 | 60 | 54 | 0.67 | 0.81 |
| 250 | Control | 90 | 69 | 56 | 31 | 44 | 2.33 | 1.22 |

* These animals were injected with Antabuse, 200 mg/kg body weight (i.p.), 2 hours and 1 hour before sacrifice.

E. Dopamine-$C^{14}$ Experiment

In these experiments the copper determinations were carried out by Mr. G. Gregoriadis. Animals were killed one hour after injection of dopamine-$C^{14}$, unless otherwise noted.

(i) Experiment No. 1

A group of 46 male albino rats were fed a diet of 100% powdered milk plus added vitamins. The results are expressed in Table XXVII and Figures 16-20. From these data, it is seen that the level of copper in the liver decreases when the animals are fed the copper-deficient diet, as does the percent conversion of injected dopamine-$C^{14}$ to norepinephrine-$C^{14}$ in the heart.

(ii) Experiment No. 2

This experiment was divided into two sections, using one group of albino rats and another group of hooded rats. Both groups were fed a basal diet of liquid milk with added vitamins.

a) Albino Rats:

The conversion in heart alone was determined. The results appear in Table XXVIII and Figures 21-23.

TABLE XXVII

Results of the First Copper-Deficiency Experiment

| Rat No. | Weight in gm. | Diet | Day* | Liver Copper µg/gm | %Norepine-phrine | | %Dopamine | | NE/DA | | %NE-Def. %NE-Con. | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Ht. | Spl. | Ht. | Spl. | Ht. | Spl. | Ht. | Spl. |
| 10 | 44 | Def. | 7** | 3.7 | 56.9 | 78 | 43.1 | 22 | 1.30 | 3.54 | | |
| 21 | 58 | Def. | 7 | 2.9 | | | | | | | | |
| 30 | 65 | Def. | 7 | 2.9 | | | | | | | | |
| 35 | 46 | Def. | 7 | 3.0 | | | | | | | | |
| 15 | 55 | Con. | 12** | 3.5 | 62 | 74 | 38 | 26 | 1.63 | 2.86 | | |
| 36 | 60 | Con. | 12 | 4.2 | | | | | | | | |
| 5 | 60 | Def. | 12 | 3.5 | 30 | 56 | 70 | 44 | 0.48 | 1.27 | 0.48 | 0.78 |
| 33 | 43 | Def. | 12 | 3.4 | | | | | | | | |
| 1 | 72 | Con. | 15** | 5.0 | 43 | 49 | 57 | 51 | 0.75 | 0.98 | | |
| 4 | 60 | Con. | 15 | 4.4 | | | | | | | | |
| 2 | 60 | Def. | 15 | 3.4 | 46 | 51 | 54 | 49 | 0.85 | 1.08 | 1.07 | 1.10 |
| 22 | 61 | Def. | 15 | 4.2 | | | | | | | | |
| 12 | 91 | Con. | 19 | 3.9 | 76 | 77 | 24 | 23 | 3.16 | 3.45 | | |
| 19 | 71 | Def. | 19 | 3.2 | 33 | 50 | 67 | 50 | 0.50 | 1.00 | 0.43 | 0.65 |
| 27 | 66 | Def. | 19 | 2.8 | | | | | | | | |

TABLE XXVII (Continued)

| Rat No. | Weight in gm. | Diet | Day* | Liver Copper µg/gm | %Norepine-phrine | | %Dopamine | | NE/DA | | %NE-Def. %NE-Con. | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Ht. | Spl. | Ht. | Spl. | Ht. | Spl. | Ht. | Spl. |
| 41 | 76 | Con. | 22 | 3.9 | 37 | 67 | 63 | 33 | 0.58 | 2.01 | | |
| 9 | 54 | Def. | 22 | 3.1 | 35 | 55 | 65 | 45 | 0.53 | 1.22 | 0.94 | 0.82 |
| 31 | 56 | Con. | 26 | 5.0 | 46 | 50 | 54 | 50 | 0.84 | 1.00 | 0.56 | 0.54 |
| 49 | 89 | Def. | 26 | 3.5 | 26 | 27 | 74 | 73 | 0.35 | 0.37 | | |
| 6 | 52 | Con. | 29 | 4.0 | 54 | 52 | 46 | 48 | 1.17 | 1.08 | 0.70 | 1.25 |
| 14 | 55 | Def. | 29 | 2.08 | 38 | 65 | 62 | 35 | 0.63 | 1.85 | | |
| 23 | 94 | Con. | 34 | 3.43 | 48 | 30 | 52 | 70 | 0.92 | 0.43 | | |
| 26 | 115 | Con. | 34 | 2.37 | 61 | 37 | 39 | 63 | 1.56 | 0.58 | | |
| 47 | 113 | Con. | 34 | 2.87 | 34 | 27 | 66 | 73 | 0.51 | 0.37 | | |
| 3 | 86 | Def. | 34 | 2.32 | 28 | 28 | 72 | 72 | 0.38 | 0.38 | 0.44 | 1.06 |
| 17 | 62 | Def. | 34 | 2.58 | 16 | 33 | 84 | 67 | 0.19 | 0.49 | | |
| 44 | 109 | Def. | 34 | 3.22 | 20 | 37 | 80 | 63 | 0.25 | 0.58 | | |

TABLE XXVII (Continued)

| Rat No. | Weight in gm. | Diet | Day* | Liver Copper µg/gm | %Norepine-phrine Ht. | %Norepine-phrine Spl. | %Dopamine Ht. | %Dopamine Spl. | NE/DA Ht. | NE/DA Spl. | %NE-Def. %NE-Con. Ht. | %NE-Def. %NE-Con. Spl. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 32 | 96 | Def. | 43 | 2.5 | 49 | 42 | 51 | 58 | 0.96 | 0.72 | | |
| 37 | 121 | Def. | 43 | 3.8 | 37 | 42 | 63 | 58 | 0.58 | 0.72 | | |
| 38 | 129 | Def. | 43 | 3.1 | 34 | 43 | 66 | 57 | 0.51 | 0.75 | | |
| 45 | 84 | Def. | 43 | 2.0 | 41 | | 59 | | 0.69 | | | |
| 50 | 108 | Def. | 43 | 3.5 | 38 | | 62 | | 0.61 | | | |
| 11 | 140 | Rep. | 33+10*** | 3.0 | 36 | 50 | 64 | 50 | 0.56 | 1.00 | | |
| 13 | 123 | Rep. | 33+10*** | 3.8 | 37 | 42 | 63 | 58 | 0.58 | 0.72 | | |
| 29 | 77 | Rep. | 33+10*** | 2.7 | 48 | 54 | 52 | 46 | 0.92 | 1.17 | | |
| 34 | 104 | Rep. | 33+10*** | 4.6 | 51 | 54 | 49 | 46 | 1.04 | 1.17 | | |

* No. of days that the animals were fed a deficient diet. The controls were fed the deficient diet for the first week and then the copper supplement was added to their diet.

** On these days the organs from each group were pooled.

*** These animals were fed the copper deficient diet for 33 days and then given the copper supplement for 10 days.

Ht - Heart

Spl- Spleen

Def.-Deficient

Con.-Control

Rep.-Repletion

TABLE XXVIII

Albino Rats - Experiment No. 2*

| Rat No. | Weight | Day No. | State | Copper (μg/gm) Liver | Heart | Heart %Norepinephrine | % Dopamine | NE/DA |
|---|---|---|---|---|---|---|---|---|
| 1c | 238 | 47 | Control | 4.00 | 6.38 | 47 | 53 | 0.88 |
| 2c | 238 | 47 | Control | 4.30 | 6.19 | 46 | 54 | 0.85 |
| 3c | 218 | 47 | Control | 4.53 | 7.80 | 44 | 56 | 0.78 |
| 4c | 208 | 47 | Control | 4.84 | 6.69 | 63 | 37 | 1.70 |
| 5c | 192 | 47 | Control | 4.63 | — | 39 | 61 | 0.63 |
| 6c | 223 | 47 | Control | 4.02 | 6.97 | 41 | 59 | 0.69 |
| 7c | 226 | 47 | Control | 4.51 | 9.38 | 40 | 60 | 0.66 |
| 8c | 172 | 47 | Control | 5.12 | 8.21 | 45 | 55 | 0.82 |
| 9c | 204 | 47 | Control | 5.41 | 9.20 | 47 | 53 | 0.90 |
| 10c | 168 | 47 | Control | 5.74 | 7.39 | 49 | 51 | 0.96 |
| Average | | | | 4.71 ±0.06 | 7.58 ±0.43 | 46.2±2.15 | 53.8 ±2.15 | 0.86 |
| 11c | 322 | 91 | Control | 5.04 | 5.78 | 39 | 61 | 0.63 |
| 12c | 315 | 91 | Control | 5.08 | 6.49 | 34 | 66 | 0.52 |
| 13c | 326 | 91 | Control | 5.00 | 6.18 | 38 | 62 | 0.62 |

* This experiment was commenced on Feb. 2, 1966.

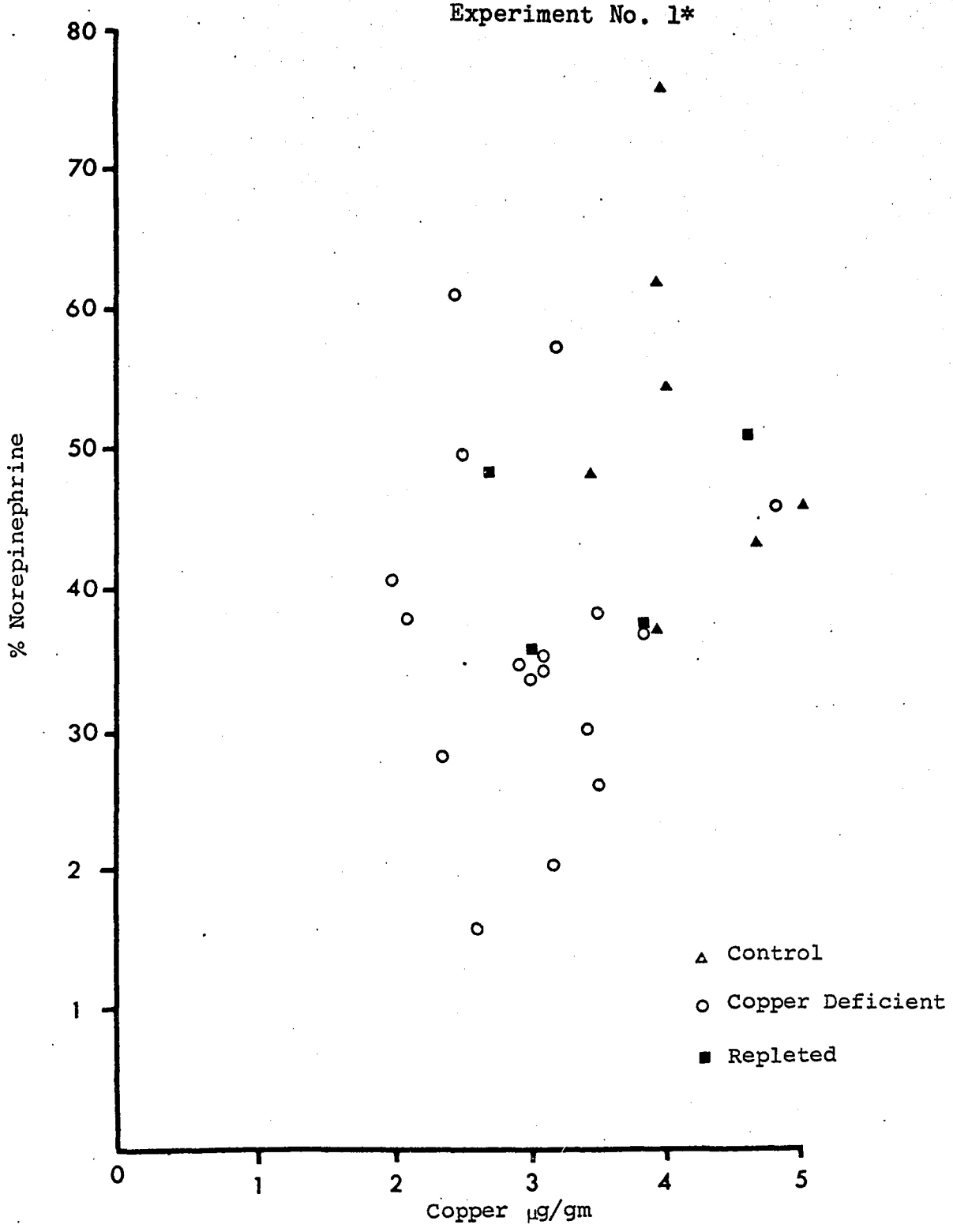
TABLE XXVIII (Continued)

| Rat No. | Weight | Day No. | State | Copper µg/gm Liver | Copper µg/gm Heart | Heart %Norepinephrine | Heart %Dopamine | NE/DA | %Inhibition |
|---|---|---|---|---|---|---|---|---|---|
| 14c | 233 | 91 | Control | 4.30 | 7.15 | 48 | 52 | 0.94 | |
| 15c | 280 | 91 | Control | 4.74 | 6.28 | 33 | 67 | 0.49 | |
| 16c | 290 | 91 | Control | 5.23 | 6.20 | 37 | 63 | 0.58 | |
| Average | | | | 4.89 ±0.15 | 6.54 ±0.23 | 38.2±2.2 | 71.8 ±2.2 | 0.63 | |
| 1D | 180 | 47 | Copper Deficient | 2.76 | —— | 31 | 69 | 0.44 | |
| 2D | 183 | 47 | Copper Deficient | 1.86 | —— | 13 | 87 | 0.15 | |
| 3D | 172 | 47 | Copper Deficient | —— | —— | 31 | 69 | 0.44 | |
| 4D | 182 | 47 | Copper Deficient | 1.74 | —— | 10 | 90 | 0.11 | |
| 5D | 209 | 47 | Copper Deficient | 1.89 | —— | 16 | 84 | 0.20 | |
| 6D | 171 | 47 | Copper Deficient | 1.82 | —— | 12 | 88 | 0.14 | |
| 7D | 152 | 47 | Copper Deficient | 1.41 | —— | 5 | 95 | 0.05 | |

TABLE XXVIII (Continued)

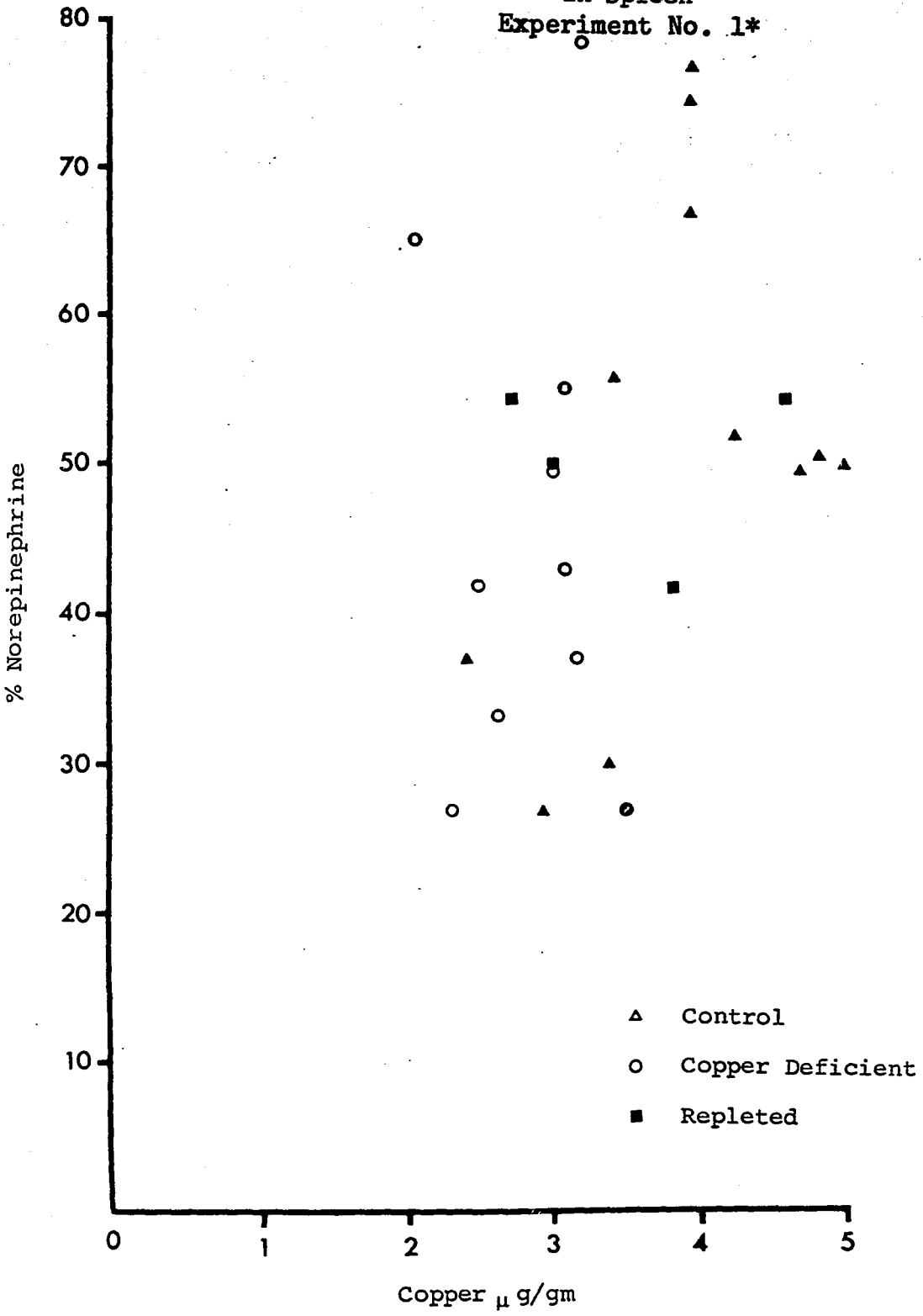
| Rat No. | Weight | Day No. | State | Copper µg/gm Liver | Copper µg/gm Heart | Heart %Norepine-phrine | Heart %Dopa-mine | NE/DA | %Inhibition |
|---|---|---|---|---|---|---|---|---|---|
| 8D | 205 | 47 | Copper Deficient | 2.06 | —— | 9 | 91 | 0.10 | |
| 9D | 178 | 47 | Copper Deficient | 2.77 | —— | 29 | 71 | 0.41 | |
| 10D | 185 | 47 | Copper Deficient | 2.82 | —— | 50 | 50 | 1.00 | |
| Average | | | | 2.10 ±0.18 | | 20.7±4.4 | 79.3 ±4.4 | 0.30 | 57.0 |
| 11D | 286 | 91 | Copper Deficient | 1.74 | 1.92 | 7 | 93 | 0.08 | |
| 12D | 312 | 91 | Copper Deficient | 2.13 | 2.53 | 7 | 93 | 0.08 | |
| 13D | 252 | 91 | Copper Deficient | 2.03 | 2.75 | 5 | 95 | 0.05 | |
| 14D | 280 | 91 | Copper Deficient | 2.24 | 1.24 | 7 | 93 | 0.08 | |
| 15D | 292 | 91 | Copper Deficient | 2.11 | 2.00 | 8 | 92 | 0.09 | |
| 16D | 232 | 91 | Copper Deficient | 2.64 | 2.15 | 12 | 88 | 0.13 | |
| Average | | | | 2.15 ±0.13 | 2.10 ±0.20 | 7.4±0.95 | 92.6 ±0.95 | 0.08 | |

Figure No. 16. Liver Copper vs % Norepinephrine in Heart
Experiment No. 1*




* Refer to text, p. 116

Figure No. 17. Liver Copper vs. %Norepinephrine in Spleen
Experiment No. 1*




* Refer to text, p. 116

Figure No. 18. Copper Content of Liver vs. Time Fed Deficient Diet, Expt. 1

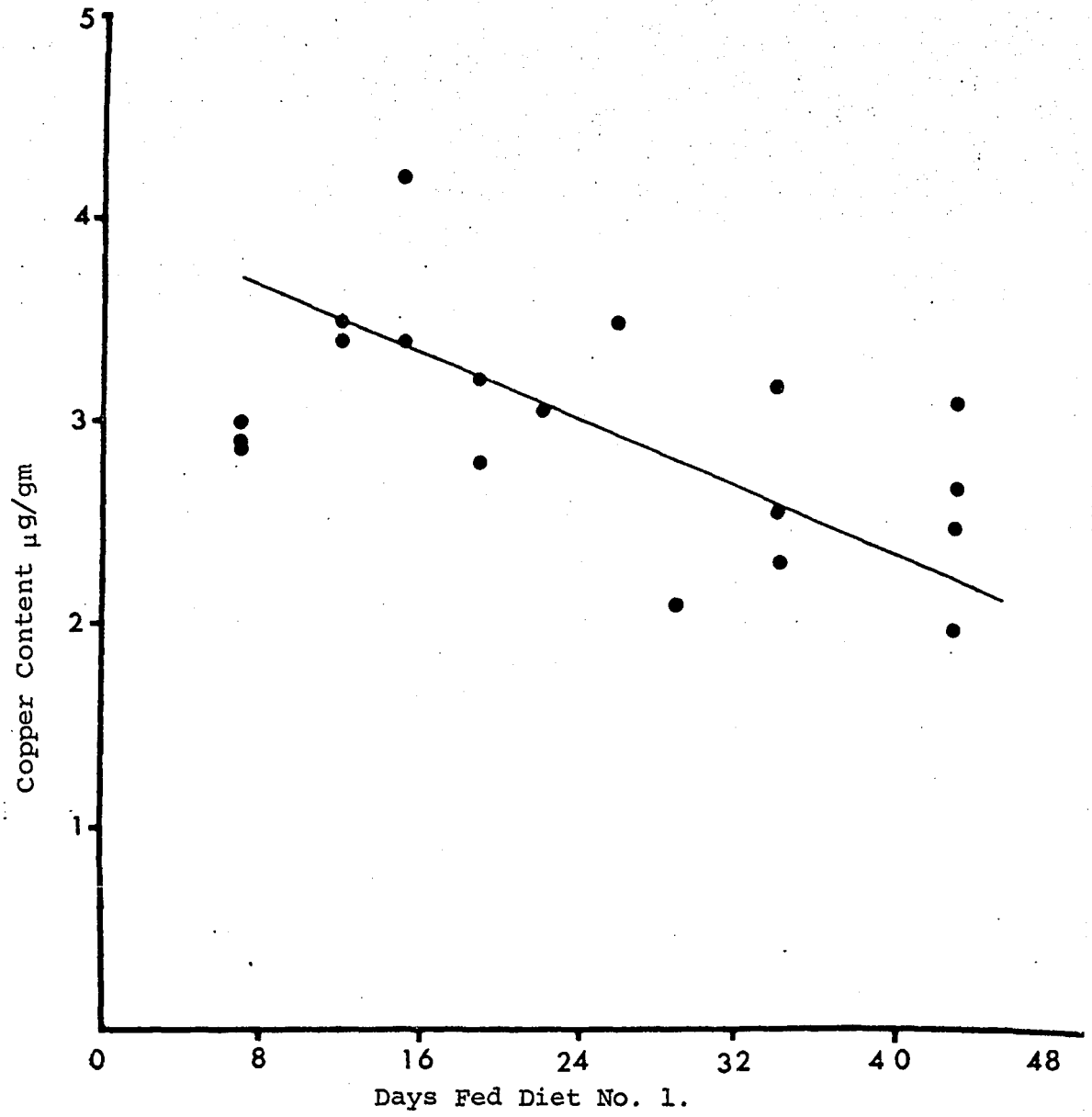
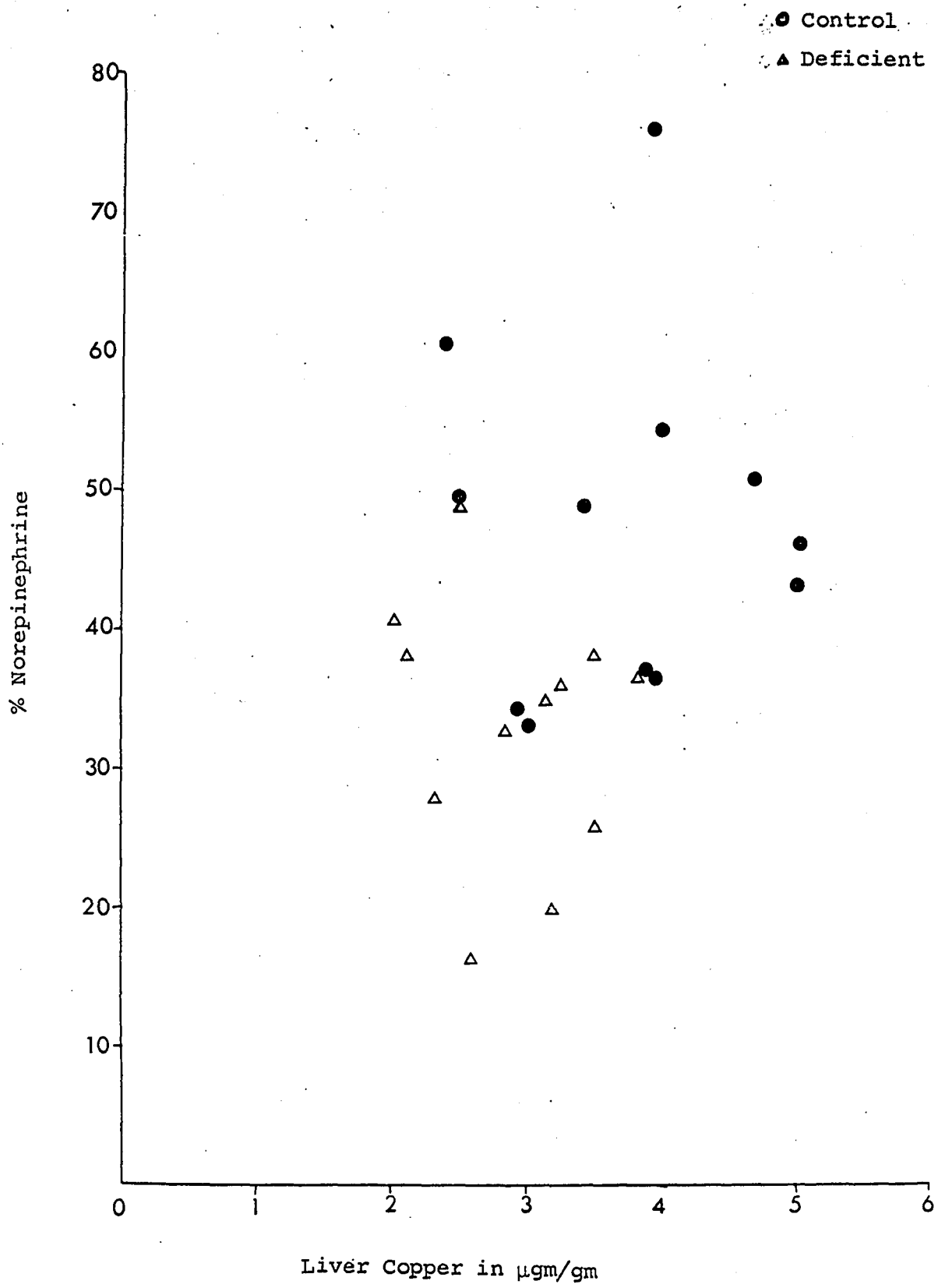


Figure No. 19 Conversion of Dopamine - $C^{14}$ in Heart vs Liver Copper, Diet No. 1*




* Refer to text, p. 116

Figure No. 20. Copper Deficient Rats. % Norepinephrine of $C^{14}$ in the Spleen vs. Days Fed Diet 1.

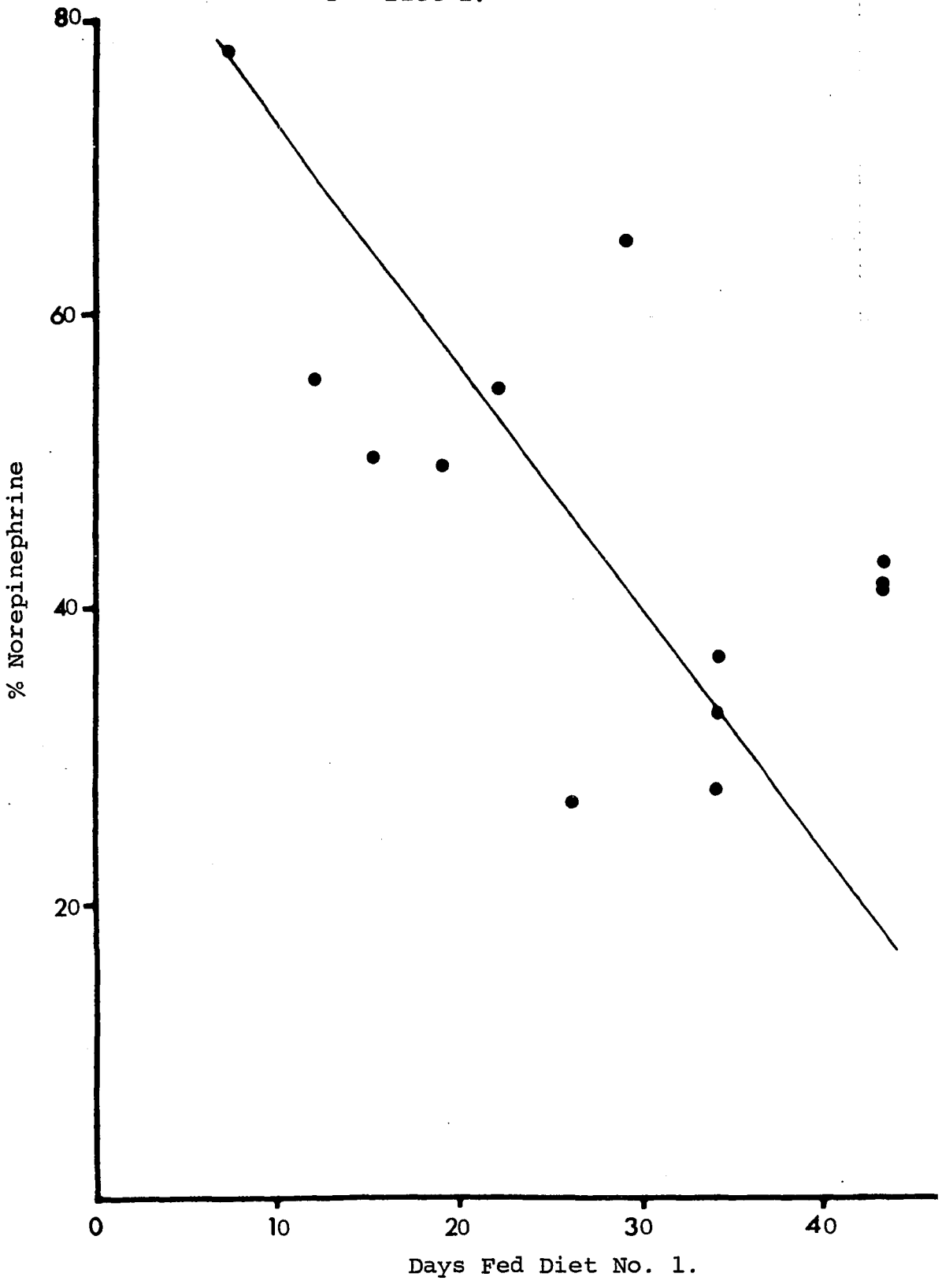
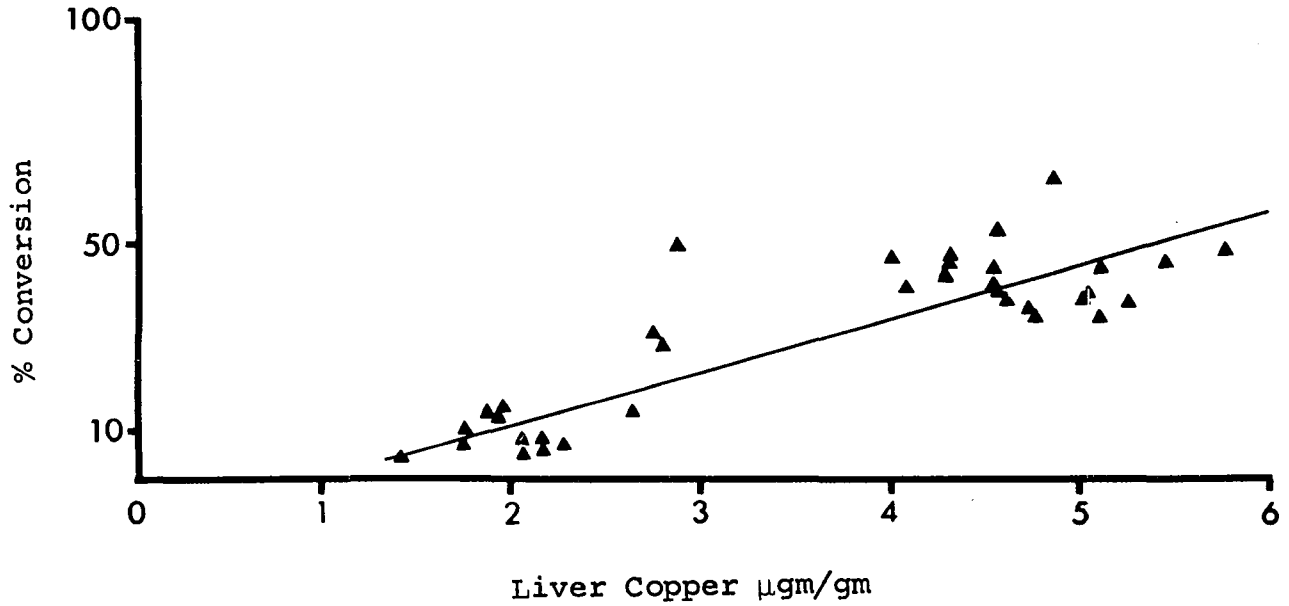
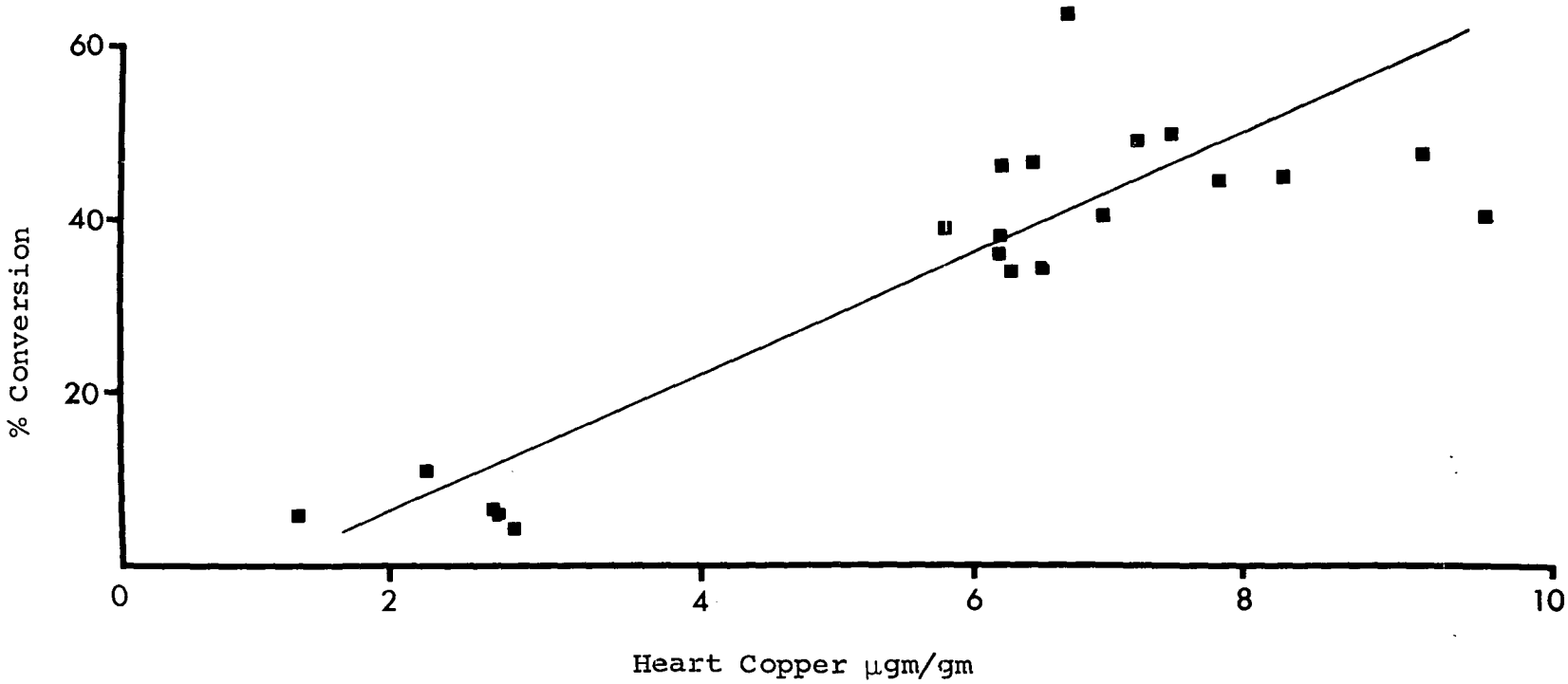


Figure No. 21 Conversion of $C^{14}$ Dopamine to Norepinephrine $C^{14}$ in the Heart vs. Liver Copper, Experiment No. 2*



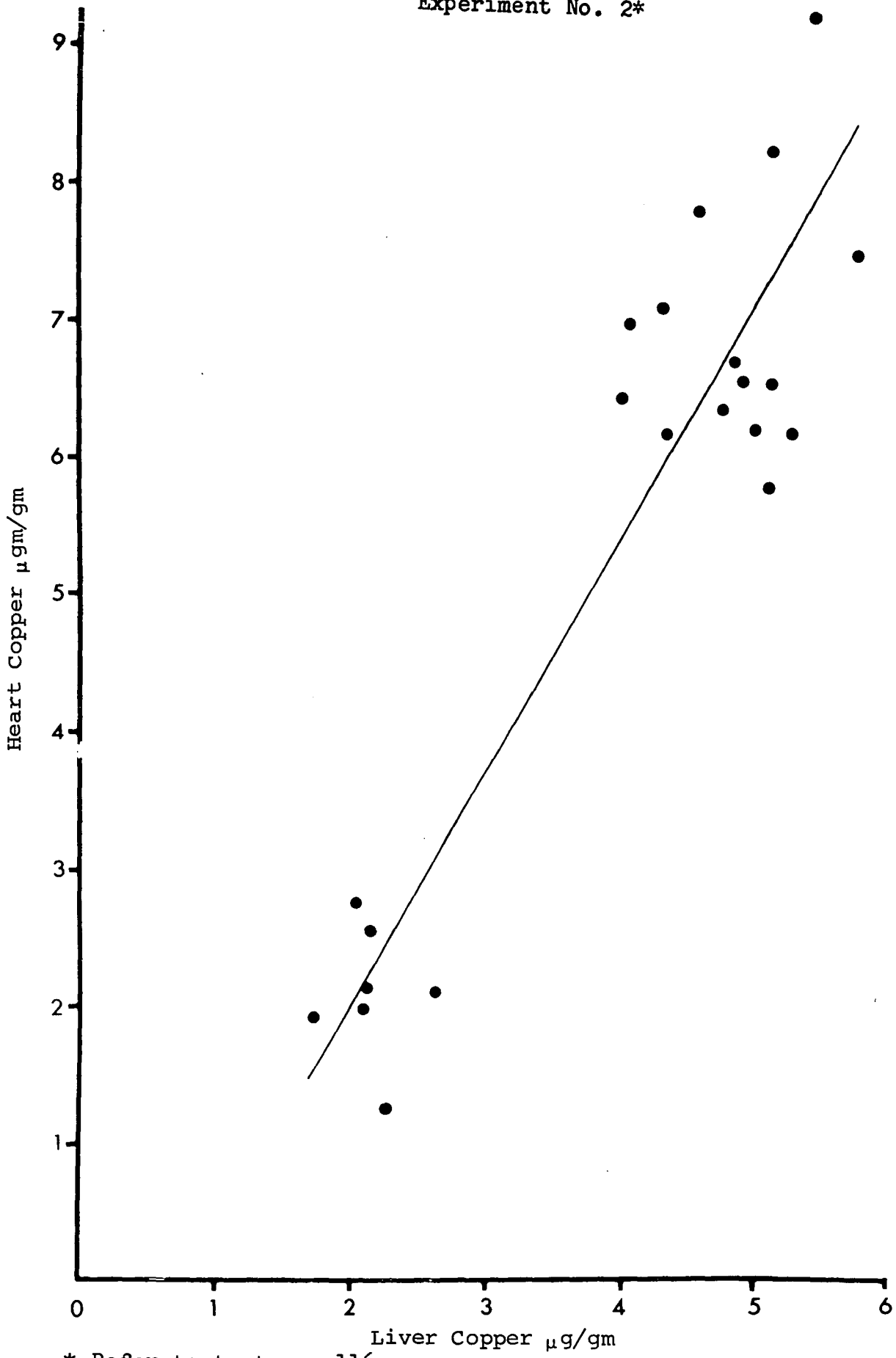

* Refer to text, p. 116.

Figure No. 22 Conversion of $C^{14}$ Dopamine to Norepinephrine $C^{14}$ in the Heart vs Heart Copper, Experiment No. 2*




* Refer to text, p. 116.

Figure No. 23. Liver Copper vs Heart Copper in Albino Rats Experiment No. 2*




* Refer to text, p. 116.

b) Hooded Rats:

The conversion of dopamine-$C^{14}$ to norepinephrine-$C^{14}$ was measured only in the heart. The results appear in Table XXIX and in Figures 24-26.

Experiment No. 2 has shown conclusively that the Dopamine-β-hydroxylase activity in the heart is dependent upon the level of body copper. The results from Experiments 1 and 2 show that it is possible, by the means of a simple dietary deficiency, to reduce the activity of Dopamine-β-hydroxylase.

Figures 23 and 26 demonstrate that the level of copper in the heart is correlated with the level of copper in the liver. It is necessary to demonstrate this in order to discuss the function of the enzyme in the heart with respect to liver copper. The sensitivity to copper in albino rats was found to be better than in hooded rats.

(iii) Time Curve in Copper Deficient Animals

A group of albino rats were fed a basal diet of 100% powdered milk plus vitamin supplement for 42 days. Dopamine-$C^{14}$ was injected and the animals were sacrificed at varying lengths of time. Both copper-deficient and control (copper-supplemented) animals were used.

TABLE XXIX

Hooded Rats - Experiment No. 2

| Rat No. | Weight | Day No. | State | Copper µg/gm Liver | Heart | Heart %Norepinephrine | Heart % Dopamine | NE/DA |
|---|---|---|---|---|---|---|---|---|
| 1 | 151 | 49 | Control | 4.52 | 7.81 | 42 | 58 | 0.72 |
| 2 | 158 | 49 | Control | 4.21 | 9.04 | 56 | 44 | 1.27 |
| 3 | 154 | 49 | Control | 4.58 | 8.10 | 53 | 47 | 1.12 |
| 4 | 182 | 49 | Control | 4.21 | 6.60 | 45 | 55 | 0.83 |
| 5 | 151 | 49 | Control | 4.39 | 8.86 | 62 | 38 | 1.62 |
| Average | | | | 4.38 ±0.08 | 8.08 ±0.41 | 51.1±3.6 | 48.3±3.6 | 1.11 |
| 1 | 210 | 89 | Control | 4.39 | — | 40 | 60 | 0.67 |
| 2 | 206 | 89 | Control | 5.32 | — | 55 | 45 | 1.23 |
| 3 | 200 | 89 | Control | 5.31 | 7.77 | 49 | 51 | 0.96 |
| 4 | 230 | 89 | Control | 5.61 | 7.04 | 57 | 43 | 1.32 |
| Average | | | | 5.16 ±0.30 | 7.40 | 50.2±4.0 | 49.8±4.0 | 1.04 |

TABLE XXIX (Continued)

| Rat No. | Weight | Day No. | State | Copper µg/gm Liver | Heart | Heart %Norepine-phrine | Heart % Dopa-mine | NE/DA | %Inhibition |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 156 | 49 | Copper Deficient | 2.08 | 4.20 | 25 | 75 | 0.33 | |
| 2 | 148 | 49 | Copper Deficient | 2.24 | 4.46 | 24 | 76 | 0.31 | |
| 3 | 136 | 49 | Copper Deficient | 2.68 | 3.72 | 30 | 70 | 0.43 | |
| 4 | 129 | 49 | Copper Deficient | 2.45 | 3.72 | 25 | 75 | 0.33 | |
| 5 | 148 | 49 | Copper Deficient | 2.26 | 3.90 | 25 | 75 | 0.33 | |
| Average | | | | 2.34 ±0.1 | 4.00 ±0.16 | 25.7±1.1 | 74.3 ±1.1 | 0.35 | 50.0 |
| 1 | 226 | 89 | Copper Deficient | 1.68 | 4.95 | 9 | 91 | 4.95 | |
| 2 | 212 | 89 | Copper Deficient | 1.00 | 5.74 | 12 | 88 | 0.14 | |
| 3 | 204 | 89 | Copper Deficient | 2.58 | 4.77 | 19 | 81 | 0.24 | |
| Average | | | | 1.75 ±0.56 | 5.15 ±0.19 | 13.7±3.0 | 86.3 ±3.0 | 0.16 | 72.8 |

TABLE XXX

Control Rats

| Rat Weight (gm) | Time of Sacrifice | State | Copper µg/gm Liver | Heart | Heart %Norepine-phrine | %Dopamine | NE/DA |
|---|---|---|---|---|---|---|---|
| 165 | 15' | Control | — | 4.1 | 8.6 | 91.4 | 0.09 |
| 135 | 15' | Control | 18.8 | 3.3 | 17.4 | 82.6 | 0.20 |
| 130 | 15' | Control | — | 7.4 | 4.6 | 93.4 | 0.06 |
| 130 | 30' | Control | 15.6 | 2.2 | 25.4 | 74.6 | 0.33 |
| 146 | 30' | Control | 5.20 | 4.0 | 26 | 74 | 0.35 |
| 105 | 30' | Control | 24.6 | 4.1 | 41 | 59 | 0.70 |
| 130 | 60' | Control | 17.1 | 5.7 | 40 | 60 | 0.67 |
| 120 | 60' | Control | 15.6 | 9.0 | 51 | 49 | 1.04 |
| 125 | 60' | Control | 37.7 | 4.0 | 60 | 40 | 1.33 |
| 134 | 15' | Copper Deficient | 1.36 | 5.6 | 4 | 96 | 0.04 |
| 144 | 15' | Copper Deficient | 0.75 | 2.2 | 3 | 97 | 0.03 |

TABLE XXX (Continued)

| Rat Weight (gm) | Time of Sacrifice | State | Copper µg/gm Liver | Copper µg/gm Heart | Heart %Norepine-phrine | Heart %Dopamine | NE/DA |
|---|---|---|---|---|---|---|---|
| 159 | 30' | Copper Deficient | — | 2.8 | 13 | 87 | 0.14 |
| 96 | 30' | Copper Deficient | 2.84 | 0.22 | 21 | 79 | 0.26 |
| 136 | 60' | Copper Deficient | — | 1.6 | 46.5 | 53.5 | 0.84 |
| 125 | 60' | Copper Deficient | 0.55 | 7.3 | 9 | 91 | 0.10 |

Figure No. 24 $C^{14}$ Norepinephrine vs Liver Copper in Hooded Rats

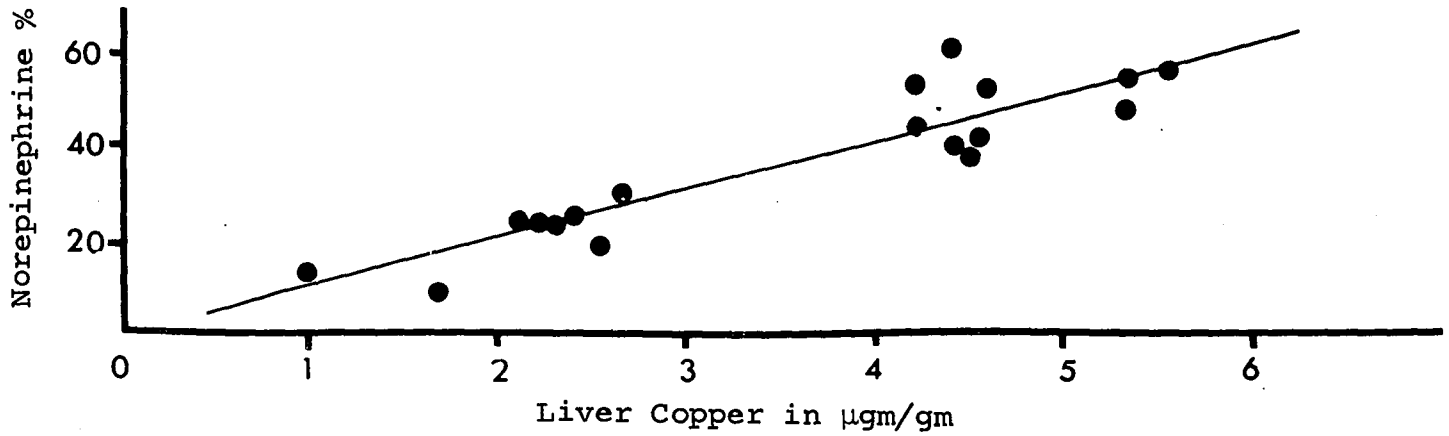



Figure No. 25 Hooded Rats $C^{14}$ Norepinephrine vs Heart Copper

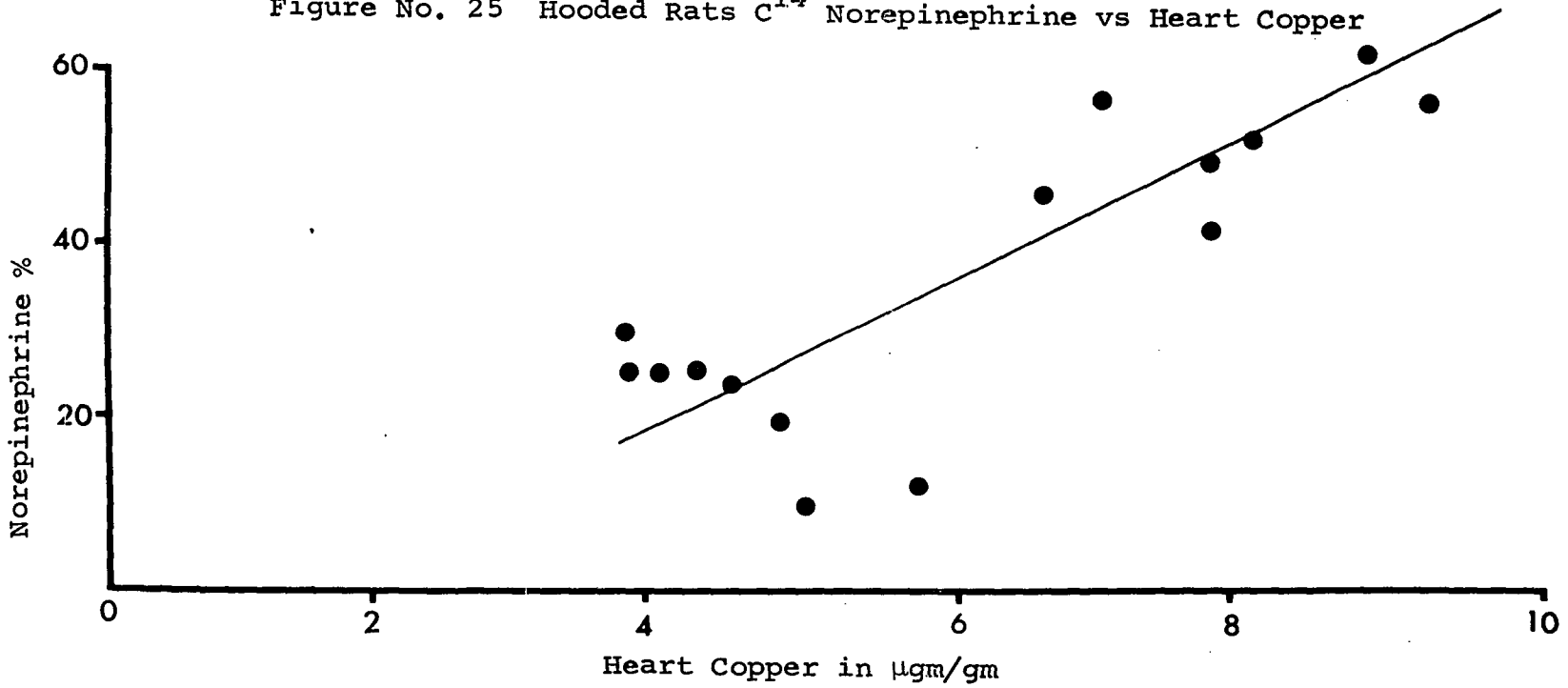


Figure No. 26. Liver Copper vs Heart Copper
Hooded Rats

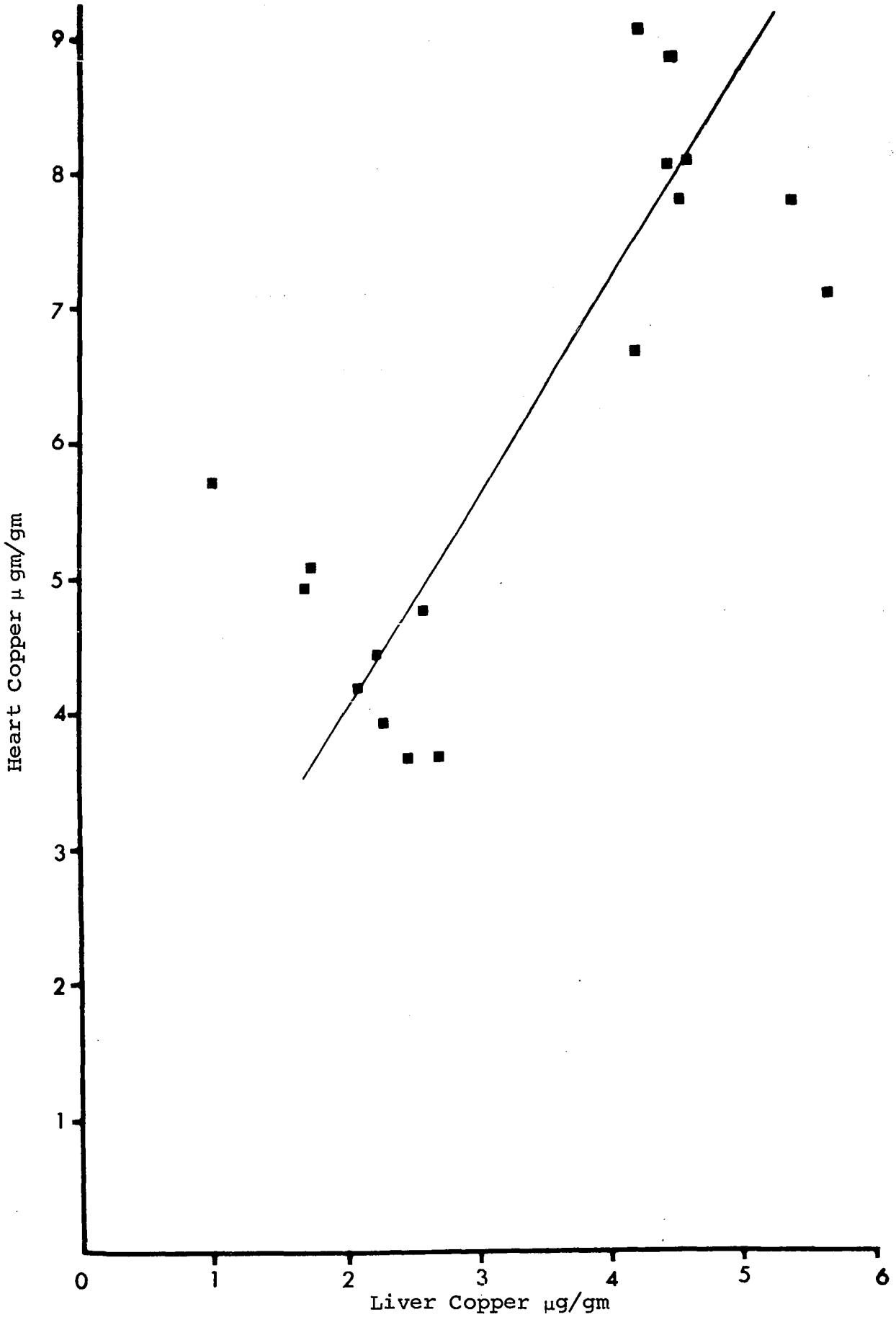
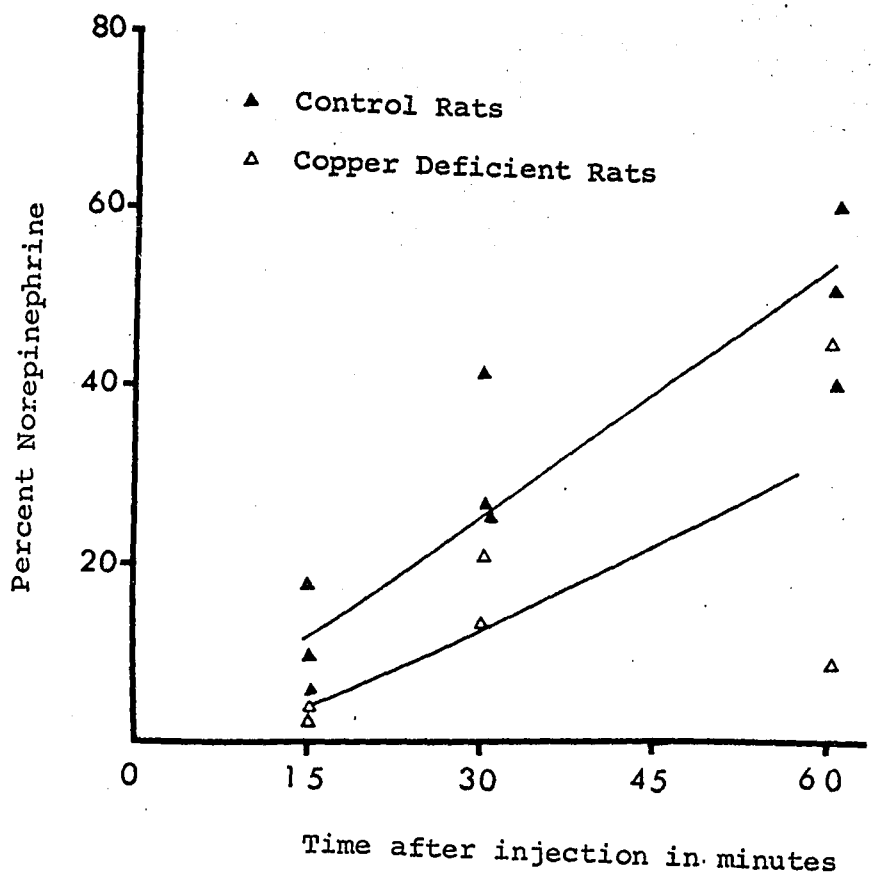


Figure No. 27 Time Study of Dopamine-β-Hydroxylase Activity



These results are expressed graphically in Figure No. 27. As can be seen, the control time curve is the same as in normal animals, i.e., animals fed Purina Laboratory Chow as compared with rats fed a stock diet.

(iv) Repletion Experiment

Three albino rats that had been fed copper deficient diet No. 2 for 84 days were repleted with copper for 19 days. The copper levels administered were the same as the control group had been receiving. The animals were sacrificed exactly one hour after injection of dopamine-$C^{14}$ and the catecholamines were extracted as in the previous experiment.

From this evidence it may be said that after 19 days of copper repletion, the formerly copper-deficient animals attain the same levels of liver copper as the controls. The rate of conversion of dopamine-$C^{14}$ in the repleted animals is the same as in the controls, i.e., dietary copper deficiency and the biochemical defects associated with it can be reversed by a supplement of copper to the diet.

TABLE XXXI

Repletion Experiment *

| Rat | Weight | Copper µg/gm Liver | Heart | %Norepine-phrine | %Dopamine | NE/DA |
|---|---|---|---|---|---|---|
| Control | 280 | 3.19 | 7.15 | 41 | 59 | 0.68 |
| Control | 352 | 3.40 | 8.32 | 45 | 55 | 0.82 |
| Control | 315 | 3.81 | 7.69 | 42 | 48 | 0.71 |
| Deficient | 265 | 3.56 | 14.8 | 41 | 59 | 0.68 |
| Deficient | 320 | 3.89 | 12.4 | 45 | 55 | 0.82 |
| Deficient | 288 | 3.78 | 6.6 | 50 | 50 | 1.00 |

* In this experiment the animals were repleted for 10 days with copper at the same level as the control group was receiving.

F. Iron and Copper Deficiency Experiment

(i) Standards

The standard curve for copper, with no iron present, is in Table XXXII and Figure No. 28.

From Table XXXII and Figure 28 the factor of 1 g. copper per optical density reading of 0.044 is obtained.

It was noticed that high values of iron interfere with the copper determination. Therefore a correction curve for iron interference was determined. This is presented in Table XXXIII and Figure No. 29.

TABLE XXXIII

Iron Interference in Copper Determination

| Iron Added μg. | 25 | 50 | 100 | 150 | 250 |
|---|---|---|---|---|---|
| Optical* Density | 0.007 | 0.034 | 0.033 | 0.047 | 0.099 |
| Value as Copper μg. | 0.16 | 0.77 | 0.75 | 1.09 | 2.25 |

* Average of two readings, corrected for blanks.

As can be seen from Table XXXIII and Figure 29 there is a linear relation between iron present in the sample and interference in the copper reading. From the

TABLE XXXII

Standard Curve for Copper-No Iron Present

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Copper Added µg. | 0.5 | 0.5 | 1.0 | 1.0 | 3.0 | 3.0 | 5.0 | 5.0 | 7.0 | 7.0 | 10.0 | 10.0 |
| Optical Density * | 0.023 | 0.021 | 0.036 | 0.035 | 0.133 | 0.114 | 0.207 | 0.205 | 0.285 | 0.286 | 0.403 | 0.384 |

* Optical density values are corrected for reagent blank in all cases.

Figure No. 28 Standard Curve for Copper

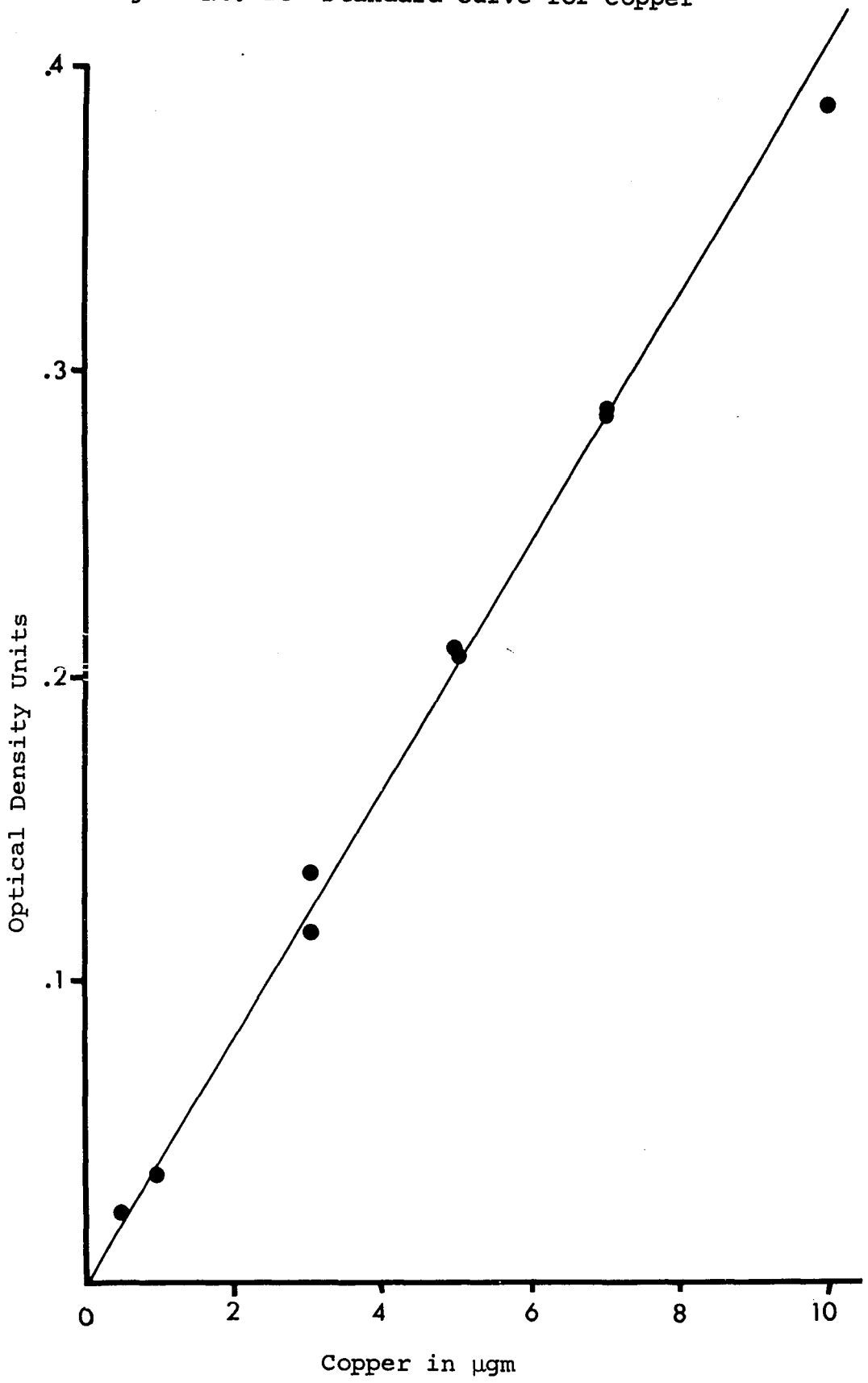
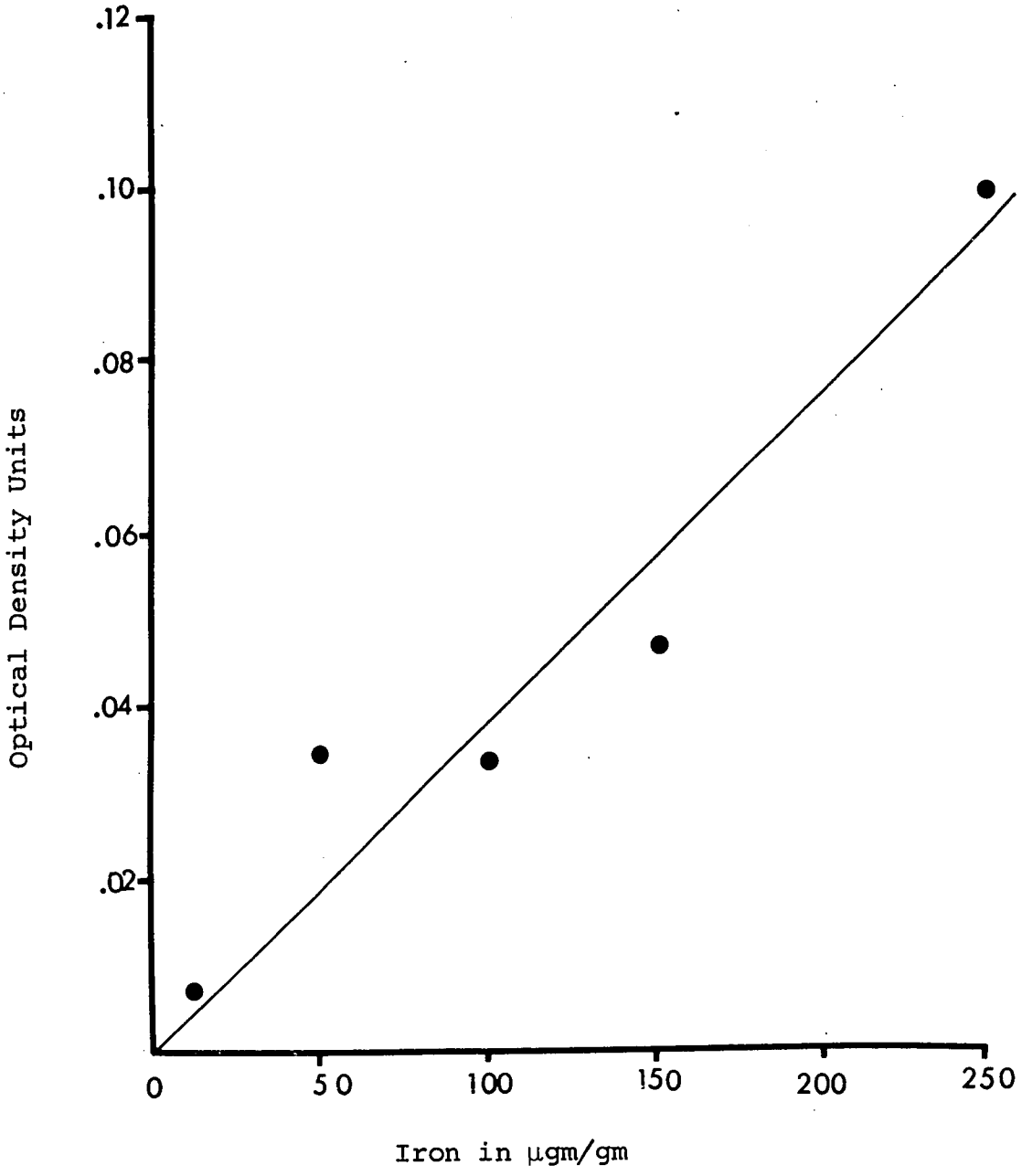


Figure No. 29 Iron Absorption Under Copper Determination Conditions



information below it is evident that the iron interference is directly additive to the copper absorption. The correction was made by subtracting the apparent copper value caused by the iron interference from the total copper value obtained. To avoid this interference in any further determinations 10 ml of saturated Na pyrophosphate was added to the sample during the acid extraction step. The results of this are in Table XXXIV.

TABLE XXXIV

Interference by Iron After Addition of Na Pyrophosphate

| Iron in μg. | 25 | 50 | 100 | 150 | 200 | 250 |
|---|---|---|---|---|---|---|
| Optical Density * | 0.008 | 0.008 | 0.000 | 0.006 | 0.000 | 0.002 |
| Value as Copper in g. | 0.18 | 0.18 | 0.000 | 0.14 | 0.00 | 0.05 |

* Optical density readings are corrected for reagent blanks, average of two readings.

To be certain of the above results, 100 g, iron + 5 g. copper were analysed when no pyrophosphate was added and then corrected for iron by means of the correction curve for iron. This was repeated with Na pyrophosphate added at the acid-extraction stage. This procedure was followed for 100 g. iron plus 2.5 g. copper. As can be seen in Table XXV, when pyrophosphate was added at the acid step, the

recovery was excellent. In the samples where there was no pyrophosphate added, the correction term brought the copper values into the correct range.

TABLE XXXV

Correction for Iron in Copper Determinations

| Metals in Solution-µg. | | µg. Copper Found | | |
|---|---|---|---|---|
| Iron | Copper | No Pyrophos-phrate | Corrected Value | Pyrophos-phrate Added |
| 100 | 5.0 | 5.6 | 4.8 | 4.93 |
| 100 | 2.5 | 3.58 | 2.77 | 2.43 |

(ii) Liver Copper and Iron Values

The iron and copper levels of the liver were measured after perfusion of the liver with 0.9% NaCl in glass distilled water. In some cases there was not enough tissue available for all determinations. The results are set out in Table XXXVI and Figures 30-32.

TABLE XXXVI

Iron and Copper Levels in Experiment No. 4. - Liver

| Day* | Rat No. | State | Iron-µg/gm | Copper-µg/gm |
|---|---|---|---|---|
| 0 | 4A | Control | 45 | 7.00 |
| 0 | 5A | Control | 42 | 6.74 |
| 0 | 6A | Control | 44 | 5.84 |
| 0 | 7A | Control | 40 | 7.93 |
| 0 | 8A | Control | 37 | 6.49 |
| 7 | 3 | Control | 35 Av. | —— |
| 7 | 9 | Control | | 4.86 |
| 7 | 52 | Iron-Deficient | 37 Av. | —— |
| 7 | 57 | Iron-Deficient | | —— |
| 24 | 4 | Control | 89 | 3.41 |
| 24 | 11 | Control | 30 | 4.45 |
| 24 | 22 | Doubly Deficient | 30 | 2.17 |
| 24 | 28 | Doubly Deficient | 41 | 4.78 |
| 24 | 34 | Copper Deficient | 57 | 5.30 |
| 24 | 39 | Copper Deficient | — | 4.02 |
| 24 | 50 | Iron Deficient | 16 | 8.57 |
| 24 | 54 | Iron Deficient | 21 | 7.27 |

TABLE XXXVI (Continued)

| Day* | Rat No. | State | Iron-µg/gm | Copper-µg/gm |
|---|---|---|---|---|
| 37 | 2 | Control | 30 | 4.60 |
| 37 | 18 | Control | 89 | 4.52 |
| 37 | 26 | Doubly Deficient | — | 3.55 |
| 37 | 27 | Doubly Deficient | 30 | 2.13 |
| 37 | 49 | Iron Deficient | 24 | 5.77 |
| 37 | 51 | Iron Deficient | 19 | 12.09 |
| 66 | 5 | Control | 77 | 5.04 |
| 66 | 12 | Control | 65 | 6.22 |
| 66 | 25 | Doubly Deficient | 21 | 3.98 |
| 66 | 30 | Doubly Deficient | — | 2.90 |
| 66 | 31 | Copper Deficient | 26 | 5.99 |
| 66 | 36 | Copper Deficient | 170 | 4.29 |
| 66 | 43 | Iron Deficient | — | 24.0 |
| 66 | 45 | Iron Deficient | — | 21.7 |
| 78 | 13 | Control | 36 | 4.60 |
| 78 | 16 | Control | 62 | 4.85 |
| 78 | 23 | Doubly Deficient | 24 | 3.33 |
| 78 | 37 | Copper Deficient | 205 | 2.45 |
| 78 | 42 | Copper Deficient | 185 | 2.53 |
| 78 | 48 | Iron Deficient | 37 | 47.3 |

TABLE XXXVI (Continued)

| Day* | Rat No. | State | Iron-µg/gm | Copper-µg/gm |
|---|---|---|---|---|
| 95 | 14 | Control | 51.4 | 4.51 |
| 95 | 15 | Control | 55 | 5.21 |
| 95 | 20 | Doubly Deficient | 45 | 1.53 |
| 95 | 32 | Copper Deficient | 155 | 2.13 |
| 95 | 40 | Copper Deficient | 188 | 2.46 |
| 95 | 58 | Iron Deficient | 25 | 22.5 |
| 109 | 1 | Control | 106 | 3.37 |
| 109 | 10 | Control | 117 | 3.71 |
| 109 | 17 | Control | 123 | 3.55 |
| 109 | 21 | Doubly Deficient | 44 | 2.59 |
| 109 | 33 | Copper Deficient | 333 | 1.35 |
| 109 | 35 | Copper Deficient | 193 | 2.19 |
| 109 | 38 | Copper Deficient | 371 | 1.63 |
| 109 | 41 | Copper Deficient | 228 | 1.67 |

* This experiment was commenced on Nov. 1, 1966.

From this Table and the figures, it is evident that copper and iron have an inverse relationship, probably associated with storage and/or utilization. In iron deficiency the copper content of the liver increases with time. In

Figure No. 30 Liver Iron vs Liver Copper
Experiment No. 4

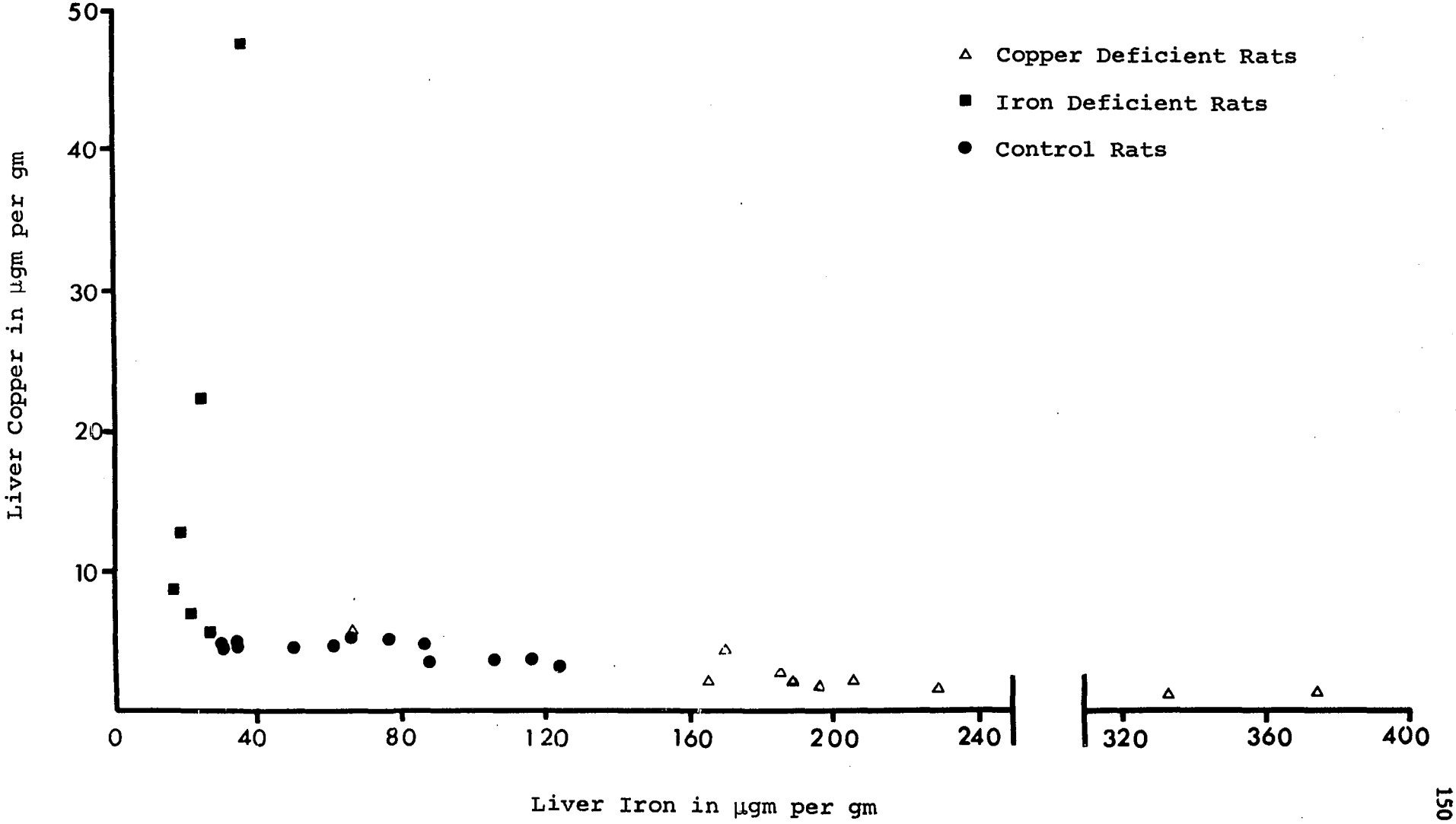


Figure No. 31 Copper-Iron Relation in Iron Deficient Rats
Experiment No. 4

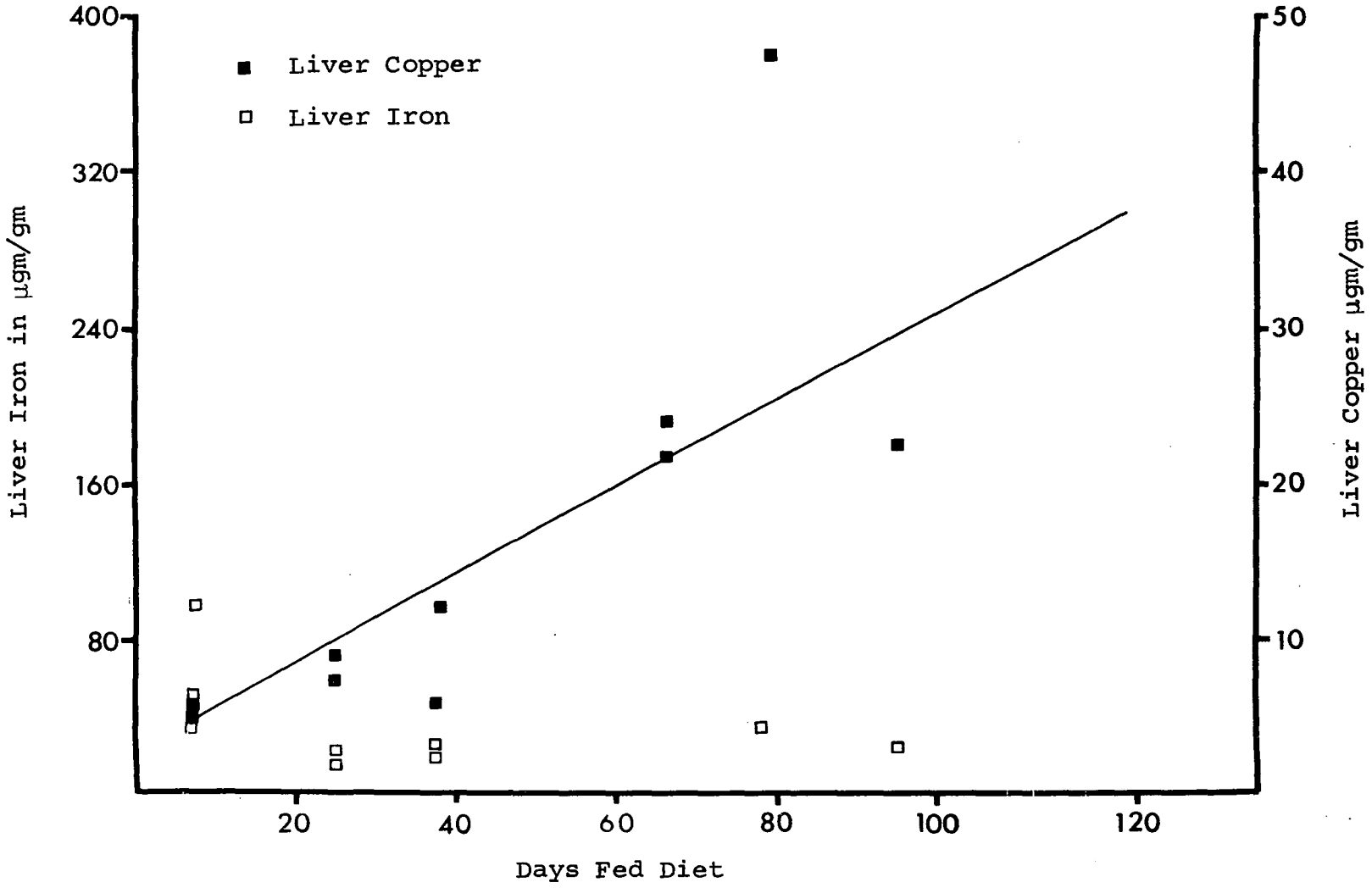
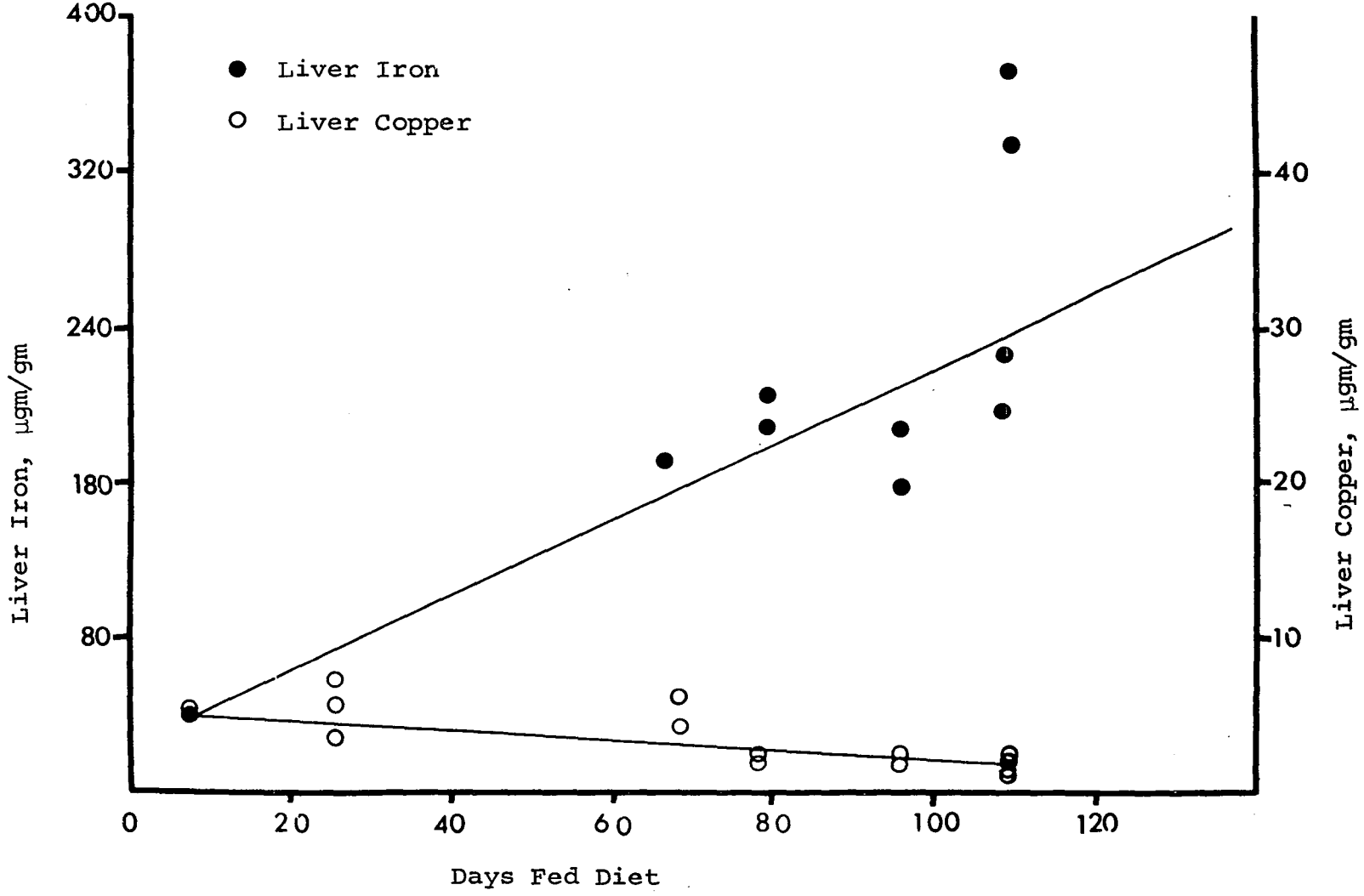


Figure No. 32 Iron-Copper Relation in Copper Deficient Rats
Experiment No. 4



copper deficiency the iron content of the liver increases with time. In the combined deficiency experiment, the levels of both metals are low, although their antagonism may prevent them from becoming even lower.

G. Heart Rate Experiments

Table XXXVII contains a good representation of the results obtained when copper deficient rats (diet No. 2) and their respective controls were examined for heart rate.

TABLE XXXVII

Heart Rates

| Rat | Weight | State | Beats per Minute* |
|---|---|---|---|
| 12 | 285 gm. | Control | 450 ± 1.0 |
| 19 | 233 gm. | Control | 523 ± 2 |
| 31 | 238 gm. | Control | 514 ± 3 |
| | | Average Control | 496 ± 22 |
| 6 | 234 gm. | Deficient | 482 ± 0 |
| 17 | 270 gm. | Deficient | 540 ± 3 |
| 31 | 238 gm. | Deficient | 530 ± 8 |
| | | Average Deficient | 517 ± 28 |

* AVERAGE of at least 4 determinations

As is evident from the above observations, there were no differences noted between the control animals and those fed the copper deficient diet.

# IV. DISCUSSION

## A. Growth Curves

In the four experiments conducted there was considerable variation between control values. This indicates that there is a difference in the nutritive value of the basal diet. This difference could not have been due to vitamin deficiency as all groups received exactly the same supplement of vitamins. As noted in the Results, the 50% powdered milk plus 50% cornstarch diet gave the best growth results in control animals. The assumed reason why this diet was so much better than the diet consisting of 100% powdered milk is that the latter has too high a protein content. It has been demonstrated than an excess of certain amino acids (246) will cause severe vitamin deficiency. This deficiency is associated with niacin, pyridoxine and vitamin $B_{12}$. The growth retardation in these cases is evidently due to an increase in the catabolism of these vitamins.

The differences in growth rate between the deficient animals and their respective controls may be considered to be a reflection of the dietary deficiency. The growth curves of the second and fourth experiments will be

discussed as in these experiments the basal diet is satisfactory, as is demonstrated in the controls.

(i) Copper Deficient Animals

These animals showed very slight difference in growth rate in one experiment (Figure·4) and none in the other (Figure 5). This is surprising as Gallagher et al. (200) reported that the difference in weight gain between control and deficient rats was noticeable after 3 weeks, and after this the deficient rats exhibited only half the weight gain of the control rats. They did not have any deaths until after the third month on the deficiency diet. The animals these authors used were hooded rats. In Experiment 2a, hooded rats were fed the deficiency and control diets and the deficient animals actually showed a slightly higher growth rate than did the corresponding controls. The copper values of Gallagher et al. were lower than those obtained in Experiments 1-4, although they continued the deficiency experiment over a much longer period of time. The values they obtained range from 0.5-1.0 µg. copper per gram tissue whereas the values in Experiment 4 range from 1.35-2.19 µg/gm. It would appear from this that the effects of copper deficiency is apparent when the animals are fed

an all liquid diet and not when they are fed a solid basal diet. However, the copper values lie within the same range with both diets (Experiment 2).

(ii) Combined Iron and Copper Deficiency

Table XII and Figure No. 5 show that the doubly deficient animals gained significantly less weight than did either the control or copper deficient animals. These animals were noticeably anemic, as verified by the Haemoglobin values (247) and had severe alopecia. This fur loss was most noticeable about the head and shoulders and on the back. These symptoms are in agreement with those reported by Morgan (207) who found that anemia developed more quickly and more severely in a combined deficiency state than in an iron deficiency state. From this information a rather high mortality rate in doubly deficient rats is expected.

(iii) Iron Deficient Rats

This was the most interesting group. These rats had a much lower rate of growth than any other group in this experiment. They exhibited the symptoms described

above even more severely and they had the highest mortality rate. The most interesting point is that after about 30 days on the diet, (Table XII and Figure 5) the growth rate of these animals dropped below that of the doubly deficient. This agrees with the findings of Morgan in that the doubly deficient animals developed the anemic symptoms faster (Table XXXVI). However, when the animals are maintained on their respective diets over a longer period of time the iron deficient group actually exhibits a lower growth than the doubly deficient group. The time at which these rates cross each other occurs at the same time (Table XXXVI, Figures 30-32), as does the increase in liver copper in iron deficiency. From these results it is suggested that the chronic copper poisoning resulting from the iron deficiency (although these rats received exactly the same amount of copper as did the controls), in conjunction with the iron deficiency, is responsible for the differences in growth rates between the doubly and iron deficient rats.

(iv) Diethyldithiocarbamate Supplemented Copper Deficient Rats

As exhibited in Table XI and Figure 4, these rats had the poorest growth rate of any group fed any diet. It had been demonstrated by other authors that diethyldithio-

carbamate had detrimental effects on the health of the animal. The most important of these appears to be nervous system changes. Edington and Howell (241) demonstrated lesions in the nervous system of rats injected daily with diethyldithiocarbamate.

The injection of Antabuse also has many marked physiological results. Some of these should also be evident in diethyldithiocarbamate-treated animals, as it is the breakdown product of Antabuse in vivo (181). Among the effects of Antabuse is peripheral neuropathy (237), which is reminescent in many respects to the nervous degeneration caused by diethyldithiocarbamate. It has been reported that Antabuse inhibits many mitochondrial enzymes (238), but it has not been demonstrated whether this is due to the parent compound or to the diethyldithiocarbamate. It seems likely that at least some of the effects observed in the Antabuse treatment should be observed in diethyldithiocarbamate treatment. This would account for the poor growth of the animals.

B. Time Curve for the Conversion of Dopamine-$C^{14}$ to Norepinephrine-$C^{14}$.

The percentage conversion has been demonstrated to increase with time after injection (see Results). The manner of increase will vary from species to species and from organ to organ (30). It was surprising not to find the rate of increase of conversion to lessen with time, (i.e. for the increase to approach a limiting value, but is possible that the studies were not carried out for a long enough period of time after injection. Bjorling and Waldeck (31) found that in the mouse heart in vivo, the norepinephrine formed from injected dopamine reaches a plateau $^3/_4$ of an hour to 3 hours after injection. No such plateau was demonstrated within 90 minutes in the present studies.

The decrease of Dopamine-β-hydroxylase activity with age is of interest (Table XXI and Figure No. 13 and 14). A similar occurrence has been reported by Burkard et al. (249) using tyramine as substrate. These authors reported that the activity of Dopamine-β-hydroxylase in 2 year-old rats had dropped to 47% of the activity in 1 month old rats. Table XXI indicates a drop in activity of 37% in 40 days (200 gram rats have 63% of the Dopamine-β-hydroxylase

activity of 50 gram rats). It is of interest to note, in connection with this, that rats have no norepinephrine in their brains 2 days before birth and it takes 6 weeks to reach adult levels (250)..

C. Copper Excess Experiments

Excessive amounts of copper have been shown to be harmful and a large injected dose (80-160 mg) will cause death within a few hours (251). The biochemical effects of copper poisoning have not been well studied. In sheep fed a high copper diet for 3 months, and then given a large (80 mg) dose of copper, as the acetate, showed elevated levels of serum lactic dehydrogenase and glutamic-oxaloacetic transaminase. Only a slight increase was noted in normal rats injected with the same dose of copper (251). Chronic copper poisoning might possibly affect Dopamine-β-hydroxylase activity for the following reasons,

(1) the enzyme is copper-dependent (21,157). For this reason a slightly larger amount of copper than normal could possibly stimulate the enzyme.

(2) excess copper inhibits the enzyme in vitro (157).

The lack of effect of excess copper on heart Dopamine-β-hydroxylase may only indicate that the heart was not sufficiently loaded with copper for inhibition of the enzyme. Two animals showed apparent stimulation of the enzyme, but the copper levels in the liver and heart (Table XXV) did not differ from any other copper excess animals.

D. Antabuse Experiment

The results of the injection of Antabuse on the inhibition of Dopamine-β-hydroxylase (Table XXVI) are in complete agreement with those of other authors (143,157, 180,181). Antabuse has been shown to inhibit the enzyme both in vivo and in vitro, apparently by chelation of the copper of the enzyme (143). Diethyldithiocarbamate also inhibits Dopamine-β-hydroxylase (114,182,183) and it is probably through this metabolite that Antabuse acts.

The dose of Antabuse used in these experiments (200 mg/kg) is only half of the dose used by other authors (157,181).

E. Copper Deficiency and the Conversion of $C^{14}$ Dopamine to $C^{14}$ Norepinephrine:

The conversion of dopamine-$C^{14}$ to norepinephrine-$C^{14}$ in the heart has been demonstrated both in vitro (20,29) and in vivo (31). It has also been shown that in the normal rat 80% of the radioactive norepinephrine found in the heart after injection has been synthesized there (87), the remainder coming from the circulation. From this it can be assumed that the intramuscularly injected dopamine was absorbed into the bloodstream and then hydroxylated by the heart.

As described in the introduction, dopamine-β-hydroxylase is a copper-dependent enzyme which is inhibited by copper chelators such as diethyldithiocarbamate and 2,9-dimethyl-o-phenanthroline (164). As is shown in the results Dopamine-β-hydroxylase activity is completely inhibited in vivo by Antabuse.

With these facts in mind it is not surprising to find that a prolonged dietary deficiency of copper, leading to low tissue levels of the metal, results in a reduction of the enzymic activity of Dopamine-β-hydroxylase.

A verification of these findings is presented by the repletion experiment. Table XXXI indicates that addition of copper only to the diet of copper deficient animals returns the level of Dopamine-β-hydroxylase activity to that of the controls. This shows that the decrease in enzymic activity is

due to lack of copper only, as if it was due to the lack of any other dietary constituent, or to a combination of copper deficiency and another dietary constituent, the levels of activity would continue to decline instead of returning to normal. i.e. there is a nutritional requirement of copper for the biosynthesis of norepinephrine and consequently epinephrine.

It is generally considered that catecholamine biosynthesis in the heart is controlled by tyrosine hydroxylase (26,30,32,54-56). As can be seen from Tables XXVII-XXXI the control in catecholamine biosynthesis in copper-deficient rats is shifted to Dopamine-β-hydroxylase. This would mean that by dietary means alone a new controlling influence can be placed on a sequence of biochemical reactions by a means other than removing the substrate for the reactions, or inhibiting the normally rate-limiting step.

The importance of this discovery cannot be underestimated. By proper regulation of diet, perhaps combined with the use of drugs, a new means of control of tissue levels of noradrenaline and adrenaline may have been found. Applications are to be found in chronic hypertension and in connection with Parkinson's Disease (see introduction).

F. Copper-Iron Interrelationships:

As noted in the introduction the effect of copper on the metabolism of iron has been studied and it is well known that a high level of iron is associated with a dietary deficiency in copper (220,227). The most likely reason for this increase in iron content of the liver is that copper is needed for the mobilization of storage iron (207,224,228) and if copper is lacking, this mobilization may be inhibited. It has been found that supplemental copper is associated with a reduction in the iron content of the liver, in previously copper-deficient animals (229).

The results in Table XXXVI and in Figures 30 and 31 are in complete agreement with the results of other authors in that low liver copper levels lead to high liver iron levels.

The effect of iron on the metabolism of copper is analagous to that of copper on iron. Table XXXVI and Figure 32 demonstrate that a dietary deficiency in iron leads to an accumulation of liver copper, with the levels increasing quite rapidly. The study of iron deficiency on copper metabolism has not been reported prior to this. It appears from the data available that the copper accumulates owing to a lack of adequate mobilization of liver copper. The cause of this may be deduced from the following reasoning:

The copper accumulation follows a time course as well as following the level of iron in the liver. i.e. in an iron deficient animal which remains at a given low level of iron the copper levels will continue to increase owing to the continued inability to mobilize the liver copper. An animal that is deficient in iron to a greater extent would accumulate copper more rapidly than an animal less deficient.

The above could well be the explanation why the iron levels in the doubly deficient animals tend to increase as the deficiency experiment progresses (Table XXXVI). The copper levels are decreasing to quite low levels and as a result the iron level in the liver begins to build up again, due to the inability for it to be mobilized, in spite of the very low level of iron in the diet. This line of thought could be extended to suggest that as the experiment is prolonged, the doubly deficient animal may actually appear to regain its health due to the rebuilding of the iron reserves as a result of the copper deficiency. This means there would be a time at which the doubly deficient group has both low copper and low iron, after which the iron begins to accumulate. From the data in Table XXXVI it appears that this point occurs after 10-11 weeks of being fed the doubly deficient diet.

The increase in liver iron in the control rats at the end of the experiment is also explained by this hypothesis. As is seen in Table XXXVI these animals have low liver copper values and would have had enough time to accumulate the amount of iron found in the liver. These values are still much below the values for the copper deficient animals (which have much lower liver copper levels) and well above those of the doubly deficient animals.

The finding of increased liver copper in iron deficiency could have far-reaching medical implications, especially in the field of iron deficiency anemia. It is possible that some of the symptoms are due to chronic copper overloading. In the case of low tissue levels of iron, but sufficient levels of iron in the diet (e.g. in malabsorption of iron) restriction of the dietary copper might be of some therapeutic value as (1) The chronic copper overloading would be halted and any abnormalities due to this would be reversed. (2) When the copper reached low enough levels the iron would begin to accumulate.

There is an alternative proposition to the inhibition of mobilization hypothesis. If both metals have the same, or a common carrier the above effects would still be observed. There are two possibilities: (i) the carrier is the same for both iron and copper. i.e. iron and copper are continually competing for the sites on the transport carrier, and when one is low the other would

therefore be absorbed, transported and stored to a greater extent than normal. If this is the case, then dietary loading of either metal should cause the liver level of the other to drop. (ii) there is a specific carrier for each metal, but there also exists a carrier which is common to both. This would be analogous to the transfer-RNA situation where there exist carriers which transfer more than one amino acid. Again in this situation depletion of one metal would lead to a build-up of the other as the common carrier would be available almost exclusively for the transport of one of the metals.

An interesting implication of this finding might be in Wilson's disease, or hepatolenticular degeneration. This is a congenital metabolic disease which is characterized by an accumulation of toxic amounts of copper in the body. There is an increased rate of absorption of copper from the intestine and consequently copper is deposited in tissues, particularly the liver, brain and cornea. The treatment of the disease is usually centred around reducing the amount of copper absorbed and removing the excess copper.

If either of the above hypotheses is correct, the iron levels in Wilson's disease should be abnormal. Also, iron added to the diet of Wilson's disease patients should lower their copper levels by loading the transport

mechanism with iron.

G. Heart Rate Measurements in Copper Deficient Animals:

It was thought that the heart of a deficient animal might respond differently from that of a normal animal, especially when the animal was stressed. The reasoning behind this is that the copper deficient animal had a chronic low level of Dopamine-β-hydroxylase activity and therefore the norepinephrine levels might be low. If the norepinephrine stores were depleted then the response to a shock might not be as great, or as rapid, as that in the normal rat.

The lack of difference in values may indicate that the norepinephrine levels are not depleted enough to effect the response of the rate to the stimulus, in spite of the chronic copper deficiency.

# V. SUMMARY

Male albino rats were fed a variety of diets deficient in copper, iron, or both. The diet consisting of 50% powdered milk and 50% cornstarch with added vitamins and minerals was found to be the most satisfactory in maintaining a proper growth rate. A diet of 100% powdered milk or liquid milk was not nearly as nutritious. When diethyldithiocarbamate was added to the basal diet a deleterious effect was soon noticed. Of all the deficiency diets tested, the iron deficiency diet incurred the highest mortality with the combined iron and copper deficiency diet also being less beneficial. The copper deficiency diet gave no noticeable differences from the control animals in terms of weight gain and mortality rate.

The effect of age on the activity of Dopamine-$\beta$-hydroxylase was investigated and it was found that for both albino and hooded rats the enzymic activity decreased with age. In a 150 gram male rat, one hour after injection of dopamine-$C^{14}$, 50% of the radioactive norepinephrine plus dopamine in the heart and spleen was found to be norepinephrine. If the animals were pretreated with disulfiram the activity of the Dopamine-$\beta$-hydroxylase was found to be completely inhibited.

The effect of copper deficiency, induced by a

copper low diet over an extended period of time, on the activity of Dopamine-β-hydroxylase was studied. The enzymic activity in the heart was found to be a direct function of the copper content in both the liver and heart. The decrease in enzymic activity was detectable after 6 weeks and was very pronounced at 10 weeks after which it became increasingly severe. A study of the change in heart rate in response to a noise showed no detectable differences between the copper deficient animals and the control animals.

A copper loading experiment was performed in which high tissue levels of copper were attained. The activity of Dopamine-β-hydroxylase was found to be unaffected.

The interrelationships of copper and iron were studied. In copper deficiency the iron levels of the liver were found to be greatly increased while the reverse was found in iron deficiency. i.e. the copper levels were much above normal. In the case of the doubly deficient animals the concentration in the liver of both metals initially decreased. However, after a period of time the iron levels began to return to normal while the copper continued to fall. The cause of these phenomena is believed to be the interdependence of the mobilization of these two metals. i.e. for either one to be utilized in a normal manner, the other

must be present in substantial amounts. It is also proposed that the transport of these two metals is interconnected and this aids in the building up of an excess of one metal when the other is low.

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