THE ROLE OF LABILE METHYL GROUPS OF CHOLINE IN DETOXICATION OF PYRIDINE

by

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SECTION I

INTRODUCTION

The purpose of this study is to investigate the role of choline in detoxication of pyridine in the albino rat. The transfer of a methyl group from some already methylated compound such as choline has been demonstrated by Riesser (45). This fact was used to explain the synthesis of creatine in animals and also the formation of alkylated (presumably methylated) derivatives of selenium and tellurium on administration of compounds of these elements to men and animals. In support of his views Riesser (45) stated that on heating betaine hydrochloride or choline hydrochloride and sodium formate with sodium selenite or tellurite, odors resembling those of dimethyl selenite and telluride were produced. No chemical identification of these compounds was carried out. The origin of Riesser's suggestion regarding the transfer of a methyl group is perhaps to be found in an article by Hofmeister (25) who referred to the formation of methylpyridinium hydroxide in the animal body. Hofmeister did not mention betaine or choline as sources of the methyl group, but only suggested that choline was a normal product of metabolism.

As early as 1887, His (22) indicated that pyridine could be detoxified by methylation in the body. In 1912 Ackermann (1) found that dogs given fairly large amounts of nicotinic acid, which is a derivative of pyridine, excreted in the urine equal amounts of trigonelline and nicotinuric acid. Further work by Najjar et al (36, 37, 38) and by Huff and Perlzweig (26, 27) indicated that in man and in rats doses of either nicotinic acid or its amide result in the excretion of N^1-methylnicotinamide as the chief end product rather than trigonelline.

Recently Heppel et al (19), showed that weanling rats maintained on low choline diets were more susceptible to ethylene dichloride poisoning than were control animals receiving choline. This was true for both chronic and acute exposure. When the low choline diets were supplemented with methionine and choline the mortality after exposure was greatly reduced. Similar results were observed by the same investigators using propylene dichloride as the toxic agent. Martin and Thompson (33), discussing detoxicants, mentioned choline and its soluble salts as good detoxicants, although no experimental evidence was presented. Keston and Wortis (30) indicated that triethylcholine, which is acutely toxic when injected into mice, is completely antagonized by simultaneous injection of an equal weight of choline chloride. Torda and Wolff (54) stated that pyridine decreases the synthesis

of acethylcholine by minced frog brain. Evidence of the fate of pyridine in the animal body is, as far as we are aware, entirely lacking.

In order to satisfy the discussion of findings in this thesis, it is necessary to review briefly the history, chemistry, and metabolism of choline.

CHEMISTRY OF CHOLINE

Historical:

Choline was isolated for the first time as a new organic base in 1852 by boiling the alkaloid derived from white mustard "Sinapis alba" seed with alkali. Babo and Hirschbrunn (3) called the new base sinkalin.

Strecker (50) in 1862 isolated a base from bile and because of this source, he named the compound choline, as the Greek word for bile is "chole." In 1867 Chaus and Keese (8) showed that sinkalin is identical with choline, the base isolated from ox bile by Strecker. Thus Babo and Hirschbrunn were really the first to isolate pure choline.

In 1865, Liebreich (32) isolated a new base from hydrolyzed brain "protagon" and named it "neurine." Baeyer (5) in 1866 suggested the identity of this compound with choline then Dybkowsky in 1867 (13) established this fact. In 1867 Wurtz (56) synthesized choline chloride, for the first time, by warming trimethylamine with ethylene chlorhydrin in a sealed tube for 24 hours on a water bath. The properties

of the chloride agree with those of a sample of a natural material obtained from Liebreich. In 1868 Wurtz (56) obtained free choline directly by treating a concentrated solution of trimethylamine with ethylene oxide at room temperature.

Properties:

The molecular weight of choline is 121.13. It is a colorless, odorless compound with a caustic bitter taste. It decomposes readily at elevated temperatures to produce trimethylamine and glycol; small amounts of β-dimethyl-amino, ethanol and dimethyl-vinylamine are also formed. Wurtz (56) found that dilute solutions were stable to heat, but that concentrated solutions gave off trimethyl-amine when boiled, leaving an oily liquid, boiling about 190° which he believed to be glycol. Solutions acidified with hydrochloric acid are more stable to heat than the free base. Pure dry choline decomposes at 40° under reduced pressure (300 mm. Hg), while choline chloride does not decompose appreciably even at 180°.

Gulewitsch (17) studied the effect of heating choline in alkaline solutions and found a negligible breakdown after boiling choline either with barium hydroxide solution for 6 hours or in 5% sodium alcoholate for 24 hours. Mathews (34) states that choline is stable in acid solution

while it breaks down in alkaline medium into trimethylamine and enthanediol.

Most choline salts are very soluble in water. Exceptions are the periodide, phosphotungstate, phosphomolydate, reineckate and the double salts of auric chloride, or mercuric chloride or with Mayer's reagent (potassium mercuric iodide) and Dragendorff's or Kraut's reagent (potassium bismuth iodide). Nitrate, sulfate, carbonate, acetate, oxalate, picrate, and picrolonate are readily soluble in both water and alcohol. The monphosphate, chloroplatinate, acid tartarate, rufianate are soluble in water, but insoluble in alcohol. This is also true of the double salts of cadmium chloride and with zinc chloride.

CHOLINE AS A DIETARY FACTOR

The role of choline in metabolism was a direct outgrowth of the discovery of insulin. Allan, Bowie, Macleod, and Robinson in Toronto (2) and Fischer in Chicago (15), observed large yellow livers in the insulin treated animals (dogs) which could not survive beyond a few months. These abnormal conditions were prevented by the addition of raw pancreas to the diet; so it became apparent that some constituents of this tissue were responsible for this curative effect. Hershey (20) alone, and then with Soskin (21), discovered that lecithin could replace the raw pancreas in the diet of depancreatized dogs. The logical

inference was, therefore, that the phospholipid fraction or some unidentified substance associated with this material in the pancreas was responsible in part at least for the effect on liver fat. Huntsman, Hershey, and Best (28) found that purified lecithin was effective in the prevention of fatty livers in rats and sometime later it was established that the efficiency of raw pancreas in Allan's experiments and of lecithin in Hershey's was primarily due to the presence of choline. Then Best, Ferguson, and Hershey (6) showed that choline, when given in sufficient amounts to diabetic dogs, prevented the development of fatty livers.

Nevertheless, Dragstedt and his colleagues (12) are of the opinion that other factors contribute to the effect of the pancreas. They claim that in spite of the fact that brain and liver contain more choline than does pancreas, feeding either brain or liver does not prevent the formation of fatty livers; also they believe that fat-free alcoholic extract of the pancreas which contains neither lecithin nor choline may be prepared which is entirely effective in controlling fatty infiltration. They report that the principle is a hormone and have named it lipocaiac. The work of Dragstedt and his colleagues, and of Chaikoff and his group, shows that there is a substance in the pancreas which affects fat metabolism. Whether

this substance is identical with choline has not been established (18).

Norris and Heuser (41) indicated that there is an interaction between manganese and choline in liptropic action, and both materials decrease the fat content of fresh bone.

Choline is essential for maintaining normal kidney function as well as preventing kidney hemorrhage (18).

Patterson and McHenry (42) presented that the primary cause of kidney degeneration in rapidly growing rats on choline precursor free diets is a result of an interference in phospholipide formation.

Choline is a constituent of lecithin and it was proved by Fishman and Artom (16) that the level of lecithin in the liver is dependent on the dietary supply of both choline and fat.

Jukes (29) showed that when the supply of manganese was adequate, perosis in chicks was not prevented unless choline was present in the diet. Sure (51) reported that choline has an influence on the lactation and normal growth of rats.

OTHER FUNCTIONS OF CHOLINE

Choline acts in the body as a detoxicant, as a methyl donor, accelerates fat absorption, decreases the prothrombin time, cures cirrhosis, helps with thiamine, riboflavin,

pyridoxine, pantothenic acid and nicotinic acid in fat synthesis from proteins. It is of major importance as a precursor of acetyl choline.

INTERMEDIARY METABOLISM OF CHOLINE

One of the most significant functions of choline is that it contains labile methyl groups which are used in detoxication reactions and synthesis of creatine and methionine in the body. It has been shown by Bach (4) that the capacity to transfer methyl groups is confined to a few nitrogen and sulfur methylated compounds; methyl groups linked to carbon are not transferable, but in some cases can be oxidized. Other N-methyl compounds cannot take part in methyl exchange, but are oxidatively demethylated or excreted unchanged. Choline, methionine, and betaine play a major part in transmethylation and by interchange of methyl groups from a "pool" of labile methyl groups which supplies $-CH_3$ for compounds such as creatine, methylnicotinamide and anserine. None of these compounds are methyl donors. The liver is the main site of methylation; muscle and kidney catalyze methylation in few cases.

The brilliant work of du Vigneaud and his colleagues have led to the conclusion that the labile methyl groups of choline are used in synthesizing creatine and methionine in the body. By using tracer elements they proved that creatine is formed in the body from glycocyamine with

methyl groups coming from choline, methionine, or betaine. They demonstrated that the administration of choline enables the rat to utilize homocystine for growth purposes in lieu of methionine. They therefore suggested that choline acts *in vivo* by methylating homocystine thus forming methionine, and that the presence of methyl groups in a utilizable form, such as in choline methionine or betaine may be essential in the diet.

Simmonds and du Vigneaud (46), using the isotope technique found that the methyl group of dietary methionine can be used by man in the synthesis of choline. It is supposed that a precursor of choline, ethanolamine, must be present in the body.

In the hands of Stetten (47, 48), guanidoacetic acid has proved to be an antilipotropic agent with properties which make it a most useful tool in the study of intermediary metabolism of choline. By using it to deplete the labile methyl supply in the body of the rat, fatty livers were produced.

However, it was found that in the presence of even larger quantities of dietary guanidoacetic acid, the rate of choline synthesis in the body, determined by methylation of isotopic ethanolamine, was not in the least impaired, probably due to available methionine. As the labile methyl supply is drained away forming creatine, with a firmly bound methyl group, which is eventually

excreted as creatine, a condition is soon reached in which the newly formed choline has to give up its methyl groups to maintain the essential metabolic reaction. Thus in turn the choline reserves are depleted.

CHOLINE AS A TOXIC AGENT

As we are going to discuss the detoxifying action of choline as the main part of this thesis, it is desirable to know something about its toxicity. Although choline is an essential metabolite, it can, like any other one, disturb the steady state of physiological processes if present in sufficiently high concentration if body tissues and fluids.

Pritzker and Jungking (44) believe that choline, rather than soluble oxalates, is the cause of the poisonous properties of beechnut residue (oil). Norris and Church (40) suggested that choline as well as isoamylamine are responsible for the toxicity of some cod-liver oils, and that they produce symptoms similar to vitamin B deficiency, which may be prevented by an increased allowance of yeast. Hodge (23) found that the amount of choline chloride required to kill the albino rat is directly proportional to the concentration when injected intrperitoneally. Expressed as milligrams per 100 grams of body weight, the LD50 (lethal dose for 50 per cent of the animals) values are 29-34 for a solution containing 200

milligrams per milliliter, 37-38 for 100 milligrams per milliliter, 41-49 for 40 milligrams per milliliter, 59-75 for 20 milligrams per milliliter. Death was preceded by respiratory paralysis, trembling, convulsive movements, alivation and hemorrhage around the eyes. Rats which survived 20 minutes after injection of any dose invariably lived and showed no symptoms of damage on the days following. Hodge (24) in another paper showed that diets or drinking water containing 1 per cent or more of choline chloride retarded growth, and 10 per cent in the diet or four per cent in the water completely checked growth. Still larger amounts caused loss in weight and sometimes death after several weeks. No particular histopathological effects were observed which could be considered characteristic of choline poisoning. Newman and Hodge (39) stated that the LD50 ranges from 6.1 grams per kilogram to 3.4 grams per kilogram depending on the concentration. Melass, Pearson, and Sherwood (35) observed that addition of one per cent, two per cent, and 24 per cent respectively but no other toxic effects were seen. With regard to the LD50 for rats, and if the same degree of toxicity is assumed for man, minimum effects would be expected somewhere between 15-70 grams of choline per day, while the LD50 dose would be of the order 200-400 grams.

REQUIREMENTS

Copeland (9) in a study of the effect of strain differences in the choline requirements of rats, stated that the minimum requirement of a selected Wisconsin strain of hooded rats was approximately 4-5 milligrams per rat per day, and that of a Wistar albino strain on the same diet was about 10 milligrams per rat per day. Tests on the progeny of each strain showed that this difference continued through the third generation. This amount is in the range of 0.1-0.3 per cent of its diet or 10-100 milligrams per kilogram per day of choline. In the chick and young turkey 0.15-0.3 per cent of choline prevents perosis and gives normal growth. If it is assumed that the requirements for choline in man are of the same order of magnitude, then 1.5-3.0 grams of choline are required daily (or 0.1-0.2 per cent of the diet) for an adult.

SECTION II

EXPERIMENTAL

The animals used in this study were young male and female rats of the Evans-Long strain. The experimental work in this thesis was run in two steps. First, a preliminary study was carried out using the stock diet, and in the second part, a choline deficient diet was used.

Preliminary Study:

This study consisted of three parts as follows:

ESTABLISHMENT OF STANDARD GROWTH CURVE FOR THE STOCK DIET.

Nine animals, 28 days old and weighing approximately 32-50 grams, were fed the stock diet and water ad lib. They were weighed daily. Table 1 shows the gain in grams per week, and Figure 1 is established by plotting the average weight in grams against time in weeks.

DETERMINATION OF THE LETHAL DOSE OF PYRIDINE.

Pyridine is a strong local irritant with low toxicity. A 10 per cent solution of pyridine has been used by atomizer in asthma and fetid bronchitis. It produces first dyspnea, then shallow respiration. Brunton and Tunnicliffe (7) refer the effects mainly to sensory paralysis. Large doses arrest the heart. Smaller amounts stimulate the bone marrow to increased production of blood-platelets. Pollock et al (43) working on the toxicity of pyridine

noted no toxic symptoms after administration of 0.31-1.54 millliliters of pyridine per day to the human. In larger doses, 1.85-2.46 millileters of the pyridine, was toxic causing death. They noted symptoms of hepatorenal disease.

Very little information was found in the literature about the lethal dose of pyridine for rats. Fifteen animals, 46-60 days old and weighing from 45-80 grams, were fed on the stock diet. They were divided into three groups; homogeneity in sex and weight was considered. Group A was given 100 milligrams of pyridine per kilogram of body weight by stomach tube, group B 150 milligrams, while group C received 200 milligrams.

None of the animals of group A and B died, but all of group C died within 24 hours after administration of pyridine. It became apparent that 200 milligrams of pyridine per kilogram of body weight under the conditions of this experiment, is a lethal dose for the average rat (Evans-Long strain).

STUDY OF THE ACTION OF CHOLINE IN PYRIDINE DETOXICATION.

Fifteen animals, 50-70 days old which ranged between 54-90 grams in weight, were used. Each animal was marked and kept in a separate small cage. Water and the diet were furnished ad lib. The animals and the food were weighed every 24 hours, and the daily food intake was

calculated. These animals were divided into three groups; control, pyridine, and pyridine plus choline. The five control animals were given 200 milligrams of pyridine per kilogram of body weight by stomach tube. The homogeneity in sex and weight was also considered. The weekly food intake, and the gain in weight per week are shown in Table V. Figure II shows the rate of growth for one group.

The author devised a method for feeding rats, using the left hand for seizing the animal and opening the mouth by the forceps, then pushing the stomach tube gently into the rat's mouth with the right hand.

The composition of the stock diet used in the departmental colony is given in Table I.

Table I

Composition of the Stock Diet

Whole yellow corn meal	38%
Whole wheat flour	32%
Ground alfalfa leaves	6%
Powdered skimmed milk	20%
Irradiated Brewer's yeast	1%
Sodium chloride	0.5%
Calcium carbonate	0.5%
Cod liver oil	2.0%

EXPERIMENTAL DIET STUDY

Twenty-five animals 36-64 days old and weighing between 28-58 grams, were used. They were fed a choline deficient, low methionine diet for about 30 days to establish the growth curve of that diet. After that, they

were divided into five groups as follows:

Group A Received 200 milligrams of water per kilogram of body weight by stomach tube.

Group B Received 150-200 milligrams of pyridine per kilogram of body weight by stomach tube.

Group C Received 150-200 milligrams of pyridine per kilogram plus 100 milligrams of choline.

Group D Received 150-200 milligrams of pyridine plus 200 milligrams of choline per kilogram by stomach tube.

The daily food intake was calculated and the animals were weighed every 24 hours; water being given ad lib.

The amount of pyridine given to the rats was 150 milligrams per kilogram in the first two weeks. This amount is less than the regular lethal dose owing to the fact that this diet is deficient in choline and low in methionine. It was the lethal dose for two rats. After two weeks it was raised to 200 milligrams per kilogram.

The weekly food intake and the gain in weight per week are shown in Table VI and Table VII. The rate of growth for the average of the five groups, is shown in Figure III.

Table II

Composition of the Experimental Diet

Compound	gms/kg food
Cerelose	730
Casein	180
Corn oil	50
Osborne and Mendel salt mixture	40

Vitamins per kilogram ration

Thiamin	20 milligrams
Riboflavin	20 milligrams
Pyridoxin	40 milligrams
Pantothenic acid	200 milligrams
Carotene	10 micrograms
Calciferol	5 micrograms

Both the stock and experimental diet were assayed for total sulfur, methionine, total nitrogen and choline. The methods used for analysis are indicated.

Table III shows the sulfur, methionine, cystine, total nitrogen and choline contents of both diets. The cystine content was calculated by subtracting the methionine content from the total sulfur.

Table III

Total sulfur, methionine, cystine, total nitrogen and choline of both stock and experimental diets

Diet	Total Sulfur	Methionine	Cystine	Total Nitrogen	Choline
	%	%	%	%	%
Stock diet					
Analysis 1	0.13	0.066	0.064	3.17	0.035
Analysis 2	0.14	0.070	0.070	3.15	0.040
Average	0.135	0.068	0.067	3.16	0.037
Experimental diet					
Analysis 1	0.144	0.024	0.120	2.81	0.0027
Analysis 2	0.150	0.028	0.122	2.81	0.0033
Average	0.147	0.026	0.121	2.81	0.0030

METHODS OF ASSAYING

DETERMINATIONS OF TOTAL SULFUR:

This method is a modification of the A.O.A.C. Method. The sample was weighed, adding 10 milliliters of magnesium nitrate to it in a pyrex beaker, mixed thoroughly and heated on a hot plate for 4-5 hours and then in an electric furnace at 600°C for 10 hours. After cooling slowly, water and hydrochloric acid were added to dissolve the precipitate, and the mixture was filtered. It was then heated to boiling, barium chloride was added (10 milliliters) and it was left 5 hours to precipitate. It was filtered by

suction (ashless filter paper, hardened by a piece of cloth), then the precipitate was put in a weighed crucible and ignited till constant weight. Sulfur was calculated as barium sulfate.

DETERMINATION OF METHIONINE:

Each diet was hydrolyzed in duplicate in 10 per cent hydrophloric acid in sealed ampules for 10 hours under steam pressure of 15 pounds. The hydrolyzate was filtered, the residue was washed, and the filtrate was neutralized to a known volume. The assay method and the basal medium were followed according to the directions of Stokes et al (49) using Streptococcus faecalis as the test organism for the microbiological assay of methionine.

DETERMINATION OF CHOLINE IN THE DIETS:

The directions of the method used by Engel (14) were followed. A sample from each diet was weighed and transferred to a fiber extraction thimble which was placed in the bottom of a Bailey-Walker extraction cylinder containing 30 milliliters of absolute methanol. After four hours extraction at the boiling temperature of the solvent, the thimble containing the sample was raised, permitted to drain, and the solvent transferred to a 125 milliliter filtering flask. The thimble was then returned to the extraction cylinder, 30 milliliters of fresh solvent

added, and the extraction continued for about 16 hours. The solvent was again removed and the sample extracted a third time for four hours with 30 milliliters of fresh solvent. This was run in duplicate.

The combined extracts were reduced to near dryness in a water bath (60-70°) under reduced pressure. The residue was treated with 30 milliliters of saturated aqueous barium hydroxide for two hours at 100°. The hydrolzate was cooled, neutralized to phenophthalein with acetic acid and then filtered by suction through an asbestos pad into another 125 milliliter filtering flask.

Ammonium reineckate was synthesized following the directions of Dakin (11). A two per cent solution of Reinecke salt in methanol was added in sufficient amount to precipitate the choline in the combined filtrate, and was then placed in a refrigerator for four hours to complete precipitation.

The precipitate of choline reineckate was filtered onto an asbestos pad, then dried by drawing air through the pad. The precipitate was dissolved by washing with acetone, filtered, and made to 25 milliliter volume. The concentration of choline reineckate was determined by means of a photoelectric colorimeter, using Filter 540.

A standard curve was made using the method of Thornton and Broome (53). As choline chloride is very hygroscopic, it was recrystallized four times from absolute methanol,

introduced into a weighed bulb, and dried at 84°C *in vacuo* for 48 hours, after which the bulb was sealed and weighed. The sample was then dissolved in distilled water and adjusted to volume. To establish the purity of choline chloride the solution was analyzed for total nitrogen by the Kjeldahl method. The Reinecke salt solution was added to the pure choline solution of different concentrations, the directions of precipitation and measurement were the same as mentioned above.

PREPARATION OF METHYLPYRIDINIUM CHLORIDE:

No information could be found in the literature regarding the preparation of methylpyridinium chloride. An indirect method of preparation was suggested. First methylpyridinium iodide was prepared by the method of Cumming et al (10) which consists of mixing equal volumes of methyl iodide and pyridine in a beaker. After the vigorous reaction had been stopped, absolute alcohol was added and warmed gently to dissolve. On cooling, the product crystallized out in flat needles, which were filtered off and washed with a few milliliters of alcohol. The theoretical melting point (117°C) was obtained.

The methylpyridinium iodide was converted to the chloride by digesting the former with silver chloride and the methylpyridinium chloride was isolated.

SECTION III

DATA AND RESULTS

Table IV

Typical weight gains of male and female rats from the Oregon State College colony when receiving stock diet *ad lib*. This is the diet given in Table I. These rats are the Evans-Long strain obtained from the University of Southern California. All animals were 28 days of age at the beginning of the experiment.

Weekly Gain in Weight of Nine Animals Receiving Stock Diet

Animal No. and sex	Initial weight	1st wk. gain	2nd wk. gain	3rd wk. gain	4th wk. gain
	grams	grams	grams	grams	grams
Animal 1 ♀	49	14	18	17	21
Animal 2 ♀	50	11	24	21	18
Animal 3 ♂	50	10	20	23	24
Animal 4 ♂	49	9	22	32	18
Animal 5 ♂	47	10	18	25	18
Animal 6 ♀	48	16	23	13	22
Animal 7 ♀	32	19	13	10	13
Animal 8 ♀	36	6	16	14	19
Animal 9 ♀	40	10	16	20	5

Animal No. and sex	5th wk. gain	6th wk. gain	7th wk. gain	8th wk. gain	Final Weight
	grams	grams	grams	grams	grams
Animal 1 ♀	4	24	12	11	170
Animal 2 ♀	-2	24	24	13	183
Animal 3 ♂	-12	35	20	37	207
Animal 4 ♂	-8	46	32	23	223
Animal 5 ♂	-2	32	16	24	188
Animal 6 ♀	2	30	10	15	179
Animal 7 ♀	15	19	3	8	132
Animal 8 ♀	19	30	14	12	166
Animal 9 ♀	19	24	8	2	144

FIGURE I

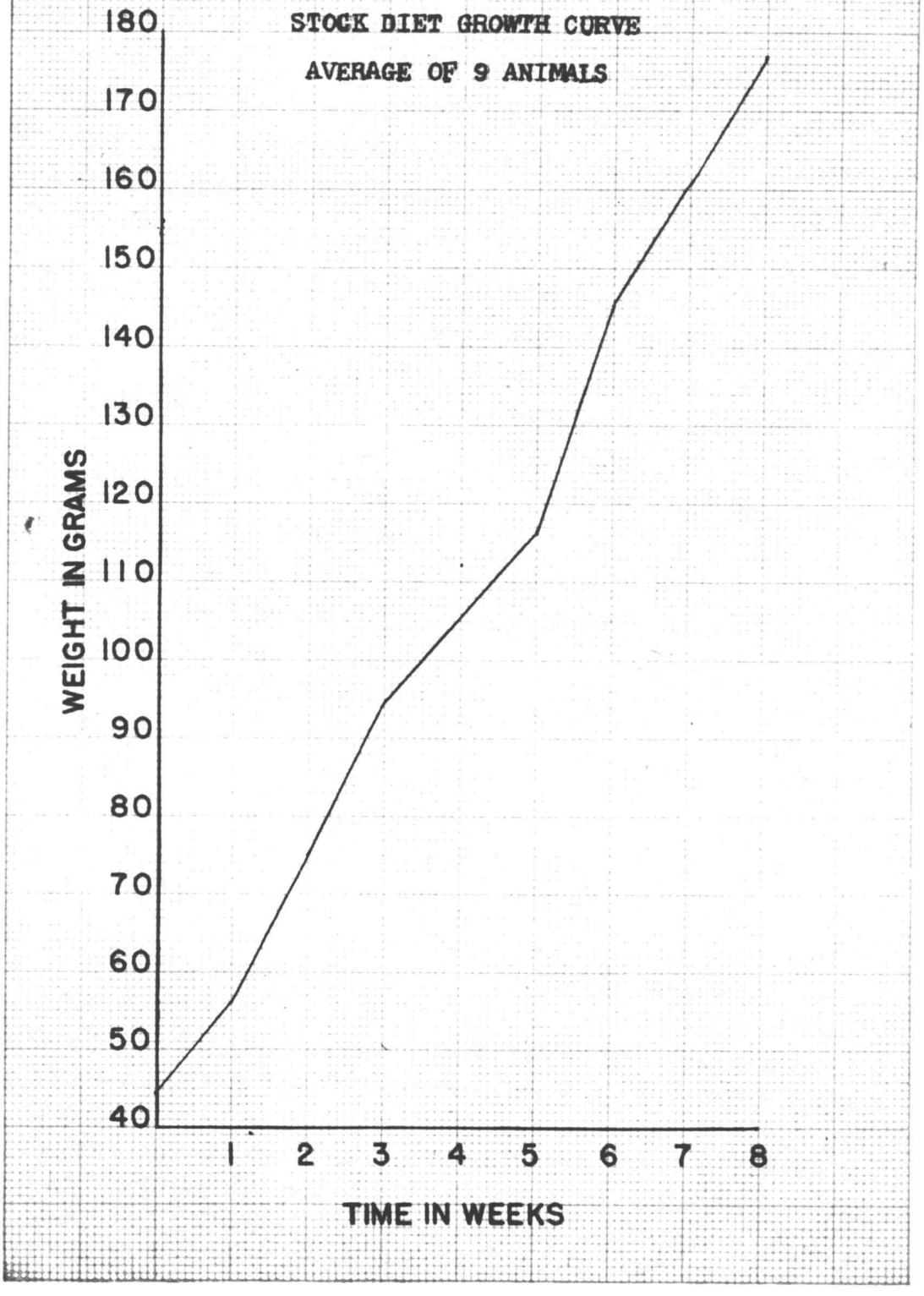

STOCK DIET GROWTH CURVE
AVERAGE OF 9 ANIMALS
WEIGHT IN GRAMS
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
1
2
3
4
5
6
7
8
TIME IN WEEKS

Table V

Typical weight gains of male and female rats (Evans-Long strain) when receiving stock diet, ad lib. This is the diet given in Table I. All animals were 50-70 days of age at the beginning of the experiment.

Animals 3, 6, 10, 16 and 18 received 200 milligrams of water per kilogram of body weight.

Animals 1, 4, 14, 13, and 8 received 200 milligrams of pyridine per kilogram of body weight.

Animals 2, 11, 7, 9 and 17 received 200 milligrams of pyridine plus 400 milligrams of choline per kilogram of body weight.

* Animal received water or pyridine or pyridine plus choline
\- (blank) Animal died.

Weekly Gain and Food Intake of 15 Animals Receiving Stock Diet Plus Water or Pyridine or Pyridine Plus Choline

Animal No. and sex	Initial weight	1st wk.		2nd wk.		3rd wk.	
		Food Intake	Gain	Food Intake	Gain	Food Intake	Gain
	grams	grams	grams	grams	grams	grams	grams
Animal 1 ♂	88	80	24	86	22	72	14*
Animal 2 ♂	101	92	30	102	28	16	-46*
Animal 3 ♂	90	90	32	95	30	101	32
Animal 4 ♂	74	94	26	93	26	65	10
Animal 6 ♂	101	87	20	86	17	89	17*
Animal 11 ♂	80	70	10	79	15	72	10
Animal 7 ♂	86	97	30	41	-16*	80	20
Animal 10 ♂	94	99	32	100	30*	81	16
Animal 14 ♂	80	93	24	87	16	29	-7*
Animal 13 ♂	92	90	20	93	18	97	20
Animal 9 ♂	86	81	16	97	24	89	18
Animal 16 ♂	96	90	24	101	28	91	22*
Animal 8 ♀	72	79	12	83	16	59	9
Animal 17 ♀	80	86	18	73	10	35	-5*
Animal 18 ♀	76	75	16	80	18	71	10*

Table V (cont'd.)

Animal No. and sex	4th wk.		5th wk.		6th wk.		7th wk.	
	Food Intake	Gain	Food Intake	Gain	Food Intake	Gain	Food Intake	Gain
	grams	grams	grams	grams	grams	grams	grams	gram
Animal 1 ♂	50	-2*	56	1	47	-2	-	
Animal 2 ♂	77	36*	83	34	80	14	49	4
Animal 3 ♂	85	18	87	15	90	21	75	6
Animal 4 ♂	29	-11*	37	-2	35	-2	-	
Animal 6 ♂	93	26	75	12	35	2	33	2
Animal 11 ♂	73	10	71	10	75	6*	53	5
Animal 7 ♂	73	10	82	20	81	18	85	22
Animal 10 ♂	77	14	69	13	67	11	65	8
Animal 14 ♂	49	7	21	-1	-		-	
Animal 13 ♂	21	-5*	15	0	-			
Animal 9 ♂	63	4*	69	6	77	14	65	4
Animal 16 ♂	96	24	89	20	72	10	63	6
Animal 8 ♀	37	-5*	51	8	47	6	30	-8
Animal 17 ♀	80	12	83	16	75	10	70	8
Animal 18 ♀	75	12	69	8	57	2	60	4

Animal No. and sex	8th wk.		9th wk.		10th wk.	
	Food Intake	Gain	Food Intake	Gain	Food Intake	Gain
	grams	grams	grams	grams	grams	grams
Animal 1 ♂						
Animal 2 ♂	53	4	51	6	48	4
Animal 3 ♂	82	10	70	6	81	14
Animal 4 ♂						
Animal 6 ♂	51	4	53	4	-	
Animal 11 ♂						
Animal 7 ♂	67	8				
Animal 10 ♂	64	8	43	4	80	16
Animal 14 ♂						
Animal 13 ♂						
Animal 9 ♂	59	4	53	4	25	-2
Animal 16 ♂						
Animal 8 ♀	36	-4	41	-2	45	-2
Animal 17 ♀	61	4	42	2		
Animal 18 ♀	51	2	59	4		

FIGURE II

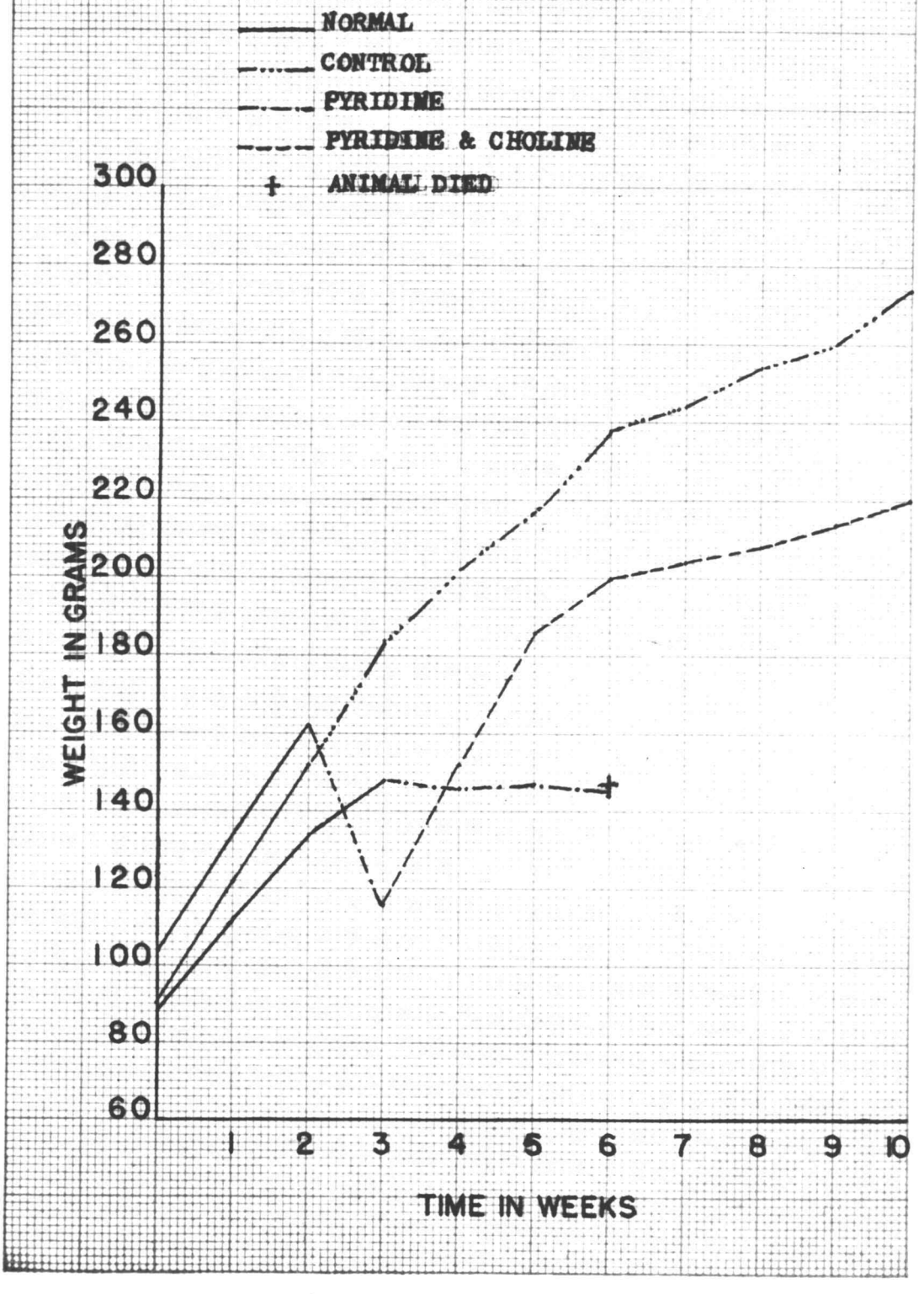

NORMAL
CONTROL
PYRIDINE
PYRIDINE & CHOLINE
+ ANIMAL DIED
300
280
260
240
220
200
180
160
140
120
100
80
60
WEIGHT IN GRAMS
1
2
3
4
5
6
7
8
9
10
TIME IN WEEKS

Table VI

Typical gains of male and female rats (Evans-Long strain) when receiving the experimental diet, ad lib. This is the diet given in Table II. All animals were 36-64 days of age at the beginning of the experiment. The period of the experiment was from March 9 to April 4, 1947.

Weekly Gain and Food Intake of 25 Animals Receiving Choline Deficient Diet

Group A

Animal No. and sex	Initial weight	1st wk. Food	1st wk. Gain	2nd wk. Food	2nd wk. Gain	3rd wk. Food	3rd wk. Gain	4th wk. Food	4th wk. Gain
	gms.	gms.	gms.	gms.	gms.	gms.	gms.	gms.	gms.
Animal 1 ♀	55	60	12	87	21	76	16	88	12
Animal 5 ♂	52	50	6	67	8	82	10	96	24
Animal 15 ♀	58	63	8	91	26	87	18	80	10
Animal 17 ♀	36	79	14	72	10	75	11	69	7
Animal 26 ♀	46	56	6	71	11	92	20	90	17

Group B

Animal No. and sex	Initial weight	1st wk. Food	1st wk. Gain	2nd wk. Food	2nd wk. Gain	3rd wk. Food	3rd wk. Gain	4th wk. Food	4th wk. Gain
	gms.	gms.	gms.	gms.	gms.	gms.	gms.	gms.	gms.
Animal 7 ♀	52	51	5	89	21	81	15	78	13
Animal 10 ♀	48	65	13	77	16	89	24	78	15
Animal 16 ♂	46	71	17	73	17	72	16	91	24
Animal 19 ♀	44	70	11	90	20	89	19	101	28
Animal 29 ♀	30	70	12	86	19	78	17	89	21

Table VI (cont'd.)

Group C

Animal No. and sex	Initial weight	1st wk. Food	1st wk. Gain	2nd wk. Food	2nd wk. Gain	3rd wk. Food	3rd wk. Gain	4th wk. Food	4th wk. Gain
	gms.	gms.	gms.	gms.	gms.	gms.	gms.	gms.	gms.
Animal 6 ♀	50	35	0	83	17	87	17	93	26
Animal 14 ♀	32	57	6	76	15	74	13	93	24
Animal 23 ♀	48	61	11	79	17	87	19	91	21
Animal 18 ♂	50	72	15	87	20	70	13	97	25
Animal 24 ♀	48	70	9	94	23	82	17	99	25

Group D

Animal No. and sex	Initial weight	1st wk. Food	1st wk. Gain	2nd wk. Food	2nd wk. Gain	3rd wk. Food	3rd wk. Gain	4th wk. Food	4th wk. Gain
	gms.	gms.	gms.	gms.	gms.	gms.	gms.	gms.	gms.
Animal 2 ♀	54	70	13	89	22	80	15	93	22
Animal 11 ♀	51	67	9	66	8	89	20	95	22
Animal 13 ♂	48	73	18	75	16	77	15	87	19
Animal 22 ♀	46	63	10	87	21	80	17	89	20
Animal 28 ♀	32	60	13	57	8	78	16	90	20

Group E

Animal No. and sex	Initial weight	1st wk. Food	1st wk. Gain	2nd wk. Food	2nd wk. Gain	3rd wk. Food	3rd wk. Gain	4th wk. Food	4th wk. Gain
	gms.	gms.	gms.	gms.	gms.	gms.	gms.	gms.	gms.
Animal 4 ♂	42	76	18	60	6	78	17	103	29
Animal 8 ♀	44	65	12	89	25	87	19	86	15
Animal 20 ♀	44	62	11	82	21	76	16	81	18
Animal 21 ♀	50	73	17	81	20	82	19	90	20
Animal 30 ♀	28	63	12	67	11	76	14	85	17

Table VII

Typical gains of male and female rats (Evans-Long strain) receiving the experimental diet, ad lib. This is the diet given in Table II. These animals were divided into five groups.

Group A received 200 mgs of water per kilo of body weight.

Group B received 150-200 mgs of pyridine per kilo of body weight.

Group C received 150-200 mgs of pyridine plus 100 mgs choline per kilo of body weight.

Group D received 150-200 mgs of pyridine plus 200 mgs choline per kilo of body weight.

Group E received 150-200 mgs of pyridine plus 300 mgs of choline per kilo of body weight.

Weekly Gain and Food Intake of 25 Animals Receiving Choline Deficient Diet Plus Water or Pyridine or Pyridine Plus Choline

Group A

Animal No. and sex	Initial weight	1st wk.		2nd wk.		3rd wk.		4th wk.	
		Food	Gain	Food	Gain	Food	Gain	Food	Gain
	gms.	gms.	gms.	gms.	gms.	gms.	gms.	gms.	gms.
Animal 1 ♀	116	75	9	93	20	87	15	83	10
Animal 5 ♂	100	79	10	90	18	95	20	103	23
Animal 15 ♀	120								
Animal 17 ♀	78	71	9	86	14	50	47	47	4
Animal 26 ♀	100	70	8	87	15	90	98	98	20

Table VII (cont'd.)

Group B

Animal No. and sex	Initial weight	1st wk. Food	1st wk. Gain	2nd wk. Food	2nd wk. Gain
	gms.	gms.	gms.	gms.	gms.
Animal 7 ♀	106	25	-9	21	-5
Animal 10♀	116	30	-4*		
Animal 16♂	120	27	-12	30	-5
Animal 19♀	122	24	-16	23	-5
Animal 29♀	99	25	-33	19	-9

* 10 lived 3 days; lost 4 gms, then died.

Group C

Animal No. and sex	Initial weight	1st wk. Food	1st wk. Gain	2nd wk. Food	2nd wk. Gain	3rd wk. Food	3rd wk. Gain	4th wk. Food	4th wk. Gain
	gms.	gms.	gms.	gms.	gms.	gms.	gms.	gms.	gms.
Animal 6 ♀	110	40	2	53	5	55	4	70	6
Animal 14♀	90	35	1	51	4	50	3	52	3
Animal 18♂	120	30	1	23	0	27	-1	42	2
Animal 23♀	116	32	1	43	-3	56	4	60	6
Animal 24♀	122	45	3	52	4	43	-2	59	5

Group D

Animal No. and sex	Initial weight	1st wk. Food	1st wk. Gain	2nd wk. Food	2nd wk. Gain	3rd wk. Food	3rd wk. Gain	4th wk. Food	4th wk. Gain
	gms.	gms.	gms.	gms.	gms.	gms.	gms.	gms.	gms.
Animal 2 ♀	126								
Animal 11♀	110	52	4	71	6	73	6	81	7
Animal 13♂	116	57	5	79	7	56	4	80	8
Animal 22♀	114	35	1	40	-2	42	2	47	3
Animal 28♀	89	41	3	52	5	49	4	77	7

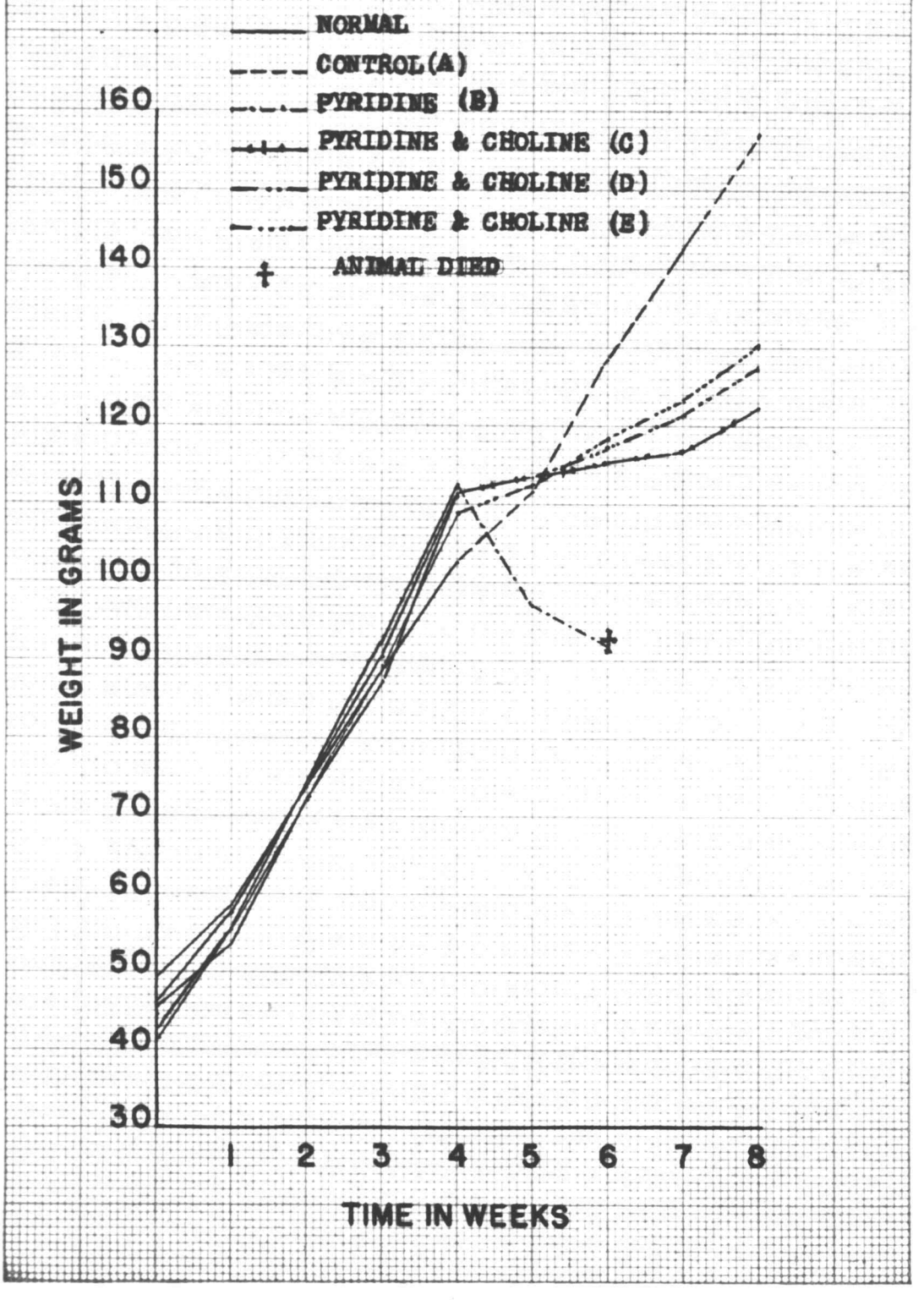
(blank) Animal died.

Table VII (cont'd.)

Group E

Animal No. and sex	Initial weight	1st wk. Food	1st wk. Gain	2nd wk. Food	2nd wk. Gain	3rd wk. Food	3rd wk. Gain	4th wk. Food	4th wk. Gain
	gms.	gms.	gms.	gms.	gms.	gms.	gms.	gms.	gms.
Animal 4 ♂	112	51	5	67	6	83	8	84	7
Animal 8 ♀	115	49	4	71	7	79	7	87	8
Animal 20♀	110	60	6	53	4	69	5	80	7
Animal 21♀	126	37	2	43	3	31	-1	52	4
Animal 30♀	82	40	2	79	8	73	5	91	9

FIGURE III


NORMAL
CONTROL (A)
PYRIDINE (B)
PYRIDINE & CHOLINE (C)
PYRIDINE & CHOLINE (D)
PYRIDINE & CHOLINE (E)
† ANIMAL DIED
160
150
140
130
120
110
100
90
80
70
60
50
40
30
WEIGHT IN GRAMS
1
2
3
4
5
6
7
8
TIME IN WEEKS

SECTION IV

DISCUSSION

As early as 1887 it was suggested that pyridine could be detoxified by methylation (22). Nicotinic acid, which is a derivative of pyridine, is methylated in the body to trigonelline or N^1-methylnicotinamide according to the genus of the animal (1, 26, 27, 37, 38, 39). Tamura (52) has showed that quinoline, an alkaloid similar to pyridine, is toxic, and is methylated in the body of the dog and rat, then excreted as methylquinolinium hydroxide. He isolated this compound, then synthesized it and fed it to his animals. He found that it was far less toxic than quinoline and it was excreted unchanged in the urine. Harrow (18) discussing detoxications, suggested that pyridine is detoxified in the body by the methyl groups of choline and methionine and is excreted as methyl pyridinium hydroxide. It seems from this suggestion and the actual finding of His (22) that methyl pyridinium chloride, rather than methyl pyridinium hydroxide is the chief end product of methylation of pyridine in the body. Although the problem is not yet completely solved, the present work definitely establishes the fact that choline detoxifies pyridine.

An examination of the data reveals that the animals receiving the stock diet were better able to tolerate pyridine than when the experimental diet was employed. It is believed that this difference is due to the higher content

of choline and methionine in the stock diet. Analytical results showed that possible methyl donors were present in a much larger amount in this diet. Some animals lived for as long as four weeks when receiving pyridine plus the stock diet. When the food low in methyl donors was fed, many of the animals were unable to tolerate a much smaller dose of pyridine. The few that lived after receiving the smaller dose of pyridine for two weeks were given the larger dose. All died within one day.

Still another observation is the fact that on the stock diet the weekly food intake and the gain in weight per week is proportionally larger. Again this is probably due to the difference in choline and methionine content of both diets.

Only one animal died from the group taking pyridine and choline which were fed the stock diet. This was due at least in part to respiratory infection. One aminal from the control group and another from the pyridine plus 200 milligrams choline group which were fed the experimental diet were dead due to the action of the stomach tube on the lungs. They died shortly after removal of the stomach tube. It seems in order to state that the stomach tube has a psychological effect of the rats, for a loss in weight and a decrease in food intake were observed in the first week after feeding by the stomach tube, even in the animals receiving water as control.

Curve II shows clearly the detoxication action of choline against pyridine. It is a unique curve in this study, for Animal 2 was left to take water ad lib, then it was given pyridine only for one week during which it lost 46 grams from its weight. It was then fed 400 milligrams of choline per kilogram by stomach tube for about 7 weeks during which it gained 102 grams, averaging 14.5 grams gain per week.

The rest of the animals taking pyridine plus choline in the two parts of the experiment were gaining, but not as much as the control animals, particularly those of the second part. It is suggested that this action may be due to the action and odor of pyridine, and perhaps the methylated pyridine has a slight toxicity.

It is interesting to note that 50 milligrams of choline per kilogram of body weight were not sufficient to keep the animals growing. After four weeks of taking pyridine plus choline in the levels mentioned above, the amount of choline was lowered to 50 milligrams per kilogram, while the amount of pyridine remained constant. There was a distinct loss in weight. Each animal lost about 15 grams per week. Two animals died after three days. No data are furnished because of the relatively short period in which this additional part was carried on.

This interesting problem is partly solved with the striking results obtained by choline detoxication of pyridine. The mechanism of this detoxication is not known with certainty, although a possible explanation has been proposed by some workers. This proposal is supported by this research. To prove this suggested mechanism, the author is extending the work further, trying to isolate methylpridinium chloride using the directions of His (22) and Kutscher (31). The next step will be to synthesize methylpyridinium chloride by a method which was discussed in the experimental part of the thesis. The final goal will be to feed this compound to the rats and study its action and metabolism.

SECTION V

SUMMARY

1. A study on the role of choline in detoxication of pyridine was carried out on a stock and a choline deficient diet.
2. Both stock and experimental diets were assayed for total sulfur, methionine, total nitrogen and choline.
3. The lethal dose of pyridine (100% mortality) for the Evans-Long strain of rats was determined to be 200 milligrams per kilogram of body weight.
4. Experimental evidence indicates that a dose of 200 milligrams of pyridine per kilogram of body weight can be detoxified by using 100 milligrams of choline per kilogram, 300 milligrams of choline gave better growth than either 200 milligrams or 100 milligrams. Improved growth could be obtained by using up to 300 milligrams of choline per kilogram. Animals receiving pyridine plus a supplement of 100 milligrams of choline per kilogram survived the entire experimental period.

SECTION VI

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