EFFECT OF THIAMINE ANALOGUES ON THE HISTOPATHOLOGY AND ON THE THIAMINE CONTENT OF RAT TISSUES

by

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I. INTRODUCTION

An increasing amount of research has been done in the last decade on structurally related compounds of thiamine. These are often referred to as analogues, some of which have been shown to produce an inhibitory effect upon the growth of animals and plants while others have produced no material change.

The literature in this field covers various aspects of the analogues in detail. There are, however, some factors that have not been fully considered. Among these are the thiamine content and the histopathology of the tissues from the laboratory animal receiving the analogue. The histopathology should be studied in order to determine the effect these analogues have upon the tissues and, more specifically, within their individual cells. If these studies are correlated, the specific reaction of these analogues within the animal body should be more easily determined.

With the advent of the microbiological method in determining growth inhibition, the possible use of metabolite analogues as chemotherapeutic agents in the treatment of infectious diseases has been encouraging. It is generally known that there is competition in many cases between normal metabolites of a cell and their structural analogues. Therefore it appears entirely possible that the normal metabolites of all microorganisms, as well as cells in complex animal

bodies, may have competitive structural analogues.

The need for more specific treatments in infectious diseases cannot be overemphasized. Today, far too many treatments are only general therapeutic measures while a specific treatment may lie at our disposal. Thus a study of the histopathology and thiamine content of tissues from animals receiving a thiamine analogue may be an important step in developing specific treatments for infectious diseases.

II. HISTORICAL

A. Analogues

1. Non-thiamine

The effect of structurally related compounds on organisms cannot be definitely determined until they have been administered to the organism in question. Many analogues have no effect whatever, while others may produce a definite competitive reaction. In order to have a competitive reaction, it is not always necessary to have the antagonist structurally related to the inhibitor.

In 1940 Woods (1) discovered that the growth inhibition produced by sulfanilamide on Streptococcus haemolyticus could be reversed by adding p-aminobenzoic acid. The investigator explained that p-aminobenzoic acid was an essential metabolite synthesized by the bacterial cell or obtained from the surrounding medium, and sulfanilamide, being so closely related, inhibited the growth of bacteria. The inhibition took place as a result of both compounds, p-aminobenzoic acid and sulfanilamide, competing for the same enzyme which prevented the normal metabolism of the bacterial cell. Rubbo and Gillespie (2) obtained similar results as Woods. They found that one molecule of p-aminobenzoic acid antagonized 23,000 molecules

of sulfanilamide.

More recent work, as summarized by Sevag (3), indicates that p-aminobenzoic acid is a non-specific sulfonamide-like inhibitor since it has actually inhibited the growth of many bacteria, molds, enzymes, and viruses. The inconsistencies in growth of the various organisms suggest that p-aminobenzoic acid may promote growth at one concentration and still be toxic at a higher level. Where p-aminobenzoic acid is considered to be essential for growth, sulfonamide therapy would be highly indicated. With such therapy, the outcome might be determined by the amount of growth inhibition that has taken place.

In 1924 Schofield (4) observed lesions in cattle, that were being fed damaged sweet clover, similar to those found in hemorrhagic septicemia and blackleg. A few years later, Roderick and Schalk (5) discovered that cattle fed improperly cured sweet clover hay or silage developed a condition in which there were severe subcutaneous hemorrhages. The blood clotting time was markedly increased. Link and co-workers (6, 7) isolated and identified the active principle from poorly cured sweet clover hay as 3,3'-methylenebis(4-hydroxycoumarin). This compound is also known as "dicumarol". The structural analogy of this compound to vitamin K has led Overman and co-workers (8) to feed dicumarol to rats. A 2.5 mg. dosage of this compound to an adult rat lowered the prothrombin activity 22 per cent of the normal in 24 hours. The

hypothrombinemia produced by the anticoagulant, dicumarol, was counteracted by the administration of vitamin K regardless of whether it had been given before or after the anticoagulant. At present very little is known regarding the action of vitamin K in the formation of prothrombin. The antagonism between dicumarol and vitamin K which occurs in nature is one of the first to be recorded. Clinically, the use of dicumarol has its merits by prolonging the clotting time of blood to prevent thrombus formation; on the other hand, hemorrhages are more likely to appear.

Woolley and co-workers (9) have shown that a number of structural analogues of nicotinic acid have anti-black tongue activity on oral administration. Among those that have this property are nicotinamide, ethyl nicotinate, nicotinic acid N-methyl amide, nicotinic acid N-diethyl amide, nicotinamide glucosidoiodide, and nicotinuric acid. Compounds that were inactive in dosages up to three and four times the effective dosage of nicotinic acid were trigonelline, nicotinamide methochloride, pyridine, pyridine-β-sulfonic acid, 6-methyl-nicotinic acid, β-acetylpyridine, quinolinic acid, picolinic acid, isonicotinic acid, and nicotinonitrile. Woolley and co-workers conclude that in addition to nicotinic acid and its amide, the anti-black tongue compounds are limited to those which are capable of producing these substances within the animal body by oxidative and hydrolytic reactions.

In working with bacteria McIlwain (10) demonstrated that

pyridine-3-sulfonic acid and its amide cause growth inhibition of those bacteria which require nicotinic acid for normal growth. There was competitive inhibition with Staphylococcus aureus and Proteus vulgaris. Escherichia coli did not exhibit growth inhibition with these analogues since this organism does not require added nicotinic acid for growth.

Pantothenic acid, a vitamin occurring in both plants and animals, is an essential factor for the growth of many organisms. Snell (11), working with Lactobacillus arabinosus, proved that the sulfonic acid analogue of pantothenic acid competed with pantothenic acid for some enzyme system in the bacterial cell. The presence of small amounts of calcium pantothenate caused inhibition; however, large amounts of calcium pantothenate reversed the inhibition and growth occurred. Yeasts gave the same results when they were subjected to the same compounds.

In a later publication, Snell (12) reported his work with sodium and barium salts of pantoyltaurine. In addition to Lactobacillus arabinosus, Snell used Lactobacillus pentosus, Brucella abortus, Staphylococcus aureus, and Escherichia coli. The results show that there was inhibition with all organisms in varying degrees with the exception of Escherichia coli. The latter organism does not require added pantothenic acid for growth. The inhibition of growth was present in all organisms which required preformed pantothenic acid. As a substitute for pantothenic acid, β-alanine was successful in preventing growth

inhibition when the organism was able to make use of it. β-alanine, a constituent of muscle, is part of the pantothenic molecule. Its presence in this molecule may partly explain why β-alanine may prevent growth inhibition in some organisms.

McIlwain (13) also worked on pantothenic acid analogues. In his work he found that pantoyltaurine (thiopanic acid) had an anti-streptococcal index of 500 and an anti-pneumococcal index of 1000. Pantoyltauramide had an anti-streptococcal index of 100 and an anti-pneumococcal index of 10,000. Homopantoyltaurine was less active. The growth of Corynebacterium diphtheriae was also inhibited. These indices refer to the concentration of the analogue that is required to prevent the growth of the organism in relation to the concentration of the pantothenate. Thus the analogues have less affinity for the enzymes than the pantothenate which accounts for the high indices. McIlwain is of the opinion that many pathogens need pantothenic acid for growth, and tissues contain more than enough pantothenate to meet this requirement. When one realizes the close structural similarity of these compounds, it becomes evident that antagonism is likely to occur. The only difference between these compounds is the replacement of the carboxyl group of pantothenic acid with the anion of sulfurous acid to form thiopanic acid.

2. Thiamine

In 1943 Woolley and White (14) reported that a deficiency disease in animals had been produced for the first time by the feeding of a structural analogue. This compound, pyrithiamine, is a pyridine analogue of thiamine. It is formed by the replacement of the sulfur atom in the thiazole ring of the thiamine molecule with -CH=CH-, the vinylene group. Pyrithiamine, 2-methyl-4-amino-5-pyrimidylmethyl-(2-methyl-3-hydroxyethyl) pyridinium bromide, was fed to mice, and it produced the characteristic symptoms of thiamine deficiency. Mice died when 20 micrograms of pyrithiamine and 2 micrograms of thiamine were given daily. However, growth was not affected during the forepart of the experiment. The ratio of pyrithiamine to thiamine essential to produce inhibition in mice was found to be 40 and 3 to 1. The addition of thiamine to the diet corrected the deficiency disease in the afflicted animal and prevented it in the control animal.

The competitive inhibition of pyrithiamine to thiamine is not restricted to animals as bacteria, yeasts, and molds will also react. Woolley and White (15) also demonstrated that bacteria requiring thiamine for growth exhibited growth inhibition with small amounts of pyrithiamine. On the other hand, those bacteria which did not require thiamine were unaffected by the analogue. Some organisms can synthesize

thiamine if the pyrimidine or thiazole portion of thiamine is supplied. Others can synthesize thiamine only if both portions are furnished. Endomyces vernalis, a yeast, was not affected by pyrithiamine when pyrimidine alone was added. The ratio of pyrithiamine to thiamine necessary to produce growth inhibition in such an organism was 100 to 1. The ratio to produce inhibition in those organisms which required the pyrimidine and thiazole portions of thiamine or thiazole portion alone was about 100 to 1. Organisms which required the entire thiamine molecule gave a ratio of 10 to 1.

Robbins (16) studied the effect of pyrithiamine with thiamine and its components on three different fungi. Phycomyces blakesleeanus and Pythiomorpha gonapodioides are able to split off the pyrimidine portion from the analogue molecule. The latter fungus can synthesize the thiazole portion of the thiamine molecule and then combine it with pyrimidine to form thiamine. The former fungus needs added thiazole to combine with the pyrimidine to make thiamine. Phytophthora cinnamomi needs molecular thiamine for growth since it cannot use the analogue. When the concentration of the analogue was 10 micromillimoles or more in the flasks, there were signs of toxicity. Here again, the concentration of the analogue appears to influence the reaction. The ability of the organism to synthesize thiamine either entirely or partially is a criterion to help determine what changes the analogue will produce.

Emerson and Southwick (17) fed 2.8 mg. of 2-n-butyl-5-(4-methyl-5-β-hydroxy-ethyl-thiazolium bromide)-methyl-6-amino-pyrimidine hydrobromide, the 2-n-butylpyrimidine analogue of thiamine, per day to rats on a suboptimal level of thiamine. This level was 5 micrograms of thiamine per day. A control group receiving the same amount of thiamine without the analogue was run. At the end of fifty day, five rats out of eight had succumbed in the group receiving the analogue and thiamine. The remaining three rats were in a dying state. The eight rats in the control group survived the fifty days. By supplementing the group receiving the analogue and thiamine with 50 micrograms more of thiamine, the growth inhibition was corrected. It was found that 1 mole of thiamine counteracted the antithiamine effect of 40 moles of the analogue.

Soodak and Cerecedo (18) prepared oxythiamine, 2-methyl-4-hydroxy-5-pyrimidylmethyl-4-methyl-5-hydroxy-ethylthiazolium chloride, by the deamination of thiamine with nitrous acid. When 25 to 50 micrograms of this thiamine analogue and 1 microgram of thiamine were given daily to mice on a thiamine-low diet, death resulted in two weeks. In a more recent report by the same investigators (19), a similar experiment was described in which the thiamine stores were depleted in the mice and then 1 microgram of thiamine was injected daily for 1 to 2 weeks. Along with the thiamine, three analogues of thiamine

were given in varying quantities, 25, 50, 90 micrograms oxythiamine, 100 micrograms oxychlorothiamine, and 130 micrograms oxybromothiamine. Growth inhibition occurred only in those animals receiving oxythiamine. The controls and those receiving oxychlorothiamine and oxybromothiamine continued to gain weight. It was concluded from this experiment that the free hydroxyl group on the side chain of the thiazole portion of oxythiamine is necessary to be antagonistic with thiamine. Oxychlorothiamine and oxybromothiamine have had this hydroxyl group replaced with chlorine and bromine, respectively. By the removal or substitution of atoms or groups of atoms from the analogue showing competitive inhibition, it is possible to determine what part of the analogue reacts with the enzyme, carboxylase, in competing with thiamine. The exact reaction between the molecules of the inhibitor and antagonist and the substance for which they are competing may not be definitely known in some cases. However, alterations within the molecule of the analogue may change the inhibition immensely.

In 1940 Buchman and co-workers (20) reported inhibition of carboxylase with thiazole pyrophosphate. Thiazole was pyrophosphorylated and then purified by converting it into a silver salt and later to a crystalline manganese salt. In checking thiazole pyrophosphate, free thiazole, thiazole monophosphate, and sodium pyrophosphate with cocarboxylase, it was found that only thiazole pyrophosphate reduced the CO_2 production from pyruvic acid. The decrease in decarboxylation

of pyruvic acid was apparently caused by the competitive inhibition between cocarboxylase and thiazole pyrophosphate for the enzyme, carboxylase. These investigators believe that the active group which joins the enzyme is the pyrophosphate group which is common to both compounds. This reaction is of great biological importance as the pyruvic acid level within the animal body increases when an insufficient amount of thiamine is present. Also, a competitive reaction between thiamine and one of its analogues for the enzyme, carboxylase, would increase the level of pyruvic acid.

In 1941 Green and co-workers (21) reported a deficiency disease of foxes produced by the feeding of uncooked fish. This disease was called "Chastek paralysis" since it occurred on the Chastek fox farm in Minnesota. The symptoms of this condition are anorexia, weakness, stiffness, incoordination, and tendency to lie down much of the time, followed by spastic paralysis, paraplegia, and death within 48 to 72 hours after the onset of the neurologic symptoms. To produce these symptoms, the ration had to contain 10 per cent or more of the uncooked fish. There was also a high mortality of fetuses and nursing pups. Large amounts of thiamine prevented the disease. These workers concluded that the foxes were suffering from avitaminosis B_1 and that fish was the main factor in producing the deficiency disease.

Sealock and Goodland (22) found that the destruction of thiamine by the active principle in fish tissues was decreased

when cupric, zinc, and ferric ions were added. Two analogues of thiamine, 3-o-aminobenzyl-4-methyl-thiazolium chloride and 3-β-aminoethyl-4-methyl-thiazolium chloride, were found to inhibit thiamine inactivation by the fish principle. The concentration of thiamine in moles per liter was 5.0×10^{-4}. To obtain 100 per cent inhibition with the o-aminobenzyl compound, the concentration was 5.0×10^{4} moles per liter. The β-aminoethyl compound produced 56.4 per cent inhibition with the same concentration and 74.6 per cent with a concentration of 20.0×10^{4} moles per liter. The inhibition with the o-aminobenzyl compound was eliminated completely when the amino group was replaced with the nitro group. With the β-aminoethyl compound, the inhibition was reduced by replacing its amino group with a phthalimido group, $C_6H_4(CO)_2N$-. Sealock and Goodland concluded that the fish principle was highly enzymatic in nature.

The work of Green, Sealock, and their co-workers proves that natural occurring inhibitors may occur in animal tissues as well as in plant tissues. This research has been of great importance to fox, mink, and ferret farmers, since avitaminosis B_1 has been reported in these animals by feeding uncooked fresh water fish. It appears likely that other inhibitors may be present in nature which may be the answer to some diseases of unknown etiology.

B. Thiamine Content

The thiamine concentration in the animal body may vary with the individual tissues and also among the different species. It is known that organs such as heart, liver, and kidney, from an animal receiving a balanced diet have more thiamine per gram of tissue than brain, skeletal muscle, and fat. However, these organs containing a greater amount of thiamine may become depleted and reach a very low level equal to the concentration in the balance of the body. On the other hand, all organs and tissues may reach a saturation point whereby excess thiamine is eliminated in the urine and feces. Thus it becomes apparent that a constant supply of thiamine is necessary to maintain the concentration for normal functioning of the animal body.

Schultz and co-workers (23) were able to determine the thiamine saturation point of the tissues in rats. The rats were fed a basal diet containing 62 per cent rice starch, 18 per cent vitamin-free casein, 14 per cent hydrogenated cottonseed oil (Crisco), 2 per cent cod liver oil, and a 4 per cent salt mix. Dry yeast, whose thiamine content was known, was added to the diet as a B-complex supplement. Analyses for thiamine were made after the excretion equilibrium had been established. According to Table 1, the maximum retention of the tissues was obtained by feeding 65 micrograms of thiamine per day. The thiamine concentration in the tissues was

Table 1

Effect of vitamin B_1 intake level on total thiamine content of the rat*

Total B_1 in microgm./day	Body wt. (gm.) av. of 3 rats	Av. microgm. B_1/gm. body wt.	Av. microgm. B_1/gm. liver
10	118	0.54	1.08
20	186	0.49	1.25
30	198	0.92	2.8
40	253	1.23	4.8
50	244	1.34	4.85
65	218	1.77	6.6
65	149	2.14	8.15
115	205	1.63	5.3
515	195	1.88	6.8

*Taken from Schultz, A. S., et al. J. Nutr. 17:145. 1939.

approximately 2 micrograms per gram of body weight. The data in Table 2 represent an average of 2 rats over a period of 28 days for the first two groups, while the last group received 215 micrograms of thiamine daily for 58 days to insure complete

Table 2

B_1 content of organs and tissues* (expressed in microgm./gm. of tissue)

Microgm. B_1/day	Av. rat wt. (gm.)	Liver	Heart	Testes	Kidney	Skeletal muscle	Blood
0	122	0.26	0.68	1.87	0.39	0.14	--
15	245	1.9	2.6	4.7	2.1	0.57	0.07
215	259	10.0	7.2	6.6	6.7	1.3	0.35

*Taken from Schultz, A. S., et al. J. Nutr. 17:146. 1939.

saturation of the tissues.

Cleland (24) found that 0.2 microgram of thiamine per gram of body weight was the minimum concentration to maintain life in the rat. Table 3 shows that the liver contains the greatest amount of thiamine per gram of tissue in the normal animals.

This finding is in agreement with Schultz. The greatest loss also occurred from the liver when the diet was inadequate.

Table 3

Weight and thiamine content of rat tissues*

Diet	Rat wt. (gm.)	Liver wt. (gm.)	Kidney wt. (gm.)	Microgm./ gm. liver	Microgm./ gm. kidney
Norm. adult	203[a]	9.04	1.55	3.00	1.78
	154-276[b]	6.81-11.65	1.40-2.0	1.96-4.83	0.81-3.14
B_1 def. adult	195	9.35	1.86	0.32	0.39
	156-255	7.17-13.10	1.57-2.17	0.2 -0.43	0.22-0.49
Norm. young	139	8.09	1.12	2.16	2.20
	124-180	6.15-11.49	0.89-1.48	1.33-3.85	0.69-3.87
B_1 def. young	56	2.49	0.93	0.21	0.17
	38- 80	1.72-3.23	0.63-1.44	0.12-0.40	0.09-0.33

*Taken from Cleland, J. B. Aust. J. Exptl. Biol. & Med. Sci. 24:228. Sept. 1946.
[a]Group average.
[b]Group range.

Cleland had 12 rats in each group. The normal rats received a normal stock diet while the abnormal rats received a thiamine-deficient diet consisting of 20 parts casein, 70 parts sucrose, 5 parts salt mixture, 10 parts dried, autoclaved yeast, and 3 parts cod-liver-oil substitute.

It is evident that the results obtained by Cleland and Schultz are in harmony. The thiamine content of the liver may vary from a mere 0.2 microgram per gram of tissue, when life is still present, to that of 10 micrograms per gram of tissue when the tissues are saturated. On the basis of thiamine per gram of body weight, the range of minimum and maximum concentrations is 0.2 to 2.0 micrograms. It is apparent that an animal will

use the last reserve of thiamine in the liver before death since the concentration in the liver and in the balance of the body is practically the same.

The thiamine concentration in rat tissues and in tissues of other mammals is comparable. There are certain variations reported, but they may be without significance when analyzed statistically. However, Waisman and Elvehjem (25) found that heart muscle from the ox and pig contained more thiamine per gram of tissue than the corresponding liver tissue. They also showed that skeletal muscle of fresh pork had a high thiamine content with an average of 15.2 micrograms per gram of ham tissue. In working with human tissues, Ferrebee and co-workers (26) found the concentration of thiamine in the heart to be 2 to 3 micrograms per gram of tissue, skeletal muscle 0.5 microgram, and brain, liver, and kidney 1 microgram. These findings are in accord with Waisman and Elvehjem, but the thiamine concentration per gram of tissue is less. Ferrebee showed that the thiamine content of tissue could be increased with vitamin therapy and reduced with inadequate diets.

Schweigert and co-workers (27) kept rats on different levels of thiamin and later analyzed the tissues for this compound. When 8 micrograms of thiamine were given to one group of rats and 25 to 50 micrograms were given to another, the thiamine content in the muscle and liver was 3 or 4 times greater in the latter group. There was no material change in the thiamine content of the tissues when the rats were placed

on high carbohydrate, protein, or fat diets. Sarett and Perlzweig (28) also found that the thiamine intake regulated the thiamine content of the various tissues. The protein level of the diet did not alter the thiamine level in the tissues. This was also confirmed by Schweigert and co-workers. Two groups of rats were given thiamine. The first group received 9 to 11 micrograms per day while the second group received 33 micrograms. When the tissues were analyzed, the first group had one-half as much thiamine in the liver and in the whole carcass as the latter group. From these data of thiamine analyses, it appears that the thiamine content of the tissues is governed to a great extent by the thiamine intake.

C. Histopathology

1. Polarized light

According to Muñoz and Charipper (29), the polarizing microscope was invented in France by Nachet between 1833 and 1855. At first it was considered a novelty; however, its use has expanded until now it is an indispensable instrument in the fields of biological chemistry, mineralogy, organic and inorganic chemistry, ceramics, botany, and other sciences. As early as 1854 Virchow and other investigators used the polarizing microscope in the study of nerve tissue. They noticed that myelin sheaths of nerve fibers were optically active.

Polarized light is known to vibrate in one plane only

while ordinary light vibrates in all directions perpendicular to the direction in which it travels. Substances appearing black under the polarizing microscope, when crossed Nicol prisms are used, are called isotropic. When substances appear light under the same conditions, they are said to be anisotropic or show the property of birefringence. A normal myelin sheath appears light when crossed Nicols are used. Cellular detail, such as nodes of Ranvier, nuclei of Schwann cells, and incisures of Schmidt-Lantermann, can be demonstrated in normal nerves with polarized light as they are isotropic. Schmitt (30) states that this sheath is really more complex than myelin. The lipid constituents of myelin are laid down in a neurokeratinogenic matrix. This albuminoid protein is isotropic; however, the abundance of lipids in the myelin sheath prevents the identification of the protein material with polarized light.

According to Schmitt and Bear (31), the myelin sheath of vertebrates is produced by the Schwann cells which are ectodermal in origin. The Speidel theory of myelin formation states that myelin is formed by the co-operative metabolic activity of Schwann cells and the axone itself. Regardless of its formation, myelin consists of phospholipids, glycolipids or cerebrosides, sulfolipids, and sterols (32). Normal myelin is practically void of true fats.

When myelin changes from its normal anisotropic appearance to an isotropic state, it is considered to be degenerated. The exact chemical change in this reaction appears to be unknown.

Sutton and co-workers (33) realized, however, that myelin may change to triglycerides, which are isotropic, thus permitting a more accurate study of myelin sheath degeneration with the polarizing microscope.

Myelin degeneration, sometimes referred to as Wallerian degeneration, is present in many nutritional deficiencies, infectious diseases, and may be produced surgically by severing a myelinated nerve. Baird (34) states that one and one-half hours after the severing of such a nerve, degeneration can be seen by using the polarizing microscope. After three hours, the fibers are swollen and the myelin has broken into masses. With ordinary staining techniques, the condition would have to be prolonged three days before degeneration would be evident. Baird was apparently referring to the fat stains, such as Marchi or the Sudan stains, but they were not specified.

Sutton and co-workers (33) produced myelin sheath degeneration of peripheral nerves in their rat experiment by feeding a vitamin A-deficient diet. The degeneration was arrested by the supplementation of vitamin A. External symptoms, such as paralysis and prostration, were not corrected with vitamin A therapy. In a later paper, Setterfield and Sutton (35) state that degeneration of the myelin sheaths of the sciatic and femoral nerves began 3 to 6 days before symptoms of avitaminosis A were present. They believe that one-fourth to one-third of the nerve fibers are affected before incoordination is present.

Lee and Sure (36) obtained results similar to those of Sutton and co-workers. They have produced myelin sheath degeneration in the sciatic nerves of rats by feeding thiamine-deficient, vitamin A-deficient, and B-complex-deficient diets. In these groups there were various degrees of myelin sheath degeneration. These results indicate that a deficiency of one or several substances may cause or contribute to the degeneration of the myelin sheathsin peripheral nerves.

Prickett and co-workers (37) found a marked difference in myelin sheath degeneration of rats by varying the thiamine intake. In acute deficiencies the nerves approached normal, while rats on a suboptimal level of thiamine over a longer period of time showed considerably more myelin sheath degeneration. Control groups receiving an adequate amount of thiamine but limited in food intake showed more degeneration than the acute deficiency. The administration of thiamine to rats showing severe symptoms proved to be of little value. Early administration of thiamine to those rats showing mild symptoms was successful. Prickett and co-workers concluded that the tissues were damaged beyond repair when the rats did not respond to thiamine therapy. Photomicrographs are shown of the sciatic nerve by using polarized light.

2. Tissue stains

The staining of tissues for histological study aids in distinguishing the abnormal tissue from the normal. These distinctions can be shown when these tissues are stained. Various stains have been employed; however, the hematoxylin and eosin method has been the principal stain used in studying histopathology. A universal stain to show maximum differentiation in all tissues of the animal body has not been developed. Thus it becomes necessary to use different stains to obtain the desired effect in each type of tissue. For instance, hematoxylin and eosin stain may be used to study vascular and cellular changes in the nervous system while it does not demonstrate degenerative changes in the sheaths of myelinated nerves. Fat stains, such as Marchi, Sudan, and Weigert, are used to detect these degenerative changes in myelin. Such stains are also used to determine fatty changes in other tissues, such as the liver and kidney. The transformation of normal myelin to triglycerides is shown by the fat stains and with polarized light in unstained myelinated nerves.

Hassin (38) states that 24 hours after a nerve is cut, the myelin becomes swollen, irregular, and breaks up into fragments. These fragments stain black with osmic acid which signifies degeneration. Baird (34) found that degeneration could be detected with polarized light one and one-half hours after the nerve had been severed. It is apparent that polar-

ized light will detect degeneration sooner than the Marchi method in myelinated nerves.

Zimmerman (39) believes that the peripheral nerve lesions in avitaminosis B_1 are non-inflammatory. He found that the primary change is medullary sheath destruction which is followed by axone disintegration. Setterfield and Sutton (35) observed black droplets in the nerves of thiamine-deficient rats by using the Marchi stain. This finding checked closely with their polarized light study.

Davison and Stone (40) observed as many lesions in the nervous system of starved rats (inanition) as those on diets deficient in B_1 and B_2. The lesions were vacuolation, liquefaction necrosis, and chromatolysis of the ganglion cells of the brain and spinal cord. The myelin sheaths of the peripheral nerves were also degenerated. In the starved rats the lesions were more marked in the peripheral nerves while the vitamin-deficient rats showed more lesions in the central nervous system. Symptoms occurring in both groups of rats were paralysis of extremities, equilibratory disturbances, priapism, convulsions, and tonic retractions of the head.

Shaw and Phillips (41) found a small amount of myelin degeneration in the peripheral nerves and spinal cord of pigeons in acute thiamine deficiency while the nerves of chicks appeared normal. Chronic thiamine deficiency produced a moderate amount of degeneration in the myelin sheaths of both pigeons and chicks. More degeneration was present in animals suffering

from chronic thiamine deficiency than from inanition. The pyruvic acid level in the blood was higher in the chronic cases than the controls.

Phillips and Engel (42) demonstrated with polarized light and by the Marchi method that a riboflavin deficiency in chicks may cause histologic changes characterized by degeneration of the myelin sheaths in the peripheral nerves and the spinal cord. Muscle fibers were also degenerated. Riboflavin therapy was successful when the degenerative changes were mild.

Various investigators have considered the Marchi method unreliable. Artifacts in the stained tissue can be produced by alterations in the technique. When the artifacts are present, the histopathology may be concealed. Duncan (43) observed black droplets in the myelin sheaths of the controls as well as the diseased animals. This pathological change was seen in the cat, rabbit, rat, and man. When the method was varied, the size, number, and general appearance of the black droplets were changed. Duncan believed that the Marchi method was valuable in denoting fatty changes in some tissues, but it was not specific for Wallerian degeneration. The diets for the animals showing degeneration were not given. There is a possibility that these diets were not balanced; therefore, the myelin degeneration may have been expected. The human tissues were obtained from aged individuals. Regardless of the conflicting results from the Marchi method, it still has its place in the staining of tissues.

Engel and Phillips (44) demonstrated that the pathological changes in the peripheral nerves of chicks on an otherwise adequate diet could be corrected by the administration of β-carotene with riboflavin. Rats receiving a balanced diet with the exception of thiamine seldom showed pathological changes in the peripheral nerves, spinal cord, brain, and kidney. When thiamine was given to B_1-deficient rats, fatty degeneration of the liver occurred. The nuclei of the liver cells remained normal.

Eveleth and Biester (45) found that severe myelin sheath degeneration in swine was not the cause of incoordination. The animals recovered from the incoordination following the supplementation of vitamin A and green feeds, but the myelin sheath degeneration was still present. Vitamin A and the B-complex vitamins were eliminated as possibilities in causing incoordination and myelin sheath degeneration in swine. Control pigs on pasture did not exhibit myelin sheath degeneration in the spinal cord. Photomicrographs by the Marchi method are shown comparing the spinal cords of the normal control group with the vitamin deficient groups. It is evident that some factor was present in the pasture and absent in the diets of those animals showing degeneration in the spinal cord. Biester, Greenwood, and Nelson (46) fed dogs a diet containing vitamins A, B-complex, D, and E which produced severe myelin degeneration without incoordination.

The primary lesions in Chastek paralysis according to

Evans, Carlson, and Green (47, 21) were found in the liver, heart, and brain. Bronchopneumonia was present in some cases. The liver showed severe fatty degeneration, congestion, hemorrhages, and necrosis of the hepatic cords. In advanced degeneration the liver had ruptured. Focal necrosis appeared in the heart along with hemorrhages, cloudy swelling, enlargement of the nucleoli in the myocardial cells, and proliferation of the connective tissue. In the brain the nerve cells were degenerated. A number of the blood vessels in the brain were dilated while in others the endothelium had proliferated. Hemorrhages were quite common in the brain.

Alexander (48) made a comparative study of the lesions occurring in B_1 avitaminosis in pigeons with those present in chronic alcoholism in man. The latter condition is known as Wernicke's disease. The syndrome resulting in Wernicke's disease is characterized by paralysis of the eye muscles, polyneuropathy, and clouding of consciousness. Alcoholic drinks are usually deficient in thiamine which contributes to the formation of the syndrome. Alexander used the benzidine stain in the tissue study. Small foci of degeneration and varicose deformity of vessels were present in the brain. Subacute necrosis of the brain parenchyma was found around the ventricular system. The pigeon brain had numerous petechial hemorrahages in the pons, medulla, and cerebellum. There was a proliferation of the glia cells, especially the oligodendroglia and later the astrocytes. Alexander concluded that chronic

alcoholism in man produced lesions similar to those in pigeons suffering from B_1 avitaminosis.

Beriberi is the thiamine-deficient disease occurring in man. The eating of whole grains in this country has prevented its appearance to any great extent. In the Orient, where polished rice is eaten, beriberi is very prevalent. Yeast extracts, dried brewer's yeast, the germs of grain, and rice bran (49) are excellent sources of thiamine.

Vedder (50) divided the pathology of beriberi into three types. They are cardiac pathology, degenerative changes in the nervous system, and anasarca. In the early stages of beriberi, the heart becomes hypertrophied, followed by dilatation and cardiac failure. Vedder observed pulmonary edema in 50 per cent of the beriberi cases. Chronic passive congestion of the liver, spleen, kidneys, and intestines was common. The livers were nutmeg in appearance due to the congestion and degenerative changes. Kidneys were usually cyanotic. The skeletal muscles showed atrophy, loss of striations, shrinkage of sarcoplasm with degenerative changes. Edema was first noticed in the legs and thighs and was soon followed by hydropericardium, hydrothorax, and ascites. The brain, meninges, and spinal cord were congested. Softening of the brain and cord was present. The myelin sheaths in the spinal cord and peripheral nerves were degenerated as evidenced by the Marchi staining method. Engel and Phillips (44) do not agree with Vedder regarding the degeneration of the myelin sheaths in the spinal

cord and peripheral nerves. They believe that in uncomplicated cases of beriberi, myelin degeneration does not occur.

III. EXPERIMENTAL

A. Objective

The pathological changes which develop in animals fed thiamine-deficient diets are well-known, but the effect of thiamine analogues on the animal has not been investigated. Although it is recognized that the feeding of some analogues inhibits growth, the microscopic changes in the tissues have not been studied.

This experiment is designed to compare the pathology of thiamine deficiency with the cellular changes that develop in thiamine-deficient animals receiving a thiamine analogue. Growth curves are established to show the effect of a thiamine analogue on rats receiving a thiamine-free diet and rats on a thiamine-deficient diet. Thiamine analyses of the liver, kidney, and skeletal muscle aid in determining any possible inhibition produced by an analogue.

B. Methods and Results

1. Growth data

Four-week-old rats were obtained from B. H. Thomas, head of Animal Chemistry and Nutrition Subsection, of the Iowa Agricultural Experiment Station. Weanling rats were selected

because such animals are likely to be normal. These rats were divided into six groups. Five groups, A, B, C, D, and E, received a basal diet ad lib., consisting of the following:

Sucrose	68.5	gm.
Casein[1]	18.0	"
Hydrogenated vegetable oil (Crisco)	6.0	"
Cod liver oil (Squibb)	2.0	"
Salt mix (Hawk-Oser)[2]	5.5	"
Riboflavin	0.0005	"
Nicotinic acid	0.01	"
Pyridoxine hydrochloride	0.0002	"
Calcium pantothenate	0.002	"
Choline chloride	0.01	"
Inositol	0.1	"

This basal diet is used in the Department of Physiological Chemistry for similar investigations. Group F received a commercial fox chow ad lib. Rats were chosen as experimental animals since they require thiamine for growth. The rats were kept in individual cages with screen floors. This prevented the animals from eating their own feces which may have contained thiamine by intestinal synthesis or excretion of excess thiamine. Fresh water and the basal diet were available to the rats at all times. Each group receiving the basal diet was supplemented with different amounts of thiamine and the o-aminobenzyl analogue of thiamine. Thiamine and its analogue have the following structural formulas:

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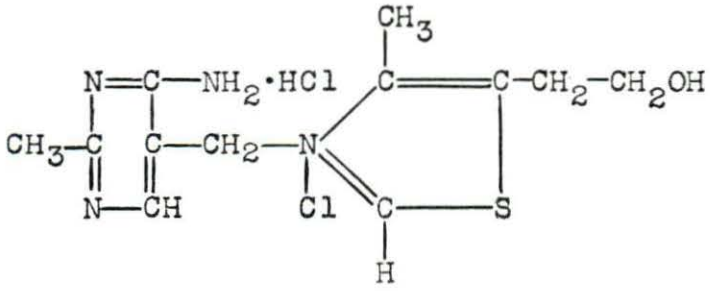
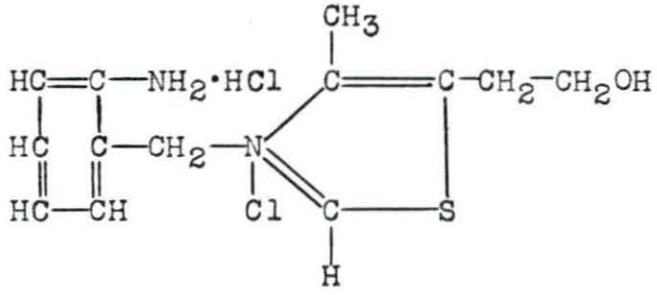
[1] Vitamin test casein was prepared by General Biochemicals, Inc., Chagrin Falls, Ohio. Fat and water soluble vitamins were removed by repeated extractions.

[2] Hawk, P. B., Oser, B. L., and Summerson, W. H. Practical physiological chemistry. 12th ed. p.1273. Philadelphia, The Blakiston Company. 1947.



Thiamine chloride hydrochloride



o-amino-benzyl(3)-4-methyl-5-betahydroxy-ethylthiazoliumchloride hydrochloride

Stock solutions of the supplements were prepared as follows:

I. Double strength diluting fluid containing no thiamine.

Ethyl alcohol	40.0 ml.
Glacial acetic acid	0.2 "
Distilled water	q. s. 100.0 "

II. Double strength thiamine solution containing 10 micrograms/0.1 ml.

Thiamine	25.0 mg.
Ethyl alcohol	100.0 ml.
Glacial acetic acid	0.5 "
Distilled water	q. s. 250.0 "

III. Double strength solution of the thiamine analogue, containing 2 mg./0.1 ml.

0.314 gm. of iodide hydroiodide analogue[1] of thiamine was converted to the chloride hydrochloride and diluted to 10 ml. with distilled water.
0.314 gm. of the I·HI form is equivalent to 0.2 gm. of the Cl·HCl form of the analogue.

IV. Concentrated thiamine solution containing 50 microgm./0.1 ml.

Thiamine	12.5 mg.
Ethyl alcohol	5.0 ml.
Glacial acetic acid	0.025 "
Distilled water	q. s. 25.0 "

Supplements for the various groups were prepared as follows:

Group A. Diluting fluid containing no thiamine. Equal quantities of stock solution I and distilled water.

Group B. Thiamine solution containing 5 microgm./0.1 ml. Equal quantities of stock solution II and distilled water.

Group C. Solution of thiamine and its analogue containing 5 microgm. thiamine and 1 mg. analogue/0.1 ml. Equal quantities of stock solutions II and III.

Group D. Solution of the analogue containing 1 mg./0.1 ml. Equal quantities of stock solutions I and III.

Group E. High thiamine solution containing 50 microgm./0.1 ml. Stock solution IV.

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[1] R. L. Taylor, Department of Physiological Chemistry, crystallized the I·HI analogue of thiamine. J. C. Picken, Veterinary Research Institute, prepared the Cl·HCl analogue from the I·HI analogue for this experiment.

Supplements of 0.1 ml. were given daily to the rats by means of a 0.5 ml. syringe and a blunt 18-gauge needle. The needle was passed through the esophagus into the stomach to prevent loss of the supplement. Groups A, B, C, and D consisted of 2 males and 2 females each. Groups E and F consisted of 3 and 2 males, respectively.

The rats in Groups A to E, inclusive, were weighed daily. Figures 1 to 5, inclusive, are individual growth records of each rat in Groups A to E, respectively. After approximately 40 days, when advanced symptoms of polyneuritis were observed in Groups A and D, two rats from each group, A to D inclusive, and one rat from Group E were sacrificed for thiamine analyses and tissue study. At approximately the same time, 5 micrograms of thiamine were supplemented to the two rats remaining in each group, A to D inclusive. The two remaining rats in Group E were continued on the same diet. One rat in Group F, receiving the commercial fox chow, was destroyed 46 days after the experiment was started. This rat had gained 199 grams in 46 days while a rat in Group E, showing the maximum rate of growth during the same time, had gained 113 grams. The rat in Group F was sacrificed to obtain normal tissues for thiamine analyses and tissue study. Twenty to 30 days after thiamine supplementation was begun, the remaining rats in all groups were destroyed for thiamine analyses and tissue study. At this time the symptoms of polyneuritis were no longer apparent in Groups A and D. The rat in Group F, receiving the commercial

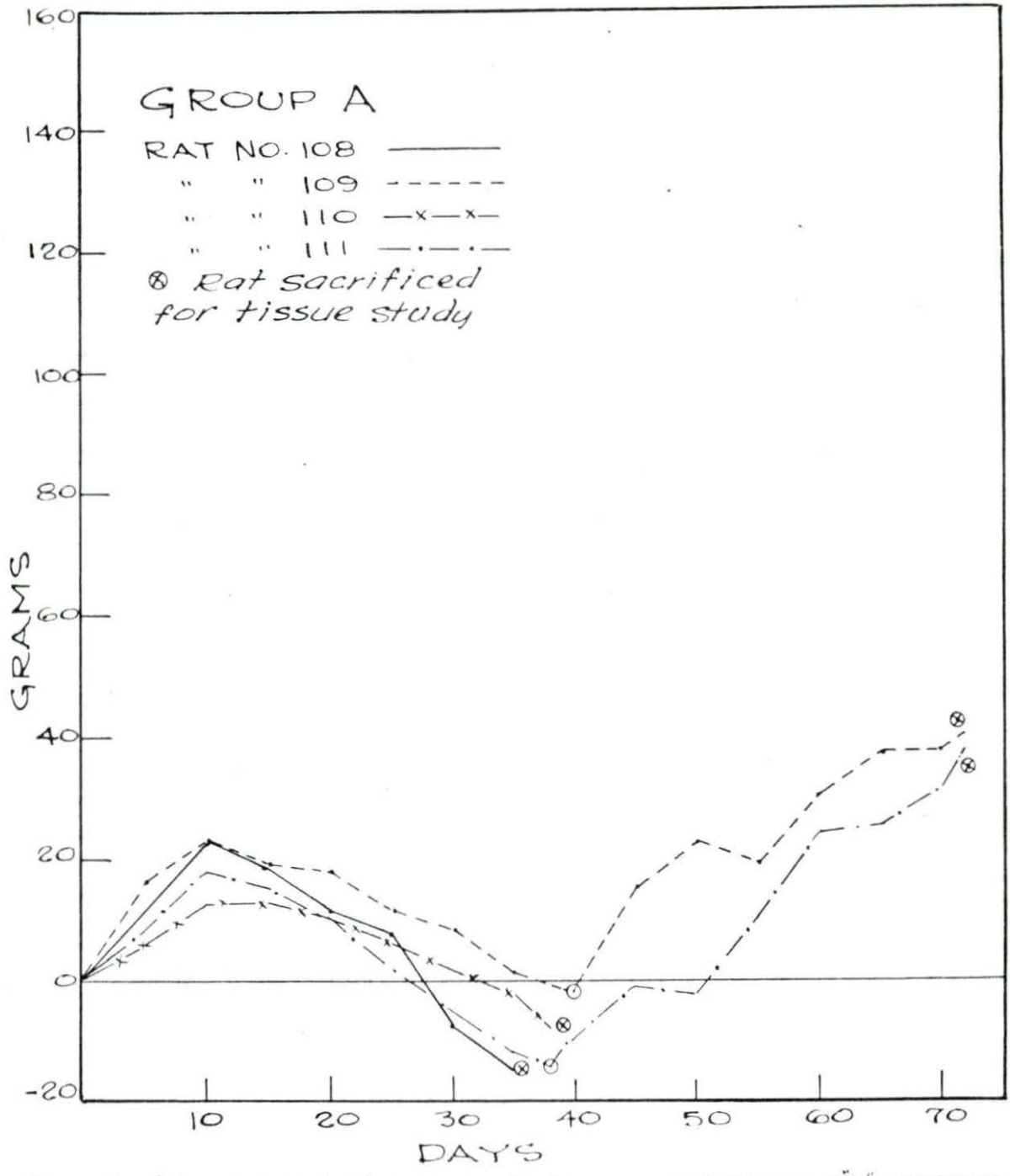


FIG. 1 INDIVIDUAL GROWTH RECORD OF GROUP "A" RECEIVING BASAL DIET PLUS DILUTING FLUID WHICH CONTAINS NO THIAMINE. O = DAILY SUPPLEMENTATION OF 5 MICROGRAMS OF THIAMINE.

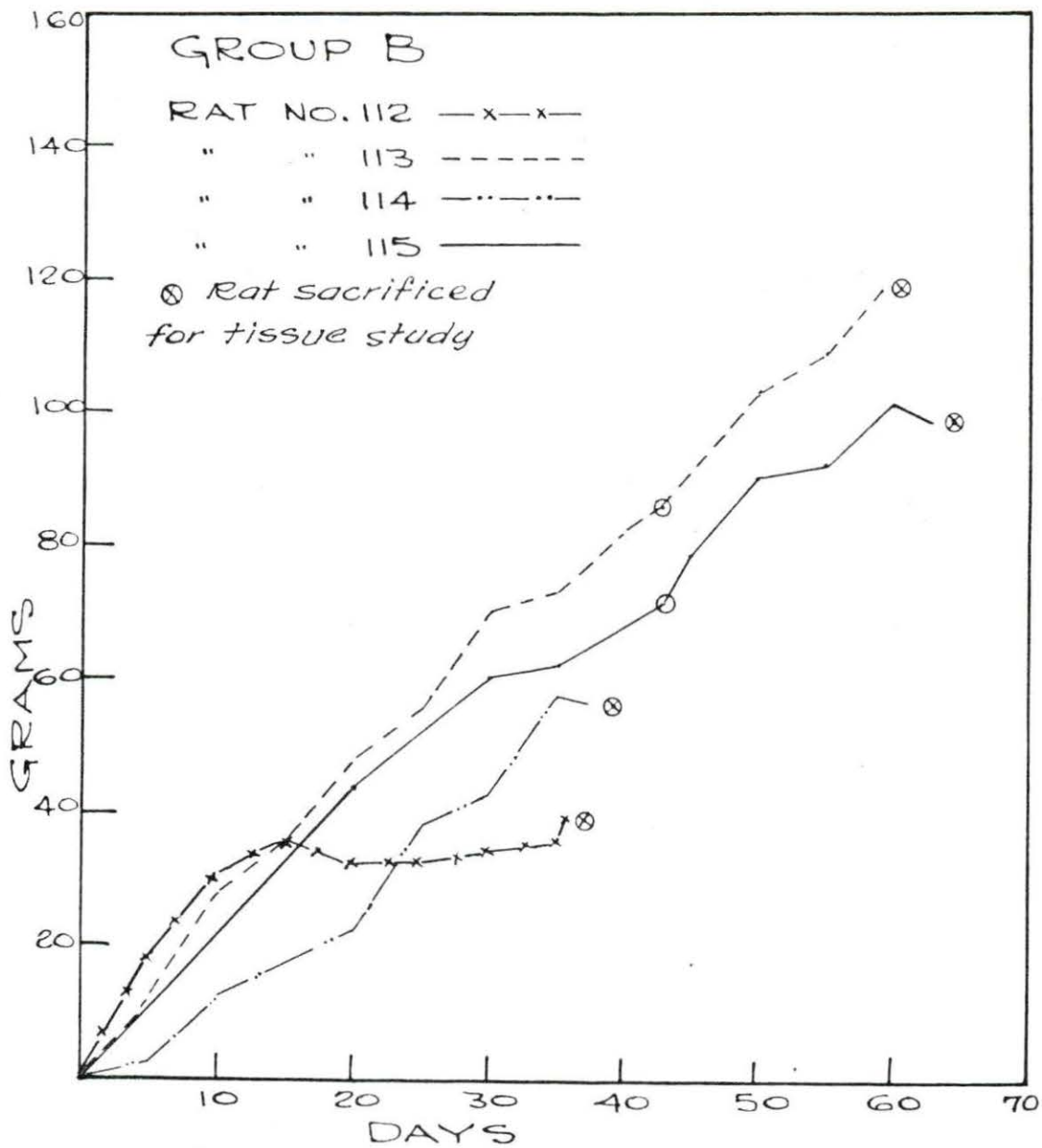


FIG. 2 INDIVIDUAL GROWTH RECORD OF GROUP "B" RECEIVING BASAL DIET PLUS 5 MICROGRAMS OF THIAMINE PER DAY. ⊗ = DAILY SUPPLEMENTATION OF 5 ADDITIONAL MICROGRAMS OF THIAMINE.

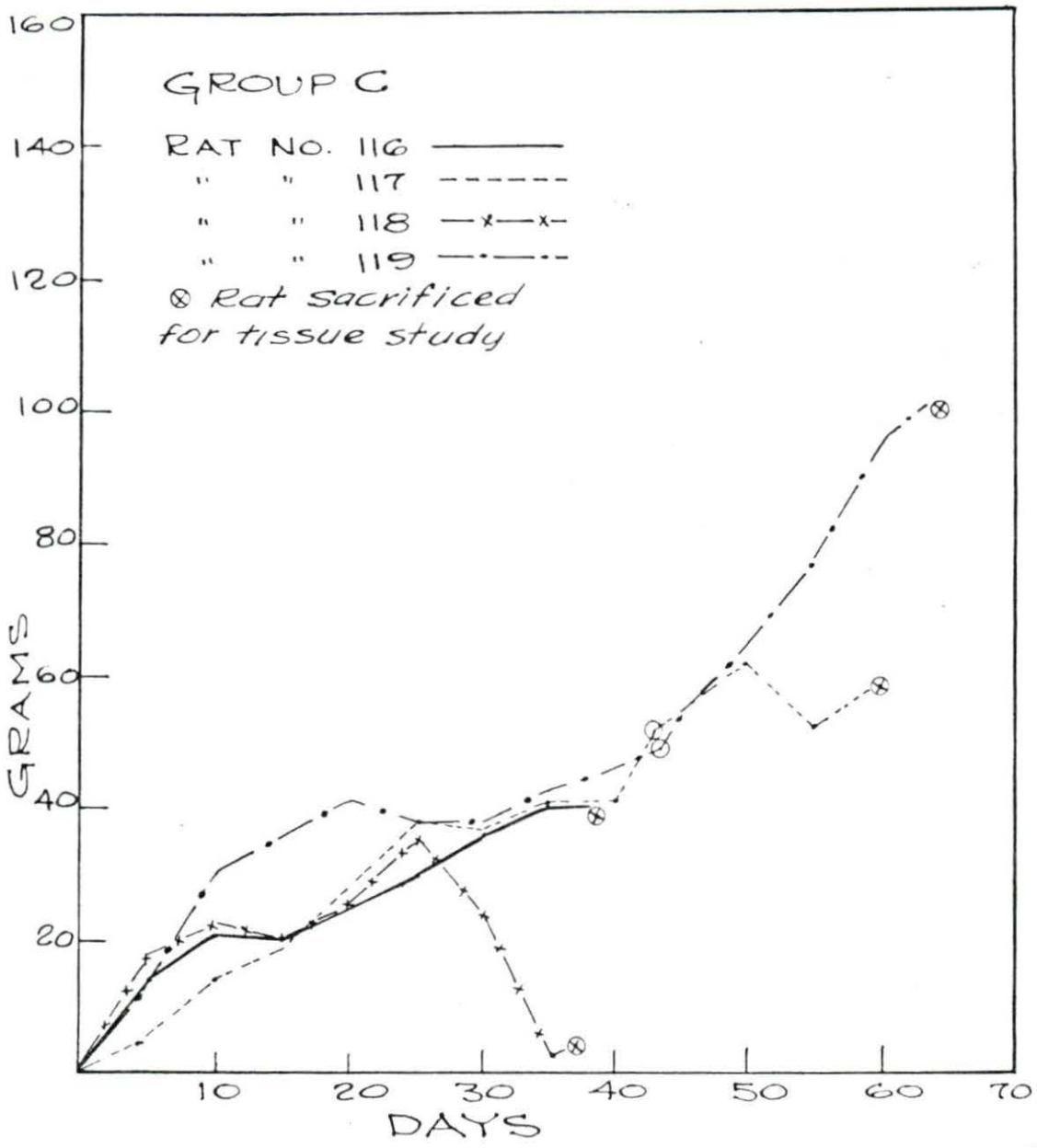


FIG. 3 INDIVIDUAL GROWTH RECORD OF GROUP "C" RECEIVING BASAL DIET PLUS 5 MICROGRAMS OF THIAMINE & 1 Mg. OF THE O-AMINOBENZYL ANALOGUE OF THIAMINE PER DAY. O = DAILY SUPPLEMENTATION OF 5 ADDITIONAL MICROGRAMS OF THIAMINE.

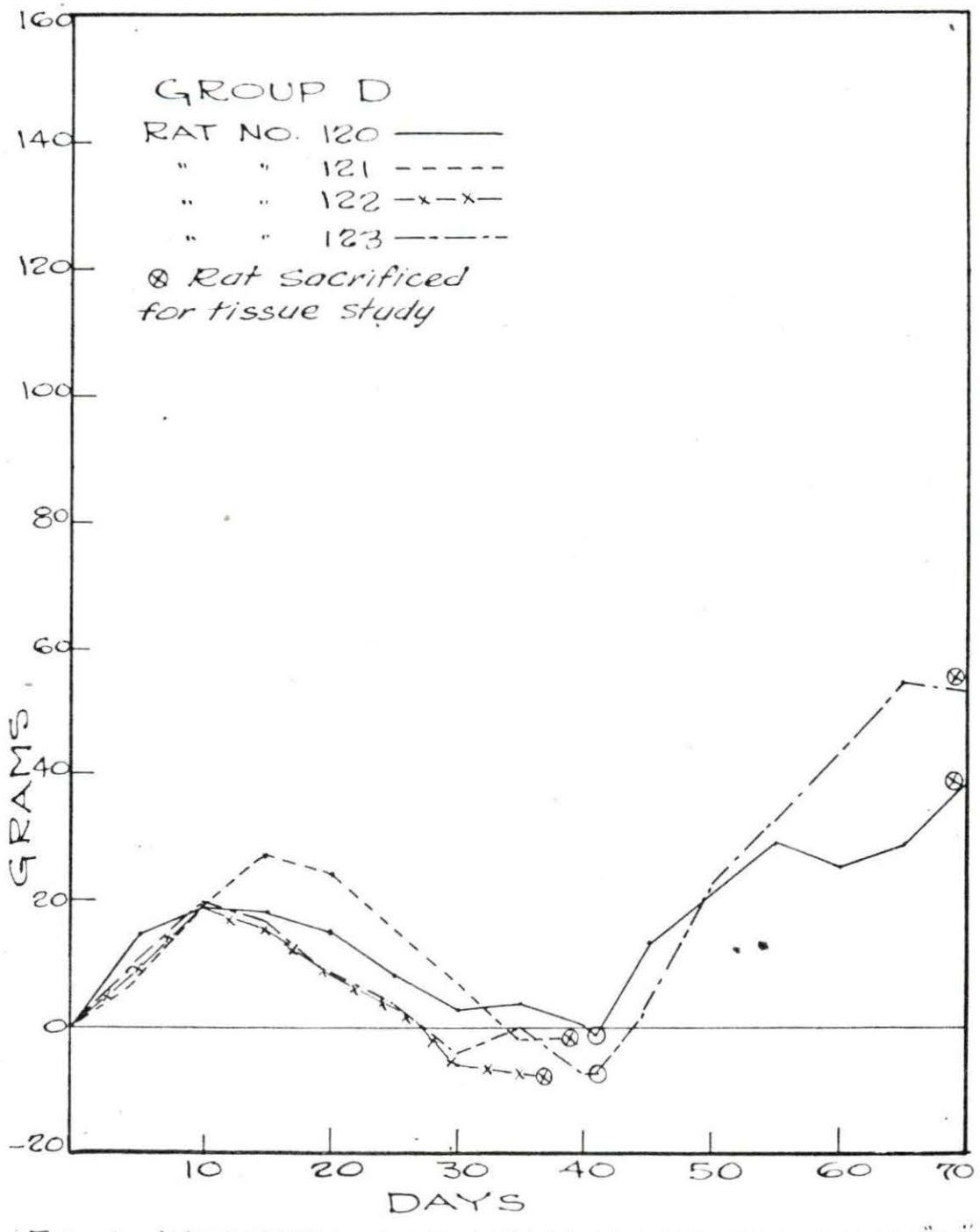


FIG 4 INDIVIDUAL GROWTH RECORD OF GROUP "D" RECEIVING BASAL DIET PLUS 1 Mg. OF THE O-AMINOBENZYL ANALOGUE OF THIAMINE PER DAY. O = DAILY SUPPLEMENTATION OF 5 MICROGRAMS OF THIAMINE.

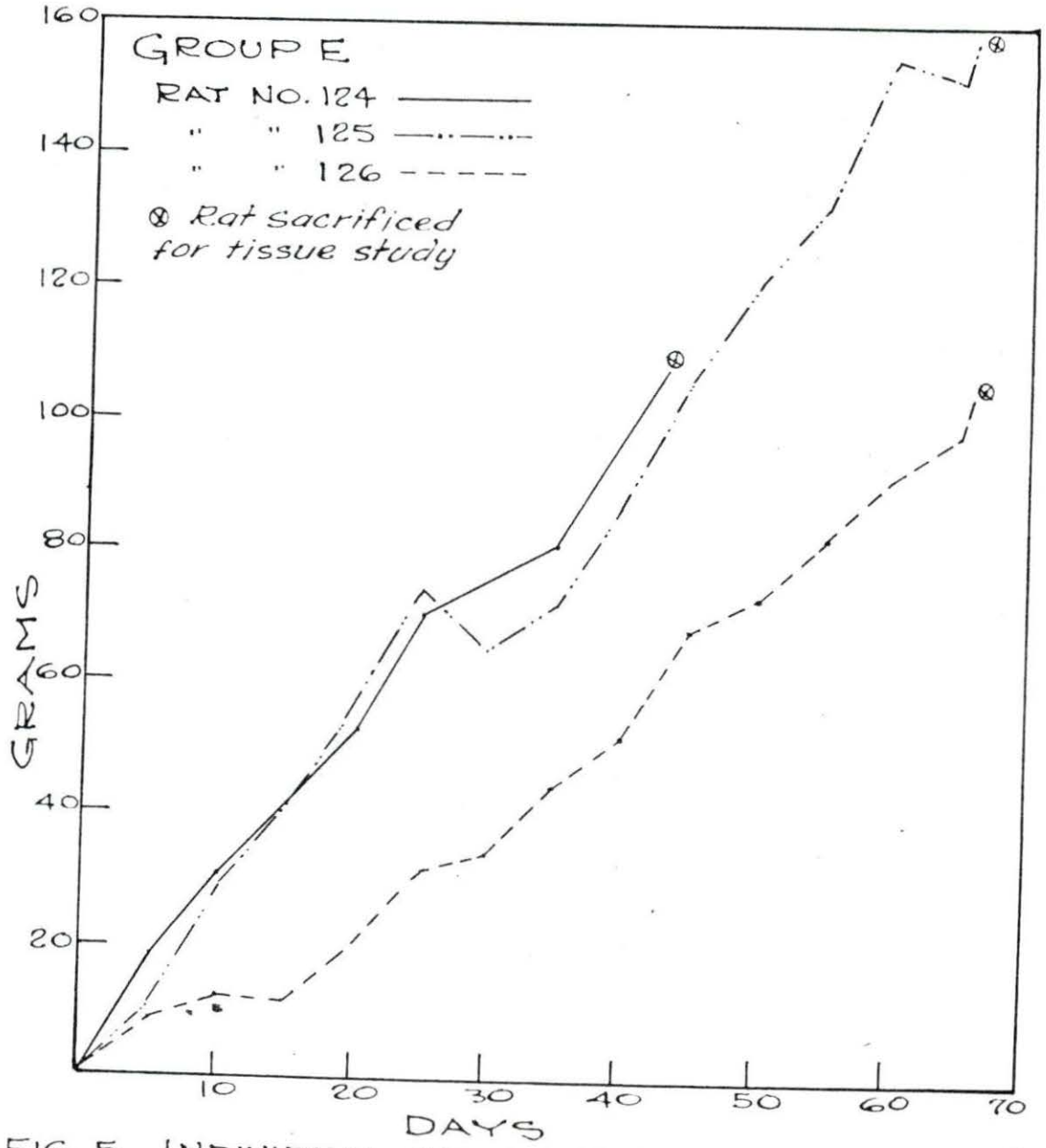


FIG. 5 INDIVIDUAL GROWTH RECORD OF GROUP "E" RECEIVING BASAL DIET PLUS 50 MICROGRAMS OF THIAMINE PER DAY.

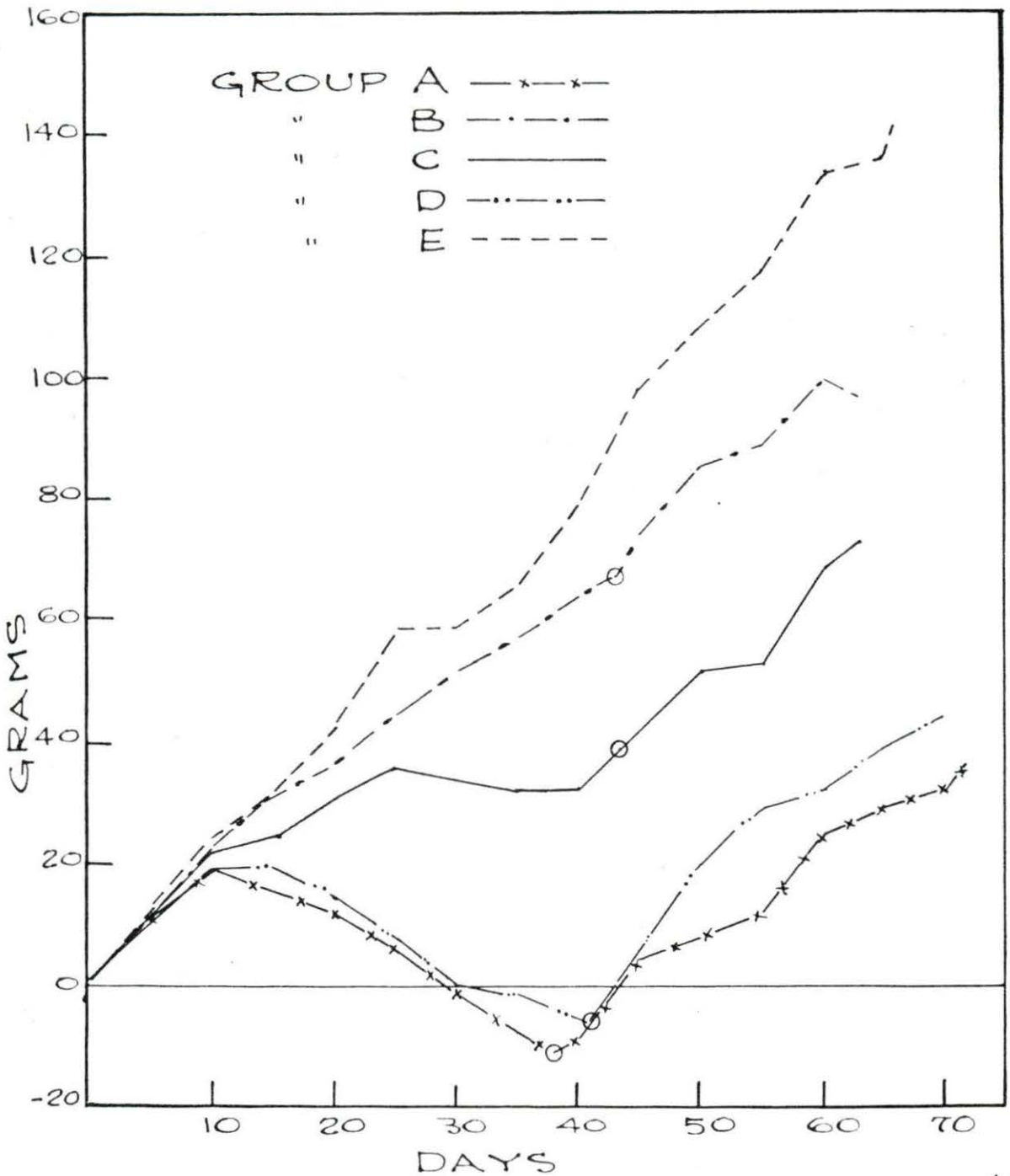


FIG. 6 COMPOSITE GROWTH RECORD OF GROUPS "A" THROUGH "E". O = DAILY SUPPLEMENTATION OF 5 MICROGRAMS OF THIAMINE PER DAY. (FOR INDIVIDUAL GROWTH RECORDS SEE FIG. 1 - 5)

fox chow, had gained 193 grams in 77 days. His weight had been constant the last 30 days.

Figure 6 is a composite growth record of Groups A to E, inclusive. Groups A and D follow closely together. Group E shows the greatest gain followed by Group B. Comparison of the growth curves of Groups B and C illustrates the inhibitory action of the thiamine analogue.

2. Thiamine analyses

The entire series of rats was destroyed by chloroform euthanasia with the exception of rat 108. This rat was found in a moribund condition. The tissues from this animal were removed shortly after death. Thiamine analyses were started on liver, kidney, and skeletal muscle from the thigh, according to the Thiochrome method of Hennessy (51). Two grams of tissue were weighed on a watch glass. The weighed sample was ground in a Waring blender with 75 ml. of 0.1 N H_2SO_4. This mixture was transferred to a 300 ml. Erlenmeyer flask. In the case of large livers weighing 8 grams or more, 150 ml. of 0.1 N H_2SO_4 were used and the mixture transferred to a 500 ml. Erlenmeyer flask. These mixtures were refluxed for 30 minutes. The Thiochrome method, which is described by the Association of Vitamin Chemists (52), was followed during the remainder of the analyses.

Table 4 shows the results of thiamine analyses with supplementary data. It is evident that rats 108, 110, 121, and 122,

Table 4

Thiamine content of rat tissues with supplementary data

Rat No.	Gp.	Tot. Days	Rat Wt. gm.	Gain or Loss gm.	Liver Wt. gm.	Liver Anal. gm.	Kidney Wt. gm.	Kidney Anal. gm.	Muscle Anal. gm.	Microgm. B_1/gm. Liver	Microgm. B_1/gm. Kidney	Microgm. B_1/gm. Muscle
108	A	35	46	-15	1.45	1.20	0.94	0.63	0.43	0.763	1.095	2.645
110	A	38	48	- 8	2.27	2.02	0.93	0.63	1.04	0.388	0.375	0.113
109	A	72	102	40	7.96	4.13	1.57	1.26	1.30	0.937	0.839	0.406
111	A	72	95	37	6.36	3.42	1.41	1.05	1.30	1.542	1.006	0.268
112	B	36	101	39	5.69	5.18	1.14	0.84	1.86	1.352	1.190	0.472
114	B	38	115	56	9.06	3.54	1.60	1.21	2.62	1.397	1.073	0.180
113	B	59	182	118	14.96	3.97	2.13	1.74	1.80		1.068	0.259
115	B	63	158	98	7.46	3.36	1.58	1.16	1.72		1.629	0.136
116	C	38	106	40	7.03	4.41	1.51	1.19	2.27	0.908	0.791	0.311
118	C	36	64	4	4.32	3.86	1.58	1.09	1.30	4.145	1.606	2.500
117	C	59	123	57	8.66	4.20	1.45	1.01	1.62		1.375	0.575
119	C	63	161	99	7.68	3.65	1.82	1.29	1.91		1.457	0.246
121	D	38	66	- 2	3.08	2.78	1.06	0.73	1.23	0.617	1.315	0.536
122	D	36	57	- 7	3.00	2.52	1.05	0.70	1.32	0.551	0.806	0.617
120	D	70	104	38	6.33	4.04	1.55	1.21	1.61	1.585	1.230	0.265
123	D	70	119	53	6.48	4.09	1.46	1.10	1.74	1.147	1.357	0.061
124	E	43	161	107	11.40	4.25	1.28	0.65	2.61	1.751	5.741	1.069
126	E	66	164	104	9.80	3.49	1.66	1.28	1.83	5.107	2.848	0.885
125	E	66	217	157	14.44	4.73	2.41	1.82	2.11	4.025	2.782	0.959
127	F	77	278	193	14.04	3.66	2.69	2.06	2.50	7.958	4.973	1.654
128	F	46	281	199	13.58	4.71	2.61	2.04	2.45	2.959	4.674	1.708

receiving a thiamine-free diet, have a very small amount of thiamine in their tissues. In these animals the thiamine concentration in the liver is equal to or less than that of the other tissues. Rats 125, 126, and 127, receiving an optimum amount of thiamine over a longer period of time, approach the cencentrations of thiamine in normal rat tissues. The remaining rats show a thiamine deficiency as a result of an insufficient amount of thiamine in their diets.

Figure 7 illustrates the effect of thiamine supplementation in Groups A to D, inclusive. The thiamine content of the tissues from rat 118 are not recorded in Figure 7. The pathological changes in the tissues, as described later, show that an unknown etiological factor caused the rapid loss in weight as seen in Figure 3. Groups E and F show the variations of thiamine concentration in the tissues of growing animals over a period of approximately 28 days (Figure 7). Thus, by this experiment, normal animals have the highest concentration of thiamine in the liver followed by that in the kidney and skeletal muscle.

3. Autopsy findings

Rats 108, 110, 121, and 122, receiving a thiamine-free diet, showed considerable dehydration atrophy. It was particularly noticeable in rat 108. The muscles in this rat were atrophied to the extent that sufficient quantities for thiamine analysis and tissue study were difficult to obtain. The heart

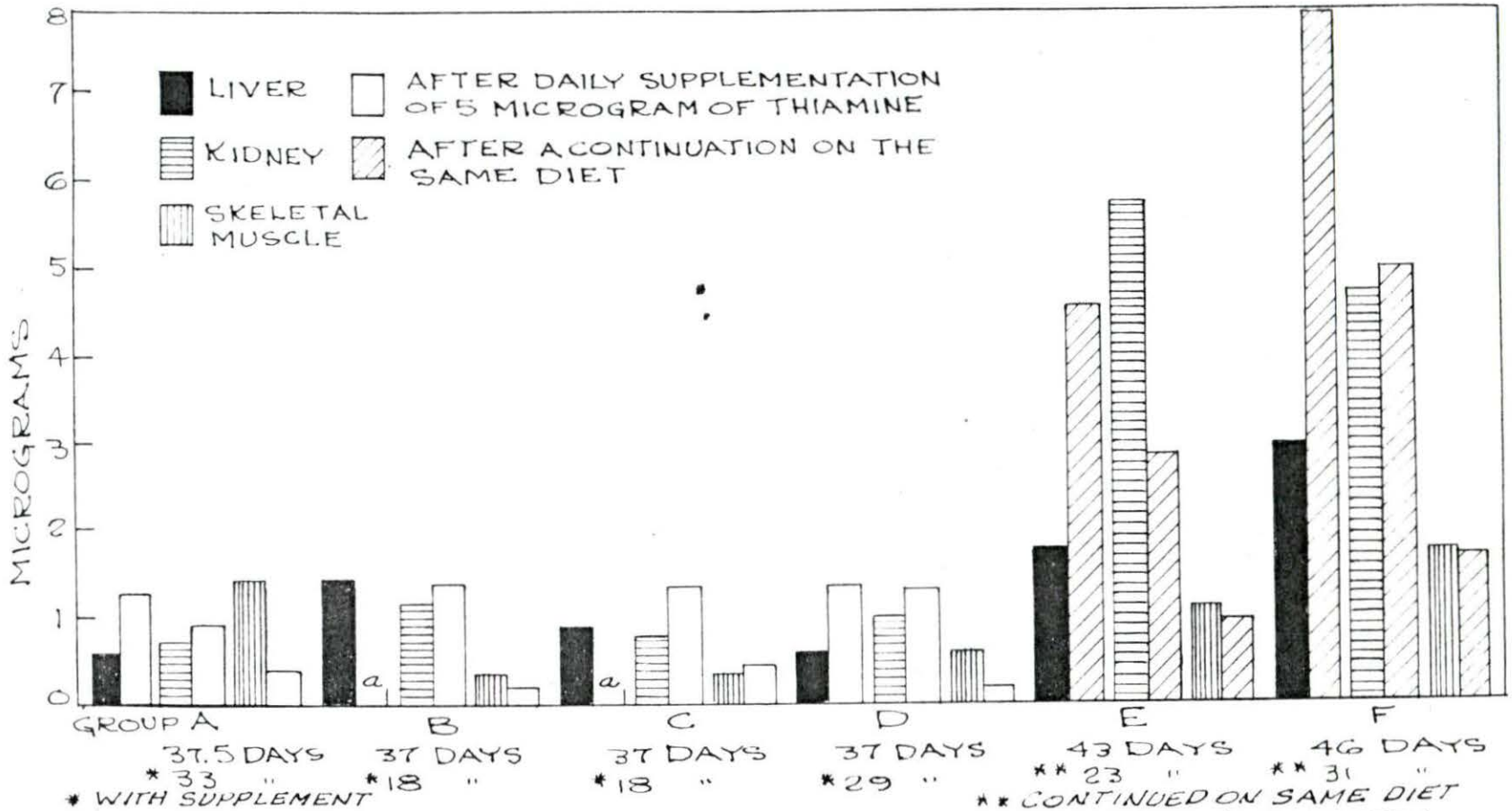


FIG. 7 COMPARATIVE THIAMINE ANALYSES OF LIVER, KIDNEY, & SKELETAL MUSCLE EXPRESSED IN MICROGRAMS PER GRAM OF TISSUE.

GROUP A - Received no Thiamine
" B - " 5 Micrograms of Thiamine Daily.
" C - " " " " " and 1 Mg. of o-aminobenzyl. Analogue of Thiamine Daily.
" D - Received 1 Mg. of o-aminobenzyl Analogue of Thiamine Daily.
" E " 50 Micrograms of Thiamine Daily.
" F " A Commercial Fox chow ad lib.
a = Low Readings on the Photo Fluorometer were obtained by using an Insufficient Quantity of Enzyme Suspension in the Analyses.

of rat 110 was soft and flabby with a rounded apex. Hydropericardium was present.

The liver of rat 112 was light brown to yellow in color. The heart of rat 119 was soft and flabby. The tissues of the remaining animals appeared normal on autopsy.

4. Polarized light

A section of sciatic nerve from each rat was placed in 10 per cent formol-saline solution. Calcium carbonate had been added to neutralize the solution. On the following day, longitudinal sections, 10 microns in thickness, were made of the nerve with the freezing microtome. The sections were floated in water, placed on a clean slide, mounted in glycerin, and covered with a clean cover glass. At this time the sections were examined between crossed Nicol prisms of a polarizing microscope. Photomicrographs were taken of the nerves at the point of greatest birefringence. Polychrome plates were used. Wratten B filter No. 58, with Eastman Kodak X-ray developer, gave the best results. It was very difficult to obtain contrast in the negative until the Wratten B filter and the X-ray developer were used. No. 4 Azo paper was used in the printing with Eastman Kodak Universal M-Q developer.

Figure 8a shows severe myelin degeneration of the nerve fibers. The normal myelin has broken down into triglycerides which are isotropic. Normal myelin, being anisotropic, appears white in the photomicrographs.

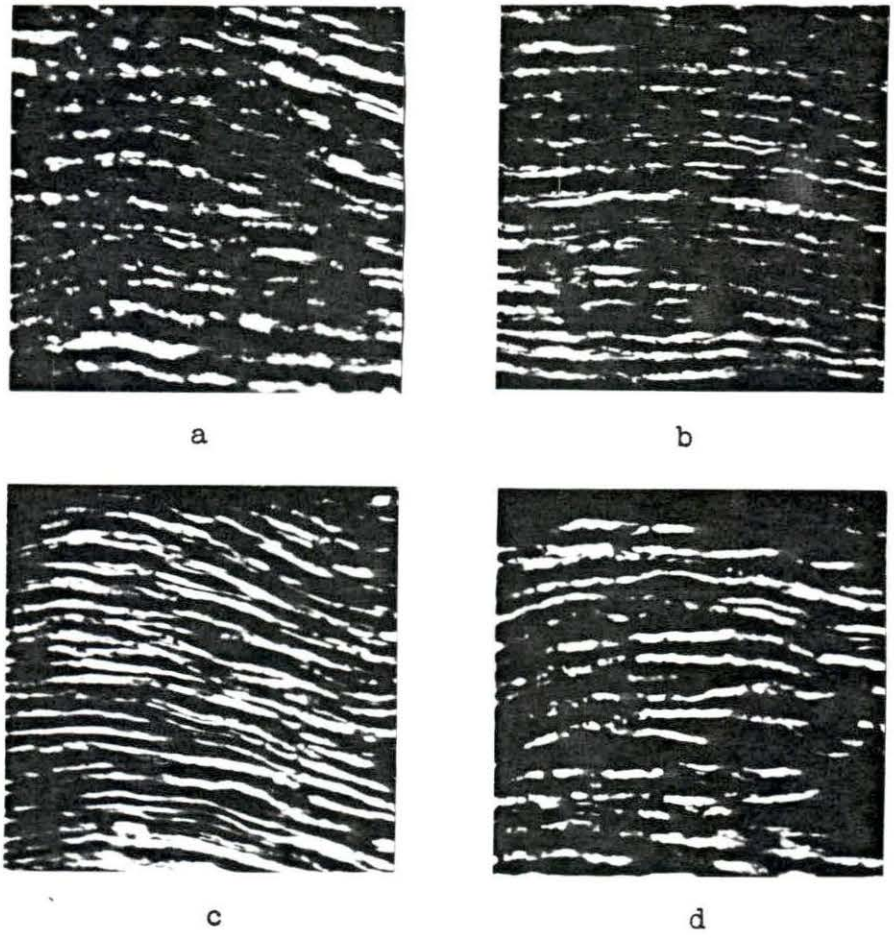


Fig. 8. Sciatic nerves from Group "A".
a and b from rats 108 and 110
c and d from rats 109 and 111
Polarized light between crossed Nicol prisms. X500.

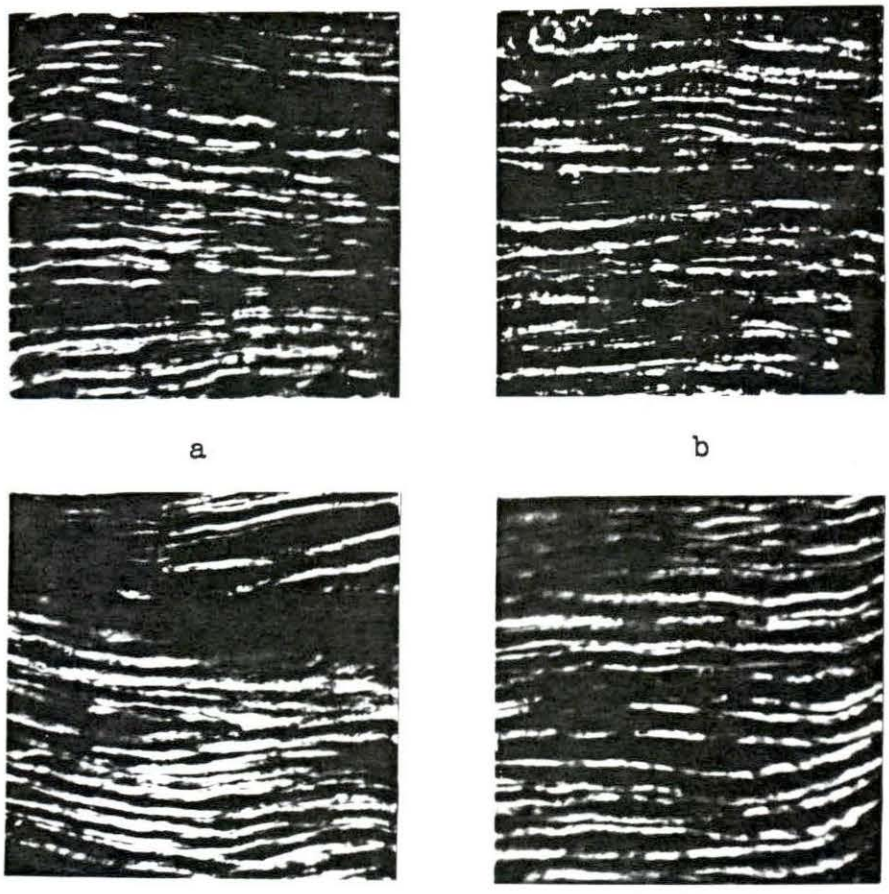
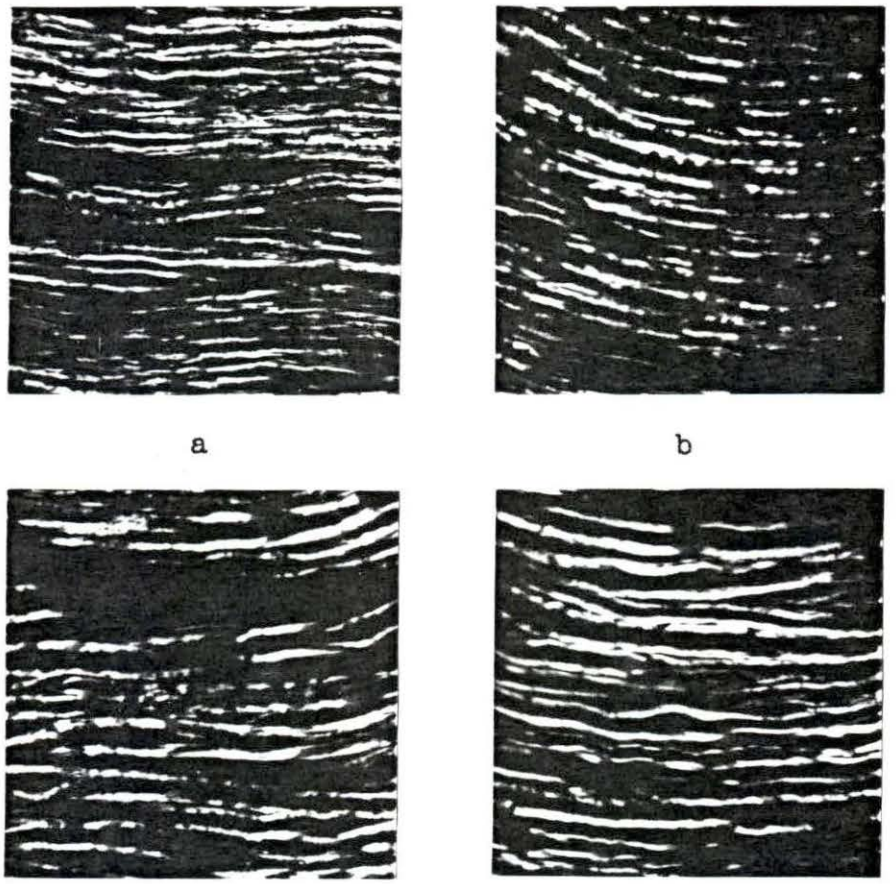


Fig. 9. Sciatic nerves from Group "B".
a and b from rats 112 and 114
c and d from rats 113 and 115
Polarized light between crossed Nicol prisms. X500.



a b

c d

Fig. 10. Sciatic nerves from Group "C".
a and b from rats 116 and 118
c and d from rats 117 and 119
Polarized light between crossed Nicol prisms. X 500.

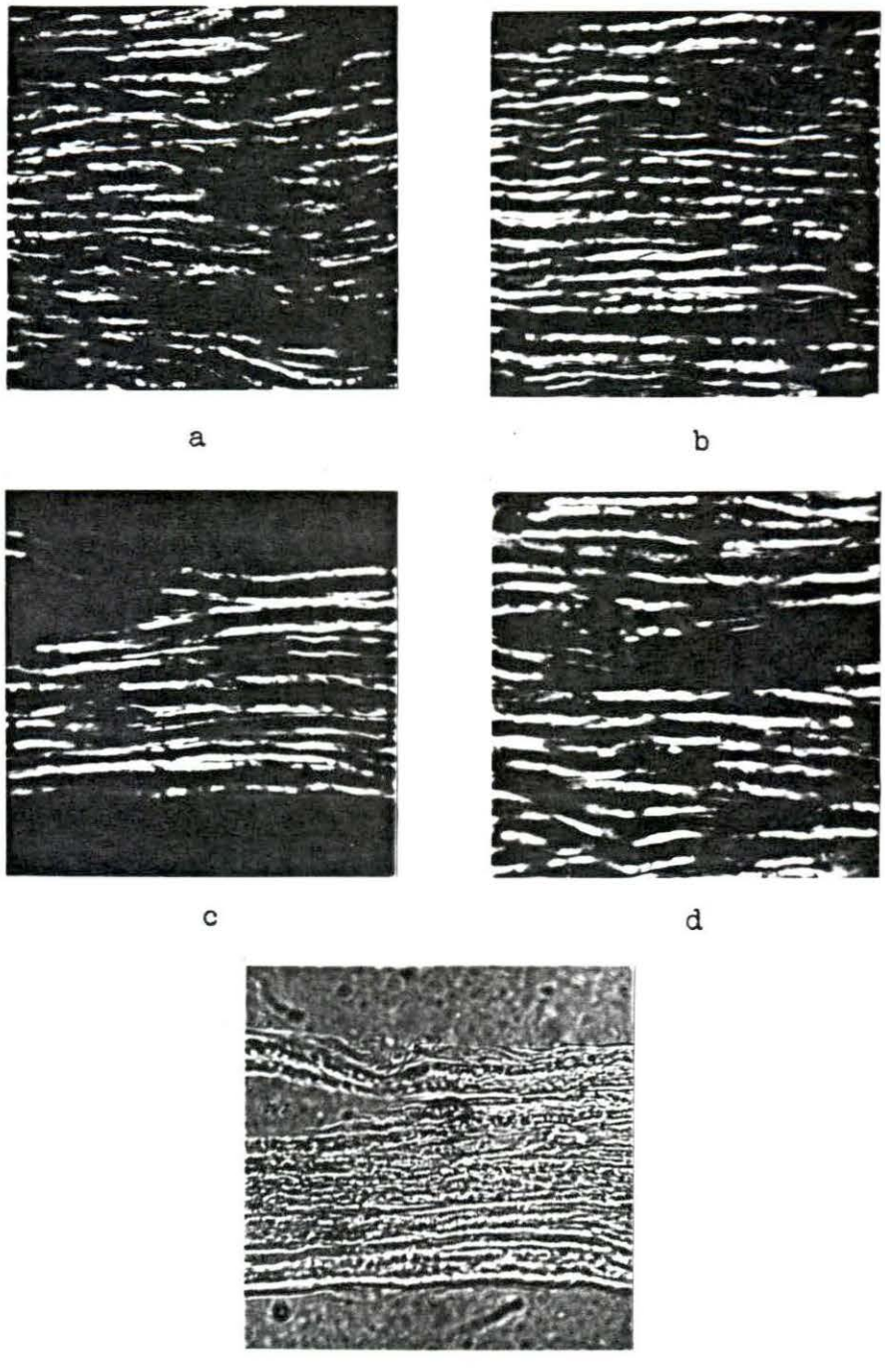


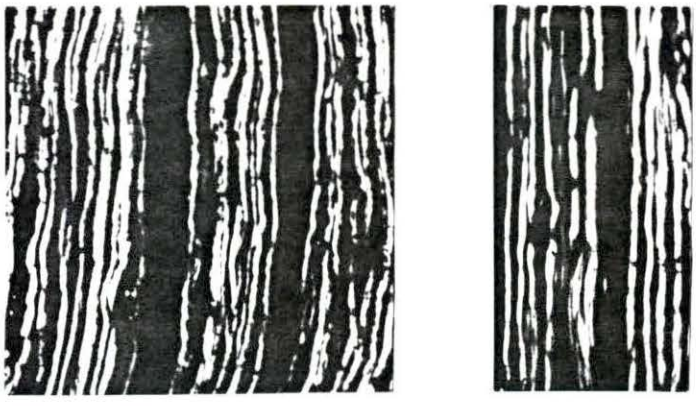
Fig. 11. Sciatic nerves from Group "D".
a and b from rats 121 and 122
c and d from rats 120 and 123
Polarized light between crossed Nicol prisms. e from rat 120 with polarized light between uncrossed Nicol prisms. X500.

a

b

c

Fig. 12. Sciatic nerves from Group "E".
a from rat 124
b and c from rats 125 and 126
Polarized light between crossed Nicol prisms. X500.



a b

Fig. 13. Sciatic nerves from Group "F".
a from rat 127
b from rat 128
Polarized light between crossed Nicol prisms. X500.

Figures 13a and 13b are photomicrographs of nerves from the rats receiving the commercial fox chow. These nerves reveal the least degeneration of the entire series of rats. There are a few breaks in the myelin sheaths other than the nodes of Ranvier. The latter are isotropic. Figure 12 of Group E reveals more degeneration than Figure 13 but less degeneration than the other groups. Supplementation with 5 micrograms of thiamine to rats 109, 111, 113, 115, 117, 119, 120, and 123 produced slight improvement in the degenerated nerves as shown in Figures 8 to 11, inclusive. Figures 11c and 11e are photomicrographs showing myelinated nerve fibers between crossed Nicol prisms at the point of greatest birefringence and between uncrossed Nicol prisms, respectively.

5. Marchi method

Small pieces of liver, kidney, heart, sciatic nerve, skeletal muscle, brain, and spinal cord were placed in Müller's fluid. The technique of Mallory (53) was followed. The tissues were kept in Müller's fluid 14 days. They were then placed in a solution containing 2 parts of Müller's fluid and 1 part of a 1 per cent aqueous solution of osmic acid for 14 days. Dioxane was used for dehydration. It was found that alcohol caused the tissues to become hard and brittle which made them unsuitable for sectioning. The tissues were embedded in Tissuemat and sectioned 20 microns in thickness. Tissuemat is a product manufactured by Fisher Scientific

Company. It is superior to paraffin as an embedding medium when thin sections are required.

Table 5 is a summary of the pathological changes occurring in the tissues that were stained by the Marchi method. The degeneration of the myelin sheaths in the sciatic nerves checks with the polarized light studies. Fatty degeneration of the liver was present in the hepatic cells around the central veins in all cases except in those rats receiving the commercial fox chow, the thiamine-free diets, and in rat 115 receiving a thiamine-low diet. Myelin degeneration was quite general in the spinal cords of all the rats. The brain showed myelin degeneration in all cases except in those animals receiving an optimum amount of thiamine and in rats 115, 116, and 119. Pathological changes in the kidney were found only in rat 118.

Figures 14, 15, and 16 are photomicrographs of the sciatic nerve, spinal cord, and medulla of rat 108, receiving no thiamine. Myelin degeneration is very prominent in these sections. Figure 17 shows fatty degeneration in the liver of rat 111 which had received a supplement of 5 micrograms of thiamine. The livers of rats in the same group receiving no thiamine did not show fatty degeneration. Figure 18 shows myelin degeneration in the medulla. This section was made of rat 112, receiving a suboptimal level of thiamine. Fatty degeneration in livers from the rats of Group E is well-marked as revealed in Figure 19. Figures 20 and 21 show a mild degeneration of the myelin sheaths in the sciatic nerve and spinal

Table 5

Results of the Marchi staining method

Rat No.	Liver	Kidney	Heart	Sciatic Nerve	Spinal Cord	Brain
108M	0	0	0	++	+++	+
110F	0	0	0	+	++	+
109M	+	0	0	Trace	+	Trace
111F	+	0	0	Trace	+	Trace
112M	++	0	0	+	++	+
114F	+++	0	0	Trace	++	Trace
113M	+	0	0	Trace	+	Trace
115F	0	0	0	Trace	+	0
116M	++	0	0	Trace	+	0
118F	+	Trace		+		Trace
117M	++	0	0	Trace	+	Trace
119F	+	0	0	Trace	0	0
121M	Trace	0	0	+	++	Trace
122F	0	0	0	+	++	Trace
120M	+	0	0	0	Trace	Trace
123F	Trace	0	0	Trace	+	Trace
124M	+++	0	0	+	+	0
125M	+++	0	0	Trace	+	0
126M	++	0	0	Trace	+	0
127M	0	0	0	Trace	Trace	0
128M	0	0	0	+	+	0

M, male; F, female.

Fig. 14. Sciatic nerve of rat 108 showing myelin degeneration. Marchi. X500.

Fig. 15. Spinal cord of rat 108 showing myelin degeneration. Marchi. X500.

Fig. 16. Medulla of rat 108 showing myelin degeneration. Marchi. X500.

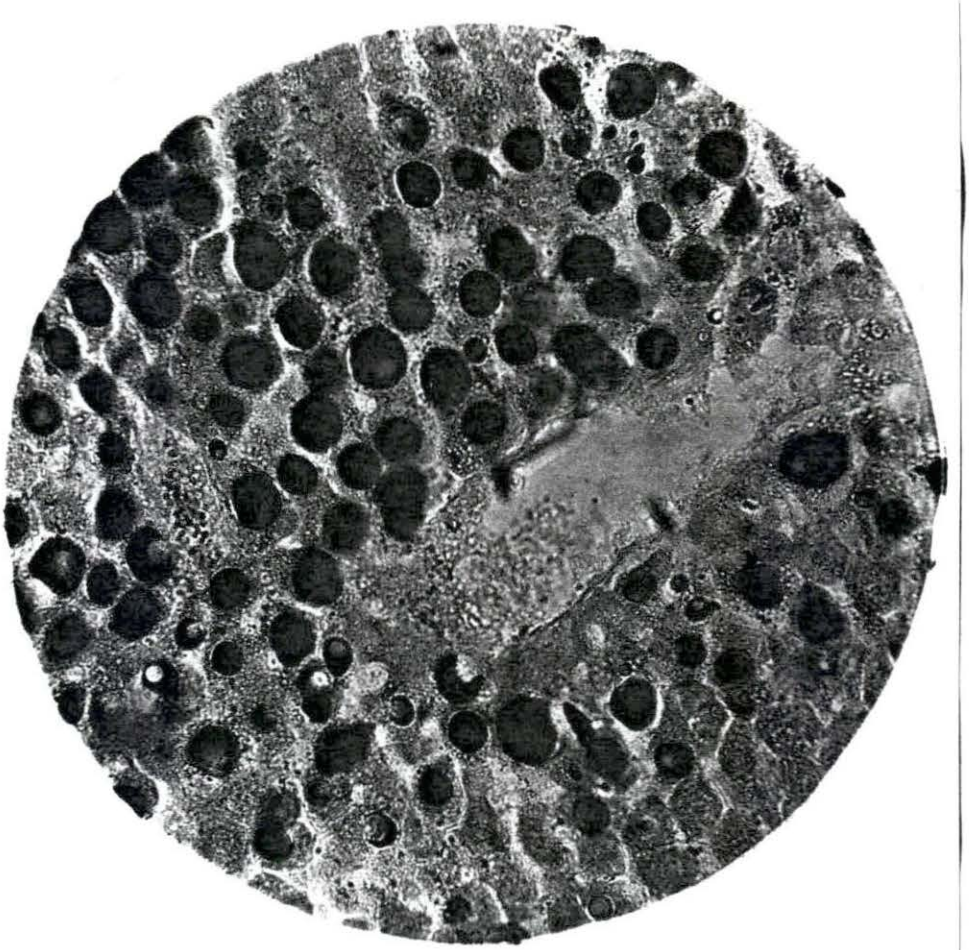


Fig. 17. Liver of rat 111 showing fatty degeneration. Marchi. X300.

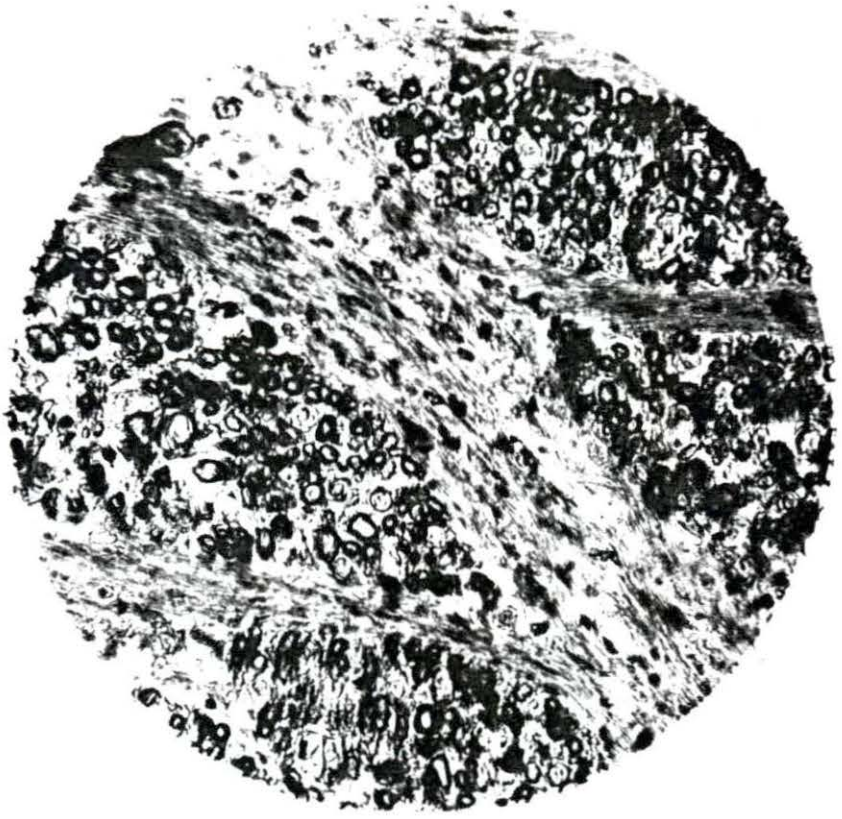


Fig. 18. Medulla of rat 112 showing myelin degeneration. Marchi. X500.

Fig. 19. Liver of rat 124 showing fatty degeneration. Marchi. X300.

Fig. 20. Sciatic nerve of rat 128 showing mild degeneration of the myelin sheaths. Marchi. X500.

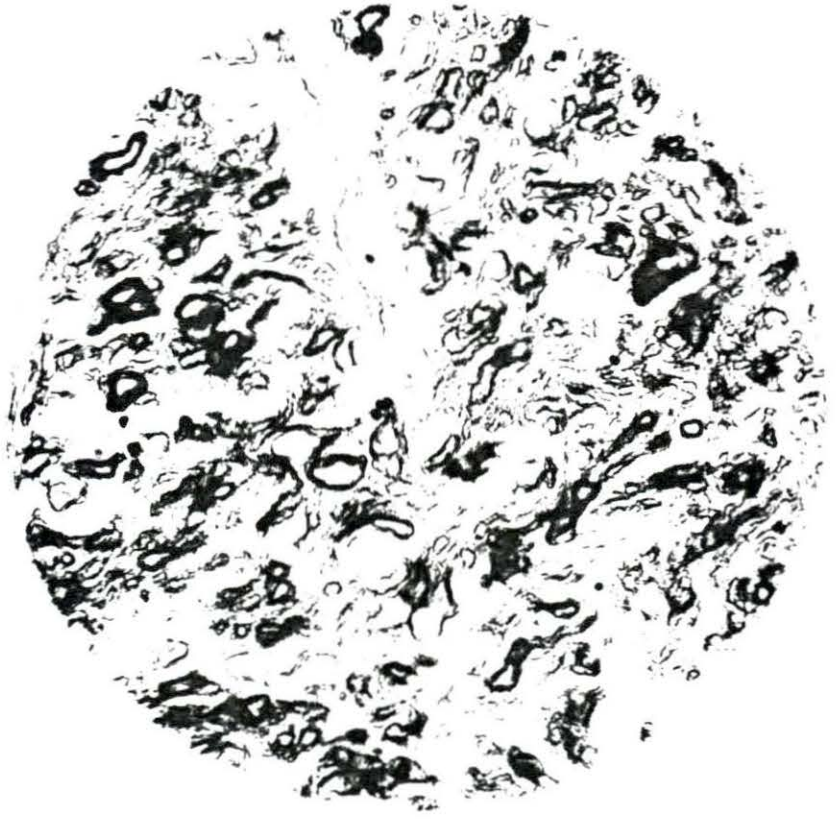


Fig. 21. Spinal cord of rat 128 showing a mild degeneration of the myelin sheaths. Marchi. X500.

cord of rat 128. The myelin degeneration in the nerve corresponds very closely to the mild degeneration that was observed with the polarized light (Figure 13b).

6. Hematoxylin and triosin stain

Pieces of tissue corresponding to those taken for the Marchi method were fixed in 10 per cent formol-saline solution. Calcium carbonate was added to neutralize the solution. After 24 hours of fixation, the tissues were dehydrated with three changes of dioxane. The tissues were embedded in Tissuemat. Sections, 6 microns in thickness, were placed over a film of distilled water on albuminized slides. The slides were oven-dried at 45° C. The sections were stained with hematoxylin and triosin as follows:

1.	Xylene #1 (to remove Tissuemat)	3 minutes
2.	" #2 " " "	3 "
3.	95% alcohol #1	3 "
4.	95% " #2	3 "
5.	70% "	3 "
6.	Wash in distilled water	5 "
7.	Stain in Delafield's hematoxylin[1] (20 parts of stock solution to 80 parts of sat. ammonium alum sol.)	10 " or until differentiated
8.	Wash in distilled water	5 minutes
9.	Wash in tap water	30 "
10.	Wash in distilled water	2 "
11.	70% alcohol	2 "
12.	90% "	2 "
13.	Triosin (1% in 90% alcohol)	45 seconds or until differentiated
14.	95% alcohol #1	1 minute

[1] Mallory, F. K. Pathological technique. p.71-72. Philadelphia, W. B. Saunders. 1938.

15.	95% alcohol #2	1 minute
16.	Carbol xylene #1	3 minutes
17.	" " #2	3 "
18.	Xylene #3	3 "
19.	" #4	10-20- "
	Mount in Clarite	

In thiamine deficiency, connective tissue is likely to replace more specialized tissue. Since triosin differentiates connective tissue from other tissue by staining the former orange-red, it was used instead of eosin.

The tissues of the rats on a thiamine-free diet showed considerable dehydration atrophy. Figure 22 illustrates this condition as it occurred in the liver of rat 108. Pathological changes in the livers of rats 110, 121, and 122 were similar.

Fatty degeneration, indicated by the appearance of large clear vacuoles in the hepatic cells, was observed in rats receiving thiamine-deficient diets and also in those receiving 50 micrograms of thiamine daily. These vacuoles correspond identically in size and location to the large black droplets found in the tissues stained by the Marchi method. Figure 24 is a photomicrograph demonstrating fatty degeneration in the liver of rat 109. This degeneration occurred when the animal was supplemented with 5 micrograms of thiamine daily for 32 days, after having been on a thiamine-free diet for 40 days.

Nerve cells in the medulla of all the rats, except those receiving the commercial fox chow, showed various degrees of degeneration. The nerve cells in the medulla of rat 108, which are shown in Figure 23, have lost all detail with the exception

of two normal cells at the right of the picture. Figure 8a is a photomicrograph of the sciatic nerve of this rat taken with polarized light, whereas Figures 14, 15, and 16 are from Marchi preparations taken at the same magnification. The degree of degeneration demonstrated by the two methods is comparable.

The skeletal muscles of the rats receiving a thiamine-free diet were atrophied, accompanied by an increase of connective tissue. The rats receiving the 5 microgram supplements of thiamine in Groups A and D showed less atrophy, an increase of connective tissue, and a loss of muscle striations. Some of the muscle fibers from the eight rats in Groups B and C were degenerated.

Degenerative changes in the muscle fibers of rat 114 are illustrated in Figure 25. The light areas in the photomicrograph represent the degenerated blue-stained fibers in contrast to the pink-stained normal fibers. Advanced degenerative changes of the skeletal muscle of rat 117 are seen in Figure 26. Calcification, hyaline degeneration, and connective tissue proliferation are present. Figure 26 suggests that the degenerating muscle fibers, as shown in Figure 25, may eventually become hyalinized and calcified. Group E, receiving 50 micrograms of thiamine daily, showed some hyalinization of the muscle cells as seen in Figure 29 (rat 124).

The kidneys from the rats in Groups A and D are atrophied. Albuminous degeneration is present in the tubules throughout the kidney. This degenerative process occurs in the entire

series of rats. The kidney tubules of the rats on the commercial fox chow reveal less albuminous degeneration than the other groups. Calcified areas in the cortex of the kidneys are found in rats 114 (Group B), 117 and 118 (Group C), and 124 (Group E). These areas are found close to the capsule. Figure 27 shows this condition in rat 117. Hyaline casts are present in the kidney tubules of the rats from Groups B, C, and E. These casts appear in the tubules of the medulla as shown in Figure 28. Many degenerative changes have occurred in the kidney of rat 118. In addition to the calcified areas in the cortex and hyaline casts in the tubules of the medulla, there are areas of necrosis in the cortex which have not become calcified. Proliferating connective tissue can be seen around the necrotic areas. The kidney from this rat shows more pathological changes than the other kidneys in this series of rats. These alterations may account for the rapid loss in weight of rat 118, as seen in Figure 3. The Marchi method demonstrates a trace of fatty degeneration in the kidney of this rat while the other kidneys reveal no fatty degeneration.

The heart appeared normal in these animals with the exception of a few hemorrhages. Pathological changes in the sciatic nerve and spinal cord could not be demonstrated with hematoxylin and triosin stain.

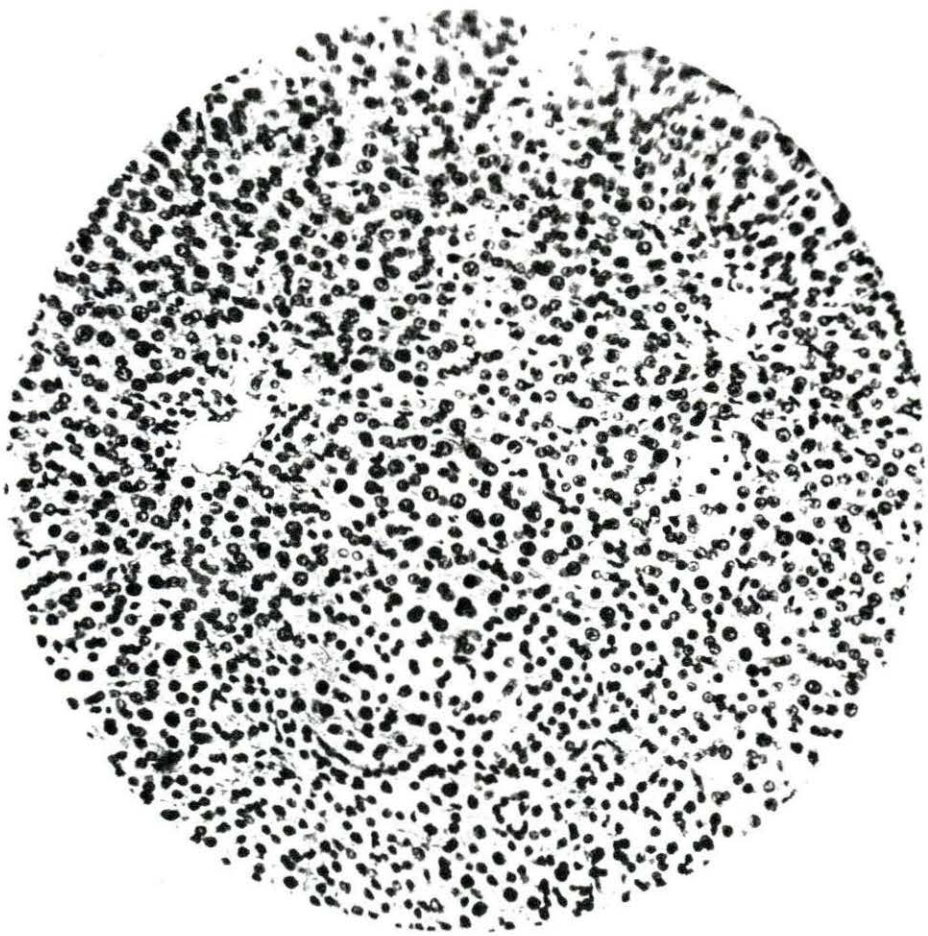


Fig. 22. Liver of rat 108 showing dehydration atrophy. Hematoxylin and triosin. X300.

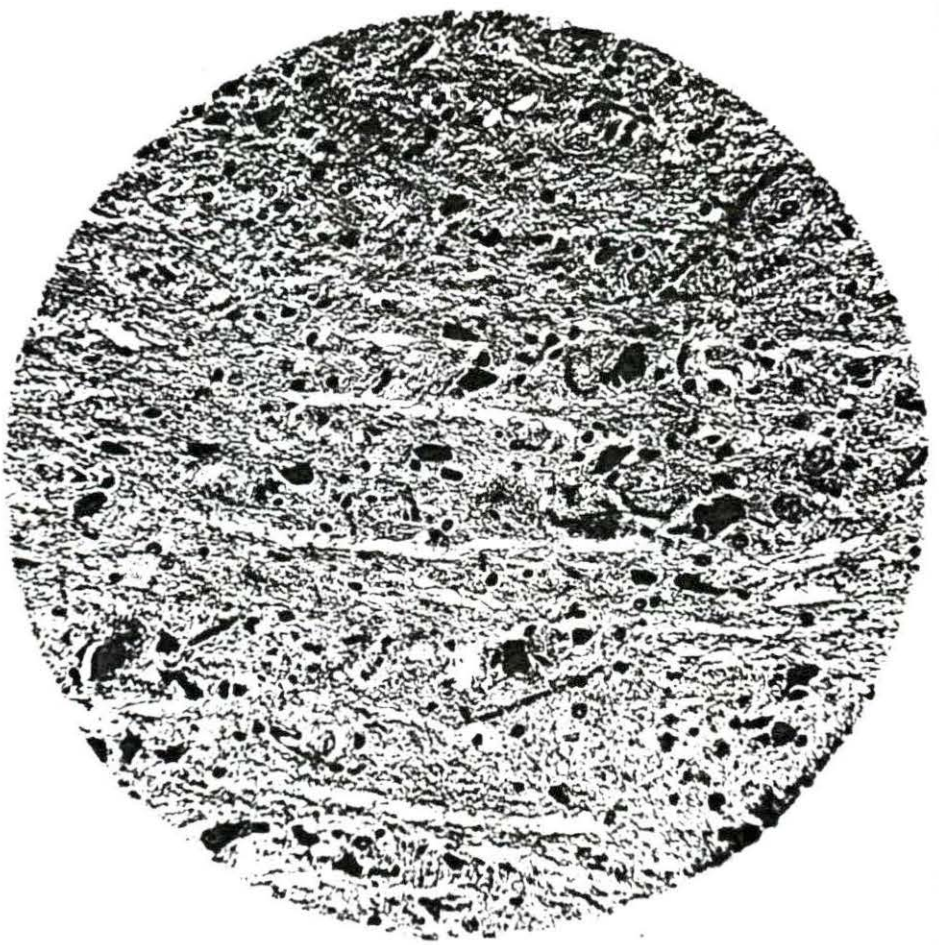


Fig. 23. Medulla of rat 108 showing degenerated nerve cells. Hematoxylin and triosin. X300.

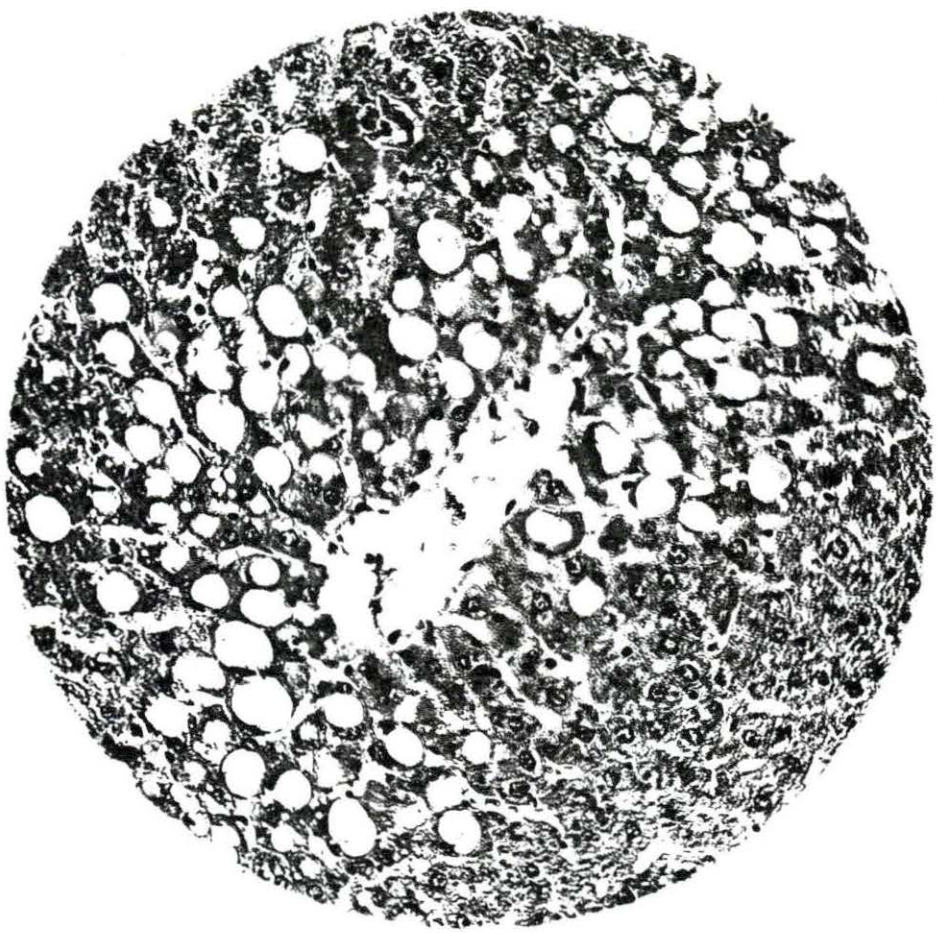


Fig. 24. Liver of rat 109 showing fatty degeneration. Hematoxylin and triosin. X300.

Fig. 25. Skeletal muscle of rat 114 showing degeneration. Hematoxylin and triosin. X300.

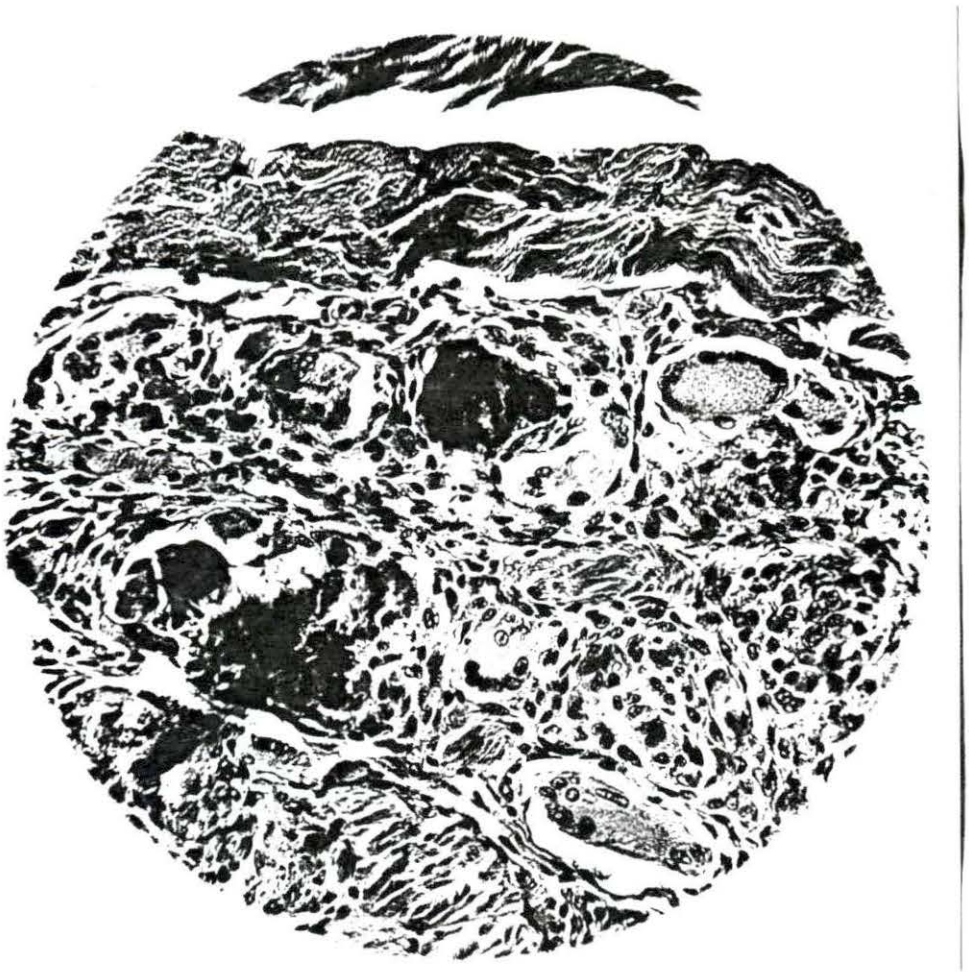


Fig. 26. Skeletal muscle of rat 117 showing hyaline degeneration and calcification. Hematoxylin and triosin. X300.

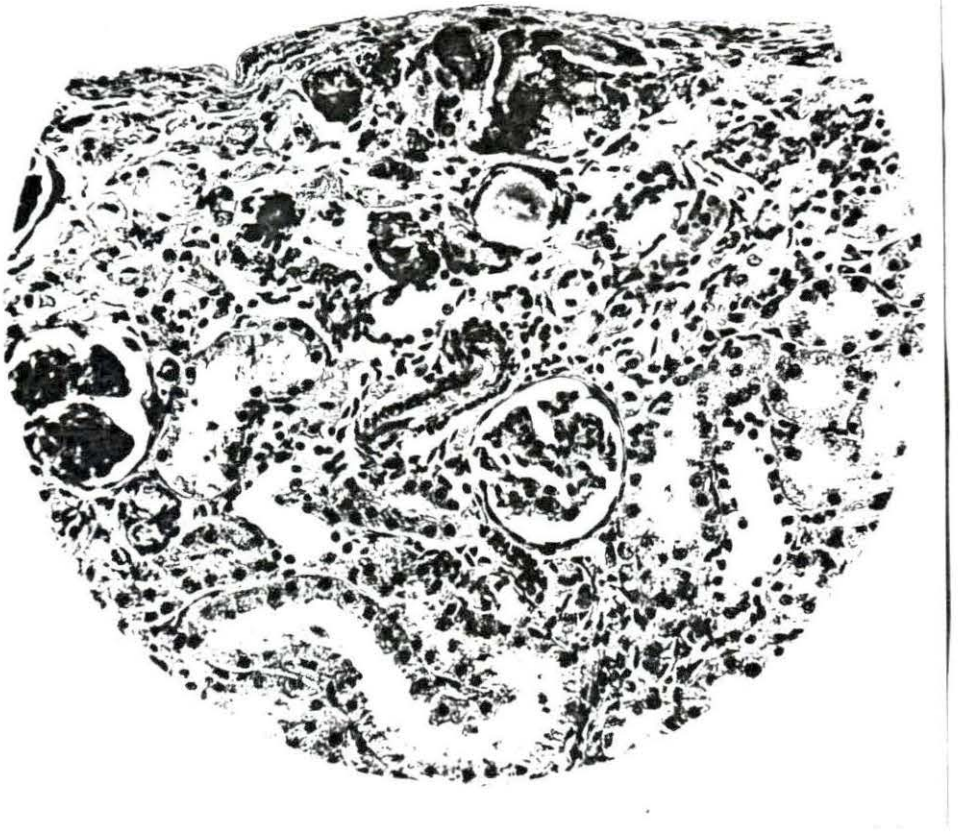


Fig. 27. Kidney (cortex) of rat 117 showing calcification. Hematoxylin and triosin. X300.

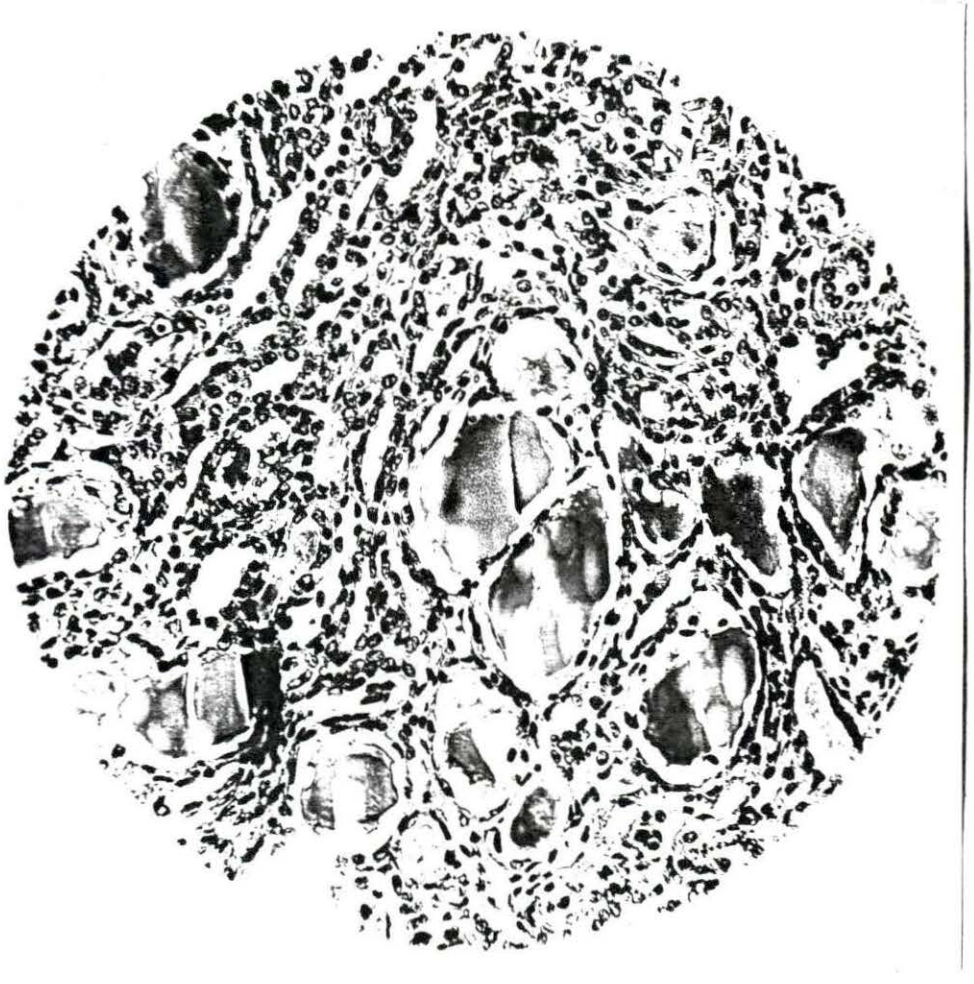


Fig. 28. Kidney (medulla) of rat 117 showing hyaline casts. Hematoxylin and triosin. X300.

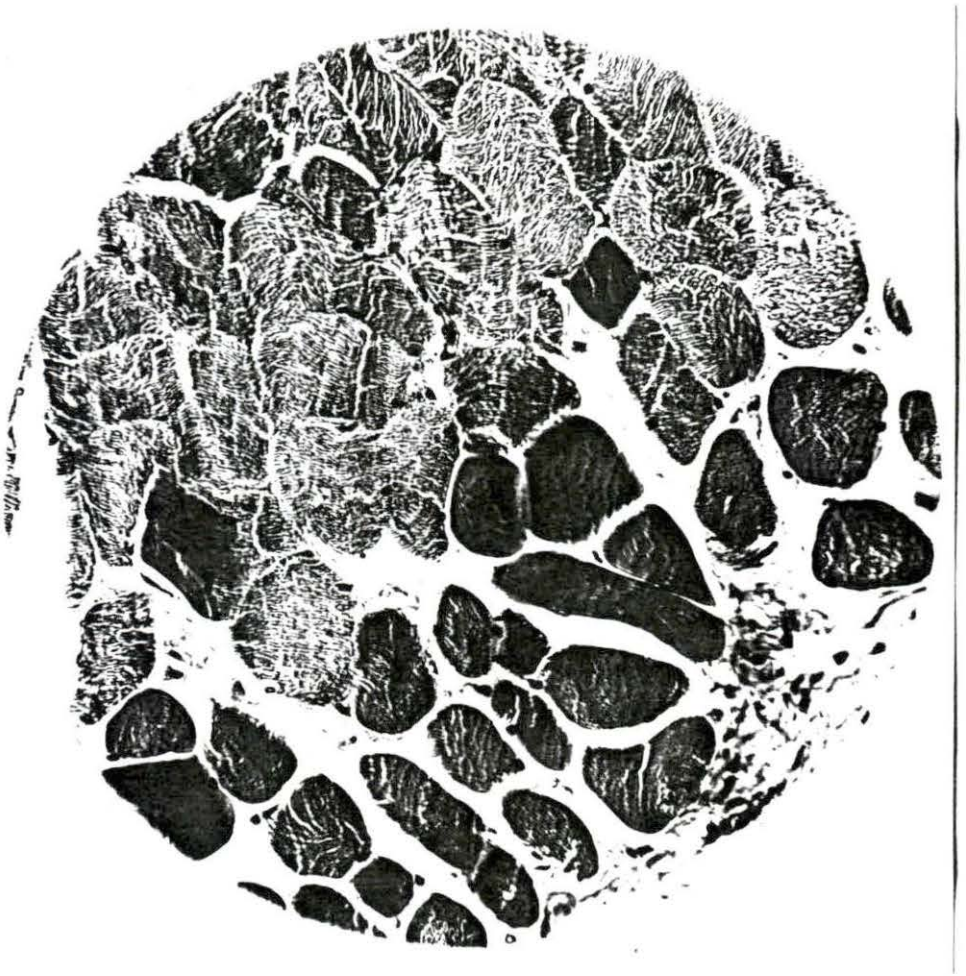


Fig. 29. Skeletal muscle of rat 124 showing hyaline degeneration. Hematoxylin and triosin. X300.

IV. DISCUSSION

Deficiency diseases in animals are associated with alterations in tissues, varying from well-marked lesions to very slight changes that can be demonstrated only by special methods. The rats in this experiment showed similar pathological changes, but the degree of change varied with each group.

This experiment was carried out in the summer of 1947. McCay (53) states that animals require more thiamine in cold weather to form body heat than in hot weather. This factor may account for the longer survival of the experimental animals as compared with those of similar investigations. The need for thiamine increases in direct proportion to the breakdown of carbohydrates. Therefore, it is possible to deplete the thiamine reserve in the animal body by feeding a high carbohydrate diet. Exercise also aids in depleting the thiamine reserve because the metabolic processes in the body are increased. It is known that a rat needs one-third more thiamine when exercising (53). The rats in this experiment receiving no thiamine were the least active.

The function of thiamine in carbohydrate metabolism is well-known. Thiamine is converted to a diphosphate ester in the animal body which is known as cocarboxylase or thiamine pyrophosphate (54). This ester acts as a coenzyme with the enzyme carboxylase in the decarboxylation of pyruvic acid.

The latter compound is an intermediate product of carbohydrate metabolism. In the absence of thimaine, the pyruvic acid level in the tissues rises above normal. This condition may occur when thiamine-deficient diets are fed. The pyruvic acid level in the tissues may also be increased artificially by feeding thiamine analogues which compete with cocarboxylase for the enzyme carboxylase. Figure 6 shows that competitive inhibition has occurred between thiamine and its analogue.

The thiamine analyses from each group of rats are correlated in Figure 7. It is obvious that rats receiving an optimum amount of thiamine have a greater concentration of thiamine in their tissues. The tissues from rats receiving thiamine-deficient and thiamine-free diets were consistently low in thiamine. Supplementation with 5 micrograms of thiamine raised the thiamine levels only slightly. Growth, however, was stimulated as shown in Figures 1 to 4, inclusive.

The results of the thiamine analyses of rat 118 in Table 4 are not recorded in Figure 7. Severe degenerative changes were present in the kidneys of this rat, but its cause could not be determined. The Marchi method revealed fatty degeneration in the kidney tubules. Considerable necrosis of these tubules was seen in the sections stained with hematoxylin and triosin. A small amount of calcification and connective tissue proliferation of recent origin was present.

The thiamine analyses are summarized in Table 4, and pertinent data regarding each animal are included. This table

shows that the weights of the livers varied from less than one and one-half grams to 14 grams. Considerable tissue atrophy was present in the animals of Groups A and D. These animals weighed less, after a period of approximately 35 days, than at the beginning of the experiment. The severe dehydration of the muscles in rat 108 may partly explain the higher concentration of thiamine in the muscle than in the liver.

Polarized light is a rapid method in examining myelinated nerves for degeneration. The method, however, has some disadvantages as compared with the Marchi method. With polarized light, photomicrographs must be taken immediately in order to obtain a permanent record of the observed field because the tissue preparations are not permanent. Another disadvantage is the fact that only one field is recorded while in the Marchi method the entire tissue section is permanent and may be examined later.

Table 5 is a summary of the pathological changes observed in the sections that were stained by the Marchi method. Plus signs indicate the degree of degeneration which was observed. Small amounts of fatty degeneration are recorded as a trace. Three plus signs indicate an extreme degree of degeneration while one and two plus signs signify intermediate changes.

The occurrence of fatty degeneration in the livers of rats on a thiamine-deficient diet and its absence in a thiamine-free diet have not been explained. However, some investigators have shown that the metabolic processes of rats receiving a thiamine-

free diet are decreased. Although the daily administration of 50 micrograms of thiamine is considered adequate for normal growth, the rats in Group E, receiving this level of thiamine daily, showed fatty degeneration of the liver. Therefore, it appears that some factor in the diet other than thiamine may have produced the fatty degeneration in the liver.

Cardiac insufficiency may produce severe congestion in the organs returning blood into the venous circulation. As the blood becomes deficient in oxygen, degenerative changes in these organs are likely to occur. It is possible that the fatty degeneration in the liver, since it is centrally located in the lobules, may be a manifestation of cardiac insufficiency caused by a thiamine deficiency. Eddy and Dalldorf (55) observed central necrosis of the liver in cases of beriberi.

The degenerative changes in skeletal muscle (Figures 25, 26, and 29) were observed in every animal except those receiving a thiamine-free diet or the commercial fox chow. Wells (56) believes that Zenker's waxy degeneration in skeletal muscle is due to an increased accumulation of acid by a defective oxygen supply. He has produced this condition in muscle fibers by the application of a weak solution of lactic acid. Since the lactic acid concentration in the tissues is increased in thiamine-deficient animals, degenerative changes in the muscles may occur. Runnells (57) states that calcification may accompany hyalinization where necrotic areas are being encapsulated. The skeletal muscle of rat 117 (Group C)

shows these degenerative changes as illustrated in Figure 26.

The myelin sheath degeneration which was observed in the rats receiving an adequate amount of thiamine (Groups E and F) indicates that some factor other than thiamine may be involved. Engel and Phillips (44) found that myelin sheath degeneration was seldom observed in a rat on a thiamine-deficient but otherwise adequate diet. The same investigators (42) demonstrated that riboflavin was necessary to prevent myelin from degenerating. Publications by Sutton, Setterfield, and Krauss (33) and Setterfield and Sutton (35) suggest vitamin A-deficiency as the causative factor in producing myelin sheath degeneration. Eveleth and Biester (45) concluded that neither vitamin A nor the B-complex was responsible for myelin sheath degeneration in the spinal cords and peripheral nerves of swine.

Many investigators have found that thiamine deificiency does produce myelin degeneration. There is a possibility that the diets of these animals may have been deficient in some factor other than thiamine. It is evident that more research should be done to determine the exact cause of myelin degeneration since the results appear to be inconsistent. The results obtained by the polarized light method and the Marchi method for demonstrating degeneration in myelinated nerves are in agreement.

V. SUMMARY AND CONCLUSIONS

1. Competition between thiamine and the o-aminobenzyl analogue of thiamine for the enzyme carboxylase is demonstrated. This is shown by the growth inhibition of Group C as compared with Group B.
2. The pathological changes observed with polarized light and by the Marchi method in the sciatic nerve are very similar.
3. Polarized light is a rapid and accurate way to check myelin sheath degeneration in peripheral nerves.
4. The o-aminobenzyl analogue of thiamine alone does not produce pathological changes in rat tissues.
5. Degenerative changes in rat tissues from thiamine deficiency are albuminous degeneration of the kidney tubules with the formation of hyaline casts, degeneration of skeletal muscles, and calcium deposition in skeletal muscles and kidneys. These changes may be caused by high concentration of lactic acid in the blood and tissues.
6. Fatty degeneration of the liver was observed in rats on a thiamine-deficient diet while it did not appear in rats on a thiamine-free diet.
7. The high concentration of thiamine in the liver and kidney of normal rats is depleted in thiamine deficiency until it reaches the thiamine level of the other tissues.
8. Chronic thiamine deficiency produces more pathological

changes in the tissues than acute thiamine deficiency.

9. Thiamine therapy is successful in rats suffering from thiamine deficiency when the tissues have not been damaged beyond repair.

10. It is believed that some factor other than a thiamine deficiency is the cause of myelin degeneration in the medulla, spinal cord, and sciatic nerve in the rat.

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