cortex is the site of the greatest concentration of C^{14} after the intravenous injection of labeled polysaccharide and protein fractions of *Klebsiella pneumoniae*. This concentration persists for two months, while the activity of liver, spleen, lung and other organs declines and disappears. In general, the bacterial fraction with the highest incorporation of C^{14} from the media yields the greatest incorporation in the adrenal.

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Received July 1, 1955. P.S.E.B.M., 1955, v90.

Thioctic Acid Content and Pyruvate Oxidation in Ethionine-Damaged Rat Livers.* (21967)

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Recent investigations have established the role of alpha-thioctic acid (lipoic acid) (6,8 dithio-octanoic acid) as essential cofactor for metabolism of alpha-keto acids in both bacteria and animal tissue. This vitamin was first described by Kidder and Dewey as "Protogen," an essential growth factor for *Tetrahymena geleii*, present in liver and yeast; more recently its identity with "Pyruvate Oxidation Factor" of O'Kane and Gunsalus and "Acetate Replacing Factor" of Guirard has been established. The compound has been crystallized from liver and chemically synthesized. Thioctic acid is essential for oxidation of pyruvate by cells of *Streptococcus fecalis*, grown on thioctic acid-free medium, and is intimately associated with pyruvic oxidase and alpha-keto glutaric oxidase recovered from pigeon breast and pig heart muscle respectively. The role of this compound has been defined in the transfer to coenzyme A of the 2-carbon acetyl fragment obtained by oxidative decarboxylation of pyruvate and the 4-carbon succinyl fragment resulting from oxidative decarboxylation of alpha-keto glutarate. These developments have recently been extensively reviewed by Reed(15) and Gunsalus(7). The role of thioctic acid in animal nutrition has not been defined. No symptoms can be induced, nor can growth be diminished, in rats, by feeding a diet deficient in thioctic acid and simultaneously depressing possible intestinal bacterial synthesis of thioctic acid by feeding antibiotics(21). Feeding of an antimetabolite, the 8-methyl analogue of thioctic acid, which inhibits growth for some bacteria, produces no symptoms in chicks and rats(22). Impaired alpha-keto acid metabolism in severe human hepatic dysfunction (1, 2,19) and in experimental hepatic necrosis in rats(12,16) has been reported, and the importance of sulfur-containing amino acids in maintaining integrity of the liver is well recognized(5,16).

* Supported in part by a grant from the Nutrition Foundation.

† Fellow of the National Foundation for Infantile Paralysis.

Therefore it was decided to investigate thioctic acid content of liver and its relationship to pyruvate metabolism in hepatic injury produced in rats by prolonged feeding of ethionine(9). This amino acid is the ethyl analogue of methionine and interferes with utilization of methionine in protein synthesis(18).

Method. Female rats of the Wistar strain, weighing 110 to 150 g, were placed on following diet, to which dl-ethionine was added to a final concentration of 0.5%: Sucrose 75%, vitamin-free casein (Nutritional Biochemical Corp.) 16%, salt mixture (HMW) 4%, and corn oil 5%. Thiamine 10 mg, niacinamide 40 mg, pyridoxine 10 mg, riboflavin 20 mg, calcium pantothenate 40 mg, choline chloride 300 mg, menadione 5 mg, Vit. A 60000 units, and irradiated ergosterol 8500 units were added to each kg of diet. Other rats were simultaneously maintained on above diet without ethionine and were either fed *ad libitum* or pair-fed in amounts equal to those consumed by ethionine-fed animals. After 21 to 35 days on the diet, and after a 16- to 20-hour fast, the rats were killed by decapitation. At sacrifice, blood was collected from the neck and pyruvic acid determined by the method of Friedemann and Haugen(4). Livers were immediately excised and homogenized in 9 parts of chilled isotonic sucrose in glass homogenizer at 4°C. The homogenate was centrifuged 10 minutes at 600 x g to sediment nuclei and cellular debris. Supernate was then centrifuged at 9000 x g for 10 minutes to precipitate the mitochondria. The mitochondrial preparation was resuspended in 2 ml of isotonic sucrose per original gram of wet liver and reprecipitated at 14000 x g for 10 minutes. Mitochondria were then resuspended in isotonic KCl. Both kidneys were also excised and homogenized in 15 ml distilled water. Mitochondrial pyruvate oxidation was measured by the Warburg method, with flask contents as in Table I. Mitochondrial protein was determined, in quadruplicate, by the method of Lowry *et al.* (11). Thioctic acid determination was accomplished, according to the method of Stokstad *et al.*(20), by turbidometric assay of the stimulation of growth of *Corynebacterium bovis*.‡ The original medium was modified to stimulate maximal growth by addition of 1.5 g of enzyme-hydrolyzed casein/liter of double strength medium. The enzyme hydrolyzed casein (Trypticase-Baltimore Biological Laboratories) and acid hydrolyzed casein (Vitamin-free Casamino Acids-Difco) were adsorbed with charcoal, extracted with benzene at pH 3, and then readsorbed with charcoal at pH 6 to remove any residual thioctic acid. Dl-methionine, 0.5 g, was added to each liter of double strength medium to counteract any inhibition of growth of test organism which might be produced by ethionine, as previously described for *Escherichia coli*(8). However, no significant inhibition of growth of *Corynebacterium bovis* due to ethionine, at concentrations ranging from 10^{-3} to 10^{-5} M, could be detected. To free biologically-inactive, bound thioctic acid(6,13), 1 ml aliquots of liver or kidney homogenate and 1 ml aliquots of mitochondrial suspension containing 5 to 10 mg of protein/ml were autoclaved with 1 ml of 12 N H_2SO_4 for 3 hours at 120°C and 15 lbs pressure. The digests were neutralized with concentrated KOH and diluted to final concentration of 0.4 to 4.0 mγ of d-alpha-thioctic acid/ml. Urines, collected from fasting rats kept in metabolism cages for 48 hours, were diluted to a similar concentration. The diluted samples were filtered and 2.5 ml aliquots were added to 2.5 ml of double strength medium, in quadruplicate, so that final concen-

TABLE I. Hepatic Mitochondrial Pyruvate Oxidation.*

Diet	No. of animals	Mean Qo_2/mg protein ± S.E.
Control, fed *ad lib.*	3	66 ± 5.8
" , pair fed	7	69 ± 2.7
Ethionine	13	36 ± 1.3

* Results expressed as μl O_2 taken up/hr/mg mitochondrial protein (based on oxygen uptake in first 10 min.).

Flask contents: Na pyruvate .02 M; Na fumarate 6.7 × 10^{-4} M; KCl .05 M; $MgSO_4$ .002 M; ATP .001 M; DPN 2 × 10^{-4} M; Niacinamide .01 M; Cytochrome C 1 × 10^{-5} M; PO_4 .02 M, pH 7.35. Mitochondrial suspension containing 8-12 mg protein.

Flask vol.: 3 ml, 20% KOH in center well.

Temperature, 37°; gas phase, O_2; thermequilibration, 5 min.

‡ We are indebted to Dr. E. L. R. Stokstad of the Lederle Division, Amer. Cyanamid Co., for the stock culture of *Corynebacterium bovis* and generous supplies of dl-α-thioctic acid.

TABLE II. Hepatic Mitochondrial Oxidation.*

Diet	No. of animals	Substrate: Pyruvate 60 μM Fumarate 2 μM	Substrate: Fumarate 2 μM	O_2 uptake due to added pyruvate
Control, pair fed	5	69 ± 2.8	32 ± 2.4	37 ± 2.7
Ethionine	6	37 ± 2.2	28 ± 3.0	9 ± 2.7

* Results expressed as mean μl O_2 uptake/hr/mg mitochondrial protein ± S.E. Flask contents as per Table I, with pyruvate omitted as indicated.

tration of d-alpha-thioctic acid ranged from 0.2 to 2.0 mγ/ml. The tubes were then inoculated with one drop of a saline-washed 48-hour culture of *C. bovis,* grown in basal medium enriched with thioctic acid. Inoculated tubes were incubated 88 hours at 30°C, and growth measured turbidimetrically in a photospectrometer. Optical density, prior to inoculation, of the relatively dark solutions obtained by liver digestion, was subtracted from the optical density following incubation, to find the increment in turbidity due to bacterial growth. A standard curve, obtained by measuring growth resulting from addition of known amounts of synthetic dl-alpha-thioctic acid‡ (having one-half the biologic activity of naturally occurring d-isomer) to the basal medium, was established simultaneously for each group of samples. At final concentrations of 0.2 to 2.0 mγ d-alpha-thioctic acid/ml, values obtained from unknowns were reproducible without a constant tendency to upward or downward "drift"; values falling outside above range were discarded.

Results. Rats fed the diet containing ethionine lost weight more rapidly than pair-fed controls. At autopsy, the livers were grossly enlarged, pale, firm, and granular. Microscopic examination revealed lobular disarrangement, marked variation in cell size, cellular swelling and cytoplasmic coagulation, with marked inflammatory cell and fibroblastic infiltration and slight fibrosis, as previously described by Koch-Weser and Popper(9). Kidneys were enlarged, soft, and pale. Microscopic examination revealed marked tubular hydropic degeneration. A similar lesion occurring following acute injection of ethionine has been described(23).

Impaired pyruvate metabolism in intact animals, following prolonged ethionine feeding, was suggested by a significant ($p<.02$) terminal elevation in fasting blood pyruvate levels from 2.9 ± .39 mg% in pair-fed controls to 4.2 ± .29 mg% in ethionine-fed animals. Pyruvate oxidation by mitochondrial preparations from livers of animals fed ethionine was markedly diminished (Table I). While mitochondria from livers of control animals maintained uniform pyruvate oxidation rate for over 40 minutes, the rate of pyruvate oxidation by mitochondria obtained from livers of ethionine-fed animals declined progressively during each 10 minutes of incubation. Hourly oxygen uptake per mg of mitochondrial protein was calculated on the basis of oxygen consumption during the first 10 minutes, to minimize above difference. The endogenous oxygen consumption by mitochondria in absence of substrate was negligible. When pyruvate was omitted from the flasks, the sole remaining substrate was 2 μmoles of fumarate "sparker" per flask. When oxygen uptake in presence of fumarate alone was subtracted from oxygen uptake in presence of fumarate plus pyruvate, the added oxygen consumption due to presence of pyruvate was markedly greater in mitochondria from livers of control animals than in ethionine-fed rats (Table II). These differences were highly significant ($p<.001$).

The thioctic acid content of liver per gram wet weight was essentially similar for rats fed chow *ad libitum,* and for rats fed ethionine-free diet *ad libitum* and in limited amounts (Table III). The mean value for control animals of 1.2 γ of d-alpha-thioctic acid per g wet liver is of the same order of magnitude as previously reported values(6,13). However, a marked decrease in thioctic acid content to 0.4 γ/g was found in livers of ethionine-fed rats. When the observed thioctic acid content was corrected for the increase in liver size noted in ethionine-fed rats, by expressing the

TABLE III. Thioctic Acid Content* of Rat Organs.

Diet	Liver			Kidney		
	No. of animals	γ/g wet wt	γ/100 g initial body wt	No. of animals	γ/g wet wt	γ/100 g initial body wt
Chow	7	1.2 ± .10	—	—	—	—
Control, fed *ad lib.*	13	1.1 ± .07	4.0 ± .27	6	1.1 ± .12	1.2 ± .11
" , pair fed	10	1.2 ± .12	4.6 ± .38	9	1.4 ± .10	1.3 ± .10
Ethionine	15	.4 ± .05†	1.7 ± .21	9	1.3 ± .11	1.3 ± .10

* Results expressed as γ of d-α-thioctic acid/g of wet organ and as γ of total liver or kidney. d-α-thioctic acid/100 g of rat body wt prior to beginning experimental diet. (Mean ± S.E.)
† For difference between livers of pair fed controls and ethionine-fed animals, $p<.001$.

results in terms of total liver d-alpha-thioctic acid/100 g original body weight, prior to starting on diet, the presence of an absolute decrease in hepatic thioctic acid from 4.6 to 1.7 γ of d-alpha-thioctic acid per 100 g of original body weight was established, at a highly significant ($p<.001$) level (Table III).

Thioctic acid content of mitochondria obtained from livers of ethionine-fed animals was also significantly ($p<.001$) diminished, from 27 to 8 mγ of d-alpha-thioctic acid/mg of mitochondrial protein (Table IV). Hepatic depletion of thioctic acid was apparently uniform in the mitochondria and in other portions of the cell, since the percentage of total hepatic thioctic acid which could be recovered from mitochondria was approximately equal in both control and experimental animals. The values of 67 and 62% respectively coincide closely with the 64% reported by Reed and Cormier(14).

Kidney was found to contain approximately 1.3 γ of d-alpha-thioctic acid/g wet tissue; this value approximately equals the amount found in liver and confirms the observations of Reed, *et al.*(13) who found approximately equal amounts of "Acetate Replacing Factor" activity in rat liver and kidney. No significant relative or absolute diminution of renal thioctic acid content could be detected in animals fed ethionine (Table III), in spite of anatomic damage to the kidneys. This is compatible with previous reports that the chemical dysfunction induced by ethionine intoxication is not as severe in kidney as in liver, the incorporation of amino acids into proteins following ethionine injection being much more impaired in liver than in kidney(18).

The 24-hour urine excretion of thioctic acid was slightly greater in fasting ethionine-fed rats than in pair-fed controls. The values of 330 and 230 mγ of d-alpha-thioctic acid per 24 hours respectively fall within the normal range of 100 to 500 mγ per 24 hours previously observed by Stokstad, *et al.*(21). The difference between the 2 groups, however, is significant only at the .05 level. Apparently, a general impairment of thioctic acid synthesis does not exist in ethionine-fed rats.

Diminished pyruvate oxidation by liver mitochondria from ethionine-fed rats could not be restored by *in vitro* addition of 1 γ of dl-alpha-thioctic acid to the flask mixture. Injection of 60γ of dl-alpha-thioctic acid into ethionine-fed rats at 96, 72, and 48 hours prior to sacrifice did not increase hepatic mitochondrial thioctic acid content above that found in untreated animals (Table IV).

Discussion. The demonstrated defect in mitochondrial pyruvate oxidation is probably not due to thioctic acid depletion. It is known that thiamine pyrophosphate, diphosphopyridine nucleotide, thioctic acid, coenzyme A, and specific protein apooxidases are all required to maintain pyruvate oxidation(15). DPN has been observed to diminish in livers of ethionine-fed rats(17). Coenzyme A originates in part from methionine and cystine (24) and is diminished in livers of rats fed a diet deficient in sulfur-containing amino acids (3, 12). Since ethionine interferes with normal metabolism of methionine, it is possible that the coenzyme A content of mitochondria is also diminished. Moreover, ethionine diminishes protein synthesis(18) and is incorporated in synthesized abnormal proteins (10); therefore the apoenzymes required for pyruvate oxidation may be deficient. In spite of the observed depletion of mitochondrial

TABLE IV. Thioctic Acid Content* of Liver Mitochondria.

Diet	No thioctic acid injection — No. of animals	Mitochondrial thioctic acid content	% of total liver thioctic acid recovered from mitochondria	Inj.† with thioctic acid — No. of animals	Mitochondrial thioctic acid content
Control, fed *ad lib.*	5	32 ± 2.7	—	—	—
" , pair fed	10	27 ± 2.6	67 ± 8	3	25 ± 3.0
Ethionine	10	8 ± 1.0	62 ± 5	3	6 ± 1.5

* Results expressed as mγ d-α-thioctic acid/mg mitochondrial protein (mean ± S.E.).
† Injected with 60 γ dl-α-thioctic acid 96, 72, and 48 hr prior to sacrifice.

thioctic acid in these animals, addition of thioctic acid *in vitro* does not increase the diminished pyruvate oxidation observed in liver mitochondria. Probably, a variety of factors other than thioctic acid depletion probably contribute to the pyruvate oxidation defect.

A general impairment of thioctic acid synthesis does not exist in ethionine-fed rats and therefore cannot account for diminished hepatic thioctic acid content. Since thioctic acid injection does not increase thioctic acid content of liver mitochondria in ethionine-fed rats, it appears possible that thioctic acid binding mechanism of liver may be damaged by ethionine feeding, possibly due to impaired synthesis of apoenzymes to which thioctic acid is bound, or due to impaired synthesis of enzymes which catalyze protein binding of thioctic acid or its conversion to the form in which it is biologically active.

Summary. Terminal blood pyruvic acid level was elevated in rats with liver lesions induced by feeding a diet containing ethionine. Pyruvate oxidation by mitochondria from livers of these rats diminished. The hepatic content of thioctic acid was decreased relative to control animals; the thioctic acid content of liver mitochondria was also reduced. *In vitro* addition of thioctic acid did not stimulate the diminished hepatic mitochondrial pyruvic acid oxidation. Undiminished urinary excretion of thioctic acid, failure of thioctic acid injection to increase the diminished hepatic mitochondrial thioctic acid content, and normal kidney thioctic acid content suggest that total body thioctic acid synthesis is unimpaired but that hepatic thioctic acid "binding" is impaired, in rats fed a diet containing ethionine.

The author wishes to thank Dr. Albert I. Mendeloff, who suggested this project, and under whose supervision the studies were carried out.

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Received July 5, 1955. P.S.E.B.M., 1955, v90.

Destruction of Virus Hemagglutination Inhibitor of Egg-White by Newcastle Disease Virus.* (21968)

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The viruses of mumps, influenza and Newcastle disease cause agglutination of certain mammalian red blood cells (RBC) by connecting these cells and forming a lattice-like matrix. If the ratio of virus and RBC is controlled, the reaction can be observed visually. In most instances the virus elutes or dissociates from the RBC after an indefinite time following mixing of the virus and red cells. The virus-treated cells are no longer capable of being "rebridged" by the virus but the virus retains its capacity to act again on untreated cells(1).

It is now known that normal ferret serum, serum from other animal species(2), a variety of tissue suspensions(3), ovalbumin(4), cow's milk(5), etc., will inhibit viral hemagglutination (HA). Inhibitor present in all of these substances seems to be very similar in composition to the "virus receptor" substance of the surface of erythrocytes which is commonly regarded as mucoprotein(6,7). Viruses of the mumps-Newcastle-influenza group can enzymatically destroy the inhibitor common to these materials. The exact role of this enzymatic action in infection has not been determined. One hypothesis is that firm adsorption to the cell surface is needed to allow the virus to enter the cell.

Since so many variations in characterized properties, *e.g.*, pathogenicity, heat stability of the hemagglutinin, of strains of Newcastle disease virus (NDV) exist, a study of the enzymatic activity of several strains of this virus seemed warranted. The following studies have utilized crude egg-white as the substrate or HA inhibitor in determining the mucinase activity of NDV.

Materials and methods. Viral strains. A repository for strains of NDV, maintained in the Department of Veterinary Science, University of Wisconsin, with the cooperation of the Agricultural Research Service, U. S. Department of Agriculture and the Newcastle Disease Technical Committee of the North Central States Region, has more than 80 strains, including 1 from Australia, 8 from Canada, 2 from Mexico, 1 from Japan, 8 from Europe and the remainder from the United States. All repository strains and the N strain of Dinter(8) were tested for their ability to destroy egg-white inhibitor (EWI). For the rate studies, however, only 7 strains were employed: Vic-Australia-1932, B1, Hert-Eng-1933, Milano-Italy-1945, GB-Texas-1948, (H)Najarian-Ohio-1948 and Roakin-NJ-1946. The Lee strain of influenza B virus was used as the indicator virus in the hemagglutination-inhibition (HI) tests. White Leghorn chick-

* The work here reported is a portion of that completed by McCollum in partial fulfillment of the requirements for the doctor of philosophy degree from the University of Wisconsin in 1954 and was supported jointly by funds from the U. S. Department of Agriculture, Agricultural Research Service, Animal Diseases and Parasites Branch; from the Regional Technical Committee on Newcastle Disease of the North Central States; and from the Wis. Agr. Exp. Station. Published with the approval of the Director of the Wis. Agr. Exp. Station, as paper NS ..... of the Dept. of Veterinary Science.

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