the Surgeon General, Department of the Army. H.A.B. held the Ayerst fellowship of the Endocrine Society, H.E.L. a traineeship from the Institute of Arthritis and Metabolic Diseases, U.S. Public Health Service, and J.E.W. a fellowship from the American Diabetes Association, during the period of the research.

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Studies on Experimental Thiamine Deficiency

2. TISSUE BREAKDOWN AND GLYOXYLIC ACID FORMATION*

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(Received 8 September 1961)

Since glyoxylic acid was detected in the blood, urine and tissues of rats suffering from thiamine deficiency and also in rats after prolonged feeding of restricted low-fat diet containing added thiamine (Liang, 1962), it was suggested that the formation of glyoxylic acid is related to excessive tissue

breakdown. It was also noted that creatinuria is a notable sign of thiamine deficiency; it is attributed to the tissue breakdown and can be correlated with loss of body weight (Sure & Dichek, 1941; Sure & Ford, 1942). Creatine synthesis apparently depends on the availability of glycine, arginine and methionine from the common metabolic pool contributed by body tissues and diet (Bloch & Schoenheimer, 1940). Studies have shown that urinary creatine was derived from liver creatine

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which was not taken up by the diseased muscle (Benedict, Kalinsky, Searrene, Werthein & Stetten, 1955), and disorders of muscular tissue are associated with increased excretion of large numbers of amino acids in the urine (Dent, 1946, 1947; Ames & Risley, 1948). Inanition resulting from thiamine deficiency, starvation and fever, or disorders of musculature, are 'wasting processes', and the occurrence of creatinuria and increased urinary amino acids might serve as the common index for those conditions mentioned above.

Though in lower organisms the enzyme isocitratase splits isocitrate into succinate and glyoxylate (Wong & Ajl, 1955), and glyoxylate may arise from the degradation of uric acid in *Pseudomonas* (Laskowski, 1951), and from the degradation of allantoin in fishes (Tolbert & Cohan, 1953), its presence in urine of women has been disputed (Beilstein, 1922; Kulonen, Carpen & Ruokolainen, 1952). However, Biserte & Dassonville (1956) found glyoxylic acid occasionally in human blood and van der Horst (1960) found glyoxylic acid in small quantity in serum and urine of man and in large amount in cattle, sheep, horse and dog serum and urine. It suffices to state that glyoxylic acid is present in the higher animals, though the condition of the animals is not definitely mentioned.

The formation of glyoxylic acid in rats has been investigated. Ratner, Nocito & Green (1944) reported the oxidation of glycine to glyoxylic acid by liver and kidney preparations. Glycine and glyoxylic acid were also found to be interconvertible (Weinhouse & Friedmann, 1951, 1952; Nakada & Weinhouse, 1953; Weinhouse, 1955). Meister (1956, 1957) suggested that the formation of glyoxylic acid was the result of deamination of glycine. Dekker (1960) has mentioned an enzyme system in rat liver which converts α -hydroxyglutamate into glyoxylate and alanine.

This paper presents an attempt to correlate the extent of tissue catabolism, assessed by urinary excretion of creatine, with glyoxylic acid formation and glycine excretion in thiamine-deficient rats and in animals on a restricted low-fat diet with added thiamine. Diversion of glycine to glyoxylate in thiamine deficiency was studied by means of [2- 14 C]glycine.

METHODS

The rats used in these experiments and the details of the feeding of thiamine-deficient and restricted diets were the same as those described by Liang (1962). Urine samples were collected for 24 hr. before analysis.

Estimation of urinary creatine and creatinine

The method was modified from the micromethod of King & Wootton (1956). The recommended pressure for hydrolysis of creatine was found to be too high and destroyed the

creatinine formed. A pressure of 10 lb./in.² for 25 min. hydrolysed creatine to creatinine without loss of the latter.

Creatinine estimation. A 24 hr. sample of urine filtrate (0.6 ml.) obtained as described by Liang (1962) was added to 0.5 ml. of 0.1 N-HCl, followed by the addition of 1.0 ml. of N-NaOH and by 0.2 ml. of saturated picric acid. The mixture was kept for 10 min. and made up to 10 ml. with water. The extinction was read at 530 m μ . The reagent was used as the blank. A calibration curve was plotted from similarly treated pure creatinine standards.

Creatine estimation. Urine filtrate (0.6 ml.) mixed with 0.5 ml. of 0.1 N-HCl was autoclaved at 10 lb./in.² for 25 min., and cooled to room temperature. N-NaOH (1.0 ml.) was added, followed by 0.2 ml. of saturated picric acid. The mixture was kept for 10 min. and then made up to 10 ml. with water. The extinction was read at 530 m μ . This gives the combined reading of creatine and creatinine, and from this the creatine may be calculated by difference.

Chromatographic estimation of urinary and blood glycine

Conventional two-dimensional paper chromatography was used. A known amount of urine filtrate or blood serum was applied to one corner of a large sheet of Whatman no. 1 filter paper. The first-way chromatography was done with the upper layer of butanol-acetic acid-water (4:1:5, by vol.) solvent of Partridge (1948). The second-way chromatography was done with phenol-aq. NH₃ soln. (200 parts of water which has been saturated with phenol and 1 part of aq. NH₃ soln., sp. gr. 0.880). After drying in a current of warm air and spraying with 0.2% (w/v) ninhydrin in acetone, the paper was hung in air for the acetone to evaporate and placed in an oven at 105° for 2-3 min. The coloured spots of individual amino acids were identified, cut out and extracted with ethanol containing 10% of N-HCl. The extinction was read at 560 m μ . The actual amounts were calculated from the calibration curve of standards treated in the same manner.

Radioisotope studies with [2- 14 C]glycine

After injection of [2- 14 C]glycine the distribution of the radioactivity in the expired CO₂ and in the keto acids in blood and urine of thiamine-deficient and normal rats was measured as described below.

Collection of expired carbon dioxide. A glass tube containing 20 ml. of N-NaOH was connected to a metabolic jar to absorb atmospheric CO₂. To the other opening of the jar a series of three absorption tubes was connected: the first tube contained 20 ml. of 0.5% 2,4-dinitrophenylhydrazine reagent in 2 N-HCl, to trap any volatile ketonic or aldehydic compounds expired, and the other two tubes each contained 20 ml. of N-NaOH for the absorption of CO₂.

A single dose of [2- 14 C]glycine (1.5-3.0 ml. containing 3-6 mg. of glycine; 1.0 ml. $\equiv$ 1 μ C) was injected intraperitoneally and the rat was at once confined in the metabolic jar. The suction pump was turned on to allow a gentle stream of air to pass through the whole system for 3 hr. Some experiments were carried out at 18°, and some at 28° in a constant-temperature water bath.

The contents of the two CO₂-absorption tubes were transferred quantitatively to a flask and made up to 250 ml. with water. Samples (1 ml.) of this were mixed with

1-9 ml. of 0.1 N- Na_2CO_3 , and 1 ml. of 5% BaCl_2 was added to each tube to precipitate the carbonate. The BaCO_3 was plated by filtration-plate on a tared circular filter paper; it was washed three times with water, twice with 95% ethanol, twice with acetone and sucked nearly to dryness. The precipitate was finally dried over anhydrous CaCl_2 in a desiccator and weighed. Specific activity was measured in terms of counts/min. Suitable absorption corrections to zero sample thickness were made on all samples by use of an empirical mass-absorption curve.

Radioautograph of chromatogram. After the collection of CO_2 the rat was taken out of the metabolic jar and killed at once. Blood was taken from the heart, and urine was collected from a watch glass in the metabolic jar and from the urinary bladder. The hydrazones of keto acids from blood and urine were chromatographed as described by Liang (1962). Some animals were kept longer in the metabolic cage for the collection of 24 hr. or 48 hr. samples.

The paper chromatogram was pressed against a sheet of X-ray photographic paper between two thick sheets of lead for 1 month, and the photographic paper was developed to show the radioactive sites of individual keto acid hydrazones.

Measurement of the radioactivity of keto acids. From the chromatogram the different keto acid hydrazones were eluted and the concentration of each was determined (Liang, 1962). Their radioactivity was measured on a planchet containing thin lens tissue soaked in the eluted solution and dried under an infrared lamp.

RESULTS

Daily urinary creatinine and creatine excretion. Fig. 1 shows that daily creatinine excretion per unit body weight declined only slightly during most of the period of thiamine depletion, but fell with sudden rapidity a few days before death. The daily creatine excretion was at first about 1 mg./100 g. body wt., and fell slightly during the first 14 days on the deficient diet. However, when the body weight began to decrease, the creatine excretion suddenly increased and reached a peak at 37-42 days on the deficient diet. From then on it continued to drop sharply, but even when the animal was moribund the daily creatine excretion was higher than that before the animals were given thiamine-deficient diet.

Fig. 2 depicts the daily creatine excretion in the urine of rats placed on restricted low-fat diet with added thiamine. It shows the same pattern of change except that creatine excretion was greater and increased from the first day of the diet. The animals steadily lost weight from the start.

Glycine in blood and in urine. Table 1 presents the blood and urinary glycine concentrations, which dropped a little in the first few days after the animals were given a thiamine-deficient diet. They suddenly rose after 2 weeks, and fall coincided with those of urinary creatine. The paper chromatogram of urine and blood of thiamine-deficient rats showed

an increased number, and usually a greater concentration, of other amino acid spots.

Radioautograph of chromatograms. Radioautography of the hydrazones of the blood and urine keto acids of animals fed with $[2\text{-}^{14}\text{C}]$ glycine showed weak radioactivity at the site of pyruvic acid in both normal rats and in rats at an early stage of thiamine deficiency. However, in later stages of thiamine deficiency the site of radio-

Fig. 1. Changes in daily urinary excretion of creatine (●) and creatinine (○) from rats fed on a thiamine-deficient diet. Each point represents the mean value from 12 rats taken at random from a large number of rats.

Fig. 2. Changes in daily urinary excretion of creatine (●) and creatinine (○) from rats fed on a restricted low-fat diet with added thiamine. Each point represents the mean value from 12 rats taken at random from a large number of rats.

Table 1. *Concentration of free glycine in blood and the daily excretion of free glycine in urine in normal rats and rats at different periods of thiamine depletion*

Condition of rats			Blood glycine (mg./100 ml.)	Urinary glycine (mg./24 hr.)
Sex	Body wt. (g.)	Days on diet		
M.	328	0	2.24	0.28
M.	274	0	1.96	0.24
F.	187	0	2.35	0.33
F.	179	0	2.66	0.38
M.	283	7	1.06	0.14
F.	193	7	1.11	0.19
M.	353	12	1.98	0.24
F.	205	12	1.15	0.28
M.	227	17	4.20	0.54
M.	182	25	5.83	0.66
M.	142	37	8.25	0.89
F.	104	40	7.84	0.79
M.	94	47	9.68	0.90
F.	84	50	4.62	0.43
M.	82	50	3.87	0.38

Table 2. *Conversion of injected [2-¹⁴C]glycine into carbon dioxide, glyoxylic acid, pyruvic acid and α -oxoglutaric acid in normal and thiamine-deficient rats*

[2-¹⁴C]Glycine was injected after varying periods on the thiamine-deficient diet. Values are expressed as percentages of the radioactivity injected. Total activity of keto acid in the whole rat is obtained from the radioactivity in 1 ml. of blood multiplied by body wt. (g.).

Condition of rat			Tempera- ture of bath	Duration of experiment (hr.)	Expired CO ₂		Total activity of keto acid in whole animal		
Sex	Body wt. (g.)	Days on diet			Vol. (ml.)	Radio- activity (%)	Glyoxylic acid	Pyruvic acid	α -Oxo- glutaric acid
F.	173	0	28°	3	342	15.70	0	4.80	2.10
M.	181	18	18	3	203	10.86	0.98	1.20	0.48
M.	252	18	18	24	—	—	1.57	0	0
F.	100	32	18	24	—	—	—	—	—
M.*	135	33	18	12	—	—	—	—	—
F.	119	40	18	3	254	62.00	1.70	0	0
M.	119	40	18	3	450	45.30	3.50	0	0
F.	102	43	28	3	133	38.95	—	—	—
F.	80	47	18	3	412	55.50	6.90	0	0

* Rat died 12 hr. after injection.

activity gradually shifted to that of glyoxylic acid in samples of blood and urine (Table 2). Also, radioactivity was detected in spots with R_f 0.72 and 0.75, which corresponded to the hydrazones of α -oxoisovaleric acid and α -oxo- β -methylvaleric acid respectively.

Radioactivity of expired carbon dioxide and keto acids. Table 2 shows that, in the normal rat, 15% of the injected radioactive glycine was eliminated in the expired CO₂ in 3 hr.; a small fraction was converted into blood pyruvate and α -oxoglutarate. No glyoxylic acid could be detected in the urine or blood. When radioactive glycine was injected into rats showing initial signs of thiamine deficiency, the percentage of radioactivity in the expired CO₂ remained small, radioactivity was detected in blood pyruvate and α -oxoglutarate and also some

in the blood glyoxylate (Table 2). When radioactive glycine was given after prolonged thiamine deficiency, more than 50% of the radioactivity of glycine was eliminated in the expired CO₂ in 3 hr., and glyoxylate of the blood showed radioactivity (Table 2). The percentage of radioactivity recovered in the form of glyoxylate of the blood was 1.7–6.9% after 3 hr. In urine glyoxylate was the only one detected to show radioactivity.

DISCUSSION

Creatinine originates solely from creatine (Bloch & Schoenheimer, 1940; Fölling, 1934a), and the urinary creatinine concentration varies with the muscular bulk and is unaffected by the dietary intake of methionine and glycine (Fölling, 1934b).

The steady decline in the urinary creatinine in thiamine deficiency may be attributed to loss of body weight, which largely results from muscular dystrophy (Hoagland, 1944). The terminal abrupt fall of creatine and creatinine concentrations in blood and in urine is apparently due to slowing of all metabolic processes (Liang, 1959).

The gain in weight during the first 14 days of thiamine-deficient diet indicates that anabolic processes predominated during this period so that little amino acid is available in the common metabolic pool for the formation of creatine. As a result, the urinary creatine concentration was low during this period. When the animal lost appetite and catabolic processes predominated, the amino acid concentration in blood and in urine increased. This could cause increased formation of creatine and result in creatinuria. The rise and fall of urinary glycine coincided with those of urinary creatine in the present study and those of urinary and blood glyoxylic acid concentrations mentioned by Liang (1962). The similar pattern of change in concentrations of these three substances, i.e. creatine, glycine and glyoxylic acid, suggests that formation of glyoxylic acid is related to tissue catabolism, whether due to thiamine deficiency or inanition.

The percentage of radioactivity from $[2-^{14}\text{C}]$ -glycine recovered as glyoxylate was small. A much greater percentage of injected glycine was disposed of in the expired carbon dioxide in thiamine-deficient rats than in normal rats, and the glycine that remained in the body was probably subjected to greater metabolic changes owing to thiamine deficiency. Furthermore, the rate of absorption, the dilution by endogenous glycine and the time of sampling of the blood may or may not coincide with the peak absorption of the injected glycine, so that the conversion of injected glycine into glyoxylate might be higher than that found in the present study.

Although we have some knowledge of the effect of thiamine deficiency on fat and carbohydrate metabolism, we know relatively little about protein metabolism in avitaminosis. The present work shows that tissue-protein catabolism plays a prominent role in the general body metabolism in thiamine deficiency. Thiamine deficiency leads to inanition with increased catabolism of body tissue, just like the wasting process in starvation, fever and muscular dystrophy. The excessive breakdown of body tissue causes an increased formation of ketone bodies and a rise in blood and urinary concentrations of creatine and glycine. Amino acids are liberated by protein catabolism and they probably follow different metabolic pathways. For glycine, a variety of pathways are open (Weinhouse, 1955), including the formation of glyoxylic acid.

The route of glycine catabolism is little known. Isotope experiments show its ready decomposition into carbon dioxide in intact animals (Olsen, Hemingway & Niar, 1948; Berlin, Tolbert & Lawrence, 1951; Ratner, Rittenberg, Kaston & Schoenheimer, 1940); however, it is poorly oxidized in isolated tissue preparations (Bach, 1939; Siekevitz, 1940). It may be that the anabolic process is favoured under physiological conditions. In the present study, the development of thiamine deficiency apparently shifted the metabolism of glycine from a route involving pyruvate to the glyoxylate route; this was probably because glycine was used mainly to provide energy under this condition, as indicated by its increased oxidation to carbon dioxide, whereas under normal conditions glycine was used for many metabolic purposes, including the transformation into pyruvate and oxidation by the Krebs cycle.

Although the present results seem to indicate glycine as a possible source of the glyoxylic acid found in thiamine-deficient rats, isocitratase, required to cleave isocitrate, has not been found in animal tissues (Smith & Gunsalus, 1955). Also, sarcosine (Woolf, Griffith & Moneriff, 1955), ethanolamine (Sakami, 1948; Weinhouse, 1955) and α -hydroxyglutamate (Dekker, 1960) are all possible precursors of glyoxylic acid.

SUMMARY

1. Urinary excretion of creatinine in rats during thiamine depletion is fairly constant until a few days before death, but the urinary creatine excretion gradually drops during the first 14 days on the deficient diet. When the body weight falls, the urinary creatine excretion rapidly increases to a peak after 37–42 days on the deficient diet and drops sharply thereafter until death. Rats given a restricted diet with added thiamine show almost the same pattern of urinary creatinine and creatine excretions, but without an initial drop of creatine excretion.

2. In rats on thiamine-deficient diet both blood and urinary concentrations of glycine fall at first and then rise steadily until a few days before death.

3. The rise and fall of concentration of glyoxylic acid in blood and urine reported previously can be correlated with the concentrations of glycine and creatine in blood and urine.

4. In normal rats, only 15% of the radioactivity of injected $[2-^{14}\text{C}]$ glycine was recovered in the expired carbon dioxide. In rats suffering from prolonged thiamine deficiency more than half of the glycine radioactivity was recovered in the expired carbon dioxide.

5. In normal rats, a small fraction of the radioactivity of injected glycine was found in the pyru-

vate and α -oxoglutarate in the blood and urine but none in glyoxylate. In thiamine-deficient rats, the glyoxylate in blood and urine contained up to 6.9 % of the radioactivity from the injected glycine, and radioactivity was not found in the pyruvate or α -oxoglutarate.

6. The present study suggests that the glyoxylate found in blood and urine of thiamine-deficient rats is derived from the glycine liberated by excessive tissue breakdown.

The work described in this paper was carried out in the Human Nutrition Research Unit, Mill Hill, London, with the aid of a research fellowship from the Sino-British Fellowship Trust. The author wishes to thank Professor B. S. Platt and all members of the Nutrition Research Unit for their encouragement and help. He also wishes to thank the University of London for permission to publish the results from a thesis presented for a Ph.D. degree.

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Toxic Liver Injury and Carcinogenesis

METHYLATION OF PROTEINS OF RAT-LIVER SLICES BY DIMETHYLNITROSAMINE IN VITRO

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(Received 10 August 1961)

Dimethylnitrosamine is a powerful liver poison in several mammalian species (Barnes & Magee,

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1954; Jacobsen, Wheelwright, Clem & Shannon, 1955). It is carcinogenic in the rat, inducing tumours of the liver (Magee & Barnes, 1956; Schmähl & Preussmann, 1959) and of the kidney (Magee & Barnes, 1959, 1962; Zak, Holzner &