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Studies on Experimental Thiamine Deficiency

TRENDS OF KETO ACID FORMATION AND DETECTION OF GLYOXYLIC ACID

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Thiamine deficiency leads to a well-defined biochemical lesion of carbohydrate metabolism with increase in pyruvate and lactate in the blood (Peters, 1936). Since Lohmann & Schuster (1937) showed that thiamine in the ester form of pyrophosphate is the coenzyme for the decarboxylation of pyruvic acid, it has been found to participate in several important reactions of carbohydrate metabolism, in the phosphogluconate oxidative pathway, the transaldolation and transketolation between ketoses and aldoses and the newly recognized oxidative decarboxylation of glyoxylic acid (Nakada & Sund, 1958).

Peters (1948, 1953) emphasized that absence of thiamine does not affect the metabolic change of glucose or glycogen to pyruvate, but that it interferes selectively with the degradation of pyruvate to carbon dioxide and water by the pyruvate-oxidase system, thereby inhibiting the liberation of the energy required to maintain the tissue in its living state. Pyruvic acid itself has not been found to be toxic, even when injected intravenously. Therefore the raised pyruvate is only a sign of thiamine deficiency and not necessarily the cause of the associated syndrome.

Methylglyoxal was considered non-toxic and was assigned a key part in carbohydrate metabolism (Dakin & Dudley, 1913), until Ströhr (1932) demonstrated its toxicity. Findlay (1921) reported that pigeons suffering from polyneuritis were deficient in liver glyoxylase and that this increased after feeding with thiamine. Vogt-Møller (1931) described thiamine deficiency as methylglyoxal intoxication caused by a decreased tissue-glyoxalase activity.

Methyglyoxal was reported to be present in the urine of polyneuritic dogs and in the urine and cerebrospinal fluid of infants suffering from apparent thiamine deficiency (Geiger & Rosenberg, 1933), in the blood and urine of man with beri-beri (Platt & Lu, 1939) and in the milk of women with beri-beri (Chiba, 1932; Takamatsu & Sato, 1934; Suzuki & Takamatsu, 1934; Orimo, 1939; Fehily, 1944). Since glutathione was the coenzyme of glyoxalase (Lohmann, 1932), Stannus (1942) suggested that absence of glutathione resulted in an accumulation of methylglyoxal. But Johnson (1936) was unable to detect any preformed methylglyoxal in the tissues of polyneuritic pigeons, and Hopkins & Morgan (1945) have pointed out the absence of satisfactory evidence for the occurrence of a substrate for glyoxalase in the cells and tissues. Kun (1950) found that methylglyoxal inhibited the succinic-oxidase system and Salem (1954) described a possible route of formation of methylglyoxal in thiamine-deficient rats. This again raised the problem of methylglyoxal in thiamine deficiency.

Though disorders resulting from lack of thiamine, in functional derangement of muscles, nerves and cardiac system, have been shown, the mechanisms underlying these abnormalities are still unknown.

Liang (1960) reported that an aldehyde acid, glyoxylic acid, was found in the urine and blood of thiamine-deficient rats and that its formation depends on the interaction of the metabolism of carbohydrate, fat and protein. Its effect on cellular functions in various organs would merit further investigation in the hope of elucidating the cause of the multiple syndromes of B_1-avitaminosis.

This paper presents a hitherto unobserved but characteristic fluctuation in pyruvic acid concentration as well as changes in the total keto acid. Furthermore, chromatographic study did not reveal the formation of methylglyoxal in experimental thiamine deficiency; nevertheless, significant amounts of glyoxylic acid were discovered in the urine, blood and tissues of thiamine-deficient rats (Liang, 1960).

EXPERIMENTAL

Animal feeding

Food materials. (*a*) Rice powder. Polished rice was washed with water by stirring and rubbing for 10 min. and left in a sink with running water overnight. The water was drained off and the rice steamed at a pressure of 5 lb./in.^{2} for 30 min. This cooking of starch prevents 'refection', which might interfere with experiments on thiamine deficiency (Kelly & Parson, 1937). The cooked rice was dried at 80° and powdered in a mill. The processing reduced the thiamine content of rice from 50 to 5 μg./100 g. of rice. (*b*) Casein. Low-vitamin casein (Genatosan Ltd.) was used. (*c*) Salt and water-soluble-vitamin mixture. Riboflavin

0·5 g., pyridoxine 0·1 g., calcium pantothenate 3·0 g., nicotinic acid 5·0 g., inositol 10·0 g., *p*-aminobenzoic acid 10·0 g., biotin 10 mg., folic acid 100 mg., vitamin B_{12} 0·25 mg., choline 30·0 g. and ascorbic acid 1·0 g., were mixed with 2 kg. of salt mixture (de Loureiro's formula, obtained from Glaxo Laboratories Ltd.), 300 g. of NaCl and rice powder as a carrier to a total of 4 kg. (*d*) Fat and fat-soluble-vitamin mixture. A mixture of peanut oil 1000 g., vitamin A 100 000 i.u., vitamin D 50 000 i.u. and vitamin E 2·5 g.

Diet mixture. Basal diet was prepared by mixing rice powder 1580 g., casein 200 g., salt and water-soluble-vitamin mixture 160 g., fat and fat-soluble-vitamin mixture 60 g. This basal diet contained carbohydrate 76·4%, protein 14%, fat 3%, NaCl 1%, salt other than NaCl 3·3% and vitamins and other materials 2·3%.

The thiamine content of this diet was 7 μg./100 g. of diet, which was too low to maintain the body weight of adult rats and the growth of young rats. To maintain the body weight of the adult rat, 0·08 mg. of thiamine had to be added to each 100 g. of diet (Arnold & Elvehjem, 1938; Byerrum & Flekstra, 1951). The control rat in paired feeding was given a diet containing 0·15 mg. of thiamine/100 g.

Feeding. Albino rats were supplied by the Human Nutrition Research Unit, National Institute for Medical Research, London. Young rats weighing from 60 to 100 g. and adult rats weighing from 170 to 350 g. were used. They were first kept on stock diet for 10 days, and then they were divided into four diet groups: group 1, on stock diet *ad lib.*; group 2, on restricted basal diet with thiamine; group 3, on basal diet *ad lib.*; group 4, on basal diet *ad lib.* with thiamine.

Restriction of food intake in group 2 consisted of feeding 8 g. of basal diet to each rat per day in the first week, and reducing the amount by 1 g./day for each following week until the rat was given 2 g./day only. Cellulose flour was added to the diet to satisfy hunger.

The rats were housed separately in wire-screen cages to prevent coprophagy. Body weights were recorded twice each week. Rats were constantly observed for any abnormal behaviour or symptoms during the experiment.

Analysis of body fluid and tissues

Preparation of biological samples. (*a*) Urine (24 hr. samples) was collected in 10 ml. of N-H_2SO_4 with a few drops of toluene and the collection funnel was washed with water. To each mixed sample and washings were added 6 g. of trichloroacetic acid and water to a total volume of 60 ml. The mixture was filtered repeatedly through Whatman no. 1 filter paper until clear.

(*b*) Blood. Blood samples were obtained at various intervals by cutting the tail, by exsanguination after killing the rat by cervical dislocation or by cardiac puncture immediately after death. A measured volume of blood was quickly mixed with 5 vol. of ice-cold 10% (w/v) trichloroacetic acid in a glass-stoppered test tube, shaken vigorously for 10 sec. to break up any lump formation, centrifuged at 1000 rev./min. for 5 min. and the clear supernatant was used for analysis.

(*c*) Brain, liver, heart, kidney and skeletal muscle. Rats reared on stock diet and rats receiving thiamine-deficient diet were killed at various intervals and tissues were removed into separate tared beakers containing ice-cold 10% (w/v) trichloroacetic acid. The tissues were minced with scissors and the beakers weighed again to obtain the weight of the removed tissue. They were homogenized and made up to a final dilution of 1:5 with 10% (w/v) trichloroacetic acid. After centrifuging at 1500 rev./min. for 15 min., the supernatant was decanted into a tube.

Estimation of total keto acids and pyruvic acid. A modified procedure based on the methods of Friedemann & Haugen (1943), Tsao & Brown (1950) and Bonting (1955) was followed.

(*a*) Estimation of pyruvic acid. A sample (0·2 ml.) of deproteinized blood or urine was allowed to react for 5 min. with 0·1 ml. of 2,4-dinitrophenylhydrazine reagent (0·1% in 2N-HCl) in a glass-stoppered test tube at 37°. The pyruvic acid dinitrophenylhydrazone was extracted by shaking the mixture with 1 ml. of xylol for 30 sec. After the mixture had been centrifuged for 3 min. at 1500 rev./min., the lower aqueous layer was removed with a fine capillary pipette, and the xylol layer was shaken for 30 sec. with 1 ml. of 10% (w/v) Na_2CO_3 solution; the mixture was centrifuged for 5 min. at 1500 rev./min. and 0·7 ml. of the carbonate extract was transferred to a comparison cell, and, after mixing with 2 ml. of 2N-NaOH, the extinction of the colour developed was measured at 520 mμ in a Unicam spectrophotometer within 5 min. The NaOH concentration in the final solution was 0·75N, which is optimum for the development of the reddish colour of the hydrazones. For more accurate estimation, the yellow colour of the carbonate extract was read at 370 mμ or at 420 mμ in a microcuvette.

(*b*) Estimation of total keto acids. The general procedure was the same as that for pyruvic acid, the differences being that reaction time with 2,4-dinitrophenylhydrazine reagent was 30 min. at 25° and ethyl acetate was used in place of xylol. The extinction was determined at 380 mμ or 420 mμ against a reagent blank, and the total keto acid content expressed in terms of pyruvic acid.

Chromatographic estimation of glyoxylic acid

Chemical markers. 2,4-Dinitrophenylhydrazine was crystallized twice in methanol and a saturated solution was made in 2N-HCl; sodium pyruvate, α-oxoglutaric acid, glyoxylic acid, phenylpyruvic acid, lithium lactate and dihydroxyacetone were obtained from L. Light and Co. Ltd.; methylglyoxal was prepared according to the method of Shroeder & Woodward (1939); acetoacetic acid was prepared by the method of Shaffer (1921); estimation of the percentage of glyoxylic acid in the sample was made according to Kaplans (1957).

Paper chromatography. A modified method of De Schepper, Parmentier & Vanderhaeghe (1958) was followed: 0·5 ml. of saturated 2,4-dinitrophenylhydrazine reagent was added to 10 ml. of the filtrate of deproteinized blood, urine or tissue. This was kept at room temperature for 4 hr. and in the refrigerator overnight. The solution was extracted four times with an equal amount of ethyl ether (AnalaR). The ether extracts were pooled in a 500 ml. beaker and evaporated in a stream of cool air to a clear yellow, syrupy fluid. The fluid was neutralized with a few drops of aq. NH_3 soln. (sp.gr. 0·880), and made alkaline by adding 2 drops of aq. 2N-NH_3 soln. It was transferred to a glass-stoppered test tube, the beaker was washed twice with a minimal amount of aq. 2N-NH_3 soln. and all the washings were pooled in the same tube. After thorough mixing and

allowing the mixture to stand for 5 min., an equal volume of $CHCl_3$ was added and the tube was shaken vigorously for 1 min. It was then centrifuged at 1500 rev./min. for 5 min. The ammoniacal layer contained the acidic ketonic acid dinitrophenylhydrazones Each layer could be examined separately by paper chromatography with butan-1-ol–ethanol–aq. 2N-NH_3 soln. (7:1:2, by vol.). After chromatography for 20 hr., the paper was dried in a warm current of air. When dry, a longitudinal strip of the paper was cut and dipped through the alkali reagent (2% of NaOH in 90% ethanol). The colour spots, which are specific for the dinitrophenylhydrazone of each keto compound, appeared immediately and each keto compound could be identified and located on the same paper. However, some colour spots slowly faded, some returning to the original yellow and others to light brown. The corresponding spots on the remaining portion of the paper could be marked off with pencil. Each of these areas was cut out, cut into small pieces and placed in a graduated glass-stoppered test tube. Sodium carbonate soln. (10%) was added to the 5 ml. mark on the test tube and the paper was macerated with a glass rod. The tube was centrifuged at 1500 rev./min. for 10 min., and the clear supernatant fluid was transferred to a comparison tube. Alternatively, the solution may be filtered by suction through Whatman no. 42 paper. The extinction of the colour solution was read at 420 mμ or 370 mμ of their absorption maxima. Areas corresponding to test spots were cut out from the reagent blank and treated in the same way to serve as the blank.

RESULTS

Life span and symptoms. Control rats fed with liberal amounts of stock diet and basal diet with added thiamine steadily gained weight. Rats on restricted basal diet with thiamine (group 2) steadily lost weight and died after 10 weeks, when the body weight was reduced to half. Young rats reared on thiamine-deficient diet survived 35–38 days, whereas adult rats on the same diet live for 47–54 days. Anorexia was the first sign of thiamine deficiency and loss of body weight was apparently due to reduced food intake. They were weakened, with ataxia, but seldom showed spastic paralysis. Body temperature fell and cyanosis was common, but convulsions rarely occurred.

Pyruvic acid and total keto acids in tissues. In blood and urine the concentrations of pyruvic acid and total keto acids increased when animals fed with a thiamine-deficient diet began to lose weight. After about 15 days the concentrations gradually fell, but after 23–28 days they rose again until a few days before death, when the concentrations again declined (Figs. 1 and 2).

Pyruvic acid and total keto acid concentrations in brain, kidney, liver, heart and skeletal muscle showed a steady rise after administration of thiamine-deficient diet. The heart pyruvic acid and total keto acid concentrations were higher than those of other tissues in both normal and thiamine-deficient rats (Table 1).

Glyoxylic acid in tissues. Glyoxylic acid was detected in the kidney and liver in minute amounts in normal rats. Feeding with thiamine-deficient diet caused gradual accumulation of glyoxylic acid in the brain, kidney, liver, heart and skeletal muscle. Highest concentrations were found in brain (Table 1).

Table 2 shows R_F values and colours of the developed hydrazones of the keto compounds of blood and markers obtained from the paper chromatograms. From the markers, the dinitrophenylhydrazone of methylglyoxal gives three coloured spots. These were supposed to be three isomeric forms of the hydrazones of this compound.

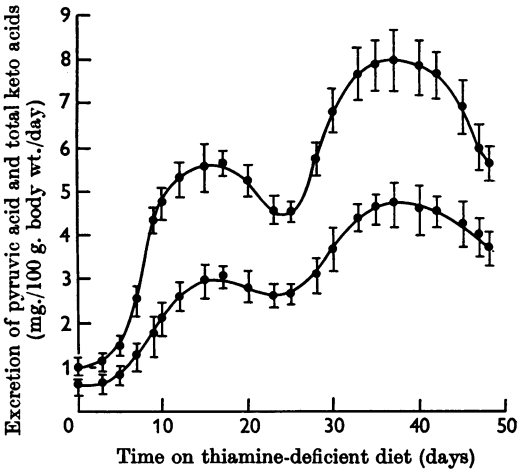

Fig. 1. Daily urinary excretion of total keto acids (upper curve) and pyruvic acid (lower curve) of adult rats fed with thiamine-deficient diet. The points and vertical lines represent mean values and S.D. from 12 rats taken at random from a large number of rats.

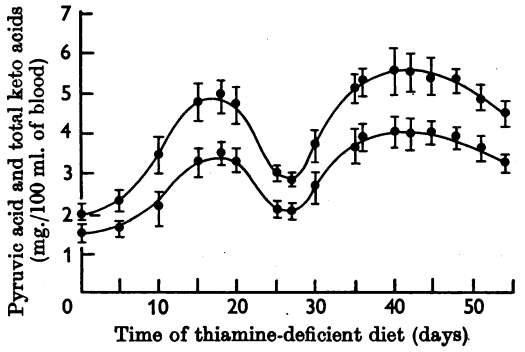

Fig. 2. Changes of total keto acids (upper curve) and pyruvic acid (lower curve) in blood of rats during the period of thiamine depletion. The points and vertical lines represent mean values and S.D. from 12 rats taken at random from a large number of rats.

Table 1. *Concentration of keto acids in tissues of normal and thiamine-deficient rats*

A, Stock diet; B, C, D, thiamine-deficient diet for 17, 37, 47 days respectively. Values of glyoxylic acid were obtained from the elution of glyoxylic acid spots from a paper chromatogram.

Keto acids	Keto acid (mg./100 g. fresh wt.) Brain	Kidney	Liver	Heart	Muscle
A. Pyruvic acid	0·62	1·97	0·57	3·73	1·39
Total keto acids	0·96	2·32	0·74	4·08	1·57
Glyoxylic acid	0·00	0·04	0·01	0·00	0·00
B. Pyruvic acid	1·33	1·37	0·79	3·57	1·25
Total keto acids	1·68	1·88	1·07	4·12	1·73
Glyoxylic acid	0·00	0·03	0·01	0·00	0·00
C. Pyruvic acid	1·30	2·13	2·02	3·95	2·34
Total keto acids	2·83	2·97	2·88	4·56	3·08
Glyoxylic acid	0·34	0·34	0·28	0·18	0·14
D. Pyruvic acid	1·55	3·62	1·72	4·83	2·80
Total keto acids	3·32	5·14	2·27	6·08	4·12
Glyoxylic acid	1·03	0·98	0·87	0·83	0·72

Table 2. *R_F values and colours of developed hydrazones of the chemical markers and of keto compounds in blood*

Colours were developed by dipping the paper chromatograms in 2% of NaOH in 90% ethanol. I, II and III, Isomeric forms of the hydrazones of the same keto compound. Those found in blood are marked with asterisks.

	R_F I	II	III
A. From chloroform layer			
Free 2,4-dinitrophenylhydrazine	0·88 Yellow–brown	0·95 Yellow–brown	—
Dihydroxyacetone	0·92 Brown	—	—
Acetone	0·97 Brown	—	—
Glyoxal	0·31 Salmon	0·50 Orange	—
Methylglyoxal	0·00 Blue–violet	0·80 Orange	0·87 Pinkish brown
B. From ammonia layer			
α-Oxoglutaric acid*	0·50 Olive–brown	—	—
Oxaloacetic acid*	0·13 Dark brown	0·38 Chocolate	0·55 Olive–yellow
Glyoxylic acid*	0·24 Salmon	0·45 Yellow–brown	—
Pyruvic acid*	0·33 Chocolate	0·55 Olive–yellow	—
Acetoacetic acid*	0·36 Yellow–chocolate	—	—
Phenylpyruvic acid	0·70 Brown	—	—
α-Oxoisovaleric acid*	0·72 Yellow–brown	—	—
α-Oxo-β-methylvaleric acid*	0·75 Brown	—	—
α-Oxoisocaproic acid*	0·75 Brown	—	—

None of these was detected in the chromatograms of blood, urine and other tissues of either normal or thiamine-deficient rats, hence the presence of methylglyoxal reported by others was not confirmed.

On the other hand, chromatograms showed the presence of glyoxylic acid in the urine of rats given thiamine-deficient diet for 12–14 days. The concentration reached its maximum after 30–40 days of the diet and then declined (Fig. 3). Glyoxylic acid also appeared in the blood of thiamine-deficient rats, usually after its appearance in the urine (Fig. 3), and the two peaks did not coincide. The blood of adult rats given restricted basal diet with thiamine contained a small amount of glyoxylic acid (Fig. 4). The identity of glyoxylic acid was confirmed by the method of Kun & Garcia-Hernandez (1957).

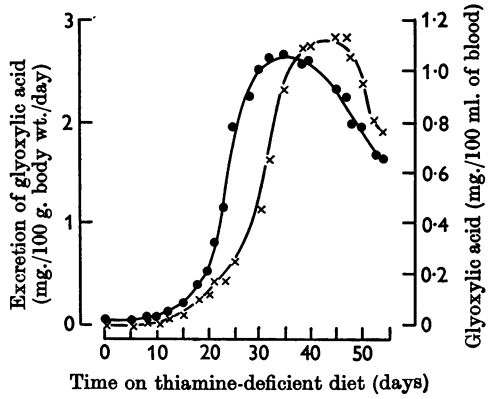

Fig. 3. Glyoxylic acid in urine (●) and blood (×) from rats fed with thiamine-deficient diet. Each point represents the average value from three rats.

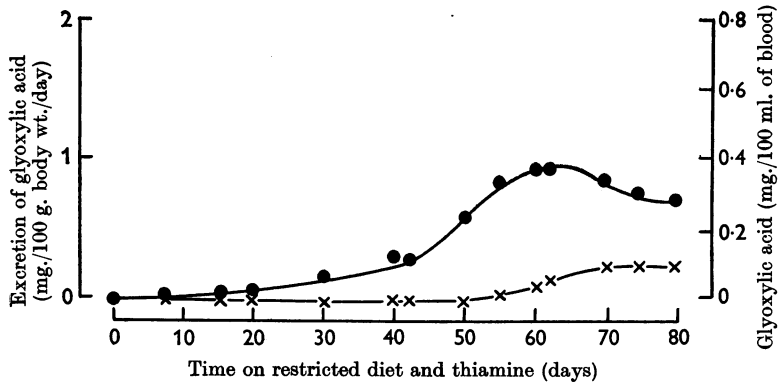

Fig. 4. Glyoxylic acid in urine (●) and blood (×) from rats fed with restricted basal diet with added thiamine. Each point represents the average value from three rats.

Other keto acids in tissues. The chromatograms of urine and blood samples showed, in addition, the presence of pyruvic acid, α-oxoglutaric acid, oxaloacetic acid and two other spots. The last-named had R_F 0·72 and 0·75 respectively and occurred in both normal and thiamine-deficient rats. They seem to correspond to the two spots with R_F approx. 0·60 reported by Kulonen, Carpen & Ruokolainen (1952) and to the X_1 and X_2 spots of De Schepper *et al.* (1958). Reduction of these hydrazones give valine, isoleucine and leucine; therefore they are apparently the dinitrophenylhydrazones of α-oxoisovaleric acid and a mixture of α-oxo-β-methylvaleric acid and α-oxoisocaproic acid. Their significance is not known.

DISCUSSION

The fall in keto acid concentrations that occurred from 15 to 22 days on the diet in experimental thiamine deficiency has not previously been reported and its mechanism is obscure. The second fall in keto acid concentrations may be related to the reduced metabolic rate, which becomes very low a few days before death (Liang, 1959).

In view of the considerable variation in the concentration of keto acids noted during the course of thiamine depletion, the timing of the collection of samples might account for the conflicting results for the pyruvic acid concentrations in the blood and urine in previous reports (Piha, 1958).

The hydrazone of glyoxylic acid possesses properties similar to those of pyruvic acid hydrazone, as, for example, in the speed of formation, solubility in organic solvents and absorption maxima (Friedemann & Haugen, 1943). Therefore it may well be that the estimations of pyruvic acid by previous workers include also glyoxylic acid. In the present experiments it was found that the second rise in total keto acid and pyruvic acid concentrations coincided with the rapid rise in concentration of glyoxylic acid in the blood and urine of thiamine-deficient rats.

Older medical books mentioned that glyoxylic acid occurs in the urine of women in the first and the last month of pregnancy (cited by Beilstein, 1921), but Kulonen *et al.* (1952) questioned this finding in some human urine samples. Meister (1956, 1957) suggested that the formation of glyoxylic acid was the result of deamination of glycine. The presence of small amounts of glyoxylic acid in the kidney and liver in normal rats may be due to the fact that these two organs are sites of active deamination or transamination of glycine (Weinhouse, 1955). From the known experimental studies and the clinical findings in the course of thiamine deficiency it seems that the nervous function is one of the first to be disturbed. In brain tissue of rats suffering for a long time from thiamine deficiency there is a high concentration of glyoxylic acid. It is known that nervous tissue has high concentrations of glutamic acid and glutamine. There may be some connexion between the high concentration of glyoxylic acid resulting from deamination of glycine and the amination of α-oxoglutaric to glutamic acid or glutamine. The effect of glyoxylic acid on nervous function remains to be investigated.

Glyoxylic acid is highly toxic to animals (Adler, 1893; Barnes & Lerner, 1943) and it inhibits oxygen metabolism (Kleinzeller, 1943; D'Abramo, Romano & Ruffo, 1958; Ruffo, Romano & Adinolfi, 1959). Therefore it may play a significant role in the abnormal metabolism in thiamine deficiency. The occurrence of small amounts of glyoxylic acid in the blood of adult rats maintained for a sufficiently long time on a thiamine-deficient diet to produce inanition strongly suggests that production of glyoxylic acid is related to excessive tissue breakdown.

SUMMARY

1. A chromatographic method for the detection and estimation of glyoxylic acid is described.

2. Pyruvic acid and total keto acid concentrations in the blood and urine of thiamine-deficient rats at first rose and then fell. After 23–28 days there occurred a second rise which lasted until 2–3 days before death. Their concentration in the brain, liver, heart and skeletal muscle gradually rose.

3. Methylglyoxal was not found in the blood, urine or tissues of either normal or thiamine-deficient rats during any stage of their life.

4. Glyoxylic acid was detected in small quantity in the kidney and liver. It accumulated in the brain, kidney, liver, heart and skeletal muscle of rats given the thiamine-deficient diet.

5. A small quantity of glyoxylic acid was found in the urine and blood of rats given prolonged restricted diet supplemented with thiamine; its concentration was much lower than that in thiamine deficiency.

6. Glyoxylic acid was present in the urine of rats fed with thiamine-deficient diet after 12–14 days, reaching its peak after 30–40 days and declining thereafter.

7. Glyoxylic acid was also detected in the blood of thiamine-deficient rats, first increasing and then declining in the same way as in the urine, but it appeared later than in the urine.

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REFERENCES

Adler, O. (1893). *Arch. exp. Path. Pharm.* **37**, 413.

Arnold, A. & Elvehjem, C. (1938). *J. Nutr.* **15**, 429.

Barnes, R. H. & Lerner, A. (1943). *Proc. Soc. exp. Biol., N.Y.*, **52**, 216.

Beilstein (1921). *Handbuch der organischen Chemie*, Auflage 4, Band 3, p. 594.

Bonting, S. L. (1955). *Biochim. biophys. Acta*, **58**, 100.

Byerrum, R. U. & Flekstra, J. H. (1951). *J. Nutr.* **43**, 17.

Chiba, M. (1932). *Tohoku J. exp. Med.* **19**, 486.

D'Abramo, F., Romano, M. & Ruffo, A. (1958). *Biochem. J.* **68**, 270.

Dakin, H. D. & Dudley, H. W. (1913). *J. biol. Chem.* **14**, 155.

De Schepper, P., Parmentier, G. & Vanderhaeghe, H. (1958). *Biochim. biophys. Acta*, **28**, 507.

Fehily, L. (1944). *Brit. med. J.* ii, 590.

Findlay, G. M. (1921). *Biochem. J.* **15**, 104.

Friedemann, T. E. & Haugen, G. E. (1943). *J. biol. Chem.* **147**, 415.

Geiger, A. & Rosenberg, A. (1933). *Klin. Wschr.* **12**, 1258.

Hopkins, F. G. & Morgan, E. J. (1945). *Biochem. J.* **39**, 320.

Johnson, R. E. (1936). *Biochem. J.* **30**, 31.

Kaplans, C. (1957). In *Methods in Enzymology*, vol. 3, p. 276. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Kelly, E. & Parson, H. T. (1937). *J. Nutr.* **13**, 453.

Kleinzeller, A. (1943). *Biochem. J.* **37**, 674.

Kulonen, E., Carpen, E. & Ruokolainen, T. (1952). *Scand. J. clin. Lab. Invest.* **4**, 189.

Kun, E. (1950). *J. biol. Chem.* **187**, 289.

Kun, E. & Garcia-Hernandez, M. (1957). *Biochim. biophys. Acta*, **23**, 181.

Liang, C. C. (1959). Ph.D. Thesis: London University.

Liang, C. C. (1960). *Nature, Lond.*, **188**, 660.

Lohmann, K. (1932). *Biochem. Z.* **254**, 332.

Lohmann, K. & Schuster, P. H. (1937). *Biochem. Z.* **295**, 188.

Meister, A. (1956). *Physiol. Rev.* **36**, 103.

Meister, A. (1957). *Biochemistry of the Amino Acids*, p. 144. New York: Academic Press Inc.

Nakada, H. I. & Sund, L. F. (1958). *J. biol. Chem.* **233**, 8.

Orimo, R. (1939). *Tohoku J. exp. Med.* **35**, 374.

Peters, R. A. (1936). *Lancet*, i, 1161.

Peters, R. A. (1948). *Brit. med. Bull.* **5**, 313.

Peters, R. A. (1953). *Brit. med. Bull.* **9**, 116.

Piha, R. S. (1958). *Acta physiol. scand.* **44**, suppl. 151.

Platt, B. S. & Lu, K. G. (1939). *Biochem. J.* **33**, 1525.

Ruffo, A., Romano, M. & Adinolfi, A. (1959). *Biochem. J.* **72**, 613.

Salem, H. M. (1954). *Biochem. J.* **57**, 227.

Shaffer, P. (1921). *J. biol. Chem.* **47**, 433.

Shroeder, E. F. & Woodward, G. E. (1939). *J. biol. Chem.* **129**, 283.

Stannus, H. (1942). *Lancet*, i, 756.

Ströhr, R. (1932). *Hoppe-Seyl. Z.* **206**, 15.

Suzuki, T. & Takamatsu, A. (1934). *Tohoku J. exp. Med.* **24**, 202.

Takamatsu, A. & Sato, A. (1934). *Tohoku J. exp. Med.* **23**, 506.

Tsao, M. U. & Brown, S. (1950). *J. Lab. clin. Med.* **35**, 320.

Vogt-Møller, P. (1931). *Biochem. J.* **25**, 418.

Weinhouse, S. (1955). In *A Symposium on Amino Acid Metabolism*. p. 637. Ed. by McElroy, W. D. & Glass, H. B. Baltimore: The Johns Hopkins Press.