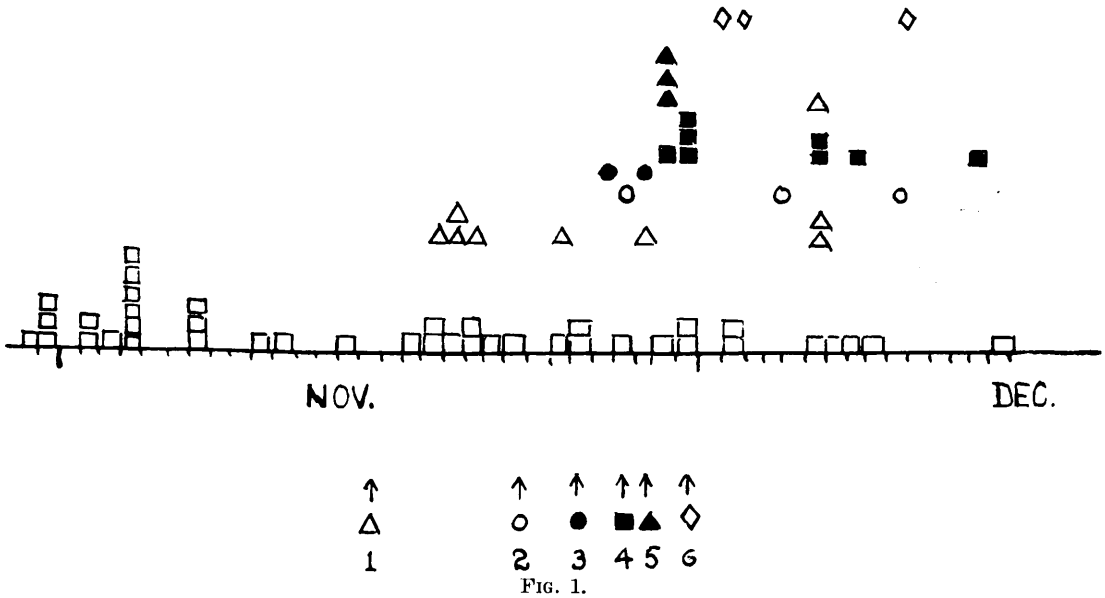

FIG. 1.

The natural epidemic among students compared with the number of induced attacks. The hollow squares represent natural attacks among 240 students in a declining epidemic; 22 attacks occurred after November 15 when the first experiment began. In the 6 experiments, 28 attacks among 53 volunteers are charted in various symbols. The 6 arrows and symbols indicate the date of exposure of volunteers to filtrates, and the corresponding symbols above indicate the day on which the respective attacks occurred.

lized stool filtrates, 11 developed symptoms. Of the combined groups of 53 volunteers, 28 (53%) developed symptoms as compared with 22 out of 240 (9%) of the rest of the student body in which the natural disease apparently occurred during the period of the tests. No attacks occurred in 6 volunteers who inhaled serum nor in 24 volunteers who were fed serum or filtrates of garglings or stools.

The results, considering the unideal circumstances during which the tests had to be made, suggest that the causative agent of the disease is filtrable, airborne, enters through the respiratory tract and is present in the oropharynx and stool but not in the blood. Tests are now being made in a nonepidemic period with material from the November epidemic preserved at −70°C.

14960

Methods for Determination of Thiamine in Blood and Tissues with Observations on Relative Contents.

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A number of methods have been proposed for the determination of thiamine in blood. Among these are the Phycomyces growth test described by Rowlands and Wilkinson,[1] and by Meiklejohn,[2] the cocarboxylase method of

* Aided by a grant from the Christine Breon Fund for Medical Research.

[1] Rowlands, E. N., and Wilkinson, J. F., *Brit. Med. J.*, 1938, **2**, 878.

[2] Meiklejohn, A. P., *Biochem. J.*, 1937, **31**, 1441.

Goodhart and Sinclair,[3] and the yeast fermentation method of Schultz, Atkin and Frey.[4] Thiochrome methods have been proposed by a number of authors, namely, Ritsert,[5] Hennessey and Cerecedo,[6] Hennessey and Lewis[7] and also Friedemann and Kmieciak.[8] The procedure of the last-named authors offers considerable simplification over the previous thiochrome methods.

We have been using a method, during the past 2 years, for the determination of thiamine in blood, which we believe represents an even more simplified thiochrome procedure than any heretofore proposed. In addition the authors' method has the advantage that it can be carried out on as little as 1 cc of blood. It yields results which are in agreement with those found by Goodhart and Sinclair,[3] with the cocarboxylase assay; with those of Goodhart and Nitzberg[9] using the fermentation method, with those of Friedemann and Kmiechiak,[8] and also with those of Benson *et al.*[10] using thiochrome procedures. The values obtained on a group of "normal" individuals (laboratory associates and medical students) with the present method range from 5.0-10 μg per 100 cc.

Our experience, in agreement with others, has been that the total thiamine of the blood of humans is relatively static or immobile and hence is not affected rapidly by the intake of thiamine. This is particularly true when the levels are above 5 μg per 100 cc.

Actually, in order to convincingly demonstrate that blood thiamine determinations are of value in the assessment of deficiency states, it would be worth while knowing if there is good correlation between the blood thiamine and tissue concentrations of man and animals. We have investigated this problem in the rat and the data is presented in this publication. At this point it appears to be timely to make some remarks concerning differences which we have observed between rat bloods and human bloods. In the first place, on an adequate intake the concentration of thiamine in rats' blood is usually somewhat higher than that found for humans; secondly, the blood of the rat appears to reflect more sensitively the changes in the dietary intake of thiamine than does the blood of man.

Method. To 10.5 cc of H_2O in a test tube is added 3.0 cc of oxalated blood, followed by the addition of 1.5 cc of 5% metaphosphoric acid. The mixture is stirred well and heated for 10-15 minutes on a boiling water bath with occasional stirring. It is cooled, 6 cc of water and 4 cc of acetate buffer, pH 4.5, are added with stirring until the whole coagulum is in a fine state of suspension. Following centrifugation the clear supernatant fluid is decanted into another test tube, 200 mg of takadiastase (Parke-Davis) is stirred into the clear liquid and a drop of toluene is introduced. The test tube is stoppered and the extract is incubated over night at 45°-50°C. Following incubation the digest is recentrifuged and 10 cc of the supernatant fluid is adsorbed on Decalso in the manner described below. With 2 cc of whole blood proportionate quantities of water and reagents are employed. The rest of the procedure is the same.

Preparation of the Filter. 500 mg of Decalso, washed and prepared according to the method of Hennessey and Cerecedo,[6] is introduced into a pyrex filter tube (30 cc cap.) with sintered glass filter of medium porosity containing 8-10 cc of 3% acetic acid. The filter tube is placed in a suction filter flask or bottle. Gentle suction is applied by means of a water pump and the acetic acid is drawn through the filter.

Adsorption and Elution of the Thiamine. 10 cc of the takadiastase-digested extract is filtered through the filter slowly (at least 10 minutes being allowed for its passage). The adsorbent is washed with 3 successive 10 cc

[3] Goodhart, R. S., and Sinclair, H. M., *J. Biol. Chem.*, 1940, **132**, 11.

[4] Schultz, A. S., Atkin, L., and Frey, C. N., *J. Am. Chem. Soc.*, 1937, **59**, 2457.

[5] Ritsert, K., *Klin. Wochschr.*, 1939, **18**, 1370.

[6] Hennessey, D. J., and Cerecedo, L. R., *J. Am. Chem. Soc.*, 1939, **61**, 179.

[7] Hennessey, D. J., and Lewis, S., Presented at September, 1941, meeting of the Am. Chem. Soc., Atlantic City, N.J.

[8] Friedemann, T. E., and Kmieciak, T. C., *J. Lab. and Clin. Med.*, 1943, **28**, 1262.

[9] Goodhart, R. S., and Nitzberg, T., *J. Clin. Invest.*, 1941, **20**, 625.

[10] Benson, R. A., Witzberger, C. M., Slobody, L. B., and Lewis, L., *J. Ped.*, 1942, **21**, 659.

portions of water and then sucked dry. The stem of the filter tube is wiped dry and a small test tube is placed under it for the collection of the eluate. The adsorbate is stirred up with 2 cc of 25% KCl in 0.1 N HCl and allowed to settle. After standing approximately 5 minutes or longer, gentle suction is applied and the KCl is sucked through the filter. When all of the first portion of the KCl solution has passed through, a second 2 cc portion is drawn through the filter followed by an additional 1 cc portion of the KCl solution. The filter is then sucked dry and the collection tube is removed from the suction bottle.

Oxidation of Thiamine. For the oxidation of thiamine, the procedure is similar to that of Harris and Wang.[11]

Two cc portions of the KCl eluate are transferred to Babcock cream test bottles. Two cc of methyl alcohol, 0.3 cc of 1% potassium ferricyanide solution, 1 cc of 30% NaOH and 10 cc of isobutyl alcohol are added in this order and the bottles are shaken vigorously for 1.5 minutes. A drop of 3% H_2O_2 is now added to destroy any excess of ferricyanide remaining in the vessels after the oxidation period. In agreement with the work of Ellinger and Holder[12] we have observed that ferricyanide causes a quenching of the thiochrome fluorescence. The blank is treated in the same way as the unknown with the exception that the ferricyanide is omitted. The mixtures are centrifuged slowly for 1 minute and 8 cc portions of the isobutyl alcohol extracts are pipetted into fluorometer tubes.

Readings of the isobutanol extracts are made with the Coleman photofluorometer model 12, standardized each time against pure thiamine samples oxidized in the same manner as the unknown. Coleman light filters (B_1 or B_1-s as the primary, and PC as the secondary) have been employed in the instrument while making fluorescence measurements.

Tissue Thiamine Methods. 0.3 to 1 g quantities of tissue are ground in a mortar with sand and distilled water to a fine mince and transferred to a test tube with water until a total of 13.5 cc of water has been used for grinding the tissue and for washing the mortar. The tissue suspension is then placed in a boiling water bath and after 1-2 minutes, 1.5 cc of 5% metaphosphoric acid is added and the suspension is mixed well. Heating is continued for 20-25 minutes with occasional stirring. The samples are cooled and centrifuged. The supernatant fluid is decanted into 25 cc volumetric flasks and the residue washed twice by centrifugation with 2 successive 5 cc portions of 0.5% metaphosphoric acid. The washings are added to the extract and the volume made up to 25 cc with distilled water.

A 5 cc portion of the tissue extract is mixed with an equal amount of acetate buffer pH 4.5 and incubated over night with 0.1 g of takadiastase. Although a 4-hour incubation period has yielded substantially the same results as over-night incubation, we have preferred the latter because of its greater convenience. Following incubation the thiamine in the whole of the takadiastase-treated sample is adsorbed, eluted and oxidized to thiochrome in the manner described for the analysis of blood thiamine. In our hands this method has yielded values which are in agreement in most instances with those reported by others.[13-16]

Experimental. 12 rats weighing 130-166 g were divided into 2 groups of 6 animals each. One group received a vitamin B_1-deficient diet, and the other the same diet supplemented with thiamine. The basal diet consisted of sucrose 68%, casein 18%, Crisco 8%, salt mixture (Hawk and Oser) 4%, and cod liver oil 2%.

The ration was supplemented by pipette daily with 2 drops (approximately 0.1 cc) of a vitamin mixture of the following composition: thiamine chloride 0.08 g; riboflavin 0.08 g; pyridoxine hydrochloride 0.08 g; nicotinic

11 Harris, L. J., and Wang, Y. L., *Biochem. J.*, 1941, **35**, 1050.

12 Ellinger, P., and Holden, M., *Biochem. J.*, 1944, **38**, 147.

13 Schultz, A. S., Light, R. F., Cracus, L. J., and Atkin, L., *J. Nutrition*, 1939, **17**, 1943.

14 Schweigert, B. S., McIntire, J. M., and Elvehjem, C. A., *Arch. Biochem.*, 1943, **5**, 113.

15 Mitchell, H. K., and Isbell, E. R., *Univ. of Texas Pub.*, No. 4237, 37.

16 Ferrebee, J. W., Weissman, N., Parker, D., and Owen, P., *J. Clin. Invest.*, 1942, **21**, 401.

TABLE I.
Blood and Tissue Thiamine of Thiamine-Deficient and Control Rats.

Rat Nos.	Deficient or control	Days on diet	Blood, μg per 100 cc	Skeletal muscle, μg per g	Heart ventricle, μg per g	Kidney, μg per g	Liver, μg per g
2, 6	control	17	10.9	0.7	4.9	4.3	5.6
1, 3	deficient	17	3.9	0.3	2.6	1.6	3.2
1_c, 4	control	45	13.1	0.8	3.7	—	4.1
4, 5	deficient	45	1.5	0.2	0.5	—	0.5

TABLE II.
Blood and Tissue Thiamine.

Rat				Thiamine				
Group	No.	Days on diet	Thiamine dosage, μg per day	Blood, μg per 100 cc	Muscle, μg per g	Heart ventricle, μg per g	Kidney, μg per g	Liver, μg per g
I	6	26	20	8.3	0.7	3.1	1.7	3.0
I	4	33	20	7.5	0.6	2.0	1.0	1.8
I	16	40	20	7.4	0.7	3.8	1.8	3.5
II	20	26	40	20.4	1.8	6.8	6.3	8.6
II	4	33	40	17.7	1.4	4.0	4.0	7.5
II	12	40	40	14.6	1.1	4.8	3.1	4.7

acid 1.0 g; choline chloride 10 g; calcium pentothenate 0.1 g; and para-aminobenzoic acid 0.15 g, dissolved in 100 cc 20% ethyl alcohol. The thiamine chloride was omitted from the supplement used for the animals on the deficient regime.

After a period of 17 days and again after 45 days on the diet 2 control and 2 deficient animals from which food and vitamin supplements had been withheld for 18-20 hours, were sacrificed on each occasion. This was carried out by anesthetization with ether and severance of the large vessels leading to the heart following opening of the thoracic cavity. The lungs were removed and the blood permitted to flow into the pleural cavity from which it was aspirated with a syringe and introduced into a test tube containing sodium oxalate. Heart, kidneys, liver and portions of skeletal muscle from the hind legs were removed and equal portions of each respective tissue from each of two animals were pooled for thiamine analysis. Blood thiamine analysis was also carried out on pooled samples.

The results of analysis of tissues and blood from animals suffering depletion of thiamine for 17 days and 45 days, along with the values for tissues of control animals are summarized in Table I. The data show that the blood thiamine level of the rat parallels the tissue concentration of the vitamin during periods of depletion.

Some additional data taken from a current, paired feeding experiment on riboflavin deficiency, in which only the data on control rats is used, demonstrates clearly the parallelism between the blood and tissue concentrations of thiamine in the rat. Blood and tissues rather sharply reflect the vitamin B_1 intake. The basal diet and supplements were the same as described earlier in this publication with the exception that each of 2 groups of control rats received 20 and 40 μg of thiamine respectively. The animals weighed 56-78 g when they were placed on the diet and were sacrificed after 26-40 days. Tissues and blood of individual animals were analyzed for thiamine by the procedures outlined in this report. In this instance 1 cc quantities of blood were subjected to analysis and proportionate amounts of reagents were used throughout with the exception that the thiamine was eluted with a total volume of 5 cc of 25% KCl in 0.1 N HCl. Two cc portions of the eluate were used for the oxidation. The results are summarized in Table II. Since these animals were restricted to their food intake, there is present a factor of partial starvation. While this might increase somewhat the thiamine values in each group, the relationship under

consideration would not be affected.

Summary. With the aid of these methods it has been shown, in the rat, that the thiamine concentration in the blood parallels that in the tissues, and both reflect the intake.

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Depression of Acetycholine Synthesis by Serum from Working Muscle. Healthy Subjects and Myasthenia Gravis Patients.*

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In the presence of serum from patients with myasthenia gravis, a persistent state of easy fatiguability,[1,2] less acetylcholine is synthesized than in the presence of serum from healthy subjects. The purpose of the following study was to investigate the effect of exercise in both healthy subjects and patients with myasthenia gravis measuring the action of serum on acetylcholine synthesis. The results are of particular interest with respect to the mechanism of the genesis of fatigue.

Method. Experiments were performed on 12 healthy subjects and 5 patients with myasthenia gravis. The subject alternately flexed his fingers, forming a fist, and extended them fully, performing the entire action rhythmically, at the rate of one contraction per second. A sphygmomanometer cuff was applied to the arm and inflated during the fourth to ninth contraction of the fingers to a pressure of 250 mm Hg. Ten cc blood was collected from the brachial vein. Blood collected from the other immobilized arm with occluded circulation served as control. The blood was centrifuged immediately after collection, oxygenated thoroughly, and the effect of serum on the synthesis of acetylcholine was tested by the method of Quastel, Tennenbaum, and Wheatley[3] with minor modifications.[2] One hundred mg fresh minced frog brain, 1 cc serum, 2 cc Ringer's solution of pH 7.4, 4.8 mg glucose, and 3 mg physostigmine salicylate were incubated and shaken aerobically for 4 hours at 37°C. The amount of acetylcholine

TABLE I.
Effect of Serum Collected During Muscle Contraction on Synthesis of Acetylcholine.

Subject	No. of first formation until fatigue	Amt acetylcholine synthesized by serum from resting arm in % of healthy control serum*	Amt acetylcholine synthesized with serum from working arm in % of own control	Total decrease of acetylcholine synthesized
Twelve healthy subjects	60-105	100	60 ± 1.6	40
Patients with myasthenia gravis:				
R	15	64	85	45
L	30	72	80	43
S	50	76	74	44
V	20	74	83	39
M	40	78	76	41

* The amount of acetylcholine synthesized in the presence of serum from the control arms of healthy subjects averaged 2.08 μ.

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2 Torda, C., and Wolff, H. G., *J. Clin. Invest.*, 1944, **23**, 649.

3 Quastel, J. H., Tennenbaum, M., and Wheatley, A. H. M., *Biochem. J.*, 1936, **30**, 1668.