Studies on the Relation Between Intake and Urinary Excretion of Thiamin in Normal Man.

Thesis

by

Erik V. Heegaard

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INTRODUCTION

The development of the Thiochrome Method for determining thiamin by Jansen (5) gave rise to a number of investigations of the content of thiamin in different organs and in body fluids of both humans and animals, and both on normal and on thiamin deficient diets (See Westenbrink and Goudsmit, 15). Another method for determining thiamin, the Phycomyces method of Schopfer and Jung (9), also contributed considerably to the progress of similar work (See Sinclar, 10 and Meiklejohn, 7).

A series of investigations of the thiamin content of different organs by application of the wellknown curvative pigeon test, had already been carried out before the above two methods were developed; due, however, to the unsystematic nature of these investigations the results must be taken with considerable reservation (See Williams and Spies, 16).

The present work was an attempt to investigate the relation between thiamin intake and its urinary excretion in normal man. The method applied is the Thiochrome Method modified by Westenbrink and Goudsmit (14) for application to determination in urine. From the experimental data an attempt will be made to deduce some information concerning the storage and destruction of thiamin in the normal human body. It is to be hoped, that this knowledge may be helpful in later studies of the metabolism of thiamin in pathological cases.

The Thiochrome Method depends on the fact that thiamin can be oxidized with ferricyanide in alkaline solution to a

strongly fluorescent substance, thiochrome. This substance can be extracted with isobutanol from the aqueous oxidation mixture, and its fluorescence may be observed and quantitavely determined with ultraviolet light.

This method has already been worked out in great detail by Jansen and Westenbrink as mentioned above, and it was not considered necessary to try to improve their method, but it was necessary to adapt the method to the limited facilities of this laboratory.

EXPERIMENTAL PART:

1. Methods:

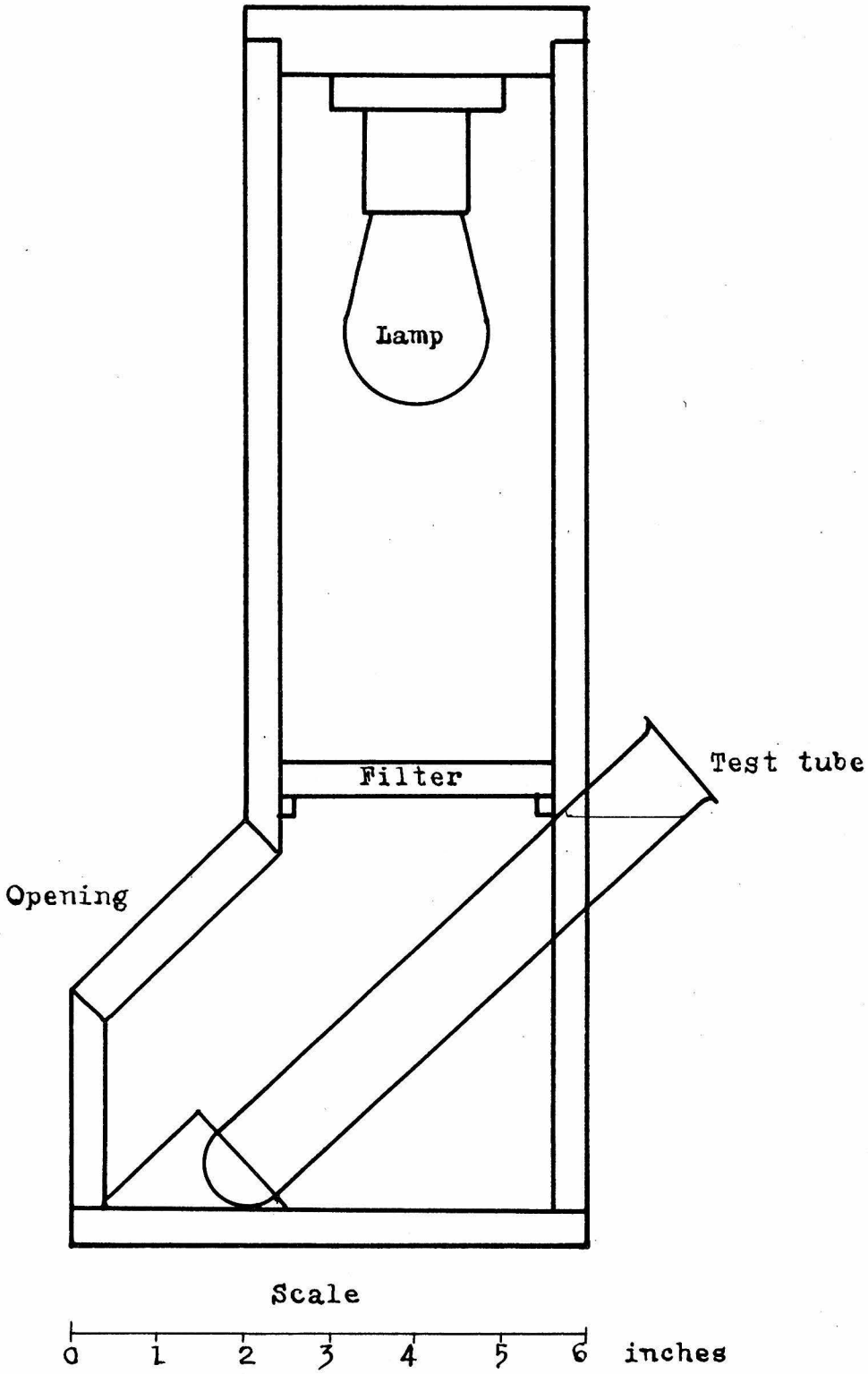
Adaptation of the Thiochrome Method to the facilities of the laboratory.

The Thiochrome Method as described by Jansen (5) and in its modification by Westenbrink and Goudsmit (14), could not be applied without modification, since no photoelectric fluorometer was available. Karrer (6) has reported the application of the Thiochrome Method without such a photoelectric fluorometer. He determined the relative fluorescence of thiochrome solutions by direct visual observation. This simplification has certain advantages because it omits the use of an expensive and sensitive galvanometer.

As a source of ultraviolet light Karrer used a Hanauer Analyse Quartz Lamp. This expensive instrument not being available, a satisfactory source of ultraviolet light was found in the "Argon Bulb" manufactured by the General Electric Vaporlamp Company. This lamp emits some visible light in the violet as well as ultraviolet and to obtain pure ultraviolet light it was necessary to transmit the light from the bulb through No. 597 Corning filter. The lamp and filter was built into a practical unit by mounting them in a wooden box, which is pictured on next page.

The main difficulty in applying the Thiochrome Method to urineis, as Westenbrink and Goudsmit already have observed, that urine contains a considerable amount of fluorescent material which tends to produce a very high blank value for the determination, and thereby decreases the accuracy of the determination,

MOUNTING OF ARGON LAMP AND FILTER IN WOODØEN BOX.



particularly when only small amounts of thiamin are present. To diminish this source of uncertainty in the determination, the method of Westenbrink and Goudsmit (14) was adopted, after other methods had been tried unsuccessfully. In particular it was attempted to extract the disturbing fluorescent substances with different solvents, and by adsorbing with activated charcoal, but in both cases only negative results were obtained.

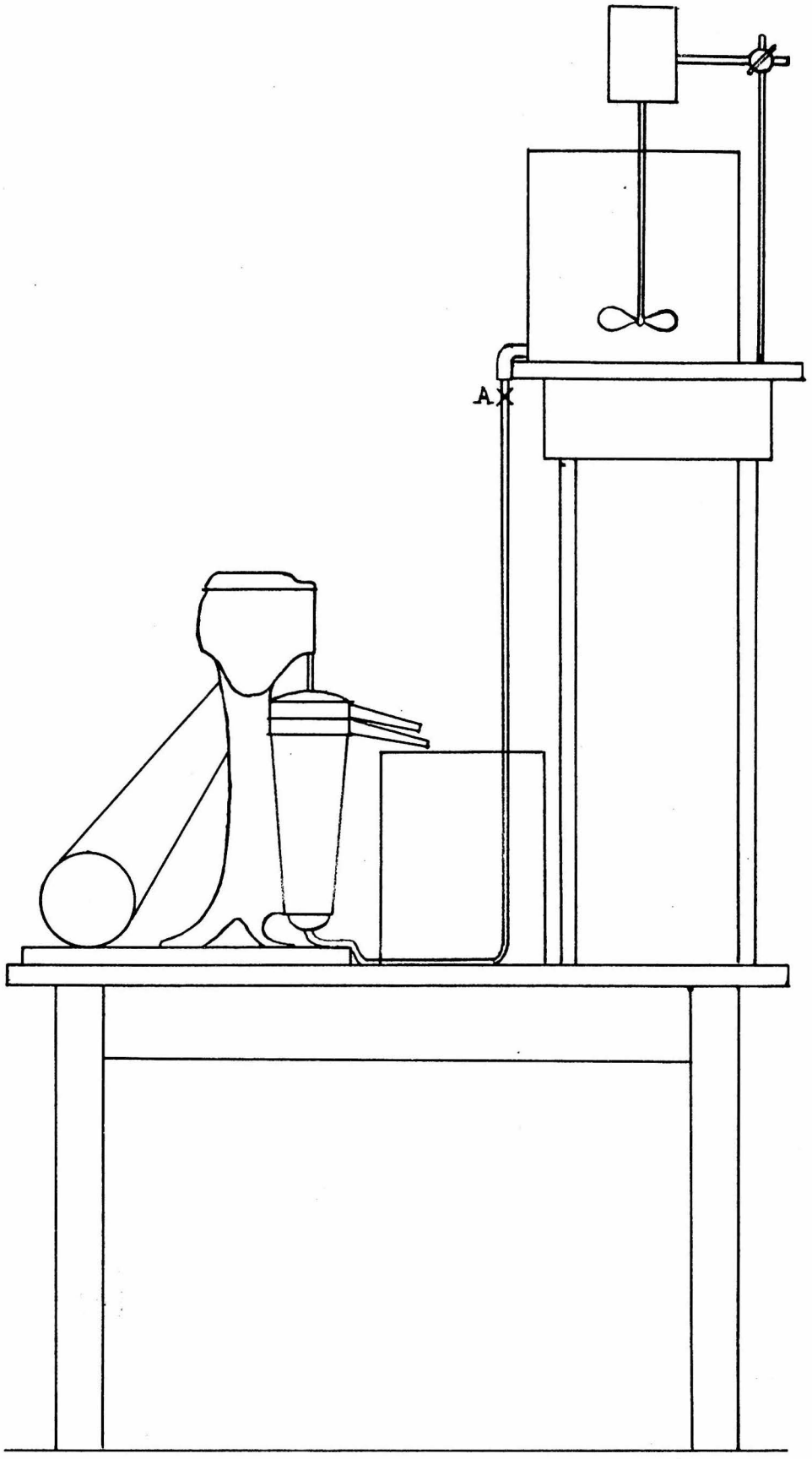
Westenbrink and Goudsmit used the following technique to make a preliminary separation of the thiamin from disturbing fluorescent substances:

"Urine was diluted with 3 to 10 times its volume of water and brought to pH= about 3 by adding hydrochloric acid. 100 mg. Franconite KL per 30 ml. urine are added and the mixture is stirred for 3 minutes. The Franconite is then separated from the liquid by centrifuging washing successively with acidulated water pH=3 and 96% alcohol and dried at 100° C."

The adsorption of thiamin on franconite is so strong that it can be accomplished even if the urine is first diluted 10 times, but the disturbing substances are not absorbed nearly as well under the conditions described. This results in a quite effective quantitative separation of the thiamin present in the urine from the disturbing substances.

When the investigation was started no Franconite KL was available, and before it could be obtained from Germany, another applicable adsorption material was obtained. It was found that Fuller's earth could be used, and it was applied throughout the investigation. With this material a pH of 4 was better than a pH of 3, and instead of 100 mg. per 30 ml. urine (as with Franconite), 4 grams of Fuller's earth per total individual daily excretion of

UNIT OF SHARPLES CENTRIFUGE AND ADSORPTION JAR.

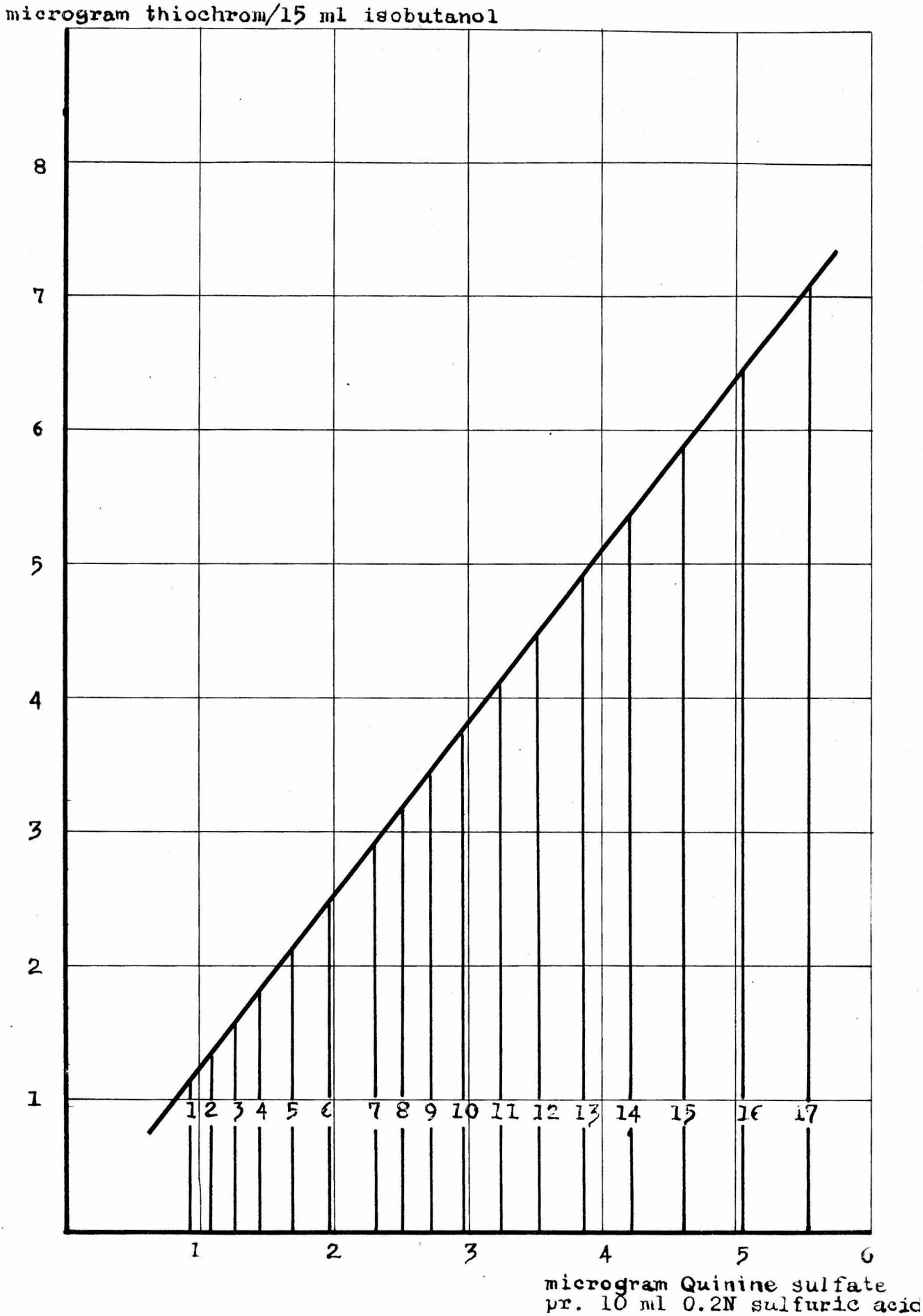


urine was used.

The use of so large an amount of urine as the total daily excretion of a man was due to the fact that in cases of very low thiamin excretion, it was necessary to have all the excreted urine to be able to determine the thiamin. Since it was desired to standardize the method throughout, the same technique was applied for urine with high thiamin content. The fact that the experimental persons excreted about 1000-1500 ml. urine in 24 hours, and that this urine had to be diluted to 10 times its own volume, gave rise to difficulties concerning the centrifugation of the Fuller's earth from the dilute urine. It was undesirable to leave the urine and Fuller's earth in contact with each other more than a short time, partly because this would prolong the procedure, and also because it would interfere with the results since the disturbing substances are adsorbed if given a sufficiently long time. A Sharples centrifuge was found very useful for the purpose of separating Fuller's earth from the dilute urine after adsorption. The centrifuge was equipped with two 20 Liters jars (see p. 6).

With the method of Jansen (5) it is possible to standardize the galvanometer readings against known amounts of thiamin, and is unnecessary to compare the unknown solutions with fresh standards as described by Karrer. The fact that the thiochrome solutions did not seem to be stable (if not made up by dissolving pure thiochrome, which was not available at this time) made it impossible to make up a known series of standards with which the unknown thiochrome solutions could be compared. To avoid the repeated making of standards an attempt was made to find a compound other than thiochrome possessing a similar fluorescence, but

RELATION BETWEEN FLUORESENCE OF QUININE STANDARDS AND THIOCHROME SOLUTIONS.



stable in solution for a considerable length of time. Such a substance was found in quinine sulfate. An aqueous acid solution of this compound has a bluish-green fluorescence, that resembles closely that of thiochrome. A series of standard solutions of quinine sulfate in 0.2N H_2SO_4 were made up, and their "thiochrome values" determined by comparing them under ultraviolet light with known amounts of oxidized thiamin. The concentrations of the standards compared with their "thiochrome values" are shown on page 10. It was found that the relative change in fluorescence with change in concentration was the same for thiochrome and quinine sulfate solutions within certain concentration limits. In other words, if the concentration is plotted against fluorescence as on page 8, we find that within the concentration limits applied, the relation is a straight line. The concentration of the quinine standards were chosen by finding the smallest possible difference in concentration it was possible to recognize with certainty by the fluorescence method. A test for the usefulness of the concentration range of the standards was to arrange the standards in the dark by their fluorescence. This was possible with the chosen concentrations. Furthermore, the difference in concentration between the standards were purposely chosen so that a given standard was exactly 10% higher than the preceding standard. In this way the difference between the standards increase with increasing number of the standards, but the maximum uncertainty on the determination is constantly 10%. (The standards below No. 7 ~~has~~ have 15% difference in concentration).

After the method had been applied for some time it was possible to determine not only ~~if~~ whether the unknown tube had a fluorescence which lay between two standards, but also to determine ~~if~~ whether it was

"Thiochrome values" of Quinine Standards.

Standard number	γ Quinine sulfate per 10 ml. 0.2N H_2SO_4	γ Thiamin per ml. i-BuOH
1	0.95 microg.	0.082
2	1.08 "	0.093
3	1.24 "	0.107
4	1.42 "	0.125
5	1.68 "	0.144
6	1.95 "	0.166
7	2.30 "	0.189
8	2.50 "	0.206
9	2.71 "	0.223
10	2.96 "	0.240
11	3.22 "	0.265
12	3.51 "	0.289
13	3.84 "	0.315
14	4.20 "	0.345
15	4.60 "	0.378
16	5.04 "	0.415
17	5.54 "	0.455

equally far from both standards or nearer to one of the two standards. Thus it was possible to increase the certainty of the standard determination to 5% of the measured amount.

Finally one more question has to be discussed, namely, the amount of ferricyanide applied in the oxidation of the thiamin. Both Jansen and Karrer found that if the amount of ferricyanide used to oxidize a given amount of thiamin was varied, the fluorescence of the resulting thiochrome solutions would also vary somewhat. This led each of them to do determinations with a series of different amounts of ferricyanide, and then select the thiochrome solution with the largest fluorescence as the one with the correct amount of ferricyanide. This method was quite laborious and it was attempted to avoid making more than one oxidation for each analysis. This was possible because Jansen (5) had found, that the addition of methyl alcohol to the oxidation mixture would cause the decrease in fluorescence with sufficient excess of ferricyanide to be almost zero. By always using a rather large excess of ferricyanide it was possible to reduce to a minimum the fluctuation of fluorescence. The fact that the standards were determined with a similar excess of ferricyanide made the results comparable, even if the maximum fluorescence was not obtained by this method. Furthermore, when such an excess of ferricyanide used, no effect of using air as a stirring agent in the extraction with isobutanol of the oxidized thiamin solutions could be observed. Such an effect was observed by Westenbrink and Goudsmit and led them to use Nitrogen as a stirring agent.

With these preliminary remarks, it will now be possible to describe the complete method of analysis, as applied to all the

determinations in the investigation:

The volume of the specimen of urine to be used for thiamin determination, consisting of the total excretion in 24 hours by one of the persons cooperating in the experiment, was first measured. The pH was then determined with a Beckman PH-meter, and the urine adjusted to pH 4 by adding 2N sulfuric acid. The urine was poured into the top jar (see page 6) and tap water was added until the total volume was 18 liters, as determined by a mark inside the jar. Four grams of Fuller's earth were then added, and the electric stirrer started. After five minutes the Sharples centrifuge was started, and the suspension run through the centrifuge by opening valve A (see page 6). The centrifugation took about 10 minutes. The rotor in the centrifuge was then taken apart and the Fuller's earth scraped out and suspended in a little acidulated water, filtered on a Büchner funnel, washed with 96% alcohol and finally dried at 100° C.

One gram of the dry Fuller's earth was now placed in each of two centrifuge tubes. To each was added 2 ml. 10% NaOH and 1 ml. methyl alcohol. After a gentle shaking, 1 ml. 1% ferricyanide solution was now added to one of the tubes and 1 ml. distilled water to the other. To both was added 15 ml. isobutanol (Eastman) from a burette and a stream of air passed through the tubes for 5 minutes. This caused a thorough mixing of the two phases. The tubes were finally centrifuged to separate the two layers clearly, and the isobutanol layer was filtered into a test tube.

We have now two test tubes, one containing the oxidized thiamin plus the remaining small amounts of interfering fluorescent substances which we shall designate as the analytical tube, the other with the unoxidized thiamin (non-fluorescent) but with the

same amount of interfering substances. This we shall designate as the blank tube. The two tubes were now compared with the quinine standards in the box (see page 4) until two standards were found between which lay the fluorescence of the unknown tube. If the concentration of the thiochrome solution in the analytical tube was too great to fit with the standards, the thiochrome solution was diluted with isobutanol until a satisfactory concentration was obtained. The appr. "Thiochrome value" of the unknown tubes could now be found from the graph on page 8. By subtracting the value of the blank, from that of the analytical tube, and taking into consideration the amount of earth used for the adsorption and analysis, and the volume of isobutanol of the thiochrome solutions, the thiamin content of the urine could be computed. However, it is necessary to extract the water layer again with isobutanol if large amounts of thiamin are present in the urine, because in this case the fraction of thiamin left behind after the first extraction is considerable. If this was the case both the analytical tube and the blank tube were extracted repeatedly with 15 ml. isobutanol and their thiochrome values determined as above. The sum of the thiochrome values for the analytical tubes minus the sum of the values from the blank tubes gives the thiamin content.

On the next page is given a data sheet as used during the work. This contains in a convenient way all the necessary data for later reference.

As a check on the method it was tried to ascertain if it is possible to recover to 100% thiamin added to urine. The following experiment shows that this is possible:

In person A's urine from the tenth day of the second period of the oral experiments, was found 345 microgram thiamin in the

analytical tube. To half of the urine, before adsorption, was added 0.05 mg. thiamin, and the analytical tube contained 450 microgram thiamin. The blank tubes were the same in both cases. The difference is 105 microgram thiamin. To half of the urine was added 50 microgram, and 52 microgram was recovered, which is inside the experimental error.

This same type of experiment was repeated once in a while to check the method, and ~~always~~ always came out quite satisfactory.

VITAMIN B_1 DETERMINATION

ies no: ____ Analysis no: ____ Date: / - 193

ject: ____

al amount of fullers earth: _____g. Used for analysis: _____g.

ume of urine: _____cc. p_H: _____ Added H_2SO_4_____cc. p_H: _____

uted to: _____ liter. Total time for adsorbtion: _______ min.

lysis:

rac-n no.	Volume cc	Quinine standard	B_1 per cc. i-BuOH	micromg B_1
1				
2				
3				
4				
5				

Total: mmg

nk:

rac-n no.	Volume cc	Quinine standard	B_1 per cc. i-BuOH	micromg B_1
1				
2				
3				
4				
5				

Total: mmg

lysis: mmg
nk: -
fer. mmg = I.U. per day

- -

arks:

The Phycomyces Method:

Stock cultures of Phycomyces Blakesleeanus were maintained on malt agar. The experimental cultures were made up with medium containing:

$MgSO_4,7H_2O$. . . . . 0.5 gram

KH_2PO_4. . . . . 1.5 "

Asparagin . . . . . 4.0 "

Dextrose . . . . . 100.0 "

Made up to 1 liter with distilled water. To 10 ml. of this medium was added the desired amount of urine to be tested for thiamin. The experimental media were then autoclaved for 15 minutes at 15 pounds pressure, and inoculated with with equal volumes of a sterile spore suspension. All cultures were allowed to remain 10 days at 25° C., the mycelium then filtered off, dried and weighed. All experiments were carried out in triplicate. The weights of the mycelium is proportional to the amount of thiamin present in the sample, if the weight of the mycelium is not more than 40 mg. Thus by knowing the weight of the mycelium grown on a known amount of thiamin (0.1 microgram thiamin gives 30 mg. mycelium) it is possible to evaluate the amount of thiamin present in the sample.

The method is not as specific as the thiochrome method, because Phycomyces is able to utilize also cocarboxylase and free pyrimidine + thiazol. However, this is what in particular makes it useful for the purpose of determining free pyrimidine or thiazol, because if urine contains one of the two, and the other is added, an increased growth of the mycelium will be observed. The response of Phycomyces to cocarboxylase is probably not of any importance in the case of urine, since the latter is reported to

contain no cocarboxylase.

It was attempted to utilize Fuller's earth adsorbates as source of thiamin for Phycomyces, but the mould seems not to be able to utilize all of the thiamin present, when it is adsorbed on Fuller's earth. Urine was, therefore, used directly in the tests.

2. Materials:

Since it was desired to approach as closely as possible to a natural diet, Galen B a concentrate of rice bran containing 150 micrograms thiamin per ml., was used instead of synthetic thiamin for the oral experiments.

Thiamin-hydrochloride was used for the intramuscular injections. It was obtained in sterile solution at a concentration of 10 mg. per ml. The thiamin was synthesized by the Merck & Co., Rahway, N. Y.

Both preparations were kindly supplied by the Galen Company, Berkeley, California.

3. Individuals Participating in the Experiments:

The three persons, designated as A, B and C in the report, were all males and in normal health. They had the following ages and body weights:

A	26 years old	Weight	177 lbs.
B	28 " "	"	160 "
C	25 " "	"	155 "

4. Experiments with Oral Intake of Thiamin in Form of Galen B.

The persons A and B participated in the experiment which lasted 48 days. The experiment consisted of six periods with different intake of Galen B. The periods followed immediately after each other. The diet was what could be designated as a normal diet, probably containing about 1 mg. thiamin per day. However, the diet was selected with special attention to uniformity from day to day, by avoiding any food particularly high in thiamin. The Galen B was taken in one dose on the days when a dose was given. With the high doses of 40 and 60 ml., this caused a pronounced feeling of hunger and some discomfort.

The urine was collected in bottles from 8 A.M. of one day until the same time the next day. Temperature changes in the atmosphere had a pronounced effect on the volume of urine excreted. The pH of the urine was quite constant at about 6.2.

Determination of the thiamin content were made daily as far as possible, and in practically all cases the adsorption on Fuller's earth was done the day after the urine had been collected.

First Period:

Intake: Normal Diet. No Extra Galen B.

Excretion:

Day	Person A	Person B
1	210 - 150 = 60 microg. B_1	190 - 140 = 50 microg. B_1
2	225 - 150 = 75 " "	240 - 160 = 80 " "
3	210 - 150 = 60 " "	210 - 150 = 60 " "
4	215 - 150 = 65 " "	200 - 150 = 50 " "
5	275 - 150 =125 " "	200 - 150 = 50 " "
6	205 - 140 = 65 " "	175 - 115 = 60 " "
7	200 - 150 = 50 " "	180 - 140 = 40 " "
8	220 - 150 = 70 " "	185 - 125 = 60 " "
9	215 - 140 = 75 " "	175 - 100 = 75 " "
10	220 - 150 = 70 " "	170 - 130 = 40 " "
	Average = 71 " "	Average = 56 " "

Second Period:

Intake: Normal Diet, plus 10 ml. Galen B daily.

Excretion:

Day	Person A	Person B
1	220 - 140 = 80 microg. B_1	370 - 180 = 190 microg. B_1
2	380 - 160 = 220 " "	420 - 150 = 270 " "
3	360 - 160 = 200 " "	405 - 200 = 205 " "
4	370 - 130 = 240 " "	450 - 200 = 250 " "
5	360 - 160 = 200 " "	340 - 200 = 140 " "
6	170 - 150 = 20? " "	410 - 200 = 210 " "
7	440 - 150 = 290 " "	555 - 200 = 355 " "
8	380 - 150 = 230 " "	545 - 200 = 345 " "
9	- - -	385 - 200 = 185 " "
10	345 - 100 = 245 " "	625 - 200 = 425 " "
	Average = 191 " "	Average = 255 " "

Third period:

Intake: Normal Diet, plus 20 ml. Galen B daily.

Excretion:

Day	Person A	Person B
1	600 - 160 = 440 microg. B_1	1010 - 200 = 810 microg. B_1
2	570 - 150 = 420 " "	680 - 200 = 480 " "
3	580 - 175 = 405 " "	695 - 200 = 495 " "
4	630 - 195 = 435 " "	685 - 200 = 485 " "
5	564 - 150 = 415 " "	715 - 200 = 515 " "
6	690 - 200 = 490 " "	700 - 200 = 500 " "
7	- - -	1000 - 200 = 800 " "
8		740 - 200 = 540 " "
9		975 - 200 = 675 " "
	Average = 435 " "	Average = 590 " "

Fourth period:

Intake: Normal Diet, plus 40 ml. Galen B daily.

Excretion:

Day	Person A	Person B
1	1215 - 340 = 880 microg. B_1	645 - 210 = 435 microg. B_1
2	1510 - 340 =1170 " "	1075 - 275 = 800 " "
3	1310 - 330 = 980 " "	1150 - 260 = 890 " "
4	805 - 250 = 555 " "	985 - 210 = 775 " "
5	1035 - 220 = 815 " "	- - -
6	1005 - 150 = 850 " "	1020 - 195 = 825 " "
7	1040 - 210 = 830 " "	1305 - 195 =1110 " "
8		1530 - 250 =1280 " "
	Average = 870 " "	Average = 875 " "

Fifth period:

Intake: Normal Diet, plus 60 ml. Galen B daily

Excretion:

Day	Person A	Person B
1	1400 - 215 = 1185 microg. B_1	1600 - 225 = 1375 microg. B_1
2	1550 - 315 = 1225 " "	1460 - 235 = 1225 " "
3	1680 - 290 = 1390 " "	1900 - 245 = 1655 " "
4	- - -	2105 - 235 = 1870 " "
5	1830 - 285 = 1545 " "	2010 - 320 = 1690 " "
6	1185 - 315 = 870 " "	
7	1450 - 235 = 1215 " "	
8	1440 - 350 = 1090 " "	
	Average = 1215 " "	Average = 1565 " "

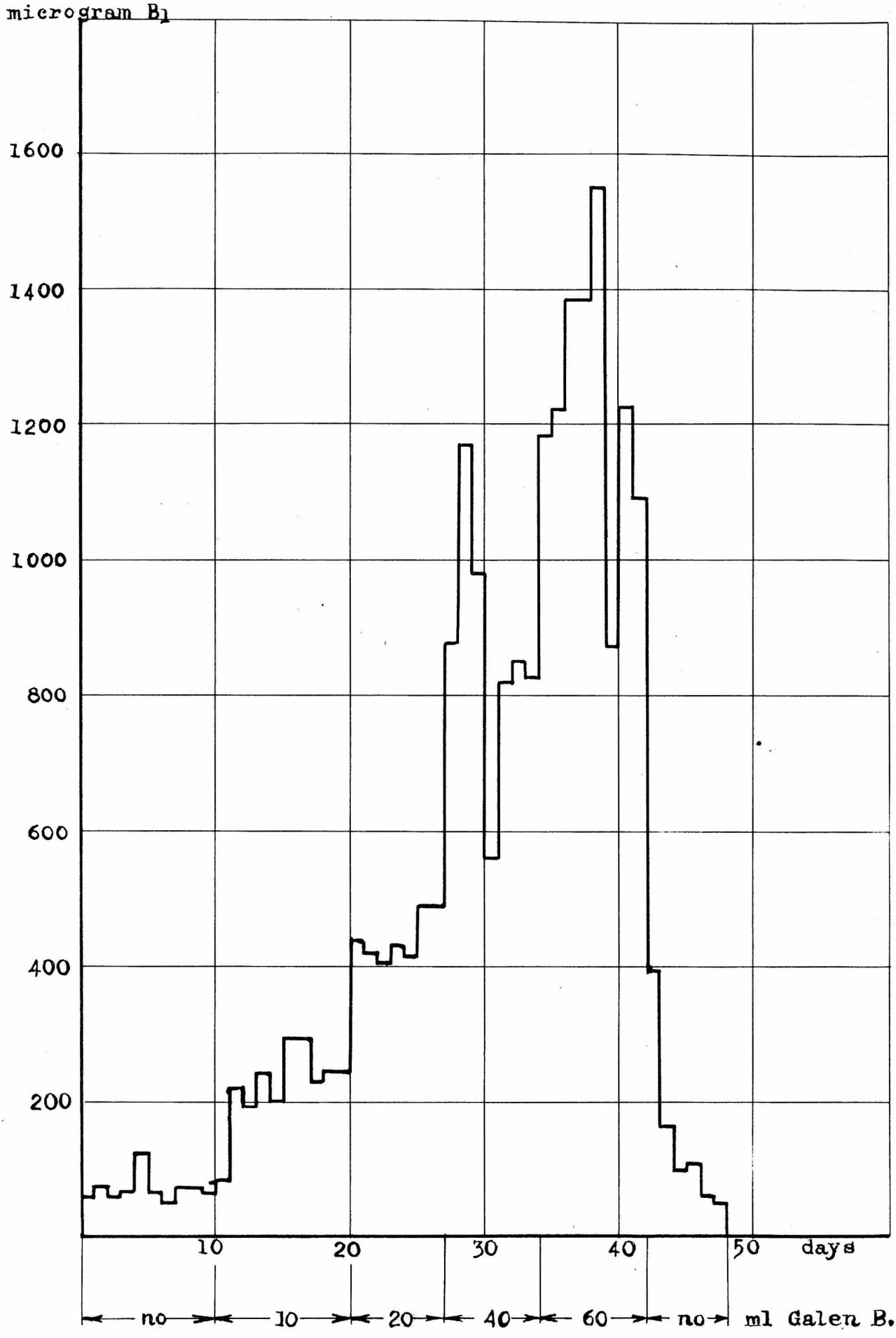
Sixth period:

Intake: Normal Diet. No extra Galen B.

Excretion:

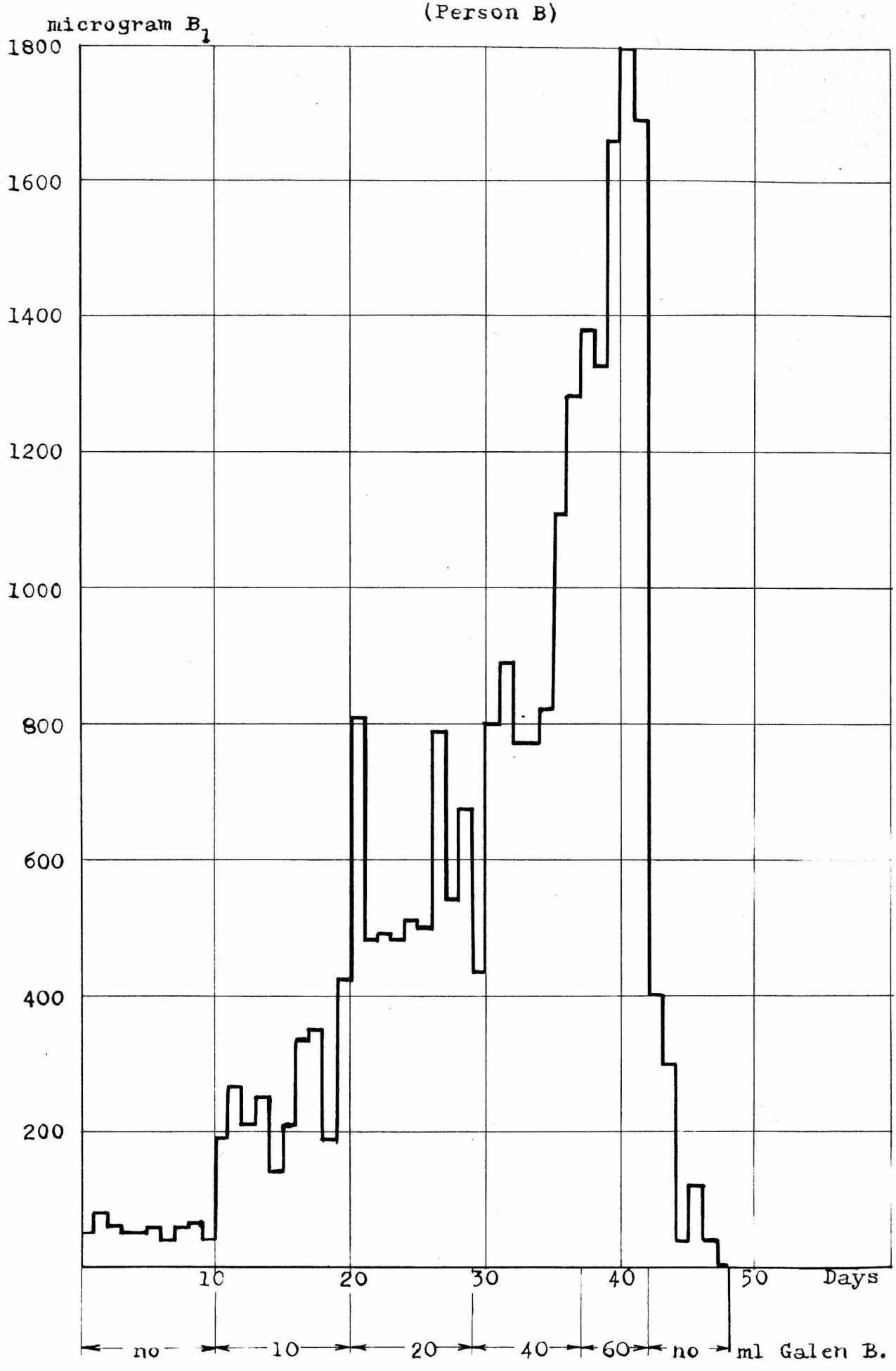
Day	Person A	Person B
1	695 - 300 = 395 microg. B_1	585 - 185 = 400 Microg. B_1
2	350 - 190 = 160 " "	505 - 205 = 300 " "
3	250 - 140 = 105 " "	245 - 210 = 35 " "
4	320 - 210 = 110 " "	255 - 135 = 120 " "
5	235 - 175 = 60 " "	210 = 165 = 45 " "
6	260 - 210 = 50 " "	180 = 180 = 0 " "

RELATION BETWEEN ORAL INTAKE AND URINARY EXCRETION OF THIAMIN.
(Person A)



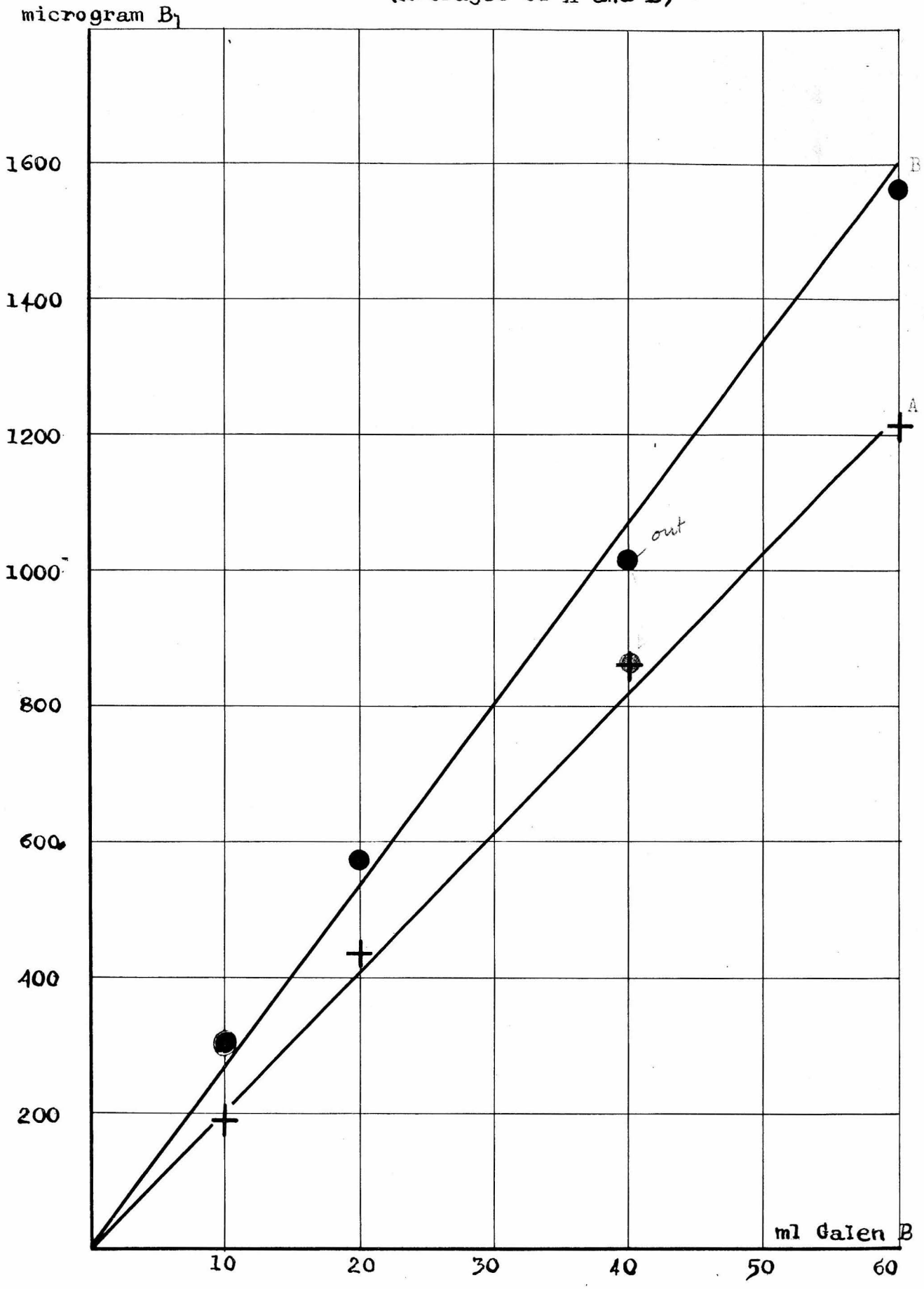
RELATION BETWEEN ORAL INTAKE AND URINARY EXCRETION OF THIAMIN.

(Person B)



RELATION BETWEEN ORAL INTAKE AND URINARY EXCRETION OF THIAMIN.

(Averages of A and B)



5. Experiments with Intramuscular Injections.

The persons A and C participated in the experiment which lasted for 30 days. The experiment consisted of four periods with different amounts of thiamin injected intramuscularly in the upper half of the left arm. The periods followed immediately after each other. A diet similar to that used during the oral experiment was maintained. Injection of a high dose of thiamin intramuscular was not followed by any discomfort. The injected thiamin seemed to have a good influence on the general well being of the persons A and C, during the experiment. The urine was collected as for the oral experiments.

The first period consisted of 10 days where only a normal diet was given, the excretion in this period averaged 60 microgram of thiamin per day for both persons.

Second period:

Intake: Normal Diet, plus 3 mg. thiamin intramuscularly.

Excretion:

Day	Person A	Person C
1	0.640 mg. thiamin	0.730 mg. thiamin
2	1.200 " "	0.930 " "
3	1.130 " "	0.940 " "
4	1.200 " "	1.110 " "
5	1.270 " "	0.990 " "
6	1.090 " "	0.630 " "
Average =	1.090 " "	0.890 " "

Third period:

Intake: Normal Diet, plus 10 mg. thiamin intramuscularly.

Excretion:

Day	Person A	Person C
1	4.26 mg. thiamin	3.15 mg. thiamin
2	4.79 " "	4.11 " "
3	5.71 " "	5.03 " "
4	8.60 " "	5.33 " "
5	11.95 " "	6.34 " "
6	8.35 " "	5.12 " "
Average =	7.28 " "	4.85 " "

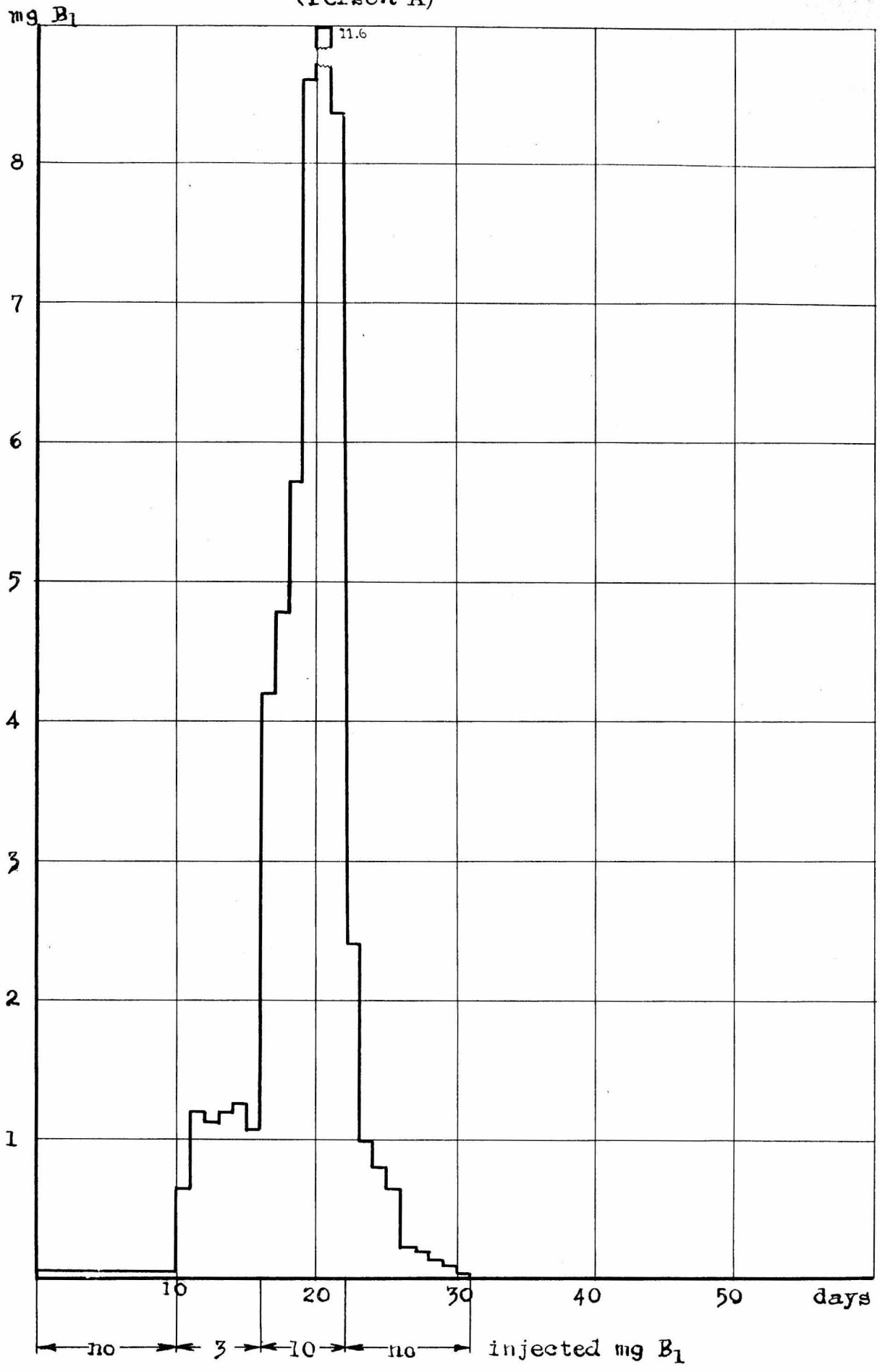
Fourth period:

Intake: Normal Diet. No injections.

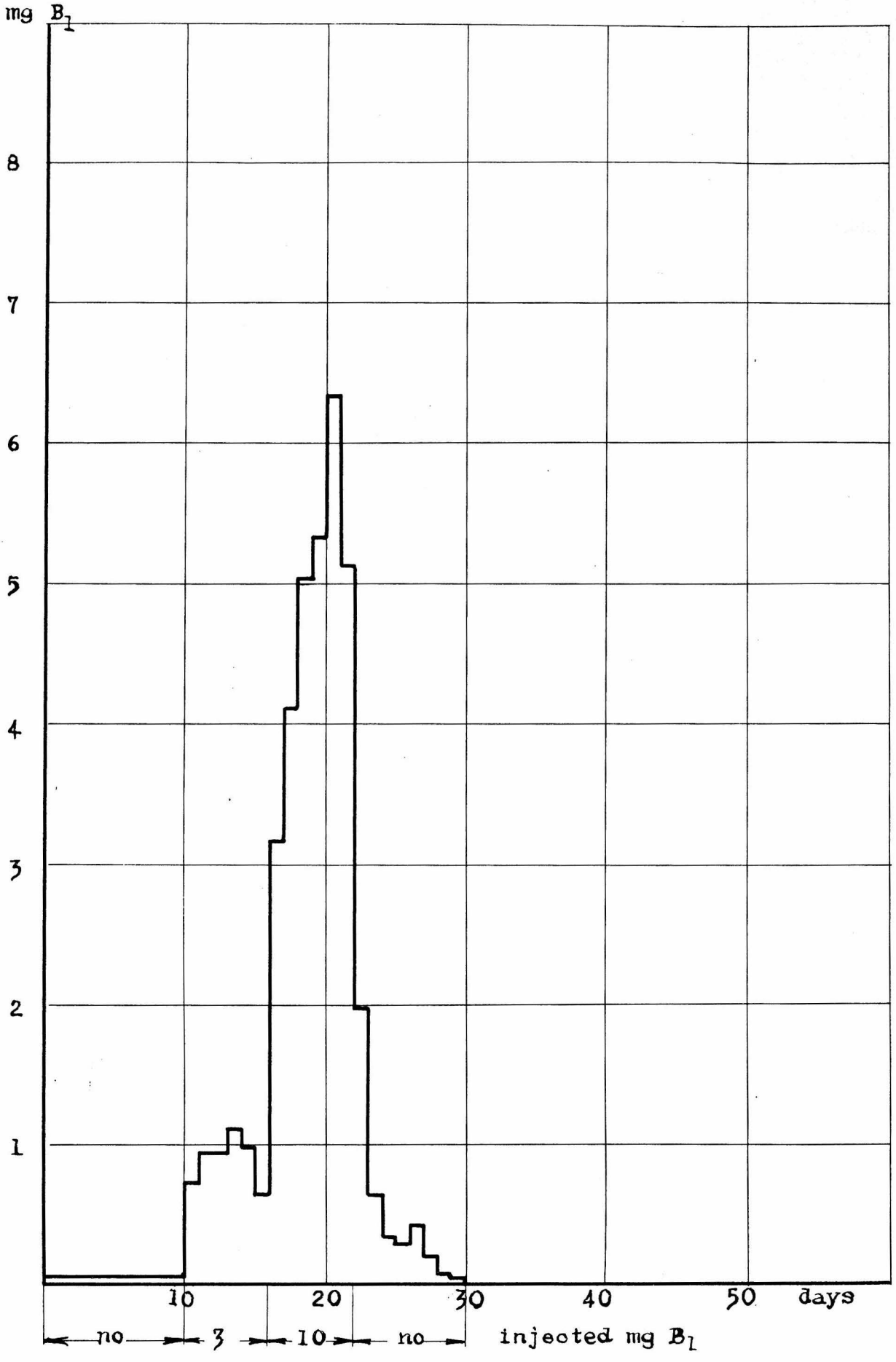
Excretion:

Day	Person A	Person C
1	2.44 mg. thiamin	1.99 mg. thiamin
2	1.02 " "	0.63 " "
3	0.80 " "	0.34 " "
4	0.65 " "	0.30 " "
5	0.22 " "	0.42 " "
6	0.21 " "	0.20 " "
7	0.15 " "	0.07 " "
8	0.11 " "	0.05 " "
9	0.09 " "	0

RELATION BETWEEN INTRAMUSCULARLY INJECTED AND URINARY EXCRETED THIAMIN
(Person A)



RELATION BETWEEN INTRAMUSCULARLY INJECTED AND URINARY EXCRETED THIAMIN
(Person C)



6. Determination of Pyrimidine in Urine.

The samples of urine was from persons A and C, both from the second periods second day in the injection experiment series.

Person A: excreted 670 ml. of urine containing 1.2 mg. thiamin or 1.79 microgram per ml.

Person B: excreted 490 ml. urine containing 0.93 mg. thiamin or 1.90 microgram per ml.

As found by the thiochrome assay (see page 24)

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The contents of the flasks and the average weights of the mycelium has been stated below:

Flask No.	Content	Weight of mycelium
1 2 3	0.05 ml. of A's urine	31 mg. diff. 17 mg.
4 5 6	0.05 ml. of A's urine + 10^{-5}mol. thiazol	48 mg.
7 8 9	0.05 ml. of C's urine	29 mg. diff. 13 mg.
10 11 12	0.05 ml. of C's urine + 10^{-5}mol. thiazol	44 mg.

Controls = 0 0.1 microgram thiamin = 30 mg. mycel.

The difference between flask No. 4-6 and flask No. 1-3 was 17 mg. This was due to the presence of free pyrimidine in the urine. The amount of pyrimidine in A's urine was 35% of the pyrimidine found bound in thiamin. The amount found in C's urine was equivalent to 29% of the pyrimidine found bound in thiamin in C's urine.

Discussion:

The results of the experiments with oral administration of thiamin have been plotted on page 21 and 22.. It is to be noted that there is a considerable variation in each period both for persons A and B. This can be explained partly by experimental errors in the analysis, but the main reason for the variation is probably to be found in the physiological conditions of the persons participating in the experiment. For example, augmented exercise on certain days give rise to an increased requirement for thiamin due to an increased carbohydrate metabolism. Furthermore, in the fourth and fifth periods, where the intake of Galen B was very high, the laxative effect of the thiamin caused in some cases Diarrhea which might have prevented the thiamin from being completely absorbed by the intestine.

On page 23 the average values for each period have been plotted against the intake of thiamin in the form of Galen B. The four points on the curve fit a straight line going through the origin reasonably well in both cases. This indicates that under the conditions of the experiment a constant proportion of the oraly administered thiamin is excreted in the urine, even when the intake is varied over very broad limits. However, the experiment does not permit of generalizing on this observation, because the preceding periods might have had an effect on the following periods. It is reasonable to assume that the bodies relative saturation with thiamin in the beginning of the experiment may also be a factor in the proportion of thiamin excreted in the urine.

The difference in the slopes of the lines for persons A and B, can possibly be accounted for by the difference in body weights

of the individuals A and B, since it is known that thiamin requirement is dependent on body weight. Person B weighed 10% less than A.

The results of the experiments with intramuscularly injected thiamin have been plotted on pages 26 and 27. The experiments were started by first examining the thiamin excretion in the urine for 10 days on a normal diet. The excretion in this period averaged about 60 gamma daily for both persons. In the next period 3 mg. thiamin were given daily intramuscularly, and it will be noted that on the first day only 20% of the injected were excreted. After this first day the excretion assumed a relatively constant level with approximately 40% of the daily injected thiamin excreted in the urine. In the next period where 10 mg. thiamin were given daily intramuscularly, the same phenomenon was observed. After a period of increasing urinary thiamin output (first day -42%, second day -48%, third day -57%), a relatively constant level was reached. In the case of A practically all of the thiamin given is excreted (average of fourth, fifth and sixth day for A was 98%). For C the average was only 58% during the same days. A and C seems to behave differently in this respect. On the twenty-second day of the experiment the injections were stopped, and the urinary output of thiamin decreased daily, until it regained the original level. This period lasted 9 days.

In totally 78 mg. thiamin were injected. A excreted a total of 50 mg. of thiamin or 65% of the administered amount during the period of injection. C excreted in the same period 34 mg. or 44% of that administered.

In the after-period of nine days A excreted a total of 5.7 mg.

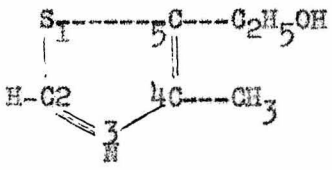
and C a total of 4.0 mg.

Comparing the results of the injection experiments with those where an oral dose of the same magnitude was given, we find that the excretion in the case of injected thiamin is much higher. This may possibly be due to an incomplete absorption of thiamin in the intestine. In this case vitamin should be found in the feces. There is evidence for the occurence of large amounts of thiamin in feces. But even if the fecal thiamin is taken into consideration, it is not possible to account for all of that administered.

Furthermore, in the case of injected thiamin, it is unlikely that fecal excretion can account for the amount not excreted in the urine. Hatcher and Borsook (2) has found only very small amounts of thiamin in the feces when the thiamin was administered by injection.

The fact that it is impossible to account for the total amount of thiamin administered by urinary and fecal excretion, suggest that the body may be able to destroy thiamin, enzymatically or otherwise. Such a possible breakdown is obscure at present, and has not been demonstrated in mammalian tissues. However, the clinical and theoretical importance which might be connected with the elucidation of the properties of such an enzyme should be expected to be considerable, so that further work along this line should be worth while.

In this connection the work of Bonner and Buchman (1) on the destruction of thiamin by the mould Phycomyces Blakesleeanus should be mentioned. Bonner and Buchman found, that the thiazol part of the thiamin molecule is broken down by an enzymatic reaction in the mycelium. They were able to show that free pyrimidine is a result of this breakdown.



The reaction products of the breakdown of the thiazol part are not yet known but their experiments suggest that the initial rupture of the thiazol ring occurs in the 2-3-position. They found that the vitamin analog which has a CH_3-group in the 2-position of the thiazol ring is apparently not broken down enzymatically. The 2-CH_3-group is able to protect the vitamin against destruction and it can, therefore, be assumed that the destruction of the thiazol ring begins with the double bond between nitrogen and carbon.

A preliminary experiment to find if urine contains any uncombined vitamin-pyrimidine has been reported on page . It was found that the two samples of urine examined contained an amount of free pyrimidine equal to about 30% of the pyrimidine combined with thiazol. This might, however, be due to the presence of free pyrimidine in the basic diet rather than to the destruction of injected vitamin, since it is known that many plants contain up to five times as much free as combined pyrimidine. It is, therefore, necessary to perform similar experiments with a diet known to be free of uncombined pyrimidine.

The injection experiments show that there is a maximum limit to the amount of thiamin which the body will absorb. When the body (in the case of A) has been "loaded up" by injection of large doses of thiamin over some time, further administration of thiamin leads to approximately 100% urinary excretion. This saturation can only be established if the blood is constantly loaded with thiamin by injection, and as soon as the injection stops, the body starts discharging thiamin in the urine, and returns to its old condition after approximately nine days.

The reason for this rejection of excess thiamin by the body is not known, and the mechanism will probably be difficult to elucidate. By taking into consideration, however, some of the facts known about the reaction of the vitamin in certain microorganisms, as for example yeast, it is possible to offer a partial explanation of the reason for and the mechanism of this rejection.

From the equilibrium constant of the oxidative pyruvic acid decarboxylation it is known that this process favors the breakdown products to such an extent that the reaction can be considered as irreversible, in the body. The fact that all the other reactions involved in the carbohydrate metabolism are reversible, makes it possible that if no other limiting factor stops the reaction, a high concentration of carboxylase would tend to make the body deplete itself rapidly of carbohydrates. In this connection should be mentioned, that an increased amount of thiamin in the body really increases the carbohydrate metabolism to a marked degree (see Hecht and Weese, 3).

It is, therefore, understandable that the body should have a protective mechanism against excessive doses of thiamin.

It is well known that thiamin does not act in the body as such, but that it must first be pyrophosphorylated to cocarboxylase. The mechanism of this phosphorylation in mammalian tissues has not yet been clearly established. Experiments of Tauber (11) in which it was reported that acetone dried intestinal mucosa tissue is able to phosphorylate thiamin have not been verified by other workers (see Ochoa, 8).

However, Weil-Malherbe (12) demonstrated that washed yeast plus adenylpyrophosphate in the presence of magnesium was able to

pyrophosphorylate thiamin, and a similar mechanism might very well be possible in mammalian tissues. It is known that brain tissues can pyrophosphorylate thiamin.

Westenbrink (13) found that washed yeast can also dephosphorylate cocarboxylase, but that large amounts of free thiamin are able to retard this reaction, possibly by a mass action effect. The same dephosphorylation of cocarboxylase was ~~observed~~ claimed by Horowitz and Heegaard (4) in an enzyme preparation of pea roots.

From a large number of determinations on the proportion between free and pyrophosphorylated thiamin in different tissues, Ochoa (8) concluded that an equilibrium must exist between the two compounds in the tissues, and this can only be explained by the presence of similar enzymes in the tissues, to those found by Weil-Malherbe and Westenbrink.

Finally the presence of the phosphate group in the cocarboxylase probably enables this compound to combine much more firmly with the tissue proteins than does free thiamin, and it seems, therefore, justified to assume that this phosphorylation is involved in the body's mechanism to absorb or reject thiamin.

One fact which emerges clearly from the present experiments is: that it is not possible to protect the body against future thiamin deficient periods, by filling it up with thiamin, because the body automatically will deplete itself rapidly. This behavior of thiamin is different from the behavior of some of the other vitamins, in particular from the behavior of the fat-soluble vitamins, which can be stored in the body for future use. This is not surprising considering the different chemical natures of these vitamins, but it constitutes an important fact in relation to the clinical use of thiamin.

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