STUDIES IN THIAMINE DEFICIENCY

IN

MAN AND EXPERIMENTAL ANIMALS.

by

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INTRODUCTION.

It has been recognised since the end of the last century that a dietary deficiency of a substance present in rice pericarp, now known to be thiamine, results in the condition of beriberi. The most convincing method of demonstrating that beriberi is due to a specific lack of thiamine, is to administer the pure thiamine to a patient suffering from beriberi and to note the speedy return to normal. This can be very dramatically shown in pigeons. A dietary deficiency in thiamine leads to neck retraction in the pigeon - an almost instantaneous recovery being made by the injection of thiamine. Beriberi was a condition very common among the rice-eating people of the East, and, since it has been recognised that this condition is due to lack of thiamine, which is present in rice pericarp, beriberi has almost disappeared.

In this country beriberi, in its severe form, is rarely met with and, if it is, the cure, and the proof that this actually was thiamine deficiency, is to administer thiamine.

However the state of sub-optimal thiamine nutrition leads eventually to a condition with various vague clinical signs and symptoms none of which are diagnostic of thiamine deficiency. It is in these cases of varying

degrees/

/degrees of mild thiamine deficiency, and in cases of sub-clinical thiamine deficiency, that biochemical evidence is of value. It was with this in mind that the following work was undertaken.

There was evidence to suggest that gastrectomy might lead to thiamine deficiency. An investigation into the state of thiamine nutrition was carried out in a group of post-gastrectomy patients and a comparable group of control subjects.

Although severe thiamine deficiency in humans is rare in this country a severe outbreak is liable to occur in animals which are being fed a standard, invariable diet. Such an outbreak occurred recently in Glasgow amongst greyhounds. This led to the opportunity of studying thiamine deficiency in greyhounds and also in other dogs.

THE DETECTION OF THIAMINE DEFICIENCY.

Five general methods have been employed in the detection of thiamine deficiency in man and experimental animals:-

(1) studies of the fasting and resting blood pyruvate concentration,

(2) examination of the blood pyruvate concentration following the administration of glucose orally or intravenously,

(3) examination of the blood pyruvate concentration following either mild or severe exercise,

(4) studies of the thiamine concentration in blood, and,

(5) examination of the thiamine excreted in the urine either on a basis of the 24-hour excretion of the vitamin, or on the basis of the amount of the vitamin excreted in the urine after a test dose.

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(1) The Fasting and Resting Blood Pyruvate Concentration.

Although the results obtained by any one worker depend on the method used for analysis, there is general agreement that the normal fasting and resting concentration of pyruvate in the blood of man and experimental animals lies in the range 0.4 to 1.2 mg. per 100 ml. with a mean of approximately 0.9 mg. per 100 per 100 ml. (See Table I).

TABLE I. Fasting and Resting Blood Pyruvate Acid Concentrations in Normal Human Subjects.

AUTHORS.	METHOD.	CONCENTRATION. mg. per 100 ml.
Platt & Lu (1936)	A	0.4 to 1.3
Platt & Lu (1939)	B	0.4 to 0.8
Bueding & Wortis (1940)	C	0.8 to 1.2
Kato & Li (1941)	B	0.6 to 1.0
Klein (1942)	C	0.6 to 0.9
Hulse et al. (1944)	C	0.8 to 1.2
Friedemann & Haugen (1943)	D	0.7 to 0.9
Friedemann et al. (1945)	D	0.6 to 0.8
Taylor & McHenry (1949)	E	0.9 to 1.3
Horwitt & Kreisler (1949)	D	0.7 to 1.1
Turnock & Welbourn (1953)	D	0.5 to 1.2
Kerppola (1953)	D	0.4 to 0.7
Bicknell & Prescott (1952)	-	0.3 to 1.3
	Means.....	0.65 to 1.05

Methods:-

A Peters & Thompson (1934).

B Lu (1939).

C Bueding & Wortis (1940).

D Friedemann & Haugen (1943) using ethyl acetate.

E Friedemann & Haugen (1943) using xylene.

The accumulation of pyruvate in the blood in thiamine deficiency was first shown by Thompson and Johnson (1935) in England using rats and pigeons, and in human beriberi by Platt and Lu (1936) in China. In the sub-acute form of human beriberi, the blood pyruvate concentrations were often found to lie within the normal range; the actual values found lying between 0.8 and 1.9 mg. per 100 ml. In severe acute beriberi in humans, much higher values were consistently obtained; values lying within the range 1.0 to 5.8 mg. per 100 ml. Lu (1939) also studied the effects of a thiamine deficient diet in experimental birds and animals. In pigeons, the blood pyruvate concentration rose from a normal value of 0.9 mg. per 100 ml. to 5.4 mg. per 100 ml. as the deficiency progressed to the severe acute stage. In rats, the corresponding figures were 1.1 rising to 3.2 mg. pyruvate per 100 ml.

Li and Kato (1941) made a large series of observations on the changes of blood pyruvate concentration during the induction of thiamine deficiency in rats. The fasting blood pyruvate concentration of normal rats was found to average 0.9 mg. per 100 ml., by the end of the second week on a low-thiamine diet it had increased to 2.0 mg. per 100 ml., and by the end of

two/

/two months, it had risen to an average of 6.0 mg. per 100 ml. - by which time the rats were clinically severely thiamine deficient. At this stage, injection of thiamine reduced the blood pyruvate concentration to about 2.5 mg. per 100 ml. within 24 hours with a simultaneous marked clinical improvement in the state of the animals.

In these experiments, Li and Kato recorded that increasing thiamine deficiency itself reduced the appetite, and consequently the food intake, of the rat. Anorexia is a typical symptom of thiamine deficiency. In another series of experiments, they found that starvation (which, of course, implies deficiency of all vitamins) increased the blood pyruvate concentration in rats, but the highest concentrations found were only 3.0 mg. per 100 ml. They concluded that this high value was due not only to lack of thiamine but to other unknown factors as well. Their most important observation was the considerable, and the very rapid, reduction in the blood pyruvate concentration of thiamine deficient rats following parenteral injection of the vitamin, thereby proving that thiamine is a major factor in the control of blood pyruvate concentration.

With/

/With human subjects, the changes in the blood pyruvate concentration in thiamine deficiency are not so great as with experimental animals, nor are the recorded results so convincing.

The effect of a low thiamine diet on 6 students was studied by Hulse, Weissman, Stotz and Clinton (1944). From an initial average value of 1.2 mg. pyruvate per 100 ml. blood, the figure rose to 1.4 mg. per 100 ml. by the seventeenth day, by which time the subjects were rather dispirited. Addition of thiamine to the diet at this point was followed by an immediate clinical improvement and the blood pyruvate rapidly returned to normal. Hulse considered these changes in the blood pyruvate concentration to be significant.

Horwitt and Kreisler (1949) also studied a series of 10 subjects on a thiamine deficient diet. Initially, the fasting blood pyruvate concentrations lay within the range 0.7 to 1.1 mg. per 100 ml. After two months on a low thiamine diet, the values had risen to 0.8 to 1.3 mg. per 100 ml., and at this point, the subjects were regarded as having developed a sub-clinical thiamine deficiency. After four months, the recorded values were 1.0 to 1.5 mg. pyruvate per 100 ml. blood, and definite signs of thiamine deficiency were apparent. These authors concluded that thiamine deficiency was present/

/present when the blood pyruvate concentration rose above 1.2 mg. per 100 ml.

In a similar series, Turnock and Welbourn (1953) agreed with this conclusion. Taylor and McHenry (1949) reported blood pyruvate concentrations within the range of 1.1 to 1.7 mg. per 100 ml. in a series of 12 cases classified as sub-clinical thiamine deficiency. Bueding, Stein and Wortis (1941), in a series of 13 cases of thiamine deficiency, found blood pyruvate concentrations around 1.8 mg. per 100 ml.

On the other hand, Klein and Elsom (1944) induced sub-clinical thiamine deficiency in a series of 5 volunteers without any significant alteration in the blood pyruvate concentration. Keys, Henschel, Taylor, Mickelsen and Brozek (1945), in a similar series, were unable to demonstrate any correlation between the degree of thiamine deficiency and the level of the blood pyruvate concentration. Berryman et al. (1947) made the comment that this disagreement was partly due to the variation in the fasting and resting blood pyruvate concentration in the normal individual, and partly due to disagreement on what constitutes the earliest clinical signs of thiamine deficiency.

In the interpretation of blood pyruvate concentrations, other factors are also involved. Since pyruvate is further metabolised in the body by oxidative processes,/

/processes, any condition leading to anoxia must necessarily lead to an increased concentration of pyruvate in the tissues and in the blood. Increased blood pyruvate concentrations have been demonstrated in congestive cardiac failure by Ochoa (1939) and by Friedemann, Haugen and Kmieciak (1945). The latter have also found increases in subjects at high altitudes, and in other forms of anoxia. In recent years, an increase in the blood pyruvate concentration has been demonstrated in conditions of stress and in other conditions where there is an increase in the circulating cortisone (Selye (1950), Rindi, Ferrari and Perri (1954), deCaro and Rindi (1955)).

It is clear that the blood pyruvate concentration in thiamine deficiency in human subjects does not increase to nearly the same extent that it does in experimental animals, and that for the purposes of detecting mild grades of thiamine deficiency in the human (which is the object of this research) it is not a very reliable criterion, although in severe cases of deficiency it undoubtedly has its place.

(2) The Blood Pyruvate Concentration following Administration of Glucose in Human Subjects.

It has long been known that the ingestion of glucose is followed by a rise in the blood pyruvate concentration. Almost identical changes are found when the glucose is given by intravenous injection. Most workers are agreed that the maximal blood pyruvate concentration is found about one hour after the administration of 50 g. glucose, when concentrations in the region of 1.6 to 2.2 mg. pyruvate per 100 ml. blood have been recorded.

Presumably this increase in the blood pyruvate concentration is due to increased glycolysis, and in any condition in which the further metabolism of glucose is interfered with (as, for example, in thiamine deficiency) a greater, and a more prolonged, rise in the blood pyruvate concentration is to be expected. This has been demonstrated by more than one research worker.

Bueding, Stein and Wortis (1941) found maximal values up to 2.8 mg. pyruvate per 100 ml. blood, with the maximum three hours after administration of glucose in cases of sub-clinical thiamine deficiency. Taylor and McHenry (1949) found maximal values in the/

/the range 1.5 to 3.3 mg. per 100 ml. again three hours after glucose ingestion. Other reported maximal values in sub-clinical thiamine deficiency include:- 1.0 to 3.0 mg. per 100 ml. after 2 hours (Turnock and Welbourn, 1953); 1.0 to 2.3 mg. per 100 ml. after 3 hours (Horwitt and Kreisler, 1949). The last two pairs of workers came to the conclusion that a delay in the return of the blood pyruvate concentration to the original fasting level was more significant than either the time taken to reach the maximal concentration or the actual blood pyruvate concentration at that point. The figures recorded by Bueding, Stein and Wortis (1941) and by Taylor and McHenry (1949) support this conclusion.

The comments of these various authors show no great enthusiasm for the test as a method of diagnosis in doubtful cases, although the results in established thiamine deficiency appear to be fairly consistent. Horwitt and Kreisler (1949) and Turnock and Welbourn (1953) both preferred to use the delay in the return of the blood pyruvate concentration to the normal level as a means of detecting sub-clinical thiamine deficiency. Taylor and McHenry (1949) regard the whole test as of some use in confirming severe deficiency, but not for detecting a sub-clinical deficiency. This view has been supported by Hulse et al. (1944) and by Florijn and Smits (1949) who have/

/have also used this method of investigation.

(3) The Blood Pyruvate Concentration following Exercise in Human Subjects.

In normal human subjects, a short bout of exercise is followed by an increase in the pyruvate concentration in the blood. During, and 10 - 15 minutes immediately following light exercise in normal subjects, blood pyruvate concentrations lying between 1.2 to 3.1 mg. per 100 ml. have been reported by Hulse et al. (1949), Platt and Lu (1939), Friedemann and Barborka (1941). Following a bout of severe exercise in normal subjects, blood pyruvate concentrations in the range 3.0 to 5.3 mg. per 100 ml. have been reported by Friedemann and Haugen (1943), Friedimann and Barborka (1941), Asmussen (1950), Johnson and Edwards (1937).

One would anticipate that exercise in thiamine deficient subjects would produce a great increase in the blood pyruvate concentration due to failure of the normal oxidative mechanism for removing pyruvate from the tissues and from the blood. Data found in the literature is exceedingly scanty and not very convincing. Only very mild cases of thiamine deficiency have been studied, and only light exercise has/

/has been tried. Platt and Lu (1939) found blood concentrations up to 1.2 mg. pyruvate per 100 ml. during the 10 minutes following a period of light exercise in mildly thiamine deficient subjects. Although this rise is less than that recorded above for normal subjects, it should be noted that the normal fasting and resting values obtained for the blood pyruvate concentrations by Platt and Lu are considerably lower than those found by most other workers (see Table I), so that an increase in concentration to 1.2 mg. per 100 ml. is quite substantial. (Platt and Lu found the normal fasting and resting values to be 0.4 to 0.75 mg. pyruvate per 100 ml. blood.) What is more important is that they found abnormally high concentrations of blood pyruvate 1 hour after the exercise had been completed in thiamine deficient subjects, whereas in normals, the concentration had returned to the original level by this time. Hulse et al. (1944) found values in the region of 1.5 mg. pyruvate per 100 ml. blood in patients with mild thiamine deficiency immediately after light exercise, and he further observed that the average value rose to a level of 1.7 mg. per 100 ml. fifteen minutes after the exercise had been stopped. Horwitt and Kreisler (1949) reported values of 1.3 to 2.5 mg. pyruvate in mildly/

/mildly thiamine deficient subjects 5 - 10 minutes after a period of light exercise.

These figures are far from convincing and do no more than suggest that the matter might be investigated further. It will have been noticed that the changes in the blood pyruvate concentration following ingestion of glucose, and following exercise, have been studied only in human subjects. There is no reported work on experimental animals.

(4) The Thiamine Concentration in Blood.

Random estimations of thiamine in blood are of little value in assessing the overall state of thiamine nutrition in human beings or in experimental animals. Such estimations mainly reflect the immediate prior intake of the vitamin. Variations in the fasting blood thiamine concentration have been reported by various workers - depending to some extent on the method and apparatus used in the estimation. For example, in normal human subjects, the following ranges have been reported:-

Blood thiamine.	Reference.
5.5 to 10.5 µg. per 100 ml.	Sinclair (1938).
9.0 to 13.5 µg. per 100 ml.	Westenbrink (1943).
6.0 to 9.0 µg. per 100 ml.	Williams (1943).

It/

/It is generally agreed that the concentration of thiamine in fasting blood cannot be used as an index of thiamine deficiency. Benson, Witzberger and Slobody (1942), for example, studied 22 children with unequivocal evidence of thiamine deficiency and found that all had blood thiamine concentrations within the normally accepted limits. This has been confirmed by other workers. In addition, there have been found to be considerable technical difficulties in the chemical method of estimation which cast some doubt on the accuracy of many of the published methods and their modifications.

(5) Excretion of Thiamine in the Urine.

(a) The 24-hour Excretion of Thiamine.

A number of workers have claimed that the 24-hour urinary excretion of thiamine is a reasonably good index of the nutritional status of the individual with respect to this vitamin.

There are three common methods of estimation of urinary thiamine, viz., the bradycardia method, the colorimetric diazo method, and the fluorometric thiochrome method, and there is some suggestion that the values found depend on the choice of method.

Harris/

/Harris and Leong (1936) used the bradycardia method and obtained urinary thiamine excretion of 72 to 105 µg. per 24 hours in the normal subject. They regard 30 µg. as the lower normal limit. Melnick, Field and Robinson (1939) and Robinson, Melnick and Field (1940) used the diazo method and recorded normal excretions ranging from 128 to 350 µg. per 24 hours. They regard 90 µg as the lower normal limit. A survey of the published data would suggest 100 to 300 µg. per 24 hours as a reasonable average normal.

As with blood, the chief objection to the urinary excretion of thiamine during a 24-hour period is that it reflects more the immediate previous intake of the vitamin. A minor, but extremely important, objection to the procedure in hospital work is the well-known hazard of attempting to collect accurate 24-hour specimens of urine in a general ward. Unless the amount of thiamine excreted in the urine is exceedingly small, significant results are not likely to be obtained, and at that stage of the condition, the diagnosis of vitamin deficiency is usually apparent clinically.

(b) Urinary Excretion of Thiamine following an Oral Test Dose of the Vitamin.

The oral dose generally used in this test is 5 mg. By collecting urine each hour following the test dose, Jowett (1940) showed that the most satisfactory results were obtained when the urine was collected over a 5-hour period following the test dose - the percentage excreted during this period being calculated. Robinson, Melnick and Field (1940) proved that thiamine was much more efficiently absorbed from the intestine when the 5 mg. test dose was given along with an ordinary mixed meal. Under these conditions they found that the normal subject excreted at least 350 µg. thiamine in the urine during the subsequent 4 hours, and this has generally been accepted as the normal standard.

As with all tests of this pattern, the possibility of errors due to malabsorption from the intestine casts doubt on the results, and this form of the test has been replaced by one using parenteral administration of the test dose.

(c) Urinary Excretion of Thiamine following parenteral Administration of a Test Dose of the Vitamin.

The general form of this modification is to give 1.0 mg. of thiamine either intravenously or intramuscularly (it does not seem to matter which) and to determine the amount of thiamine excreted in the urine during the next 4 hours. The following data appear to be generally accepted standards:-

Urinary Thiamine (4 hours).	Reference.
Minimal excretion of 110 µg.	Najjar and Holt (1940).
Minimal excretion of 180 µg.	Pollack et al. (1940).
Minimal excretion of 180 µg. (Mean = 160 µg).	Mason and Williams (1942

Any excretion over 160 µg. per 4 hours is regarded as showing adequate thiamine nutrition. Values between 160 and 60 µg. per 4 hours indicate varying degrees of sub-clinical thiamine deficiency, while values below 60 µg. per 4 hours are always accompanied by signs and symptoms of thiamine deficiency.

EXPERIMENTAL.

(1) The Estimation of Blood Pyruvate.

General Considerations.

Since Berzelius first isolated pyruvic acid in 1835, there have been over 50 methods, or modifications of methods, recorded in the literature for its estimation. Nearly all the methods in general use depend on some reaction common to all compounds containing a carbonyl group and quite a number of such compounds are present in blood, viz., acetoacetic acid, oxaloacetic acid, α-ketoglutaric acid, methyl glyoxal, dihydroxyacetone and acetone.

For example, the reaction of carbonyl compounds with bisulphite was used by Clift and Cook (1932) to estimate the total concentration of carbonyl ("bisulphite-binding") compounds in blood. Excess bisulphite was removed with iodine and the bound bisulphite, after being set free by hydrolysis with disodium hydrogen phosphate, was estimated by titration with iodine. A micro-chemical method using this procedure was developed by de Jong and Picard (1937) and was suitable for the analysis of small volumes of blood. Thompson and Johnson (1935) later showed that pyruvate comprised about 25% of the total "bisulphite-binding" substances in blood. And they also/

/also showed that the blood "bisulphite-binding" substances were increased in cases of thiamine deficiency, and that this increase was due almost entirely to an increase in pyruvate. They further demonstrated that administration of thiamine reduced the blood "bisulphite-binding" substances to the original normal limits.

About the same time, others were studying the reaction between carbonyl compounds and 2:4-dinitrophenylhydrazine and many attempts were made to make this reaction specific and quantitative for pyruvic acid. This is the basis of most of the modern methods. It has been extensively studied.

Neuberg and Kobel (1929) and Case (1932) were among the first to use this reaction as a basis for the estimation of blood pyruvate. 2:4-Dinitrophenylhydrazine was added to a trichloracetic acid filtrate of whole blood and the hydrazones thus formed were quantitatively extracted into ethyl acetate together with the excess dinitrophenylhydrazine reagent. After neutralisation with solid calcium carbonate, the ethyl acetate extract was evaporated to dryness and the residue taken up in toluene. The pyruvic acid hydrazone was extracted from the toluene with 10% sodium carbonate solution, and then precipitated from solution by neutralisation with/

/with concentrated hydrochloric acid. The precipitated hydrazone was dissolved in alcoholic potassium hydroxide and the red colour of the solution estimated directly by colorimetry.

This original method had certain advantages and certain disadvantages. Case proved that the crystalline substance precipitated from the sodium carbonate extract by acid was indeed pure pyruvic acid hydrazone. It was also satisfactorily shown that methyl glyoxal, dihydroxyacetone, and acetone (when present) did not significantly affect the analysis. On the other hand, acetoacetic acid, laevulinic acid, and α-ketoglutaric acid did interfere - the last of these to the extent of 13%. The method required a large volume of blood (10-20 ml.) and was slow and time-consuming, thus limiting its clinical applications.

Jowett and Quastel (1937) took the analysis the length of the ethyl acetate extract, and without any further extraction, added the alcoholic potassium hydroxide directly to this extract. The red colour of the resulting mixture was determined and used as a measure of the pyruvate present. No attempt was made to remove excess dinitrophenylhydrazine but allowance was made for its colour by the provision of/

/of suitable "blanks".

The main objections to this modification were (a) lack of specificity chiefly due to the fact that only one extraction if employed, and (b) the high "blanks" obtained.

In 1939, Lu published a "rapid, specific and sensitive method for the estimation of blood pyruvate". This method followed exactly the techniques described above up to the stage of obtaining the ethyl acetate extract. The ethyl acetate extract, which contained the hydrazones of a variety of aldehydes and ketones together with the excess dinitrophenylhydrazine, was then extracted with 10% sodium carbonate solution. It was shown that the hydrazones of pyruvic acid and other α-keto acids were extracted into the sodium carbonate layer, while the aldehyde hydrazones plus the excess dinitrophenylhydrazine remained in the ethyl acetate layer. The sodium carbonate extract was then made strongly alkaline with 4% sodium hydroxide and the resulting red colour was estimated directly. It was shown that, on addition of the sodium hydroxide, all the α-keto acid hydrazones gave a red colour which was maximal in 2 minutes. The colour due to the main interfering substances (the hydrazones of α-ketoglutaric and acetoacetic acids) faded rapidly, and at the end of 10 minutes, the/

/the colour remaining was almost entirely due to the hydrazone of pyruvic acid.

Bueding and Wortis (1940) introduced a slight modification by using 2N in place of N sodium hydroxide for the final colour development. According to them, this modification produced a more stable final colour and reduced interference from other keto acids. The value of this modification has been confirmed in the present studies.

In all the modifications of the method described above, maximal extraction of the hydrazones from the reaction mixture has been achieved by use of repeated extractions with ethyl acetate. Friedemann and Haugen (1943) have examined the extraction of these hydrazones in great detail. Using standardised conditions, they found a constant, but incomplete, extraction of the pyruvic acid hydrazone into ethyl acetate. In view of the fact that this extraction was neither specific nor quantitative, other solvents were tested out. Of those tried, xylene (in place of the ethyl acetate in Lu's modification) was the most useful in that it appeared to increase the specificity of the subsequent sodium carbonate extraction, and gave results which were more reproducible and/

/and more quantitative. The use of xylene appeared to greatest advantage when the blood contained relatively high proportions of keto acids other than pyruvic acid. In normal blood, where the concentration of these other keto acids is low, there is no great advantage to be gained in substituting xylene for ethyl acetate, and Friedemann and Haugen themselves continued to use the ethyl acetate extraction procedure for blood analyses.

Friedemann and Haugen also attempted to make the final stage more specific by determining colour densities at two different wavelengths selected by filters. They showed that the ratio of the extinction of the hydrazone of pyruvic acid at 420 and at 520 mμ was 1.3, whereas with the hydrazone of α-ketoglutaric acid, this ratio was 1.9. With this information, they determined the relative proportions of pyruvate and α-ketoglutarate in blood, and at the same time, confirmed their previous observations on the relative merits of ethyl acetate and xylene as extraction solvents.

Other workers do not agree with the absorption maxima quoted by Friedemann and Haugen. Hammarsten (1948), Taylor and McHenry (1949), and Asmussen (1950) all find that an alkaline solution of the dinitrophenylhydrazone of pyruvic acid has a peak about 450 mμ, and this figure has been confirmed in the present work. (see figure I)

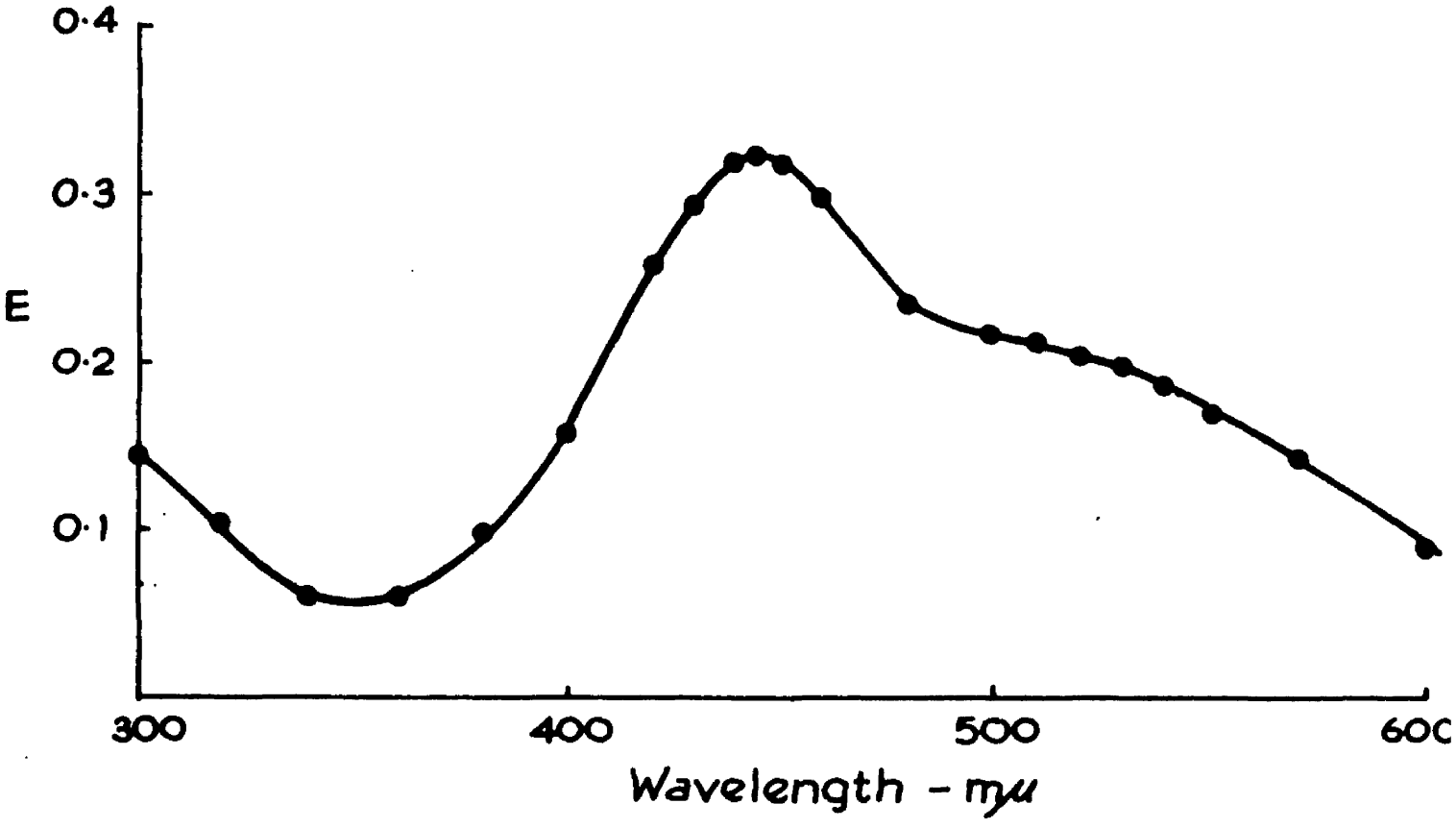

Figure 1. Absorption spectrum of pure pyruvic acid 2:4 dinitrophenylhydrazone.

(2) The Estimation of Blood Pyruvate.

Details of the Method used.

The method is essentially that of Lu (1939) and certain modifications and details have been further investigated.

Blood was obtained from the median basilic vein with the minimal amount of stasis. Immediately, 2.00 ml. of blood were transferred to 6.00 ml. of ice-cold 10% trichloracetic acid which had been freshly prepared. The mixture was centrifuged as soon as possible, but it was found in later experiments (when it was not possible to centrifuge immediately) that the mixture could be kept overnight at 0°C. without any appreciable change in the pyruvate concentration. Likewise, the protein-free supernatant could be stored for several days at 0°C. without appreciable change in the pyruvate concentration.

In shed blood there is an initial fall, followed by a rise in the pyruvate concentration. The initial fall is due to the pyruvate reacting with triose phosphate to give lactate (plus 3-phosphoglycerate). The later rise is due to the fact that pyruvate is the end-product of glycolysis. These processes have been studied by Long (1944). Both changes are inhibited by adding the blood, without delay, to the ice-cold trichloracetic acid./

/acid.

2.00 ml. of the trichloracetic acid supernatant (equivalent to 0.50 ml. blood) was added to 1.00 ml. of 0.1% 2:4-dinitrophenylhydrazine dissolved in 2N hydrochloric acid. The mixture was allowed to stand for half an hour at room temperature during which time the formation of the hydrazones of pyruvic acid and other carbonyl compounds is complete. The only precautions to take at this stage are to use freshly prepared dinitrophenylhydrazine reagent and to filter it immediately before use, otherwise high and non-reproducible blanks may be obtained.

The hydrazones, together with the excess dinitrophenylhydrazine, were then removed by 3 extractions each with 3 ml. of ethyl acetate. This gives the maximal extraction. The ethyl acetate extract can be stored overnight in the refrigerator without loss of pyruvic acid, but if left overnight at room temperature considerable losses (up to 33%) can occur.

The ethyl acetate layers were combined and then extracted twice with 3 ml. of 10% aqueous sodium carbonate solution. The combined extracts were centrifuged to separate off the last traces of ethyl acetate. This sodium carbonate extract could also be stored overnight at refrigerator temperature without loss/

/loss of pyruvate, but at room temperature, considerable losses (up to 50%) can occur.

5.00 ml. of this sodium carbonate extract, which must be completely free from any trace of the ethyl acetate layer, was added to 5.00 ml. of 2N sodium hydroxide, and the intensity of the red colour was measured at 445 mμ in a Hilger "Uvispek" Photoelectric Spectrophotometer. The colour develops immediatly and is stable for at least one hour.

Some further experiments were undertaken to investigate the completeness of the extraction of the hydrazone of pyruvic acid under different conditions. All organic solvents retain a greater or lesser amount of the hydrazone which cannot be extracted into the aqueous sodium carbonate layer. Friedemann and Haugen (1943) stated that with ethyl acetate as the solvent, 81% of the hydrazone of pyruvic acid could be extracted into the sodium carbonate layer, while with xylene as the solvent, 95% could be extracted. But no protocols were given in their paper.

This was confirmed in the present work. When standard pyruvic acid solutions (1.00 mg. per 100 ml.) were treated by the method described above for the estimation/

/estimation in blood, the extinction of the final colour at 445 mμ using ethyl acetate as solvent, was found to be E = 0.165, but using xylene as solvent a value of E = 0.180 was obtained. Both results were highly reproducible.

The 2:4-dinitrophenylhydrazone of pyruvic acid was next prepared as follows:- Pure pyruvic acid was redistilled in vacuo and then added to the calculated amount of 2:4-dinitrophenylhydrazine in 2N hydrochloric acid. The latter reagent had been purified by recrystallisation twice from ethanol. A yellow precipitate of pyruvic acid 2:4-dinitrophenylhydrazone was obtained. This crystalline precipitate was collected on a sintered glass filter, washed free of reagents, and finally recrystallised from ethanol. The M.P. was 218°C. which agrees with the figures quoted in the literature. A standard solution of this hydrazone was prepared corresponding to an equivalent concentration of 1.00 mg. pyruvic acid.per 100 ml., and the colour development carried out in the usual way. The density of this solution at 445 mμ was E = 0.195. Comparing this figure with those quoted in the preceding paragraph, it will be seen that when ethyl acetate is the solvent, 85% of the hydrazone can/

/can be extracted into the sodium carbonate layer, and when xylene is the solvent, 92% can be so extracted. These figures agree well with the data given by Friedemann and Haugen.

It was also confirmed that, although recovery was not 100% complete, the use of either ethyl acetate or xylene as solvent for the extraction gave recoveries which were highly reproducible.

When the blood pyruvate concentration was within normal limits, i.e. around 1.0 mg. per 100 ml., the differences between results obtained using ethyl acetate and xylene as the extraction solvent are insignificant, provided that the standard has been extracted with the same solvent as the blood. But when the blood pyruvate is high, i.e., of the order of 10.0 mg. per 100 ml., the results using ethyl acetate as solvent were higher than those obtained using xylene by about 10%. According to Friedemann and Haugen (1943) this difference is due to the retention of keto acid hydrazones other than pyruvic acid hydrazone by the xylene during the aqueous sodium carbonate extraction. This is a big analytical difference but when the blood pyruvate concentration reaches these high levels, an error of 1 mg. per 100 ml. is of little clinical significance.

Friedemann/

/Friedemann and Haugen, using ethyl acetate and xylene, found an analytical difference of 0.12 mg. pyruvic acid per 100 ml. blood in fasting and resting samples, and a difference of 0.69 mg. pyruvic acid per 100 ml. blood in samples collected after a bout of severe exercise. They regard this difference as insignificant, chiefly because it was no greater than the difference found in two successive samples of blood, drawn within a few minutes from the same subject after exercise - so rapidly does the blood pyruvic acid concentration changes when the concentration is high.

There is no doubt that the figures quoted by Friedemann and Haugen (1943) for the absorption maxima of pyruvic acid 2:4-dinitrophenylhydrazone are in error (see above p. 22) presumably due to these authors having used a series of coloured filters to determine the absorption spectrum. All other workers have used instruments with a monochromatic device for selecting wavelengths and are agreed that the maximal absorption peak occurs at 445 mμ (see Figure I, p.23). At concentrations within the normal and abnormal biological range, the colour obeys the Beer-Lambert Law.

(3) The Estimation of Thiamine.

General Considerations.

The methods which have been used for the estimation of thiamine have been (1) biological, (2) microbiological, and (3) chemical.

The earliest biological assay was based on the amount of the crude vitamin preparation required to cure the neck retraction of thiamine deficient pigeons (Funk, 1911) and dates back to the beginning of the history of this vitamin. Sherman and Spohn (1923) later used the rat for assay. When all other known growth factors have been supplied in adequate amount, the rate of increase of weight in rats, previously on a thiamine deficient diet, is proportional to the amount of thiamine supplied. Coward (1936) showed that the accuracy of the rat method is very high for assays of this type. The Oxford School under Peters (1933, 1935, 1938) was the first to develop an in vitro method of assay - the "Catatorulin Effect" - which depended on their discovery of the fact that thiamine increases the oxygen uptake of avitaminotic pigeon brain tissue. The last of the important biological methods was the "Bradycardia Method" developed by Drury, Harris and Maudsley (1930) and by Birch and Harris (1934). This

was/

/was based on the finding that the thiamine deficient rat constantly exhibited a bradycardia which could be cured by administration of the vitamin, and the increase in the heart rate was proportional to the dose of vitamin administered. The test is highly specific, and comparatively rapid for a biological assay.

The microbiological methods have all depended on some activity of the cell requiring thiamine when the concentration of thiamine is the limiting factor in controlling that activity. Schopfer (1935) measured the rate of growth of Phycomyces blakesleeanus which is dependent on the thiamine concentration of the medium. Sarret and Cheldelin (1944) used Lactobacillus fermentum. Others have used yeast following the observation that addition of thiamine to the medium increases the rate of production of carbon dioxide during fermentation. And so on. The microbiological methods have been proved to be extremely sensitive, but unless great precautions are taken in the control of the conditions, they lack specificity (Meiklejohn, 1937, and Sinclair, 1939).

There are two chemical methods in common use; the fluorometric method and the diazo method.

Peters/

/Peters, Rydin and Thompson (1935) discovered that thaimine could be oxidised by alkaline ferricyanide to give a substance (thiochrome) which has an intense blue fluorescence in ultraviolet light. The thiochrome could be extracted from the aqueous reaction mixture by isobutanol and the degree of fluorescence was found to be proportional to the original thiamine content. Various modifications of the method have been introduced with the object, in most cases, of adapting the technique to the type of material under examination. In the anlysis of urine, Hennessy and Cerecedo (1939) found that the thiamine was quantitatively absorbed on sodium aluminium silicate ("Decalso") from which it could be eluted by an acidified aqueous solution of potassium chloride. Hills (1939) introduced the use of a blue filter during the measurement of the fluorescence, a modification which considerably increased the specificity. The most important analytical improvement was the discovery by Mawson and Thompson (1948) that the fluorescence due to thiochrome was destroyed by the addition of strong acid, while the fluorescence due to certain interfering substances, such as methyl-nicotinamide, was not. The difference between the fluorescence before and after the addition of/

/of strong acid was therefore a much more specific measure of the original thiamine content. Thiamine is present in blood and tissues partly as the free thiamine, and partly in the form of thiamine diphosphate (cocarboxylase). Before the total thiamine can be estimated, an enzymic hydrolysis of the ester must first be carried out (Harris and Wang, 1941, Burch, Bessey, Love and Lowry, 1952).

The diazo method is an older method and was first introduced by Jansen and Donath (1926). When thiamine is coupled with diazotised sulphanilic acid, a pink colour is produced and the depth of colour is proportional to the thiamine present. This method has been widely used and numerous modifications have been introduced. Kinnersley and Peters (1934) used a formaldehyde-azo complex of sulphanilic acid. Probluda and McCollum (1936, 1939) used diazotised p-amino-acetophenone which reacted with thiamine to give a red dye which was stable, insoluble in water, but which could be extracted quantitatively into xylene. They found that it was the 4-methyl-5-hydroxy-thiazole part of the thiamine molecule which entered into this reaction. This reagent was specific for thiamine, and did not estimate cocarboxylase. The method has been extensively examined by Melnick and Field/

/Field (1939).

In the estimation of thiamine in urine, the vitamin was adsorbed on "Decalso" (previously converted to its "potassium" form by suspending it in potassium chloride solution). The thiamine displaced the potassium and was itself retained by the resin. After thorough washing, the thiamine was eluted by hot 25% aqueous potassium chloride solution containing N/10 hydrochloric acid.

Melnick and Field (1939) found that the large amount of inorganic salts in a 24-hour collection of urine inhibited the displacement of potassium by thiamine, and that a preliminary extraction of the urine with benzyl alcohol was necessary. Hochberg and Melnick (1944) found that there was not sufficient inorganic salts in a 1-hour collection of urine (approximately 100 ml.) to inhibit this exchange, but, on the other hand, there was not sufficient thiamine in a 1-hour collection to estimate accurately. They therefore devised a 4-hour excretion test for thiamine deficiency in which 1 mg. thiamine is injected intravenously and the urine collected over the following 4 hours. In one-quarter of this specimen (equivalent to a 1-hour sample of urine) there was sufficient thiamine to estimate/

/estimate accurately, but not enough inorganic salt to interfere with the action of the "Decalso".

To the acidic potassium chloride eluate is added alcoholic phenol (which was found by Melnick and Field to increase the sensitivity of the reaction) and the pH is adjusted to 7.0 with N/10 sodium hydroxide using an internal indicator. The diazo reagent is then added, the mixture allowed to stand for 2 hours, and the red dye is then extracted (once) into 3.0 ml. xylene. The intensity of the colour is then measured in a Hilger Uvispek Photoelectric Spectrophotometer. This is the basis of the method which has been used throughout the present work.

(4) Estimation of Thiamine.

Details of the Method used.

Although the diazo method of estimation of thiamine is not so sensitive as the fluorimetric method, it is claimed to be more specific. Melnick and Field (1939) and Hochberg, Melnick and Field (1944) found the method most suitable for the estimation of quantities of the order of 100 µg. thiamine. Briefly, the method of Melnick and Field (1939) is as follows:-

31.8 mg. of p-amino-acetophenone in 5 ml. N hydrochloric acid is diazotised in the cold with 25 ml.

N/

/N sodium nitrite. 20 ml. of the diazotised p-amino-acetophenone solution is added to 274 ml. of an alkaline mixture containing equal volumes of N sodium hydroxide and N sodium bicarbonate. The reagent is at first a faint pink colour, but the colour fades after standing for 20 minutes at room temperature, and the reagent is then ready for use.

A standard thiamine solution (pH = 1.0) is prepared by dissolving thiamine in a solution containing equal volumes of (a) 25% potassium chloride in N/10 hydrochloric acid, and (b) 0.88% phenol in 95% ethanol. Immediately prior to use, the pH is brought to 7.0 by the addition of a few drops of 2N sodium hydroxide (using Thymol blue as internal indicator). A volume of the alkaline diazotised p-amino-acetophenone, equal to the volume of the thiamine solution is then added and the reaction is allowed to proceed in the dark for at least 2 hours. The thiamine-diazo complex is then extracted into 3.0 ml. xylene and the colour intensity measured in a microcolorimeter using a green filter.

Only two minor improvements were introduced at this stage.

(i) All pH measurements and adjustments were made by/

/by means of the Marconi glass electrode pH-meter in place of thymol blue indicator, and,

(ii) The colour density of the xylene extract containing the thiamine-diazo complex was read at 520 mμ, the wavelength being selected by a monochromating device. It is considered that this is more accurate than the use of a colour filter.

Reproducibility of the Method: 20 estimations, each using 10.0 μg. thiamine, were carried out by the above technique. The mean extinction was E = 0.140 (S.D. = 0.007) so that the variation is less than 5%.

Standard Graph for the Estimation of Thiamine: An attempt was made to contruct a standard graph covering the range 3 to 100 μg. thiamine. The following results were obtained using the technique described above.

μg. Thiamine.	Extinction.
3	0.032
5	0.064
10	0.140
20	0.265
30	0.450
50	0.890
75	1.36
100	1.93

See also Figure 2.

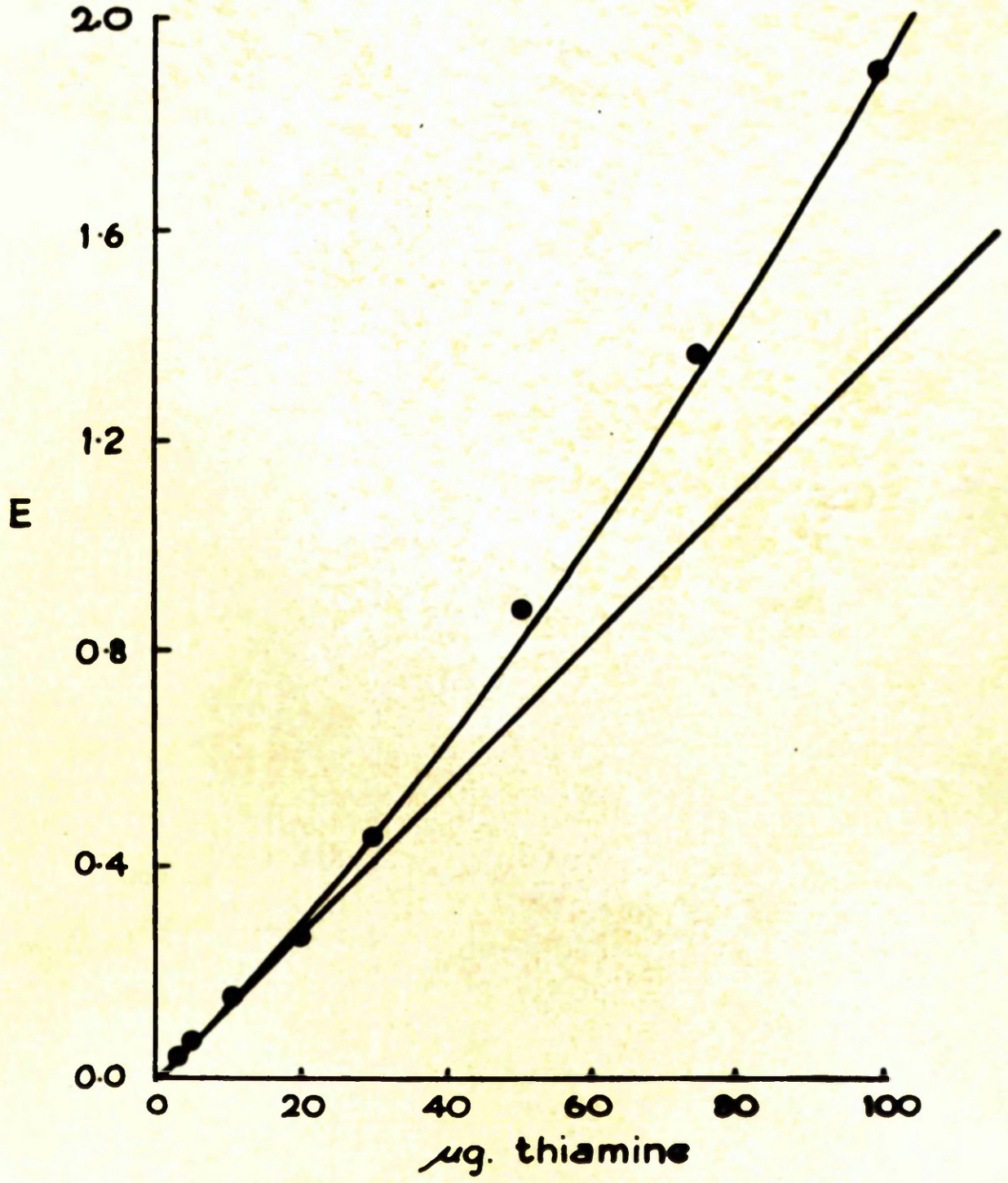

Figure 2. Relationship between the extinction at 520 mu and the quantity of thiamine present, using the original method of Melnick and Field (1939). (Protocols on p. 37.)

Over the range 0 - 30 µg. thiamine, the extinction is proportional to the amount of thiamine present, but beyond this point, the graph ceases to be a straight line and the deviation is quite marked. This deviation from the Beer-Lambert Law attracted attention because the extinction at the upper limits, was greater (not less) than was expected. It was decided that this was worth investigating further.

A reference solution of the thiamine-diazo complex in xylene was prepared. This solution had an extinction of E = 1.60, i.e., the extinction is at a point on the graph (Figure 2) where the deviation was quite marked. This reference solution was then diluted with varying quantities of xylene and the extinctions measured.

Volume of Thiamine -diazo complex.	Volume of xylene added.	Relative amount of Thiamine.	Extinction
1.0 ml.	0	10.00	1.60
1.0 ml.	1.0 ml.	5.00	0.800
1.0 ml.	2.0 ml.	3.33	0.540
1.0 ml.	3.0 ml.	2.50	0.397
1.0 ml.	4.0 ml.	2.00	0.320
1.0 ml.	5.0 ml.	1.67	0.246
1.0 ml.	7.0 ml.	1.25	0.198
1.0 ml.	9.0 ml.	1.00	0.162
1.0 ml.	11.0 ml.	0.83	0.130

See also Figure 3.

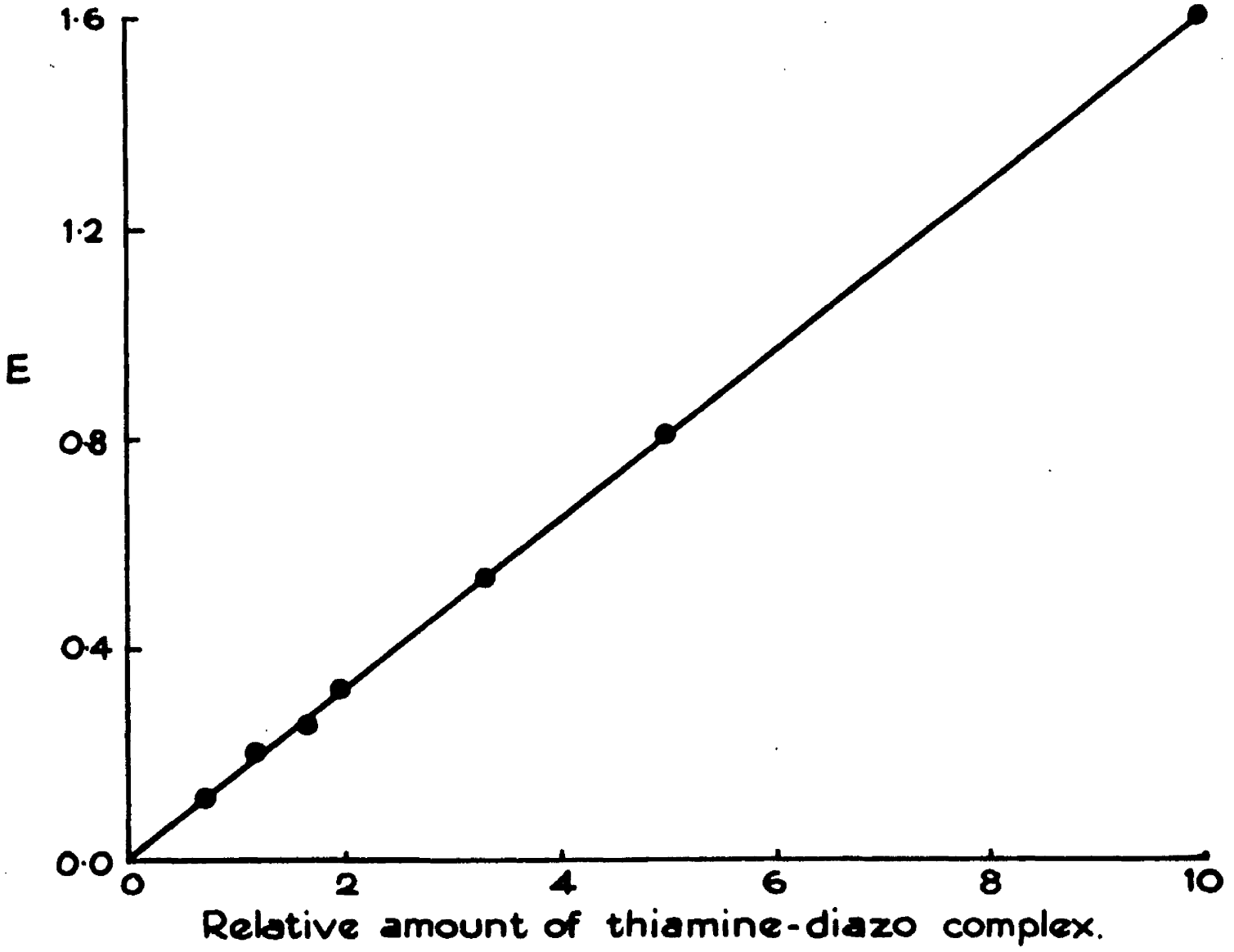

Figure 3. Relationship between the extinction at 520 mu and the relative amount of thiamine-diazo complex in pure solution.
(Protocols on page 39.)

It will be seen from Figure 3 that under these experimental conditions, the colour obeys the Beer-Lambert Law at least up to an extinction of E = 1.60.

In order to test the stability of the final red diazo complex under the conditions in which it is formed, serial samples were taken at various times after the final reagent had been added - the method having been carried through as described on p. 35 - and these samples were then extracted with xylene and the extinction of the xylene solution determined. The following results were obtained:-

Time Interval.	Extinction.
2 min.	0.602
5 min.	1.16
10 min.	1.49
15 min.	1.60
30 min.	1.71
1 hr. 0 min.	1.82
2 hr. 0 min.	1.82
4 hr. 0 min.	1.82
16 hr. 0 min. (overnight)	1.82

See also Figure 4.

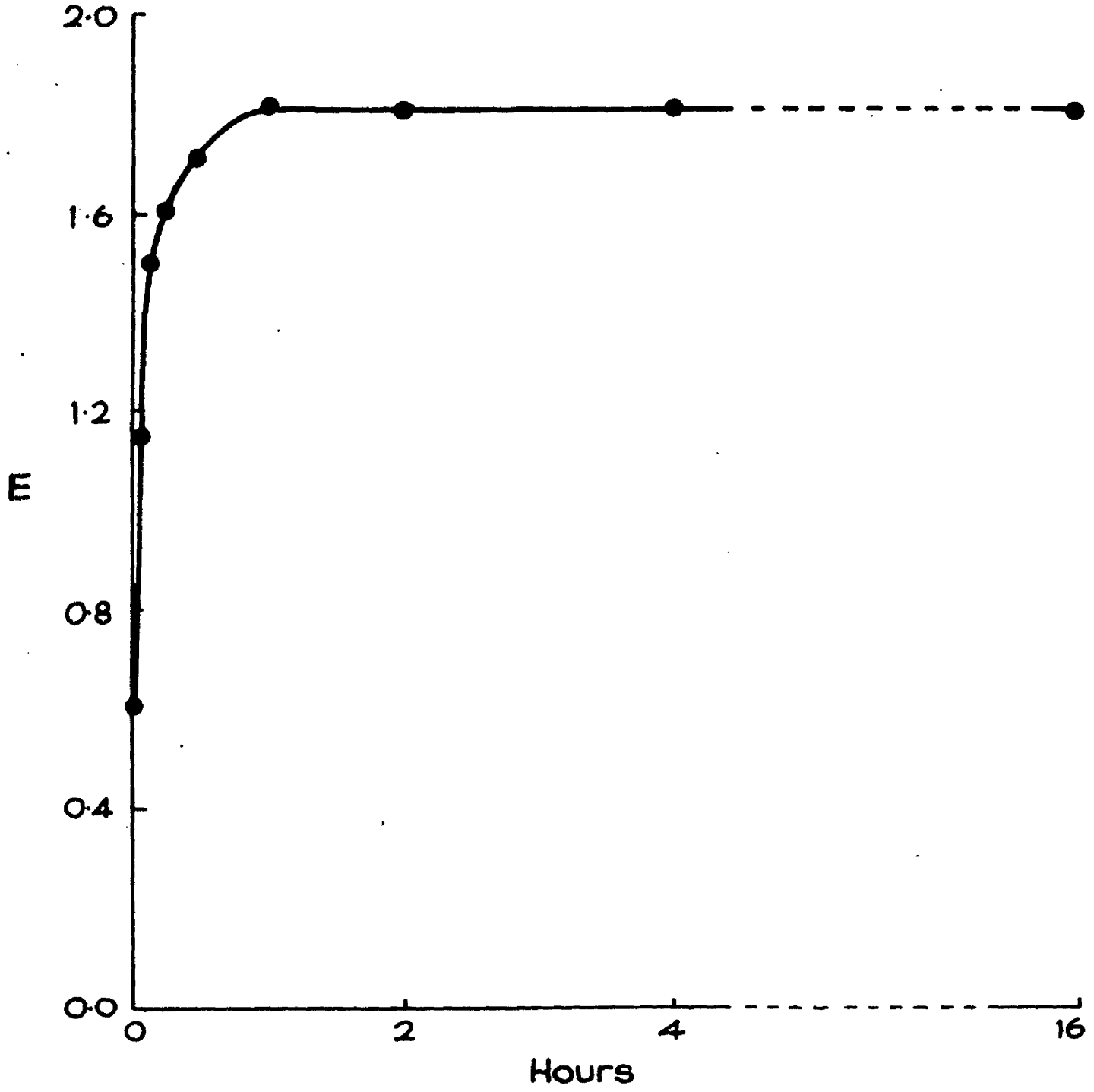

Figure 4. Graph showing the rate of colour development, and the stability of the coloured complex.
(Protocols on p. 41.)

It appears from these experiments that the lack of linearity in Figure 2 is not due to failure of the red diazo complex to follow the Beer-Lambert law over the range of quantities employed, or to instability of the complex under the conditions in which it is formed during the reaction. The only remaining explanation is that the conditions of the reaction lead to incomplete formation of the diazo complex. And this conclusion opened up the whole question of the mechanism of the reaction and the factors on which it depends. All of these factors were examined seriatim and as a considerable amount of experimental work was involved, only the main conclusions will be summarised here.

(a) Since the alkaline diazo reagent is made up in relatively concentrated alkali, it is unnecessary to neutralise the comparatively small amount of acid in the standard thiamine solution before proceeding to add the diazo reagent.

(b) The pH of the reaction mixture, i.e., the thiamine plus diazotised p-amino-acetophenone plus phenol, is immaterial provided that it is at least 10.5. If the pH is lower than this, only a yellow colour, due to coupling with phenol, is produced. Increasing the pH above 10.5 does not result in increased colour formation.

(c)/

/(c) In absence of phenol, the extinctions found are approximately one-third of the values found when the amount of phenol recommended by Melnick and Field has been added. The explanation of this is not known, but experiments have suggested that the function of the phenol is to destroy excess diazotised p-amino-acetophenone.

(d) Also, in absence of phenol, it was found that the amount of diazotised p-amino-acetophenone in the reaction mixture was critical. Using quantities of thiamine of the order of 10 to 100 µg., it was found that the optimal amount of diazotised p-amino-acetophenone for maximal production of colour, was 300 ± 20 µg. Greater or lesser amounts gave sub-optimal colour production.

(e) The presence of phenol increases the colour production, and again, the amount of phenol in the reaction mixture is critical. Using quantities of thiamine of the order of 10 to 100 µg., it was found that the optimal amount of phenol for maximal production of colour was 25 ± 2 mg. Greater or lesser amounts also gave sub-optimal colour production.

(f) In alkaline solution, phenol itself reacts with diazotised p-amino-acetophenone to give a yellow compound which, however, cannot be extracted from the/

/the reaction mixture by xylene.

(g) It was also found that the presence of phenol considerably improved the reproducibility of the results.

The details of the method finally employed are:-

13.5 mg. p-amino-acetophenone in 5 ml. N hydrochloric acid, is diazotised in the cold with 20 ml. N sodium nitrite. To this mixture is added 200 ml. of a solution containing equal volumes of N sodium hydroxide and N sodium bicarbonate. The reagent must be prepared immediately before use.

Varying quantities of thiamine (10 to 100 µg.) are added to 3.0 ml. of 0.88% phenol in alcohol (26.4 mg. phenol). 2.2 ml. of the alkaline diazotised p-amino-acetophenone solution is then added (300 µg. diazotised p-amino-acetophenone) and the mixture allowed to stand overnight at room temperature. It is then extracted (once) with 3.0 ml. xylene and the optical density of this xylene extract is read at 520 mµ.

Reproducibility of the modified Method: 27 estimations, each using 10.0 ug. thiamine in aqueous solution, were carried/

/carried out using the modifications of the technique described above. The mean extinction was now found to be E = 0.178 (S.D. = 0.004) indicating a variation of ±2%.

By strict control of the quantities of reagents, the sensitivity of the method has been appreciably increased, and the random error has been halved. (Cf. the figures quoted on p. 37).

Standard Graph for the Estimation of Thiamine: Modified Method.

The following results were obtained using the modified method described on p. 37.

µg. Thiamine.	Extinction.
10	0.178
20	0.350
30	0.520
50	0.855
75	1.25
100	1.59

See also Figure 5.

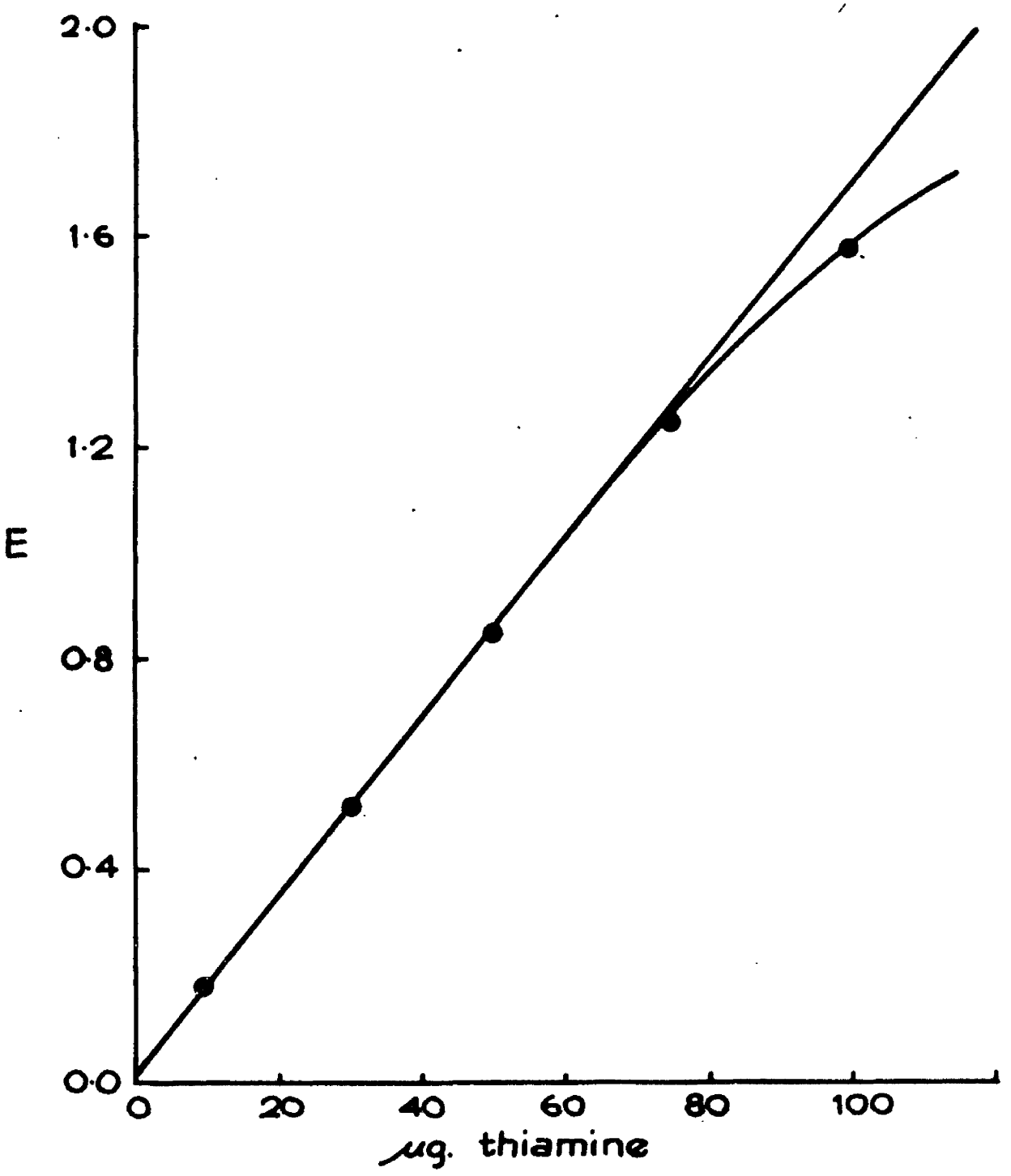

Figure 5. Relationship between the extinction at 520 mu and the quantity of thiamine present, using the final modification of the method described on p. 45.
(Protocols on p. 46.)

The optical density is directly proportional to the amount of thiamine in the range 0 - 65 µg. thiamine, thereafter, the colour intensity no longer obeys the Beer-Lambert Law, the extinctions being smaller than theoretical - which is the usual deviation from the law at high optical densities. (Cp. Figure 2, p. 38). This is the standard graph for the estimation of thiamine which has been used throughout the following work.

Thiamine Deficiency in Post-gastrectomy Patients.

Nutritional deficiencies in patients with gastro-intestinal complaints is comparatively common, e.g., the "dumping syndrome" after partial or total gastrectomy, the scurvy which develops on a so-called "ulcer diet", and so on. This section of the work deals with an investigation into the possible occurrence of sub-clinical thiamine deficiency in a series of post-gastrectomy cases; it is not so much concerned with those cases in which frank signs and symptoms of thiamine deficiency are apparent on clinical examination. Many cases of thiamine deficiency occurring during the course of gastro-intestinal upsets, and following surgical interference with the gastro-intestinal tract, have been recorded in the literature.

Ungley (1938) reported neuritis complicating some cases of gastric ulcer, pyloric stenosis, and neoplasms of the intestinal tract. Scott (1938) and Laurent and Sinclair (1938) made similar observations. In these cases, there was no evidence that the neuritis was a direct sequel of thiamine deficiency. Field, Robinson and Melnick (1940) examined two series of patients, (1) a series receiving/

/receiving alkaline therapy in the course of treatment for peptic ulceration, and (2) a miscellaneous series of cases of achlorhydria, and in both, he found a sub-normal urinary excretion of thiamine following a test dose of the vitamin. A similar response to a test dose of thiamine was found by Pollack, Dolger, Ellenberg and Cohen (1940) in a group of patients with various forms of gastro-intestinal upset.

More definite evidence of a correlation between gastro-intestinal disease, neuritis, and thiamine deficiency was produced by Muir (1949). He followed up 124 post-gastrectomy patients of whom 20 complained of varying degrees of neuritis; of these 9 rapidly responded to administration of thiamine while the other 11 did not. Two of Muir's cases were also found to be suffering from mild scurvy which responded immediately to ascorbic acid.

Welbourn, Hughes and Wells (1951) reported 10 cases of thiamine and riboflavine deficiency out of 100 cases of sub-total gastrectomy one or more years after operation. On the other hand, Blake and Rechnitzer (1953) found no concrete evidence of vitamin B deficiency in 104 post-gastrectomy cases.

During/

During the last five years, there seems to have been no published work on the subject, and the literature prior to that contains no adequate scientific examination of the possible occurrence of sub-clinical thiamine deficiency in this type of case.

This investigation was carried out at the request of Professor A. W. Kay and the post-gastrectomy cases were from the unit of Professor C. F. W. Illingworth in the Western Infirmary, Glasgow. Seventeen cases were selected at random as they appeared at the Out-patient Clinic and it was believed that they formed a reasonable cross-section of post-gastrectomy patients. They were thoroughly examined by the clinical staff, and on a basis of this examination, they were classified as follows:-

Group 1. 9 patients. These patients had no clinical evidence whatever of any degree of thiamine of other vitamin deficiency.

Group 2. 5 patients. In this group, the patients complained of at least two minor symptoms suggestive of thiamine deficiency, and the symptoms included neurasthenia, lassitude, anorexia, limb pains, and loss of weight.

Group 3./

/Group 3. 2 patients. In addition to various symptoms, these two patients showed objective signs suggestive of vitamin B deficiency, angular stomatitis, glossitis and cheilosis.

Group 4. 1 patient. While it was not the original intention to study florid thiamine deficiency, this patient appeared while the cases were being collected and was included as a matter of interest. She was a typical case of severe thiamine deficiency showing all the classical mental, neurological, ocular and cardiovascular signs of the condition.

Control Group. The control group consisted of 24 subjects selected from ward patients who had been admitted for relatively minor surgical treatment - mostly cases of uncomplicated hernia repair. Tests were all carried out on the day before discharge. The subjects were all in good general health, free from any gastro-intestinal symptoms or signs, had been receiving a general mixed hospital diet without any vitamin supplement, and, as far as possible, were matched in age and social status with the patients of the post-gastrectomy group. In selecting these controls, the variable factor was the absence of a gastrectomy. Many of these control subjects/

/subjects came from the poorer districts around the hospital, and there is no certainty that their past dietary history had been adequate with respect to thiamine.

In endeavouring to detect sub-clinical thiamine deficiency, efforts were concentrated on (1) the fasting and resting blood pyruvate concentration as this quantity is frequently requested by clinicians as a measure of thiamine depletion, and (2), the urinary thiamine excretion following a test dose of the vitamin.

A. The Fasting and Resting Blood Pyruvate Concentration.

Control Group: The mean fasting and resting blood pyruvate concentration of the 24 control subjects was 0.74 mg. per 100 ml. (S.D. = 0.20, Range, 0.25 to 1.15 mg. per 100 ml.).

Post-gastrectomy Patients: The results are shown in Table II (see p. 55).

The mean fasting and resting blood pyruvate concentration in the 17 post-gastrectomy patients was 1.03 mg. per 100 ml. (S.D. = 0.23). Using the Student "t" test, these mean values are significantly different at the 1% level. It is clear/

/clear, however, from an examination of the data in Table II that there is no relationship whatever between the level of the blood pyruvate concentration and the degree of thiamine deficiency. Hence, other factors must be operating.

The fasting and resting blood pyruvate concentration of the patient Jo (Group 4) was determined on several occasions and consistently fell within the normal range. After intravenous administration of thiamine, there was a marked fall (see p. 55).

TABLE II. Fasting and Resting Blood Pyruvate Concentrations.

Controls and Post-gastrectomy Patients.

(All results in mg. Pyruvate per 100 ml. blood).

Group and Patient.		Blood Pyruvate.	Mean.	Range.
Control Group. (24 subjects).		-	0.74	0.25 to 1.15
Group 1.	St.	1.03		
	Mi.	0.93		
	Ca.	0.72		
	Ni.	0.91		
	Br.(1)	1.25		
	Du.	0.93		
	McN.	0.74		
	Br.(2)	1.00		
	Gr.	1.15	0.96	0.72 to 1.25
Group 2.	Ba.	1.60		
	Sh.	0.90		
	Shi.	0.96		
	Ha.	1.30		
	McM.	1.15	1.18	0.90 to 1.60
Group 3.	Gi.	0.80		
	St.	1.10	-	-
Group 4.	Jo.	1.00	-	-

B. Excretion of Thiamine in the Urine following a Test Dose.

The response to the administration of a test dose of thiamine was measured while the subjects, patients and controls, were in a fasting and resting (basal) state.

The test was carried out in the morning. Immediately before the experiment was started, the bladder was completely emptied and the urine discarded. 1.00 mg. thiamine was then injected intravenously, and all urine passed during the following 4 hours was collected, the bladder being emptied again completely at the end of that time and the urine added to the specimen. The urine was collected in a brown bottle containing 10 ml. glacial acetic acid as preservative. The thiamine content was estimated by the method described above.

In a few preliminary experiments, it was found that these subjects excreted very small amounts of thiamine in the urine without having been given any of the vitamin in the form of a test dose. The maximum output observed was 15 µg. thiamine per 4 hours, and since this is very small compared with the amount excreted after the test dose,/

/dose, it has been ignored in the calculations.

Since the amount of the test dose which is retained by the subject is related to the body weight, this factor of body weight must be taken into account in assessing the value of the data. The 24 control subjects could all be described as normal in size. None could be classed as obese. The post-gastrectomy patients were all either normal in size, or, in a few cases, considerably underweight as might be expected from the nature of their medical histories.

The fate of the injected thiamine has therefore been followed in two ways:- (1) the amount of thiamine excreted in the urine in the 4 hours after the test dose, and (2), the thiamine retained per kg. body weight up to the end of the 4-hour period which the experiment lasted.

Control Group: The mean urinary excretion of thiamine following injection of 1.00 mg. of the vitamin in the 24 control subjects was 210 µg. per 4 hours (S.D. = 62, Range 126 to 340 µg. per 4 hours).

The average body weight of the control subjects was 63 kg. (Range 47 to 80 kg.).

When the thiamine retention was related to body weight/

/weight in each of the individual control subjects, it was calculated that the mean retention was 12.6 µg. thiamine per kg. body weight (S.D. 2.0, Range 10.5 to 17.1 µg. thiamine per kg. body weight).

Post-gastrectomy Patients: The results are shown in Table III (see p. 60).

The mean urinary excretion of thiamine following injection of 1.00 mg. of the vitamin in the 17 post-gastrectomy patients was 128 µg. per 4 hours (S.D. = 64, Range 50 to 290 µg. per 4 hours). Using the Student's "t" test, this mean value is significantly different from the control mean value of 210 µg. per 4 hours. However the results of this test do not altogether agree with the conclusions of Najjar and Holt, Pollack et al., and Mason and Williams (see p.16). The scatter in the group of 17 post-gastrectomy patients is much too wide for any conclusion to be drawn, and in 8 of the 17 cases, the amount of thiamine excreted falls withinthe normal mean ± 1 S.D. Nor is there any strict correlation between the amount of thiamine excreted and the clinical assessment of the degree of severity of the thiamine depletion.

Examination/

/Examination of the amounts of thiamine retained per kg. body weight in the 4-hour period after injection of thiamine, gives a much better classification of the patients. The mean retention of thiamine was 15.8 µg. per kg. body weight (S.D. = 2.3, Range 11.8 to 20.5 µg. thiamine per kg. body weight). Once again this mean value is significantly different from the control mean. With few exceptions, the amount retained per kg. body weight corresponds to the clinical and dietetic evaluation of the case. Since thiamine is used and stored only in tissues which are metabolically active (e.g., muscle, liver, kidney, etc.) the amount of thiamine retained under the conditions of the experiment should properly be correlated with the lean body weight to which the total body weight is the first approximation. And since only 2 of the 17 post-gastrectomy patients were heavier than the mean of the controls, this factor should be taken into account.

TABLE III. Urinary Excretion of Thiamine.

Post-gastrectomy Cases.

Group and Patient.	Weight. kg.	EXCRETION µg. Thiamine per 4 hours.	RETENTION µg. Thiamine per kg. body weight.
Control Group (Mean values).	63	210	12.6
Group 1. St.	58	135	15.0
Mi.	53	141	16.2
Ca.	64	183	12.7
Ni.	54	150	15.7
Br. (1)	56	66	16.7
Du.	54	89	16.8
McN.	67	167	12.4
Br. (2)	47	155	18.0
Gr.	56	92	16.3
Means for Group 1.	56	131	15.5
Group 2. Ba.	56	50	17.0
Sh.	65	230	11.8
Shi.	57	150	14.6
Ha.	56	114	15.7
McM.	46	290	15.4
Means for Group 2.	56	167	14.9
Group 3. Gi.	49	210	16.2
St.	44	78	20.5
Group 4. Jo.	50.	90	18.2

Further information concerning the relative value of these three methods of detecting sub-clinical thiamine deficiency has been obtained by examining the effects of systematic treatment of a proportion of the patients with thiamine in various therapeutic preparations. Since the whole experiment involved hospitalisation, not all of the 17 post-gastrectomy patients were available for follow-up purposes, but extended observations were carried out on 6 of the cases.

C. Effect of Vitamin Therapy on Post-gastrectomy Patients.

According to the American National Research Council (1953), the recommended daily intake of thiamine is 1.0 to 1.5 mg. 6 of the post-gastrectomy patients were placed on an ordinary mixed diet with a supplement of 2.0 mg. thiamine daily and further observations were made after 4-8 weeks. The effects of this regime on the fasting and resting blood pyruvate concentration are shown in Table IV, and on the excretion and retention of a test dose of thiamine in Table V.

TABLE IV. Effect of Thiamine Therapy on the Fasting and Resting Blood Pyruvate Concentration.

(Each patient had 2 mg. thiamine daily for 4-6 weeks.)

Group and Patient.		Blood Pyruvate Concentration. mg. per 100 ml. Before Therapy.	After Therapy.
Group 1.	Mi.	0.93	0.70
	Ca.	0.72	0.55
	Br. (1)	1.25	1.10
Group 2.	Sh.	0.90	0.65
	Na.	1.30	0.70
Group 4.	Jo.	1.00	0.55

TABLE V. Effect of Thiamine Therapy on the Urinary Excretion and Retention of Thiamine following a Test Dose.

(Each patient had 2 mg. thiamine daily for 4–8 weeks).

Group and Patient.		EXCRETION. µg. Thiamine per 4 hours.		RETENTION. µg. Thiamine per kg. body weight.	
		Before Therapy.	After Therapy.	Before Therapy.	After Therapy.
Group 1.	Mi.	141	180	16.2	13.2
	Ca.	183	220	12.7	10.3
	Br. (1)	66	87	16.7	15.4
Group 2.	Sh.	230	230	11.8	11.8
	Ha.	114	110 (a)	15.7	15.9 (a)
			200 (b)		14.2 (b)
Group 4.	Jo.	90	110 (a)	18.2	17.7 (a)
			145 (b)		17.3 (b)

(a) after 4 weeks.

(b) after 8 weeks.

The results set out in Table IV are no more convincing than those in Table II. While the blood pyruvate concentration falls in all 6 cases after a course of thiamine treatment, the results are neither striking nor convincing, and it is clear that this extension of the study of the blood pyruvate concentration can never be of diagnostic importance.

The same argument applies to the data in Table V.

D. Blood pyruvate changes following Ingestion of Glucose.

This test was attempted in two of the post-gastrectomy cases (Mi. and Sh.). In both cases, the patients were violently sick and nauseated after swallowing 50 g. glucose, and this line of investigation was therefore abandoned.

Discussion.

Fasting and Resting Blood Pyruvate Concentrations (See Table II, p. 55).

The only conclusion that could be reached was that, if the blood pyruvate concentration were above the upper normal limit, the patient was probably suffering from some degree of thiamine deficiency, provided that other causes (see p. 7) of a raised blood pyruvate concentration could be excluded. This upper limit may be taken as 1.1 mg. pyruvate per 100 ml. blood. But the level of the blood pyruvate concentration was no index of the degree of thiamine deficiency, and a normal blood pyruvate concentration did not exclude even a severe degree of thiamine depletion.

Thiamine Excretion after a Test Dose. (see Table III, p. 60.)

Clearly, there is no strict correlation between the occurrence or severity of thiamine deficiency and the amount of thiamine excreted in the urine in the 4 hours after injection of a test dose of the vitamin.

Thiamine Retention after a Test Dose. (see Table III, p. 60.)

When/

/When the amount of thiamine retained in the body after injection of a test dose is calculated, and is then correlated with the weight of the patient, the results obtained agree reasonably well with the clinical assessment of the case. The results suggest that, of the tests selected, this is the most reliable and the most likely to give positive confirmation of the clinical diagnosis. But, taken as a whole, the test adds little to the diagnosis of sub-clinical thiamine deficiency, for the same patients could be equally well classified on a basis of the clinical examination.

Thiamine Therapy. Effect on Blood Pyruvate Concentration (see Table IV, p. 62).

Since the blood pyruvate concentration was not markedly elevated before therapy, it is hardly surprising that the effects of therapy are limited. Yet it is of some interest that in all cases examined, administration of thiamine brought about a reduction (and in some, a marked reduction) in the level of the blood pyruvate concentration. On the other hand, the majority of the analytical results fall within the normal range of variation, and doubtless, similar results, although perhaps not so marked,/

/marked, could be obtained in a series of normal subjects. It was not felt that the point justified further investigation.

Thiamine Therapy. Thiamine Excretion and Retention following a Test Dose. (See Table V, p. 63.)

The effects of prolonged thiamine administration both on the excretion and retention of a test dose of the vitamin are disappointing. It was expected that the course of therapy employed would have had a much greater effect on the individual patients who had not only been receiving supplementary vitamin, but had also been advised about their general diets.

Perhaps the most disturbing feature of these results is the serious lack of correlation between the clinical assessment of the patient's condition and the biochemical findings, e.g., the basal blood pyruvate concentrations in Table II, and especially the findings in the patients in Groups 3 and 4. The patients Jo. was undoubtedly a severe case of vitamin B deficiency and a very brief summary of her clinical history has been inserted at this point.

Resume of the Clinical History of the Patient in Group 4 (Jo.).

This patient was of special interest as she was diagnosed clinically as a severe case of vitamin B deficiency, but the biochemical tests which were applied showed no outstanding abnormality.

M. Jo. Female. Married. Born 1904.

The patient's symptoms began in 1942, and she complained of increasing dyspepsia for the next ten years.

September 1952:	Partial gastrectomy followed by gastro-jejunostomy.
November 1952 - January 1955:	Patient complained of numerous attacks of vomiting; epigastric pain beginning in November 1953; lassitude and loss of weight, a feeling of fullness after meals, and general malaise.
January 1955:	Repeated gastroscopic examinations did not show any stomal ulcer, but the appearances are described as "not those of a normal gastrectomy".
February 1955:	Laparotomy. Numerous adhesions were found in the upper abdomen. There was also a stomal ulcer. The anastomosis was undone, and a further portion of the stomach was removed. A new anastomosis was made.
August 1955:	The patient was re-admitted with symptoms strongly suggestive of a severe vitamin deficiency. She complained of sore tongue, mouth and gums, and had an angular stomatitis. There were no symptoms of peripheral neuritis. She was disorientated. Myocardial changes were detected by the E.C.G. The patient had circum-orbital oedema, excoriation of the canthi, slight photophobia, and a slight superficial/

/superficial keratitis. She also had a histamine-fast achlorhydria.

August 1955 - September 1955: The various biochemical tests reported here were carried out.

December 1955: A course of 2 mg. thiamine daily by mouth had failed to effect any improvement in the patient's condition. She was then started on a course of 5 mg. thiamine (+10 mg. riboflavine) once weekly by intramuscular injection, and by December 1955, all signs and symptoms of vitamin deficiency had disappeared.

The proof that her symptoms in the middle of 1955 were, in fact, due to thiamine deficiency is shown by the complete remission following large intramuscular injections of thiamine. At no time was her blood pyruvate concentration even up to the upper limit of the normal range, yet she was sufficiently ill to make the response to glucose or the exercise tolerance-tests inadvisable. Although she retained a very high percentage of an injected dose of thiamine (see Table III, p. 60) she was still able to excrete almost 10% of the injected dose at a time when her deficiency was so severe that she was exhibiting psychosomatic disturbances.

The failure of these patients to exhibit either a reduced excretion or an increased retention of thiamine after 4-8 weeks on a diet containing added thiamine, may well be due to an excessively high rate of destruction of the vitamin in the alimentary tract following removal of the source of gastric acidity. The fact that the patient Jo. did not respond to oral administration of thiamine, yet was completely cured of her signs and symptoms by parenteral administration of the vitamin, strongly suggests this.

While/

/While in individual cases these results (apart from the thiamine retention test) are not conclusive, they do confirm the strong possibility of thiamine depletion developing in post-gastrectomy cases and the need for vitamin supplements in the diet. It may be that in severe cases such as the case Jo., this vitamin supplement may have to be given parenterally although the reason for such therapy in this particular case was not discovered.

EXPERIMENTS ON THIAMINE DEFICIENCY IN DOGS.

When this work on human subjects was almost completed, an opportunity arose of studying the effects of thiamine deficiency in dogs, and in view of the rather unsatisfactory results of experiments on post-gastrectomy patients, this chance provided facilities for a more thorough examination of the effects of prolonged thiamine deficiency.

A number of unaccountable deaths of racing greyhounds was reported in Glasgow in 1955. These deaths occurred suddenly either during racing or immediately after a bout of severe exercise. The chief post-mortem finding was extensive cardiac lesions. On investigating the general management of these animals, it was discovered that they had been fed a diet consisting of 1½ lb. meat plus 1 lb. rusked white bread per day, but that this mixture had been simmered for about 6 hours before it was fed to the animals. The possibility of death being due to vitamin deficiency led to the matter being referred to this Department. There was no record in the veterinary or scientific literature of any biochemical investigations of thiamine depletion in dogs leading to illness and death.

EXPERIMENTAL.

Eight dogs were available for the experiments. Six were from kennels where the original deaths had been reported, and the other two came from kennels where no deaths had occurred. In an attempt to bring all the animals to the same nutritional status, the eight dogs were transferred to the University of Glasgow Veterinary Hospital, Garscube, Glasgow, where they were maintained on the normal hospital dog diet for several weeks before the experiments were started. The animals were housed separately.

Before the experiments began, the fasting and resting blood pyruvate concentrations were determined on each dog on two separate occasions. The mean concentration was found to be 1.02 mg. per 100 ml. (S.D. = 0.18) in the 16 estimations. The results all fell evenly about the mean and it was concluded that the dogs were all comparable with respect to thiamine nutrition.

To make sure of this important point, each animal was then given 5 mg. thiamine intramuscularly, and the fasting and resting blood pyruvate concentrations were determined three days later. The mean concentration found in these 16 analyses was 0.94 mg.

pyruvate/

/pyruvate per 100 ml. blood (S.D. = 0.14). Two days later, the mean concentration was 1.10 mg. per 100 ml. (S.D. = 0.14). These changes were not regarded as significant. Having established these points, the dogs were then divided into three groups as follows:-

Group 1. Dogs: C/5 C/6 F/2. (Control group).

Dogs C/5 and F/2 were fed what will be referred to as the "lethal diet" which was similar to the diet fed at the kennels where the deaths had occurred. It consisted of meat and rusked white bread which had been simmered together for 6 hours before being fed to the animals. In addition, these two dogs were given 0.5 mg. thiamine daily by intramuscular injection.

Dog C/6 was fed meat plus unrusked white bread. In addition, this animal was coprophageous and so had a second source of thiamine.

These three animals all received adequate daily amounts of thiamine and therefore served as controls for the other two groups.

Group 2. Dogs: C/3 C/4. (Hospital diet).

Dogs C/3 and C/4 were fed the hospital diet of knackery meat and rusked white bread. Both meat and bread therefore had been previously subjected to a/

/a short heat treatment. No further heating of food was carried out before being fed to the dogs. It was believed that this diet was adequate in thiamine.

Group 3. Dogs: C/1 C/2 F/1. ("Lethal Diet").

These animals were fed on the "lethal diet" and were not given any supplementary thiamine.

1. Fasting and Resting Blood Pyruvate Concentrations.
2. Blood Pyruvate Changes following mild Exercise.

The changes in blood pyruvate concentration in these experiments in each of the 8 dogs are shown graphically in Figures 6 - 13, pp. 74 - 81.

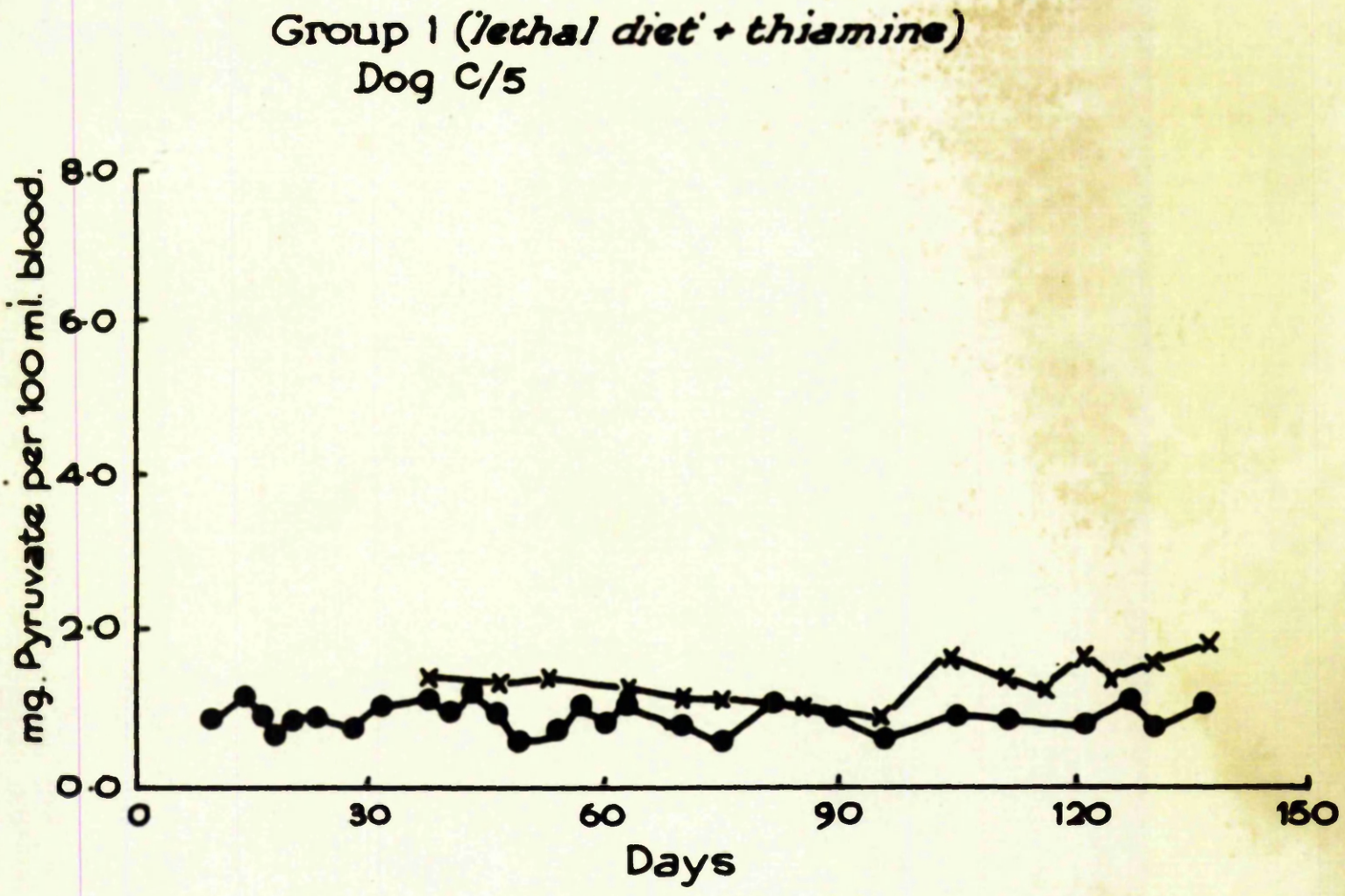

Figure 6. Graph showing the changes in blood pyruvate concentrations over the course of the experiment.

o-o fasting and resting

x-x following exercise

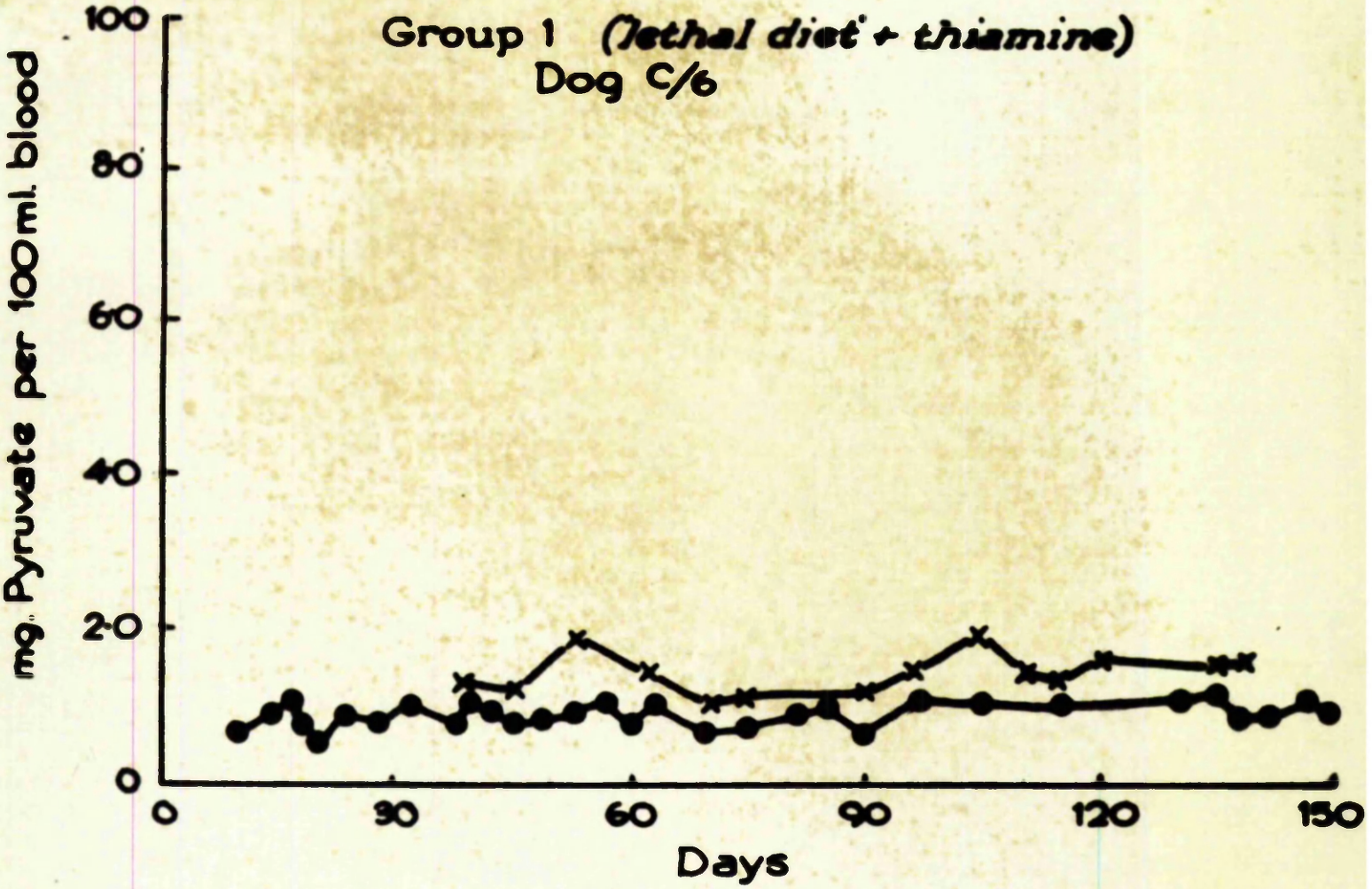

Figure 7. Graph showing the changes in blood pyruvate concentrations over the course of the experiment.

o-o fasting and resting

x-x following exercise

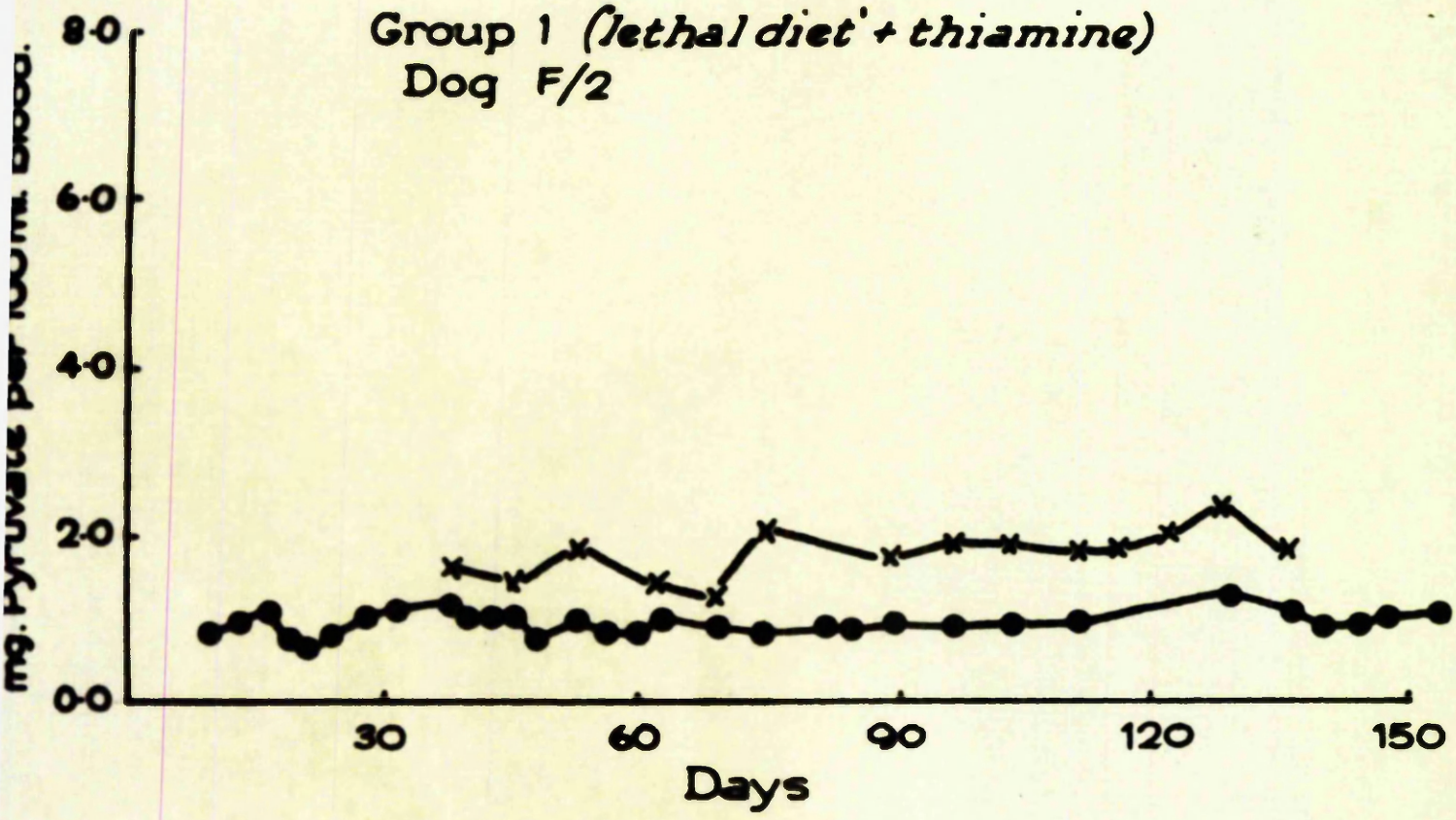

Figure 8. Graph showing the changes in blood pyruvate concentrations over the course of the experiment.

o-o fasting and resting

x-x following exercise

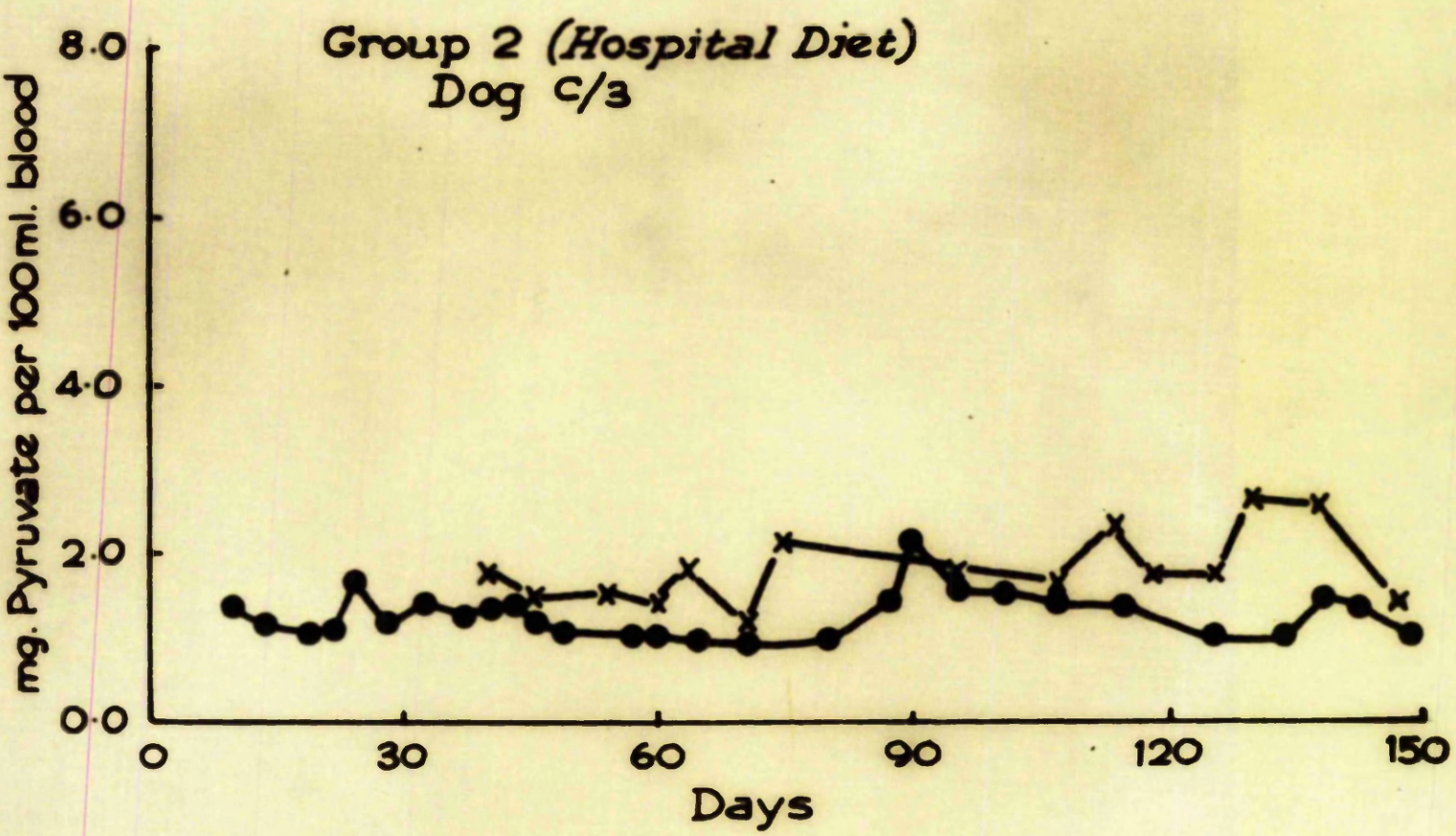

Figure 9. Graph showing the changes in blood pyruvate concentrations over the course of the experiment.

o-o fasting and resting

x-x following exercise

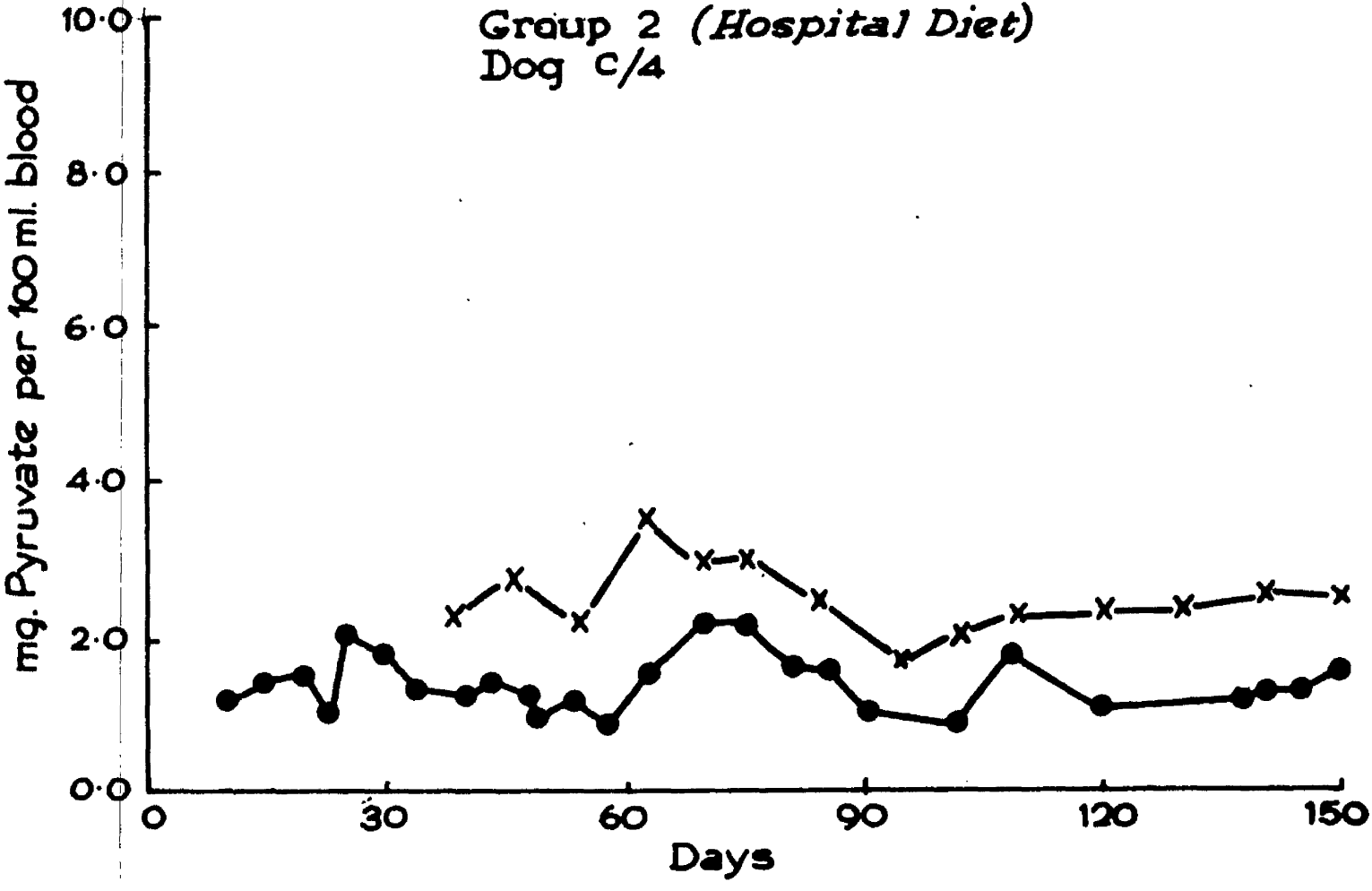

Figure 10. Graph showing the changes in blood pyruvate concentrations over the course of the experiment.

o–o fasting and resting

x–x following exercise

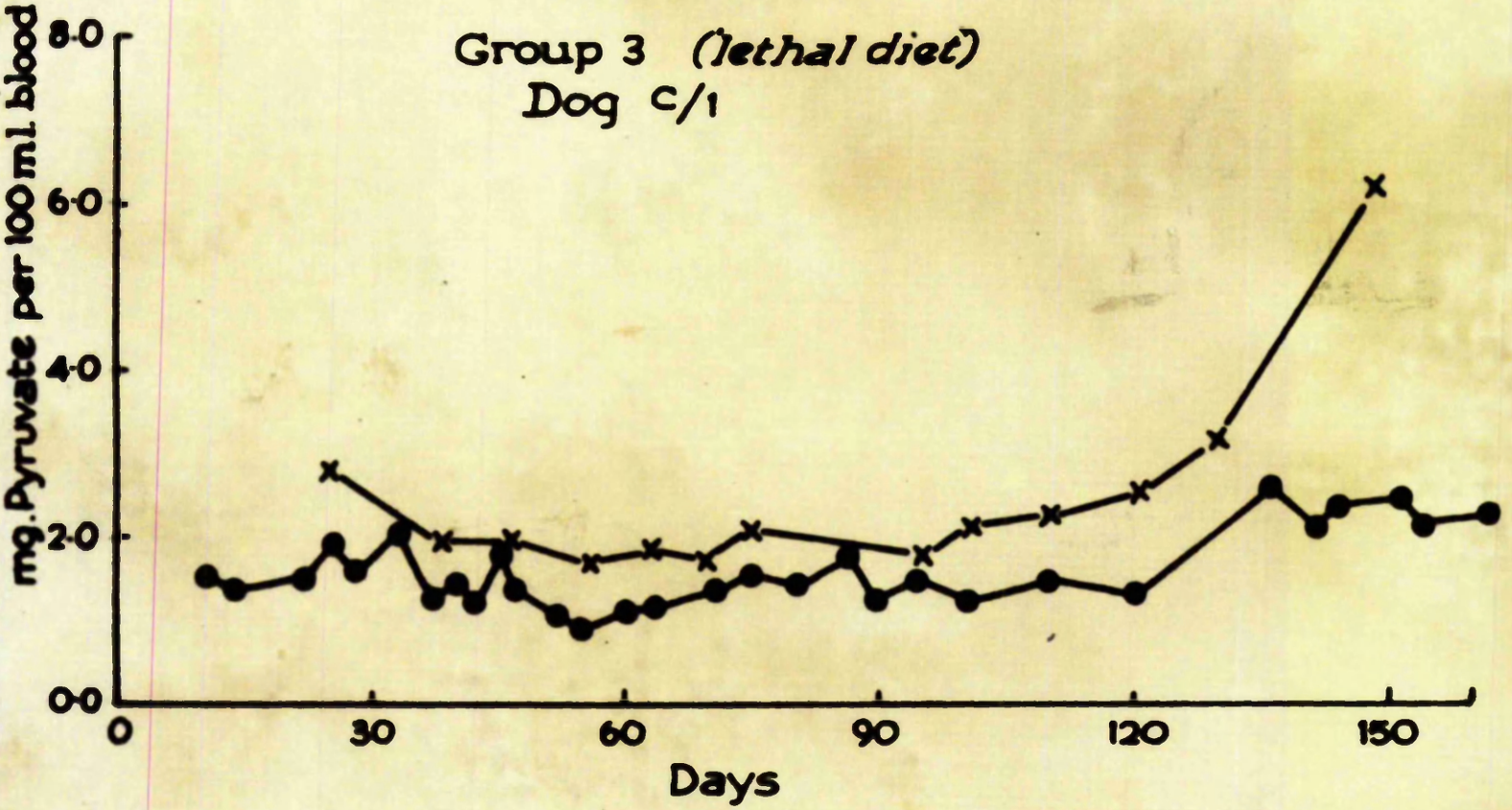

Figure 11. Graph showing the changes in blood pyruvate concentrations over the course of the experiment.

o-o fasting and resting

x-x following exercise

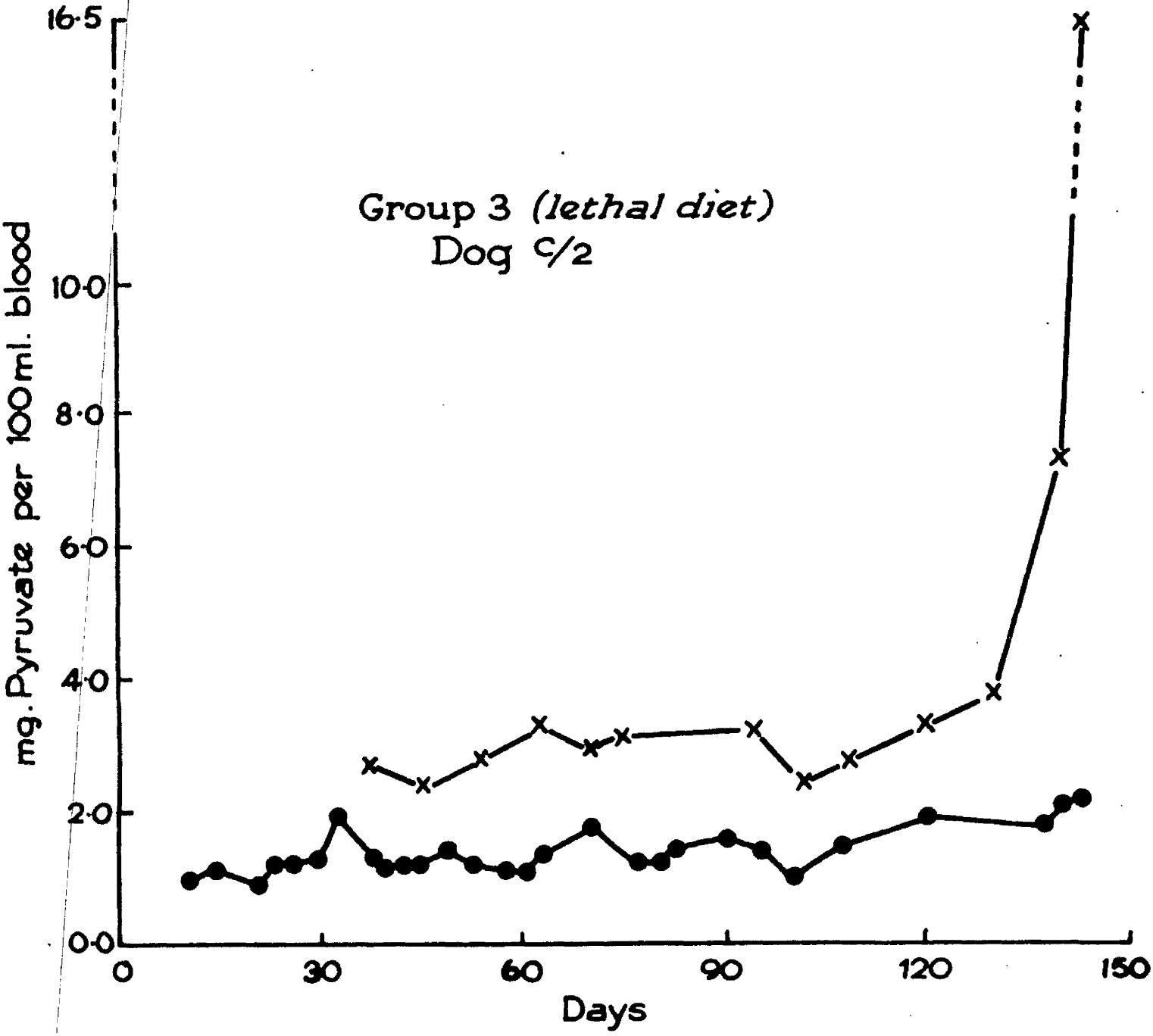

Figure 12. Graph showing the changes in blood pyruvate concentrations over the course of the experiment.

o–o fasting and resting

x–x following exercise

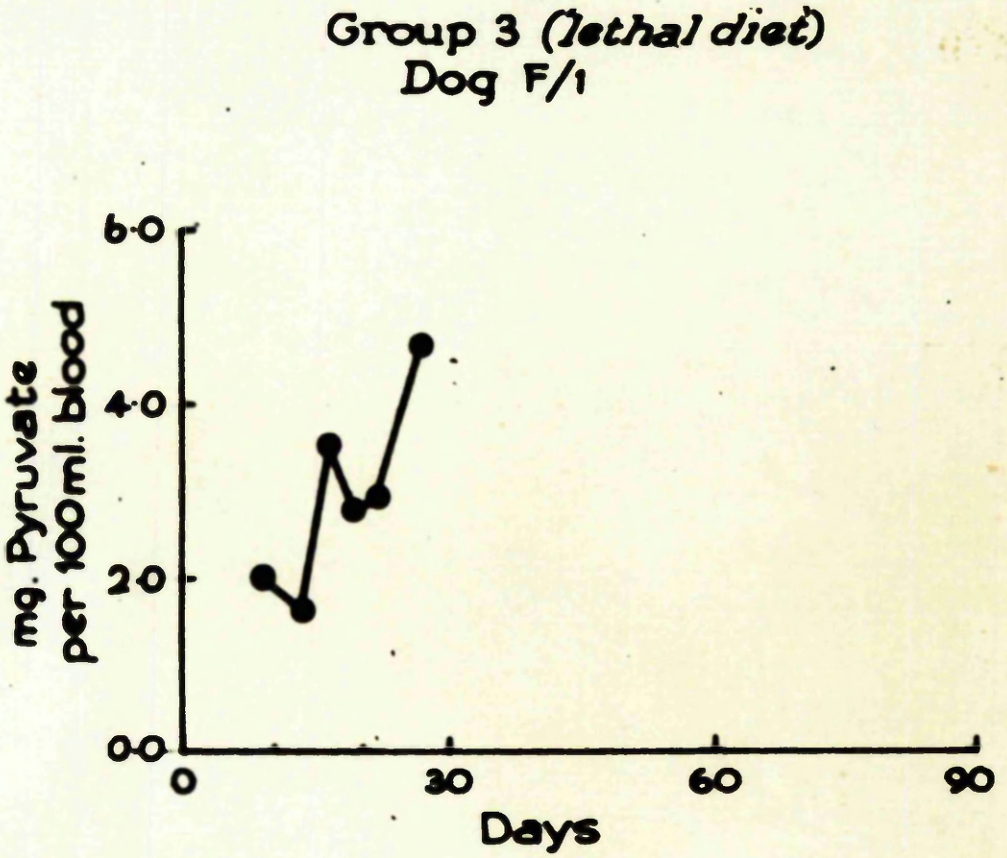

Figure 13. Graph showing the changes in blood pyruvate concentrations over the course of the experiment.

o-o fasting and resting.

1. Fasting and Resting Blood Pyruvate Concentrations.

Group 1. ("lethal diet" plus thiamine).
(see Figures 6, 7, and 8).

Starting 10 days after these dogs had been placed on their diets, fasting and resting blood pyruvate concentrations were determined at intervals over a period of 150 days. The mean of 91 estimations on these three dogs in this control group was 0.94 mg. pyruvate per 100 ml. blood (S.D. = 0.15). The differences between the three animals were not significant, nor was there any significant day-to-day variation during this period. In particular, the blood pyruvate concentration remained within normal limits until the end of the experiment.

Group 2. (standard hospital diet).
(see Figures 9 and 10).

After 10 days on the standard hospital diet, fasting and resting blood pyruvate concentrations were determined at suitable intervals over a period of 150 days. The mean of 58 estimations on these two dogs was 1.25 mg. pyruvate per 100 ml. blood (S.D. = 0.26). This mean is significantly higher than the mean for the animals in group 1. The standard deviation is also greater. This data would suggest that the standard hospital diet is

not/

/not particularly rich in thiamine, although it should be noted that there is no tendency for the blood pyruvate concentration to increase even at the 150th day.

Group 3. ("lethal diet").

After 10 days on the "lethal diet", the fasting and resting blood pyruvate concentrations were determined at intervals for periods up to 160 days. It soon became apparent that, while the dogs C/1 and C/2 were reacting in the same way, the dog F/1 was responding quite differently. It is of some interest that this dog (and dog F/2) had been obtained from different kennels than the other six dogs and had quite a different dietary history.

Dog C/1. (see Figure 11).

The mean of 25 estimations of the fasting and resting blood pyruvate concentration during the first 120 days on the "lethal diet" was 1.33 mg. per 100 ml. (S.D. = 0.28) which is significantly higher than the mean values for the control dogs in group 1. After 120 days, the blood pyruvate concentration began to rise - the highest recorded figure being 2.50 mg. per 100 ml. This animal survived.

Dog C/2. (see Figure 12).

The mean of 24 estimations carried out at intervals/

/intervals during the first 108 days on the "lethal diet" was 1.30 mg. pyruvate per 100 ml. blood (S.D. = 0.26) which is also significantly higher than the mean values for the controls of group 1. After 108 days, the blood pyruvate concentration began to rise - the highest recorded figure for this animal being 2.05 mg. per 100 ml. This dog collapsed and died soon after a bout of exercise, and the post-mortem findings were consistent with death due to thiamine deficiency. Death occurred after 142 days on the "lethal diet".

Dog F/1. (see Figure 13).

This animal rapidly developed biochemical abnormalities characteristic of acute thiamine deficiency. The mean of 6 determinations of the fasting and resting blood pyruvate concentration carried out at intervals during the 29 days which it survived on the "lethal diet" was 2.63 mg. per 100 ml. The highest recorded figure was 4.60 mg. per 100 ml. on the 27th day. Post-mortem findings were again consistent with death due to thiamine deficiency.

For reasons which are not obvious, this dog (F/1) developed a sudden acute thiamine deficiency corresponding to the fulminating beri-beri described in/

/in human beings in China by Platt and Lu (1936). In the human, fulminating beri-beri appears to be due to a complete lack of vitamin B_1 over a relatively short period of time, and is not necessarily associated with severe symptoms of vitamin deficiency although Platt and Lu found that the blood pyruvate concentration was markedly increased.

In the other two dogs of this group (C/1 and C/2) the general condition of the animals remained quite good. C/1 showed signs of fatigue after exercise on the 120th day, but C/2 appeared to be quite healthy until its sudden collapse and death after exercise on the 142nd day on the "lethal diet".

From these results it would appear that the basal blood pyruvate concentration is not a very delicate method of determining the thiamine status of the animal. Two of the three animals on the "lethal diet" died as the result of thiamine deficiency. In F/1, the basal blood pyruvate concentration increased steadily over the period of thiamine depletion and just before death a very high level was obtained; in C/1 and C/2, on the other hand, blood pyruvate concentrations were never grossly raised - even just before death in C/2. It is clear that such analyses are of little help in the/

/the detection of the early stages of a sub-clinical thiamine deficiency.

2. Blood Pyruvate Changes following mild Exercise. (see Figures 6 to 12).

The following experiments were carried out between the 38th and the 142nd days on the special diets, i.e., soon after dog F/1 had died but while the two remaining dogs on the "lethal diet" were showing no signs or symptoms of thiamine deficiency and before their basal blood pyruvate concentrations had become permanently elevated. The procedure was as follows:-

Blood was withdrawn while the dogs were still in a basal condition in order to determine the fasting and resting blood pyruvate concentrations (see above). The animals were then taken out, walked around for ten minutes, allowed to run freely for ten minutes, and then walked back to their cages - the whole procedure occupying a total of thirty minutes. As soon as they returned, a second specimen of blood was taken for analysis. The results are shown in Figures 6 - 12.

Group 1. ("lethal diet" plus thiamine). (see Figures 6, 7, and 8).

The mean of 42 estimations of the blood pyruvate concentration/

/concentration following exercise was 1.55 mg. per 100 ml. (S.D. = 0.37). Examination of the graphs of Figures 6 - 8 shows that the blood pyruvate concentration is consistently higher after exercise, although the average increase was only 0.61 mg. per 100 ml. The day-to-day variation after exercise is slightly greater than the day-to-day variation of the basal concentrations.

Group 2. (standard hospital diet).
(see Figures 9 and 10).

The mean of 30 estimations of the blood pyruvate concentration following exercise was 2.35 mg. per 100 ml. (S.D. = 0.51). Again, the blood pyruvate concentration is consistently higher than the corresponding figure for the basal state, and also shows a greater day-to-day variation.

Group 3. ("lethal diet").

Dog F/1 had died of thiamine deficiency before this series of experiments was started.

Dog C/1. (see Figure 11).

The mean of 10 estimations of the blood pyruvate concentration following mild exercise during the first 120 days on the "lethal diet" was 1.90 mg. per 100 ml. (S.D. = 0.25). Thereafter, the blood pyruvate/

/pyruvate concentration after exercise rose very steeply - the maximum recorded value being 6.2 mg. per 100 ml. on the 144th day of the experiment.

Dog C/2. (see Figure 12).

The mean of 8 estimations of the blood pyruvate concentration following mild exercise during the first 108 days on the "lethal diet" was 2.83 mg. per 100 ml. (S.D. = 0.31). Thereafter, the blood pyruvate concentration after exercise also rose steeply - the maximum recorded value of 16.5 mg. per 100 ml. on the day of death.

The mean values quoted above for the blood pyruvate concentration following standard mild exercise are no more help in the diagnosis of the early stages of sub-clinical thiamine deficiency than were the basal blood pyruvate concentrations. Indeed, for the first 100 days, dog C/1 ("lethal diet") is nearer to the control series than dog C/4 (standard hospital diet). For reasons which will be explained in connection with later experiments (see p.92) it might have been possible to detect thiamine depletion at an earlier stage by determining the blood pyruvate concentration after 15 minutes mild exercise, but the necessary information

was/

/was not available at the time when the above experiments were carried out.

The gross elevation of the blood pyruvate concentration after exercise in the two remaining dogs of group 3 leaves no doubt that, at that time, the dogs were severely depleted. But it will have been noticed that the basal blood pyruvate and the blood pyruvate concentrations after exercise, begin to rise almost exactly at the same time, viz., after 120 days in the case of dog C/1 and after 108 days in the case of dog C/2, so that either type of estimation would suffice for diagnostic purposes although the increase after exercise is much the more dramatic.

3. Further, more detailed, Experiments on the Blood Pyruvate Changes following mild Exercise.

In order to gain more information on these changes, an exercise tolerance test was carried out in the following way:-

A specimen of blood was taken while the animal was still fasting and resting to determine the basal blood pyruvate concentration. The animal was then allowed to run freely for a total period of 1½ - 2 hours. Blood specimens were taken at intervals/

/intervals during the exercise, the exact time of venepuncture being determined largely by the ability of the kennelman to catch a dog. The changes in the blood pyruvate concentration are shown in Figure 14. (see p.94).

These experiments were carried out between the 144th and the 161st days on the diets by which time, the remaining dog in group 3 (C/1) was severely depleted of thiamine, and the two dogs in group 2 were slightly or moderately depleted.

The graphs in Figure 14 show that the maximal blood pyruvate concentration in the dogs of groups 1 and 2 occurs about 15 - 20 minutes after exercise is started and thereafter falls even although the dogs are still exercising. It would therefore have been a better experiment if the blood pyruvate changes following mild exercise (above) had been studied 15 - 20 minutes (instead of 30 minutes) after the exercise had commenced. There was, however, a good reason for the tardiness of this discovery. For some time after the greyhounds had been assembled at the Veterinary Hospital, they were very vicious animals with a marked objection to intravenous work, and it was not until they had become thoroughly accustomed to handling that it

was/

/was considered prudent to attempt 6 - 7 venepunctures in the course of 90 minutes. After 140 days of the experiment the dogs were sufficiently tame to handle in this way and co-operated to the extent of lifting a paw to assist with the venepuncture.

Returning to Figure 14, it will be seen that the exercise tolerance curves correspond well with the nutritional status of the dogs.

The exercise tolerance test has shown that the greatest differences in blood pyruvate concentrations, amongst the 3 groups of dogs, were to be found at the end of 15 - 20 minutes' exercise. During this initial period the dogs were unable to metabolise the pyruvate as rapidly as it was being formed with a consequent steady increase in the concentration in blood. This had no apparent effect on the ability of the dogs of groups 1 and 2 to take fairly strenuous exercise. By the end of 30 minutes these dogs were able to maintain their blood pyruvate within narrow limits - around 2 mg. per 100 ml. for group 1 dogs and 3 mg. per 100 ml. for group 2 dogs. The same could not be said about C/1. At the end of 15 minutes' exercise this dog showed signs of weakness, ataxia and unwillingness to run. The further exercise which C/1 took was very light and very spasmodic. By the end of 1 hour, however, the above signs/

/signs disappeared, and the dog willingly took moderate exercise once again but with no return of these signs. It is interesting that the blood pyruvate concentrations closely followed these observations (see Figure 14), and it could be that the early signs of weakness and ataxia were in fact due to the pyruvate accumulated in the body. Eventually, after about 1½ hours, this animal was able to maintain the blood pyruvate around 5 mg. per100 ml., while continuing to run freely, though much less strenuously than the other dogs. This concentration, though greatly elevated, did not appear to be harmful to the dog. As a test of thiamine deficiency the blood pyruvate concentration following 15 minutes' exercise might be expected to be the most satisfactory in diagnosing sub-clinical thiamine deficiency.

Furthermore, the fluctuations in the blood pyruvate concentration closely follow the activity of the animal. On the 166th day on the "lethal diet" the dog C/1 was taken out and allowed to exercise freely. As before, the blood pyruvate concentration rose during the first 15 minutes by which time the signs of ataxia and weakness were apparent and the dog was unwilling to run any more. While the dog rested, over the following 10 minutes, blood/

/blood pyruvate concentrations started to decrease. The dog was then forcibly run for 10 minutes when the blood pyruvate once more increased but without a return of the above clinical symptoms. The dog then willingly continued to run slowly while the blood pyruvate decreased again to remain steady at about 5 mg. per 100 ml. (see Figure 15, p. 95).

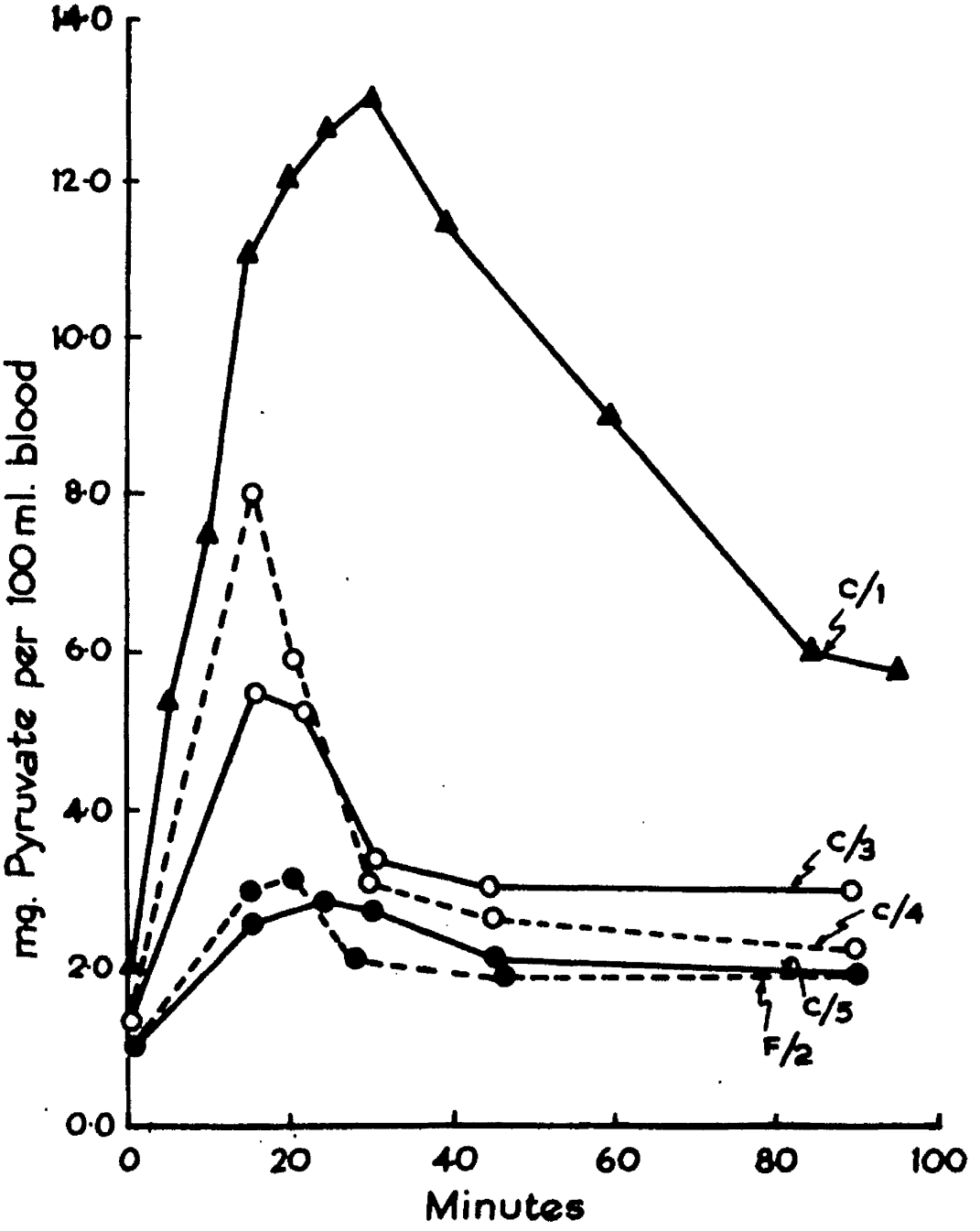

Figure 14. Graph showing the changes in blood pyruvate concentrations during exercise.

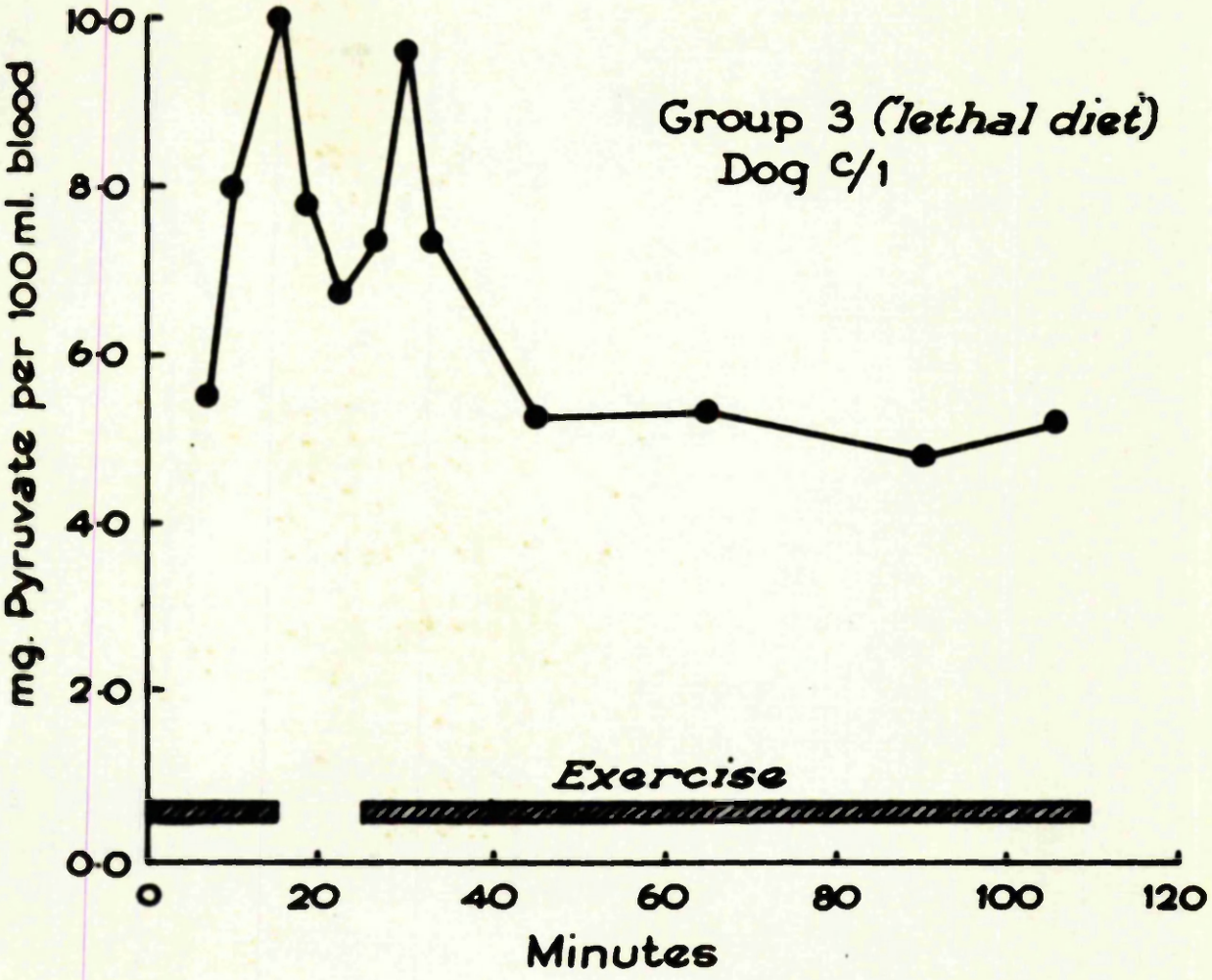

Figure 15. Graph showing the changes in blood pyruvate concentrations during periods of exercise and rest.

4. Effect of Thiamine Therapy.

On the 168th day of the experiment, the dog which had been fed the "lethal diet" (dog C/1) was given intravenous thiamine (20 mg.) after a short bout of exercise. The effects of the exercise and the thiamine on the blood pyruvate concentration are shown in Figure 16 (see p. 97).

Clinically, the animal became much more lively within 20 - 30 minutes of the injection, exercised voluntarily, and, in spite of the exercise, maintained a blood pyruvate concentration comparable with the dogs in group 1 (the controls).

5. Blood Pyruvate Changes following strenuous Exercise.

After the basal blood pyruvate concentration and the blood pyruvate concentration after mild exercise had started to increase in the 2 remaining dogs of group 3 ("lethal diet"), the effects of a sharp bout of strenuous exercise was examined. This experiment was carried out on two separate occasions.

Blood was taken for pyruvate estimation while the animals were fasting and resting. The dogs were then allowed to race 380 yards at a licenced race track under standard racing conditions. Immediately after racing, a second specimen of blood was taken for analysis. Results are shown in Table VI, p. 98.

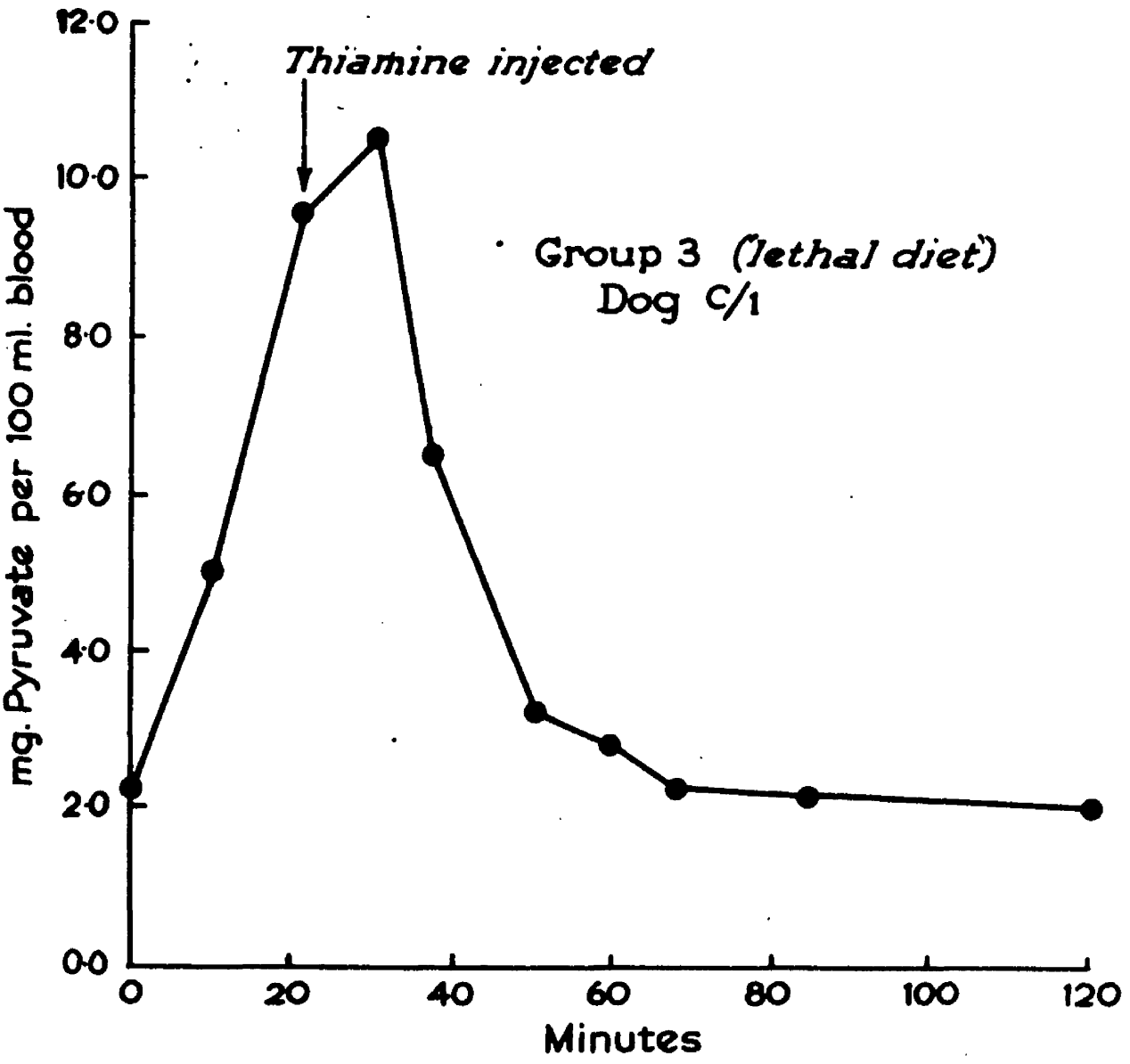

Figure 16. Graph showing the changes in blood pyruvate concentrations before and after injection of thiamine.

TABLE VI. The Effect of Strenuous Exercise on the Blood Pyruvate Concentration.

(All values in mg. pyruvate per 100 ml. blood.)

Group and Dog.		Day 137.		Day 160.	
		Basal State.	After Exercise.	Basal State.	After Exercise.
Group 1.	C/5.	1.1	3.9	(dead)	
	C/6.	1.1	4.7	0.9	4.1
	F/2.	1.0	4.6	1.0	5.0
Group 2.	C/3.	1.2	8.3	1.3	10.4
	C/4.	1.3	9.1	1.4	10.4
Group 3.	C/1.	2.5	12.5	2.3	17.0
	C/2.	2.0	13.8	(dead)	

The effects of strenuous exercise are merely a magnification of the changes produced by the mild standard exercise, but it is worth noting in passing, that the blood pyruvate concentrations of the dogs in group 3 are the highest that have ever been recorded in the literature. As a test of possible thiamine deficiency in racing greyhounds this would be the test of choice. It is simple to carry out as no special precautions or conditions are necessary and the results are clear cut.

In these determinations, the keto-acid hydrazone was extracted by ethyl acetate before photometric estimation. It has been stated (see p. 21) that this extraction method is relatively unspecific in that the hydrazones of several keto-acids may be extracted simultaneously, and that the use of xylene is more specific for pyruvic acid (see p. 22). The specimens obtained from the dogs in group 3 after strenuous exercise were analysed in duplicate using xylene as extraction solvent in the second estimation. The results using xylene were 8 to 12% lower than the figures recorded in the Table, showing that the increase after exercise is almost entirely due to the accumulation of pyruvate.

In the second series (160th day), the CO_2 combining/

/combining power of the blood of dog C/1 in the basal state was 55 vols. per 100 ml. After racing, the value fell to 37 vols. per 100 ml.

The dog apparently has a fairly wide range of alkali reserve with values rather lower than the human. Data is very scanty, but the Handbook of Biological Data (Wright Air Development Center, 2nd Ed., 1956, Table 41) quotes a range of 17 - 27 meq. per litre (37 - 60 vols. per 100 ml.) for dog plasma.

6. Changes in Blood Pyruvate following oral Administration of Glucose.

The experiments were carried out on the 144th day, by which time the dog C/1 was the only survivor in group 3. For this experiment, the dogs were kept in the resting state throughout. A fasting specimen of blood was taken for determination of pyruvic acid. Immediately afterwards, the animals were given 25 g. glucose mixed with a little meat as prepared for the "lethal diet", i.e., devoid of thiamine. In this way the dogs ingested the glucose quickly and completely. Further blood specimens were collected 40, 80, 120 and 160 minutes after the glucose had been taken. Results are shown in Figure 17, p. 101.

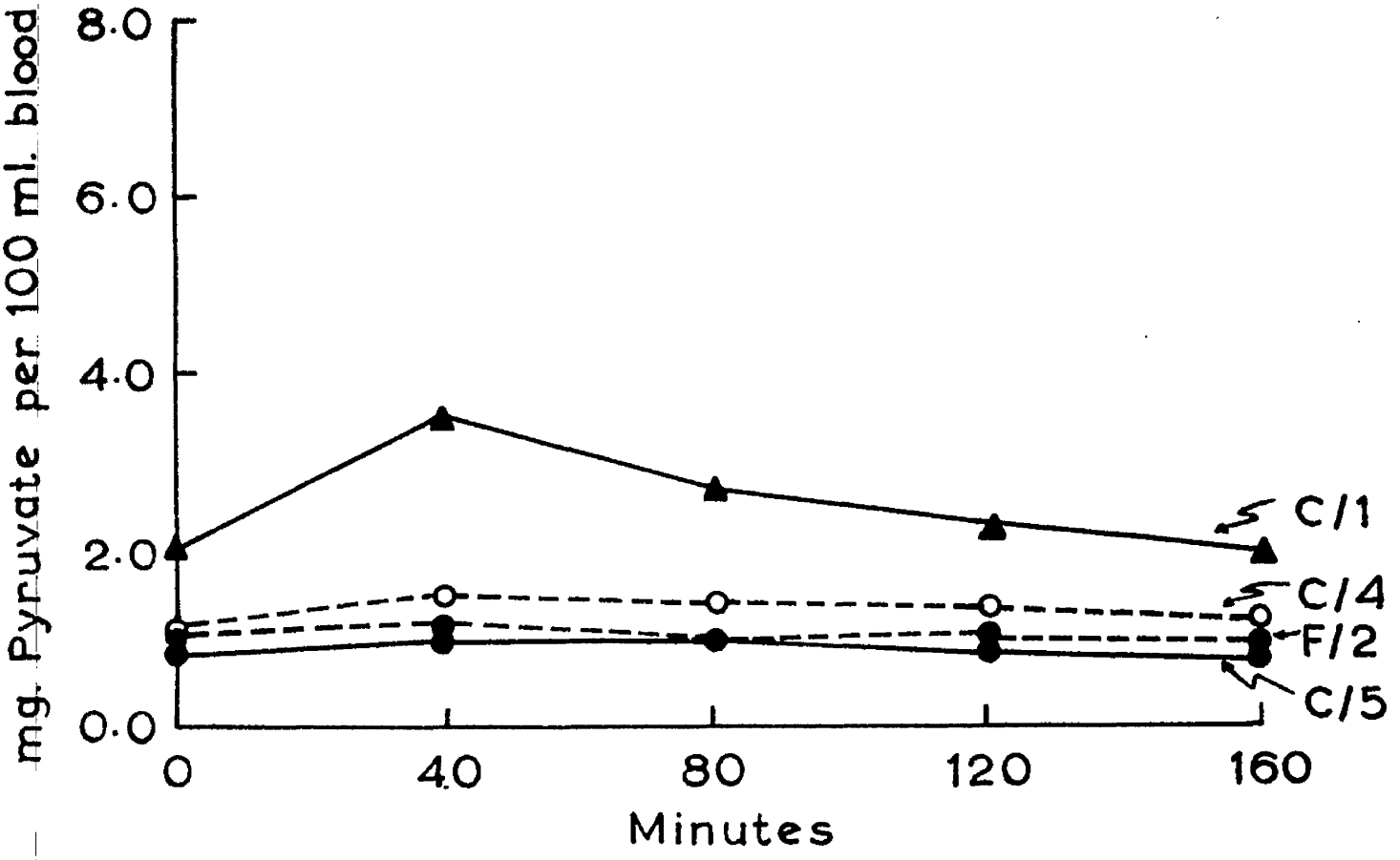

Figure 17. Graph showing the changes in blood pyruvate concentrations following the oral ingestion of glucose.

The control group show no significant change in blood pyruvate concentration following glucose. The increase in the dog C/4 (group 2) is more obvious and is possibly a further indication that the standard hospital diet is thiamine-deficient, while with dog C/1 (group 3) the increase in blood pyruvate is considerable.

By the time that this experiment was carried out (144th day) the dog C/1 had a raised basal blood pyruvate concentration, and showed considerable increases in blood pyruvate after exercise, (e.g., see Table VI, p. 98). In spite of this, C/1 showed no clinical symptoms of vitamin deficiency, so that this test can be used to demonstrate sub-clinical thiamine deficiency although at how early a stage, has yet to be demonstrated.

7. Excretion of Thiamine in the Urine following a Test Dose.

Since this test involves the parenteral administration of thiamine, its regular use would have upset the main experiment, and therefore it was carried out only when the other biochemical findings showed the need of more information about the dogs on the "lethal diet" (group 3). In all, the test was applied on four occasions; once before the/

/the start of the experiment proper, and three times during the experiment, viz., on the 35th, 90th and 150th days.

The experiments were carried out during the forenoon with the animals in a fasting condition. The bladder was first completely emptied by catheterisation and the urine was discarded. 500 µg. thiamine was then given by intravenous injection and all the urine passed during the following 4 hours was collected by catheter. The volume of urine was measured and the thiamine concentration determined by the method described above (p. 45). The results are shown in Table VII, p. 104.

TABLE VII. Excretion of Thiamine in the Urine following Administration of a Test Dose of the Vitamin.

(Results in µg. thiamine excreted in the urine in 4 hours).

Group and Dog.		Control Period.	35th day.	90th day.	150th day.
Group 1.	C/5	131	152	140	150
	C/6	170	167	200	180
	F/2	190	170	150	160
Group 2.	C/3	120	140	76	60
	C/4	131	102	66	65
Group 3.	C/1	134	160	77	10 Ca.
	C/2	153	98	54	dead
	F/1	128	dead	-	-

Control period:

Mean excretion = 144 µg. thiamine per 4 hours (S.D. = 24).

Note: The experiment during the control period is not to be confused with the experiment (see p.) where the blood pyruvate concentration was studied following injection of 5 mg. thiamine.

Dog F/1 (group 3) had died of acute thiamine deficiency after 29 days on the "lethal diet", and it was considered advisable about this time to have an independent assay of the thiamine status of the remaining dogs. Hence the first thiamine excretion test was carried out on the 35th day. After 90 days, the remaining 7 dogs showed no clinical signs of thiamine deficiency and a study of the basal blood pyruvate and the changes in blood pyruvate following standard mild exercise showed no consistent difference between the three groups. The question then arose whether an examination of the urinary thiamine excretion might disclose biochemical abnormalities which were not shown by blood pyruvate examinations, since it was felt that, after 3 months on the "lethal diet", the two dogs in group 3 must be very depleted in thiamine. The third occasion, on the 150th day, was shortly after the dog C/2 had died of chronic thiamine deficiency.

The difference between the three groups on the 35th day is not sufficient to be able to draw any definite conclusions, but if larger numbers of animals had been available there are indications that there might have been statistically significant differences. On the 90th day, the difference between the/

/the control dogs (group 1) and the other two groups of dogs is very marked, and the results show that this test of sub-clinical thiamine deficiency gives positive results at a much earlier stage than the tests involving changes in the blood pyruvate concentration.

The urinary excretion of thiamine after injection of a test dose of the vitamin again indicates that the standard hospital diet was deficient in thiamine, and that the dogs in group 2 were demonstrably thiamine-deficient by the 90th day.

No calculations were made of the fraction of injected dose which was retained per kg. body weight (see p. 59). But it is of interest to note that on the 150th day, the dog C/1 retained something of the order of 98% of the injected dose.

Post-Mortem Results in Greyhounds.

Dog.	Post-Mortem Results.
C/1	Well marked lesions of cardiac dystrophy.
C/2	Congestive cardiac failure.
F/1	Cardiac dystrophy.
C/3	1 small focus of myocardial degeneration.
C/4	1 small dystrophic focus in left auricle.
C/5	2 small dystrophic foci in right ventricle.
C/6	Marked lesions of cardiac dystrophy in one auricle.
F/2	1 small focus of myocardial degeneration.

Further Observations on the Relationship between Thiamine Deficiency and the Blood Pyruvate Concentration in Dogs.

Certain considerations led to a repetition of part of the work described above. The main biochemical reason was the belated observation that the maximal increase in blood pyruvate concentration occurred not after 30 minutes, but after 15 minutes of mild exercise (see p. 90 above), and a further reason was that the pathologists wished to have more detailed information about the morbid anatomy and histology of cardiac muscle in thiamine deficiency. It was also thought advisable to study some breed of dog other than the greyhound which, through in-breeding for racing purposes, has a very highly developed cardio-vascular system.

Ten animals were chosen at random for this experiment. These consisted of 4 mongrel terriers and 6 mongrel collies. The dogs were paired, as well as possible, and divided into two groups each consisting of 2 terriers and 3 collies. Each dog was housed separately. They were all fed the standard hospital diet for two weeks before experiments began.

Group 4. Dogs A/1 A/2 A/3 A/4 and A/5. (Control group.) One of these dogs (A/5) was found to have a physical deformity of the jaw which led to difficulty in eating. Being a nutritional experiment, therefore, this dog was discarded leaving 2 terriers and 2 collies in the group.

These dogs were fed the "lethal diet" (see p. 72) but were also given a daily intramuscular injection of thiamine (0.5 mg.).

Group 5. Dogs B/1 B/2 B/3 B/4 and B/5. ("Lethal diet"). These dogs were fed the "lethal diet" but were allowed only 1 lb. of food (meat and bread).
They did not receive any supplementary thiamine.

The immediate object of this experiment was to follow the fasting and resting blood pyruvate concentration and the changes in blood pyruvate after 15 minutes of mild exercise. It was hoped that this method would be more successful in detecting the earliest stages of thiamine deficiency than the best of the methods used in the greyhound experiments, viz., the much more laborious urinary thiamine excretion after a test dose of the vitamin. However, with this collection of dogs it proved impossible to collect reliable basal specimens of blood owing to the very/

/very lively and excitable nature of the animals. As the experiment proceeded, and the dogs came to know the staff concerned, so their excitement increased. Although attempts were made to compare the blood pyruvate concentrations in the basal state and after exercise, sometimes the former turned out to be higher than the latter.

Group 4 dogs. Control group.

The results are shown in Table VIII (see p. 110) where the two sets of analyses are more correctly referred to as "before exercise" and "after exercise". The experiment was continued for a total of 140 days with each dog, and it will be seen that the difference between the two sets of figures is negligible, and both, in effect, are comparable to the previously recorded (p. 87) blood pyruvate changes after mild standard exercise. The mean value for the blood pyruvate concentration of all 109 analyses was 1.25 mg. per 100 ml. (S.D. = 0.42).

Throughout the whole experiment, the dogs remained in good physical condition and none showed any signs of thiamine deficiency.

TABLE VIII. Blood Pyruvate Concentrations before and after mild standard Exercise.

Control group dogs (Group 4) only.

Results in mg. pyruvate per 100 ml. blood.

	Before Exercise.			After Exercise.		
Dogs.	No. of Analyses.	Means.	S.D.	No. of Analyses.	Means.	S.D.
A/1	17	1.30	0.43	13	1.26	0.41
A/2	16	1.12	0.37	11	1.41	0.49
A/3	15	1.19	0.39	11	1.28	0.47
A/4	15	1.19	0.34	11	1.31	0.40
Means.		1.21			1.31	

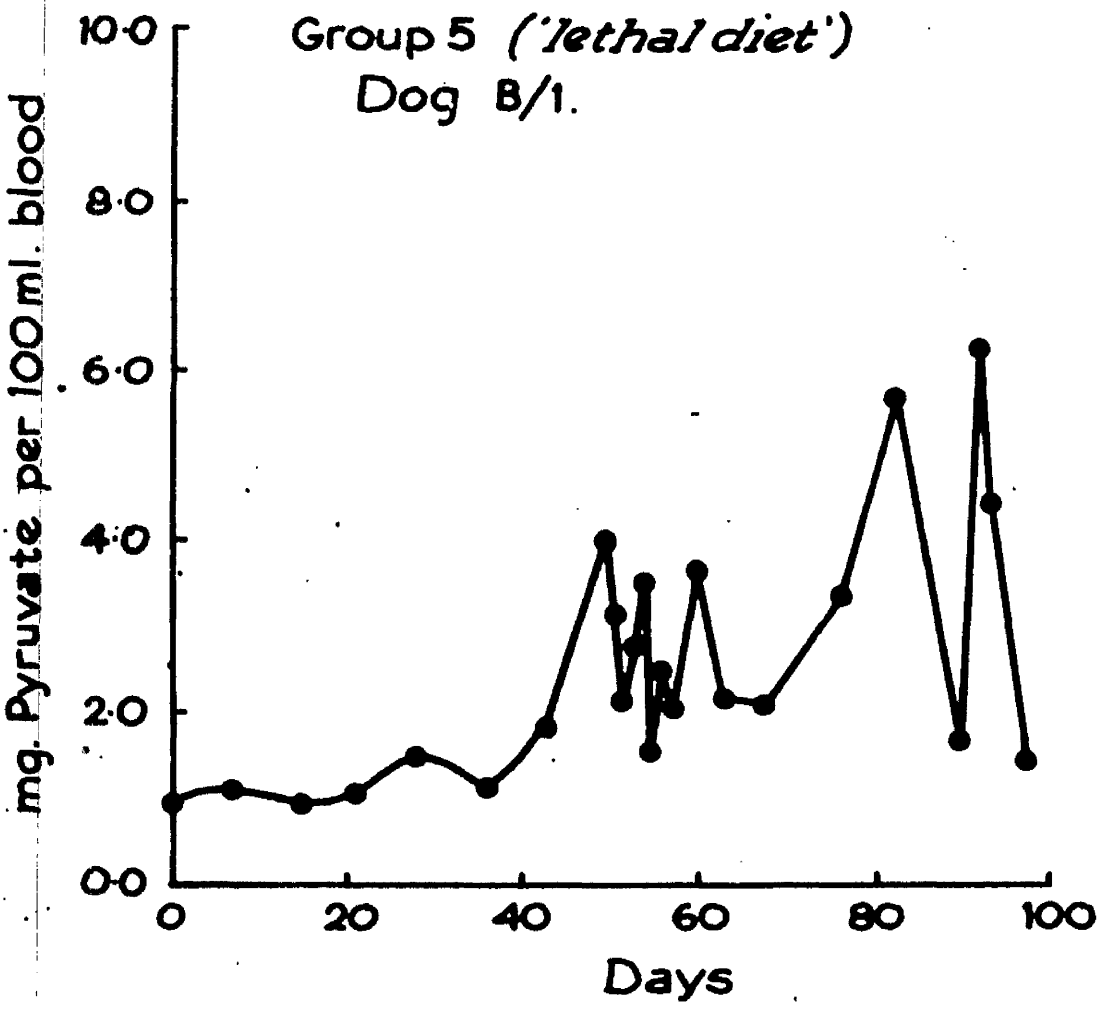

Figure 18. Graph showing the changes in blood pyruvate concentrations over the course of the experiment.

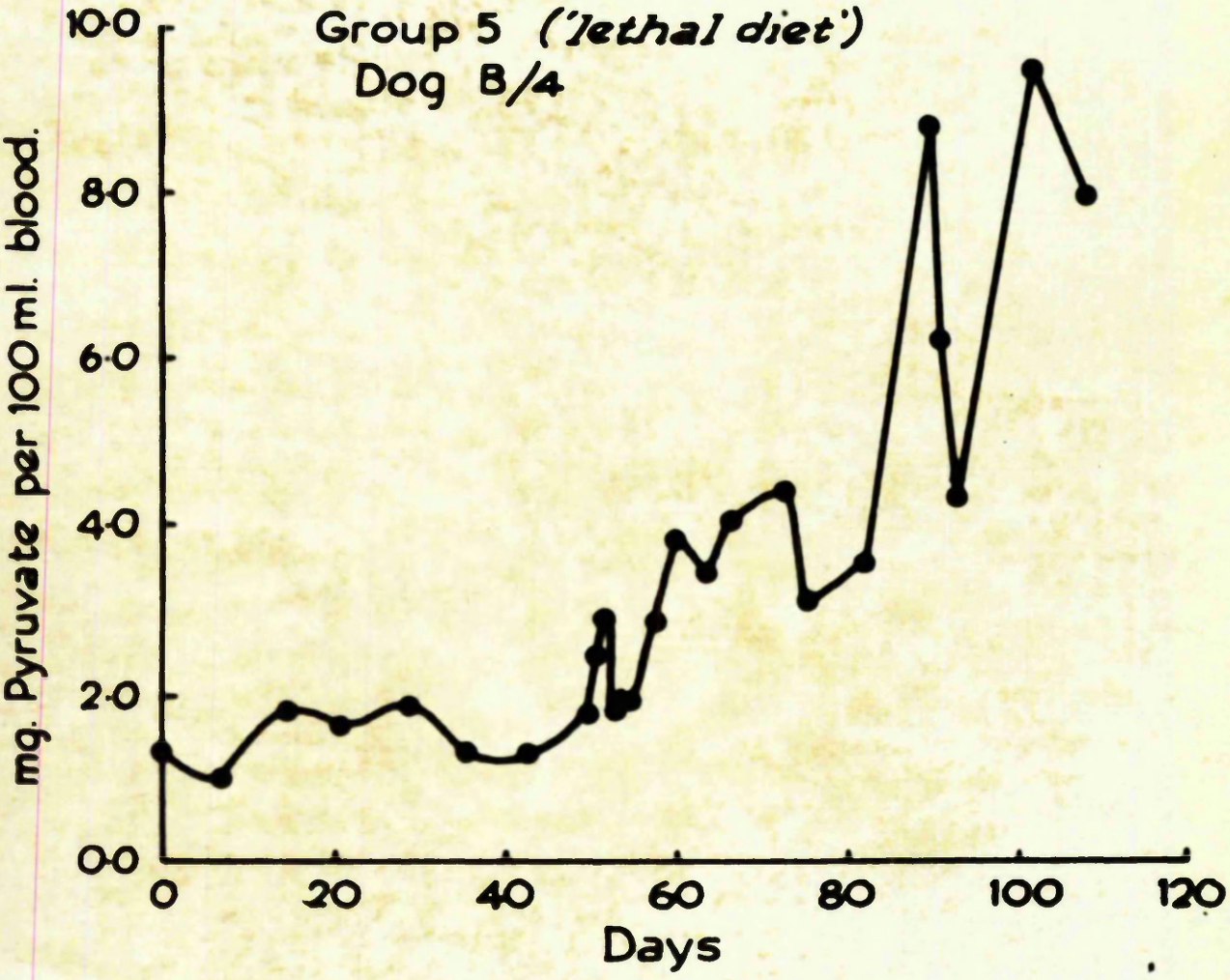

Figure 19. Graph showing the changes in blood pyruvate concentrations over the course of the experiment.

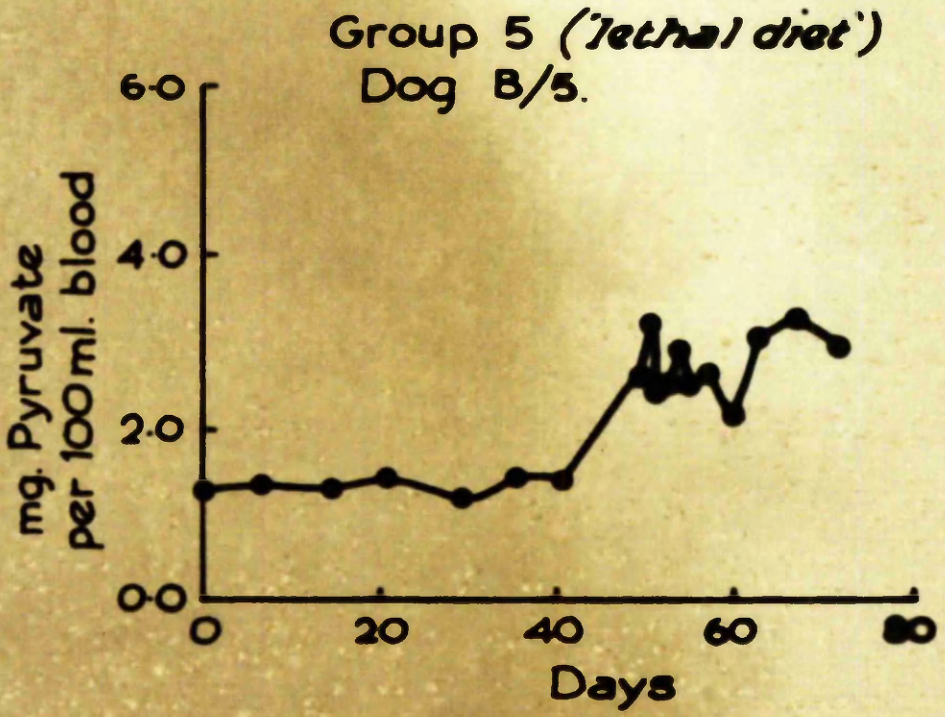

Figure 20. Graph showing the changes in blood pyruvate concentrations over the course of the experiment.

Group 5 dogs. "Lethal diet".

(See Figures 18, 19, 20, pp. 111, 112, 113.)

Each point on each graph is the mean pyruvate concentration obtained on that day, i.e., the average of the values "before exercise" and "after exercise". The two sets of analyses fluctuated in exactly the same way as the analyses in the control group (see above).

Dog B/1. (see Figure 18). This dog died after 97 days on the "lethal diet". Up to the 43rd day the blood pyruvate concentration remained within normal limits, the mean value being 1.20 mg. per 100 ml. (S.D. = 0.37). Thereafter, the blood pyruvate increased considerably, the maximum figure being 6.1 mg. per 100 ml. There was also a very great day-to-day variation. Clinically, this dog was bright and lively until 3 days before death. It then became unwilling to exercise, became weaker, comatose, and finally died. Death was due to cardiac failure, but post-mortem examination did not reveal any signs of thiamine deficiency.

Dog B/2. Dog B/3. These two dogs remained fit and well throughout the 140 days which the experiment lasted./

/lasted. They showed no signs or symptoms of thiamine deficiency. The mean blood pyruvate concentrations after exercise were:-

B/2 1.58 mg. per 100 ml. S.D. = 0.50 (22 estimations).

B/3 1.47 mg. per 100 ml. S.D. = 0.40 (23 estimations).

And even in the last few days of the experiment, the blood pyruvate showed no tendency to increase in either animal.

Dog B/4. (See Figure 19). This dog died after 107 days on the "lethal diet". Up to the 50th day, the mean blood pyruvate concentration was 1.06 mg. per 100 ml. (S.D. = 0.44) which is actually less than the mean values for the controls of group 4. After 50 days, the blood pyruvate began to rise with tremendous day-to-day fluctuations, the highest recorded value being 9.5 mg. per 100 ml. Clinically, this dog remained healthy until a few minutes before death. Again, death was due to heart failure and post-mortem examination did not reveal any of the classical findings of thiamine deficiency.

Dog B/5. (See Figure 20). This dog died after 72 days on the "lethal diet". Up to the 43rd day, the blood pyruvate was not significantly increased, the mean value being 1.37 mg. per 100 ml. (S.D. = 0.18), with/

/with very little day-to-day variation. Thereafter, the blood pyruvate began to rise, but the highest observed concentration was only 3.2 mg. per 100 ml. Clinically, the dog remained well until 2 days before death. It then became unwilling to exercise, and showed signs of weakness and ataxia, and eventually died in cardiac failure. The post-mortem findings in this cases were compatible with death due to thiamine deficiency.

The considerable day-to-day fluctuation in the blood pyruvate concentrations in these animals is probably due to two factors.

(a) The animals in group 5 occasionally went off their food. On three occasions this anorexia was associated with a fall in blood pyruvate. However a fall in the blood pyruvate was also noted on other occasions when the animals were apparently feeding normally.

(b) By reference to graph 14, it can be seen that over the first 30 minutes' exercise blood pyruvate concentrations are rapidly changing in the animal. As it was impossible in these experiments to obtain blood samples at exactly 15 minutes large fluctuations were to be expected.

The failure of two of the dogs (B/2 and B/3) in group/

/group 5 to develop any symptoms of thiamine deficiency or to exhibit an increased blood pyruvate concentration, is not regarded as anything other than a normal biological variation. In the last few weeks of the experiment, these two dogs must necessarily have been seriously thiamine-depleted, and the findings of persistently normal blood pyruvate concentrations merely confirms the conclusions previously reached (p. 88) that a normal blood pyruvate concentration either before or after mild exercise does not exclude sub-clinical thiamine deficiency even when the estimations are carried out on successive days. Although in the other 3 dogs (B/1, B/4 and B/5) thiamine deficiency was picked up at an early stage by means of the above biochemical analysis.

The Stability of Thiamine.

The results which have been obtained in this work naturally led to two further investigations relating to the mechanism of thiamine depletion, (1) the stability of thiamine in relation to achlorhydria and alkalinity, and (2) the rate of destruction of thiamine under the conditions in which the "lethal diet" (p. 72) was prepared.

The Stability of Thiamine and the pH.

Melnick, Robinson and Field (1939, 1941) showed that thiamine is stable in gastric juice between pH 1.5 and pH 8.0, but is destroyed by the presence of antacids, bile or pancreatic juice. It has long been known that many of the B group of vitamins (e.g. thiamine, riboflavine, pantothenic acid) are unstable in alkaline solution. It was also found by Melnick et al. (loc. cit.) and confirmed by Sinclair (1939) and by Goodhart and Sinclair (1940), that absorption of thiamine is deficient in cases of achlorhydria. Robinson, Melnick and Field (1940) also showed that thiamine was much more efficiently absorbed from the intestine when a test dose of the vitamin was given along with an ordinary mixed meal, presumably due to simultaneous liberation of gastric acid./

/acid.

But there is no precise data in the literature on the rate of destruction of thiamine at alkaline physiological pHs.

Indeed, owing to the difficulties of obtaining material and the obvious variations which can occur, there is little precise data on the pH of the intestinal canal and the various alimentary secretions. The following data are taken from the Handbook of Biological Data (1956 edition):-

		Human.	Dog.
Gastric juice.	pH	1.49-8.38	-
Bile. (hepatic)	pH	6.2 - 8.5	7.4 - 8.5
Bile. (gallbladder)	pH	5.6 - 8.0	-
Pancreatic juice.	pH	7.0 - 8.0	7.1 - 8.2
Duodenal secretion.	pH	5.8 - 7.6	6.30- 7.28

Post-gastrectomy patients usually have a long medical history with frequent reference to the use of alkaline therapy which may, or may not, be continued after operation. Surgical removal of the source of gastric acidity, together with the continued use of alkalis, will result in pH values considerably higher than any quoted as a normal range. While the highest physiological pH quoted for any intestinal secretion is 8.5, the use of alkali therapy in post-gastrectomy patients/

/patients may involve pH values in the upper jejunum as high as 9.0 perhaps higher. There is no available data. The commonest household source of "alkali" is baking soda which, in pure aqueous solution, begins to lose carbon dioxide at about 20°C. and at 37°C. the decomposition is much more rapid. N/10 sodium bicarbonate (initial pH = 8.2) decomposes at 37°C. to give a solution of pH 8.9 after 40 minutes.

The rate of destruction of thiamine in alkaline solution is shown in Figure 21 (p. 121). 100 µg. thiamine was dissolved in 100 ml. of buffer solution (NaH_2PO_4-Na_2HPO_4-NaOH) of varying pH and incubated at 37°C. Samples were withdrawn for analysis at times up to 60 minutes.

Below pH = 8.0, thiamine is completely stable, but the higher the pH above this value, the greater the rate of destruction.

In post-gastrectomy patients, who presumably have a complete achlorhydria, some destruction of thiamine is certain to occur in the alimentary tract and if alkali is taken before or after food, the amount of vitamin destroyed may be considerable. This is therefore one factor which may induce thiamine deficiency in such patients.

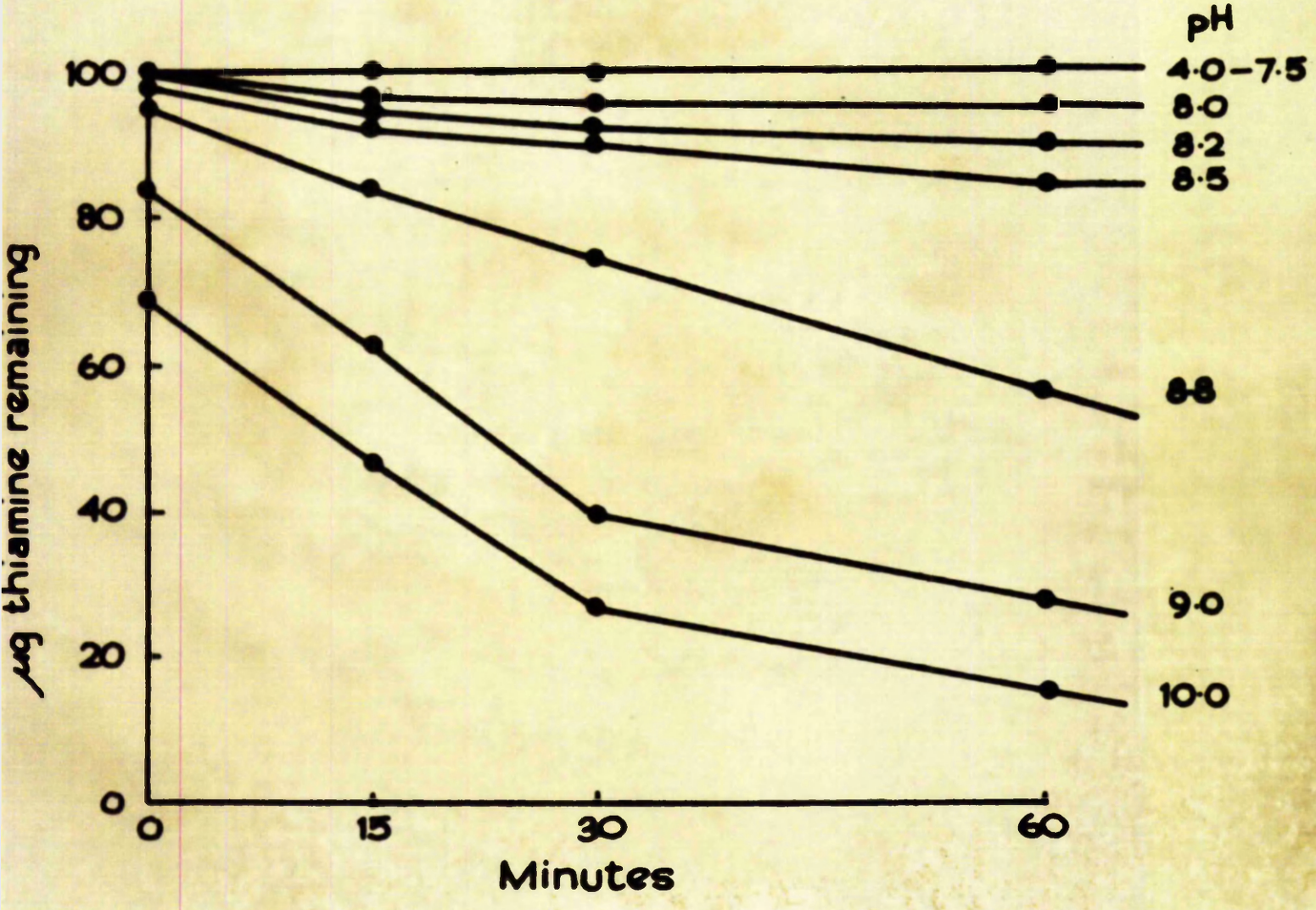

Figure 21. Graph showing the rate of destruction of thiamine at various pH values.

The Stability of Thiamine and the Effect of Heat.

It is known that many of the B group of vitamins are partially or totally destroyed by heat depending on the conditions. In the case of the "lethal diet" described in a previous section, the food was subjected to heat at three stages in the preparation of the diet.

(1) The meat obtained from commercial sources for conversion into dog food is obtained from condemned carcasses, and must be sterilised by heat before being sold,

(2) The bread of the diet has been rusked, i.e., exposed to dry heat for varying lengths of time, and finally,

(3) The mixture of meat and bread was subjected to simmering for a period of 6 hours before being fed to the animals.

There was therefore ample opportunity for destruction of thiamine.

But under any conditions of heat, alkalinity (e.g., see Figure 21) etc., treatment is seldom so drastic that the thiamine is completely destroyed. Even after the treatment described above, it is unlikely that the final product was entirely devoid of all thiamine, and it became a matter of interest, first, to discover how much of the thiamine was destroyed

by/

/by this pre-treatment, and secondly, exactly how much thiamine was contained in the so-called "lethal diet."

The "lethal diet", when homogenised and extracted with water, was always on the acid side of neutrality; the pH in different experiments varying from 5.0 to 5.4. In this range of pH, thiamine is completely stable, so that any destruction of the vitamin in the "lethal diet" must be due to heating or to the length of time of heating.

Analysis of Diets.

The food was prepared in the ratio of 1½ lb. meat (heat sterilised) to 1 lb. whitebread (rusked). Water was added to allow for evaporation and the mixture analysed for thiamine before and after 6 hours' simmering.

300-400 g. lots were taken for analysis, homogenised (pH of the homogenate = 5.3), acidified to pH 4.5 with hydrochloric acid, and treated with a preparation of acid phosphatase of proven activity prepared from dog prostate in order to liberate thiamine from thiamine diphosphate. The mixture was then deproteinised with 10% trichloracetic acid, filtered, the residue thoroughly extracted with water, and/

/and the filtrate re-adjusted to pH 4.5.

Decalso was added and stirred for about 2 hours in the solution. The resin was then centrifuged, washed several times with water, and the thiamine eluted with 25% (w/v) potassium chloride containing N/10 hydrochloric acid. Thiamine was determined in this purified extract by the method described in a previous section. Results are shown in Table IX.

TABLE IX. The Effect of Heating for 6 Hours on the Thiamine Content of the Diet.

Results in µg. thiamine per 100 g. (wet wt.) of mixed food.

Experiment.	Before heating.	After heating.
1	10.0	2.5
2	8.0	1.8
3	9.2	1.7
4	6.5	1.5
5	7.8	2.0
6	8.0	2.1
7	6.0	1.9
8	7.5	2.6
Means.	7.8	2.0

The simmering process caused destruction of about three-quarters (average, 74%) of the original thiamine in the diet.

This point was further investigated in the following experiments. Pure crystalline thiamine was added (a) to an extract of homogenised meat plus bread at pH 5.3, and (b) to a phosphate buffer (NaH_2PO_4-Na_2HPO_4) at pH 5.3. Both systems were boiled under a reflux condenser for 6 hours, and, after allowing for the original thiamine content of the food, the percentage destruction in each case was calculated. Results are given in Table X.

TABLE X. Destruction of Thiamine by Heat. pH = 5.3

	Percentage destruction.	
µg. Thiamine Added.	Heated with Food Extract.	Heated with Buffer.
200	44, 48, 49%	47, 49%
100	50, 51, 52%	52, 52%
50	56, 58, 58%	55, 56%
30	60, 64, 65%	60, 60%

At pH 5.3, thiamine is completely stable at 37°C., hence the destruction on boiling is purely a heat effect. (Cp. Figure 21, p. 121). The smaller the amount of thiamine present, the greater the relative amount destroyed, so that on a low thiamine diet (such as the standard hospital diet, p. 72) the percentage thiamine destroyed may be considerable, and the diet readily/

/readily made thiamine deficient by heating. The diet before heating corresponds to the standard hospital diet (see p. 72), and the diet after heating is the "lethal diet". From these figures, the following data has been calculated:-

	µg. Thiamine.	
Group of dogs.	Thiamine received daily in the diet.	Thiamine received daily per kg. body wt.
Group 2. Standard hospital diet.	200 to 340	8 to 13
Group 3. "Lethal diet".	50 to 85	2.0 to 3.5
Group 4. Standard hospital diet.	80 to 140	8 to 13
Group 5. "Lethal diet".	20 to 35	2.0 to 3.5

Street, Zimmerman, Cowgill, Hoff and Fox (1941) found that thiamine deficiency could be produced in dogs on a diet containing approximately 2 µg. thiamine per day per kg. body weight. Swank, Porter and Yeomans (1941) stated that no symptoms of thiamine deficiency developed in dogs when the diet contained more than approximately 4 µg. thiamine per day per kg. body weight. Swank et al. did not carry out any biochemical investigations, and the fact that their animals/

/animals showed no symptoms does not exclude a thiamine deficiency which can be demonstrated by biochemical methods. The dogs of group 2 in this work did not show any symptoms of thiamine depletion (see pp.) but the development of thiamine deficiency could be demonstrated in more ways than one, in spite of the fact that these dogs were receiving 8 - 13 µg. thiamine per day per kg. body weight.

DISCUSSION.

Thiamine Deficiency in Post-gastrectomy Patients.

Biochemically, the experiments were disappointing in that they did not differentiate clearly between the post-gastrectomy patients and the control subjects. However, the experiments confirmed the general view that post-gastrectomy patients do tend to be thiamine deficient, and the observations made on the effect of thiamine therapy suggested that an oral thiamine supplement of 2 mg. daily was not adequate to protect all the patients against a sub-clinical thiamine deficiency.

Avery Jones (1952) in his book, Modern Trends in Gastro-enterology (p. 462) suggested three main factors responsible for thiamine deficiency following gastrectomy, (1) lack of gastric hydrochloric acid, (2) intestinal hurry, and (3), decomposition of thiamine by bacteria in the upper jejunum.

Reference has been made above (p.49) to the work of Melnick et al. (1940) and of Sinclair (1938) on thiamine deficiency associated with achlorhydria and on the instability of thiamine in presence of antacids, bile, and pancreatic juice. The experiments recorded here have led to an examination of the stability of thiamine/

/thiamine at different pHs. (see p. 121). Thiamine was found to be unstable above pH 8.0 - the extent of the destruction depending mainly on the pH, but also on the total amount of thiamine present in the system.

Bile and pancreatic juice both contain bicarbonate in a concentration of the order of 100 meq. per litre. Normally, this is neutralised by the gastric acidity but in achlorhydria, decomposition of the bicarbonate with loss of carbon dioxide at 37°C. may result in a pH as high as 8.9 (see p. 120). In post-gastrectomy cases, where there is a gastro-jejunostomy, the fluid aspirated through a Ryle's tube is a mixture of saliva, plus oesophageal secretion, plus any gastric juice secreted by the remnant of the stomach, but contaminated in every case with varying amounts of bile, pancreatic juice, duodenal secretion and secretion from the upper jejunum. The pH of this mixture is usually quoted as 6 - 8 units, and if sodium bicarbonate is taken in quantity, pH values above 8.0 are assured. This will lead to considerable destruction of thiamine.

The possibility that excessive intestinal hurry, leading to malabsorption of thiamine, is a factor that has been put forward by Avery Jones and while it must remain a theoretical possibility there is little evidence that it is of practical importance. For example, none of/

/of the patients of the post-gastrectomy series complained of diarrhoea.

Frazer (1949) has shown that achlorhydria, and to a lesser extent, hypochlorhydria, leads to bacterial invasion of ileum, jejunum, duodenum, and even the stomach itself. He has suggested the possibility of competition between bacteria and the host for vitamins of the B group in general. But achlorhydria is by no means unknown in normal healthy subjects, and is regularly found, for example, in cases of pernicious anaemia, and neither of these two types appears to be especially prone to the development of thiamine deficiency. Nevertheless, this may be a factor in certain cases.

Of the three possibilities, the destruction of thiamine by an abnormally high pH seems the most important. The optimal intake of thiamine is variously quoted as 1.0 to 1.5 mg. per day. Since the great majority of the subjects were from the poorer classes, it is most unlikely that any reached this optimal level, and partial destruction due to an increased pH in the jejunum might well have brought about a state of sub-clinical thiamine deficiency.

It is only proper that some of the difficulties encountered in work of this type should be referred to at this point.

Multiple/

/Multiple vitamin deficiency is the rule, not the exception, and it is clear from the case history (p. 67a) and the response to vitamin therapy, that the patient M.J. was also suffering from a considerable deficiency of riboflavin which was probably the main source of most of her symptoms.

Although Muir (1949) reported 20 post-gastrectomy cases complaining of peripheral neuritis, only 9 of these responded clinically to administration of thiamine, and the remainder eventually responded to various forms of physiotherapy. Gilder (1950) concluded that, although it was very difficult to produce experimentally, a deficiency in any one of the B group of vitamins in humans, in general vitamin B deficiency it was a thiamine deficiency which was most rapidly produced and led to the initial symptoms.

The clinical difficulties of selecting probable cases of thiamine (or vitamin B) deficiency are considerable for no signs and symptoms are specific in the early stages of the condition, and in this work, attempts were being made to diagnose the sub-clinical stage of the deficiency. Vague symptoms and signs such as lassitude, neurasthenia, anorexia, limb pains, glossitis, cheilosis, angular stomatitis, etc., occur in many conditions other than vitamin B deficiency.

The/

/The post-gastrectomy patients formed a heterogeneous group of patients whose only common factor was a previous partial gastrectomy. The gastrectomies had been performed 2 months to 10 years previously.

Thiamine Deficiency in Dogs.

This is the first recorded biochemical investigation of an outbreak of thiamine deficiency in dogs. The objects of these experiments were,

(1) to verify that the sudden deaths of racing greyhounds were in fact due to a deficiency of thiamine,

(2) to study clinically and biochemically, the effects of the "lethal diet" which was believed to be the cause of these sudden deaths, and,

(3) to study under more controlled conditions, the various tests of thiamine deficiency in an attempt to assess their relative merits in the investigation of sub-clinical thiamine deficiency.

The "lethal diet" fed to the dogs provided 2.0 to 3.5 ug. thiamine per kg. body weight per day which would appear, from a comparison with the findings of other workers (p. 126), to be on the border-line of inadequacy. But these other workers, such as Street et al. (1941) and Swank et al. (1941) were trying to observe nothing more than the development of clinical symptoms due to thiamine deficiency; they carried no biochemical tests.

Now, two points have been made clear in the present/

/present work, (1) that biochemical tests can detect sub-clinical thiamine deficiency, and (2) that dogs may be near the point of death due to thiamine deficiency without exhibiting any marked clinical symptoms.

There can be no doubt that a diet containing 2-4 µg. thiamine per kg. body weight per day is severely deficient in thiamine. Even on the "standard hospital diet", which contributed 8-13 µg. thiamine per kg. body weight per day, biochemical methods could elicit signs of thiamine deficiency while the animals were apparently quite healthy. While no experiments have been carried out to determine the minimal daily intake of thiamine compatible with health, these figures would suggest that the minimum must be of the order of 15-20 µg. per day - a figure which is of the same order (per unit of body weight) as that required by humans. The Committee on Food and Nutrition of the National Research Council, U.S.A. (1952) recommend a daily intake of 20 - 30 µg. thiamine per kg. body weight. This is probably a very generous estimate. Minimal requirements for health, established in conjunction with biochemical data, are considerably lower. Stiebeling and Phipard (1939) found 13 µg. per kg. adequate/

/adequate to prevent biochemical lesions of thiamine deficiency - a finding which has been supported by the work of Melnick (1942). Keys and Henschel (1943) found that a diet providing 10 µg. thiamine per kg. just adequate.

The satisfactory behaviour of the dogs of the control groups (which were given thiamine daily by injection) both clinically and biochemically, proves the point that death was due to thiamine deficiency when the dogs were fed the "lethal diet". The cause of the thiamine deficiency of the "lethal diet" was the stupid way in which the food was prepared by the kennelmen. Destruction by alkalinity does not come into the argument. The pH of the prepared "lethal diet" was always on the acid side of neutrality (average pH about 5.3), and dog gastric juice is reported to have 150 meq. of hydrochloric acid per litre (Handbook of Biological Data (1956), p.61). The initial diet is not particularly rich in thiamine (see Table IX, p. 124), and 6 hours' continuous heating destroys about three-quarters of the vitamin present.

As a result of these findings, the various kennels were advised to change the diet fed to their dogs. The following suggestions were made:-

White/

/White bread in the diet should be replaced by whole meal bread, and it should not be rusked before being mixed with meat,

The final mixture of whole meal bread and meat should not be heated before the final preparation of the food, and,

Yeast tablets should be added occasionally to the diet.

The other important point which has been observed is the total lack of all clinical signs and symptoms of thiamine deficiency in dogs fed the "lethal diet" until a few days before death. In the dogs of both groups 3 and 5 ("lethal diet") the animals remained apparently healthy and in good spirits, e.g. dog B/4 (p. 115) which appeared to be quite normal until a few minutes before death.

Assessment of the different Methods of investigating Thiamine Deficiency.

Fasting and Resting Blood Pyruvate. Human Subjects.

Statistically, the post-gastrectomy patients had higher fasting and resting blood pyruvate concentrations than the control group of subjects. It could reasonably be concluded that these post-gastrectomy patients, as a whole, tended to be thiamine deficient. Individually, however, there was considerable overlapping between the two groups, patients and controls, so that it would be quite impossible to decide if an individual patient were thiamine deficient on the strength of a single blood pyruvate analysis, or even a series of such analyses. The only conclusions that could be reached were that if the basal blood pyruvate concentration were above the normal upper limit, the patient was probably suffering from some degree of thiamine deficiency, provided that the other cases of a raised blood pyruvate concentration (p. 7) were excluded, but that the level of the blood pyruvate concentration was no index of the degree of thiamine deficiency, and that a normal blood pyruvate concentration did not exclude even a moderately severe degree of thiamine deficiency.

Fasting and Resting Blood Pyruvate. Experimental Animals.

The difficulties of correlating results on human subjects and on experimental animals are too well known to require emphasis here. From the laboratory point of view, the outstanding difference was that the 8 dogs on the "lethal diet" were allowed to become so thiamine deficient that 5 of them died of this deficiency.

Only the animals in groups 1, 2, and 3 will be considered here; no true basal blood pyruvate concentrations could be obtained with the animals in groups 4 and 5 (see p. 109).

The mean fasting and resting blood pyruvate concentration for the animals in group 2 (standard hospital diet, which was slightly deficient in thiamine) was higher than that of the animals in group 1 (controls), and the mean value for the dogs in group 3 ("lethal diet") was in turn, higher than that of the dogs in group 2. But again, there was considerable overlapping in all three groups, and a biochemical separation could not be made on the basis of a single analysis except in the dogs of group 3, and then, only toward the end of the experiment when they were grossly depleted of thiamine. Even then, the basal blood pyruvate concentration did not always/

/always show any striking abnormality and reached only 2.05 mg. per 100 ml. in dog C/2 shortly before death.

The conclusions reached in theanimal experiments are much the same as those reached with the post-gastrectomy patients, viz, that a raised basal blood pyruvate is diagnostic of thiamine deficiency, other causes having been excluded, and that a normal blood pyruvate concentration does not necessarily exclude a state of thiamine deficiency.

In both human subjects and experimental animals, the analysis is quite useless in the detection of sub-clinical thiamine deficiency and should not be used clinically as a diagnostic procedure.

Blood Pyruvate Changes following Exercise.

Exercise, even in normal animals, increases the concentration of blood pyruvate (Figures 6, 7, 8, pp. 74, 75, 76). On a slightly thiamine deficient diet, the increase after exercise is greater (Figures 9, 10, pp. 77, 78) and there is a greater variation. This variation may be due to several factors, e.g., the impossibility of reproducing accurately a "standard" bout of exercise with experimental animals, slight variations in the time at which the blood specimen was withdrawn/

/withdrawn, and so on. But when there is advanced thiamine depletion, very high values for the blood pyruvate concentration after exercise are invariably found and the increase is far beyond any possible experimental error or variation in procedure. But this increase occurs at the same time as the increase in the fasting and resting blood pyruvate concentration, so that it does not help in the early diagnosis of the condition. Strictly speaking, it can be argued that these analysis establish a diagnosis of "sub-clinical" thiamine deficiency for at the point when the blood pyruvate concentration was significantly elevated both before and after exercise, the animals still appeared quite healthy on clinical examination. But an examination of figures 11, 12, 18, 19 and 20 (pp. 79, 80, 111, 112, 113) makes it clear that this increase in blood pyruvate is a late event in the development of thiamine deficiency.

These changes in blood pyruvate concentration in dogs after mild exercise, corroborate the findings of others in human subjects suffering from thiamine depletion (see p. 11) and suggest that this might be the basis of a standard biochemical test of thiamine depletion.

Blood Pyruvate Changes following Ingestion of Glucose.

The changes in blood pyruvate after ingestion of 25 g. glucose successfully separated the dogs into their respective groups (see Figure 17, p. 101) but the experiment was not carried out until the 144th day on the various diets. It is not known at how early a stage this test will give positive results, but the findings recorded here are in general agreement with the observations that have been recorded by others in human thiamine deficiency (see p.9).

Urinary Excretion of Thiamine after Injection of a Test Dose.

Human Subjects.

Post-gastrectomy patients excrete in the urine a significantly smaller proportion of a test dose of thiamine than do the corresponding group of controls (see Table III, p. 60). But again, there is considerable overlapping of the results of the two groups and one would be unlikely to be able to classify any given individual accurately by this test. When the results are recalculated as the weight of thiamine retained per kg. body weight, a better correlation is found with the clinical assessment of the case, but again there is still much overlapping of/

/of the "control range". With neither method of expressing results did the biochemical findings agree accurately with the clinical assessment of the case.

Nevertheless, taken as a group of patients, the results lead to the conclusion that post-gastrectomy patients tend to suffer from thiamine deficiency which should be avoided by recommendation of a diet rich in vitamin B_1 or by therapeutic administration of vitamins, or both. The results in Table V (p. 63) show that a considerable excess of thiamine over the normal intake may be necessary, and the case of M.J. again stresses the importance of multiple vitamin deficiency.

Experimental Animals.

The excretion of thiamine in dogs after administration of a test dose (see Table VII, p. 104) gives more clear-cut results, and, more important, there is a very significant difference between the dogs on the "lethal diet" and the controls by the 90th day of the experiment. For the diagnosis of the earlier stages of thiamine depletion, this would seem to be the method of choice.

SUMMARY.

1. Chemical methods involved in the detection of thiamine deficiency, pyruvic acid and thiamine, have been studied in detail. Some modifications and improvements of existing methods have been introduced.

2. The thiamine status of a group of post-gastrectomy patients has been investigated by measuring the fasting and resting blood pyruvate concentration and also the urinary excretion of thiamine following an intravenous test dose of the vitamin. Compared with a similar group of control subjects post-gastrectomy patients tend to be thiamine deficient.

3. Thiamine deficiency has been produced experimentally in dogs. The various tests of thiamine deficiency, viz. blood pyruvate concentrations under different experimental conditions, and urinary excretions of thiamine following an intravenous test dose of the vitamin, have been studied in these animals during the course of thiamine depletion and also when severely thiamine deficient. The relative merits of these various tests have been discussed.

4./

/4. The effects of the variation of pH and of temperature on the stability of thiamine have been investigated.

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STUDIES IN THIAMINE DEFICIENCY IN MAN AND EXPERIMENTAL ANIMALS.

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Summary.

This thesis deals with the biochemical manifestations of thiamine deficiency in man and in dogs, and with the problems of detecting sub-clinical thiamine deficiency and of assessing the extent of clinical deficiency by biochemical means.

The first section is a resume of the literature pertaining to the chemical estimations involved in the detection of thiamine deficiency viz. pyruvic acid and thiamine. The various tests of thiamine deficiency, involving these two estimations, as used by the different workers in this field have been discussed.

The chemical estimations of pyruvic acid and thiamine have been studied in detail and certain modifications have been introduced.

A/

/A measure of the fasting and resting blood pyruvate concentration, and of the urinary excretion of thiamine in the 4 hours following a 1 mg. intravenous dose - the most widely used tests of thiamine deficiency - have been employed in the investigation of thiamine nutrition in post-gastrectomy patients. A comparison of the thiamine status of 17 post-gastrectomy patients and 24 normal subjects has been made.

An outbreak of thiamine deficiency in greyhounds led to a controlled study of this condition in dogs. The deficiency was due to the destruction of thiamine in the preparation of the food prior to its being fed to the dogs. This diet was shown by chemical analysis to have a very low thiamine content.

The course of thiamine depletion and the subsequent states of sub-clinical and clinical thiamine deficiency were studied biochemically. Blood pyruvate concentrations in the fasting and resting state, and following mild exercise, were followed throughout the duration of the experiment. Blood pyruvate concentrations have also been studied at intervals during exercise. The effect of the ingestion of glucose on the blood pyruvate concentration has been studied. Once before, and three times during the course of the experiment, urinary thiamine excretions were estimated following an/

/an intravenous test dose.

The result of feeding this same thiamine deficient diet was also studied using collies and terriers as experimental animals.

The stability of thiamine at various pH values was next investigated; the effect of heat was also studied.

Thiamine deficiency in post-gastrectomy patients was discussed in relation to the stability of thiamine at the pH of the gastro-intestinal tract. Thiamine deficiency in the greyhounds was discussed in relation to the stability of thiamine under the conditions of the preparation of food.

Finally the various methods of detecting thiamine deficiency have been critically reviewed in the light of the work recorded in this thesis.