THE CARBOXYLASE SYSTEM OF YEAST AND ITS USE IN THE MANOMETRIC METHOD FOR DETERMINING DIPHOSPHOTHIAMINE IN ANIMAL TISSUE EXTRACTS

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CHAPTER I

INTRODUCTION

I. THE PROBLEM

Statement of the problem. The purpose of this study was to appraise the manometric method for measuring diphosphothiamine (DPT) in animal tissues. In this method DPT is estimated by its cocarboxylase activity in the decarboxylation of pyruvic acid by yeast. Certain properties of the carboxylase system were also investigated in an effort to explain or eliminate the defects of the method.

Importance of the study. In a review of the significance of diphosphothiamine, Ochoa (1) has pointed out that it appears to be the active form of thiamine in the catalysis of pyruvate utilization (2 - 13), as well as a coenzyme in numerous other biological reactions involving decarboxylation, (14 - 18). In view of the great metabolic significance of DPT, the method used for estimating it is of considerable importance. Consideration of this method involves a study of the carboxylase system used.

II. ORGANIZATION OF THE REMAINDER OF THE THESIS

The remainder of the thesis falls into four main divisions: a review of the literature, a description of the methods used, a report of the results, and a summary of the results and discussion of their relation to the literature.

Review of the Literature. Chapter II contains a review of the literature bearing upon the manometric method of determining DPT. As yeast carboxylase is employed in the method, a review of the properties of the enzyme is presented first.

Methods. Chapter III contains a description of the general methods used repeatedly throughout the investigation. In some instances special modifications of the methods have been described in conjunction with the results.

Results. The results of the investigation are presented in three chapters: Chapter IV contains the results bearing upon the appraisal of the method; in Chapter V are reported the results of an investigation of certain defects of the method; Chapter VI deals with the inhibition of carboxylase by iodoacetate.

Summary and Discussion. In Chapter VII the findings are summarized and discussed in the light of previous investigations.

CHAPTER II

REVIEW OF THE LITERATURE

Introduction. The review of the literature deals with four main topics:

I. The discovery of the carboxylase system and the identification of its components.

II. The properties of carboxylase.

III. The properties of yeast relevant to the carboxylase system.

IV. The manometric method in its various modifications.

I. THE DISCOVERY OF THE CARBOXYLASE SYSTEM OF YEAST.

Discovery of carboxylase. In 1911 Neuberg and Karczag (19, 20, 21) reported the presence in yeast of an enzyme which catalyses the decarboxylation of pyruvic acid to acetaldehyde and carbon dioxide according to the equation:

$$CH_3\text{-}CO\cdot COOH \longrightarrow CH_3\cdot CHO + CO_2$$

This carboxylase was not identical with zymase, since it was not inactivated by a temperature of 50°C, or inhibited by chloroform (22). As dialysis had no effect upon the enzyme it was believed to act without a coenzyme.

Discovery of cocarboxylase. However, Auhagen showed in 1932 (23) that the washing of dry brewer's bottom-yeast in an alkaline medium was accompanied by a loss of carboxylase activity which could be restored by the addition of boiled yeast extract.

The boiled yeast extract could not be replaced by Mg + cozymase + hexose-diphosphate + inorganic phosphate, or by the ash of boiled extract, adenylic acid, adenosine triphosphate, glutathione, or cysteine. He concluded that the boiled yeast extracts contained a new coenzyme acting as a cocarboxylase, and was able to purify it somewhat by precipitation with lead acetate and with barium acetate, (24). Dialysis of a neutral suspension of yeast through a collodion membrane did not reduce the carboxylase activity, (25), suggesting that the cocarboxylase was in combination with a substance of high molecular weight, the combination being less strongly dissociated in neutral than alkaline solution. The coenzyme appeared to be present in animal tissues, for small amounts of cocarboxylase activity were found in kidney, heart and blood extracts, (26).

Identity of cocarboxylase. While attempting to isolate the antineuritic vitamin from yeast, Kinnersley and Peters (27) reported a form of active material which, unlike most of the vitamin, was precipitated by lead acetate. This finding suggested the presence of two forms of vitamin B_1 in yeast. In support of this view they reported that the active material extracted from a charcoal adsorbate of yeast by 50% acid alcohol differed in some ways from that extracted by N/10 HCL. In 1932, Simola (28) showed that the cocarboxylase content of brain and liver was lower for rats maintained on a diet deficient in Vitamin B Complex than for rats

that were normal. Reports that yeast synthesized cocarboxylase in the presence of thiamine and inorganic phosphate (29, 30) further suggested a relationship between cocarboxylase and vitamin B_1. Finally, in 1937, Lohmann and Schuster (2) isolated cocarboxylase from yeast as the hydrochloride, and showed it to be the pyrophosphoric acid ester of vitamin B_1. These workers also found that a manganese or magnesium salt was necessary for the carboxylase activity of alkaline-washed yeast. Cocarboxylase has since been synthesized chemically from thiamine in various ways (31, 32, 33).

II. PROPERTIES OF CARBOXYLASE

Components of the system. Green et al. (34) have reported that the active holoenzyme comprises a thermolabile apoenzyme, a thermostable coenzyme which is identical with DPT, and magnesium. Zima and Williams (35) discovered in yeast a thiol form of thiamine. The thiol form of DPT was later synthesized (36) and found to possess approximately the same cocarboxylase activity as DPT.

Dissociation of carboxylase. Auhagen (23) discovered that the enzyme could be split by repeated washings with alkaline phosphate solutions to yield an inactive apoenzyme. D.E. Green (34) accomplished the dissociation of his partially purified enzyme by the addition of ammoniacal $(NH_4)_2SO_4$ solution. Weil-Malherbe, (37), also working with a partly purified carboxylase, split the enzyme by acidification with HCl in the presence of $(NH_4)_2SO_4$.

The methods used by most investigators (6, 38 - 41) for preparing apocarboxylase were based upon Auhagen's method, modified as to the number and length of washings, and the pH and temperature of the washing fluid. The dissociation has been reported to be rapid at pH 8.0, being almost complete in two minutes (72).

Stability of apocarboxylase at pH 8.0. Steyn-Parvé and Westenbrink (42) studied the stability of the apoenzyme at pH 8.0 by measuring the carboxylase activity after recombination with DPT at pH 6.8. Five minutes incubation at pH 8.0 resulted in a permanent loss of activity of about 50%.

Resynthesis of carboxylase. Steyn-Parvé and Westenbrink (42) reported that the resynthesis of carboxylase from alkaline-washed yeast, DPT, and magnesium ions, was most rapid at about 27.5°C and about pH 6.8. Nearly maximum resynthesis occurred during the first five minutes. The presence of pyruvate during reconstitution of the holoenzyme lowered the carboxylase activity. Additional experiments suggested that the inhibition was due to acetaldehyde formed during the incubation period.

Green, Herbert and Subrahmanyan (34) in studying the kinetics of recombination of their partially purified enzyme, found that with a given amount of enzyme the decarboxylation reaction was proportional to the concentration of DPT within certain limits. The presence of some divalent metal such as manganese or magnesium was necessary in the reconstructed enzyme system. Magnesium was the only metal found in the native enzyme,

and although several other divalent metals could replace it in a reconstructed system, the substitution usually lowered the activity. Iron was found to be active in the ferrous form but practically inactive in the ferric form. These workers suggested that "the metal acts as a chemical bridge between the specific protein and DPT." For maximum activity it was necessary to add the magnesium to the apoenzyme before the DPT.

Activators of carboxylase. Ochoa and Peters (6, 43) reported the stimulation of carboxylase activity of alkaline-washed yeast by thiamine and structurally related substances, such as monophosphothiamine and certain pyrimidines with a 6-amino group. Westenbrink and Van Dorp (44, 45) suggested that this was due to thiamine inhibiting a phosphatase in yeast (46) which dephosphorylates free DPT, rather than a direct effect upon the carboxylase activity. In agreement with this suggestion were the findings that the 'stimulating' action of thiamine occurred only in the presence of DPT (14, 47) and disappeared when the DPT concentration was high (48). More recently (37, 34), partially purified carboxylase preparations were obtained upon which thiamine had no effect, confirming the view that the 'stimulating' action of thiamine was not a direct effect upon the enzyme.

Hexose diphosphate has been found to stimulate the carboxylase activity of alkaline-washed yeast in the presence of either boiled tissue extracts (38), or cozymase plus DPT (6). The mechanism of the effect is discussed under Section III of this chapter.

Inhibitors of carboxylase. Acetaldehyde (2, 34), bisulfite (49), adenosine triphosphate in very large amounts (37), $AgNO_3$, $CuSO_4$ and $HgNO_3$ (34) have all been reported to depress the carboxylase activity of the native enzyme. The effect of bisulfite was prevented by the addition of a large excess of pyruvate (49). Inhibition of the reconstituted system by calcium chloride was reported by Goodhart and Sinclair (39), but this depression has since been shown to occur only if the calcium salt is present before the reconjugation of the holoenzyme (50).

Other inhibitors of the reconstituted system have been reported with no mention as to whether these substances were added before or after the addition of DPT. Among these are potassium oxalate (39), p- aminobenzoic acid (51), sulfathiazole (52), and compounds of the Wurster type[1] (48). Inhibition by Wurster type compounds was reversed by an increase in DPT concentration (48), whereas sulfathiazole inhibition was partly reversed by p-aminobenzoic acid, (51).

Green et al (34) reported that concentrations of iodoacetic acid up to 1 molar had no effect upon their purified holoenzyme. Kensler, Young, and Rhodes (48) found that 5×10^{-4} M iodoacetate resulted in 5% inhibition of the reconstituted system but felt that this was of no significance.

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1. Diamines which form free radicals upon oxidation in an acid medium.

Effect of substrate concentration. The curve of carboxylase activity against pyruvate concentration, reported by Green et al. (34), approximated a rectangular hyperbola. Maximum velocity was obtained at a pyruvate concentration of about M/6, and K_M[2] was about M/33.

Effect of enzyme concentration. A straight line relationship between enzyme concentration and activity has been observed by Green et al. (34) for their purified preparation.

Effect of pH. Steyn-Parvé and Westenbrink (42) measured the effect of pH upon the activity of native carboxylase, upon a mixture of alkaline-washed yeast, magnesium ions, and DPT, and upon the holoenzyme after resynthesis at pH 6.8. These workers claimed that the optimum pH of both the reconstituted and native enzymes was 5.7 to 5.8, while that of the mixture was 6.2. They concluded that the latter pH optimum was the net result of two pH optima: that for carboxylase activity at pH 5.7 to 5.8, and that for reconstitution of the enzyme at pH 6.8. However, their results were obtained by interpolation of pH optimum curves which contained too few points to warrant any conclusions other than that the optimum pH in all cases was about 6.0. Green et al. (34) reported the pH optimum for decarboxylation by his partly purified enzyme to be about 6.0

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2. The Michaelis constant: the substrate concentration giving half maximum reaction velocity. The conditions in this case were: 0.3 ml. of 0.5M citrate buffer, pH 6.0; final volume 3.3 ml.; temperature = 30°C.

Nature of the link between DPT and the apoenzyme.

Speculation regarding the link between the coenzyme and apoenzyme began when Auhagen (23) discovered cocarboxylase activity in yeast and indicated that the coenzyme was attached to a high molecular weight substance in neutral solution (25). Westenbrink et al. (53) reported that an amount of unwashed dried yeast containing 0.2 μgm DPT was about 45 times as active as the same amount of alkaline-washed dried yeast to which 0.2 μgm. DPT had been added. They interpreted this to mean that the native enzyme differed from the reconstituted one, and suggested that the coenzyme was bound to a protein by two groups, one highly dissociated and one highly undissociated, and that during reconstitution only the undissociated link was reformed. However, subsequent work from the same laboratory (54, 42) indicated that the apparent discrepancy between the activity of the native and reconjugated enzymes was due to the destruction of unbound DPT by a phosphatase in yeast, and close scrutiny revealed no difference between the native and reconstituted enzyme with regard to activity per unit of DPT or stability.

The possibility of a link between DPT and apocarboxylase through a sulfhydryl group has not been investigated. However, such a link has been suggested by Baer (55) for pyruvic oxidase, a bacterial enzyme which resembles carboxylase in that it contains DPT and magnesium (56). Baer based this hypothesis on a study of the effect of iodoacetate and other compounds which bind the sulphydryl group (55). When iodoacetate was added to a DPT-free

preparation of pyruvic oxidase, the activity following the addition of DPT and manganese was lowered. As the inhibition could be prevented by incubating the protein with DPT before adding the inhibitor, he concluded that the DPT was attached to an SH group of the protein.

III. PROPERTIES OF YEAST RELEVANT TO THE CARBOXYLASE SYSTEM

Introduction. The reactions in yeast which have been investigated with regard to their influence upon carboxylase are the synthesis and the destruction of DPT. They are considered here because of their bearing on the manometric method of determining DPT.

DPT destruction. Melnick and Field (46) extracted a phosphatase from dried yeast which brought about the quantitative hydrolysis of diphosphothiamine. Westenbrink and Van Dorp (44) observed a similar phosphatase in alkaline-washed yeast. They suggested the competitive inhibition of this phosphatase by structurally related compounds as an explanation of the stimulation of carboxylase by thiamine, monophosphothiamine, and certain pyrimidines which was reported by Ochoa and Peters (6). In support of this view Westenbrink and Van Dorp reported that when the pyrimidyl portion of the thiamine molecule (2-methyl-4-aminopyrimidyl-5-methylaminodihydrochloride) was added to a yeast suspension, the production of thiamine from added DPT was lowered. Westenbrink et al. (57) attempted to ascertain whether monophosphothiamine (MPT)

was an intermediate in the dephosphorylation of DPT. They followed the cocarboxylase disappearance by a manometric method and the disappearance of DPT plus MPT by a thiochrome method. By subtracting the DPT as estimated by the manometric method, from the sum of DPT plus MPT as estimated by the thiochrome method, Westenbrink obtained some measure of the MPT present. Since the results of the thiochrome method were much higher than those of the manometric method, it was concluded that a large amount of MPT was present, as an intermediate in the reaction. In order to determine whether both steps of this reaction were catalyzed by one enzyme, the effect of pH upon the breakdown of DPT and upon the breakdown of MPT was measured. In both cases the optimum was approximately pH 3.7, suggesting that both reactions were catalyzed by one enzyme. At this pH the destruction of DPT was extremely rapid. One hundred micrograms of added DPT were completely broken down in sixteen minutes. In the presence of sufficient thiamine the yeast did not destroy any DPT over a period of thirty minutes. It was further concluded that the DPT attached to its apoenzyme was attacked less readily than free DPT added to the yeast, since incubation at pH 3.7 resulted in a relatively small reduction of DPT content; from 15 μgm. to 9 μgm. in fifteen minutes.

DPT synthesis by whole yeast. The synthesis of cocarboxylase by whole yeast during aeration in phosphate buffer at pH 6.2 with pyrophosphate and vitamin B_1 was reported by Kinnersley

and Peters (30). The cocarboxylase could be precipitated from a hot water extract of the yeast by the addition of lead acetate. Since thiamine is not precipitated by lead acetate (27) it was probably not present in their cocarboxylase. In a later paper (58) pyrophosphate was stated to be unnecessary for the synthesis. When dried yeast was used instead of fresh yeast no synthesis was observed.

DPT synthesis by dried yeast was reported by Euler and Vestin (29). Dried yeast was incubated in 0.02M phosphate solution for 20 minutes with thiamine, ATP and inorganic phosphate, and the mixture was heated and centrifuged to remove the proteins. The supernatant fluid was found to stimulate the carboxylase activity of alkaline-washed yeast to a greater extent than did an extract of controls to which no thiamine had been added. However, since thiamine itself was later shown (43, 6, 47, 14) to stimulate the carboxylase activity of alkaline-washed yeast in the presence of DPT and magnesium, the work of Euler and Vestin cannot be considered as good evidence of DPT synthesis by alkaline-washed yeast.

Westenbrink and his co-workers (59, 60) investigated the DPT synthesis by fresh yeast incubated under various conditions. The synthesis was followed by measuring the DPT contained in aliquots withdrawn at time intervals from the incubating material. For the estimation of DPT a modification of the manometric method was used which was not affected by thiamine. In the first experi-

ment (59) 300 µgm. DPT was added to 4 gm. yeast. Almost all of the added DPT disappeared in the first few seconds, after which there was a gradual but marked resynthesis. In another experiment 4 mgm. thiamine were incubated with 14 gm. yeast in succinate buffer pH 3.7 for 16 hours at 27.5°C. The yeast was then washed three times with water and dried at room temperature. The carboxylase activity was only 80% of that of untreated yeast although analysis of a boiled extract indicated that the yeast incubated with thiamine contained 560 µgm. DPT per gram, whereas the untreated yeast contained only 34 µgm. per gram. From these results it was concluded that the synthesized DPT was inactive as cocarboxylase, but was attached to some kind of protein, as it was not removed by washing.

DPT synthesis by alkaline-washed yeast. Lipschitz, Potter and Elvehjem (38) reported that the addition of thiamine increased the CO_2 production of a mixture of alkaline-washed yeast, pyruvate, and boiled tissue extract. When hexose diphosphate was added with the thiamine, the stimulation of gas production was greater. Iodoacetate, at a concentration of 2.5×10^{-3}M, prevented the increase in CO_2 output almost completely when added with the thiamine, but not when added one half hour after the reaction was started. In the absence of tissue extract no thiamine effect could be observed. Lipschitz et al interpreted these observations to indicate a synthesis of DPT by alkaline-washed yeast plus tissue extract, stimulated by hexose diphosphate and inhibited completely by iodoacetic acid.

However, Ochoa and Peters (6) reported that thiamine, monophosphothiamine, or certain pyrimidines could stimulate the cocarboxylase activity of DPT even in the absence of hexose diphosphate or tissue extracts, and attributed the synthesis reported by Lipschitz et al. (38) to this type of stimulation. Moreover, hexose diphosphate was shown (6) to enhance the CO_2 output in the absence of thiamine if both DPT and cozymase were present, indicating that the hexose diphosphate effect observed by Lipschitz et al. (38) was not due to the phophorylation of thiamine. Ochoa and Peters offered the alternative explanation that "the yeast enzymes form triosephosphate which dismutes with acetaldehyde in the presence of cozymase to give ethyl alcohol and phosphoglyceric acid, so that acetaldehyde is removed and the decarboxylation can proceed at a higher rate. (. . .) When tissue extracts are added they supply the necessary cocarboxylase and cozymase." This explanation would account for the inhibition by iodoacetate of the apparent synthesis of DPT, in view of the extreme sensitivity of the alcohol dehydrogenase of yeast to iodoacetic acid (61). Although Lipschitz et al. (38) discounted any significant inhibition by acetaldehyde in their experiments, very little detail was reported. Their sole statement in this regard was, "In these experiments considerable acetaldehyde is formed which would be expected to have some effect on the manometer readings. We have checked this point by placing semicarbazide at pH 7.0 in the inner cup. The manometric readings were affected but slightly."

Lipton and Elvehjem (62) later replied to the criticism (6) that the DPT synthesis in their experiments (38) could be accounted for by thiamine 'stimulation' of carboxylase. They demonstrated that the degree of thiamine 'stimulation' varied with different kinds of yeast, and did not occur appreciably in the case of the particular yeast they had used in their experiments(38). Furthermore, the DPT synthesis by this yeast had been prevented by iodoacetate, which had no effect upon thiamine stimulation.

DPT synthesis by a purified enzyme preparation. Evidence of DPT synthesis by a yeast carboxylase preparation, purified to the extent that thiamine alone had no activating effect, was reported by Weil-Malherbe (37). He showed that after the removal of DPT from his purified enzyme preparation, cocarboxylase activity could be restored by ATP plus either thiamine or monophosphothiamine. Monophosphothiamine was not as active as thiamine, indicating that it was not an intermediate in the synthesis.

IV. THE MANOMETRIC METHOD FOR DETERMINING DPT

The first attempt to use carboxylase to estimate the cocarboxylase content of tissues was made by Auhagen (24) who added muscle extract to alkaline-washed yeast, but observed no reactivation of carboxylase. Extracts of liver, kidney, heart, and blood contained some cocarboxylase activity (26). Simola (28) used a similar method to investigate the cocarboxylase content of

tissues of vitamin B deficient rats. With the discovery by Lohman and Schuster (2) that a divalent metal as well as cocarboxylase was necessary for the reactivation of alkaline-washed yeast, results of previous workers could be considered only qualitative. As a result of their work showing DPT to be identical with cocarboxylase, pure DPT has been since used as the standard in the estimation of cocarboxylase.

Lipschitz et al. (40) suggested the use of iodoacetic acid to block the phosphorylation of thiamine during the course of the analysis.

Ochoa and Peters (6) were the first to fully describe a workable method for the manometric determination of DPT in animal tissues. The tissues were weighed, chopped, and ground in a mortar with two volumes of distilled water, and the resulting suspension heated for three to five minutes in a boiling water bath. After centrifugation, the resulting supernatant was analyzed for cocarboxylase. An aliquot was placed into the main compartment of a Warburg flask with the alkaline-washed residue from 100 mgm. yeast, 10 µgm. of thiamine and 0.1 mgm. of magnesium. The amount of thiamine used was found to give maximum activation of carboxylase for the quantity of cocarboxylase used. The reaction was started, after ten to twelve minutes equilibration, by tipping in 5.0 mgm. of pyruvic acid from the side bulb. The gas produced in thirty minutes was measured, and in this way, the cocarboxylase activity was

compared with that of solutions containing known amounts of DPT. Since Ochoa and Peters did not believe that synthesis of DPT could occur under these conditions, they did not include iodoacetic acid in the assay. The alkaline-washed yeast was prepared by washing 1.0 gm. of dried baker's yeast three times with 50 ml. of 0.1 M Na_2HPO_4, and once with distilled water, at room temperature. The washed yeast was suspended in 10 ml. of 0.1 M phosphate buffer pH 6.3, and 1 ml. of this suspension employed in each Warburg flask.

The procedure of Ochoa and Peters (6) was modified by Goodhart and Sinclair (39) for use in the estimation of DPT in blood. Oxalated blood (1.0 ml.) was added to a Warburg vessel with 0.1 mgm. magnesium, 3 µgm. manganese, and 0.96 mgm. calcium, all as chloride salts. The calcium chloride was added to precipitate oxalate which, they found, inhibited the system. The flasks were then placed in a boiling water bath for two minutes because "This undoubtedly destroys many substances which would interfere, and does not destroy the cocarboxylase." To the flasks were added 10 µgm. thiamine, 100 mgm. of alkaline-washed yeast, and sufficient HCl to bring the pH to 6.2. The flasks were equilibrated in a nitrogen atmosphere at 28°C and 5 mgm. of pyruvic acid tipped in from the sidearm. The gas production was read at the end of thirty minutes and compared with the gas production in flasks containing standard solutions of DPT in place of blood. The standard flasks were not placed in the boiling water bath, nor did they contain

any calcium. The alkaline-washed yeast was prepared from dried baker's yeast by the same washing procedure as that of Ochoa and Peters (6), then washed twice with acetone and dried in vacuo. They found the activity of this preparation to remain constant for at least three weeks.

In America, Goodhart (41) found it necessary to modify this method, due to the use of a different yeast. He reported that a substance in plasma contributed to the CO_2 production in the absence of pyruvate, and resorted to the practice of washing the cells three times with 0.8% NaCl and suspending them to the original volume in phosphate buffer pH 6.2. This was based upon the observation that all of the DPT in the blood was to be found within the cells.

Westenbrink et al. (63) introduced the procedure of precipitating the protein and extracting the DPT from the blood for analysis. As this step lowered the DPT concentration, the procedure for measuring cocarboxylase activity was modified in an attempt to increase the sensitivity. The chief alteration was the separate execution of the resynthesis of carboxylase and the decarboxylation of pyruvic acid so that these processes could be carried out at their respective pH optima (42). In this way, Westenbrink et al. (63) were able to obtain a gas production of 164 µl. in thirty minutes with 0.200 µgm. DPT, while the blank during the same period of time produced 19 µl. The difference in

volume they considered to be sufficient to enable a sensitive estimation of DPT in blood extracts. The details of the method are as follows:

In order to liberate the DPT, a sample of blood was acidified with HCl to pH 3.0 and placed in a boiling water bath for one and one half minutes. Sufficient KOH solution was then added to precipitate the denatured proteins, and centrifugation yielded a clear supernatant extract. The cocarboxylase activity of this extract was compared with that of solutions containing known amounts of synthetic DPT. Samples of the blood extract or standards were incubated at pH 6.8 for fifteen minutes at 27.5°C. with alkaline-washed yeast in the presence of thiamine and manganese to permit resynthesis of the holoenzyme. The yeast residue containing the reconstituted carboxylase was then spun down in a centrifuge and resuspended in 0.1 M acetate buffer pH 5.6. An aliquot of this suspension was transferred to the main compartment of a 15 ml. Warburg flask, equilibrated at 27.5°C, and 5 mgm. of sodium pyruvate was tipped in. The total volume of the reaction mixture was 1.7 ml. Carbon dioxide production at the end of thirty minutes was used for comparison of the cocarboxylase activity of the extracts with that of the standard solutions.

Blanchaer and Cohen (64) reported that the manometric method as applied to blood was unreliable because of a marked upward drift[3] of the results during the period of measurement. In this work, blood extracts were prepared by the method of Westenbrink

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3 The word 'drift' signifies the change in the results of the estimation dependent upon the interval at which the gas production was measured.

et al. (63) but their practice of carrying out reconstitution of carboxylase separately from the measurement of carboxylase activity was not adopted. The drift in cocarboxylase activity of the blood extracts was prevented by the presence of 10^{-3}M iodoacetate.

The appraisals by various authors of their own modifications of the manometric method for the estimation of DPT in animal tissues. In only two cases have the authors given some account of the accuracy and sensitivity of their methods. Ochoa and Peters (6) report one experiment in which 0.75 and 3.28 μgm. of DPT were added respectively to two aliquots of a sample of minced normal pigeon's muscle and the recoveries were found to be 141% and 93% respectively. Three similar experiments with liver were reported, from which were obtained the following results:

Experiment no.	μgm. DPT added	% Recovery
1	1.30	167
	5.00	94
2	5.00	83
	2.50	117
3	4.00	99

Westenbrink et al. (61) stated that determinations were carried out in duplicate but gave no figures regarding the number of estimations. They reported that duplicate estimations of the DPT in a sample of blood agree within "0.6%". Determinations

carried out upon the same blood samples at different times were found to "often agree within 0.6%, but sometimes also the divergence amounts to 1.2%". In one experiment recovery values for 0.1, 0.2, and 0.4 μgm. of DPT, added to 9.0 ml. of blood, were reported to be 70%, 100% and 100% respectively.

CHAPTER III

METHODS

The same general procedure has been used by most workers (24, 28, 40, 6, 39, 64) for estimating DPT manometrically, although variations have been introduced from time to time to avoid difficulties resulting from interfering substances, or to increase the sensitivity and precision of the method. Since authors of the various modifications have been somewhat vague in appraising their methods, no one modification was demonstrably superior to the rest. In the method adopted, therefore, the most reasonable modifications were chosen, especially those of Ochoa and Peters (6) and of Westenbrink et al. (64).

I. PRINCIPLE OF THE MANOMETRIC METHOD FOR ESTIMATING DPT IN ANIMAL TISSUES

Diphosphothiamine (DPT) was estimated by its cocarboxylase activity in catalyzing the reaction:

$$CH_3\text{-}CO\text{-}COOH \xrightarrow[\text{yeast apocarboxylase}]{\text{DPT}} CH_3\text{-}CHO + CO_2$$

To determine DPT, a water extract of the tissue was made and added to an excess of yeast apocarboxylase in the presence of magnesium and manganese ions, which are activators of the system. In order to inhibit the phosphatase in yeast which catalyzes the hydrolysis of DPT, large amounts of thiamine were included. Under these conditions DPT combines with the apocarboxylase, yielding the holoenzyme

which catalyzes the decarboxylation of pyruvic acid.

$$\text{Apocarboxylase} + \text{DPT} \longrightarrow \text{Carboxylase (holoenzyme)}$$

In the presence of excess apoenzyme and activating ions, the quantity of carbon dioxide released in the decarboxylation of pyruvic acid is limited by the amount of DPT present. The DPT may be measured by comparing the cocarboxylase activity of the tissue extract with that of known amounts of DPT.

II. DETAILS OF THE MANOMETRIC METHOD FOR ESTIMATING DPT

Preparation of tissue extract. To obtain tissue for DPT estimations, a rat was killed by decapitation, and the tissue to be analyzed was excised and rolled on slightly moistened filter paper to remove adherent blood. Pieces of tissue of about 300 mgms. were weighed to the nearest milligram on a torsion balance and the weight was designated by t. Each sample was homogenized with (0.28 x t) ml. of 0.14 N HCl, by the method of Potter and Elvehjem (65). The homogenate was then heated in a boiling water bath to release combined DPT and to denature the protein matter. The pH was brought to 6.4 by adding (0.0133 x t) ml. of 0.4 M phosphate buffer pH 6.2, and (0.120 x t) ml. of KOH solution, with stirring after each addition. For this procedure 0.17% KOH was made up roughly and the concentration adjusted to give good protein precipitation. The tubes were stoppered with rubber bungs, were inverted several times, and the contents were centrifuged to separate the precipitated protein. The extract was stored at -15°C

in small portions so that aliquots could be used for subsequent experiments without thawing the remainder. It was assumed that one milliliter of the final extract contained the DPT from 0.01845 gm. of tissue in 0.1 M phosphate buffer. Tissue extracts were prepared as rapidly as possible to avoid any loss of DPT which might occur by autolysis.

Effect of storage of tissue extract upon DPT content. As it was sometime necessary to store tissue extracts for some time before measuring the DPT content, information regarding the effect of storage at - 15°C was obtained. Two experiments were performed in each of which a tissue extract was prepared and stored in small portions at - 15°C. At intervals of one or two days, a portion was thawed and analyzed for DPT. The data for these two experiments, presented in Table I, show that storage in this manner had no effect upon the DPT content of liver extract.

Preparation of standards. Merck's synthetic cocarboxylase was used as a standard in all estimations. A standard solution was made by dissolving 10.0 mgm. of crystalline compound and diluting to 500 ml. with distilled water. To one milliliter of this solution was added 0.333 ml. of 0.4 M phosphate buffer pH 6.2, and the volume was brought to 100 ml. with 0.1 M phosphate buffer pH 6.4. The final solution contained 0.200 µg. DPT/ml. in 0.1 molar phosphate buffer pH 6.4. From this standard, solutions were prepared containing 0.050, 0.100, and 0.150 µg. DPT/ml. in 0.1 M phosphate buffer, pH 6.4. All standard solutions were prepared daily.

TABLE I

EFFECT OF STORAGE UPON DPT

CONTENT* OF RAT LIVER EXTRACT

Experiment no.	Days of Storage	Gm. tissue analyzed	μgm. DPT per gm. tissue
1	1	0.00614	13.2
	2	0.00790	12.9
	3	0.00691	13.6
	4	0.00614	13.2
	6	0.00614	13.2
	8	0.00614	12.7
2	1	0.0079	9.9
	2	0.0079	9.9
	14	0.0079	10.4

* These values are based upon the gas volumes at 30 minutes, and are calculated as outlined on page 31.

Preparation of salts solution. A solution of the magnesium and manganese ions used as activators of the system was prepared as follows: 364 mgm. $MnCl_2 \cdot 4H_2O$ and 835 mgm. $MgCl_2 \cdot 6H_2O$ were dissolved in water, adjusted to pH 6.2, and the volume brought to 100 ml. with water. One milliliter of this solution contained 1.0 mg. of magnesium and 1.0 of manganese ions.

Preparation of Sodium pyruvate. Pyruvic acid was prepared by the pyrolysis of tartaric acid (66). The crude product was fractionally distilled at a pressure of 25 mm. Hg, and the fraction from 75°C. to 80°C. was collected. For use as substrate, the free acid was converted to its sodium salt (67).

Preparation of dried yeast. Dried brewer's bottom-yeast, treated to remove native DPT, was used as the source of apocarboxylase. It was prepared as follows: Fresh brewer's bottom-yeast was filtered by suction and fan-dried in shallow pans at room temperature. When stored over calcium chloride in a vacuum desiccator at 4 - 8°C., the dried yeast maintained its cocarboxylase activity for at least one year. The removal of native DPT from this preparation was delayed until immediately before the estimation, since the final product was only moderately stable (39).

Removal of DPT from dried yeast. The DPT was removed from the dried yeast in the following manner: Dried yeast (1.2 gm.) was rapidly suspended in 20 ml. of 0.1 M phosphate

buffer pH 9.2 in a 90 ml. centrifuge tube by means of a stainless steel or glass pestle of the Potter-Elvehjem type (65). An additional 70 ml. of the same buffer was added and the suspension stirred vigorously. The yeast was then separated by centrifuging at 3500 r.p.m. in a #2 International centrifuge. This procedure was repeated three more times: once with another portion of the same phosphate buffer, once with water, and finally with 0.1 M phosphate buffer pH 6.4. To the centrifugate was added:

1. 2 ml. of 0.1 M phosphate buffer, pH 6.4, containing 2 mg. thiamine.
2. 2 ml. of the 0.1 M phosphate buffer pH 6.4, containing 2 mg. of manganese and 2 mg. of magnesium ions.
3. Sufficient 0.1 M phosphate buffer pH 6.4 to bring the final volume to 12 ml.

The residue was then resuspended by homogenization for about three minutes. The final pH was 6.4. The procedure is summarized in Table II.

Use of glass redistilled water. Since calcium interferes with the estimation (39, 50) all water used in washing the yeast and preparing reagents was redistilled in glass.

Measurement of carbon dioxide evolution. The carbon dioxide produced by the decarboxylation of sodium pyruvate was measured manometrically in the conventional Warburg apparatus.

The Warburg apparatus comprises essentially an open end manometer to which a reaction flask can be attached by means

TABLE II

PROCEDURE FOR THE REMOVAL OF DPT FROM DRIED YEAST

Step no.	Wash medium	*Time of washing	Time of centrifugation
1.	0.1 M phosphate buffer pH 9.2	5½ mins.	2½ mins
2.	"	3 mins.	2½ mins.
3.	Glass redistilled water	2½ mins.	2½ mins.
4.	0.1 M phosphate buffer pH 6.4	3 mins.	2½ mins.
5.	resuspension in 0.1 M phosphate buffer pH 6.4		

* Time from beginning of suspension to beginning of centrifugation.

of a ground glass joint. The flask may have side arms from which reactants can be added to the main compartment. By means of a three way stopcock located on the manometer the system may be opened or closed to the air. The manometer contains Brodie's solution, a fluid of known density. By pressure of a screw clamp on a rubber reservoir, the level of the fluid in the manometer arms may be adjusted in order to maintain a constant volume of gas in the system. When the volume and temperature are held constant, changes in the amount of gas present in the system may be measured by changes in pressure. The pressure is read in millimeters of Brodie's solution and converted by an appropriate factor to the amount of gas released (microliters CO_2 at N.T.P.). The conversion factor, which is calculated individually for each flask and its respective manometer, takes into account the volume of the gas space, the temperature and the solubility of carbon dioxide. In deriving this factor it is assumed that the amounts of gas released other than CO_2 are negligible. The flask, sealed with lanolin to the manometer, may be immersed in a constant temperature water bath and shaken to promote rapid gas exchange between the fluid and the gas above it. Fourteen such manometers may be shaken simultaneously. Of these, two are required to measure changes due to variations in temperature and pressure, and are referred to as thermobarometers. The apparatus and techniques of manometric measurement are described in detail by Umbreit et al. (68).

Determination of cocarboxylase activity. The cocarboxylase activity of the tissue extract was measured by comparison

with the activity of 0.00, 0.050, 0.100, 0.150, and 0.200 μg. DPT.

The details are as follows: Into the main compartment of each Warburg flask was placed 1.00 ml. of tissue extract or standard DPT solution. Forty milligrams of sodium pyruvate in 0.3 ml. of 0.1 M phosphate buffer pH 6.4 were pipetted into each side arm, which was then sealed. The yeast apocarboxylase was prepared and 1.0 ml. of the final suspension was added to the main compartment of each vessel. The vessels were then attached to the manometers, placed in the constant temperature bath (27.5°C), and shaken for ten minutes to permit gaseous equilibration and reconstitution of the holoenzyme. The substrate was then tipped from the sacs into the main compartments and the cocks were closed. Gas production was usually measured at ten minute intervals for one hour. Addition of the yeast to the Warburg vessels, tipping of the substrate, closing of the manometer cocks and reading of the manometers, were all carried out at half minute intervals, thereby enabling one person to perform the entire operation.

Standard curves were constructed on the basis of gas production in the flasks containing standard amounts of DPT. The apparent DPT content of each flask containing tissue extract was read directly from the standard curves.

Recovery estimations. In recovery experiments the DPT content of 1.00 ml. of tissue extract fortified with an added

known amount of DPT, was compared with that of 1.00 ml. of tissue extract alone. Details of the procedure are as follows:

An aliquot of the tissue extract was diluted with an equal volume of the standard DPT solution containing 0.200 μgm. DPT. One milliliter of this mixture (A) contained 0.100 μgm. DPT, plus an unknown amount supplied by the extract. A second aliquot of the extract was mixed with an equal volume of 0.1 M phosphate buffer pH 6.4. One milliliter of this mixture (B) contained only the DPT present in the extract. The DPT concentration in A and B were estimated and the per cent recovery was calculated from the equation:

$$\% \text{ recovery} = \frac{\mu\text{gm. DPT in 1 ml. A} - \mu\text{gm. DPT in 1 ml. B}}{0.100} \times 100.$$

The 0.200 μgm./ml. DPT solution used in the recovery experiments was also used in preparing the series of standards in each experiment in order to eliminate the effect upon recovery values of impurities and inaccuracies in weighing. Complete (100%) recoveries were considered to imply an absence of extraneous factors influencing the estimation, or, alternatively, a balance between interfering factors acting in opposing manners.

Variations from the standard technique in early experiments. In early experiments the procedure differed from that described above in the following respects:

1. During the treatment used in removing native DPT, the dried yeast was suspended in the first portion of alkaline buffer

by means of a mortar and pestle and in subsequent steps by stirring. The use of a homogenizing pestle was later adopted for this procedure to obtain rapid and even suspension of the yeast.

2. The residue from 50 mgm. of dried yeast was used in each flask, a quantity which has been used in the determination of DPT in blood (64). The quantity of yeast residue was doubled when it became apparent that 50 mgm. provided insufficient apoenzyme to give maximum activity with 0.2 μgm. DPT (Table III, Experiment 1). The quantity of carboxylase obtained from 100 mgm. dried yeast gave maximum activity with 0.2 μgm. DPT (Table III, Experiment 2), so that small errors in pipetting the viscous yeast suspension had little effect upon the gas production.

3. Originally, only 5 mgm. of sodium pyruvate was employed in each flask, as indicated by previous investigators. However, this resulted in a final concentration of only M/50, whereas Green et al. reported that the substrate concentration for maximum velocity was about M/6. In order to insure that the sodium pyruvate concentration was at no time a limiting factor in the determination, it was increased to about M/6 by employing 40 to 45 milligrams. This quantity gave maximum velocity with 0.200 μgm. DPT and the washed residue from 100 mgm. dried yeast (Table IV).

The incorporation of these changes not only lessened the probability of variations due to inaccuracies in measuring the substrate

TABLE III

EFFECT OF APOENZYME CONCENTRATION UPON CARBOXYLASE ACTIVITY

		Experiment #1*			Experiment #2**	
mgm. dried yeast		50	200	100	150	200
µl. CO_2 produced in 30 min.	0.200 µgm. DPT added	112	222	210	234	244
	0 µgm. DPT added (blank)	13	29	24	35	55
	Due to added DPT	99	193	186	199	189

* Each flask contained 40 mgm. Na pyruvate.

** Each flask contained 50 mgm. Na pyruvate.

TABLE IV

EFFECT OF SUBSTRATE CONCENTRATION

UPON CARBOXYLASE ACTIVITY

Each flask contained 0.200 μgm. DPT and the alkaline-washed residue from 100 mgm. dried yeast.

Mgm. Na pyruvate	25	33.3	40	50
Microliters CO_2 in 30 mins.	232	228	231	225

and apoenzyme, but resulted in greater carboxylase activity, and therefore greater sensitivity.

Data of an illustrative recovery experiment. The data of a typical recovery experiment is presented to illustrate the calculations of results (Table V) from the gas volumes at successive time intervals (Table VI). It can be seen that the volume of gas produced in 30 minutes by the 0.200 μgm. standard was much greater than that reported by Westenbrink et al. (63)[1], whereas the blank was about the same. The standard curves are shown in Fig. 1.

Statistical analyses. Where warranted the results were analyzed statistically by either the t-test or the Analysis of Variance (69). The standards adopted as a guide for significance were:

$P < 0.05$ .....significant.

$P < 0.01$ .....highly significant.

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[1] See Chapter II, page 19.

TABLE V

RESULTS OF AN ILLUSTRATIVE RECOVERY EXPERIMENT

Tissue gms/flask	µgm. DPT added	µgm. DPT found after intervals of:				
		20 min.	30 min.	40 min.	50 min.	58 min.
0.0079	0	0.074	0.077	0.080	0.081	0.081
"	0	0.078	0.078	0.078	0.082	0.087
Mean		0.076	0.078	0.079	0.082	0.084
Mean µgm. DPT per gm. tissue		9.64	9.88	10.0	10.4	10.6
0.0079	0.100	0.158	0.160	0.165	0.168	0.167
"	0.100	0.152	0.154	0.162	0.162	0.163
Mean		0.155	0.157	0.164	0.165	0.165
Recovery (%)		79	79	85	83	81

TABLE VI

GAS PRODUCTION IN A TYPICAL EXPERIMENT

DPT μgm.	Tissue (gm.)	Microliters CO_2 at:				
		20 min.	30 min.	40 min.	50 min.	58 min.
0.00	---	14	21	25	36	40
0.050	---	59	84	107	129	148
0.100	---	98	138	173	204	230
0.150	---	131	182	225	265	296
0.200	---	158	219	269	313	346
0.000	0.0079	79	114	149	177	201
0.100	"	136	190	239	282	314
0.000	0.0079	82	116	146	179	210
0.100	"	132	185	235	277	310

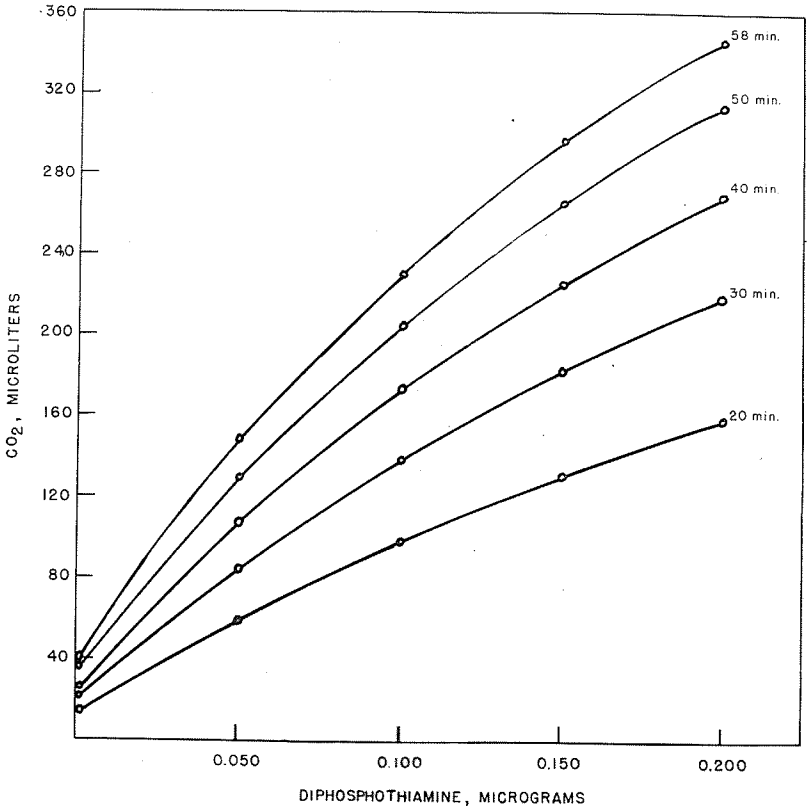

FIGURE 1.

STANDARD CURVES IN A TYPICAL ESTIMATION

CHAPTER IV

RESULTS OF EXPERIMENTS BEARING ON THE APPRAISAL OF THE METHOD

In establishing criteria for the assessment of a method of analysis based upon biological activity, the following considerations were felt to be relevant:

I. The accuracy of the method. This may be influenced by extraneous materials in the tissue to be analyzed.

II. The precision of the method.

The results of experiments bearing on the appraisal of the method are presented under these two headings.

I. THE ACCURACY OF THE METHOD

The influence of material in the tissue extract upon accuracy might be expected to manifest itself in at least one of the following effects:

1. A variation of the results dependent upon the duration of the estimation. As has been described in Chapter III, results of an estimation may be calculated from the gas produced during any time interval after the addition of pyruvate to the reaction mixture. The length of the interval would be expected to affect the results only if a progressive change were occuring in the unknown and not in the standard flasks.

2. A variation of the results dependent upon the concentration of the tissue extract used.

3. Recoveries of more or less than 100% of DPT added to the tissue or tissue extracts.

Each of these effects was investigated and the results are reported separately under the appropriate headings.

1. Effect of the Duration of the Estimation Upon the Results

Forty-five experiments were carried out to determine how the results varied with the length of time of the estimation. Estimations were carried out in which the gas volumes were recorded at successive time intervals after the beginning of the reaction. The initial reading was made 20 or 25 minutes after the addition of pyruvate. The times at which the final readings were made varied from 35 to 80 minutes after the addition of the pyruvate. In 39 estimations the values for DPT content of the tissue extracts increased during the experiment; in three estimations they decreased; and in three estimations there was no change. For convenience, the change in DPT values during the course of the experiment will be referred to hereafter as 'drift'.

During the course of the investigation certain changes in technique were made.[1] Since the method as used in the earlier experiments was felt to be the less reliable, some of the earlier

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[1] See Variations from the standard technique in early experiments, Chapter III, page, 32.

work was repeated under the altered experimental conditions. The detailed results of the earlier work are reported separately.

The occurrence of drift in early experiments. Of the 45 DPT estimations in which drift was studied, 23 were carried out under the conditions prevailing in the earlier experiments.[1] The increase in DPT over the periods from 20 to 30 minutes, 20 to 40 minutes, and 20 to 50 minutes, are shown in Table VII, expressed in per cent of the 20 minute value. The average drifts over these periods were + 2.3, + 3.4, and + 6.0 respectively. Statistical analysis indicated that these increases were highly significant.

The occurrence of drift in later experiments. In 22 later experiments larger quantities of apoenzyme and substrate were used.[1] In 20 estimations, a significant mean drift of 3.85% occurred between 20 and 30 minutes of gas production (Table VIII). The drift was very large in those experiments which were continued for a long period of time.

2. Effect of Tissue Extract Concentration Upon the Results of the Estimation

The dependence of the results upon the duration of the estimation suggested the presence of an interfering substance in the tissue extracts. Further evidence in this regard was sought by a study of the relation between the results of the estimation and the concentration of the tissue extract.

TABLE VII

DRIFT IN EARLY EXPERIMENTS

Gm. Tissue Analyzed	Increase in DPT as % of the 20 minute value at:		
	30 min.	40 min.	50 min.
.0369			9.4
.0369			11.1
.0246			4.9
.0246			6.4
.0184			2.6
.0184			5.6
.0369	6.7	8.4	
.0246	2.1	2.1	
.0246	2.9	1.0	
.0184	3.8	2.5	
.0184	1.4	1.4	
.0369	2.8	5.8	
.0369	4.7	6.5	
.0369	2.9	4.3	
.0211	2.0	4.8	
.0211	0.7	4.6	
.0211	2.0	4.8	
.0211	5.6	7.0	10.5
.0211	0.0		
.0211	2.1	+1.4	3.4
.0369	0.0	+0.4	3.9
.0369	-0.7	-0.4	3.5
.0369	0.0	0.4	4.6
Mean	+2.3%	+3.4%	+6.0%
Standard deviation of the mean.....	0.4990	0.6713	0.9059
t	4.59	5.12	6.61
P ..... (for significance of the mean)	< 0.01	< 0.01	< 0.01

TABLE VIII

DRIFT IN LATER EXPERIMENTS

Grams tissue analyzed	µgm. DPT Found at 20 min	30 min	% increase	µgm. DPT Found at 60 min	70 min	82 min	Increase as % of 20 min. value
0.0079	0.077	0.076	-1.3				
0.0079	0.081	0.080	-1.2				
0.0158	0.154	0.155	0.6				
0.0158	0.153	0.153	0.0				
0.0079	0.078	0.080	2.6				
0.0079	0.082	0.083	1.2				
0.0158	0.153	0.173	13.1				
0.0158	0.158	0.163	3.2				
0.0079	0.093	0.091	2.2				
0.0079	0.091	0.091	0.0				
0.0158	0.157	0.163	3.8				
0.0158	0.157	0.158	0.6				
0.0079	0.074	0.077	4.1	0.81			9
0.0079	0.078	0.078	0.0	0.87			11
0.0158	0.148	0.153	3.4	0.163			10
0.0158	0.143	0.150	4.9	0.156			9
0.0164	0.145				0.26*		79
0.0082	0.071				0.085		20
0.0079	0.095	0.096	1.1			0.3*	>200
0.0079	0.095	0.098	3.2			0.3*	>200
0.0158	0.195	0.253*	30.0			0.5*	>150
0.0158	0.200	0.220*	10.0			0.5*	>150

Mean.....	3.85
Standard deviation of the mean.....	1.606
t.....	2.4
P.....(for significance of the mean).....	<0.05

* Obtained by extrapolation of the standard curve, since the gas production exceeded the range of the standard series. The extrapolation was done by extending the standard curve as a straight line, so that the DPT values marked by asterisks are lower than the actual results.

In each of eleven experiments, a sample of liver extract was diluted with an equal volume of 0.1 M phosphate buffer, pH 6.4, and simultaneous estimations of DPT were carried out on aliquots of the diluted and undiluted extract. Table IX contains the data of these experiments. The results are expressed in micrograms DPT per gram of tissue to facilitate comparison. The DPT values, calculated from the gas production at 20 minutes, were higher for the lower concentrations of extract. Although the difference appeared to be slight, statistical analysis indicated that it was significant.

3. Recoveries of DPT Added to the Tissue Extracts.

Recovery experiments constituted the third method of investigating the accuracy of the estimation.

Recovery experiments with liver extract. Table X contains the recovery values, calculated from thirty minute readings, for eighteen experiments with rat liver extract. All estimations were made in duplicate and the recovery values were calculated from the means. The mean recovery was 89.8%, which differed from a mean of 100% by 5.02 times the standard deviation of the mean, a highly significant difference.

Recovery experiments with kidney extracts. Six recovery experiments were carried out in which extracts of rat kidney were used.(Table XI). The mean recovery of 92.5% differed significantly from a mean of 100%, although it was slightly higher than that obtained with liver extracts.

II. PRECISION OF THE METHOD

Precision was the second factor considered in appraising the method, and was investigated by a study of the deviations from the mean of duplicate estimations. In 49 estimations (Table XII) the mean deviation from the mean of duplicates was 1.65%, with a standard deviation of 1.55%. These figures apply to the estimation of DPT in duplicate samples of a tissue extract, and do not take into account variations incurred in preparing the extract. Two tissue extracts could be considered to differ significantly in DPT content if the mean deviation from their mean were more than $\pm$ 4.69%.[2]

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[2] i.e. the mean $\pm$ (1.96 x standard deviation)

TABLE IX

EFFECT OF TISSUE CONCENTRATION UPON DPT VALUES

Gm. tissue analyzed in 'A' (x)	μgm. DPT found per gram tissue* at 20 minutes	
	A (x gm. tissue analyzed)	B (2x gm. tissue analyzed)
0.00790	9.95	9.71
0.00790	9.62	9.21
0.00790	10.13	9.86
0.00790	11.60	9.95
0.00790	12.02	12.51
0.00395	10.76	10.50
0.00790	8.68	9.00
0.00614	12.61	12.39
0.00691	13.48	12.72
0.00614	13.32	12.09
0.00922	9.82	9.63
Mean	11.1	10.7

$t = 2.26$

P (for 'A' vs. 'B') < 0.05

* All values shown are the means of duplicate estimations.

TABLE X

DATA OF RECOVERY EXPERIMENTS WITH LIVER EXTRACT

gm. tissue analyzed	µgm. DPT added	Recovery* (%)
0.00790	0.100	84
0.00790	0.100	79
0.00790	0.100	83
0.00790	0.100	85
0.00790	0.100	90
0.00790	0.100	71
0.00790	0.100	94
0.00395	0.050	92
0.00614	0.066	101
0.00790	0.100	88
0.00614	0.066	102
0.00790	0.100	105
0.00691	0.100	80
0.00614	0.066	96
0.00614	0.100	89
0.00614	0.066	90
0.00614	0.100	83
0.00790	0.100	94

Mean.....	89.8%
Standard deviation from the mean.....	2.035
t	5.02
P (For significance of the difference of this mean from a mean of 100%).....	< 0.01

* The mean of duplicate experiments.

TABLE XI

RESULTS OF RECOVERY EXPERIMENTS WITH KIDNEY EXTRACTS

No. of estimations	mgm. tissue	µgm. DPT added	% Recovery
2	0.00923	0.100	88
1	0.00895	0.133	89
1	0.00447	0.133	97
1	0.00246	0.133	97
1	0.0123	0.133	88
1	0.00615	0.133	96

Mean	92.5
Standard deviation of the mean	1.850
t	4.05
P (for the significance of the difference of the mean from a mean of 100%)	< 0.01

TABLE XII

MEAN DEVIATIONS OF DUPLICATES FROM THE MEAN

Gm. tissue analysed	γgm. DPT added	Mean deviation of duplicates from the mean (%)
0.0079	0	2.56
0.0079	0.100	4.63
0.0158	0	0.65
0.0079	0	0.64
0.0079	0	1.91
0.0158	0	2.69
0.0079	0	1.83
0.0079	0.100	1.16
0.0158	0	1.81
0.0079	0	0.00
0.0079	0.100	0.31
0.0158	0	1.56
0.0079	0	1.03
0.0079	0.100	4.71
0.0158	0	1.26
0.0079	0.100	2.14
0.0158	0	1.01
0.0040	0	5.50
0.0040	0.050	0.55
0.0079	0	1.11
0.0061	0	4.48
0.0061	0.067	2.24
0.0079	0	1.32
0.0079	0.100	1.22
0.0158	0	1.30
0.0061	0	0.00
0.0061	0.067	0.00
0.0092	0	1.64
0.0079	0	4.39
0.0069	0	1.06
0.0069	0.100	0.00
0.0138	0	2.52
0.0061	0.067	1.43
0.0061	0	2.88
0.0061	0	0.00
0.0061	0.100	1.18
0.0092	0	0.44
0.0061	0	1.94
0.0061	0.067	0.00
0.0061	0.100	0.00
0.0079	0.100	0.552
0.0079	0	2.68
0.0092	0	6.38
0.0092	0.100	0.00
0.0184	0	1.67
0.0074	0	1.81
0.0092	0	0.72
0.0092	0.100	0.95
0.0184	0	1.10
Mean.....		1.65
Standard		1.55

CHAPTER V

FACTORS AFFECTING THE DEFECTS IN THE METHOD

The results of the previous chapter indicated the following defects in the manometric method of estimating DPT in animal tissues:

I. Drift.

II. Low recoveries.

III. Variation of results dependent upon the concentration of tissue extract.

In this chapter are presented the results of a study of the effects of certain factors upon the degree of drift and low recovery values. This work was undertaken in an effort to eliminate or explain these defects.

I. DRIFT

The factors studied in relation to drift were:

1. The effect of iodoacetate upon drift.
2. The effect of the concentration of tissue extract upon drift.
3. The effect of DPT concentration upon drift.
4. The relation of acetaldehyde to drift.

1. The Effect of Iodoacetate upon Drift.

During the investigation of the effect of iodoacetate upon drift, the technique was altered to some extent. Since the

method as used in the earlier experiments was felt to be the less reliable, some of the earlier work was repeated under the altered experimental conditions. The results of the earlier work are reported separately.

Effect of iodoacetate upon drift in earlier experiments. A drift similar to that observed in the present investigation has been reported to occur during the estimation of DPT in blood (64), and to have been prevented by the presence of 10^{-3} M iodoacetate. An estimation of DPT in a rat liver extract was therefore carried out, in which sufficient iodoacetate was added to the alkaline-washed yeast to give a final concentration of 10^{-3} M in each flask. This concentration of iodoacetate did not appear to prevent drift, for in three experiments, the drift between 20 and 40 minutes ranged from 5.9% to 10.7% (Table XIII).

The iodoacetate concentration was increased to 5×10^{-3} M in a series of 22 estimations of DPT. In these experiments the iodoacetate was added to all the solutions involved, rather than to the alkaline-washed yeast alone, so that the concentration in contact with the apoenzyme was at no time greater than 5×10^{-3} M. For all estimations a single tissue extract was prepared containing 5×10^{-3} M iodoacetate and stored in small portions at -15°C. Samples of this tissue extract were used throughout this experiment to eliminate variability due to differences in source and preparation of the material. It may be seen from Table XIV that

TABLE XIII

DRIFT IN THE PRESENCE OF 10^{-3} M

IODOACETATE

Tissue analyzed (gm.)	µgm. DPT found at:		Drift as % of 20 min. value
	20 min	40 min	
.0211	.152	.163	7.2
"	.131	.145	10.7
"	.152	.161	5.9

TABLE XIV

DRIFT IN THE PRESENCE OF 5×10^{-3} M IODOACETATE

Gm. tissue per flask	Increase in per cent of the 20 min values at:		
	30 min.	40 min.	50 min.
.0211	0.9	4.4	1.8
.0211	0.00	3.0	3.0
.0369	-3.3	-1.7	-3.3
.0369	-1.1	+0.5	-2.2
.0211	-5.1	-0.9	-5.9
.0211	-3.7	-1.9	-2.8
.0211	-3.4	-0.9	-6.7
.0369	3.2	+5.8	+4.2
.0246	6.4	8.0	8.0
.0185	4.9	2.4	3.7
.0246	5.9	4.2	7.6
.0185	13.4	9.8	12.9
.0211	0.0	1.3	0.9
.0211	3.8	3.8	0.0
.0211	-1.7	0.0	-2.6
.0222	5.2	2.6	3.4
.0246	5.5	6.3	7.1
.0222	6.1	3.5	4.3
.0246	11.9	7.6	8.5
.0205	0.9	-1.8	-1.8
.0205	3.3	0.8	+1.7
.0205	13.5	10.6	12.5
Mean	+3.03%	+3.06%	+2.47%
t	2.6568	3.829	2.1244
P*	>0.01 <0.02	<0.01	>0.02 <0.05

* For significance of the mean.

under these conditions a significant drift of + 3.03% occurred between 20 and 30 minutes. Unlike the experiments carried out in the absence of iodoacetate, no further drift occurred between 30 and 50 minutes (Fig. 2). Although the mean per cent drift at 50 minutes was lower in the presence of 5×10^{-3} M iodoacetate than in its absence[2], the difference was not statistically significant.[3]

The effect of iodoacetate upon drift in later experiments. Since the results of early experiments indicated that 5×10^{-3} M iodoacetate did not fully prevent drift, an experiment was carried out in which the iodoacetate concentration was doubled to 10^{-2} M. The iodoacetate was added to the reaction mixture along with the sodium pyruvate, since certain undesirable effects of iodoacetate were avoided in this way.[4] The cocarboxylase activity of a tissue extract and that of a standard solution of DPT were measured in the presence of iodoacetate added in one of two ways: placed into the centre compartment before the addition of yeast, or tipped into the centre compartment from a sidearm with the sodium pyruvate. The tissue extract was prepared so as to have approximately the same activity as the standard, 1 ml. of which contained 0.200 µgm. DPT. Under these conditions it was felt that the change in the ratio

$$\frac{CO_2 \text{ evolved in the flask with tissue extract.....(T)}}{CO_2 \text{ evolved in the flask with standard.....(S)}}$$

was a measure of drift.

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2. See Table VII.

3. By the Analysis of Variance, $P > 0.05$.

4. See Chapter VI. The effect of Iodoacetate upon Carboxylase.

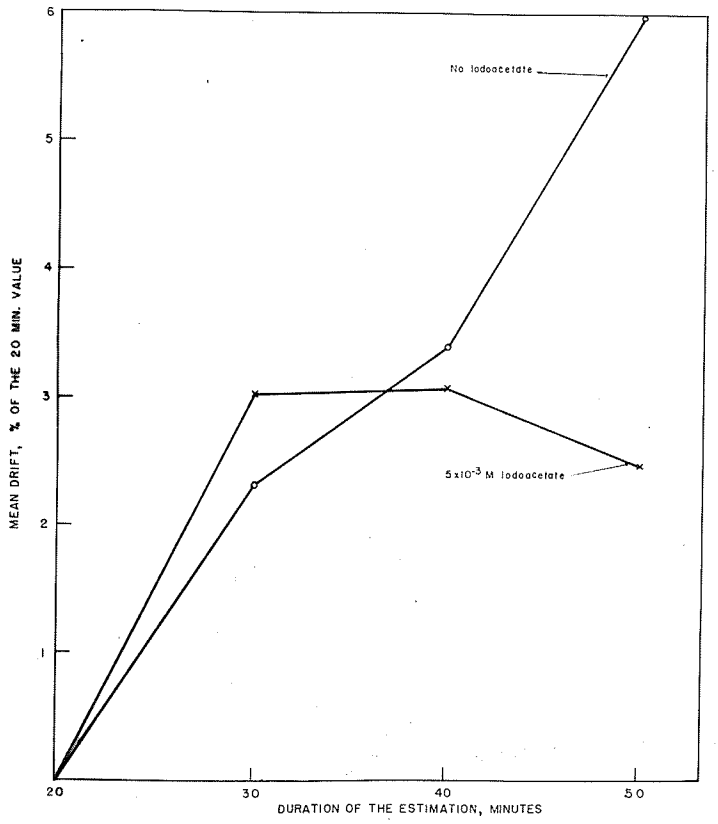

FIGURE 2.

EFFECT OF IODOACETATE

ON DRIFT

From the results reported in Table XV, it may be seen that the increase in the T/S ratio observed in the control experiment did not occur in the presence of 10^{-2} M iodoacetate. Furthermore, the T/S ratio was the same whether the iodoacetate was added before the period of reconstitution of carboxylase, or with the sodium pyruvate. Any change, therefore, which occurred before the addition of sodium pyruvate, was either negligible or not affected by iodoacetate.

The data of the above experiment indicated that 10^{-2} M iodoacetate, added with the pyruvate, would suppress drift. To obtain a quantitative picture of the drift under these conditions, 14 estimations were carried out, the results of which are shown in Table XVI. The average drift between 20 and 30 minutes, expressed in per cent of the 20 minute value, was 1.4%. Statistical analysis did not indicate this degree of drift to be significant. With two exceptions, no large increases in DPT occurred during the estimations, even over a period of 85 minutes.

Precision of the method in the presence of 10^{-2} M iodoacetate. Some measure of the precision of the method in the presence of iodoacetate was obtained from four duplicate estimations of the DPT in rat liver extract, in which 10^{-2} M iodoacetate was used. The mean deviation from the mean in these estimations was 2.02%, with a standard deviation of 1.11 (Table XVII). Two tissue extracts could be considered to differ significantly in DPT content if the mean deviation from their mean was more than $\pm$ 4.2%.[5]

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[5] i.e. 2.02+(1.96 x standard deviation).

TABLE XV

EFFECT OF 10^{-2} M IODOACETATE UPON DRIFT

t (min.)	No iodoacetate			10^{-2} M Iodoacetate introduced:					
				Before add'n of DPT			With pyruvate, 35 min. after add'n of DPT.		
	S	T	T/S	S	T	T/S	S	T	T/S
6	49	49	1	32	30	0.94	48	47	0.98
12	110	115	1.4	67	64	0.96	108	102	0.94
19	170	179	1.05	101	96	0.95	162	155	0.96
25	216	221	1.02	126	121	0.97	204	198	0.97
40	313	331	1.06	184	180	0.98	298	291	0.98
* 79	89	125	1.41	53	55	1.04	86	85	0.99

t = time interval after addition of sodium pyruvate to the main compartment.

T = CO_2 evolved in the flask containing tissue extract.

S = " " " " " " standard solution.

* The gas volumes at 79 minutes resulted from 19 minutes gas production, for the cocks were opened at 50 minutes and closed again at 60 minutes.

TABLE XVI

DRIFT IN THE PRESENCE OF 10^{-2} M IODOACETATE

Gm. tissue analyzed	µgm. DPT found at 20 min.	30 min.	% increase	µgm. DPT found at 65 min.	80 min.	increase as % of 20 min. values
0.0092	0.095	0.095	0			
"	0.099	0.099	0			
0.0184	0.190	0.199	4.7			
"	0.184	0.198	7.6			
0.0092	0.092	0.092	0		0.097	5.4
"	0.100	0.098	-2.0		0.100	0.0
0.0184	0.174	0.167	-4.0		0.163	-6.32
"	0.174	0.175	0.6		0.175	0.6
0.0082	0.063	0.071	12.7	.073		14
0.0164	0.122	0.127	4.1	.133		9
0.0082*	0.097	0.092	-5.2			
0.0082	0.084	0.084	0			
0.0092	0.092	0.093	1.1			
0.0184	0.180	0.179	-0.06			

Mean 1.40

Standard deviation of the mean 1.24

t..... 1.13

P (for significance of the mean) > 0.2

* This estimation took place with a nitrogen atmosphere in the Warburg vessel.

TABLE XVII

MEAN DEVIATIONS OF DUPLICATES FROM THE MEAN
IN THE PRESENCE OF 10^{-2} M IODOACETATE

Gm. tissue analyzed	Mean deviation from the mean (%)
0.0092	2.06
0.0184	0.505
0.0092	3.16
0.0184	2.34
Mean.....	2.02
Standard deviation....	1.11

2. Effect of the Concentration of Tissue Extract Upon Drift

The effect of the tissue extract concentration upon drift was studied in the following experiment:

An aliquot of tissue extract, prepared as described in Chapter III, was diluted with an equal volume of 0.1 M phosphate buffer pH 6.4, and simultaneous DPT estimations were carried out on the undiluted and diluted portions. For nine such experiments, the drift between 20 and 40 minutes, expressed in per cent of the 20 minute value, is shown in Table XVIII. The per cent drift was significantly higher in the case of the higher concentration of tissue extract.

3. Effect of DPT Concentration

To ascertain whether the effect upon drift of increased tissue concentration was due to increased DPT concentration, drift was measured in tissue extracts containing added DPT and compared with controls (Table XIX). In 15 experiments added DPT evoked no change in absolute drift. However, expressed in per cent of the 20 minute value, the drift was significantly lowered by the addition of DPT.

4. Relation of Acetaldehyde to Drift

The drift observed in the present investigation, resembled the apparent synthesis of DPT by alkaline-washed yeast in the presence of boiled tissue extracts reported by Lipschitz et al. (38),

TABLE XVIII

PER CENT DRIFT IN THE ESTIMATION OF DPT IN TISSUE EXTRACT AT TWO CONCENTRATIONS

% Drift during estimation of DPT in	
'A': Tissue extract	'B': diluted tissue extract
3.9	0.7
5.9	4.1
10.1	5.1
13.9	10.7
14.8	14.6
7.8	-1.1
7.6	1.2
29.0*	10.1
27.8	17.2
Mean 13.4	Mean 6.96

$t = 3.324$

P (for A vs. B) < 0.02
> 0.01

* Single estimation. All others are means of duplicate experiments.

TABLE XIX

EFFECT OF ADDED DPT UPON DRIFT

Added DPT (µgm.)	Absolute drift*		Difference	% Drift *		Difference
	Without Added DPT	With Added DPT		Without Added DPT	With Added DPT	
0.100	.001	.002	.002	0.70	-0.6	1.3
0.100	.003	.008	.005	4.1	5.0	-0.9
0.100	.004	.004	.000	5.1	5.4	-0.3
0.050	.004	.006	.002	10.7	7.5	3.2
0.067	.008	.010	.002	12.7	8.2	4.5
0.100	.010	.010	.000	14.6	6.4	8.2
0.067	.011	.008	-.003	14.2	5.2	9.0
0.100	-.001	.006	.007	-1.1	3.5	4.6
0.067	.002	.002	.000	3.2	1.1	2.1
0.067	.002	.002	.000	1.9	1.5	0.4
0.067	.008	.016	.008	11.9	11.6	0.3
0.100	.008	.006	-.002	11.9	7.4	4.5
0.100	.001	.006	.005	1.2	3.5	-2.3
0.100	.008	.005	-.003	10.1	3.1	7.0
0.100	.012	.010	-.002	17.2	8.5	8.7
	$t = .0000047$			$t = 2.5382$		
	$P >> 0.05$			$P < 0.05$		

* Means of duplicate experiments.

which Ochoa and Peters (6) suggested to be due to the removal of acetaldehyde formed in the decarboxylation of pyruvate. Although Lipschitz et al had reported that the removal of acetaldehyde, as effected by semicarbazone formation, did not appreciably affect the carboxylase activity, the conditions of their experiment did not appear to be suitable for rapid acetaldehyde removal. The experiment was therefore repeated under conditions more suitable for semicarbazone formation (70). The procedure was as follows:

The cocarboxylase activity of 0.200 μgm. DPT was measured with semicarbazide in the centre cup (110 mgm. semicarbazide in 0.3 ml. of 0.38 N citrate buffer, at pH 4.9), and compared with a control in which citrate buffer was placed in the centre cup. The results in Table XX show that, although little effect was observed during the early part of the experiment, after 84 minutes the gas production in the flask containing semicarbazide was markedly higher than that in the control flask. This indicated that the removal of acetaldehyde exerted an appreciable effect upon carboxylase.

II. LOW RECOVERIES

Introduction. The study of factors affecting recovery values was made in an effort to provide an explanation for low recoveries. Two possible explanations were considered:

1. A greater CO_2 retention in the vessels containing tissue extract than in those containing pure DPT.

TABLE XX

EFFECT OF ACETALDEHYDE REMOVAL

UPON CARBOXYLASE

Each flask contained: the alkaline-washed residue from 100 mgm. yeast; 0.200 μgm. DPT; 100 μgm. Mg^{++}; 100 μgm. Mn^{++}.

Time from start of reaction		20	30	74	84	136	151
*Time of gas production		20	30	20	30	20	35
μl. CO_2	Semicarbazide in Centre cup	155	226	103	151	87	160
	No Semicarbazide in Centre cup	155	220	92	120	75	132

* At intervals the stopcocks were opened and closed again. These intervals are marked off by double vertical lines.

2. A greater oxygen uptake in the presence of tissue extract than in the presence of pure DPT. Oxidizable substrates and the co-dehydrogenases concerned in their oxidation, present in tissue extract, might stimulate oxygen uptake by alkaline-washed yeast. The apparently lower CO_2 production would result in false low values for the DPT content of the tissue. However, unless the oxygen uptake depended also upon the quantity of DPT the recoveries would not be affected, as in the recovery experiments the amount of tissue extract was the same in all flasks.

1. The retention of CO_2

Experimental. The possibility that the presence of tissue extract resulted in a greater retention of CO_2 was investigated by an experiment in which recovery values were calculated from the volumes of total acid-labile CO_2. A recovery experiment was carried out as usual, and 30.5 minutes after the beginning of the reaction, 0.20 ml. of 10 N H_2SO_4 was tipped into the main compartment of the Warburg vessel from the side arm. This amount was calculated to be sufficient to bring the pH to between 2 and 3, and so release CO_2 bound as bicarbonate. The results shown in Table XXI indicate that the retention of CO_2 in an acid-labile form did not appreciably alter the results of the estimation or the recovery values.

2. Oxygen Uptake

Introduction. In order to investigate the possibility that low recoveries were due to differences in oxygen uptake, the

TABLE XXI

RECOVERIES CALCULATED FROM

TOTAL ACID-LABILE GAS

Calculations based upon:	μgm. DPT found in:		Recovery
	0.0079 gm. liver	0.0079 gm. liver plus 0.100 μgm. DPT	
Gas produced in 30 min.	.091	.162	
	.091	.161	
Mean	.091	.162	71%
Gas produced after addition of H_2SO_4	.093	.169	
	.087	.167	
Mean	.090	.168	78%

effect of anaerobic conditions upon the estimation was studied.

Experimental. Two simultaneous recovery experiments were carried out, one in which the atmosphere in each flask at the beginning of the experiment was air, the other in which the atmosphere was nitrogen. The nitrogen atmosphere was obtained by flushing out the air with nitrogen for ten minutes, while shaking the Warburg vessels in the constant temperature bath to insure equilibrium of the dissolved oxygen with the atmosphere in the flask. The estimation of DPT was then carried out in the usual manner.

After forty minutes the conditions were reversed in the following way: the stopcocks were opened, nitrogen flushed through the flasks which had contained air at the start of the experiment, and air sucked through the vessels which had contained nitrogen. The cocks were closed and the CO_2 production read after 39 minutes. Table XXII contains the gas volumes in the various flasks. In each of the paired estimations the gas production was higher in the air-free flask, indicating that oxygen uptake occurred under aerobic conditions. However, from the results in Table XXIII, it may be seen that the presence or absence of oxygen during the estimation had little effect upon the DPT content or recovery values.

Similar results were obtained from a series of five separate recovery experiments with samples of a single tissue extract: three under aerobic conditions, and two under anaerobic conditions. As can be seen from the data in Table XXIV, there was no appreciable difference in the DPT content or the recovery values as determined

TABLE XXII

GAS PRODUCTION IN

PRESENCE OF NITROGEN

μgm. DPT per flask	0		.1		.15		.2		0		.1	
Tissue	-		-		-		-		+		+	
Nitrogen	+	-	+	-	+	-	+	-	+	-	+	-
A*	44	38	174	168	228	207	274	252	170	152	258	233
Nitrogen	-	+	-	+	-	+	-	+	-	+	-	+
B*	35	38	88	117	114	142	131	163	92	113	128	153

* A: Microliters of carbon dioxide produced in 40 minutes.

B: Microliters of carbon dioxide produced in 39 minutes after reversing the conditions in the Warburg vessels.

TABLE XXIII

EFFECT OF A NITROGEN ATMOSPHERE UPON

RECOVERY ESTIMATIONS

μgm. DPT added	DPT found after intervals of:	
	40 min.	39 min.
0	.095	.103
0.1	.183 N_2	.190 Air
Recovery %	88	87
0	.085	.093
0.1	.164 Air	.171 N_2
Recovery %	79	78

TABLE XXIV

EFFECT OF NITROGEN

Each determination was carried out on a quantity of tissue extract equivalent to 0.0079 mgm. Results were calculated from gas produced in 30 minutes. DPT added - 0.100 µgm.

Age of extract (days)	Atmosphere	DPT found per flask		Recovery
		In tissue extract	In recovery	
1	Air	0.076	0.170	
	"	0.080	0.155	
Mean		0.078	0.162	84%
2	Air	0.077	0.160	
	"	0.078	0.154	
Mean		0.078	0.157	79%
13	Nitrogen	0.083	0.168	
	"	0.082	0.162	
Mean		0.082	0.165	83%
14	Nitrogen	0.080	0.168	
	"	0.082	0.164	
Mean		0.081	0.166	85%
14	Air	0.080	0.174	
	"	0.083	0.170	
Mean		0.082	0.172	90%

under the different conditions. As these experiments were not done simultaneously, the number of days between preparation of the tissue extract and the performance of the experiment are also shown.

CHAPTER VI

THE EFFECT OF IODOACETATE UPON CARBOXYLASE

Inhibition of apocarboxylase by iodoacetate. During the study of drift the use of iodoacetate appeared to be associated with lower standard curves than usual, suggesting a partial inhibition of the carboxylase system itself. The effect of various concentrations of iodoacetate upon carboxylase activity was therefore investigated.

Iodoacetate solutions of varying strength were prepared, and to 10 ml. of each was added 10 ml. of either DPT solution or liver extract. Two ml. of the resulting solution, containing either 0.200 μgm. DPT or the extract of 0.0184 gm. liver, was added to a mixture of 50 mgm. alkaline-washed yeast, 100 μgm. thiamine, and 100 μgm. each of magnesium and manganese ions in the main compartment of a Warburg flask. After 10 minutes equilibration in the bath at 27.5°C., the reaction was started by adding from the side arm 5 mgm. sodium pyruvate in phosphate buffer. Two such experiments, the results of which are shown in Table XXV, revealed a marked inhibition of carbon dioxide production whether in the case of tissue extract or synthetic DPT. The inhibition increased with increasing concentrations of iodoacetate.

Table XXVI contains the results of two further experiments on the effect of graded amounts of iodoacetate upon the

TABLE XXV

EFFECT OF IODOACETATE UPON YEAST CARBOXYLASE

Expt. no.	Iodoacetate concentration ($\times 10^{-3}$ M)	μl. CO_2 evolved by 50 mgm. alkaline-washed yeast in 50 minutes in presence of:	
		0.0200 μgm. DPT	Extract corresponding to 0.0184 gm. Rat liver.
1	0	220	120
	3.85	158	74
	7.6	129	62
2	5	137	58
	6.67	132	60
	7.50	122	55

TABLE XXVI

EFFECT OF IODOACETATE UPON

YEAST CARBOXYLASE

Each flask contained 0.200 μgm. DPT, 50 mgm. alkaline-washed yeast, and 5 mgm. sodium pyruvate.

	Experiment I	Experiment II
Iodo. conc. ($\times 10^{-3}$ M)	μl. CO_2 evolved in 50 min.	μl. CO_2 evolved in 32 min.
0	159	101
3.33	128	83
5.00	119	77
5.55	110	73
6.25	102	77
7.15	100	68
8.34	93	67
10.0	92	60
15.0		48

cocarboxylase activity of a DPT solution, and shows definitely that the degree of inhibition depended upon the concentration of iodoacetate used.

However, an alternative explanation for this inhibition of gas production was possible: the yeast might synthesize DPT from thiamine even in the absence of tissue extract, and the addition of iodoacetate might merely prevent this synthesis. To investigate this possibility a study was made of the effect of iodoacetate upon carboxylase activity with and without thiamine added to the reaction mixture. Inhibition in the absence of thiamine was only slightly less than that in its presence (Table XXVII). As no synthesis of DPT by yeast in the absence of thiamine has been reported, these results indicate that iodoacetate inhibited the carboxylase system, rather than systems synthesizing DPT.

Effect of iodoacetate upon holocarboxylase. As Green et al. (34) reported no inhibition of their partly purified holoenzyme by iodoacetate, the effects of iodoacetate on the apoenzyme and the holoenzyme were compared. Iodoacetate was added in three ways to a mixture of 100 mgm. alkaline-washed yeast, magnesium and manganese salts, and 0.200 μgm. DPT:

1. Iodoacetate was mixed with the apoenzyme before the addition of DPT.
2. Iodoacetate was tipped from the side arm into the main compartment after a 18 minute period of incubation of the mixture.

TABLE XXVII

INHIBITION OF CARBOXYLASE BY 10^{-2} M IODOACETATE IN THE PRESENCE AND ABSENCE OF THIAMINE

Time (mins.)	Thiamine			No Thiamine		
	Iodo.	No Iodo.	i*	Iodo.	No Iodo.	i*
20	42	74	43	16	24	33
25	52	91	43	20	29	31
32	65	112	42	25	36	31
35	69	120	43	26	38	32
37	74	126	41	28	41	32
44	83	146	43	30	47	36

* i = % Inhibition

3. The iodoacetate was mixed with the pyruvate and the two were tipped together from the side arm into the main compartment after a 35 minute period of incubation of the mixture. The final concentration of iodoacetate in each case was 10^{-2} molar.

The gas production in each flask was compared with that of a control into which buffer was added instead of iodoacetate solution.

From the data of this experiment (Table XXVIII), it is evident that inhibitions of the order of 40% were brought about by the addition of iodoacetate before reconstitution of the holocarboxylase, whereas the inhibition was slight when the inhibitor was added sufficiently long after the reconstitution of the holoenzyme. That the diminished inhibition was not simply due to a shorter period of contact of the inhibitor with the enzyme is demonstrated by the fact that there was no increase in inhibition during the 79 minute period of gas production.

TABLE XXVIII

EFFECT UPON CARBOXYLASE OF IODOACETATE ADDED BEFORE AND AFTER RECONSTITUTION OF THE HOLOENZYME

i = % inhibition.

I Iodoacetate added before DPT.

II Iodoacetate added after 18 minutes incubation of alkaline-washed yeast with DPT.

III Iodoacetate added with pyruvate, after 35 minutes incubation of alkaline-washed yeast with DPT.

Each flask contained: alkaline-washed residue from 100 mgm. dried yeast; 0.200 μgm. DPT; 40-45 mgm. pyruvate.

Time of gas prod'n.	No Iodo-acetate	10^{-2} M Iodoacetate					
		I		II		III	
		CO_2	i	CO_2	i	CO_2	i
6	49	32	35	49	0	48	2
12	110	67	39	100	9	108	2
19	170	101	41	152	11	162	5
25	216	126	42	191	12	204	4
40	313	184	41	276	12	298	5
79*	89	53	40	82	9	86	3

*The gas volume at 79 minutes was obtained after 19 minutes of gas production, since the cocks were opened at 50 minutes and closed again at 60 minutes.

CHAPTER VII

DISCUSSION AND SUMMARY

I. DISCUSSION

A study has been made to assess the manometric method for estimating DPT. As applied to tissue extracts the method was found to be precise within ± 5%. With regard to accuracy, certain defects have been revealed which will be discussed in turn. Observations on the effect of iodoacetate on carboxylase will also be considered.

Drift.

Occurence of drift. The values obtained by this method were found to drift upwards during the period of gas production. Between 20 and 30 minutes the apparent DPT content increased by as much as 30%. Over longer periods of time the drift became much greater, prohibiting entirely the use of long periods of gas production for the calculation of results. The degree of drift was increased by doubling the concentration of the tissue extract analyzed. When DPT alone was added to the tissue extract no significant effect was observed. It would appear that the degree of drift depended upon some substance in tissue extract other than DPT.

Cause of drift. Two possible explanations for drift are:

1. the tissue extract stimulates the removal of acetaldehyde formed in the carboxylase reaction;

2. the tissue extract stimulates a synthesis of DPT.

The chief objection to the first explanation is the report by Lipschitz et al. (38) that the acetaldehyde formed in the reaction had but little effect upon carboxylase. However, their experiment has been repeated in the present investigation under more suitable conditions (70), and the results suggest that an appreciable increase in carboxylase activity may be brought about by removing the acetaldehyde produced in the reaction. Whether or not acetaldehyde is removed during a DPT estimation was not investigated, but alcohol dehydrogenase, the enzyme catalyzing the hydrogenation of acetaldehyde (71), is present in yeast. The coenzyme of alcohol dehydrogenase[1] is removed from the yeast during the washing process employed for the removal of DPT (6), so that hydrogenation of acetaldehyde might be expected to occur when a tissue extract containing this coenzyme, but not a pure DPT solution, is added to the yeast. The progressive inhibition by acetaldehyde in the standard flasks would cause a gradual upward drift in the results of the estimation. As iodoacetate powerfully inhibits alcohol dehydrogenase (61), the inhibition of drift by iodoacetate is in accord with this explanation.

The second explanation, that tissue extract stimulates a synthesis of DPT was not investigated. However, that tissue extract supplies the necessary coenzymes or substrates for the

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[1] Diphosphopyridine nucleotide (71).

formation of high energy phosphate bonds, is not unreasonable, so that this possibility cannot be discounted.

Effect of iodoacetate. The use of 5×10^{-3} M iodoacetate did not prevent drift. In the presence of 10^{-2} M iodoacetate, however, the average drift was not significant, though appreciable drift occurred in some individual instances. High concentrations of iodoacetate have the disadvantage of markedly inhibiting carboxylase activity, which may be overcome by adding the iodoacetate after the reconstitution of the holoenzyme. This may be done conveniently by using a solution of iodoacetate and pyruvate, and tipping both into the main compartment together. In this way drift, but not carboxylase, is inhibited. In cases where the DPT content of a tissue is very low, therefore, the period of gas production may be extended in order to obtain gas volumes large enough for a sensitive estimation.

Low recoveries.

It has been seen that the recovery of DPT added to tissue extracts was significantly low, the average being about 90%. This suggests that the values for the DPT content of tissues obtained by this method are correspondingly lower than the true values. In studies comparing the DPT content of various materials therefore, recovery experiments should be done with each. If similar recoveries were obtained throughout, the use of this method for the study would be justified, since the comparison would not be affected. For absolute studies, values obtained for the DPT content

of tissues by this method can be only tentative, until verified by a method in which good recoveries are obtained.

The low recoveries did not appear to be related to the retention of carbon dioxide in an acid-labile form, nor to the uptake of oxygen by the yeast.

Dependence of the results upon the amount of tissue analyzed.

The results of analyses by this method, expressed in micrograms DPT per gram tissue, were found to be lowered by doubling the concentration of tissue extract analyzed. Although this difference was too slight to cause serious errors due to variations in the amount of tissue analyzed, it suggests the presence in tissue extracts of an inhibitor of the carboxylase system. This might provide an explanation for low recoveries, in that low concentrations of an inhibitor might produce a marked inhibition, whereas doubling the concentration need not cause a great increase in effect.

Inhibition of carboxylase by iodoacetate.

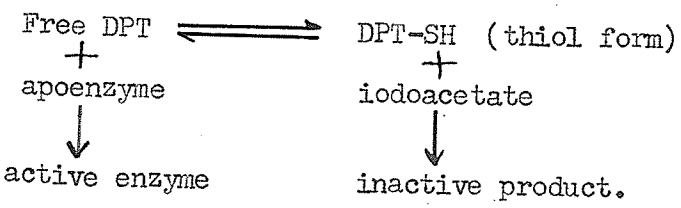
Aside from its importance in the manometric estimation of DPT, the inhibition of carboxylase by iodoacetate carries theoretical implications regarding the structure of holoenzyme. From the present investigation it may be concluded that iodoacetate acts upon either the coenzyme or the apoenzyme so as to prevent their joining to form an active preparation, but that once the two are linked they are resistant to the inhibitor. There are at least

three possible explanations for this phenomenon:

1. If the link between DPT and the apoenzyme were through an SH group of the protein, the SH group of the split enzyme would be free for reaction with iodoacetate. Having reacted with iodoacetate, this SH group could no longer bind DPT. Such an explanation has been suggested for pyruvic oxidase, a bacterial enzyme containing DPT.

2. The thiol form of DPT has been reported to possess the same cocarboxylase activity as the thiazole form (36). If the link to the apoenzyme were through the SH group of the thiol form of DPT, iodoacetate would bind the free coenzyme, but not that attached to the apoenzyme.

3. Assuming that yeast is capable of the reversible conversion of DPT to the thiol form, one may consider the following reactions to occur:



By binding the thiol form, iodoacetate would effectively remove DPT from solution, thereby inhibiting the system.

No one of these explanations appears to be more favourable than the others, and further experimentation is required to determine which is correct.

II. SUMMARY

1. The manometric method for determining DPT in animal tissues has been found to possess certain defects, apparently due to interference by substances present in tissue extract. Changes have been suggested which make the method usable with certain reservations.

2. Iodoacetate inhibits the formation of an active enzyme from a mixture of alkaline-washed yeast, magnesium, and DPT. The relation of this finding to the structure of carboxylase has been discussed.

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