1955

The investigation of succinic and malic dehydrogenases in serum.

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Thesis

THE INVESTIGATION OF SUCCINIC AND MALIC DEHYDROGENASES IN SERUM

by

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(A.B., Lincoln University, 1953)

Submitted in partial fulfilment of the requirements for the degree of Master of Arts

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## ABSTRACT
Succinic and malic dehydrogenases are respiratory enzymes, which form an important link in the chain of reactions concerned with oxidation of carbohydrates, lipids, and proteins.

They catalyse the transfer of protons from succinic and malic acids respectively through hydrogen acceptors to molecular oxygen to form water and thereby liberate a certain amount of energy. This energy is used for body heat and for synthesis of high energy phosphates.

Although succinic and malic dehydrogenases are enzymes of the cyclophorase system, usually associated with the mitochondria of cells, the possibility that they may find their way into blood plasma is suggested by the following considerations:

First, the presence of several enzymes that occur intracellularly in animal tissue have been demonstrated in the blood—for example, alkaline phosphatase (21) and acid phosphatase (24).

Secondly, there was a time when enzymes concerned with oxidation and reduction were thought to be as a rule absent from plasma, but Warburg and Christian (68) (69) and more recent workers (36) (15) have demonstrated not only the presence of glycolytic enzymes, but also of non glycolytic oxidative enzymes like xanthine oxidase, peroxidases, catalase, and glutamic dehydrogenase.

In the third place, the demonstration of isocitric dehydrogenase in the soluble fraction of the liver cell by Recknagel (50) and of aconitase, malic, and isocitric dehydrogenases in the soluble fraction of chicken erythrocyte by Rubinstein et al (52) suggests that some of
the enzymes of the cyclophorase system may not always be insolubly attached to the particulate fraction of mitochondria, but that they may, depending on the cell membrane condition, be able to leach out into the surrounding fluid medium.

The phenomenon of oxidative phosphorylation in non mitochondrial systems observed by Green and others (7) during succinate oxidation by heart muscle weakens the structural barrier theory of cellular particulates.

Fourth, blood plasma contains proteins and enzymes in equilibrium with proteins and enzymes of formed elements and also of tissue cells. While this explains why some tissue changes are reflected in the blood plasma, it also suggests that many tissue-cell enzymes may be demonstrated in the plasma.

Fifth, there is even no clear connection between metabolic activity and succinic oxidase activity, since there are some tissues with normal respiratory activity but with practically no succinoxidase activity, for example spleen and several tumors (58). On the other hand, absence of succinic oxidase is not characteristic of cancer tissue, since some like Jensen sarcoma (58) always have definite activity. The important point is that absence of respiratory activity in the plasma does not exclude the possibility of the enzymes, malic and succinic dehydrogenases, being there.

There is no doubt that the presence or absence of these enzymes in normal serum, if definitely ascertained, would be of significance. If they are absent in normal plasma, then their occurrence in patients' plasma may be of diagnostic value, bearing in mind that certain diseases are sometimes characterized by hyperactivity or hypoactivity of the cellular metabolic system. If they are present in normal plasma, then their absence
in patients' plasma may be still diagnostically important. Finally, the relative concentrations of the two in normal and pathological sera may be of some use in diagnosis and possibly in therapy.
HISTORY

Malic dehydrogenase was discovered in 1911 by two independent workers viz Thunberg (63) and Battelli et al (12,13a). Substrate of malic acid was found to be dehydrogenated with animal tissue as a source of malic dehydrogenase. Anderson (5,6), working with dried yeast and Holmberg (25) with extracts of washed muscle, discovered and proved that a coenzyme is required in the reduction of methylene blue (MB) by the malic dehydrogenase. This coenzyme now known as diphosphopyridine nucleotide (DPN) seems to be required in all biochemical reactions involving the transfer of hydrogen from malic acid to a hydrogen acceptor (a dye, or molecular O2).

Malic dehydrogenase has a very wide distribution. It is found in brain, heart, liver, muscle, kidney and other animal tissue (64,65). It is found also in yeast (6), in certain bacteria (22) such as E. Coli, and in higher plants (5). Green (23) found high concentrations in the tissues of rat, rabbit and pigeon. He precipitated an extract of pig heart muscle at pH 4.6 and used the fraction for an extensive study of malic dehydrogenase.

Straub (61) isolated malic dehydrogenase by extracting acetone-dried pig heart muscle with .1M phosphate buffer at pH 7.3. It was demonstrated by Sisakyan (59) that there is considerable malic dehydrogenase activity in the chloroplast of clover leaf. McGShan et al (64) reported increased activity of malic dehydrogenase in lutein tissue of rat during pregnancy. The malic dehydrogenase activity of copora lutea during the course of pregnancy rose to a maximum on the 11th day, while
there was significant rise in malic dehydrogenase activity on the 20th day of lactation.

On the other hand, Agather et al (1) reported that total dehydrogenase activity was independent of sex, strain, and age of the normal and tumor bearing rats she tested. Addition of coenzyme I accelerated the total dehydrogenase activity of muscle, liver, and brain of normal and tumor bearing animals.

The presence of malic dehydrogenase in dolphin tissues was demonstrated by Kenneth et al (34) who found that the heart muscle contained the greatest concentration of the respiratory enzymes (cytochrome dehydrogenase, succinic and malic dehydrogenase, etc.) as is also the case with rat tissues. The dolphin tissues however, have less of these enzymes than the corresponding rat tissues. Whether this reflects a lower metabolic activity as compared with rats is not certain. In contrast to the situation present in the rat, Ca++ affects very little the adenosinetriphosphatase (ATP) activity in dolphin tissues, while Mg++ and Mn++ definitely have a stimulating effect on the hydrolysis of ATP by tissue homogenates.

With the methylene blue method of Thunberg the malic (also succinic) dehydrogenase activity of the pupas of calliphora erythrophalal was found by Agnell (3) to be correlated with morphological changes which take place with increasing age of pupas.

**Succinic Dehydrogenase.** The presence of succinic dehydrogenase was first discovered by Batelli and Stern (13b). Elliot et al (18) investigated the distribution of these enzymes in various animal tissues and reported a considerable quantity of succinic dehydrogenase in rat kidney, liver and heart. They showed that when cytochrome concentration is low in
the homogenate, that the addition of cytochrome c accelerates the reaction of succinate oxidation.

Breusch (14) reported small or moderate amounts of the same enzyme in brain, testes, skeletal muscle, lungs, adrenals, spleen and retina. On the other hand hemolysates of pigeon blood and chicken erythrocytes have a fairly large amount of succinic dehydrogenase as opposed to rabbit and human erythrocytes which have little or none, (Ashwell (8)), (Baker & Hunter (11)) and (Rubenstein et al (52)).

A method of determining the activity of tissue homogenate was reported by Schneider and Potter (54) (55) who, using the Warburg apparatus, found that it was necessary to add Al\(^{+++}\) and Ca\(^{++}\) for maximum activity of the homogenate. They determined the activity in heart, kidney, liver, brain, skeletal muscle, spleen, lung, and Jensen Sarcoma of the rat. They also determined the optimum concentration of cytochrome c, Al\(^{+++}\) and Ca\(^{++}\).

In connection with the activation of succinic dehydrogenase reaction by certain cations, Horecker, Stotz, et al (27) were the first to show that Al\(^{+++}\) activates the reaction of succinic dehydrogenase. Axelrod et al (9) found that Ca\(^{++}\) does the same. They postulated that Ca\(^{++}\) accelerates the destruction of DPN, which if not destroyed, might promote the formation of an inhibitory oxaloacetate by the malic dehydrogenase present in the homogenate. This would be the case when malic acid happens to be also in the homogenate.
Succinic dehydrogenase activity in endocrine tissues were reported by McShan (4,2) while Fawcett (20) reported more succinic dehydrogenase activity in the brown adipose tissue than in the white.

Several reports have recently appeared on the correlation of changes in succinic dehydrogenase activity of various tissues with changes in the function of these tissues. According to Potter et al (49) the activity in the liver of the rat embryo increases rapidly during late embryonic and early post-natal life, and reaches the adult level 15 days after birth. It increases during pregnancy in functional corpora lutea of rat ovaries being 2½ times than in non functional diestrous corpora. It has also been reported that the activity of the enzyme increases in developing chick embryo (4) just as it does in developing young dolphin as compared with the adult (34).

Schneider and Hogeboom (53) fractionated liver and hepatoma of mouse by differential centrifugation into a nuclear, a mitochondrial, a submicroscopic particulate, and a supernatant soluble fraction. The distribution of total nitrogen and of succinic oxidase and cytochrome oxidase activities were studied in the various fractions. The succinic oxidase and cytochrome oxidase activities of hepatoma were only about one-fifth as great as the activity of the same enzymes in the liver homogenate.

Just as in the case of malic dehydrogenase, the distribution of succinic dehydrogenase in dolphin tissues was studied by Kenneth and Geiling et al (34). The results were comparable to those of malic dehydrogenase already discussed (1).

William and Denton (70) found that methionine deficiency in rats reduced liver succinic dehydrogenase slightly, but completely abolished
xanthine dehydrogenase activity.

In his study of succinic oxidase and dihydrocozymase (DPNH) oxidase systems in heart muscle and kidney preparations, Slater (60) found that different enzymes are involved in activating the substrates (succinate and DPNH2), but that subsequent reactions of the H atoms follow similar patterns. Malonate inhibits oxidation of succinate but not that of dihydrocozymase. Factors which affect the British Anti-Lewisite (BAL) sensitive factor cytochrome c inhibit oxidation of both. Also phenylurethan, carbon monoxide, and cyanide have the same effect.

More recently Cooperstein et al (16) reported a microspectrophotometric method for determination of succinic dehydrogenase. The kidney, heart, liver, brain, lung and striated muscle of rats were assayed and results were discussed. His result was essentially in agreement with the results of previous workers.

Besides methylene blue, quinones and phenol cresyl blue, the tetrazolium salts are new additions to vital dyes that are of use in biochemical investigation of dehydrogenase activity. The works of Kuhn and Abood (37) and those of Yoshia Saweda et al (71) bear testimony to this. Along similar lines Yoshio (71) reported procedures for and results of histochemical demonstration of succinic dehydrogenase activity in kidneys using blue tetrazolium salts. Malonic acid (M/80), maleic acid (M/80) and urethan had a marked inhibitory effect on the enzyme in aerobic conditions, but had no effect in anaerobic conditions.

More recently, Koppel et al (35) reported moderate dehydrogenase activity in a versene-collected freshly prepared human platelets. Using the method of Kuhn and Ababod (37) they noticed that completely lysed platelets have no dehydrogenase activity. It seems that the platelets show
dehydrogenase activity only when they are morphologically intact. Addition of gluthathione resulted in 253% activation of the dehydrogenase activity. Cysteine, mercaptoethanol, thiomalic acid, thiyglylolic acid, respectively, caused 166%, 62%, 32%, and 5% activation.

It has since been indicated by Neish (46a) that serum from rats with transplanted tumors were less effective than normal rat serum for reducing 2,3,5,-triphenyltetrazolium chloride as determined polarographically. There was a negative linear correlation between the percentage of tumor weight and the dye reduced. Cancer serums mainly gave a red color with the dye while normal serum gave a purple color.
BODY OF THE THESIS

I. The TTC Reduction Method.


There are at least four established methods for the determination of succinic and malic dehydrogenases. They are:

1) Measurement of O$_2$ uptake in a Warburg apparatus (55).
2) Measurement of the time of decolorization of methylene blue (MB) using the Thunberg Technique (66), (47).
3) Measurement of the amount of 2,3,5-triphenyl tetrazolium chloride (TTC) which is reduced to the formozan using the method of Kuhn and Abood. (29), (37), (71), (36), (33).
4) Measurement of the rates of reduction of cytochrome C at 550 mu for succinic dehydrogenase (16), and of reduction of (TPN) or diphosphopyridine nucleotide (DPN) at 334 mu for malic dehydrogenase (25).

From the methods listed, two methods were chosen. They are:

1) Measurement of the amount of 2,3,5-TTC which is reduced to the formozan using the method of Kuhn and Abood.
2) Measurement of the time of decolorization of MB using the Thunberg Technique.

The two methods were chosen because they involve the handling of inexpensive and time saving equipment; at the same time they do not sacrifice accuracy for simplicity.

2,3,5-TTC is a stable dye, inexpensive and commercially available. It dissolves in water, acetone and toluene and the reduced formozan stays reduced in the presence of air. Unlike MB, it does not require cytochrome C, diaphorase, KCN. It is a comparatively new tool in the
investigation of biological oxidation.

Measurement of Dehydrogenase activity by the use of 2,3,5-T.T.C.

TTC, first synthesized by Pechman and Runge in 1894 (46) extensively investigated by Kuhn and Jarcel (38), and proposed for a living tissue test by Matson (41), is a colorless water soluble dye, with apparent redox potential of -.08 volt at pH 7 (33).

Kuhn and Abood and more recently, Yoshio Sawada et al (71) have adapted the reduction of the dye to the quantitative colorimetric determination of dehydrogenase activity. The dye accepts hydrogen (proton) from a substrate in the presence of the specific enzyme and in doing so becomes reduced to a red water-insoluble formozan. This formozan can be dissolved by acetone, toluene, xylene, etc., and in this way can be measured and estimated colorimetrically in a suitable photometer. The postulated reaction is as follows:

\[
\text{N} = \text{N} \quad \text{C} \quad \text{N} \quad + \quad \text{H}^+ \quad + \quad \text{Co}^-
\]

The importance of careful control in reaction with 2,3,5-triphenyltetrazolium chloride, cannot be overemphasized. This is because of the sensitivity of the dye to bright sunlight, to change in pH and to reducing agents of various types. A very recent polarograph study (28) showed that above the pH of 6 the dye is reduced mainly to the red water-insoluble formozan. In a very strongly alkaline pH (12.5-14) and in absence of light it is spontaneously reduced to the red formozan. More recently the reduction of TTC
(by normal serum) has been reported and has been correlated with the serum alkaline and phosphatase activity (67). The accuracy of the results from the differential reduction that would be expected from reaction with serum will depend on the effectiveness of the control used.

**Measurement of Enzyme Activity by use of Methylene Blue (Thunberg Technique)**

Thunberg demonstrated the presence in frog muscle of dehydrogenases capable of bringing about the oxidation of not only lactic, citric, glutamic, and glycerophosphoric acids, but also of succinic and malic acids. His method (63),(64),(65), consisted essentially of reacting the tissue homogenate with MB in the presence of the desired substrate in an evacuated Thunberg tube. MB accepts hydrogen from the substrates in the presence of the specific enzymes and becomes reduced under anaerobic conditions to leuco-methylene blue. In dilute tissue homogenates diphosphopyridine nucleotide (DPN), a necessary hydrogen acceptor in this reaction, deteriorates very fast; it is usual therefore to fortify the reaction mixture with DPN. Diaphorase is a flavoprotein which catalyses the reaction of hydrogen from reduced DPN (DPNH) to MB and is also required in this reaction. Straub, (61) and Green (23) used KCN and NaCN respectively in the determination of malic dehydrogenase, using MB with Warburg vessels. KCN was supposed to inhibit the reconversion of oxaloacetate acid (formed as a result of oxidation of malate and pyruvate) to malate either by inhibiting the enzyme mediating this reaction or by fixing the oxaloacetate formed. Cyanide also inhibits cytochrome dehydrogenase and the reaction which it catalyses.
2. Preliminary Investigation

Having decided on the two methods, certain questions arose in connection with the use of TTC, especially in relation with malic dehydrogenase determination. Since the expected amount of the two enzymes in plasma, if any, may be very small (because of the low values reported for whole blood) it is necessary to work under optimal condition. A preliminary investigation was made to ascertain the following:

1) The effect of varying concentrations of TTC on malic and succinic dehydrogenase activity.

2) The effects of the absence or presence of DPN, KCN, and diaphorase in the reduction of TTC by the malic dehydrogenase system.

3) The effect of varying the concentration of DPN, on the reduction of TTC by malic dehydrogenase.

4) The pH curve of malic and succinic dehydrogenase system in the reduction of TTC.

5) The effect of varying the concentration of buffer in the reaction of TTC by malic and succinic dehydrogenases.

Rat kidney and liver were used as sources of the two enzymes. The works and methods of Kuhn and Abood (37) and of Jansen (29) were arbitrarily taken as a starting point.

The concentration of the factors, the effects of which were to be determined, was varied over a reasonable range. The degree of the reduction of the dye in comparison with the arbitrary basis or standard, gives in each case an idea of how the enzyme reaction was influenced by the variation in concentration.
The result of this investigation was most useful in the case of the malic dehydrogenase system where there were no previous data comparable to those of Kuhn and Abood who used succinic dehydrogenase.

The results of these investigations are summarized in Table I.

**TABLE I**

**OPTIMUM CONDITIONS FOR ENZYME ASSAY**

<table>
<thead>
<tr>
<th>Factor</th>
<th>System</th>
<th>Optimum Cone and pH Range</th>
<th>Final Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTC</td>
<td>Both</td>
<td>0.12%</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>DPN</td>
<td>Malic</td>
<td>0.072%</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>Phosphate</td>
<td>Both</td>
<td>m/2—m/32 (range tested)</td>
<td>(range tested)</td>
</tr>
<tr>
<td>pH</td>
<td>Malic</td>
<td>8.1—8.6</td>
<td>2.5 ml</td>
</tr>
<tr>
<td></td>
<td>Succinate</td>
<td>7.5—7.8</td>
<td>2.5 ml</td>
</tr>
</tbody>
</table>

The concentrations of substrates, dye buffer, homogenate, with the exception of those factors that are being varied, are those used by Kuhn and Abood, (succinic) and Jensen (malic). The preceding tabulated summary, (Table I) was obtained from the experimental data in Table II, III, and IV.
TABLE II

EFFECT OF VARYING THE CONCENTRATION OF THE DYE (TTC) ON MALIC AND SUCCINIC DEHYDROGENASES ACTIVITY USING THE METHOD OF KUHN AND ABOOD AS BASIS.

<table>
<thead>
<tr>
<th>Concentration of dye (in %)</th>
<th>Malic Dehydrogenase</th>
<th>Succinic Dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>.005%</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>.01%</td>
<td>.010</td>
<td>0</td>
</tr>
<tr>
<td>.025%</td>
<td>.025</td>
<td>.065</td>
</tr>
<tr>
<td>.05%</td>
<td>.155</td>
<td>.123</td>
</tr>
<tr>
<td>.1%</td>
<td>.365</td>
<td>.133</td>
</tr>
<tr>
<td>.2%</td>
<td>.350</td>
<td>.140</td>
</tr>
<tr>
<td>.25%</td>
<td>.350</td>
<td>.120</td>
</tr>
<tr>
<td>.3%</td>
<td>.350</td>
<td>.260</td>
</tr>
<tr>
<td>.35%</td>
<td>.470</td>
<td>.650</td>
</tr>
<tr>
<td>.4%</td>
<td>.550</td>
<td>.375</td>
</tr>
<tr>
<td>.45%</td>
<td>.450</td>
<td>.275</td>
</tr>
<tr>
<td>.50%</td>
<td>.295</td>
<td>.375</td>
</tr>
</tbody>
</table>

Figure I is the Graphical Representation of These Data

TABLE III

THE EFFECT OF VARYING THE CONCENTRATION OF DPN IN RAT KIDNEY MALIC DEHYDROGENASE ACTIVITY USING THE METHOD OF JANSEN.

<table>
<thead>
<tr>
<th>DPN Concentration</th>
<th>Total % of Rx mixture</th>
<th>Optical Density 334 mu</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of one ml added</td>
<td></td>
<td></td>
</tr>
<tr>
<td>to Rx mixture of 2.5 ml.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>.016%</td>
<td>.0071%</td>
<td>0</td>
</tr>
<tr>
<td>.02%</td>
<td>.0088%</td>
<td>.120</td>
</tr>
<tr>
<td>.04%</td>
<td>.0176%</td>
<td>.225</td>
</tr>
<tr>
<td>.08%</td>
<td>.0352%</td>
<td>.600</td>
</tr>
<tr>
<td>1.2%</td>
<td>.0428%</td>
<td>1.132</td>
</tr>
<tr>
<td>1.6%</td>
<td>.0704%</td>
<td>1.150</td>
</tr>
<tr>
<td>1.8%</td>
<td>.0792%</td>
<td>1.185</td>
</tr>
</tbody>
</table>

The Graphical Representation is Shown in Figure II.
METHOD OF KUHN AND ABBO, AS BASIS
DEHYDROGENASE ACTIVITY USING THE
OF THE DE (L-L, G) ON MALIC AND SUCCHINIC
EFFECT OF VARYING THE CONCENTRATION

OPTICAL DENSITY

OPTICAL DENSITY

SUCCHINIC DEHYDROGENASE
FIG II

THE EFFECT OF VARYING THE CONCENTRATION OF DPN IN RAT KIDNEY MALIC DEHYDROGENASE ACTIVITY USING THE METHOD OF TIPSÖN

DPN (PERCENT DPN)

OPTICAL DENSITY
TABLE IV

THE EFFECT OF pH VARIATION ON MALIC AND SUCCINIC DEHYDROGENASES
OF RAT KIDNEY.

<table>
<thead>
<tr>
<th>Tubes</th>
<th>Tubes</th>
<th>pH Malic</th>
<th>pH Succinic</th>
<th>Optical Density 485mu Malic</th>
<th>Optical Density 485mu Succinic</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>A</td>
<td>6.3</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>B</td>
<td>7</td>
<td>6.2</td>
<td>.082</td>
<td>.075</td>
</tr>
<tr>
<td>C</td>
<td>C</td>
<td>7.3</td>
<td>6.695</td>
<td>.100</td>
<td>.095</td>
</tr>
<tr>
<td>D</td>
<td>D</td>
<td>7.4</td>
<td>7</td>
<td>.125</td>
<td>.2</td>
</tr>
<tr>
<td>E</td>
<td>E</td>
<td>7.65</td>
<td>7.5</td>
<td>.225</td>
<td>.475</td>
</tr>
<tr>
<td>F</td>
<td>F</td>
<td>7.8</td>
<td>7.6</td>
<td>.375</td>
<td>.480</td>
</tr>
<tr>
<td>G</td>
<td>G</td>
<td>8</td>
<td>7.7</td>
<td>.455</td>
<td>.460</td>
</tr>
<tr>
<td>H</td>
<td>H</td>
<td>8.5</td>
<td>7.8</td>
<td>.460</td>
<td>.465</td>
</tr>
<tr>
<td>I</td>
<td>I</td>
<td>8.6</td>
<td>8</td>
<td>.455</td>
<td>.320</td>
</tr>
<tr>
<td>J</td>
<td>J</td>
<td>9</td>
<td>8.3</td>
<td>.223</td>
<td>.095</td>
</tr>
<tr>
<td>K</td>
<td>K</td>
<td>9.5</td>
<td>8.5</td>
<td>.125</td>
<td>.010</td>
</tr>
<tr>
<td>L</td>
<td>L</td>
<td>10</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>M</td>
<td>M</td>
<td>11</td>
<td>11</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The Graphical Representation is Shown in Figures IIIa and IIIb.

The effect of omission of DPN, KCN, and Diaphorase respectively on the reduction of TTC by rat liver malic dehydrogenase is shown in Figure IV.

FIGURE IV

THE EFFECT OF OMISSION OF DPN, KCN, AND DIAPHORASE IN THE REDUCTION OF 2,3,5-TTC BY RAT LIVER MALIC DEHYDROGENASE.

Test Tubes

Factors Missing (Diaphorase) KCN DPN Nothing DPN Diaphorase

Optical Density .680 .595 1.30 1.37 .165 .155 .780 .790 .05
dil X4

.680 .710
THE EFFECT OF pH VARIATION ON MALIC AND SUCCINIC DEHYDROGENASES OF RAT LIVER.

OPTICAL DENSITY

FINAL pH

6 7 8 9 10

MALIC DEHYDROGENASE
SUCCINIC DEHYDROGENASE
From the results in Fig. IV, it appears that KCN inhibits the reduction of the dye by malic dehydrogenase. The importance of DPN on the other hand is very strikingly shown by the results. It can be safely postulated that the slight reaction without added DPN is due to DPN in the tissue homogenate.

Diaphorase fortified the reaction in a way but does not seem to be necessary for the reaction. Since the previous workers (37) (29) reported that diaphorase is not required for the reaction, the prepared diaphorase was tested, and found to show a little dehydrogenase activity in the presence of potassium malate. It was concluded therefore that the fortifying effect was due to this dehydrogenase activity.

The result of the preceding preliminary investigation suggested the slight modification of the procedures of Kuhn et al, and Jenson, as follows:

Kuhn and Abood worked at final pH of 7.2 - 7.4 but the preceding results suggested a higher pH - 7.5 - 7.7 - for succinic dehydrogenase. Jensen worked at pH of 7, while a still higher pH of 8.0 - 8.6 seemed to be suggested by the results obtained in the pH curve. The concentration of the dye which gave highest reduction was not .04% of total final reaction mixture (which was used by Kuhn and Abood) but .12% of the total final reaction mixture. Instead of "equal volume" of .1% DPN suggested by Jensen, "equal volume" of .2% DPN was found to give greater reduction.

Finally, the reaction was run anaerobically since Kuhn and Abood reported better yield of reduction under this condition. The slight modification seems to be justified by the results obtained after modification as compared by the results before. These results are shown in Table V. The results were obtained by testing both methods using as sources of enzymes rat liver, kidney, brain and blood.
3. Testing of Method.

Using the following procedure which is outlined in five steps, rat liver, kidney, blood and brain homogenates were used as sources of enzymes for testing the malic and succinic dehydrogenase activity.

Step I: Addition of reaction mixture minus homogenate:

Eight Thunberg tubes were lined up in a rack which was half immersed in a vessel containing plenty of ice cubes. To six of the eight Thunberg tubes the following were added:

1. .5 ml. of .5% TTC (modified) or 1 ml. of .1% TTC (unmodified)
2. .5 ml. of .2 M Na succinate or K malate.
3. .5 ml. of M/10 phosphate buffer (7.8 pH)

Total final pH 7.7 modified or 7.3 unmodified
8.3 for malate modified
or 7 for unmodified

Two of the tubes had buffer of pH 7.4 substituted for succinate or malate.

Step II: Preparation and addition of homogenate:

When all the components of the reaction mixture were ready and added adult white rats which were normally fed, were decapitated. The liver, kidney, brain or blood were collected and weighed in a beaker containing M/30 phosphate buffer (about 5-10 ml.). The weighed organ was homogenized in 9 volumes of M/30 phosphate buffer containing an equimolar quantity of K and Na and at pH 7.4. The homogeniser consists of a pyrex test tube (13 x 100 mm) and a snug fitting pestle beaded at the tip for cutting. It was operated by hand. The time of homogenization was between 2 and 5 minutes. The homogenate was filtered through cheese cloth and the filtrate collected.
.5 ml. of this homogenate was pipetted into each of the eight Thunberg tubes except for two of the first six which substituted buffer (pH 7.4) for the homogenate as enzyme controls. Thus there were four controls: two without substrates, and the other two without enzymes.

Step III: Evacuation, Incubation:

In cases of anaerobic determination of enzyme activity, the tubes were evacuated for 5 - 10 minutes to a pressure of 6 mm Hg. The tubes were then incubated for 60 - 90 minutes in a water bath at 38 C.

Step IV: Precipitation with Acetone:

After 60 - 90 minutes, the reaction was stopped by the addition to each tube of 7.5 ml. of acetone. The mixtures were shaken vigorously and stored in the dark at 0.5 C for 20 - 24 hours.

Step V:

The acetone precipitated the protein of the reaction mixture. At the same time it dissolved the water insoluble formozan resulting from the reduction of TTC by the enzyme or other reducing agent present. The precipitated protein was centrifuged off and the clear transparent orange red color of the formozan read in a Beckman DU spectrophotometer at 485 μm.

Step VI: Calculation:

The amount of reduced formozan in micrograms was calculated from a standard curve made by plotting varying concentration of reduced formozan (400 μ - 25 μ) against the corresponding optical density. This is essentially the method of Yoshio Sawada et al (71) and Kuhn and Abood (37).

Preparation of Standard Curve:

Reduction of TTC by sodium hydrosulfite.

The formozan used to prepare the standard curve was prepared by
reducing 0.8 gms of TTC with 10 ml. of 20% sodium hydrosulfite. The reaction was at room temperature for thirty minutes with constant stirring. The precipitated formozan was filtered by gravity, and washed several times with distilled water. Two ml. of 75% acetone were used to redissolve the formozan and 15 to 20 ml. of distilled water to recrystallize it. After purification by recrystallization and washing several times with distilled water, the formozan was weighed and dried. The yield was about 60%. 25–400 mg of formozan were serially dissolved in 10 ml. each of 75% acetone and 25% water. The corresponding optical densities were read in a Beckman DU spectrophotometer at 485 mu. The standard curve was drawn from the data in Table V.

**TABLE V**

**OPTICAL DENSITIES OF KNOWN AMOUNTS OF FORMOZAN.**

<table>
<thead>
<tr>
<th>formozan dissolved in 10 ml. of mixture of 1 part of H₂O and 3 parts of acetone</th>
<th>Optical density</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>25</td>
<td>.125</td>
</tr>
<tr>
<td>50</td>
<td>.250</td>
</tr>
<tr>
<td>100</td>
<td>.400</td>
</tr>
<tr>
<td>150</td>
<td>.700</td>
</tr>
<tr>
<td>200</td>
<td>.720</td>
</tr>
<tr>
<td>250</td>
<td>.820</td>
</tr>
<tr>
<td>300</td>
<td>1.15</td>
</tr>
<tr>
<td>350</td>
<td>1.20</td>
</tr>
<tr>
<td>400</td>
<td>1.23</td>
</tr>
</tbody>
</table>

The graph follows (Fig. V).
The standard curve has an optimum range between $25\gamma (0.125)$ and $250\gamma (0.820)$ formozan concentration. When the concentration of the reduced formozan in the reaction mixture was above the optimal range, the colored solution was diluted in order to bring it within the optimal range. This optimal range concentration times the dilution factor gave the original concentration of the formozan in the reaction mixture.

Since Fahmy and Walsh suggested that the formozan is not stable in acetone (19), toluene was tried as an extracting solvent. This involved some difficulty. In the first place the protein of the reaction mixture was not precipitated. Therefore one could not be sure that the reaction was stopped. In the second place, there was a much lower yield of the formozan when compared to the yield when acetone was used. Finally, contrary to the conclusion of Fahmy and Walsh, a solution of 10 mgm formozan (which was prepared as described under Preparation of Standard Curve) dissolved in 100 ml. of 3 parts of acetone and 1 part of water was stored in this laboratory for six months, (three months in the dark and three in light) without any noticeable change of color intensity.

Calculation: According to Kuhn and Abood (37) the number of units of enzyme was calculated as follows:

$$\text{No of units of enzyme} = \frac{\text{dye reduced for the first 10 minutes}}{\text{mgn of tissue (wet)}}$$

$$= \frac{\text{formozan} \times 10}{55 \times 60}$$

Explanation:

(a) Since 15 ml. of 10% homogenate was used, this homogenate contained about 55 mgm of tissue (wet).

(b) Since the incubation time was 60 minutes, the first 10 minutes
(assuming linear relationship) could be roughly estimated by taking 1/6 of 60 minutes. (This assumption may not be entirely correct in a strict quantitative work, but suffices for the present purpose of the experiment.)

(c) formozan was calculated directly from the standard curve already referred to.

(d) The mean optical density, which corresponds to formozan of the standard curve, is the average of the optical densities of the four tubes minus the average optical densities of the two control tubes. The remaining two control tubes without homogenate (enzyme controls) were used as blanks by setting them at zero in the spectrophotometer before measuring the other tubes. The details of a typical experiment are summarized in Table VI.

**TABLE VI**

**SET-UP OF A TYPICAL EXPERIMENT**

<table>
<thead>
<tr>
<th>Tube</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
</tr>
</thead>
<tbody>
<tr>
<td>.5% 2,3,5 TTC</td>
<td>.5ml</td>
<td>.5</td>
<td>.5</td>
<td>.5</td>
<td>.5</td>
<td>.5</td>
<td>.5</td>
<td>.5</td>
</tr>
<tr>
<td>M/10 phosphate buffer</td>
<td>.5ml</td>
<td>.5</td>
<td>.5</td>
<td>.5</td>
<td>.5</td>
<td>.5</td>
<td>.5</td>
<td>.5</td>
</tr>
<tr>
<td>.2 M K malate</td>
<td>.5ml</td>
<td>.5</td>
<td>.5</td>
<td>.5</td>
<td>.5</td>
<td>.5</td>
<td>.5</td>
<td>.5</td>
</tr>
<tr>
<td>.2% DPN</td>
<td>.5ml</td>
<td>.5</td>
<td>.5</td>
<td>.5</td>
<td>.5</td>
<td>.5</td>
<td>.5</td>
<td>.5</td>
</tr>
<tr>
<td>10% homogenate</td>
<td>.5ml</td>
<td>.5</td>
<td>.5</td>
<td>.5</td>
<td>.5</td>
<td>.5</td>
<td>.5</td>
<td>.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total final pH</td>
<td>8.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time of incubation</td>
<td>60 minutes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temperature of incubation</td>
<td>38 C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total final volume</td>
<td>2.5 ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

After incubation the reaction was stopped by the addition of 7.5ml of acetone, the mixture was shaken vigorously and left at +0.5C for 20-24 hours. The precipitated protein was centrifuged and the optical den-
sities of the supernatant residue of the tubes A-F were read in the spectrophotometer with G and H tubes serving as blanks.

The reading of the original 10 ml. of the reaction mixture (2.5 ml. reaction mixture plus 7.5 ml. acetone) was so much above the optimal range of the spectrophotometric readings that a dilution factor of four was necessary to bring it to the optimal range. The data are shown in Table VII.

**TABLE VII**

**DATA USED IN CALCULATION OF KUHN AND ABOOD UNIT**

<table>
<thead>
<tr>
<th>Tube</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final volume after addition of 7.5 ml. acetone</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Optical density after dilution to 20 ml.</td>
<td>1.20</td>
<td>1.35</td>
<td>1.20</td>
<td>1.35</td>
<td>1.620</td>
<td>0.600</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Optical density after dilution to 40 ml.</td>
<td>0.550</td>
<td>0.680</td>
<td>0.650</td>
<td>0.680</td>
<td>0.300</td>
<td>0.290</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Formozan of 10 ml of the 40 ml. dilution (std. curve)</td>
<td>145</td>
<td>185</td>
<td>173</td>
<td>185</td>
<td>81</td>
<td>80</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Multiplication of the above X 4 gives total formozan in Rv as follows</td>
<td>580</td>
<td>740</td>
<td>692</td>
<td>740</td>
<td>334</td>
<td>330</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Correcting for endogenous and other extraneous reduction, the average difference = 688 - 332 = 356

Unit of enzyme according to Kuhn and Abood = \( \frac{356 \times 10}{55 \times 60} = 1.08 \text{ Dye reduced mgm of tissue} \) 10 minutes (wet)

Table VIII is the tabulation of results obtained by testing the two methods with fourteen normal white rats. In each case the results were obtained by calculations similar to those already described.
TABLE VIII

MALIC AND SuccINIC DEHYDROGENASE ACTIVITY OF NORMAL RAT TISSUES - TTC REDUCTION METHOD.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Substrate</th>
<th>Result using the method of Kuhn and Abbo d and C.O. Jensen</th>
<th>Result using modified method.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Average Enzyme units dye reduced formazan. units</td>
<td>Average Enzyme units mgm of tissue.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>formazan. units units 10 minutes</td>
<td>etc.</td>
</tr>
<tr>
<td>Kidney</td>
<td>K malate</td>
<td>A 282 .854</td>
<td>575 1.74</td>
</tr>
<tr>
<td>Rats A, B, C, D, E, F</td>
<td></td>
<td>B 289 .875</td>
<td>600 1.88</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C 275 .833</td>
<td>520 1.675</td>
</tr>
<tr>
<td></td>
<td>Na succinate</td>
<td>A 229 .685</td>
<td>D 500 1.54</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B 259 .660</td>
<td>E 505 1.54</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C 249 .750</td>
<td>F 490 1.48</td>
</tr>
<tr>
<td>Liver</td>
<td>K malate</td>
<td>G 356 1.07</td>
<td>J 644 1.94</td>
</tr>
<tr>
<td>Rats G, H, I, J</td>
<td></td>
<td>H 367 1.11</td>
<td>K 680 2.06</td>
</tr>
<tr>
<td></td>
<td>Na succinate</td>
<td>G 204 .618</td>
<td>J 490 1.48</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H 175 .530</td>
<td>K 450 1.39</td>
</tr>
<tr>
<td></td>
<td></td>
<td>I 209 .635</td>
<td>L 500 1.54</td>
</tr>
<tr>
<td>Brain</td>
<td>K malate</td>
<td>M 22 .069</td>
<td>O 240 .72</td>
</tr>
<tr>
<td>Rats M, N, O, P</td>
<td></td>
<td>N 27 .081</td>
<td>P 230 .870</td>
</tr>
<tr>
<td></td>
<td>Na succinate</td>
<td>M 44 .13</td>
<td>O 300 .9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N 32 .09</td>
<td>P 290 .875</td>
</tr>
<tr>
<td>Blood</td>
<td>Both</td>
<td>0 0 0 0</td>
<td>0 0 0 0</td>
</tr>
</tbody>
</table>

The only results available in the literature pertaining to the reduction of TTC are those of Kuhn and Abbo d in the determination of succinic dehydrogenase in rat kidney, liver and brain, viz., Table IX.
TABLE IX

ENZYME UNITS REPORTED IN THE LITERATURE. KIDNEY, LIVER AND BRAIN, SUCCINIC AND MALIC DEHYDROGENASES. TTC REDUCTION METHOD.

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Kuhn and Abood (43)</th>
<th>Jensen (35)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Enzyme units</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Succinic dehydrogenase</td>
<td>Malic dehydrogenase</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.72</td>
<td>No quantitative data</td>
</tr>
<tr>
<td>Liver</td>
<td>1.56</td>
<td>&quot;</td>
</tr>
<tr>
<td>Brain</td>
<td>.75</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

There is good agreement between the literature data and the experimental results, although the experimental results are in some cases somewhat lower than the literature data. This is true only with the modified method. With the direct method only approximately half the result reported by Kuhn and Abood could be obtained. Since these experiments were done with different batches of rats on different days, it is difficult to say whether the remarkable difference in results due to the use of the modified method was due to the change of method per se, or to individual variation in enzyme content of the rat tissues used. This is especially so with the succinic dehydrogenase system where there was hardly any modification.

There has also been justifiable doubt as to the validity of calculating Kuhn and Abood units from the modified method. Accordingly a new unit system was adopted. A unit of enzyme, according to this new system is the amount of enzyme necessary to reduce one gamma of dye in a given time. This unit is a simpler one and since all the ten percent
homogenates contain approximately 55 mgm of wet tissue, it is not necessary to divide by this weight. Therefore using an incubation period of one hour, the column under Average Formozan Units becomes the units of enzyme. (Table VIII)

4. Sensitivity of the Method:

The next question is how sensitive is this method. In other words, how far can the homogenate be diluted before there ceases to be any noticeable reduction of the dye? This was tested for by using serially diluted homogenate as source of enzyme. Table X is the result of an experiment with malic dehydrogenase of rat kidney.

**TABLE X**

<table>
<thead>
<tr>
<th>Concentration of homogenate</th>
<th>Optical Density</th>
<th>New Enzyme Unit</th>
<th>Unit of Kuhn &amp; Abood</th>
</tr>
</thead>
<tbody>
<tr>
<td>10%</td>
<td>1.38</td>
<td>400</td>
<td>1.21</td>
</tr>
<tr>
<td>5%</td>
<td>0.680</td>
<td>190</td>
<td>0.57</td>
</tr>
<tr>
<td>2 1/2%</td>
<td>0.335</td>
<td>90</td>
<td>0.27</td>
</tr>
<tr>
<td>1 1/2%</td>
<td>0.137</td>
<td>40</td>
<td>0.12</td>
</tr>
<tr>
<td>5/8%</td>
<td>0.065</td>
<td>22</td>
<td>0.06</td>
</tr>
<tr>
<td>5/16%</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

From the preceding data it could be concluded that this method can pick up at least a concentration of enzyme units of .06 (Kuhn & Abood) or 22. (new unit). In other words the enzyme in this tissue is detectable colorimetrically or visually provided that at least 22 formozan per 10 ml. of solution are formed by the system.

Preparation of plasma and serum.

Blood was drawn from subjects and put into a large test tube (2 cm X 25 cm) containing a few mgms of Liquoid (Hoffman LaRoche Co.) as the anticoagulant. Mixing was done gently to avoid hemolysis. Centrifugation at top speed (1000 xg) for 60 minutes, essentially the method of Straubia and McGraw et al (61a) was used to separate the formed elements from the plasma. After centrifugation, a distinct buffy white coat separated the clear transparent plasma above from the red, thick formed elements below. The plasma was removed with a dropper, care being taken to stay as far as possible away from the buffy coat.

To test for the presence of leucocytes, platelets, etc., in the plasma, the plasma was recentrifuged. The bottom sediment was smeared on slides and Wright-stained. The stained slides showed no evidence of contaminating formed elements.

Serum was finally used, in order to minimize the chances of contamination by formed elements, most of which are known to contain malic and succinic dehydrogenases (35).

Direct application of the modified method using 0.5 ml of serum in place of 10% homogenate did not show any reduction under aerobic conditions. Anaerobically, however, there was reduction. The reduction increased much more when the time of incubation was lengthened from 6 to 12 hours. Increased concentration of serum (from 0.5 ml to 1.0 ml) also increased the reduction but did not increase it two fold.

The much higher reduction obtained anaerobically led to the suspicion that one might be dealing with bacterial contamination. The possibility
of bacterial contamination was, however, eliminated by:

(1) Preparing the serum under aseptic condition, and
(2) setting up controls containing everything but serum
(which was substituted for by a buffer with pH 7.8).

The controls in six of seven experiments did not show any reduction while samples containing serum reduced.

Since the reduction of the dye seemed greater in the tubes with potassium malate and sodium succinate than in the tubes without those substrates, it appeared that succinic and malic dehydrogenases might be present in the serum or plasma. With Na succinate, the difference was very slight. However, with K malate the difference was more marked and deserved further investigation. These results obtained from about 20 subjects appear in Table XI.

6. Inhibition Test:

It was decided therefore, to test the presence of whatever enzyme is responsible for the reduction in a negative way by use of specific enzyme inhibitor. NaF (45) in the concentration range of .01 M to .1 M was added to the reaction mixture. This did not inhibit the reduction of TTC by the serum with potassium malate as substrate as compared with the same serum without the inhibitor. Since NaF of this concentration range was known to inhibit malic dehydrogenase at least in reactions with methylene blue, failure to reduce in this case led to the suspicion that the entire dye-reduction in the serum might not be enzymatic.

7. Heat Test:

Heating first to 65 C for 30 minutes and later to 70 C for 15 minutes did not inhibit or stop the reduction of the dye. In some cases the boiled samples actually reduced more than the unboiled samples. Heating
to 70 C for 15 minutes caused coagulation of the serum. Distilled water was used to suspend the coagulum in solution before it could be used for the experiment. In a few cases there was no reduction. The explanation of this is not known.

Possible explanation:

Several theories may be offered to explain what was happening in the test tubes of the reaction mixtures. The first theory is that perhaps the enzyme present was destroyed, but that heating liberated sulfhydryl groups which still reduced this sensitive dye.

The second theory is that the enzymes are too stable to be destroyed by heating at 65 C for 30 minutes or at 70 C for 15 minutes.

The third theory is that the reaction as a whole is not enzymatic, but that the addition of potassium malate might have produced a redox potential more favorable to the reduction of the dye than should have been the case were potassium malate omitted.

The second explanation is the weakest. The first and third theories would be an interesting field for further investigation. The results which led up to these conclusions are shown in Tables XI and XII.
### TABLE XI

**MALIC AND SUCCINIC DEHYDROGENASE ACTIVITY OF HUMAN BLOOD**

<table>
<thead>
<tr>
<th>Subject</th>
<th>Malic Dehydrogenase</th>
<th>Succinic Dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Optical Densities</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A B C D A B C D A B C D A B C D A B C D</td>
<td></td>
</tr>
<tr>
<td>R.B.</td>
<td>0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0</td>
<td></td>
</tr>
<tr>
<td>A. A.</td>
<td>0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0</td>
<td>0.085 0.060 0.075 0.06 0.040</td>
</tr>
<tr>
<td>A. A.</td>
<td>0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0</td>
<td>0.125 0.105 0.125 0.095 0.095</td>
</tr>
<tr>
<td>O. R.</td>
<td>0.590 0.550 0.420 0.460 0.055 0.065 0.045 0 0 0 0.085 0.040 0.020 0 0 0 0 0 0 0 0 0 0 0 0</td>
<td></td>
</tr>
</tbody>
</table>

**Anaerobic**

<table>
<thead>
<tr>
<th>Subject</th>
<th>Malic Dehydrogenase</th>
<th>Succinic Dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Optical Densities</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A B C D A B C D A B C D A B C D A B C D</td>
<td></td>
</tr>
<tr>
<td>R. B.</td>
<td>0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0</td>
<td>0.175 0.145 0.04 0.04</td>
</tr>
<tr>
<td>O. R.</td>
<td>0.200 0.195 0.06 0.06</td>
<td></td>
</tr>
<tr>
<td>R. R.</td>
<td>0.230 0.220 0.08 0.08</td>
<td></td>
</tr>
<tr>
<td>M. R.</td>
<td>0.360 0.350 0.250 0.225 0.175 0.09 0.08 0.100 0.090 0.03 0.150 0.130 0.110</td>
<td></td>
</tr>
<tr>
<td>M. R.</td>
<td>0.340 0.300 0.215 0.100 0.090 0.03</td>
<td></td>
</tr>
<tr>
<td>A. A.</td>
<td>0.650 0.636 0.650 0.686 0.550 0.085 0.150 0.06 0.075 0.05</td>
<td></td>
</tr>
<tr>
<td>A. A.</td>
<td>0.236 0.235 0.100 0.100 0.050 0.065 0.06 0.030 0.030</td>
<td></td>
</tr>
<tr>
<td>O. R.</td>
<td>0.100 0.085 0.0 0.0</td>
<td></td>
</tr>
<tr>
<td>O. R.</td>
<td>0.105 0.095 0.01 0.0</td>
<td></td>
</tr>
<tr>
<td>J. C.</td>
<td>0.355 0.340 0.250 0.030 0.045 0.0</td>
<td></td>
</tr>
<tr>
<td>M. N.</td>
<td>0.265 0.295 0.1 0.030 0.045 0.0</td>
<td></td>
</tr>
<tr>
<td>J. C.</td>
<td>0.250 0.200 0.150 0.030 0.045 0.0</td>
<td></td>
</tr>
</tbody>
</table>

**Pooled Serum**

<table>
<thead>
<tr>
<th>Determination #</th>
<th>Optical Densities</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>0.200 0.205 0.120</td>
</tr>
<tr>
<td>#2</td>
<td>0.180 0.170 0.105</td>
</tr>
<tr>
<td>#3</td>
<td>0.160 0.170 0.090</td>
</tr>
<tr>
<td>#4</td>
<td>0.245 0.275 0.147</td>
</tr>
<tr>
<td>#5</td>
<td>0.085 0.075 0.0</td>
</tr>
</tbody>
</table>
TABLE XII

Malic and Succinic Dehydrogenase Activity of Human Blood Condensed From Table XI.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Malic Dehydrogenase</th>
<th>Optical Densities</th>
<th>Succinic Dehydrogenase</th>
<th>Plasma (serum)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R.B.</td>
<td>.045</td>
<td></td>
<td>.035</td>
<td></td>
</tr>
<tr>
<td>A.O.</td>
<td>.04</td>
<td></td>
<td>.04</td>
<td></td>
</tr>
<tr>
<td>O.R.</td>
<td>.045</td>
<td></td>
<td>.025</td>
<td></td>
</tr>
<tr>
<td>A.C.</td>
<td>.055</td>
<td></td>
<td>X</td>
<td></td>
</tr>
</tbody>
</table>

Anaerobic

<table>
<thead>
<tr>
<th>Subject</th>
<th>Optical Densities</th>
<th>Plasma (serum)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R.B.</td>
<td>.120</td>
<td>.070</td>
</tr>
<tr>
<td>O.R.</td>
<td>.137</td>
<td>.030</td>
</tr>
<tr>
<td>R.R.</td>
<td>.140</td>
<td>.030</td>
</tr>
<tr>
<td>M.R.</td>
<td>.110</td>
<td>.040</td>
</tr>
<tr>
<td>M.R.</td>
<td>.105</td>
<td>.050</td>
</tr>
<tr>
<td>A.A.</td>
<td>rec.=.05</td>
<td>*rec.=.065</td>
</tr>
<tr>
<td></td>
<td>whole=07</td>
<td>whole=.075</td>
</tr>
<tr>
<td>A.A.</td>
<td>.135</td>
<td>.030</td>
</tr>
<tr>
<td>O.R.</td>
<td>.092</td>
<td>.045</td>
</tr>
<tr>
<td>O.R.</td>
<td>.100</td>
<td>.035</td>
</tr>
<tr>
<td>J.C.</td>
<td>.097</td>
<td>.06</td>
</tr>
<tr>
<td>M.N.</td>
<td>.105</td>
<td>.08</td>
</tr>
<tr>
<td>J.C.</td>
<td>.119</td>
<td>.04</td>
</tr>
</tbody>
</table>

Pooled serum

A (boiled) .070
B (not boiled) .085
C (boiled) .099
D (not boiled) .065
E (Boiled) .085
F (NaF) .095
G (no NaF) .097

W.M.
Fresh (not boiled) .110
1st boiled .094
2nd boiled .115

* The figures in Table XII were obtained by calculating the average of the duplicate samples in Table XI. The values for reconstituted blood were the dehydrogenase activity obtained when the packed cells were isolated from their supernatant plasma and reconstituted to original volumes with isosmotic albumin solution. These values were compared with values obtained with hemolyse whole blood in order to see how much difference the absence of plasma would make
II THE THUNBERG TECHNIQUE (M B REDUCTION METHOD)

The measurement of dehydrogenase activity by the use of the Thunberg technique was adopted as a means of checking the comparatively new method of TTC. The works of Elliot et al (18), Green (23), Stread (61), and of Gale and Stephenson (22) are important references in this connection.

1. Preparations:

Since according to these workers, cytochrome c and diaphorase are necessary in the MB reduction method for succinic and malic dehydrogenases assay, their preparation was undertaken.

(a) Cytochrome C:

This was prepared from frozen horse and ox heart respectively. The method of Keilin and Hartree (31, 32, 33) was used.

Test for property and purity of cytochrome c.

The spectra of the dark red solution prepared were followed in the visible region between the wave lengths of 470 - 800 μm. According to Keilin and Hartree (30), four different bands of cytochrome c appear at different pH's; three of them between the pH's 4 and 8. These maxima are at 530, 560, 695 μm. In the sample prepared, only one peak appeared at 530 μm. Ox heart was tried and again only one peak appeared at 530 μm. Then a few mgs from a commercial product (Nutritional Biochemicals) was tested, and again only one peak appeared at 530 μm. Since the Beckman DU spectrophotometer is very sensitive and presumably capable of separating the various wave lengths, this anomaly could not be accounted for.
Since the commercial product behaved in all other respects, (color, solubility, etc.), with the prepared sample it was decided to pass the preparation as good. The results of the tests are graphically illustrated in Fig. VI.

The concentration of the prepared cytochrome was calculated from a standard curve (graph of optical densities vs. concentration) made with a few mgms of commercial product diluted serially. The concentration was found to be about 185 mgm/100 ml of solution; the measured optical density being 0.810 as shown below. (Fig. VII)

(b) Diaphorase

This was prepared from rabbit skeletal muscle according to the method of Dewan and Green (17b). Measurement of diaphorase activity was done by following dehydrogenation of DPNH₂ by disappearance of its typical absorption band at 334 μm. The activity is expressed as decrease of extinction calculated for 0.1 ml enzyme during the first 10 minutes. (See Table XIV)

The DPN was reduced with sodium hydrosulfite according to the method of Lehinger (40). The extent of reduction was tested for by following the spectra in the ultra violet region between the wave lengths of 240 μm and 370 μm. The absorption peak of reduced DPN appeared at 334 μm, replacing to a great extent the absorption peak at 260 μm, characteristic of DPN. (See Fig. VIII) The reaction mixtures which were used to test the diaphorase activity are described in Table XIII.
FIG VII

OPTICAL DENSITY OF KNOWN AMOUNT OF CYTOCHROME C

OPTICAL DENSITY

mg of cytochrome C

2.0
# TABLE XIV

Measurement of Diaphorase Activity

<table>
<thead>
<tr>
<th>Mixing Time</th>
<th>1st Reading Time</th>
<th>1st Reading Time</th>
<th>2nd Reading Time</th>
<th>Optical Density</th>
<th>1st 10 minutes A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>Optical Densities</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>9.56</td>
<td>9.58</td>
<td>10.03</td>
<td>5 min.</td>
<td>.225</td>
<td>.450</td>
<td>.775</td>
<td>.550</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>10.05</td>
<td>10.8</td>
<td>10.13</td>
<td>5 min.</td>
<td>.195</td>
<td>.390</td>
<td>.620</td>
<td>.420</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>10.28</td>
<td>10.30</td>
<td>10.38</td>
<td>8 min.</td>
<td>.380</td>
<td>.475</td>
<td>.640</td>
<td>.260</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>10.33</td>
<td>10.35</td>
<td>10.42</td>
<td>7 min.</td>
<td>.320</td>
<td>.450</td>
<td>.650</td>
<td>.330</td>
<td></td>
</tr>
</tbody>
</table>

Control without diaphorase

<table>
<thead>
<tr>
<th>X</th>
<th>Y</th>
<th>Z</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.00</td>
<td>11.02</td>
<td>11.12</td>
</tr>
<tr>
<td>11.05</td>
<td>11.07</td>
<td>11.16</td>
</tr>
<tr>
<td>11.20</td>
<td>11.22</td>
<td>11.37</td>
</tr>
</tbody>
</table>

Average - for 1st 10 minutes = .4425 (with enzyme)
Average - for 1st 10 minutes = .1860 (with enzyme)
Enzyme activity = .2565

Ref.—Corran, Strub, and Green, Nature 143:337 (1939)
The absorption peak of reduced DPN (DPNH₂) appeared at 334 μm.
TABLE XIII

SET-UP FOR TESTING DIAPHORASE ACTIVITY

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/1000 MB</td>
<td>1/1000 MB</td>
</tr>
<tr>
<td>Diaphorase prep.</td>
<td>H2O</td>
</tr>
<tr>
<td>DPNH</td>
<td>DPNH</td>
</tr>
<tr>
<td>M/10 phosphate buffer (pH 7.4)</td>
<td>M/10 phosphate buffer (pH 7.4)</td>
</tr>
<tr>
<td>Total volume</td>
<td>Total volume</td>
</tr>
<tr>
<td>Temperature 24° C</td>
<td></td>
</tr>
</tbody>
</table>

Table XIV shows the results obtained.

2. Test of the Tunberg Technique with Rat Kidney and Liver as Sources of Enzymes.

Table XV shows the systems used to assay the malic and succinic dehydrogenase content of rat liver and kidney.

TABLE XV

<table>
<thead>
<tr>
<th>Succinic dehydrogenase</th>
<th>Tube</th>
<th>Malic dehydrogenase</th>
<th>Tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>b</td>
<td>c</td>
<td>a</td>
</tr>
<tr>
<td>1/1000 MB</td>
<td>.5ml</td>
<td>.5ml .5ml</td>
<td>1/1000 MB</td>
</tr>
<tr>
<td>.2M sodium succinate</td>
<td>1.5ml</td>
<td>1.5ml 0</td>
<td>.1M malate</td>
</tr>
<tr>
<td>M/15 phosphate buffer</td>
<td>1 ml</td>
<td>1 ml 1 ml</td>
<td>KCN M/33</td>
</tr>
<tr>
<td>10% tissue homogenate</td>
<td>1 ml</td>
<td>1 ml 1 ml</td>
<td>Diaphorase</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>DPH (2%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Buffer (8.5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10% Tissue Homogenate</td>
</tr>
</tbody>
</table>
TABLE XV continued.

Temperature -- 38° C (water bath)

Liver homogenate -- decolorization time = 43-45 mins. 6-8 min.
Kidney homogenate -- decolorization time = 20-22 mins. 15-16 min.
Control -- decolorization time = Liver 16-18 min. Kidney

Three test tubes A, B, and C were used for each system. The tube
C is the control in either case. The homogenates were prepared as in
the case of 2,3,5-triphenyltetrazolium chloride.

The liver succinic dehydrogenase took 43 - 45 minutes to decolorize,
while the control did not decolorize, at least after one day. The kidney
succinic dehydrogenase took 20 - 22 minutes to decolorize, while the con-
trols did not decolorize after a day. 6 - 8 minutes were required for
the malic dehydrogenase of the liver to decolorize while the control took
16 - 18 minutes. The kidney malic dehydrogenase took 15 - 16 minutes to
decolorize while the control took an infinite time to decolorize. Rat
blood tested with the above system did not decolorize MB for at least a
day.

Calculation:

\[
\text{Substrate } MB = \frac{ul \text{ O}_2 \text{ equivalent of total MB reduced}}{hr \times mgm \text{ tissue}} \\
= \frac{MX (t - t_{sub}) \times 60}{t_{sub} \times t}
\]

Where M = O$_2$ equivalent of the total MB reduced

\[
t = \text{time in minutes for reduction in absence of substrate}
\]

\[
t_{sub} = \text{time in minutes for reduction in presence of substrate}
\]
\[ t - t_{\text{sub}} = \text{fraction of total MB which is reduced by } t \text{ substrate} \]

Substrate MB = oxygen consumption (measure of dehydrogenase activity) of MB in presence of the specific substrate. Actually MB takes off (H)

Moles of MB = .001

Oxygen equivalent = .001 M

Oxygen equivalent in ul = \(22.4 \times 0.001 \times 1000\)

\[+ 22.4 \text{ ul} \]

Liver

Succinic MB = \(22.4 \times \frac{60}{45.5} = 59 \text{ mgm/tissue}\)

Kidney

Succinic MB = \(22.4 \times \frac{60}{22 \times 0.5} = 119 \text{ mgm/tissue}\)

The two values for succinic dehydrogenases in liver and kidney were 59/ mgm of tissue and 119/ mgm of tissue respectively, which compares well with the literature value, (18,53) of 66.4 and 138 per mgm of protein respectively.

The values for malic dehydrogenases were calculated to be 185/mgm of tissue and 176/mgm of tissue for liver and kidney respectively. These values are in the relative order of values obtained with the reduction of the TTC, viz:

<table>
<thead>
<tr>
<th>MB</th>
<th>(185)</th>
<th>(176)</th>
<th>(119)</th>
<th>(59)</th>
</tr>
</thead>
<tbody>
<tr>
<td>liver</td>
<td>malic</td>
<td>kidney</td>
<td>malic</td>
<td>kidney</td>
</tr>
<tr>
<td>dehydrogenase</td>
<td>dehydrogenase</td>
<td>succinic</td>
<td>succinic</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TTC----</th>
<th>(727)</th>
<th>(600)</th>
<th>(500)</th>
<th>490</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.20</td>
<td>1.88</td>
<td>1.54</td>
<td>1.48</td>
</tr>
</tbody>
</table>
3. Application of the method in the investigation of malic and succinic dehydrogenases in plasma:

It was found that the diaphorase prepared showed a little malic dehydrogenase activity. This might tend to make any positive results with plasma or serum ambiguous and equivocal. In an effort to solve the difficulty, heated serum was used as a control. This assumed that heating destroyed the enzyme and that any observed activity would be due to the malic dehydrogenase present in the diaphorase. On boiling, the serum turned somewhat milky white due to protein coagulation. This made it difficult to compare with the unboiled serum. Apart from this difficulty, there was no noticeable difference between decolorization times of the two samples. In each case this was about 45 minutes. This meant that the reduction could be attributed solely to the enzyme in the diaphorase. The succinic dehydrogenase system did not show any reduction when tested in plasma either with or without cytochrome c.

(a) Slight Modification

The concentration of methylene blue was reduced from M/1000 to M/5000. Since the reaction should take place in 14 minutes without diaphorase provided that the DPN was appreciably reduced in that time, diaphorase was entirely omitted. With this modified system there was very, very slight decolorization of methylene blue which was discernible only against a white background. Furthermore, the slight decolorization appeared after 3 hours incubation, not after 45 minutes.

(b) Inhibition Test

Once again, a specific enzyme inhibitor test was used to see whether the reduction could be due to this specific enzyme (malic dehydrogenase). NaF in the concentration range of
.001 M to 1 M could not inhibit this slight decolorization — just as in the case of formozan reduced by the serum.

The serum of two human subjects and pooled human serum were tested by the MB technique, all with the same results.
CONCLUSIONS AND DISCUSSION

This investigation can hardly be regarded as conclusive, though it strongly indicates that if the enzymes are present in human serum, their concentration is extremely low. Certain complex problems present themselves. While cyanide may inhibit cytochrome dehydrogenase or fix oxaloacetic acid and thereby promote reduction by inhibiting oxidation, it may also form reducing substances, one of which would be cystine from cysteine. While heating may destroy malic dehydrogenase of the plasma and thereby serve as adequate check or control, it may also liberate sulfhydryl groups which are good reducing agents. These reducing substances can and do vitiate the significance of the experimental results in certain oxidation reduction reactions.

Even if experimental results of this type show that plasma contains malic and succinic dehydrogenases, it would be difficult to prove that the enzymes do not result from mechanical friction or rupture of the formed elements. The platelets and leucocytes which are known to contain these enzymes are easily ruptured. It is possible that the force of centrifugation could do this. The fact that completely lysed platelets lose their dehydrogenase activity leaves the leucocytes alone to consider.

Tetrazolium salts, in spite of their inherent weakness, like sensitivity to pH, high alkalinity, sunlight and oxidation potential, are powerful new tools in biochemical investigations. Recent advances in the use of this group of dyes were reported recently by contemporary workers in the field at the February 14th meeting of the New York Academy of Science (1955). The discussions at that session showed that many of these investigations are beset with comparable unsolved problems.


ABSTRACT OF THESIS

Introduction

The aim of the experiment is to search for malic and succinic dehydrogenases in normal plasma or serum. The role which these two enzymes play in cellular metabolism, the unique way in which plasma reflects many cellular metabolic changes, the discovery of so many cellular enzymes in plasma, and the vagueness of knowledge about the relation of cellular enzymes to plasma proteins in general, seem to justify the search.

History

Malic and succinic dehydrogenases are widely distributed in certain tissues of most forms of life, from the bacterium to the primate.

Starting with Batelli (13a, 12, 13b) and Thunberg (63) who first discovered these enzymes, most workers however reported results of investigation of different tissues of the rat. Elliot and Greig (18) reported a considerable quantity of succinic dehydrogenase in rat kidney, liver, and heart. He found that addition of cytochrome c accelerates reaction. Potter, Schneider et al (49) reported rapid increase in succinic dehydrogenase activity during late embryonic and early post natal life. The activity in liver tumors was reported (56) to be one quarter normal. Meyer (166) disclosed that the activity in functional copora lutea of rat ovaries during pregnancy is two and one-half times greater than in non functional diesterous copora. Agather et al (2) reported that total dehydrogenase activity of tissue from normal and tumor bearing mice is independent of sex, strain, and age of animals. Neish (164) found that sera from rats with transplanted tumors were less effective than normal rats serum for reducing 2,3,5-TTC.
Rat thymus, pancreas, spleen, and blood were reported to be lacking in succinic dehydrogenase by Laskowski (39). Among more diversified workers are; Straub (61) who isolated malic dehydrogenase from pig heart muscle; Albaum (4) who reported increased dehydrogenase (succinic) activity in chick embryo; Anderson (5,6) who proved the importance of DPN in dehydrogenase reaction with washed yeast; Koppel (35) who reported activity in human morphologically intact platelets; Waldo (67) who showed inverse relation between TTC reduction and phosphatase activity in human plasma; and Fawcett (20) who reported that brown adipose tissue has more dehydrogenase activity than white.

EXPERIMENTAL

There are at least four established methods for the determination of succinic and malic dehydrogenases, namely;

(1) Measurement of O₂ uptake in a Warburg apparatus (55).

(2) Measurement of time of decolorization of MB (66,47).

(3) Measurement of TTC reduced (29,33,37,71,31).

(4) Measurement of reduction of cytochrome c (16) at 550 μm and of reduced DPN at 334 μm (25).

The methods of Kuhn et al (37) and Thunberg (66) were chosen for this work because of their simplicity and accessibility.

METHOD OF KUHN ET AL.

Kuhn et al (37) and Yoshio et al (71) adapted the reduction of TTC to quantitative colorimetric determination of dehydrogenase activity. The dye is reduced to water insoluble, acetone soluble formozan by the enzymes in the presence of specific substrates. Thunberg designed a special tube
for anaerobic reduction of methylene blue by the enzyme in the presence of the necessary substrate. An anaerobic condition is necessary for the demonstration of leuco-methylene blue which is auto oxidizable in the presence of O₂. Before testing the presence of malic and succinic dehydrogenase in rat tissue and plasma by the method of Kuhn and Abood, it was necessary to determine the optimum concentrations of the dye, the buffer, the coenzyme I (DPN), and H⁺. These were found to be .12% of TTC, .072% DPN, of the total reaction mixture. The optimum pH of malic is 8 - 8.6 and of succinate is 7.5 - 7.8. The buffer concentrations of M/2 to M/32 have the same effect.

KCN was found to inhibit reduction of TTC, DPN accelerates, increases it. Diaphorase has no effect on it. Such findings as preceded led to slight modification of methods of Kuhn and Jensen by working at higher pH, and higher concentrations of dye and DPN.

Basically, 5 steps were involved in the test. The first is the addition of reaction mixture at optimal concentrations with controls provided. Equal volumes of .5% TTC, .2M substrate and M/10 phosphate buffer were mixed. The final pH was 7.7 for succinate and 8.3 for malate.

The second step is the preparation of homogenate by removing the liver, kidney, or brain from the decapitated rats and homogenizing in 9 volumes of M/30 phosphate buffer for 2 to 5 minutes. Equal volumes of the filtered homogenate were added to the reaction mixtures.

In the third step the tubes of the reaction mixture were evacuated for 5 - 10 minutes to a pressure of 6 mm and incubated for 60 - 90 minutes in a water bath at 38°C. At least four control tubes were run concur-
ently, two for enzyme control (without homogenate), and two for substrate control (without substrate).

The fourth step. After incubation, the reaction was stopped by addition to each tube of three volumes of acetone. The mixtures were shaken vigorously and stored in the dark at 5°C for 20 - 24 hrs.

The fifth step. The precipitated protein sediment was centrifuged off, and the clear transparent orange-red color of the formozan was read in a Beckman DU spectrophotometer at 485 μm. The amount of reduced formozan in micrograms was calculated from a standard curve made by plotting varying concentration of reduced formozan (400μg - 25μg) against the corresponding optical density. The reduction was done by sodium hydrosulfite, the yield was about 60%.

The enzyme unit was calculated by the equation:

\[
\text{no. of unit of Enzyme} = \frac{\text{μg of dye reduced for the 1st 10 min.}}{\text{mgm of tissue (wet)}}
\]

Sensitivity test indicated that the lower limit of formozan concentration that can be measured colorimetrically was 22 μg/10 cc of solution.

Rat kidney, liver, brain, and blood were tested for malic and succinic dehydrogenase activity as a control and for the purpose of testing the TTC method before its use in the investigation of human plasma. The results of values obtained by such test shows good agreement with results of values obtained by other workers as the tabulated results show.

The measurement of dehydrogenase activity by the use of the Thurberg (66,47) technique was adopted as a means of checking the comparatively new method of TTC. Cytochrome c and diaphorase needed for this reaction
were prepared and tested for their properties and approved. The test system by the Thunberg technique is as follows: .5 ml of m/1000 MB 1.5 ml of .2M substrate, 1 ml 10% tissue homogenate and 1 ml M/15 phosphate buffer were reacted in an evacuated Thunberg tube and time of decolorization ascertained. For malic dehydrogenase DPN (.5 ml of 2%) and diaphorase .5 ml were added to the above test system.

The enzyme units were calculated from the equation:

\[
\text{Sub} \_\_\text{MB} = \frac{\text{ul } \text{O}_2 \text{ equivalent of total MB reduced}}{\text{hrs} \times \text{mgm tissue}}
\]

The two methods checked fairly well as can be shown by the following comparison of the relative magnitude of units of enzymes calculated by the two systems:

<table>
<thead>
<tr>
<th>MB (Thunberg technique)</th>
<th>(185)</th>
<th>(176)</th>
<th>(119)</th>
<th>(59)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver malic dehydrogenase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidney malic dehydrogenase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidney dehydrogenase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver Succinic dehydrogenase</td>
<td></td>
<td></td>
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<tr>
<td>TTC (Kuhn &amp; Abood)</td>
<td>727</td>
<td>600</td>
<td>500</td>
<td>490</td>
</tr>
<tr>
<td></td>
<td>2.20</td>
<td>1.88</td>
<td>1.54</td>
<td>1.48</td>
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</tbody>
</table>

The two methods were used in the investigation of human plasma from about twenty subjects. The plasma was prepared according to the method of Strumia (61b) and serum by essentially the same method, except that no anticoagulant was used, and clotting was allowed to take place for one hour. There was more reduction of the dye in the serum with malate than in the serum without malate, but the reduction could not be proved to be enzymatic since heating, and specific enzyme inhibitor (NaF) could not lessen or inhibit this substrate specific reduction. The extent of
reduction however, was very low. Although the result of the investigation is hardly conclusive, the experience gained in the use of TTC, the many problems raised, and the solution of those problems may eventually be of some aid in the future final verdict.