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Collie B. Shaw
University of Central Arkansas

Tara L. Chronister
University of Central Arkansas

John D. Peck
University of Central Arkansas

OPTIMAL CONDITIONS FOR KINETIC STUDY OF SUCCINATE DEHYDROGENASE IN RAT LIVER

COLLIE B. SHAW, TARA L. CHRONISTER*
and JOHN D. PECK
Department of Biology
University of Central Arkansas
Conway, AR 72032

ABSTRACT

Succinate dehydrogenase (SDH) commonly is assayed as a marker enzyme for mitochondrial activity. The literature presents numerous conditions for conducting this assay due to the fact that, it has been difficult to get sufficient reduction of the acceptor dye, 2,3,5-triphenyl-2H-tetrazolium chloride (TTC). This study was undertaken to optimize the SDH-catalyzed reduction of TTC dye by evaluation of a greater range of molor ratios of TTC to succinate and by further evaluation of additives reported as beneficial. Improvement in enzyme specific activity was achieved by liver perfusion via the left cardiac ventricle with homogenizing solution. Increase in TTC from 1 to 10 mM and further increase to 20 mM resulted in major improvement in color production. The greatest improvement in apparent activity was achieved by addition of 1 mM phenozine methosulfate, a hydrogen transfer mediator. Use of $CaCl_2$, EDTA, Triton X-100, NaN_3 and KCN was not beneficial. The above modifications of the SDH assay resulted in greater sensitivity, the conduct of a greater number of assays with less tissue and the sacrifice of fewer animals.

INTRODUCTION

Succinate dehydrogenase (SDH) commonly is used as a marker enzyme for the mitochondrion because it is bound to the inner membrane of the mitochondrion. Frequently, its activity is used to assess tricarboxylic acid cycle activity as well as electron and hydrogen transfer to the electron transport system. The activity of SDH usually is measured spectrophotometrically by following the reduction of an artificial acceptor dye such as 2,3,5-triphenyl-2H-tetrazolium chloride (TTC).

Numerous conditions have been presented in the literature for conducting this assay, due to difficulty in obtaining sufficient dye reduction and adequate absorbance values when using reasonable amounts of enzyme. For determination of Michaelis-Menton constants for inhibited and uninhibited reactions catalyzed by SDH, it is important to obtain high absorbance values for the uninhibited reaction. Otherwise inhibitors may decrease the formation of reduced dye and absorbance values to such a point that instrumental error becomes too great to provide reliable measurements.

This study was initiated to optimize the SDH catalyzed reduction of TTC dye by evaluating a greater range of molar ratios of succinate to TTC dye and by further evaluation of additives mentioned in the literature. These include EDTA, a chelator, $CaCl_2$ and Triton X-100, substances reputed to increase membrane permeability, KCN and NaN_3, electron transport inhibitors, and phenazine methosulfate, a putative electron transfer mediator between $FADH_2$ and TTC.

This paper presents improvements in the assay that will increase the SDH catalyzed reduction of TTC, give high dye absorbance values for uninhibited reactions and adequate absorbance for inhibited reactions. This will facilitate future kinetic studies of SDH.

MATERIALS AND METHODS

Chemicals used in this study were obtained from 4 major chemical companies. 2,3,5-triphenyl-2H-tetrazolium chloride monohydrate (TTC) and sodium dithionite were obtained from Aldrich Chemical Company. Phenazine methosulfate, sodium azide, and sucrose were obtained from Sigma Chemical Company. Disodium succinate was obtained from National Biochemical Corporation. Sodium phosphate, ether, acetone, and potassium cyanide were obtained from Fisher Scientific Company. The necessary aqueous solutions were made with distilled, deionized water.

The animals, black-hooded derived rats, were housed in environmentally controlled conditions and were provided food and water ad libitum. The animals were sacrificed by stunning and cervical dislocation or by etherization, and exsanguination. Exsanguination was achieved by perfusion with ice-cold homogenizing solution (.25 M sucrose) via left cardiac ventricle until the liver became more lightly colored.

The liver was excised, weighed, placed in a 0°-5°C solution of homogenizing fluid, diced and transferred to a prechilled homogenizing vessel. Homogenization was achieved by 5 passes of a teflon pestle into a glass vessel (size C Thomas). The homogenizer was powered by a Talboys Instrument Corporation Model 102 electric motor operated at full speed. The homogenate was maintained at less than 5°C during this procedure.

The homogenate was then placed in a refrigerated Sorvall RC2-B centrifuge and spun at 800 xG for 10 minutes. The supernatant (S1) was collected, diluted with 0.25 M sucrose and recentrifuged at 20,000 xG for 20 minutes to pellet the mitochondria. The supernatant (S2) was removed and the pellet (P2) was resuspended in .25 M sucrose in the ratio of 1-2 gm wet weight of liver to 1 ml sucrose. This suspension was centrifuged at 600 xG for 5 minutes to remove all large particles not resuspended in the earlier step. The supernatant (S3) was kept chilled for subsequent use.

If appropriate, the homogenate was sonicated using 10-second bursts at maximum power from an Ultrasonic System Model 1000 Insonator. During this procedure care was taken to maintain a temperature below 10°C, and following this procedure the homogenate was returned to 0-5°C.

A determination of the homogenate's protein concentration was conducted, using the Bio-Rad protein assay kit, with bovine serum albumin as the standard. Absorbance was measured at 595 nm.

Typical reaction tubes were prepared by the addition of 0.5 ml of 0.1 M phosphate buffer, 0.5 ml of succinate and 1.0 ml of TTC (Kun and Abood, 1949). The final concentrations of these solutions were 16.6 mM, 5-10 mM, and 1-10 mM, respectively. After prewarming these reagents and liver homogenate at 38°C in a Forma Scientific water bath/circulator, the reactions were initiated by addition of 1.0 ml of liver homogenate and mixing. The reactions were terminated 15-20 minutes later by addition of 7 ml of acetone. The tubes were then centrifuged at 3000 rpm for 5 minutes and the absorbance of the supernatant was determined at 490 nm.

The standard curve for TTC was obtained by combining increasing amounts of TTC from 30-300 ug with approximately 7 mg of dry sodium dithionite. The reaction proceeded for 5 minutes, then was terminated by acetone addition and the absorbance of the solution was measured at 490 nm.

*Present address: University of Arkansas for Medical Sciences, 4301 West Markham, Little Rock, Arkansas 72205.

RESULTS

TTC Standard Curve

In setting up a standard curve for TTC, difficulty was encountered in obtaining linearity when a few crystals of sodium dithionite were used as specified in the literature (Kun and Abood, 1949). Four to 10 mg of reductant were found to be necessary to achieve full reduction of TTC dye (Figure 1). Less sodium dithionite caused incomplete reduction. Greater amounts of sodium dithionite caused less absorbance to be recorded and a change in the absorption spectra was noted.

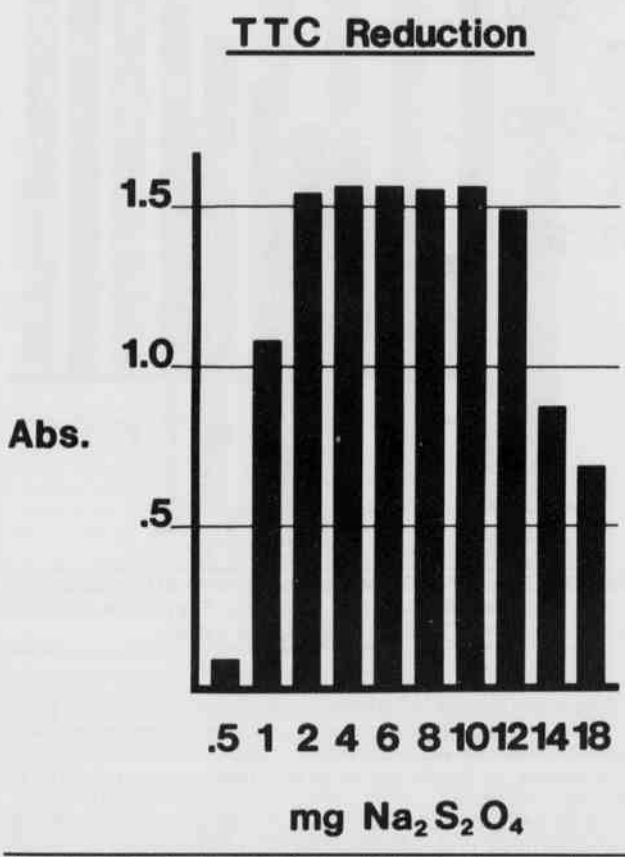

Figure 1. TTC reduction with Sodium Dithionite. Known weights of Sodium Dithionite were added to 3 ml aliquots containing 300 micrograms TTC. The solution was mixed, incubated for five minutes, and 7 ml acetone was added. Absorbance of the reduced TTC was then measured.

Test of Additives

EDTA at 1 mM (Melnick and Schiller, 1985), Triton X-100 at 0.1% (Tushurashvili et al., 1985), and $CaCl_2$ at 1-2 mM (Minatoguchi et al., 1984) are reported in the protocols of SDH assays in the literature. In the present system the above concentrations of EDTA and Triton X-100 caused decreases in dye reduction in the amounts of 32% and 91% respectively (Table 1). $CaCl_2$ was tested in TRIS buffer at the same pH as used above, because in phophate buffer it forms a $CaHPO_4$ precipitate. 1-2 mM $CaCl_2$ caused decreases in dye reduction of 16% to 17%.

Test of Perfusion

Centrifugation of nonperfused liver homogenate resulted in a perceptible blood cell pellet following the first and second centrifugations, and the final mitochondrial suspension was darkly colored. Perfusion of the liver led to a decrease in the quantity of the blood cells in the first and second pellets and produced a more lightly colored mitochondrial suspension. Perfusion led to a 19% increase in specific activity of SDH (Figure 2) and was used in all subsequent experiments.

Table 1. The effect of various chemicals on succinate dehydrogenase activity.

Buffer	Additive	Concentration	% Activity
Phosphate	None	___	100
Phosphate	EDTA	1 mM	68
Phosphate	Triton X-100	0.1%	9
TRIS	None	___	100
TRIS	$CaCl_2$	1 mM	83
TRIS	$CaCl_2$	2 mM	84

Enzyme activity was determined at +20 minutes. The reaction contained 5 mM succinate, 1 mM TTC, 1.33 mg protein/ml and buffer

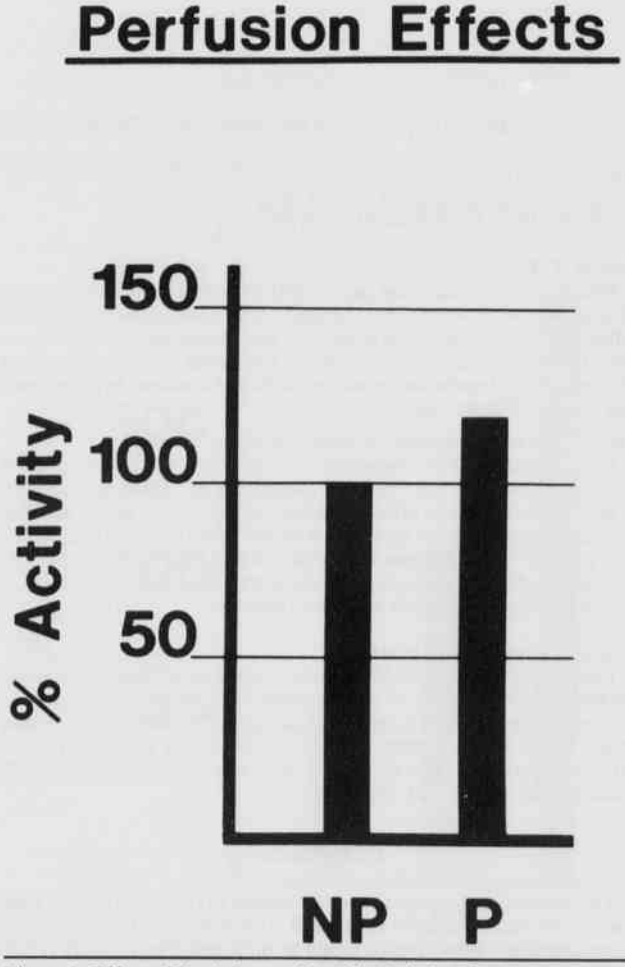

Figure 2. Effect of Perfusion on Specific Activity of SDH. Enzyme activity was determined at +20 minutes. The reactions contained 5 mM succinate, 1 mM TTC, 1.33 mg protein, and buffer.

Test of Sonication

Sonication of the mitochondrial suspension was conducted to determine if some disruption of the mitochondrial membranes would lead to greater dye access to and reduction by SDH. Minor improvement in dye formation occurred following 10 to 20 seconds of sonication while extended sonication hampered product formation slightly (Table 2). Ten seconds of sonication was conducted in all subsequent experiments.

Sonication time (sec)	% Activity
0	100
10	104
20	103
30	97
40	99
50	94
60	90

Enzyme activity was determined at +20 minutes. The reaction contained 5 mM succinate, 1 mM TTC, 1.33 mg protein/ml and buffer.

Test of TTC

The literature records the use of 10:1 or greater ratio of succinate to acceptor dye (Rondina, 1971; Futai, 1973). Tests of succinate to dye ratios up to 1:1 increased the amount of absorbance up to 278% (Figure 3A). Further increases of succinate to dye ratios up to 1:2 increased the amount of absorbance by another 48% and a plateau in absorbance was evident (Figure 3B).

Test of Electron Transport System Inhibitors

With regard to TTC reduction by SDH, the additive cyanide is reported to increase dye reduction (Nachlas et al., 1960; Minatoguchi et al., 1984), to have no effect (Kun and Abood, 1949), and to decrease reduction because of its action as a protein denaturant (Tsou, 1951). The present work demonstrated that cyanide and sodium azide, inhibitors of electron transport at cytochrome aa_3, caused decreases in dye reduction by as much as 95% (Table 3).

Test of Phenazine Methosulfate

PMS is mentioned in the literature as a mediator of hydrogen transfer between FAD and TTC. In these reports PMS usually is presented to the reaction in a ratio of 2 parts PMS to 1 part TTC (Futai, 1973; Massa et al., 1985). In the present work in which 10 mM TTC was used, a much smaller proportion of PMS, 1 mM, was found to improve TTC reduction by a factor of four (Figure 4).

DISCUSSION

The optional amount of sodium dithionite to reduce TTC for a standard curve was found to be 4-10 mg. It is interesting that many publications stated that a few crystals would be sufficient (Rendina, 1971; Hall and Hawkins, 1975). Too few crystals resulted in incomplete reduction and greater than 12 mg of sodium dithionite resulted in decreased color formation and a visible change in the absorption spectrum. This change in absorption suggests that an additional reaction may be involved that is not described in the literature.

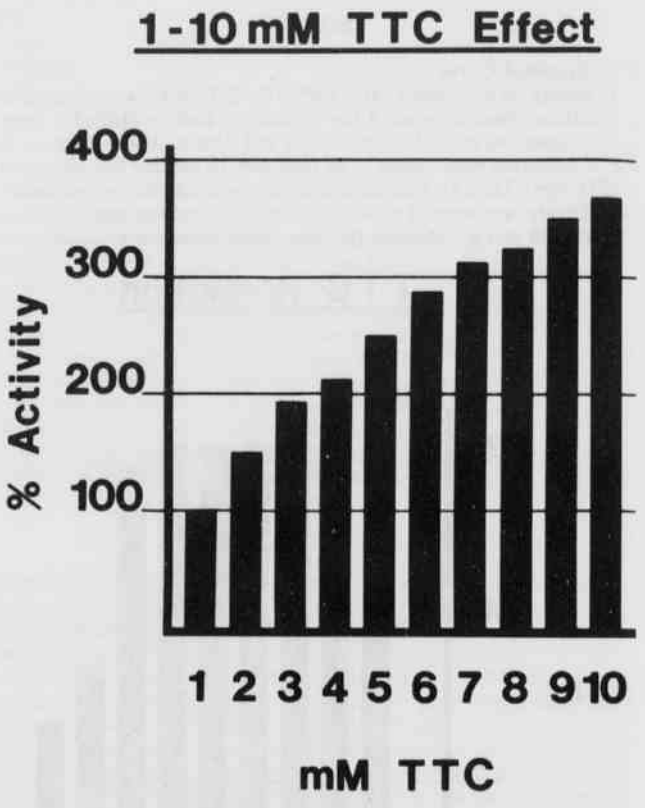

Figure 3A. Effect of 1-10 mM TTC on Apparent SDH Activity. Enzyme activity was determined at +15 minutes. The reaction contained 10 mM succinate, 1.33 mg protein/ml, and buffer.

Contrary to findings in the literature, we found the detergent, Triton X-100, and $CaCl_2$ did not improve dye reduction. These reagents were reputed to increase the permeability of the outer membrane of the mitochondria, so that the dye would have better access to the SDH on the inner membrane.

The perfusion of the liver decreased the amount of intact and lysed blood cells found in the mitochondrial pellet and increased the specific activity of SDH by 19%. This rather time-consuming technique may continue to be selected for the above reasons but other modifications of the procedure gave such improvement in dye reduction that a 19% increase in specific activity may not be necessary.

Ten to 20 second sonication of the mitochondrial suspension gave very minor differences that were not different from control. But sonication was so easy to conduct and it may have improved access of dye to SDH, we found it worthwhile to continue.

The optimal amount of TTC to monitor the SDH reaction was found to be 20 mM. Unfortunately, the solubility limit of TTC is just greater than 30 mM, hence, the set up of this assay required 2 ml of 30 mM TTC in a reaction having a total volume of 3 ml. The remaining reagents were prepared at twice the typical concentration and were delivered in half the typical volume. Delivery of these smaller volumes and preparation of liver homogenate at twice the usual concentration was found to be less than desirable for routine work. Therefore, the reagent volumes described in the materials and methods section were used in all subsequent work. The amount of TTC achieved in the remaining assays was 10 mM. This provides almost 90% of the absorbance obtainable with 20 mM TTC.

Cyanide has been reported to improve the reduction of dye in the SDH reaction (Minatoguchi et al., 1984), to have no effect (Kun and

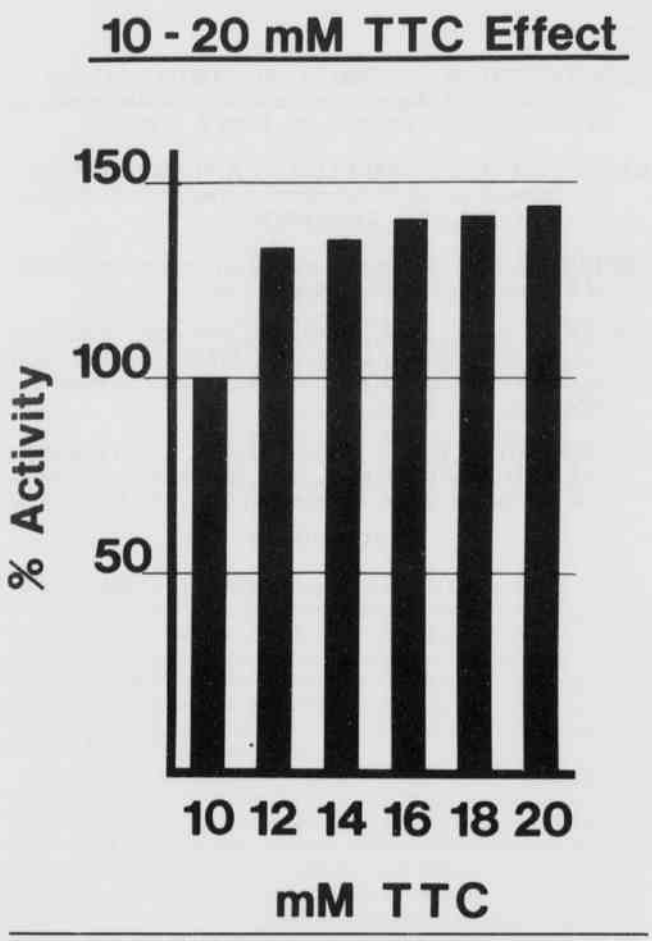

Figure 3B. Effect of 10-20 mM TTC on Apparent SDH Activity. Enzyme activity was determined at +20 minutes. The reaction contained 10 mM succinate, 1.33 mg protein/ml, and buffer.

Abood, 1949), and to harm the reaction due to its ability to denature protein (Singer and Kearney, 1957; Hatefi and Stiggal, 1976). Data herein demonstrate that when the reaction is run with 10 mM TTC, cyanide and the other tested ETS inhibitor, sodium azide, inhibited dye reduction. This and the EDTA chelation results, suggests that TTC may accept hydrogens from a number of sites that are fed by succinate metabolism and substantiates others findings (Nachlas et al., 1960).

The greatest improvement in dye reduction was brought about by the addition of 1 mM PMS. This agreed with the literature (Futai, 1973; Massa et al., 1985), but also indicated that the oft published ratio of 2:1 PMS to TTC is not important under the present conditions. The 1 mM PMS used here is in a 1:10 ratio to TTC.

It is thought that the future use of 10 mM TTC and 1 mM PMS will facilitate a greater number of assays to by conducted on less tissue and may then require use of fewer animals.

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Table 3. The effect of electron transport inhibitors on succinate dehydrogenase activity.

Additive	Concentration	% Activity
None	——	100
KCN	10^{-2} mM	50
KCN	10^{-1} mM	11
KCN	10^{0} mM	13
KCN	10^{+1} mM	5
NaN_3	10^{-3} mM	95
NaN_3	10^{-2} mM	97
NaN_3	10^{-1} mM	95
NaN_3	10^{0} mM	72
NaN_3	10^{+1} mM	51

Enzyme activity was determined at +15 minutes. The reaction contained 1 mM succinate, 10 mM TTC, 1.2 mg protein/ml and buffer.

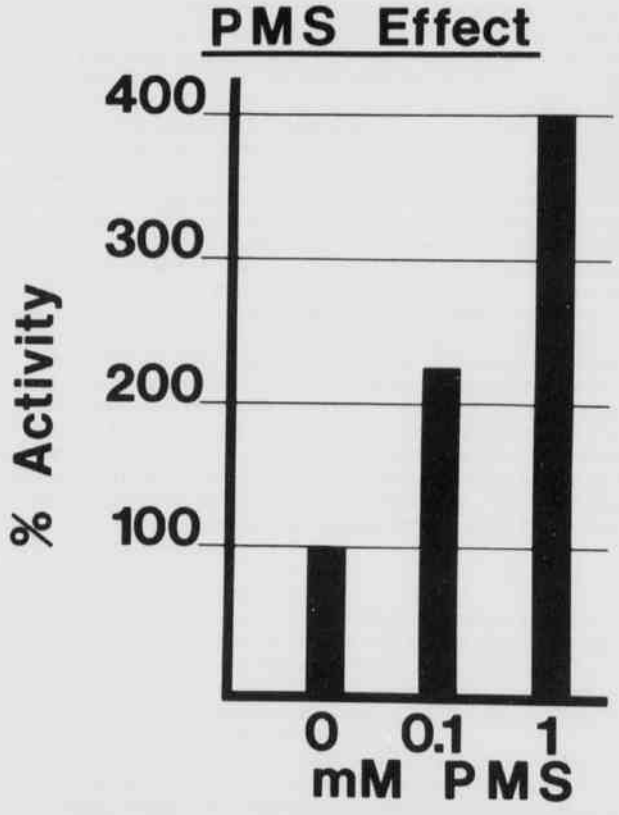

Figure 4. Effect of PMS on TTC Reduction. Enzyme activity was determined at +15 minutes. The reaction contained 1 mM succinate, 10 mM TTC, 1.2 mg protein/ml, and buffer.

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