Bio 122 Lab; Section 073; Group 01:

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The Effects of Malonate Inhibiting Succinate Dehydrogenase (SDH) Activity in the Krebs Cycle

Introduction

The aim of this project was to measure enzyme activity by analyzing succinate dehydrogenase (SDH) reactions in the experiment. Through cell fractionation, the mitochondrion from a bovine liver sample was isolated to produce a more detailed analysis (Bradford, 249). Succinate dehydrogenase (SDH) is an enzyme found within the inner mitochondrial membrane that, in a normal reaction during the Krebs cycle, oxidizes to fumerate. This reaction releases an electron, which is deposited into the electron transport chain (ETC) via $FADH_2$ reduction (Mason et. al., 132).

Methodology was based on the Bradford and SDH assays. The Bradford assay was implemented to measure protein concentration as well as to create a standard curve. The curve represented the relationship between Bovine Serum Albumin (BSA) concentration and light absorbance. Establishing a standard for enzyme activity allowed comparison of enzyme reactions and observation of inhibitory enzyme reactions while calculating for protein concentration (Bradford, 248; Thorn, 540). In addition to succinate, reagents were added to the solutions to maintain integrity of the data and isolate activity for accurate interpretation (Bradford, 248). Dichlorophenolindophenol (DCPIP) acted as an artificial electron acceptor in the solutions. DCPIP also colored the solution blue indicating oxidation and a lack of color when the solution is reduced (Bradford 249). Several samples with reagents and varying dilutions were measured to distinguish the relationship.

Similar to the Bradford assay, the SDH assay uses a combination of reagents and controls to illustrate enzyme activity (Thorn, 541). In the SDH Assay the assigned reagent, Malonate, was included in the experiment. Succinate was added to the solutions to act as the substrate for SDH and/or malonate to measure the competitive nature of the inhibitor enzyme (Thorn, 541). The SDH assay demonstrated the correlation between light absorbance with respect to time for eight samples used with varying dilutions and the assigned reagent. The light absorbency represented SDH oxidation. As SDH was reduced to $FADH_2$, electrons were accepted by DCPIP making the solution colorless, and thus the light absorbency decreased (Bradford, 251-252).

Succinate dehydrogenase is a flavoprotein including the prosthetic group FAD. It is the reductant, combining with succinate (the electron receptor) (Dervartanian and Veeger, 233) to reduce $FADH_2$. In the cell, SDH activity occurs as part of the Krebs Cycle. Organisms can generate ATP through cellular respiration. The most basic breakdown is glycolysis which ultimately generates pyruvate. The Krebs cycle is a series of nine reactions in which an acetyl group from pyruvate is oxidized (Mason et. al., 129-132). Within the third segment of the Kreb's cycle, reaction 7 states succinate is oxidized to fumerate by an enzyme located in the inner mitochondrial membrane (Mason et. al., 132). In this process, the free energy change is not great enough to reduce NAD+. Also, in this case, FAD is the electron acceptor and is not free to diffuse within the mitochondrian. FAD is tightly crossed with the enzyme once again in the mitochondrian membrane. FAD's reduced form $FADH_2$ can only lend electrons to the electron transport chain within the membrane. For FAD to convert to $FADH_2$, oxidation must occur between the enzyme and the reactant (Mason et. al., 132).

The focus of this experiment was the effect of malonate on the succinate dehydrogenase complex. The reagent malonate is a competitive inhibitor enzyme that competes directly with

succinate dehydrogenase in the Krebs cycle. Inhibitor enzymes have similarly designed structures to compete with the original enzyme by combining with the desired substrate (Krantz, 1327). This competitive inhibitor is one that is reversible depending on its mode of action. Reversible inhibitors are those that can be removed from an enzyme thus allowing the enzyme to regain its activity. The inhibitor, in this case malonate, only attempts to copy one substrate—whereas irreversible inhibitors cannot be removed from the enzyme to restore activity. When these inhibitors tightly bind to the substrate, activity is not restored between the enzyme and the solution when separated (Krantz, 1327). Malonate fits in with the reversible inhibitors that are called transition state analogs. These inhibitors are structurally similar to the transition state to their enzyme catalyzed reactions. These inhibitors are potent with values of the inhibitor association constants several orders of magnitude higher than the enzyme-substrate association constant (Krantz, 1327- 1329).

Malonate is characterized by an activated methylene group making it an electron donor. Therefore, the enzyme malonate would affect light absorbency in the experiment; comparatively malonate is not as competitive as other enzyme inhibitors (Dervartanian and Veeger, 238-239). When an enzyme is categorized as a competitive enzyme, the degree to which the inhibitor is effective must be quantified to determine potency (Thorn, 540). Malonate, like succinate dehydrogenase is also a dicarboxylate that binds to cationic amino acid residues in the active site of the succinate dehydrogenase complex (Hajjawi, 135-137). Malonate will not oxidize because it lacks the $CH_2 – CH_2$ group necessary for dehydration. The missing group is necessary for enzyme activity to take place within this reaction because this is postulated as the state of highest energy activated complex through which reactants (substrates) must pass on their way to becoming products (Hajjawi, 137). Malonate inhibits fumerate production within the Krebs cycle when the enzyme outcompetes SDH.

Thus, we hypothesize that the reagent malonate will act successfully as a competitive enzyme inhibitor, decreasing succinate dehydrogenase (SDH) activity by not allowing the reaction of converting FAD to $FADH_2$,within the Krebs cycle, to occur.

Results

Bradford Assay:

Table 1. Light wave absorbance by cellular fractions isolated from the bovine liver at specific concentrations.

Concentration(mg/mL)	Absorbance(Au) @ 595 nm
0	0
0.050	0.056
0.100	0.124
0.200	0.152
0.400	0.558
1	1.158

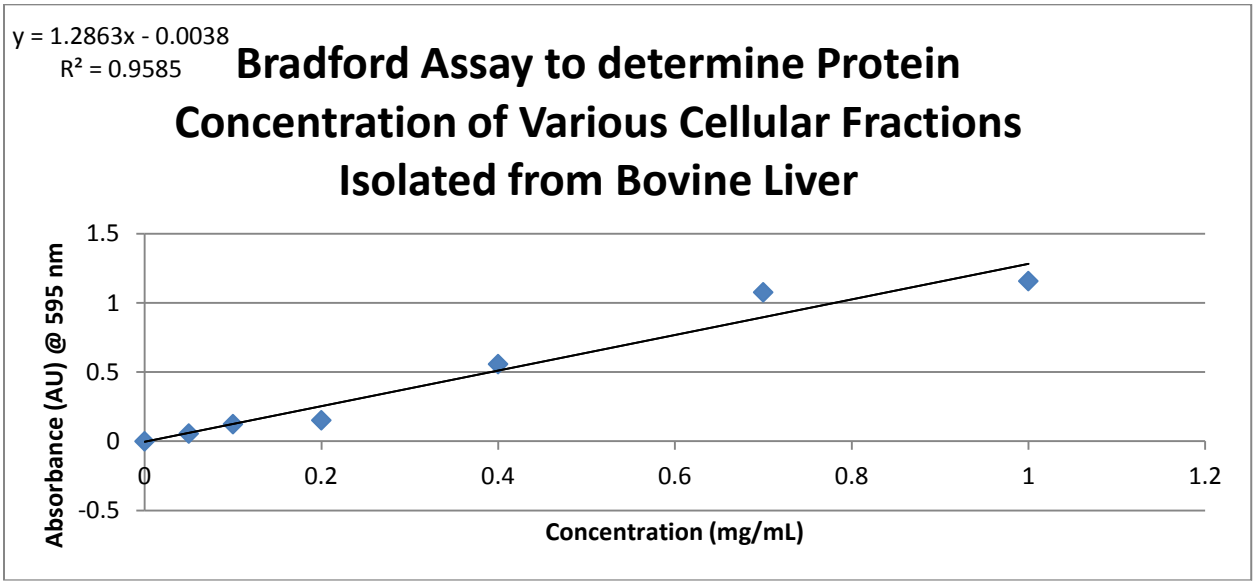

Figure 1.BSA standard curve of protein concentration in various cellular fractions isolated from bovine liver in the Bradford assay.

Table 1. Calculated protein concentration of crude and mitochondrial fractions taken from the bovine liver.

Dilution Factor	Sample	Absorbance(Au)	Sample Concetration (mg/mL)
20	Mitochondria	0.217	3.433
50	Crude	0.304	11.964

Week 3 SDH Assay:

Table 3. Protein absorbance readings over a 21 minute span.

	0	3	6	9	12	15	18	21
Blank	0	-0.100	-0.002	-0.002	-0.002	-0.003	-0.004	-0.006
Succinate Control	0.752	0.751	0.738	0.732	0.728	0.724	0.721	0.720
Enzyme Control	0.600	0.481	0.409	0.352	0.315	0.288	0.273	0.261
mito extract(1)	0.581	0.478	0.400	0.352	0.321	0.291	0.277	0.269
mito extract (2)	0.772	0.628	0.534	0.467	0.414	0.377	0.350	0.338
crude extract(1)	0.570	0.447	0.364	0.301	0.251	0.217	0.187	0.172
crude extract(2)	0.765	0.593	0.480	0.394	0.327	0.217	0.243	0.222
crude extract(3)	0.539	0.413	0.331	0.273	0.227	0.199	0.176	0.164

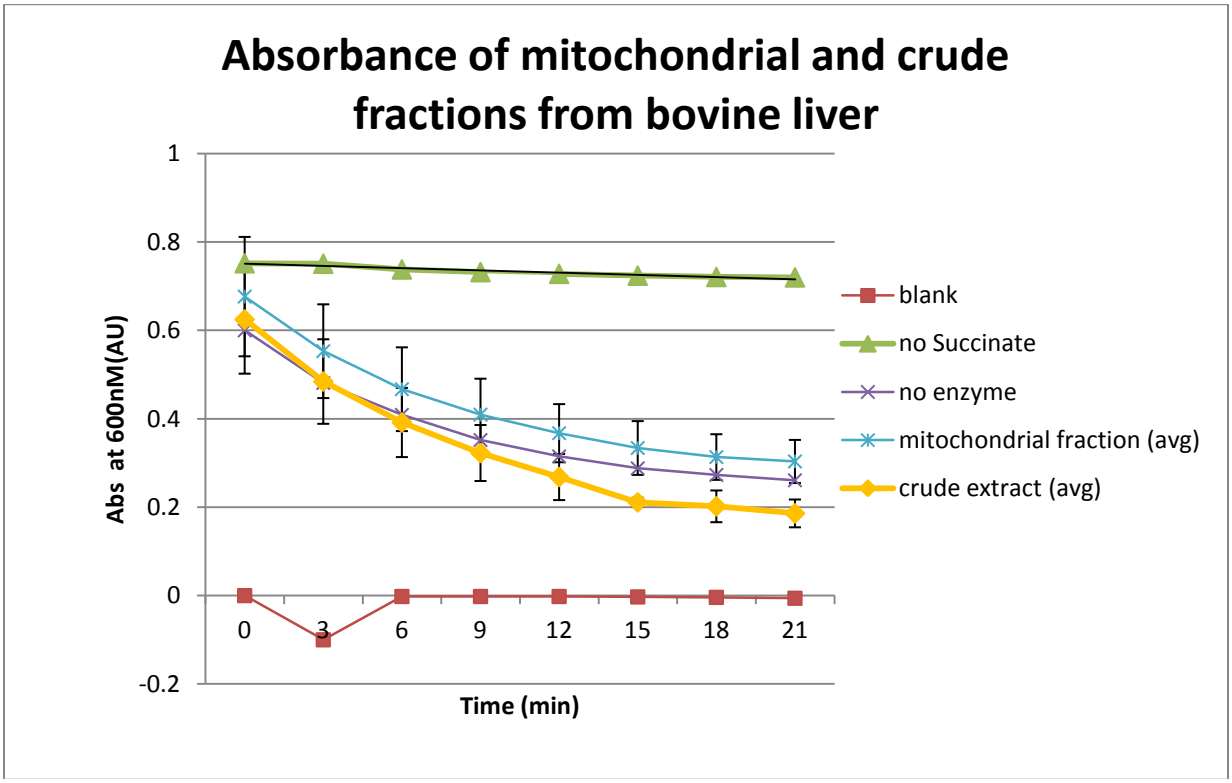

Figure 2. Absorbencies of the mitochondrial and crude fractions over 21 minutes. The absorbance change is a reflection of the reduction of DCPIP.

Table 2. Average mitochondrial fractionation compared to average crude fractionation.

Time (mins)	0	3	6	9	12	15	18	21
Average mitochondrial	0.675	0.553	0.467	0.409	0.367	0.334	0.313	0.303
sd mitochondrial	0.136	0.106	0.095	0.081	0.065	0.060	0.051	0.048
Average crude	0.625	0.484	0.391	0.322	0.268	0.211	0.202	0.186
sd crude	0.122	0.095	0.078	0.063	0.052	0.010	0.035	0.031

Table 5. Specific Activity of first SDH assay of crude and mitochondrial fractions taken from bovine liver.

	SDH Assay (mito.)	Assay Trial (crude)
Dividing Au (at 3 minutes) by Au(at 15 minutes)	1.54941	2.29541
Taking the natural log of the quotient	0.43787	0.83091
Dividing the natural log of the quotient by the time span	0.03648 min^{-1}	0.06924 min^{-1}
Dividing the above quotient by 1 unit of enzyme activity	.36 Units	.69 Units
Dividing Units by total amount of fraction added to reaction	3.6 Units/ml	6.9 Units/ml
Dividing the Unit/ml by the Protein concentration(mg/ml)	1.0486 Units/mg	0.57674 Units/mg

- Specific Activity of mitochondrial fractions were greater than the Specific Activity of the crude fractions.

Week 4 Assay 1:

Table 6. Absorbencies of SDH assay including reagent malonate within a 21-minute span.

	0	3	6	9	12	15	18	21
Blank	0	-0.001	0	0.001	0.004	0.003	0.001	0.001

No Succinate	0.933	0.904	0.884	0.871	0.861	0.851	0.845	0.837
No Enzyme Control	0.795	0.708	0.679	0.661	0.631	0.612	0.601	0.581
mito extract(1)	1.183	1.008	0.862	0.743	0.645	0.563	0.493	0.440
mito extract (2)	1.148	0.982	0.840	0.722	0.626	0.541	0.473	0.419
mito extract with reagent(1)	1.080	1.029	0.988	0.954	0.924	0.892	0.862	0.835
mito extract with reagent(2)	1.085	1.037	0.997	0.964	0.935	0.904	0.875	0.849
mito extract with reagent(3)	1.017	0.969	0.928	0.895	0.862	0.831	0.800	0.775

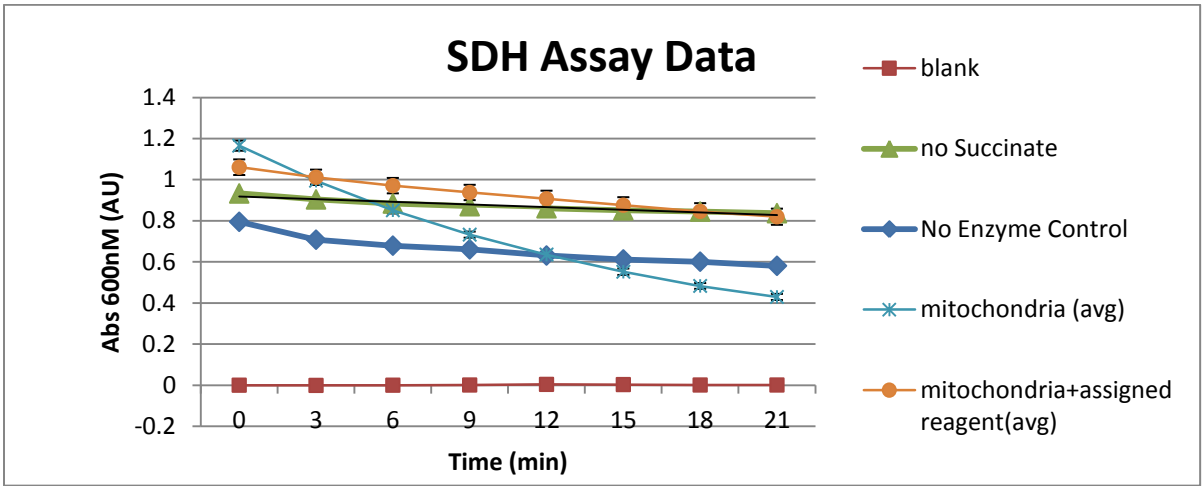

Figure 3. The average mitochondrial absorbencies on SDH Assay with malonate reagent.

Table 7. Average mitochondrial fractionation with and without reagent.

Time (mins)	0	3	6	9	12	15	18	21
mito extract (Average)	1.165	0.995	0.851	0.732	0.635	0.552	0.483	0.429
mito extract (SD)	0.024	0.018	0.015	0.014	0.013	0.015	0.014	0.014

mito extract with reagent (Average)	1.060	1.011	0.971	0.937	0.907	0.875	0.845	0.819
mito extract with reagent	0.037	0.037	0.037	0.037	0.039	0.039	0.040	0.039

Week 4 Assay 2:

Table 8. Second SDH Assay data with assigned reagent (malonate).

	0	3	6	9	12	15	18	21
Blank	0	0.003	0	0.001	0	0	0.001	0
No Succinate	1.073	1.059	1.048	1.039	1.032	1.024	1.019	1.014
No Enzyme Control	1.133	0.925	0.851	0.806	0.785	0.755	0.719	0.702
mito extract(1)	1.438	1.179	1.047	0.893	0.775	0.756	0.646	0.619
mito extract(2)	1.353	1.104	0.978	0.830	0.721	0.650	0.606	0.581
mito extract with reagent(1)	1.360	1.290	1.256	1.205	1.160	1.121	1.084	1.049
mito extract with reagent(2)	1.291	1.226	1.192	1.146	1.105	1.068	1.031	0.997
mito extract with reagent(3)	1.256	1.183	1.143	1.094	1.050	1	0.968	0.933

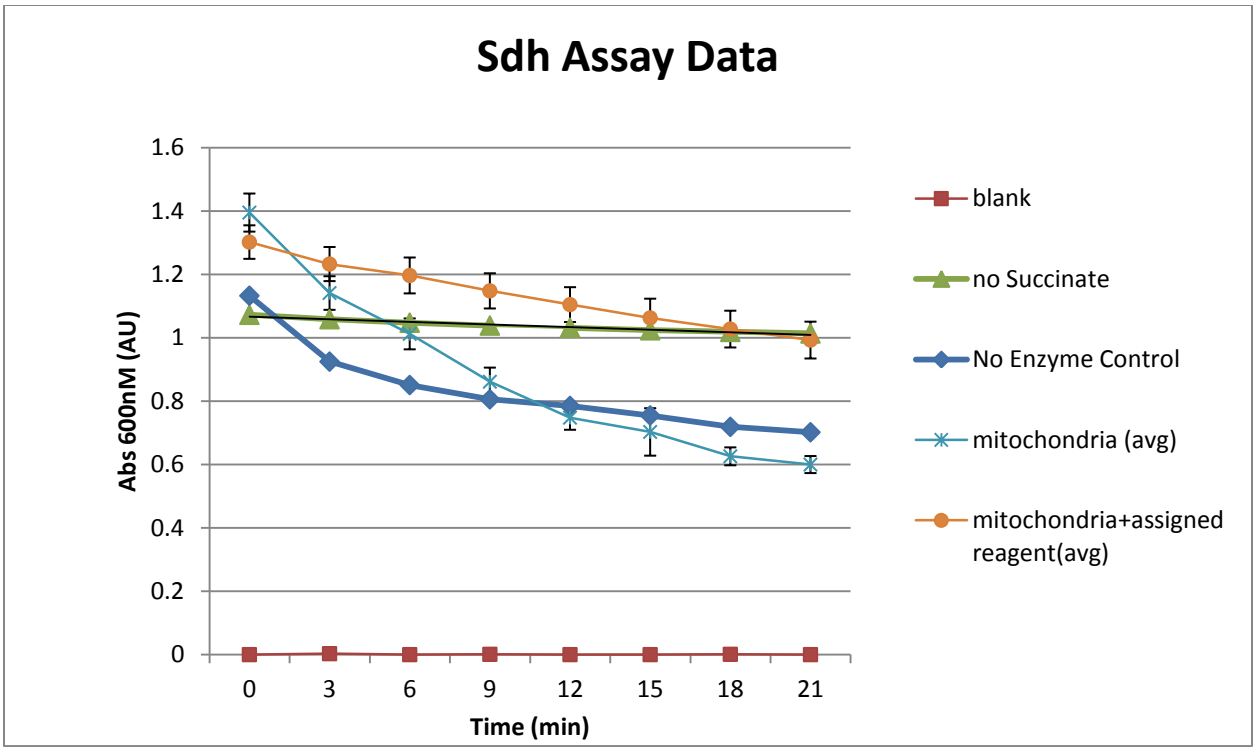

Figure 4. Average mitochondrial absorbance from second SDH Assay with assigned reagent (malonate).

Table 9. Average mitochondrial fractionation with and without reagent.

Time (mins)	0	3	6	9	12	15	18	21
mito extract (Average)	1.395	1.141	1.012	0.861	0.748	0.703	0.626	0.600
mito extract (SD)	0.060	0.053	0.048	0.044	0.038	0.074	0.028	0.026
mito extract with reagent (Average)	1.302	1.233	1.197	1.148	1.105	1.063	1.02	0.993
mito extract with reagent	0.052	0.053	0.056	0.055	0.055	0.060	0.058	0.058

Table 3. Comparison between SDH Assays with and without reagent malonate.

	SDH Assay 1 (without reagent)	SDH Assay 1 (with reagent)	SDH Assay 2 (without reagent)	SDH Assay 2 (with reagent)
Dividing Au (at 3 minutes) by Au(at 15 minutes)	1.80253	1.15520	1.62376	1.15992
Taking the natural log of the quotient	0.58919	0.14433	0.48474	.14836
Dividing the natural log of the quotient by the time span	0.04909 min^{-1}	0.01203 min^{-1}	.04039 min^{-1}	0.01236 min^{-1}
Dividing the above quotient by 1 unit of enzyme activity	.49 Units	.12 Units	.403 Units	0.12 Units
Dividing Units by total amount of fraction added to reaction	4.9 Units/ml	1.2 Units/ml	4.03 Units/ml	1.2 Units/ml
Dividing the Unit/ml by the Protein concentration(mg/ml)	1.43 Units/mg	0.34957 Units/mg	1.17673 Units/mg	0.36013 Unit/mg

- The reagent slowed down the enzymatic activity of the SDH.

Conclusions

- In this experiment, the Bradford assay was implemented to determine the protein concentration of the mitochondrial fraction from bovine liver cell fractions.
- From the Bradford assay, the experiment produced a standard curve, showing the absorbency readings of the tested solutions at different concentrations. The standard curve from the Bradford assay was used in comparison to the SDH assay to examine enzyme activity.
- The blank and negative solutions served as controls for the experiment. The blank and negative solutions were compared with the other solutions that contained the various reagents referenced in the introduction.
- The blank cuvette contained no DCPIP, the artificial electron receptor, and did not present a change to the data compared to any of the other cuvettes.
- The first negative control in the second cuvette contained the assigned reagent and no succinate. DCPIP did not collect any electrons because malonate is incapable of oxidizing. Since malonate is unable to give any electrons to form $FADH_2$, the product was not formed.
- The third cuvette was the no-enzyme control. The mitochondrial fraction was boiled, destroying the enzyme. This control had very low Specific Activity and incremental decrease in light absorption indicating slow oxidation rate.
- The reaction differences between the cuvettes that did and did not contain the reagent malonate were slightly different. From the results, the absorbencies of the cuvettes that did not contain the reagent reached a zero-value more quickly. The approach of zero within this experiment meant the DCPIP oxidized and the experiment had completed.
- The cuvettes that contained the malonate reagent showed a delayed decrease to a zero-value, which indicated malonate was partially successful in slowing down the reaction. However, malonate was not completely successful in fully stopping the reaction.
- The enzyme inhibitor malonate slowed the reaction between DCPIP and the substrate. The activity was demonstrated between the cuvette, which contained the mitochondrial extract and the cuvette, which contained the mitochondrial extract with the reagent.
- Specific Activity from the Bradford assay and SDH assay are the units of activity of an enzyme per milligram of protein.
- The Specific Activity of the crude fraction compared to Specific Activity of the mitochondrial fraction was lower. Enzyme activity occurs in the mitochondrion; as the mitochondrial fraction was further isolated, enzyme activity increased.
- The Specific Activity of the SDH assay with the reagent was lower than the Specific Activity of the SDH assay without the reagent. Malonate acted as a competitive inhibitor thus blocking the activity of SDH. This is shown in the decrease in Specific Activity when the reagent was added.
- Overall the collective results of the experiment were consistent— enzyme activity decreased in the presence of malonte. These results supported our hypothesis.

References

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