THE EFFECT OF CENTRIFUGAL FIELDS
ON ENZYMATIC REACTIONS

by

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B.S., Kansas State University, 1965

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Biochemistry

KANSAS STATE UNIVERSITY
Manhattan, Kansas
1966

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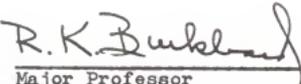
Major Professor

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INTRODUCTION

The conventional methods by which enzyme systems are investigated include studies of the effect of varying the concentration of the enzyme, concentration of the substrate, pH, and temperature. These studies are indeed valuable and necessary in our understanding of enzyme action. However, studies involving other variables such as pressure and centrifugal fields also may well be of value.

Several experiments dealing with the effect of pressure have been published. Eyring's group (8) found that they could inhibit the denaturation of luciferase by applying a hydrostatic pressure. Campbell and Johnson (1) applied a hydrostatic pressure of 10,000 pounds per square inch and showed that they could almost entirely retard the precipitation of a protein by an antibody. Eyring, Johnson, and Gensler (3) studied the effect of pressure on invertase and found that at pH 4.75 a hydrostatic pressure of 476 atmospheres increased the activity 5%. The same pressure caused greater increases at more alkaline and more acid pH (58% increase at pH 7.5 and 38% at pH 1.5).

The effect of pressure on enzymatic reactions has been attributed to the fact that there is a volume increase (positive $\Delta V^{\neq}$) when the enzyme goes to an activated state. The theoretical significance of this can be seen from the following equation[1] (9):

$$\left(\frac{\partial \ln K}{\partial P} \right)_T = -\frac{\Delta V}{RT}$$

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[1]When the activated state is being considered, $K^{\neq}$ refers to the equilibrium constant between the initial reactants and the activated state while $\Delta V^{\neq}$ refers to the volume change as a mole of the molecules go from the initial volume to the activated volume.

It follows from the above equation that for reactions which occur with an increase in volume (ΔV positive) the equilibrium constant decreases with increasing pressure. If ΔV is negative the equilibrium constant increases with increasing pressure. Hence, the equilibrium concentration of products is increased or decreased by pressure as the volume change is negative or positive, respectively.

When a solution is spun in the centrifuge a hydrostatic pressure will be produced. This hydrostatic pressure can by calculated from the following equation (14):

$$\frac{dP}{dx} = \rho \omega^2 x$$

Where ρ is the density of the solution, ω is the angular velocity in radians per second, and x is the distance from the center of rotation.

The use of this equation can be illustrated by the following example. Five ml. of water is placed in a centrifuge tube in a Serval SS-34 rotor and then subjected to centrifugation. The maximum radius of this rotor is 11.5 cm. The radius at the meniscus of the water would be 10.1 cm. Assuming the density of the water to be 1.0 gm. per cm.^{3}, the maximum hydrostatic pressure at 5,000 rpm (3,200 x g maximum) is 4 atmospheres, and at 18,000 rpm (41,300 x g maximum) 53 atmospheres.

Few papers have been published about the effect of centrifugal fields on chemical reactions. However, van Calcar and Lobry de Bruyn (15) found they could bring about the precipitation of sodium sulfate in a centrifuge, from a solution which was unsaturated under ordinary conditions. They theorized that when the solution is rotated, the concentration of the salt at the periphery increases.

Also, the pressure at the periphery is quite high. Both of these factors increase the chemical potential of the salt in solution. The chemical potential of the salt in solution becomes greater than the chemical potential of the solid salt under the pressure. Hence, precipitation of the salt occurs.

Just as the studies of enzymes under pressure were valuable in the visualization of the nature of enzyme action, so also could the effect of centrifugation be useful. If indeed there is an effect on the activity, due to a centrifugal field, this could be of assistance in the study of space travel since enzyme systems in a living organism might be affected in a manner similiar to the systems studied. One might, therefore, be able to predict at the molecular level, how reactions of the animal body would be affected by the changes in gravitational force needed to lift a space craft into orbit.

In preliminary work, Whitehead (16) found that mitochondrial activity was greater in centrifuged systems than in control systems that were not centrifuged. However, she did not determine if this was due to centrifugation or some other factor. There was also an insufficient number of trials to determine the actual magnitude of this effect. Therefore, it was decided to pursue this observation. Accordingly a systematic study was made to develop an analytical method for studying enzymatic reactions in systems subjected to centrifugal fields. If enzymatic reactions were then found to be affected by centrifugation, further studies would be made to determine the cause of this effect.

An inhomogeneous mixture may separate when spun in the centrifuge, while a homogeneous mixture will not. For this reason two

enzyme systems were chosen. In one system the enzyme would separate and be spun down. The other system was chosen so that it would not form a pellet when centrifuged. Mitochondria were selected as the system that would form a pellet at the forces that would be used in the centrifuge. Invertase was chosen as the system that would not separate upon centrifugation.

EXPERIMENTAL

Invertase Assay Procedure

In order to study the invertase system in the centrifuge, an assay procedure was needed that would satisfy two requirements. (1) The activity should not vary with small changes in substrate concentration. (2) The reaction should proceed linearly for the duration of the trial. Also, the change in temperature in the centrifuge needed to be measured as accurately as possible and the dependence of invertase activity on temperature determined.

The enzymatic activity of invertase (EC 3.2.1.26), was followed by determining the rate of production of reducing sugar when invertase acted upon the nonreducing disaccharide sucrose. The reducing sugar was measured with the dinitrosalicylic acid (DNS) reagent which was developed by Sumner and Sisler (13) and modified by Miller (11). A standard curve was prepared using from 1 to 5 micromoles of glucose.

Procedure. Four ml. of 0.4 M sucrose, in 0.1 M acetate buffer (pH 4.5), were incubated at the desired temperature for 5-10 min.

The reaction was started by adding 1.0 ml. of the diluted enzyme[1] and stopped after 15 min. by adding 5.0 ml. of 0.1 N NaOH. A 1.0 ml. aliquot was diluted with 1.0 ml. of water. This solution then was analyzed for reducing sugar by adding 5 ml. of DNS reagent and heating for 5 min. The total volume was adjusted to 17 ml. with water and the absorbance read at 560 mμ in a Bausch and Lomb Spectronic 20 Spectrophotometer. A blank was prepared by adding 0.1 N NaOH before the reaction was started. The specific activity was expressed as micromoles of glucose per min. per mg. of protein.

Sucrose Concentration. The sucrose concentration should be high enough to give zero order kinetics and avoid substrate concentration affects on the reaction velocity. Accordingly, concentrations of sucrose from 0.2 M to 0.8 M were employed in the procedure outlined above and the activity plotted against sucrose concentration.

Reaction Time. The proposed method for studying effects of centrifugal fields required long reaction times. For this reason, a study was made in which the reaction was performed for different lengths of time employing 0.4 M sucrose. This was done to show that the amount of product formed increased linearly during the assay period.

Temperature Dependence of Activity. Since there was a small change in temperature in the centrifuge, the dependence of invertase activity on temperature was investigated. A control reaction was run at 25°C with each reaction at a given temperature. The relative

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[1] Invertase (0.3 mg.) was diluted in 100 ml. of 0.05% gelatin. Two different lots of invertase were used: Nutritional Biochemicals Company, lot 3024; and Sigma, lot 15B-2180.

rate of the reaction at temperature $t^{\circ}C$ was given by the ratio:

$$\frac{\text{micromoles glucose produced at } t^{\circ}C}{\text{micromoles glucose produced at } 25^{\circ}C}$$

Measurement of Invertase Activity in the Centrifuge

When invertase activity was measured in a centrifugal field the diluted enzyme was added to start the reaction as in the procedure above. The reaction tubes were put in the centrifuge 1 min. after the reaction was started and the centrifuge was run at the desired speed for 10 min. The centrifuge required 35-40 sec. to come to speed at 3,200 x g (maximum) and 1½-2 min. for the rotor to come to a stop. The 10 min. were measured from the time the centrifuge was started until the rotor started to decelerate. As soon as the rotor stopped, the reaction tubes were set in a rack and 0.1 N NaOH added 15 min. after the reaction was started. At the same time as the centrifuged systems were run, a set of control tubes were reacting. The rate of reaction in a centrifugal field, relative to the rate of the control, was expressed as a ratio:

$$\frac{\text{micromoles of glucose produced in the centrifuged system}}{\text{micromoles of glucose produced in the control system}}$$

Two centrifuges were used in these studies: a Serval Super-speed centrifuge, type SS-3 (type SS-34 head) and a Lourdes Beta-fuge A2 (9 RA head). The Lourdes Beta-fuge was equipped with a refrigeration unit so that a set temperature could be maintained. The Serval Super-speed centrifuge did not have a refrigeration unit.[1]

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[1] Reaction systems that were spun in the Serval centrifuge are denoted as "centrifuged reactions at ambient temperature", and those in the Lourdes Beta-fuge as "centrifuged reactions at controlled temperature".

For reaction systems that were spun in the Lourdes Beta-fuge the temperature control on the centrifuge was set at 20°C and the controls were run in a water bath at 20°C. When reaction systems were spun in the Serval Super-speed centrifuge the controls were put near the centrifuge to eliminate as much temperature difference as possible.

Determination of the Change in Temperature in the Centrifuge

A temperature rise due to air friction was anticipated in the Serval Super-speed centrifuge. Therefore, a study was made to determine this rise in temperature. A copper-constantan thermocouple was used with a Leeds and Northrup millivolt potentiometer as a "read out" device. The reference junction was placed in an ice-water bath at 0°C.

Into a 50 ml. polyethylene centrifuge tube was pipetted 4.9 ml. of 0.1 M phosphate buffer. The solution was allowed to come to equilibrium at room temperature and the temperature was recorded. The centrifuge tube containing the solution was then placed in the centrifuge and spun for ten min. at the desired speed. The temperature was again recorded after the rotor had stopped. Another centrifuge tube with 4.9 ml. of 0.1 M phosphate buffer was used as a control by setting it next to the centrifuge. The temperature of the control was measured at the same time as the centrifuged solution. From these measurements a net change in temperature was calculated.

According to the specifications for the Lourdes Beta-fuge, a

deviation of only 1°C from the temperature setting was to be expected. However, at higher speeds this was found not to be true. Therefore, a study similar to that described above was made. The temperature of the centrifuge was set at 20°C. The buffer solutions were spun for 10 min. in the centrifuge and the temperature was measured after the rotor stopped. The change in temperature from 20°C was then calculated.

Isolation of Mitochondria

Mitochondria were isolated from fresh beef heart by the procedure of Green and Zeigler (6). The beef hearts were cut up and homogenized with 5 volumes 0.25 M sucrose in a Waring blendor. This homogenate was centrifuged at 1,000 x g (maximum) for 10 min. to sediment the cell debris, nuclei, etc. The supernate was then centrifuged at 15,000 x g (maximum) for 10 min. to obtain a mitochondrial pellet. The mitochondrial pellets were resuspended in 4 volumes of 0.25 M sucrose. Such mitochondrial preparations had a protein concentration of 10-12 mg. per ml.

Procedure for the Measurement of Mitochondrial Activity

The same considerations were taken into account in setting up a procedure for the measurement of mitochondrial activity in the centrifuge as were previously mentioned for the invertase study.

The procedure for measuring mitochondrial activity was a modification of the method used by Lester and Smith (10). The change

in absorbance was determined as the yellow neotetrazolium was reduced to the red formazan using succinate as the hydrogen donor substrate. Triton X-100 was used to solubilize both the mitochondria and the insoluble formazan, so that a homogenous solution was formed that could be read in the spectrophotometer.

Procedure. Into 50 ml. centrifuge tubes was pipetted 2.0 ml. of 0.1 M sodium phosphate buffer (pH 7.4), 0.5 ml. of 0.01 M ethylene-diaminetetraacetate[1] (EDTA), 1.5 ml. of neotetrazolium chloride solution[2], 0.5 ml. of 0.01 M sodium cyanide[1], and 0.2 ml. of 0.1 M sodium succinate (pH 7.4). This solution was incubated 5-10 min. at the desired temperature. To start the reaction 0.2 ml. of mitochondria (2-3 mg. protein) was added and the solution immed-iately homogenized in the centrifuge tube using a Potter-Elvejhem homogenizer. The reaction was allowed to proceed for 20 min. and was then stopped by adding 25 ml. of a Triton-formalin-PCMB mixture[3]. This solution was homogenized again in the Potter-Elvejhem homogenizer. A suitable blank was prepared by adding the Triton-formalin-PCMB mixture before the reaction was started. The absorbance was read on a Bausch and Lomb Spectronic 20 Spectrophotometer at 505 mμ.

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[1]Solutions were made up in 0.1 M sodium phosphate buffer (pH 7.4).

[2]Commercial neotetrazolium chloride contained a small amount of water insoluble substance. For this reason 0.5 g. of the commercial prepar-ation was dissolved in 100 ml. of 0.1 M sodium phosphate buffer and stirred on a magnetic stirrer of 20-30 min. and filtered before use. Three lots of neotetrazolium chloride were used in this study; Nutritional Biochemicals, lot 9407; Sigma, lot 55B-0160; Sigma, lot 104B-0830.

[3]The Triton-formalin-PCMB mixture was prepared by mixing one liter of 0.4 M sodium formate buffer (pH 3.5), 125 ml. of 6% Tirton X-100, 125 ml. of 14.8% formaldehyde, and 40 ml. of saturated p-chloro-mercuribenzoic acid (PCMB).

The specific activity was expressed as the change in absorbance
per min. per mg. of protein.

Sodium Succinate Concentration. As with sucrose in the inver-
tase procedure, it was desirable to have a sodium succinate concen-
tration high enough to achieve zero order kinetics. Reactions were
studied using concentrations of sodium succinate from 0.025 M
to 0.4 M.

Reaction Time. Mitochondrial activity was measured for
different lengths of time to show that absorbance increased linearly
during the assay period.

Neotetrazolium Chloride Concentration. It was also of interest
to study neotetrazolium chloride concentration, since during the
assay or on long standing the neotetrazolium is slowly reduced and
it's concentration changes. The activity therefore, was measured
at $30^{\circ}C$ using neotetrazolium concentrations from 0.001 M to 0.014 M.

Temperature Dependence of Activity. A study was made to deter-
mine how mitochondrial activity was affected by a change in
temperature. A control reaction, run at $25^{\circ}C$, was run along with
each reaction at a given temperature. The relative rate of reaction
at temperature $t^{\circ}C$ to that at $25^{\circ}C$ was expressed as the ratio:

$$\frac{\text{change in absorbance at temperature } t^{\circ}C}{\text{change in absorbance at } 25^{\circ}C}$$

Measurement of Mitochondrial Activity in a Centrifugal Field

When mitochondrial activity was measured in a centrifugal
field, mitochondria were added to start the reaction and the
solution was homogenized as in the procedure above. Reaction

tubes were placed in the centrifuge[1] after 1½ min. and were spun at the desired speed for 10 min. After the rotor stopped, the reaction tubes were taken out of the centrifuge and placed in a rack. The reaction was stopped 20 min. after the mitochondria were added by adding 25 ml. of Triton-formalin-PCMB mixture and the absorbance determined. At the same time as the centrifuged reactions were run, a set of control tubes were reacting. The rate of the reactions run in a centrifugal field, relative to that of the control, was expressed as a ratio:

$$\frac{\text{change in absorbance of the centrifuged system}}{\text{change in absorbance of the control system}}$$

Procedure for Running a Reaction on Mitochondrial Pellets

Since a pellet is formed when mitochondria are spun in the centrifuge, it was thought that diffusion of substrate into the pellet might be a limiting factor in the reaction. To test this, reactions were run on mitochondrial pellets that had been spun at the appropriate speeds.

Sodium phosphate buffer, EDTA, neotetrazolium chloride, sodium cyanide, and mitochondria were added to the centrifuge tubes and homogenized as before. These tubes were then centrifuged at the desired speed for 10 min. in the Lourdes Beta-fuge (20°C). The reaction was started by adding sodium succinate and stopped after 20 min. by addition of the Triton-formalin-PCMB mixture as in the

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[1]The centrifuges used in this study were the same as those used in the measurement of invertase activity in the centrifuge.

procedure for measuring mitochondrial activity. The reactions
were run in a water bath at 20°C.

Study of Mitochondrial Activity in the Centrifuge
Employing a Viscous Reaction Media

To eliminate the effects of forming a mitochondrial pellet
in the centrifuge, the reaction was run in a viscous sucrose solution.
The sucrose solution prevented the mitochondria from sedimenting
to the bottom of the tube.

The components of the reaction mixture were made up in sucrose
solution such that the final reaction mixture had a sucrose concen-
tration of 39.2% (w/w). Mitochondria were added to start the
reaction which was then centrifuged at 3,200 x g (maximum) in the
Lourdes Beta-fuge for 10 min. at 20°C. The Triton-formalin-PCMB
mixture was added 20 min. after the reaction was started. The con-
trols were run in a water bath at 20°C.

Recrystallization of Neotetrazolium Chloride and
Determination of the Molar Absorbancy Index

The commercial preparation of neotetrazolium chloride seemed
to contain two types of crystals. One was a yellow, water soluble
type and the other was a reddish-brown, water insoluble type.
Both types dissolved in ethanol to give a reddish-brown solution.
Neither type of crystal dissolved in ether, while the formazan
of neotetrazolium was readily soluble in ether.

To obtain a homogeneous sample, the commercial neotetrazolium
was recrystallized first from water and then from absolute ethanol

after adding dry ether to the cloud point and cooling. The crystals
were washed twice with dry ether, air dried, and finally dried over
phosphorus pentoxide. This yielded yellow, water soluble crystals
of neotetrazolium chloride.

The recrystallized neotetrazolium chloride was weighed on a
Mettler Microbalance, Model M, and dissolved in 0.1 M phosphate
buffer (pH 7.4). The absorbance was determined by the use of the
Beckman DU spectrophotometer and the molar absorbancy index calculated.
At 320 mμ the molar absorbancy index was 1.73×10^{4}.

Biuret Determination of Protein

Protein in mitochondrial preparations was analyzed using the
biuret reagent of Gornall, Bardawill, and David (5). Their pro-
cedure was modified in the following way; 5 ml. of the reagent was
added to the protein sample and the total volume adjusted to 10 ml.
with distilled water. This solution was placed in a boiling water
bath for 1 min., cooled in an ice bath for 5 min., allowed to come
to room temperature, and read at 540 mμ in a Bausch and Lomb
Spectronic 20 Spectrophotometer. To dissolve insoluble protein,
0.1 ml. of 5% deoxycholate was added. A standard curve was prepared
using bovine serum albumin.

Folin-Ciocalteu Determination of Protein

Protein in the invertase solution was analyzed by the method
of Folin and Ciocalteu (4) as modified by Miller (12). The solid
invertase was accurately weighed and dissolved in distilled water.

Protein analysis was then run on this solution and the milligrams of protein were calculated per milligram of solid invertase. A standard curve was prepared using bovine serum albumin. Some precipitate was formed when high levels of invertase were assayed by this procedure. However, this precipitate could not be detected at lower levels.

RESULTS AND DISCUSSION

Measurement of the Temperature Change in the Centrifuge

The net temperature increase in the centrifuge was calculated from measurements of temperature before and after centrifugation. In the Serval Super-speed centrifuge, measurements were made at 1,000 rpm (128 x g maximum), 5,000 rpm (3,200 x g maximum), and 10,000 rpm (12,800 x g maximum). The net increases in temperature were $1.5^{\circ}C$, $2.2^{\circ}C$, and $5.2^{\circ}C$, respectively. A similiar measurement was made in the Lourdes Beta-fuge at 18,000 rpm (41,300 x g maximum) with a temperature setting of $20^{\circ}C$. The increase in temperature was $5.4^{\circ}C$. The increase in temperature in all cases was probably due to the friction between the rotor and the air.

Study of Conditions for Invertase Procedure in a Centrifugal Field

In setting up the procedure for the study of invertase in the centrifuge, several factors were considered: (1) What concentration of sucrose is necessary for the reaction to proceed according to zero order kinetics and hence, proceed linearly for the duration of

the reaction period; (2) What is the change in relative rate of invertase activity per degree change in temperature? These factors were investigated and the results are shown in Fig. 1-3. These graphs show that (1) 0.4 M sucrose was of sufficiently high concentration to give zero order kinetics and permit the reaction to proceed linearly for 15 min.; and (2) the change in relative rate of invertase activity was approximately 0.05 per degree.

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Study of Invertase Activity in a
Centrifugal Field

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The change in invertase activity, as a result of centrifugation, was expressed as the ratio of the activity of a centrifuged system to that of a control.

The average relative rate of 10 reactions spun at 3,200 x g (maximum) in the centrifuge at ambient temperature was 1.10 with a standard error of 0.03 and average specific activity of 4210 micromoles of glucose per min. per mg. of protein. If it is assumed that the centrifuged system was $2.2^{\circ}C$ higher for the entire period of the reaction, a relative rate of 1.11 would be expected, since the relative rate of invertase activity increased 0.05 per degree temperature rise. Therefore, the increase in temperature would account for the increase in activity. Hence, no effect of centrifugation on invertase activity was observed. The assumption that the temperature rise was effective for the entire period of the reaction was confirmed by later experiments.

The average relative rate of 10 reactions spun in the centrifuge at 3,200 x g (maximum) with the temperature controlled ($20^{\circ}C$) was

Specific Activity
(Micromoles of
glucose per min.
per mg. protein)
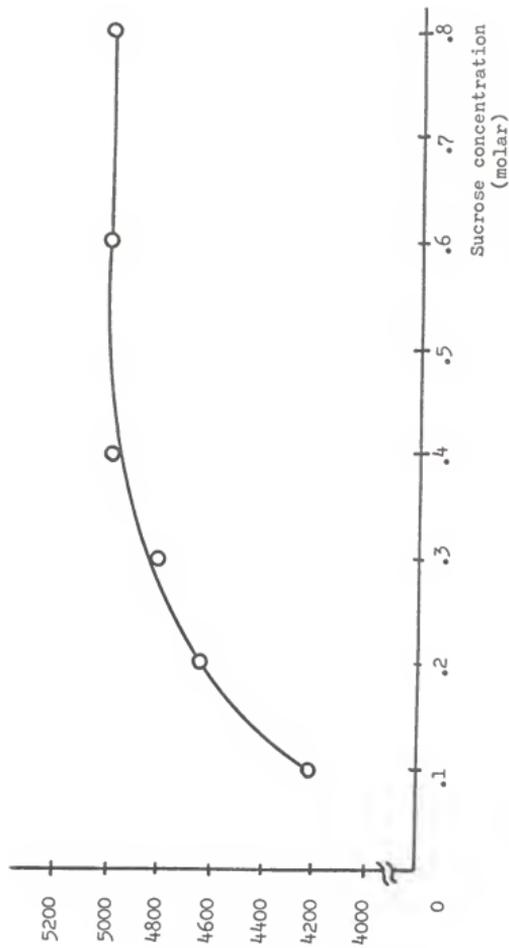
Fig. 1. The dependence of the specific activity of invertase on sucrose
concentration. Temperature of the reaction was 30°C.
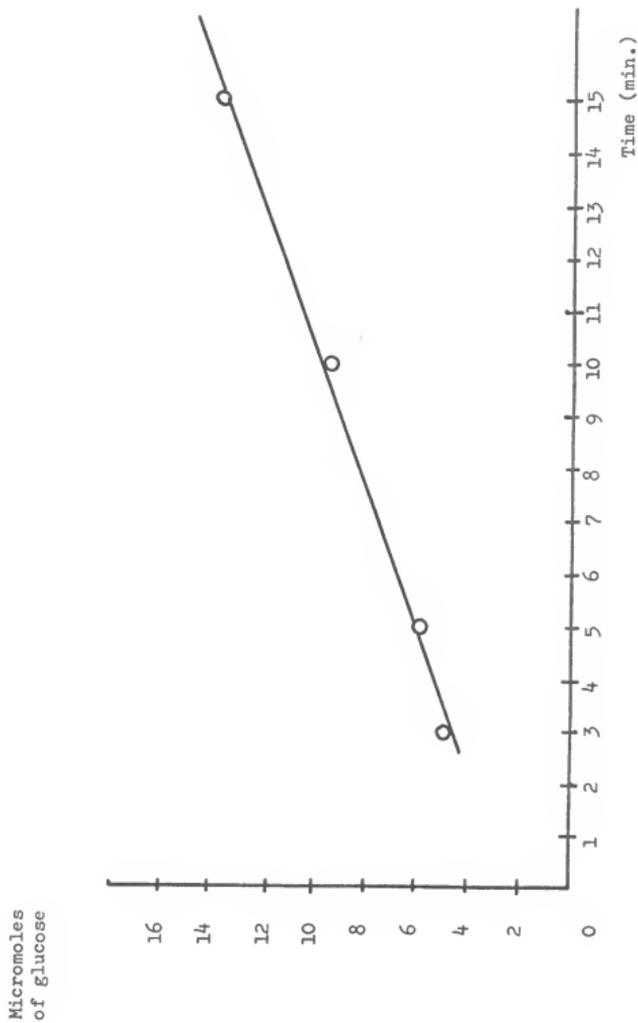
Fig. 2. The relationship between the formation of reducing sugar and time. The temperature of the reaction was $30^{\circ}C$.
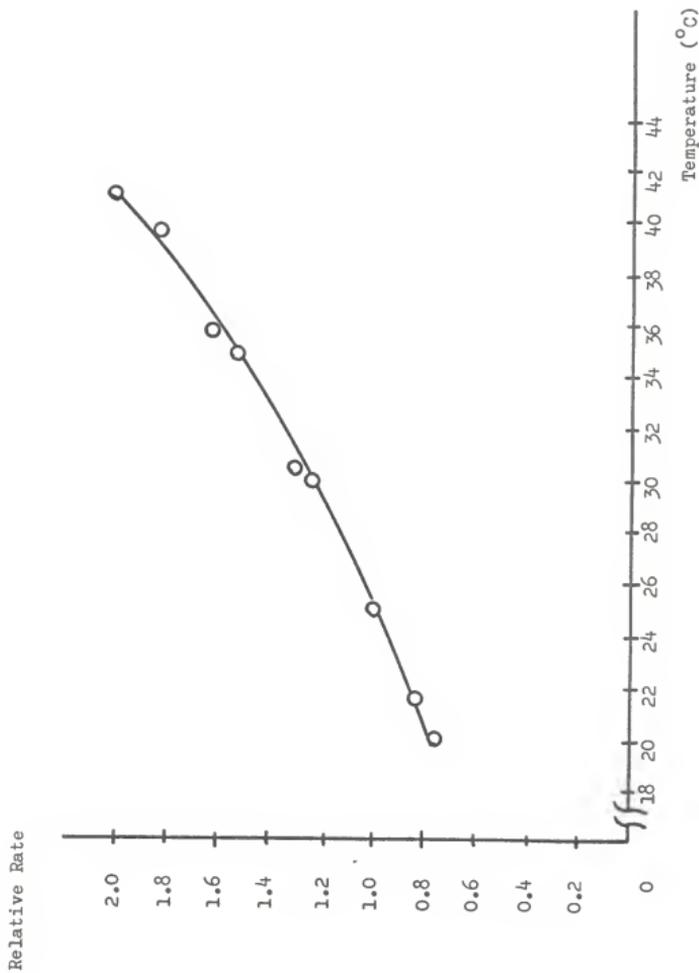
Fig. 3. Dependence of the relative rate of invertase activity on temperature.

1.02 with a standard error of 0.01 and an average specific activity of 2,983 micromoles glucose per min. per mg. protein. This observation supports the above statement that at 3,200 x g (maximum), there is no effect of centrifugation on invertase activity.

Five sets of reactions were spun at 41,300 x g (maximum) in the Lourdes Beta-fuge with a temperature setting of 20°C. The average relative rate was 1.33 with a standard error of 0.02 and an average specific activity of 2878 micromoles glucose per min. per mg. protein. Since there was a 5.4°C increase in the centrifuge, a relative rate of 1.30 would be expected due to the increase in temperature. Therefore, the increase in the activity of invertase in the centrifuge at 41,300 x g (maximum) can be explained by the increase in activity if the increase in temperature is effective for the entire period of the reaction.

To eliminate the increase in temperature in the Lourdes Beta-fuge an experiment was performed where the empty rotor was spun at 41,300 x g (maximum) for 10 min. to bring the temperature of the rotor to an equilibrium temperature of 25.4°C. As soon as the rotor stopped a reaction was started and spun at 41,300 x g (maximum) for 10 min. A control was run in a water bath at 25.5°C. The relative rate of 5 such reactions was 1.05 with a standard error of 0.01 and an average specific activity of 3926 micromoles glucose per min. per mg. protein.

When all of the data are considered, there appears to be no effect of centrifugation on invertase at forces up to 41,300 x g (maximum) or the effect is so small that it cannot be detected with confidence by these methods.

As mentioned in the introduction, there is a hydrostatic

pressure produced when a solution is centrifuged (4 atmospheres at 3,200 x g maximum and 53 atmospheres at 41,300 x g maximum). Eyring (4) found that invertase activity increased 5% at a hydro-static pressure of 476 atmospheres. This increase was observed using conditions that were approximately the same as those used in the study of invertase activity in the centrifuge. From these results, a very small increase in activity would be expected due to the pressure produced by centrifugation of the solution. However, this increase is probably so small that it cannot be detected.

Study of the Conditions for Measuring the Rate of Mitochondrial Activity in the Centrifuge

The same considerations were used in setting up conditions for measuring mitochondrial activity in the centrifuge, as those used in the invertase study. The results of using different concentrations of succinate, different concentrations neotetrazolium, different lengths of reaction time, and varying temperatures are shown in Fig. 4-7. These graphs show that a succinate concentration of 0.1 M is sufficiently high to obey zero order kinetics and the reaction proceeds linearly for at least 20 min. (Fig. 4-5). The increase in the relative reaction rate is 0.14 per degree increase in temperature (Fig. 6). Even though the activity is maximal at a neotetrazolium concentration of approximately 0.002 M (Fig. 7), this condition was not used in the assay. To measure mitochondrial activity in the centrifuge, approximately 0.008 M neotetrazolium was used to avoid changes in activity due to small changes in concentration during the reaction. Since autoreduction takes
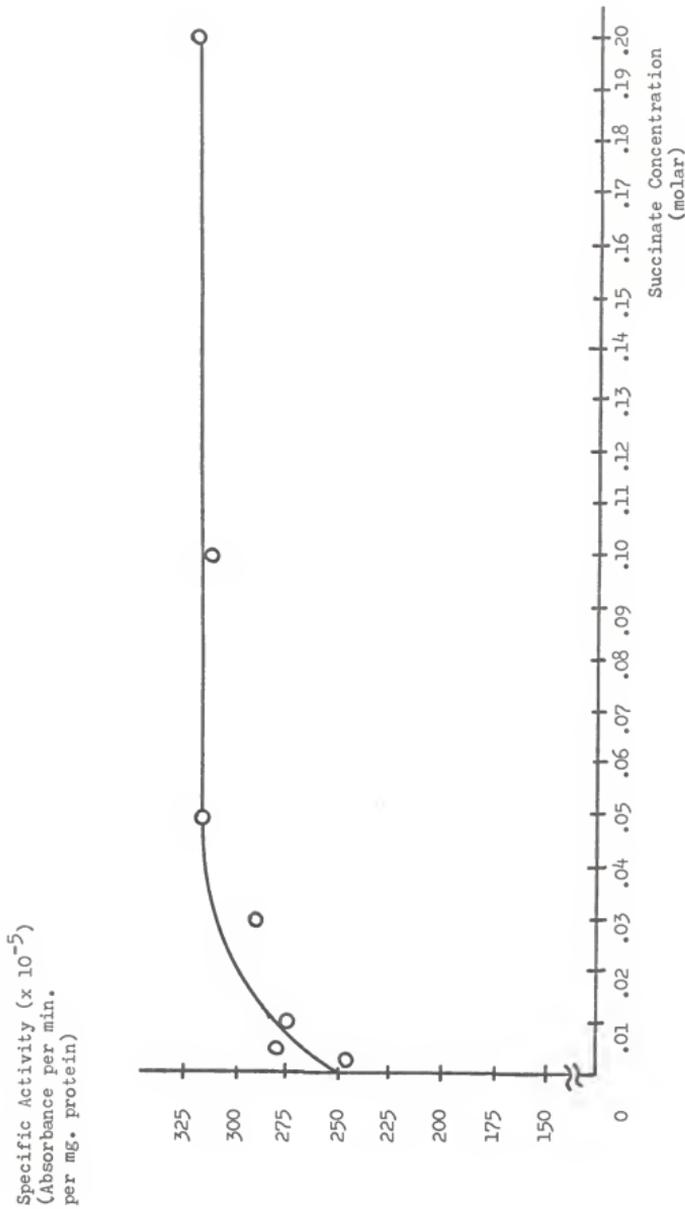
Fig. 4. The dependence of the specific activity of mitochondria on succinate concentration. The temperature of the reaction was 30°C.
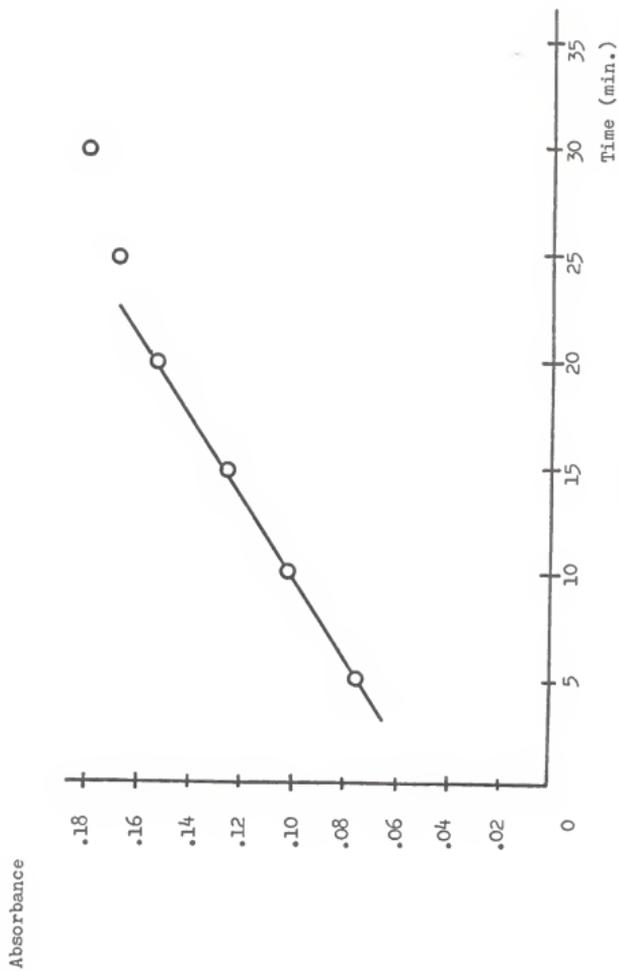
Fig. 5. Mitochondrial activity as it progresses with time. The temperature of the reaction was 30°C.
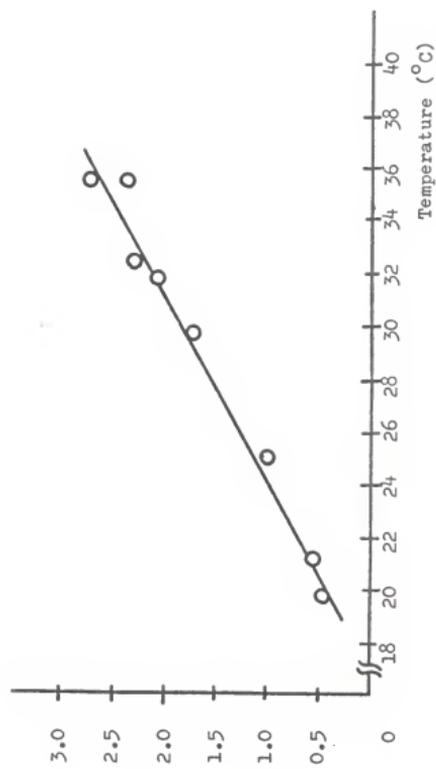
Fig. 6. Dependence of the rate of mitochondrial activity on temperature.

Specific Activity (x 10^{+5})
(Absorbance per min. per mg. protein)
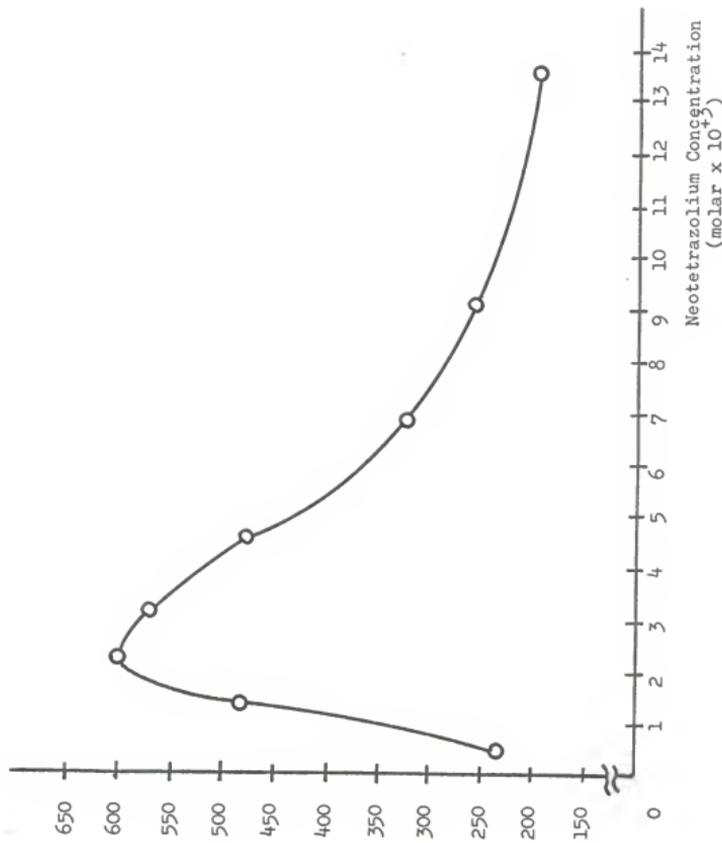
Fig. 7. Dependence of the specific activity of mitochondria on neotetrazolium concentration. The temperature of the reaction was $30^{\circ}C$.

place when neotetrazolium is in solution a truely constant concen-
tration is difficult to obtain. However, this changing concentration
is compensated for, since the concentration of neotetrazolium is
the same in the control as in the centrifuged reactions.

Results of the Study of Mitochondrial
Activity in a Centrifugal Field

The increase in mitochondrial activity in the centrifuge was
expressed as the ratio of the activity of the centrifuged system
to the activity of a control.

The results of trials where mitochondrial systems were spun
in the Serval Super-speed Centrifuge are given in Table 1. They
are listed in chronological order and the lot number of neotetra-
zolium that was used is noted. As can be seen from Table 1, the
results from trials 13-32 are lower than the results of the first
12 trials. The first 12 trials have an average of 1.7 and a stand-
ard error of 0.1. Trials 13-32 have an average of 1.2 with a standard
error of less than 0.1. When all of the trials are considered the
relative rate is 1.4 with a standard error of less than 0.1. Since
there is a $2.2^{\circ}C$ increase in temperature in the centrifuge and the
increase in activity per degree rise in temperature was 0.14, a
relative rate of 1.3 would be expected due to the rise in temperature.
Therefore, the increase in temperature would account for most of the
increase in activity, when all of the data are considered. However,
if only the first 12 trials are considered, the relative rate is
too high to be caused by only the increase in temperature.

Ten sets of reactions were spun in the Lourdes Beta-fuge at
3,200 x g (maximum) with a temperature setting of $20^{\circ}C$.

Table 1. Rate of centrifuged reactions employing
mitochondria at ambient temperature.

Trial Number	Date	Specific Activity of the Control	Rate
1	March 9	.00460*	1.4
2	March 10	.00355*	2.0
3	March 12	.00299*	1.9
4	March 12	.00329*	1.9
5	March 15	.00250*	1.6
6	March 15	.00252*	1.7
7	March 19	.00259*	1.6
8	March 22	.00329*	1.6
9	March 22	.00228*	1.9
10	March 26	.00250*	1.7
11	June 24	.00171*	1.3
12	June 25	.00186*	1.5
13	July 20	.00254**	1.1
14	July 20	.00272**	1.1
15	July 20	.00245**	1.2
16	July 20	.00250**	1.2
17	July 21	.00257**	1.0
18	July 21	.00257**	1.2
19	July 21	.00336**	1.2
20	July 21	.00179**	1.4
21	July 22	.00176**	1.1
22	July 22	.00158**	1.2
23	July 26	.00328**	1.1
24	July 26	.00477**	1.3
25	July 27	.00835**	1.2
26	July 28	.00755**	1.3
27	July 28	.00680**	1.1
28	July 28	.00562**	1.2
29	July 29	.00171**	1.1
30	July 30	.00705**	1.1
31	July 30	.00800**	1.0
32	August 4	.00612**	1.2
33	August 4	.00375*	1.5
34	August 5	.00448*	1.3
35	August 5	.00614**	1.2
36	August 5	.00460*	1.4
37	August 5	.00573**	1.2
38	August 5	.00398*	1.4
39	August 5	.00535**	1.2

* Neotetrazolium from Sigma, lot 104B-0830.
**Neotetrazolium from Sigma, lot 55B-0160.

The average relative rate was 0.92 with a standard error of 0.02 and an average specific activity of .00296 change in absorbance per min. per mg. of protein.

Reactions were spun in the Lourdes Beta-fuge after the rotor had first been spun for 10 min. at 41,300 x g (maximum) to bring the temperature to equilibrium at 25.4°C. The controls were run in a water bath at 25.5°C. The average relative rate for 5 such trials was 1.00 with a standard error of 0.03 and an average specific activity of 0.00276 change in absorbance per min. per mg. protein.

The above observations indicate that there is little or no effect on mitochondrial activity due to centrifugation up to 41,300 x g (maximum).

Measurement of Activity as Observed From a Reaction on a Mitochondrial Pellet

Reactions were run on mitochondrial pellets to determine if diffusion into the pellet was a limiting factor in the reaction. Pellets were formed in a solution containing phosphate buffer, neotetrazolium chloride, EDTA, and NaCN, at 3,200 x g (maximum). The reaction was then started by adding the requisite amount of succinate to the pellet-containing system. The average relative rate of 5 reactions was found to be 1.08 with a standard error of 0.01. When pellets were formed in the same manner as above, but at 41,300 x g (maximum), the average relative rate of 5 reactions was found to be 0.70, with a standard error of 0.03. These results suggest that succinate can easily enter a pellet formed at 3,200 x g, but not a pellet formed at 41,300 x g.

Pellets were also formed in the absence of neotetrazolium chloride by centrifuging 0.2 ml. of mitochondria for 10 min. in 30 ml. of 0.155 M KCl. After the pellet was formed, the KCl was carefully decanted. The reaction mixture containing both succinate and neotetrazolium chloride was poured onto the pellet to start the reaction. The controls were treated in the same way except that the pellet was resuspended in the reaction mixture.

Pellets formed in KCl at 3,200 x g (maximum) possessed an average relative rate of 0.37 with a standard error of 0.01 (5 trials). The average relative rate of 5 trials, where pellets were formed in KCl at 105,651 x g (maximum) was 0.22 with a standard error of 0.02.

The above results indicate that when both succinate and neotetrazolium chloride must diffuse into a mitochondrial pellet the reaction is markedly slower than the control which contains no pellet. When neotetrazolium is packed into the pellet by centrifuging the mitochondria in a solution containing neotetrazolium the reaction rate is not decreased unless a very hard pellet is formed.

In all of the experiments which were designed to study the effect of centrifugal fields on mitochondrial oxidation of succinate, both succinate and neotetrazolium chloride were packed into the pellets formed, because they were present in the solution from which the pellets were formed. The above results indicate that while experimental conditions can be devised in which entry of succinate and neotetrazolium chloride into a pellet can be a limiting factor, this was not a limiting factor in the usual centrifugation experiments.

Results of Mitochondrial Activity in the Centrifuge
Employing a Viscous Reaction Media

To eliminate the effects of forming a mitochondrial pellet
in the centrifuge, reactions were spun in the centrifuge employing
a reaction medium containing 39.2% (w/w) sucrose. The viscosity
of 39.2% (w/w) sucrose at $25^{\circ}C$ is 4.88 centipoise (2,7). At
3,200 x g (maximum) a pellet did not form. The average relative
rate of 5 such reactions was 1.0 with a standard error of less
than 0.1 and an average specific activity of 0.00187 change in
absorbance per min. per mg. protein.

Reactions spun at 3,200 x g (maximum) in either 39.2% sucrose
solution or reaction media with no sucrose gave the same results.
This indicated that forming a pellet did not effect the activity
of mitochondria.

Considerations of Certain Inconsistant Results

An inconsistancy was observed in the results of reactions spun
in the centrifuge at ambient temperature. This can best be seen
in Table 1, where values obtained in the first 12 trials appear to
be larger than in the remaining trials. This inconsistancy prob-
ably was not due to technique, since the deviation among data in
any one assay of quadruplicate samples was quite small. Examples
of the results obtained in quadruplicate assays are shown in
Table 2.

Table 2. Comparison of quadruplicate samples.

Date	Activity of the Control	Mean	Standard Error	Activity of the Centrifuged Reaction	Mean	Standard Error
March 9	.143 .156 .153 .153	.151	.003	.203 .206 .206 .210	.206	.001
March 10	.110 .113 .115 .115	.113	.001	.215 .235 .230 .240	.230	.005
March 12	.095 .098 .100 .100	.098	.001	.185 .190 .190 .190	.189	.001
March 12	.102 .110 .108 .110	.108	.002	.210 .202 .208 .202	.206	.002
March 15	.080 .082 .082 .082	.082	.001	.132 .135 .130 .125	.131	.002
March 15	.085 .085 .080 .080	.083	.002	.145 .140 ---- .145	.142	.001

By assuming that the distribution of the relative rate is normal, statistical calculations were made using the "Student-t" distribution at the 5% level that show trials 1-12 to be a different statistical population than trials 13-32. This indicates that probably some factor other than the sampling error caused the incongruent results.

Several attempts to determine the cause of the inconsistant results were made. The results of these studies are given in the following paragraphs.

Spectra of Neotetrazolium. Since one lot of neotetrazolium was used in trials 1-12 and another in trials 13-32 (Table 1), it was possible that the lots of neotetrazolium differed. The spectra of three lots of neotetrazolium were taken on a Cary Model 14 Spectrophotometer. The spectra of these lots of neotetrazolium coincided when plotted on a relative absorbance scale. The absorption spectra of neotetrazolium chloride after recrystallization from water and alcohol is shown in Fig. 8.

Reactions in the Centrifuge Using Three Lots of Neotetrazolium. A comparison was made of the activity of mitochondria in the centrifuge using three lots of neotetrazolium. The mean relative rate for neotetrazolium from Sigma, lot 104B-0830, was 1.4; the mean rate for neotetrazolium from Sigma, lot 55B-0160, was 1.2; and the mean rate for neotetrazolium from Nutritional Biochemical Company, lot 9407, was 1.4. Statistical calculations were made using the "Student-t" distribution, showing at the 5% level that the trials, using three lots of neotetrazolium, were not from different populations. This would indicate that the different lots of neotetrazolium were not the cause of the incongrous results.

Reactions Run With Recrystallized Neotetrazolium. Neotetrazolium from Sigma, lot 55B-0160, was recrystallized first from water and then absolute alcohol. The crystals were washed twice with dry ether and dried over phosphorous pentoxide. A comparison was made of the relative rates using the recrystallized neotetrazolium and the commercial preparation of neotetrazolium. The recrystallized neotetrazolium gave a lower relative rate than the commercial neotetrazolium. This result suggests that the low relative rates in trials 13-32 were not caused by impurities in the neotetrazolium.

Molar Absorbancy
Index (x 10⁻⁴)
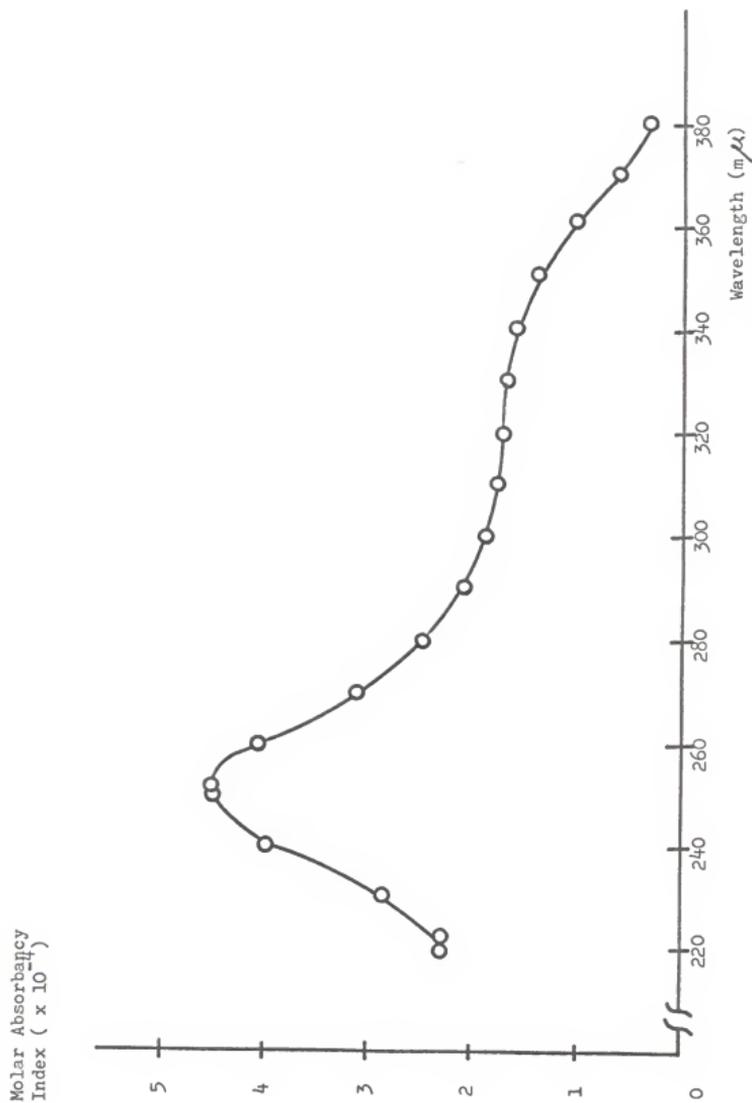
Fig. 8. Spectra of recrystallized commercial preparation of neotetrazolium.

Wavelength (mμ)

Reaction in the Centrifuge Using Varying Concentration of Neotetrazolium. Mitochondrial activity was measured in the centrifuge using varied concentrations of neotetrazolium to determine if the concentration of neotetrazolium was the factor causing discordant results. Concentrations from 0.0005 M to 0.0145 M were used and relative rates from 1.1 to 1.3 were obtained.

The conclusion drawn from these results was that the concentration of neotetrazolium had no appreciable effect on the relative rate.

Reaction on a New Batch of Mitochondria. To determine whether differences in mitochondrial preparations were the cause of the low relative rates in trials 13-32, a fresh batch of mitochondria was prepared and used. The relative rate from three trials with these new mitochondria gave an average relative rate of 1.2. This would suggest that the mitochondrial preparations were not the cause of the inconsistant results.

Reactions Using All New Reagents. Mitochondrial activity was measured in the centrifuge using newly prepared reagents. A mean relative rate of 1.2 was observed suggesting that impure reagents did not cause the inconsistant results.

SUMMARY

A systematic study was made to develop an analytical method for studying enzymatic reactions in systems subjected to centrifugal fields. An assay procedure was set up for both a homogenous system (invertase) and an inhomogenous system (mitochondria).

From the study of invertase activity in the centrifuge, the
statement can be made, that at centrifugal forces up to 41,300 x g
(maximum) there is no effect on invertase or the effect is so
small that it cannot be detected with confidence by the methods used.

The study of mitochondrial oxidation of succinate in the centri-
fuge also showed no significant change relative to a control at
centrifugal forces up to 41,300 x g (maximum).

From studies involving pellets, the observation was made that
when succinate and neotetrazolium chloride are both required to
diffuse into the pellet the rate of reaction is markedly decreased.
However, if the mitochondria are spun down from the reaction mixture,
succinate and neotetrazolium seem to be packed into the pellet with
the mitochondria and diffusion is not a limiting factor as the
reaction proceeds.

ACKNOWLEDGMENT

LITERATURE CITED

1. Campbell, D. H. and F. H. Johnson.
        Pressure and Specific Precipitation. J. Am. Chem. Soc.
        68, 725. 1946.

2. de Duve, C., J. Berthet and H. Beaufay.
        Gradient Centrifugation of Cell Particles; Theory and
        Applications. Prog. Biophys. and Biophys. Chem. 9,
        357. 1959.

3. Eyring, H., F. H. Johnson, and R. L. Gensler.
        Pressure and Reactivity of Proteins, with Particular
        Reference to Invertase. J. Phys. Colloid Chem. 50,
        453. 1946.

4. Folin, O. and V. Ciocalteu.
        On Tyrosine and Tryptophane Determination in Proteins.
        J. Biol. Chem. 73, 627. 1927.

5. Gornall, A. G., C. J. Bardawill and M. M. David.
        Determination of Serum Proteins by Means of the Biuret
        Reaction. J. Biol. Chem. 177, 751. 1949.

6. Green, D. E., and D. M. Zeigler.
        Electron Transport Particles. In Methods in Enzymology.
        (Edited by Colowick, S. P., and N. O. Kaplan),
        Vol. VI, 416. Academic Press, New York. 1963.

7. Hodgman, C. D., R. C. Wease, R. S. Shankland, and S. M. Selby.
        Handbook of Chemistry and Physics, 44th Edition, 2270.
        1946.

8. Johnson, F. H., H. Eyring, R. Stelblay, H. Chaplin, C. Huber,
    and G. Gherardi.
        Nature and Control of Reactions in Biolumenescence.
        J. of Gen. Physiol. 28, 463. 1945.

9. Laidler, K. J.
        The Chemical Kinetics of Enzyme Action.
        Oxford University Press, London, 1958.

10. Lester, R. L., and A. L. Smith.
        Studies on the Electron Transport System XXVIII.
        The Mode of Reduction of Tetrazolium Salts by Beef Heart
        Mitochondria; Role of Coenzyme Q and Other Lipids.
        Biochim. et. Biophys. Acta, 22, 475. 1956.

11. Miller, G. L.
        Use of Dinitrosalicylic Acid Reagent for Determination
        of Reducing Sugar. Anal. Chem. 31, 426. 1959.

12. Miller, G. L.
          Protein Determination for Large Numbers of Samples.
          Anal. Chem. <u>31</u>, 964. 1959.

13. Sumner, J. B., and E. B. Sisler.
          A Simple Method for Blood Sugar. Arch. Biochem.
          <u>4</u>, 333. 1944.

14. Svedberg, T., and K. O. Pedersen.
          The Ultracentrifuge. Oxford University Press,
          London, 1958.

15. van Calcar, R. P., and C. A. Lobry de Bruyn.
          Concentratieveranderinger in en Kristallisatie uit
          Oplossinger Door Centrifugaalkracht. Verhandel.
          Akad. Amsterdam <u>XII</u>, 936. 1904.

16. Whitehead, K.
          The Effect of Gravitational Force on the Reactions of
          Mitochondria. Senior Research Report, Department of
          Chemistry, Kansas State University. 1965.

THE EFFECT OF CENTRIFUGAL FIELDS
ON ENZYMATIC REACTIONS

by

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B.S., Kansas State University, 1965

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AN ABSTRACT OF MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Biochemistry

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1966

A systematic study was made to develop an analytical method for studying enzymatic reactions in systems subjected to centrifugal fields. An assay procedure was set up for both a homogenous system (invertase) and an inhomogenous system (mitochondria).

The effects of centrifugal fields on invertase and mitochondrial systems were studied by spinning them in a centrifuge for 10 min. The activities of the centrifuged systems were then compared with control systems that were not centrifuged. The rate of the reaction run in a centrifugal field, relative to the rate of the control, was expressed as a ratio:

$$\frac{\text{activity in the centrifuged system}}{\text{activity in the control system}}$$

The relative rate of invertase in a centrifuge at 3,200 x g (maximum) was 1.02. At 41,300 x g (maximum) the relative rate was 1.05. From the study of mitochondrial activity in the centrifuge at 3,200 x g (maximum), a relative rate of 0.92 was obtained. At 41,300 x g (maximum) the relative rate was 1.00.

Since a pellet is formed when mitochondria are spun in the centrifuge, reactions were run on mitochondrial pellets to determine if diffusion into the pellet was a limiting factor in the reaction. When pellets were formed at 3,200 and 41,300 x g (maximum) in solutions containing neotetrazolium chloride, the relative rates were 1.08 and 0.70 respectively. When pellets were formed at 3,200 and 105,651 x g (maximum) in 0.155 M KCl the relative rates were 0.37 and 0.22 respectively.

To eliminate the effect of forming a pellet, reactions were run in a viscous sucrose solution. The relative rate, when such

reactions were spun at 3,200 x g (maximum), was 1.0.

The conclusion was drawn that centrifugal fields up to
41,300 x g (maximum) have no effect on the activity of invertase
or the oxidation of succinate by mitochondria. Also observed was
the fact that if succinate and neotetrazolium chloride must enter
a mitochondrial pellet the rate of succinate oxidation was decreased.
However, sufficient succinate and neotetrazolium chloride apparently
is packed into a pellet when mitochondria are sedimented in the
presence of these two chemical substances, so that the reaction
proceeds at a maximum rate even though the mitochondria are removed
from the bulk of the solution.