To the Graduate Council:

I am submitting herewith a dissertation written by Donald R. Bransford entitled "Effects of Training on Free Fatty Acid Metabolism during Prolonged Exercise in Women." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Education, with a major in Physical Education.

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EFFECTS OF TRAINING ON FREE FATTY ACID METABOLISM DURING PROLONGED EXERCISE IN WOMEN

A Dissertation

Presented for the

Doctor of Education

Degree

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Donald R. Bransford

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1332601

TO DIANE, DARCIE AND SCOTT,

Who did without while I endured. To them

I owe and dedicate this work.

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ABSTRACT

The purpose of this study was to investigate the effects of training on free fatty acid (FFA) metabolism during prolonged work in women. Subjects were randomly placed into two groups: 5 in a control group and 5 in an experimental group.

All subjects were given a pretest in which they pedalled at 55 percent of their maximal aerobic power for 60 minutes. Blood samples and heart rate were taken at rest, every 10 minutes during exercise and 10 minutes post exercise. The blood samples were later analyzed for FFA, lactate and glycerol. Ventilatory and gas exchange measurements were taken every 20 minutes during the exercise period. Following the pretest the experimental group trained one hour a day, three days a week for four weeks (12 sessions) on a bicycle ergometer. The control group maintained normal daily living habits. At the end of four weeks the same test was again administered to both groups using the same absolute workload.

The increased maximal aerobic power ($P < 0.05$), decreased resting and exercise heart rates ($P < 0.05$) and depressed respiratory exchange ratio ($P < 0.05$) indicate an increased level of fitness in the experimental group following training. No significant differences ($P > 0.05$) were observed in the control group. Submaximal oxygen uptake did not change ($P > 0.05$ in either group following the posttest.

FFA levels were significantly lower ($P < 0.05$) during exercise in the experimental group following training. A gradual but significant increase ($P < 0.05$) in FFA levels was observed during the 60 minute work

period. No significant changes ($P > 0.05$) were noted in lactate levels between groups. No significant differences ($P > 0.05$) were observed in the glycerol levels between the two groups although the post exercise group had consistently lower levels ($P < 0.06$) than its pretest levels and the two tests of the control group.

The lower FFA and glycerol levels observed during exercise for the experimental group following training were probably the result of a decreased rate of lipolysis and an increased rate of FFA oxidation by the muscle. The lower FFA and glycerol concentrations, and lower R values of the experimental group in the presence of the same oxygen uptake further support this hypothesis.

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CHAPTER I

INTRODUCTION

The concept of substrate utilization by exercising muscle has changed drastically in recent years. In the early part of the 1900's carbohydrate was considered the predominant, if not the only, source of fuel for energy production by the muscle. This view was changed by the growing awareness of the physiological importance of fat, especially plasma free fatty acids (FFA). However, the identification of circulating FFA as energy-yielding substrates has overshadowed the importance of the role of glucose in muscle energy metabolism. It is now clear that in addition to FFA, stored muscle glycogen and blood-borne glucose contribute substantially to the energy needs induced by exercise. The relative contributions of each of these fuels at any given time is largely determined by the duration and intensity of exercise.

Prolonged exercise at a low work intensity (30-60 percent of maximal oxygen uptake) utilizes primarily FFA and glucose as substrates. Ahlborg, et al. (2) reported that the combined uptakes of glucose and FFA by the exercising muscle could account for approximately 65 percent of the total metabolism after 40 minutes while at 4 hours of exercise the figure had increased to more than 90 percent. This study also demonstrated that during prolonged exercise blood-borne substrates, particularly FFA, play an increasingly important role in the supply of fuel to exercising muscle.

The metabolic response to prolonged exercise of well-trained athletes differs from untrained subjects. Trained subjects have lower blood concentrations of lactate and pyruvate and a lower RQ during submaximal exercise (32,42,89,94,121). Conversely, the rate of FFA uptake by the cell is greater. Armstrong, et al. (5,6,32,42) have proposed that this increased uptake of plasma FFA by the exercising muscle is due to a mass-action effect, i.e., the higher the plasma concentration of FFA, the greater the uptake by the muscle. Based on these results, one would assume that the plasma FFA concentration would be higher in well-trained subjects, especially with lower lactate and RQ values.

Although the plasma FFA concentration increases during prolonged work (9,110,117,41) comparisons between trained and untrained subjects are equivocal. The same is true of the plasma FFA level during prolonged work before and after physical training. A number of studies reported lower FFA levels in trained than untrained subjects (29,90,91,114,115, 40). Others have reported either no difference or a higher FFA concentration in the trained subjects (22,68,70,93).

The apparent discrepancies described above might be attributed to the differences in design of the various experiments. Due to the highly interactive nature of substrate metabolism, metabolite production and work load, interpretation of these results is limited and comparisons are difficult. A more carefully designed study, using a control and an experimental group and a light work load would clarify the present picture of FFA metabolism during prolonged work of low intensity.

The light work load, approximately 55 percent of maximal aerobic power, would minimize lactate production (59) and thus eliminate its inhibitory effect on free fatty acid mobilization (3,79,87,100). Using a control and experimental group allows for pretest and posttest comparisons of both groups and makes the design much stronger.

The purpose of this study was to investigate changes in plasma free fatty acid concentration during prolonged work in trained and untrained women. Specifically, the purposes of the study were: (1) To critically evaluate the effects of prolonged exercise on free fatty acid metabolism; (2) To critically evaluate the effects of prolonged exercise on free fatty acid metabolism after four weeks of training; and (3) To critically evaluate lactate and glycerol concentration under the same experimental conditions.

CHAPTER II

REVIEW OF LITERATURE

Introduction

In 1913 Lafon gave a report to the Academy of Science entitled, "On the consumption of fat in the animal organism" in which he stated:

> It has long been established that . . . it is carbohydrates and body fat which furnish the organism the energy spent for maintenance and activity, nitrogenous substances having another role, that of serving renewal of the intrinsic substances of tissues. . . . The investigations by Chauveau and Kaufman . . . have shown the preponderant role of glucose as immediate nutrient for . . . muscle activity, but the role of fats is no less apparent (139).

Despite Lafon's report there seems to have developed in his time, and during the next four or five decades, an acceptance of the doctrine that glucose was the main fuel, perhaps even the essential fuel, for oxidative metabolism by muscle. In 1938 Lundsgaard (97) summarized the available information responsible for this viewpoint. Most of the physiologists had approached this question by measuring ventilatory exchanges of oxygen and carbon dioxide. Respiratory quotients near unity were often observed, indicating carbohydrate utilization.

At the same time, a few studies were also appearing that showed ventilatory RQ's near 0.8 at rest and during exercise (52,74). These authors concluded that both carbohydrate and fat were substrates for muscular contraction. Gemmill (53) reviewed other preisotope experiments which were designed to investigate the fuels for muscular exercise. While it was concluded from the available information that carbohydrate

was predominantly important, data were available which suggested that fat could be used by muscle.

Free Fatty Acid Turnover and Oxidation

In 1946 Lehninger (96) succeeded in demonstrating that cardiac muscle possessed the enzymatic capabilities necessary for the direct oxidation of fatty acids. Similar capabilities were demonstrated in skeletal muscle by Fritz (49). At about the same time several researchers reported that skeletal muscle was capable of extracting free fatty acids (56,130,134). Subsequent research left little doubt that plasma free fatty acids (FFA) are readily oxidized by the muscle in vitro (14,32,51, 102,103,133) as well as in vivo (42,44,45,47,88,126).

In 1956 Andres, et al. (4) demonstrated that carbohydrate utilization could account for less than 17 percent of the oxygen consumption of resting skeletal muscle in normal postabsorptive subjects. The RQ of the muscles at rest was about .75 which was in agreement with conclusions from measurements of glucose uptake. The results also indicated that more than 80 percent of the substrate oxidized was lipid. They concluded: "Purely on the basis of relative ease of transcapillary exchange, it would appear most probable that the small fraction of fatty acids which are unesterified might be the major substrate." The development of methods to quantify the FFA fraction in plasma enabled researchers to examine actual uptake and release of FFA by the muscle. Dole (30) demonstrated that FFA are released from adipose tissue and Gordon (60) reported a positive arteriovenous difference in plasma FFA concentration

in humans across the myocardium and in some instances the forearm. The latter results were somewhat inconsistent.

Using radioisotopes, more direct evidence was presented which indicated FFA utilization by the muscle. Friedberg, et al. (47) measured arteriovenous differences of ^{14}C palmitate in the forearm during contralateral intravenous infusion of the tracer. They demonstrated that muscle removed the labelled FFA from the arterial blood irrespective of the simultaneous arteriovenous differences for endogenous FFA. Subsequent reports from other laboratories gave convincing evidence that FFA's are a major energy substrate for oxidative metabolism in skeletal muscle (2,63, 70,71,82,84,86,100,108,110,126).

Intra- and Extramuscular Adipose Stores (Rest)

Muscle cells always have a lower FFA concentration than the surrounding medium, and there is, thus, a diffusion gradient. This gradient is a necessary condition for a sufficient influx of FFA since no active transport mechanism for FFA is known (98). Free fatty acids that are oxidized by the muscle appear to come from two sources: an intramuscular pool and extramuscular adipose tissue. Entry of FFA into the blood from extramuscular adipose cells is controlled by the activity of an intracellular lipase which hydrolyzes triglycerides to form FFA and glycerol (66). Both of these products then enter the blood with FFA transported in association with albumin. Although FFA make up only 5 percent of the total plasma lipids, there is a rapid turnover rate of FFA (42) and they can provide a substantial portion of the energy needs during rest (30,61).

The concentration of FFA in plasma is very important since it has an influence on turnover, uptake, and oxidation of FFA. A straight line correlation exists between plasma FFA concentration and their turnover, uptake, and oxidation (6,51,54,78,125,126,133). As the concentration of FFA increases, the turnover rate and oxidation also increases.

Dagenais, Tancredi and Zierler (26) provided evidence for an intramuscular supply of FFA and proposed a model to help explain FFA uptake and oxidation by skeletal muscle at rest. They observed that FFA's were extracted from arterial blood by the resting muscle in amounts more than sufficient to account for all the oxygen uptake. There was a 30 minute delay before $^{14}CO_2$ was detectable in forearm venous blood even though uptake of ^{14}C oleic acid by the muscle was stable within 10 to 15 minutes after the onset of infusion. They concluded that because of the long delay none of the FFA was oxidized directly. The favored, or perhaps only, pathway for plasma FFA oxidation in the muscle was by way of prior esterification and incorporation into intramuscular lipid granules. Subsequently, the FFA are hydrolyzed and oxidized by the mitochondria.

Intra- and Extramuscular Adipose Stores (Exercise)

Participation of intramuscular fat in exercise metabolism was proposed when the oxidation of plasma ^{14}C-FFA proved to be much too low to explain the RQ measured on exercising dogs and humans (19,63,68,70,71, 80,85,110). For exercising human muscle, the recovery of $^{14}CO_2$ after infusion of ^{14}C-FFA averaged 46-60 percent of the ^{14}C-FFA uptake at moderate to heavy steady state exercise (63,65,70,71) and almost identical

results were obtained in studies on the hind limbs of dogs during electrical stimulation (127). Paul and Issekutz (110) reported that 20-30 percent of the energy during heavy work was derived from extramuscular sources. In contrast, a more complete oxidation of labelled FFA (70-90 percent) was observed for humans and dogs during exercise at low work loads (63-65,68,70,71,110). Thus, the intramuscular lipid stores may explain the imbalance between the total amount of lipid oxidized and the amount supplied by plasma FFA (18,69,102).

Havel, Ekelund, Holmgren (69) have proposed that there are two intramuscular pools of esterified fatty acids in which FFA equilibrate during exercise. It appears there is a smaller pool which has a rapid turnover and a larger one which is much slower. Hagenfeldt and Wahren (63) have presented supporting data for this hypothesis.

Paul and Issekutz (85,86,108) added further support to this hypothesis. They proposed that the rapid turnover in plasma FFA was dependent not only on the plasma FFA concentration but also on the metabolic rate during work. As long as the oxygen supply was adequate, i.e., no lactate production above a critical level, the FFA turnover rate and oxidation rate will increase. This increase will be in such a way that the percentage of the total energy derived from plasma FFA oxidation would be the same at increasing workloads. Below this critical lactate level up to 90 percent of the plasma FFA were directly oxidized to $^{14}CO_2$ (110).

Free Fatty Acids and Lactate

A large body of evidence is now available to substantiate that plasma FFA can serve as a direct fuel for the oxidative metabolism of working muscles (45,50,51,71). Whether the FFA level will decrease, increase, or remain unchanged depends on the response of the adipose tissue. Work of low intensity and long duration is characterized by a gradual increase in FFA levels during 4-5 hours of exercise (9,20,110, 117). In heavy work the situation is entirely different. Due to the probable switch in fiber type, from slow twitch oxidative to fast twitch glycolytic, the blood lactate concentration rises (24,57,58,111), and as it rises, the FFA level decreases (20,46,48,65,108,113,117).

Experiments using radiotracer techniques revealed that the decrease of plasma FFA was caused by inhibition of the rate of release of FFA from the adipose tissue by lactate levels which were well within the range measured during moderately heavy or heavy exercise (81-83). It was demonstrated that heavy exercise itself could inhibit the release of FFA to the extent that the FFA turnover becomes less than it was at rest (81-83). This relationship between plasma FFA concentration and plasma lactate appears to be inverse (22,82).

As a consequence, during heavy work, which is characterized by an increase in lactate concentration, a small proportion (on the range of 25-50 percent) of the total energy requirement is derived from FFA (68,70). In contrast, virtually all of the fuel of working muscle and up to 90 percent of oxidative metabolism is supplied by FFA during prolonged exercise of low intensity (2,63,65,67,117,137,138). A lower level of lactate accompanies this level of exercise (113).

These observations and conclusions are consistent with the findings that lactate infusion inhibits FFA levels and turnover (3,16,79,82,87, 100). It has also been demonstrated that there is a reduction in FFA release from adipose tissue perfused by lactic acid (41,81,99,100). Lactate had the same effect in vitro on isolated epididymal fat pads (11,12) and on in situ perfused subcutaneous adipose tissue (40).

Training and Free Fatty Acid Metabolism

Because there is an inverse correlation between FFA level and plasma lactate, it is very important that any study measuring these substrates during prolonged work be critically evaluated. This is especially true when comparing trained and untrained subjects during prolonged work or during prolonged work before and after physical training. Variables to be considered in such an evaluation include: (1) a description of the work load (relative or absolute), (2) the subject's fitness level, (3) the intensity of the work, and (4) duration of the work period. All of these affect lactic acid production and as a consequence, FFA mobilization.

The effects of training on FFA metabolism in human subjects are limited to two studies. Karlsson, Nordesjo, and Saltin (93) tested five male subjects before and after two months of physical training. The same absolute work load was used before and after training despite an increase in maximal oxygen uptake following training. There was no significant change in FFA concentration either at rest or during exercise even though lactate concentration was significantly lower after training.

Rennie and Johnson (115) trained eight men for 30 minutes, at moderate speeds, three times a week for three weeks. The subjects ran

over a fixed course of 7.5 kilometers for 60 minutes. Blood samples were taken by venepuncture at rest and 30 minutes after the start of exercise. At the end of exercise a nylon catheter was inserted and recovery samples were taken at 30-minute intervals for 90 minutes. The same test was repeated at the end of the training program, again the subjects running the course at their own speed. A significant decrease ($P < 0.05$) in plasma FFA levels was observed after training. Lactate levels were also significantly lower ($P < 0.05$) following the training program.

Studies reporting data on plasma FFA and lactate levels in athletes and untrained subjects have also yielded conflicting results. Cobb and Johnson (22), using subjects with sedentary occupations and a group of physically active men (mountain climbers), had all subjects work at the same absolute work load of 1.0-1.1 liters oxygen/min m^2. The sedentary subjects had a greater rise in lactate and a lower FFA concentration throughout the exercise period than the trained subjects (an average exercise period of 24 minutes for the sedentary and 63 minutes for the trained).

Havel, et al. (68,70) concluded from two of their studies that untrained subjects receive less of their energy requirements during exercise from FFA than the athletes who presumably worked at similar rates. This was chiefly the result of a smaller increase in mobilization of FFA into the blood and to a decrease in oxidation of FFA in the untrained (68). The work load of the untrained subjects consisted of pedaling on a bicycle ergometer at a rate of 60 rpm for two hours. The

load was selected to increase the heart rate to 130 beats per minute. The trained subjects walked on a horizontal treadmill at a constant rate of three to four miles per hour for a period of two hours.

Johnson, Walton, Krebs and Williamson (91) had 9 trained runners and 18 sedentary subjects run for a period of 1-1/2 hours on an outdoor track at a speed of their own choosing. Blood samples were drawn by venepuncture which interrupted work every 30 minutes. Interestingly enough, the untrained subjects had both the highest lactate and FFA concentrations during the sampling periods. In fact, the great increase in lactate observed during the first 30 minutes of the run did not depress FFA levels—they increased. These results seem strange in view of the inverse relationship between lactate concentration and FFA mobilization reported elsewhere (22,82).

In another study with the same design, Johnson and Walton (90) studied trained and untrained subjects following the oral administration of sodium acetoacetate. Their results were the same as the previous study.

Devlin, Varma and Prendergast (29) exercised 10 unfit subjects at 50 watts for 30 minutes and 10 fit subjects at 100 watts for a similar period. Their results agreed with those of Johnson, et al. (90,91) that fit subjects have a lower concentration of FFA than untrained subjects during prolonged work.

Rennie, Jennett and Johnson (114) compared untrained subjects and racing cyclists. The subjects pedaled on a bicycle ergometer at a load expected to raise the heart rate to 150-170 beats per minute for 20

minutes. The mean work loads of the cyclists were 1070 kpm/min and 715 kpm/min for the noncyclists. Again, as in their previous studies, they reported that FFA levels in the trained were lower than the untrained ($P < 0.01$). However, the heart rate of the trained was higher than the untrained which might indicate that they were working harder. The respiratory exchange ratio reported was well above 1.0 for the trained cyclists while the untrained group was slightly below this value. Oxygen uptake never leveled off, indicating a steady state was never reached during the work period.

The most recent paper from that laboratory (13) used a relative work load in a progressive work test. Contrary to their previous results, they reported that the FFA concentration was higher in trained cyclists than in untrained individuals. The authors were unable to explain these results in view of their previously published data.

A study using dogs made comparisons between the fit and unfit based on the lactate response to work. Issekutz, et al. (82) ran dogs at the same absolute work load of 100 meters per minute on a 15 percent grade for 30-40 minutes. The "fittest" dog had the highest plasma FFA level during exercise. This was attributed to the lower lactate response during the same work bout. Paul (106) on the other hand reported no change in FFA and lactate concentrations in dogs before and after an eight-week training program.

Summary

Evidence is now available to substantiate that plasma FFA can serve as a direct fuel for the oxidative metabolism of working muscles (45,50, 51,71).

There is a straight line correlation between plasma FFA concentration and its turnover, uptake and oxidation (6,51,54,78,125,126,133), i.e., the higher the plasma FFA level the greater the turnover, uptake and oxidation.

An intramuscular source of FFA participates in exercise metabolism (19,63,68,70,71,80,85,110) because the amount of plasma FFA oxidized by the muscle is much too low to explain the RQ measurements on exercising dogs and humans.

There is an inverse relationship between plasma FFA levels and lactate (22,82). Such a relationship is found at rest and during exercise(22,81-83).

Data on FFA metabolism during exercise are equivocal although the most recent studies (89-91,114,115) suggest that plasma FFA are lower in trained subjects than untrained during prolonged work. Earlier studies reported either higher plasma FFA levels in trained subjects (22,68,70, 82) or no differences between trained and untrained (106).

CHAPTER III

METHODS

Subjects

This investigation utilized 10 inactive female subjects ages 19-31. Experimental protocol was explained to each subject and informed consent was obtained prior to their participation in the study. All subjects then filled out personal history forms and underwent a physical examination by a physician. Those diagnosed as healthy by the physician were allowed to participate in the study.

Following the physical, five subjects were randomly placed in a control group and five in a training group.

Procedure

Cost of cycling and maximal oxygen uptake test. A preliminary oxygen cost of cycling and maximal oxygen uptake test was determined on each subject prior to the prolonged work test. The results were used to compute each individual's work load for the 60 minute prolonged work test. All submaximal and maximal tests utilized a Monarch bicycle ergometer calibrated prior to each testing day (7).

To determine the oxygen cost of cycling, subjects rode at three different submaximal loads, each lasting 10 minutes. One two minute gas sample collected from minutes 8-10 was used to calculate the oxygen uptake for that particular work load. Heart rate and an electrocardiogram

were recorded ruring the last 15 seconds of each work load. During each ride a fan was directed on the subjects.

Following the cost of cycling test the subject rested 10 minutes before the maximal oxygen uptake test. The progressive maximal test used a constant pedal frequency of 50 rpm with the resistance increased 0.5 kilogram every two minutes until the subject was unable to continue. Gas samples were collected every 30 seconds during the last two to three minutes of the test. The usual criteria of a plateau or a drop in oxygen uptake in response to an additional work level was applied in determining the maximal oxygen uptake (8). The highest value attained was used as the subject's maximal aerobic power. During the entire test, heart rate and an electrocardiogram were recorded.

Following the four week training or control period, all subjects again took a maximal oxygen uptake test using the procedure described above.

Prolonged work test. Subjects reported to the laboratory in the morning after an overnight fast. A catheter was placed in a vein in the antecubital area and the subject rested for 10 minutes. At the end of the rest period (just prior to the start of exercise), a blood sample was drawn for later analysis. Heart rate was also recorded at this time (see Table 1).

Following the rest period the subject mounted the bicycle ergometer and began the prolonged work test. She pedalled for 60 minutes at 50 rpm against a resistance that required about 55 percent of her maximal

TABLE 1

Prolonged Work Test Protocol

Test Phase	Sampling Period (Minute)	Physiological Measurement
Rest	10*	Blood, HR
Prolonged Work	10	Blood, HR
	20	Blood, HR, Gas Exchange
	30	Blood, HR
	40	Blood, HR, Gas Exchange
	50	Blood, HR
	60	Blood, HR, Gas Exchange
Recovery	10	Blood, HR

*Just prior to the start of exercise.

aerobic power. The absolute work rates ranged from 225-375 kilogram-meters per minute. Throughout the work period fans were directed at the subject.

Blood samples were drawn at minutes 10, 20, 30, 40, 50, and 60 during the work period (Table 1). Heart rate was recorded at the same time the blood was being drawn. A two-minute gas sample was collected at 20 minute intervals to measure oxygen uptake. At the conclusion of the 60 minute exercise period, subjects again rested for 10 minutes. Heart rate was recorded and a blood sample was drawn 10 minutes after exercise.

All subjects followed the same procedure when the prolonged work test was repeated four weeks later. Work loads were also the same as those used in the pretest.

Catheterization. The catheterization sequence outlined below was the same for each test.

A 20 gauge disposable needle was placed on a sterile 5 ml disposable plastic syringe (Becton, Dickinson and Co., Rutherford, N.J.) and 3.6 ml of heparin (Panheprin, 1000 U/ml, Abbott Laboratories, North Chicago, Ill.) was withdrawn from the heparin vial. This volume was added to a vial containing 16 ml of sterile saline (Bacteriostatic Sodium Chloride). The 5 ml syringe was then discarded. Next, a new sterile 5 ml syringe was fitted with a 20 gauge needle and 5 ml of the sterile heparinized saline was withdrawn into the syringe. Air was forced out of the syringe by gently tapping the side of the syringe; the needle was discarded and a new sterile three-way stopcock (MS-411, Medex Inc., Hilliard, OH) was

attached. The syringe and stopcock were placed on a sterile barrier on the table.

A velcro tourniquet was wrapped around the upper arm and the antecubital area was examined for a vein to be punctured. After scrubbing the skin with alcohol, an angiocath (Desert Radiopaque Teflon Catheter, 2 inches, 18 gauge, Desert Pharmaceutical Co., Dandy, UT) was removed from its sealed container and inserted about 1/4 to 1/2" into the vein. If blood could be withdrawn into the syringe the catheterization was considered acceptable and the catheter was further inserted by sliding the catheter adapter along the needle until about 1-1/2" of the catheter had entered the vein. The catheter was then held in place and the needle gently withdrawn.

The three way stopcock with attached 5 ml syringe of heparinized saline was quickly fitted onto the neck of the teflon catheter to prevent blood leakage and contamination. Blood was withdrawn into the syringe to be certain the catheter was still properly placed. About 0.5 ml of heparinized saline was then infused into the vein to clear the catheter of blood and the stopcock was turned to seal the catheter. Gauze pads were placed underneath and on top of the exposed catheter. Tape was placed on the gauze covering, the catheter and the 5 ml syringe to hold them securely in place.

The minute before blood collection, the cap covering the withdrawal port was removed and a disposable 20 ml plastic syringe (Becton, Dickinson and Co., Rutherford, N.J.) was placed onto the port to clear the catheter of its heparin and saline. The stopcock was turned to

allow blood to flow from the catheter into the 20 ml syringe and a small amount of blood was withdrawn. The stopcock was turned to stop blood flow, the syringe removed and capped, and a new sterile 20 ml syringe put in its place for sampling. The stopcock was opened again and 12-15 ml of blood were withdrawn. The stopcock was turned to seal the catheter, the syringe removed and the drawing port capped. The stopcock was turned to the 5 ml syringe containing the heparinized saline, and 0.5 ml of the solution was again infused into the catheter. The stopcock was turned back to reseal the catheter.

The sequence was repeated each sampling period. The 20 ml syringe used for withdrawing heparinized saline and clearing the catheter was replaced by a new syringe after three sampling periods.

Ventilation and gas exchange. Oxygen uptake was measured by the open circuit method utilizing meteorological balloons and a Daniel's valve. Expired gasses were measured with a Beckman Model E-2 O_2 Analyzer and Model LB1 CO_2 Analyzer. The Beckman analyzers were calibrated with standardized gases previously measured by the Scholander method (122).

Inspired minute ventilation was measured with a Parkinson-Cowan CD4 dry gas meter and recorded with a Narco Biosystems PMP-4A recorder. The dry gas meter was calibrated against a 13.5-liter Collins Respirometer and 120-liter Tissor gasometer with the respirometer taken as the standard. Oxygen uptake was calculated according to standard procedures.

Heart rate and electrocardiogram. Heart rate and an electrocardiogram were monitored and recorded with surface electrodes on an Electronics for

Medicine Model IR Monitoring System. Both heart rate and the electrocardiogram were recorded during the oxygen cost of cycling and the maximal oxygen uptake test. The monitoring system was used only as a cardiotachometer to monitor heart rate during the 60 minute submaximal work period.

Blood samples. Blood samples were taken via an indwelling catheter previously placed in the arm. During each sampling period, approximately 12-15 ml of blood was withdrawn into a 20 ml disposable plastic syringe and immediately divided into two aliquots for later analysis. About 1 ml of blood used for the lactic acid analysis was placed on a spot plate containing 0.05 ml of 4 percent sodium fluoride. The blood and sodium fluoride were mixed and 50 µl of the sample placed in 0.4 ml of perchloric acid, thoroughly mixed and placed in an ice bath for later analysis.

The remaining aliquot of blood to be used for FFA and glycerol analyses was placed in a heparinized centrifuge tube (10 U heparin/ml blood) and stored in an ice bath.

At the completion of the 60 minute exercise test all blood samples were centrifuged at 4000 rpm for 10 minutes at 0-5°C. The tubes containing perchloric acid and 50 µl of blood were decanted off into another chilled tube and stored in the refrigerator until analysis. The plasma for FFA and glycerol analysis was aspirated into a Pasteur pipette delivered into plastic tubes, capped and stored in the refrigerator.

Analysis. All chemical analysis for lactic acid, FFA and glycerol were done in triplicate and were carried out the same day of the experiment. Lactic acid was analyzed by the method of Gutmann and Wahlfeld (62), FFA by Dole and Meinertz (31) and glycerol by Bucolo and David (17). See Appendix for detailed description of the procedures.

Training. After completion of the initial 1 hour work test those subjects in the experimental group began a four week (12 exercise sessions) training program. The subjects rode a bicycle ergometer 3 days a week for one hour in each session. The work load started at 300 kilogram-meters per minute and was progressively increased to 525 kilogram-meters per minute during the last week of training.

The control group did not participate in any of the training sessions. They did no extra work outside of their normal daily activities during the four week period.

Statistics. A three Analysis of Variance with repeated measures on two factors was used to test for significant differences (135) and is diagramed in Figure 1. A probability less than 0.05 was used to reject the null hypothesis. The Tukey HSD Test was utilized *a posteriori* to find where the significant differences existed (132).

Some of the variables had missing data and a formula for estimating the missing values was used (28). The values computed had no influence on interaction terms in the analysis.

			TEST															
			PRETEST								POSTTEST							
Group	Subject	Sample	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
EXPERIMENTAL	1																	
	2																	
	3																	
	4																	
	5																	
CONTROL	6																	
	7																	
	8																	
	9																	
	10																	

Figure 1. Block Diagram of Three Factor Analysis of Variance with Repeated Measures on Two Factors.

CHAPTER IV

RESULTS

Descriptive Statistics

Data on the physical characteristics of the control and experimental groups are listed in Table 2. No significant differences ($P > 0.05$) were observed in the pre- and posttest weights of the control and experimental groups (Table 3).

Maximal Aerobic Power

Data on the maximal oxygen uptake of the control and experimental groups are listed in Table 2. A training effect was observed in the experimental group as evidenced by their 14-percent increase in maximal aerobic power. Aerobic power was significantly higher ($P < 0.05$) than their pretest values and also higher than those of the control group (Tables 4 and 5). The maximal oxygen uptake of the control group did not differ ($P > 0.05$) from the pre- and posttest scores (Table 5).

Heart Rate (Figure 2)

Data on the heart rate response of the control and experimental groups are listed in Table 6. There was a significant decrease ($P < 0.05$) in both the resting (Tables 7 and 8) and exercise (Tables 9 and 10) heart rates of the experimental group following training. Heart rates decreased 8 beats per minute at rest and approximately 15 beats per minute during the prolonged exercise test. No significant change ($P > 0.05$) was

TABLE 2

Descriptive Statistics of Experimental and Control Groups

Group	Age (years)	Height (cm)	Weight (kg)		Max VO_2 (ml/kg min^{-1})	
			Pre	Post	Pre	Post
Control (n = 5)	20.4 ± 0.6	157.5 ± 1.5	53.0 ± 1.5	53.3 ± 1.8	33.3 ± 1.7	31.8 ± 1.5
Experimental (n = 5)	22.4 ± 2.3	167.1 ± 1.5	59.8 ± 4.9	59.9 ± 5.3	34.4 ± 3.4	39.1 ± 3.5*

Values are $\overline{X}$ ± SE.

*$p < 0.05$.

TABLE 3

Two Way ANOVA for Variable Weight

Source	DF	Sum of Squares	Mean Squares	F	Prob F
Group	1	1089.2880	1089.2880	1.5547	0.2468
Subject(Group)	8	5604.9520	700.6190		
Test	1	0.8000	0.8000	0.5224	0.5042
Subject(Group) × Test	8	12.2520	1.53150		
Group × Test	1	0.3380	0.3380	0.2207	0.6539
Subject(Group) × Test	8	12.2520	1.5315		

TABLE 4

Two Way ANOVA for Variable Maximal Aerobic Power

Source	DF	Sum of Squares	Mean Squares	F	Prob F
Group	1	85.6980	85.6980	1.2439	0.2975
Subject(Group)	8	551.1620	68.8952		
Test	1	13.1220	13.1220	4.5527	0.0635
Subject(Group) × Test	8	23.0580	2.882		
Group × Test	1	48.0500	48.0500	16.6710	0.0038
Subject(Group) × Test	8	23.0580	2.8822		

TABLE 5

Tukey HSD Test for Maximal Aerobic Power (Group × Test)

Comparison Mean	Comparison Mean Minus Smallest Mean	Comparison Mean Minus Second Smallest Mean	Comparison Mean Minus Third Smallest Mean
39.08 (Posttest Experimental)	39.08-31.84=7.24*	39.08-33.32=5.76*	39.08-34.36=4.72*
34.36 (Pretest Experimental)	34.36-31.84=2.52	34.36-33.32=1.04	
33.32 (Pretest Control)	33.32-31.84=1.48		
31.84 (Posttest Control)			

Values are maximal aerobic power in ml/kg min^{-1} expressed as $\overline{X}$.

$$t = (q_{\alpha};\ r;\ N\text{-}r)\ (\sqrt{MS_E/n})$$

$$t = (q_{.05};\ 4;\ 16)\ (\sqrt{2.88/5})$$

$$t = (4.05)\ (.759)$$

$$t = 3.07$$

*$p < 0.05$.

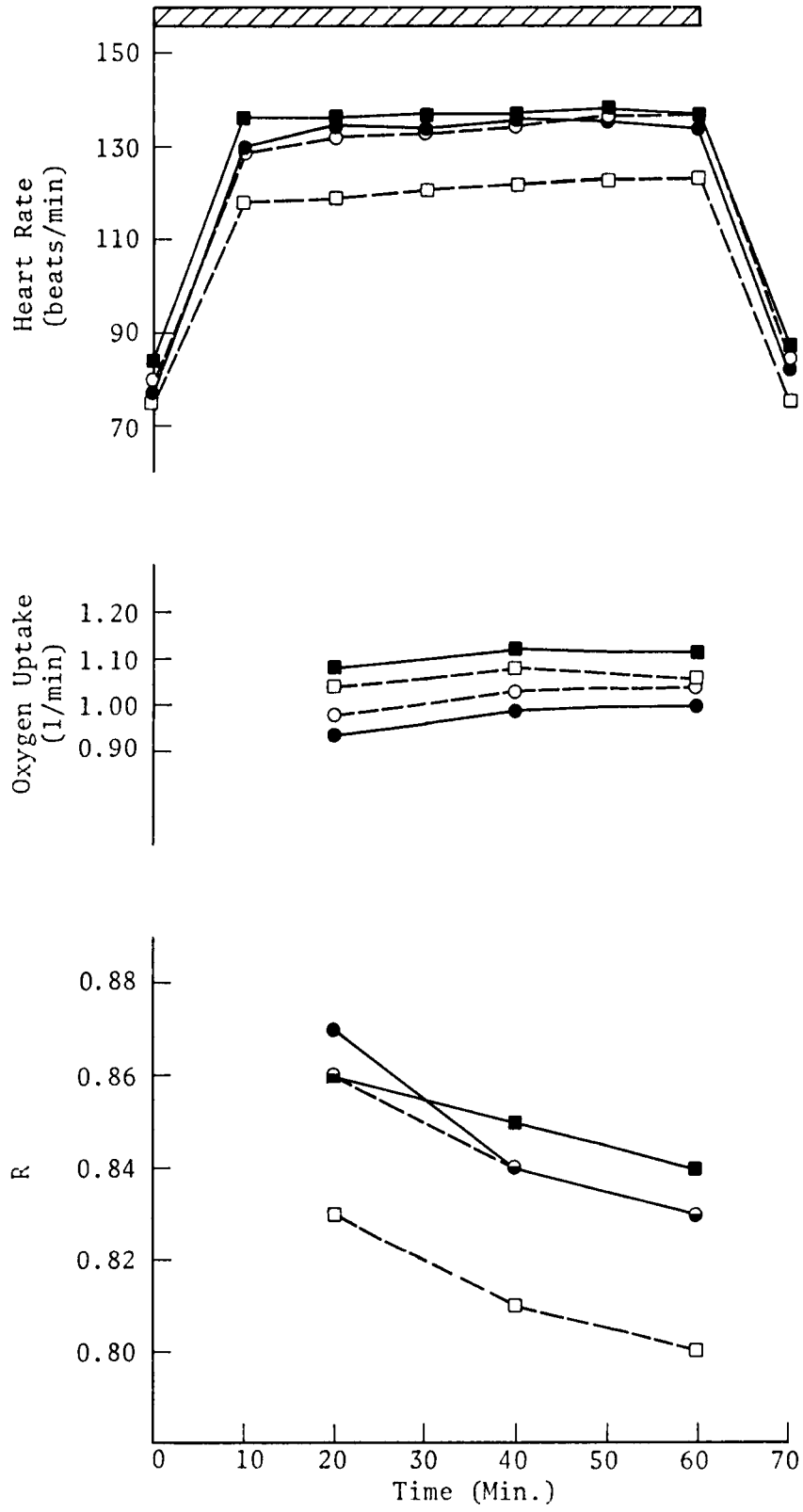


Figure 2. Pre- and Posttest Changes in Heart Rate, Oxygen Uptake and R during 60 Minutes of Exercise in the Experimental and Control Groups. Closed circles and squares indicate pretest, open circles and squares post test. Squares represent the experimental group and circles the control group. The hatched bar represents the exercise period.

TABLE 6

Pre- and Posttest Heart Rate Response of the Control and Experimental Groups during Prolonged Work

		Rest	Exercise						Recovery
Time (Min)		0	10	20	30	40	50	60	70
CONTROL	Pre n=5	76.0±4.3	130.0±6.5	135.2±6.8	133.6±6.8	136.4±6.9	136.2±6.2	134.2±6.9	82.0±6.0
	Post n=5	79.4±6.7	128.6±5.4	131.6±5.3	133.4±6.5	134.8±6.9	137.0±7.0	137.4±7.9	84.4±8.2
EXPERIMENTAL	Pre n=5	82.4±313	135.6±7.0	136.0±7.2	136.6±7.2	137.0±8.1	137.6±8.1	137.4±8.1	86.6±4.2
	Post n=5	74.6±2.6	117.6±4.4	119.4±4.6	121.4±4.6	122.2±4.6	123.4±5.0	123.4±3.5	75.4±2.9

Values are heart rate in beats per minute expressed as $\overline{X}$ ± SE.

TABLE 7

Two Way ANOVA for Variable Resting Heart Rate

Source	DF	Sum of Squares	Mean Squares	F	Prob F
Group	1	3.200	3.200	0.017	0.893
Subject(Group)	8	1458.600	182.325		
Test	1	24.200	24.200	1.202	0.305
Subject(Group) × Test	8	161.000	20.125		
Group × Test	1	156.800	156.800	7.791	0.023
Subject(Group) × Test	8	161.000	20.125		

TABLE 8

Tukey HSD Test for Resting Heart Rate
(Group × Test)

Comparison Mean	Comparison Mean Minus Smallest Mean	Comparison Mean Minus Second Smallest Mean	Comparison Mean Minus Third Smallest Mean
82.4 (Pretest Experimental)	82.4-74.6=7.8	82.4-76.0=4.4	82.4-79.4=3.0
79.4 (Posttest Control)	79.4-74.6=4.8	79.4-76.0=3.4	
76.0 (Pretest Control)	76.0-74.6=1.4		
74.6 (Posttest Experimental)			

Values are heart rate in beats per minute expressed as $\overline{X}$.

$$t = (q_{\alpha};\ r;\ N\text{-}r)\ (\sqrt{MS_E/n})$$

$$t = (q_{.05};\ 4;\ 16)\ (\sqrt{20.125/5})$$

$$t = (4.05)\ (2.01)$$

$$t = 8.1$$

TABLE 9

Three Way ANOVA for Variable
Exercise Heart Rate

Source	DF	Sum of Squares	Mean Square	F	Prob F
Group	1	770.133	770.133	0.361	0.570
Subject(Group)	8	17069.033	2133.629		
Test	1	1904.033	1004.033	7.152	0.027
Subject(Group) × Test	8	2129.633	266.204		
Group × Test	1	1687.500	1687.500	6.339	0.035
Subject(Group) × Test	8	2129.633	266.204		
Sample	5	430.800	86.160	14.000	0.001
Subject(Group) × Sample	40	246.167	6.154		
Group × Sample	5	41.867	8.373	1.360	0.259
Subject(Group) × Sample	40	246.167	6.154		
Test × Sample	5	79.367	15.873	1.152	0.349
Subject(Group) × Test × Sample	40	551.167	13.779		
Group × Test × Sample	5	18.300	3.660	0.266	0.928
Subject(Group) × Test × Sample	40	551.167	13.779		

TABLE 10

Tukey HSD Test for Exercise Heart Rate (Group × Test)

Comparison Mean	Comparison Mean Minus Smallest Mean	Comparison Mean Minus Second Smallest Mean	Comparison Mean Minus Third Smallest Mean
136.7 (Pretest Experimental)	136.7-121.2=15.5*	136.7-133.8=2.9	136.7-134.3=2.4
134.3 (Pretest Control)	134.3-121.2=13.1*	134.3-133.8=0.5	
133.8 (Posttest Control)	133.8-121.2=12.6*		
121.2 (Posttest Experimental)			

Values are heart rate in beats per minute expressed as $\overline{X}$.

$$t = (q_{\alpha};\ r;\ N-4)\ (\sqrt{MS_{\varepsilon}/n})$$

$$t = (q_{.05};\ 4;\ 116)\ (\sqrt{266.204/30})$$

$$t = (3.70)\ (2.98)$$

$$t = 11.02$$

*$p < 0.05$.

observed in the control group either at rest (Table 8) or during exercise (Table 10).

Submaximal Oxygen Uptake (Figure 2, page 29)

Data on the oxygen uptake of the control and experimental groups are listed in Table 11. No significant difference ($P > 0.05$) in the submaximal oxygen uptakes was observed between the pre- and posttest results of the control and experimental groups (Table 2, page 25). A slight but significant increase ($P < 0.05$) was observed in the samples during the work period (Table 12). This increase was observed at 40 minutes with no further increase at 60 minutes (Table 13).

Respiratory Exchange Ratio (R) (Figure 2, page 29)

Table 14 lists the R data for the control and experimental groups. The posttest R of the experimental group was significantly lower ($P < 0.05$) than their pretest values and was also lower than those of the control group (Tables 15 and 16). The R decreased significantly ($P < 0.05$) at 40 minutes of exercise and continued to decrease until the end of exercise although the decrease was not significant (Table 17).

Plasma Free Fatty Acids (Figure 3)

Data on the plasma FFA levels of the control and experimental groups are listed in Table 18. There was no significant difference ($P > 0.05$) between the pre- and posttest resting FFA levels of the control and experimental groups (Table 19). The pretest FFA concentration of the experimental group was significantly higher ($P < 0.05$) during exercise

TABLE 11

Pre- and Posttest Oxygen Uptake Values of the Control and Experimental Groups during Prolonged Work

		Rest	Exercise					Recovery
Time (Min)		0	10	20	30	40	50	60	70
CONTROL	Pre n=5			0.94±0.03		0.99±0.02		1.02±0.02	
	Post n=5			0.98±0.03		1.03±0.02		1.04±0.03	
EXPERIMENTAL	Pre n=5			1.08±0.7		1.12±0.08		1.11±0.10	
	Post n=5			1.04±0.04		1.08±0.04		1.04±0.03	

Values are oxygen uptake in liters per minute expressed as $\overline{X}$ ± SE.

TABLE 12

Three Way ANOVA for Variable Oxygen Uptake

Source	DF	Sum of Squares	Mean Squares	F	Prob F
Group	1	0.100	0.100	1.746	0.222
Subject(Group)	8	0.458	0.057		
Test	1	0.001	0.001	0.108	0.748
Subject(Group) × Test	8	0.090	0.011		
Group × Test	1	0.025	0.025	2.236	0.171
Subject(Group) × Test	8	0.090	0.011		
Sample	2	0.027	0.013	5.699	0.013
Subject(Group) × Sample	16	0.037	0.002		
Group × Sample	2	0.009	0.005	1.975	0.170
Subject(Group) × Sample	16	0.037	0.002		
Test × Sample	2	0.003	0.001	1.084	0.363
Subject(Group) × Test × Sample	16	0.022	0.001		
Group × Test × Sample	2	0.0007	0.0003	0.025	0.976
Subject(Group) × Test × Sample	16	0.0222	0.0014		

TABLE 13

Tukey HSD Test for Oxygen Uptake (Sample)

Comparison Mean	Comparison Mean Minus Smallest Mean	Comparison Mean Minus Second Smallest Mean
1.05 (60 minutes)	1.05-1.05=0.0	1.05-1.01=0.04*
1.05 (40 minutes)	1.05-1.01=0.04*	
1.01 (20 minutes)		

Values are oxygen uptake in ml/kg min^{-1} expressed as $\overline{X}$.

$$t = (q_{\alpha};\ r;\ N\text{-}r)\ (\sqrt{MS_E/n})$$

$$t = (q_{.05};\ 3;\ 57)\ (\sqrt{0.0023/20})$$

$$t = (3.40)\ (0.01)$$

$$t = 0.036$$

*$p < 0.05$.

TABLE 14

Pre- and Posttest R Values of the Control and Experimental Groups during Prolonged Work

		Rest	Exercise					Recovery
Time (Min)		0	10	20	30	40	50	60	70
CONTROL	Pre n=5			0.87±0.02		0.84±0.02		0.83±0.02	
	Post n=5			0.86±0.02		0.84±0.02		0.83±0.01	
EXPERIMENTAL	Pre n=5			0.86±0.01		0.85±0.01		0.84±0.01	
	Post n=5			0.83±0.01		0.81±0.01		0.80±0.01	

Values are respiratory exchange ratio expressed as $\overline{X}$ ± SE.

TABLE 15

Three Way ANOVA for Variable R

Source	DF	Sum of Squares	Mean Squares	F	Prob F
Group	1	0.0028	0.0028	0.4521	0.5256
Subject(Group)	8	0.0496	0.0062		
Test	1	0.0070	0.0070	13.9669	0.0059
Subject(Group) × Test	8	0.0040	0.0005		
Group × Test	1	0.0034	0.0034	6.6942	0.0311
Subject(Group) × Test	8	0.0040	0.0005		
Sample	2	0.0095	0.0048	9.8132	0.0020
Subject(Group) × Sample	16	0.0078	0.0005		
Group × Sample	2	0.0002	0.0001	0.2502	0.7841
Subject(Group) × Sample	16	0.0078	0.0005		
Test × Sample	2	0.0001	0.0001	0.3032	0.7462
Subject(Group) × Test × Sample	16	0.0027	0.0002		
Group × Test × Sample	2	0.0003	0.0001	0.7922	0.5265
Subject(Group) × Test × Sample	16	0.0027	0.0002		

TABLE 16

Tukey HSD Test for R (Group × Test)

Comparison Mean	Comparison Mean Minus Smallest Mean	Comparison Mean Minus Second Smallest Mean	Comparison Mean Minus Third Smallest Mean
.85 (Pretest Experimental)	.85-.81=.04*	.85-.84=.01	.85-.85=0
.85 (Pretest Control)	.85-.81=.04*	.85-.84=.01	
.84 (Posttest Control)	.84-.81=.03*		
.81 (Posttest Experimental)			

Values are respiratory exchange ratio expressed as $\overline{X}$.

$$t = (q_{\alpha};\ r;\ N\text{-}r)\ (\sqrt{MS_E/n})$$

$$t = (q_{.05};\ 4;\ 56)\ (\sqrt{.0005/15})$$

$$t = (3.75)\ (.0058)$$

$$t = .02$$

*$p < 0.05$.

TABLE 17

Tukey HSD Test for R (Sample)

Comparison Mean	Comparison Mean Minus Smallest Mean	Comparison Mean Minus Second Smallest Mean
0.86 (20 minutes)	0.86-0.83=0.03*	0.86-0.84=0.02*
0.84 (40 minutes)	0.84-0.83=0.01	
0.83 (60 minutes)		

Values are respiratory exchange ratio expressed as $\overline{X}$.

$$t = (q_{\alpha};\ r;\ N-4)\ (\sqrt{MS_E/n})$$

$$t = (q_{.05};\ 3;\ 57)\ (\sqrt{0.0005/20})$$

$$t = (3.40)\ (0.005)$$

$$t = 0.017$$

*$p < 0.05$.

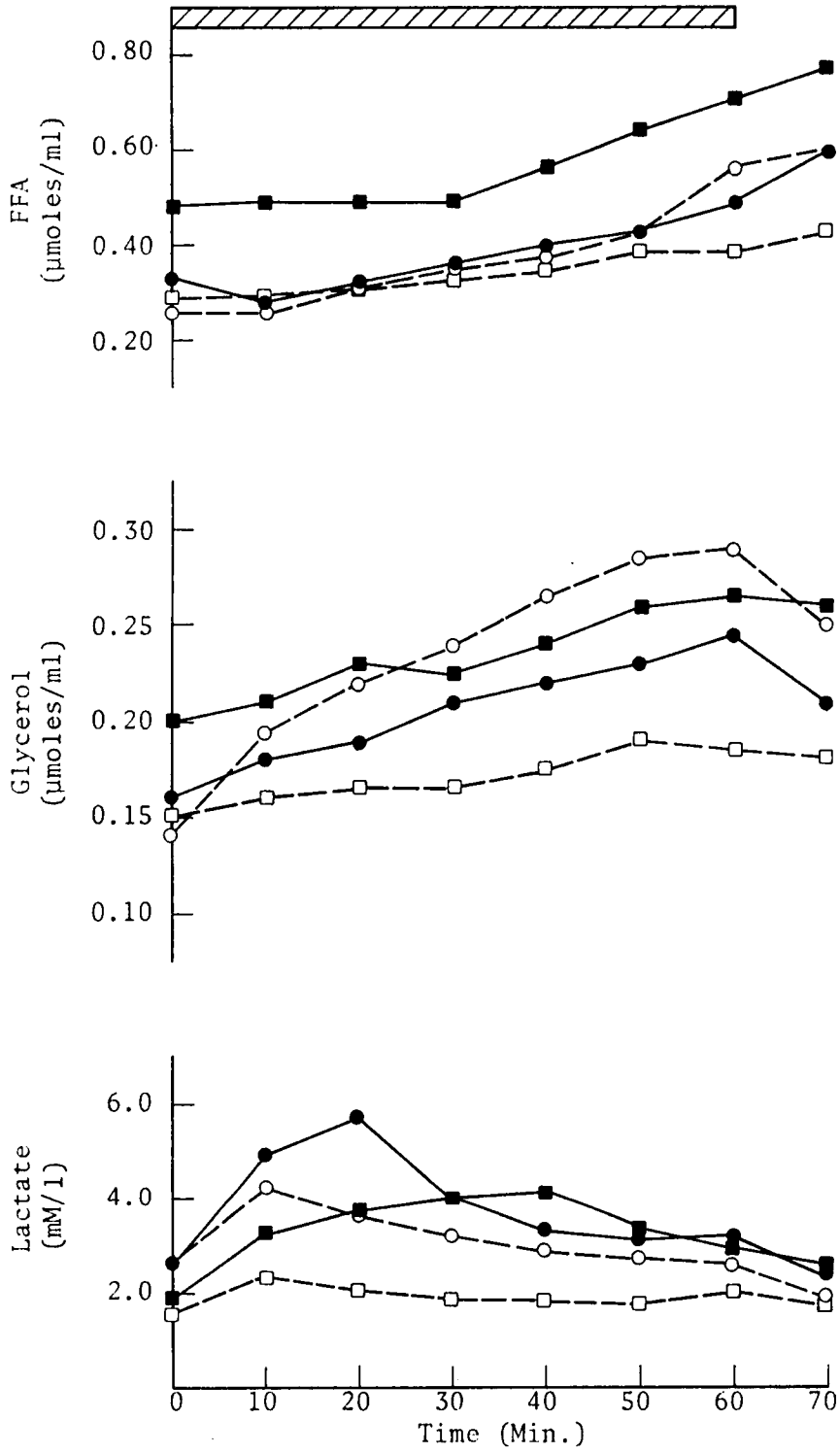


Figure 3. Pre- and Posttest Changes in FFA, Glycerol and Lactate during 60 Minutes of Exercise in the Experimental and Control Groups. Closed circles and squares indicate pretest, open circles and squares post test. Squares represent the experimental group and circles the control group. The hatched bar represents the exercise period.

TABLE 18

Pre- and Posttest FFA Levels of the Control and Experimental Groups during Prolonged Work

		Rest	Exercise						Recovery
Time (Min)		0	10	20	30	40	50	60	70
CONTROL	Pre n=5	0.33±0.03	0.28±0.04	0.32±0.03	0.36±0.04	0.40±0.06	0.43±0.07	0.49±0.08	0.60±0.11
	Post n=5	0.26±0.04	0.26±0.04	0.31±0.07	0.35±0.08	0.38±0.08	0.43±0.08	0.56±0.13	0.60±0.16
EXPERIMENTAL	Pre n=5	0.48±0.06	0.49±0.09	0.49±0.09	0.49±0.11	0.56±0.12	0.64±0.12	0.70±0.13	0.77±0.13
	Post n=5	0.29±0.03	0.29±0.04	0.31±0.04	0.33±0.04	0.35±0.04	0.39±0.04	0.39±0.05	0.43±0.05

Values are plasma FFA levels in μmoles per milliliter expressed as $\overline{X}$ ± SE.

TABLE 19

Two Way ANOVA for Variable Resting FFA

Source	DF	Sum of Squares	Mean Squares	F	Prob F
Group	1	0.0461	0.0461	4.3318	0.0689
Subject(Group)	8	0.0851	0.0106		
Test	1	0.0871	0.0871	11.3401	0.0098
Subject(Group) × Test	8	0.0615	0.0077		
Group × Test	1	0.0168	0.0168	2.1894	0.1752
Subject(Group) × Test	8	0.0615	0.0077		

than the posttest value and both the pre- and posttest values of the control group (Tables 20 and 21). There was a 65-percent decrease in the plasma FFA levels of the experimental group following training. A gradual but significant ($P < 0.05$) increase in plasma FFA during the exercise and recovery period was observed (Table 20).

Glycerol (Figure 3, page 43)

Data on the plasma glycerol concentrations of the control and experimental groups are listed in Table 22. Glycerol did not differ significantly ($P > 0.05$) between the control and experimental groups in either testing session (Table 23) although the levels were lower in the experimental group ($P < 0.06$). The glycerol concentration tended to steadily increase ($P < 0.05$) during the exercise period and decrease during recovery.

Lactate (Figure 3, page 43)

Table 24 lists the lactate concentrations of the experimental and control groups during the work tests. No significant differences ($P > 0.05$) in lactate were observed between the pre- and posttest results of the two groups (Table 25). Lactate concentration tended to rise during the first 10-20 minutes of exercise and then decrease toward resting values for the remainder of the period.

TABLE 20

Three Way ANOVA for Variable Exercise FFA

Source	DF	Sum of Squares	Mean Square	F	Prob F
Group	1	0.1577	0.1577	0.7024	0.5693
Subject(Group)	8	1.7959	0.2243		
Test	1	0.3575	0.3575	5.9579	0.0391
Subject(Group) × Test	8	0.4801	0.0600		
Group × Test	1	0.3597	0.3597	5.9943	0.0386
Subject(Group) × Test	8	0.4801	0.0600		
Sample	5	0.5812	0.1162	10.2681	0.0001
Subject(Group) × Sample	40	0.4528	0.0113		
Test × Sample	5	0.0070	0.0014	0.3500	0.8793
Subject(Group) × Test × Sample	40	0.1599	0.0040		
Group × Test × Sample	5	0.0393	0.0078	1.9659	0.1042
Subject(Group) × Test × Sample	40	0.1599	0.0040		

TABLE 21

Tukey HSD Test for Exercise FFA (Group × Test)

Comparison Mean	Comparison Mean Minus Smallest Mean	Comparison Mean Minus Second Smallest Mean	Comparison Mean Minus Third Smallest Mean
0.563 (Pretest Experimental)	0.563 - 0.344 = 0.219*	0.563 - 0.381 = 0.182*	0.563 - 0.381 = 0.182*
0.381 (Posttest Control)	0.381 - 0.344 = 0.037	0.381 - 0.381 = 0	
0.381 (Pretest Control)	0.381 - 0.344 = 0.037		
0.344 (Posttest Experimental)			

Values are plasma FFA in μmoles per milliliter expressed as $\overline{X}$.

$$t = (q\alpha;\ r;\ N-r)\ (\sqrt{MS_E/n})$$

$$t = (q\ .05;\ 4;\ 116)\ (\sqrt{0.0600/30})$$

$$t = (3.69)\ (.045)$$

$$t = .165$$

*P < 0.05.

TABLE 22

Pre- and Posttest Glycerol Levels of the Control and Experimental Groups during Prolonged Work

		Rest	Exercise						Recovery
Time (Min)		0	10	20	30	40	50	60	70
CONTROL	Pre n=5	0.159±0.007	0.179±0.012	0.190±0.018	0.208±0.020	0.220±0.025	0.232±0.027	0.245±0.030	0.212±0.007
	Post n=5	0.141±0.013	0.196±0.028	0.222±0.025	0.241±0.040	0.266±0.033	0.286±0.036	0.289±0.039	0.252±0.043
EXPERIMENTAL	Pre n=5	0.200±0.017	0.210±0.009	0.228±0.013	0.225±0.021	0.242±0.017	0.259±0.015	0.267±0.013	0.257±0.014
	Post n=5	0.153±0.018	0.161±0.014	0.167±0.616	0.167±0.013	6.174±0.017	0.188±0.018	0.187±0.014	0.181±0.019

Values are glycerol in μmoles per milliliter expressed as $\bar{X}$ ± SE.

TABLE 23

Three Way ANOVA for Variable Glycerol

Source	DF	Sum of Squares	Mean Squares	F	Prob F
Group	1	0.01151	0.0115	0.8521	0.6140
Subject(Group)	8	0.1080	0.0135		
Test	1	0.0108	0.0108	0.5611	0.5194
Subject(Group) × Test	8	0.1536	0.0192		
Group × Test	1	0.0896	0.0896	4.6681	0.0608
Subject(Group) × Test	8	0.1536	0.0192		
Sample	7	0.1111	0.0159	34.7857	0.0001
Subject(Group) × Sample	56	0.0255	0.0004		
Group × Sample	7	0.0178	0.0025	5.5608	0.0002
Subject(Group) × Sample	56	0.0255	0.0004		
Test × Sample	7	0.0020	0.0003	0.3760	0.9124
Subject(Group) × Test × Sample	56	0.0419	0.0007		
Group × Test × Sample	7	0.0097	0.0014	1.8604	0.0932
Subject(Group) × Test × Sample	56	0.0419	0.0007		

TABLE 24

Pre- and Posttest Lactate Concentration of the Control and Experimental Groups during Prolonged Work

		Rest	Exercise						Recovery
Time (Min)		0	10	20	30	40	50	60	70
CONTROL	Pre n=5	2.64±0.18	4.90±0.70	5.67±0.98	3.97±0.40	3.32±0.32	3.13±0.27	3.19±0.31	2.43±0.13
	Post n=5	2.57±0.32	4.20±0.53	3.57±0.51	3.19±0.50	2.85±0.39	2.73±0.36	2.56±0.25	1.95±0.11
EXPERIMENTAL	Pre n=5	1.77±0.35	3.16±0.56	3.74±1.18	4.01±1.31	4.11±1.29	3.34±1.02	2.95±0.82	2.51±0.66
	Post n=5	1.52±0.18	2.27±0.27	2.04±0.31	1.80±0.24	1.76±0.25	1.66±0.22	1.96±0.36	1.72±0.30

Values are lactate in millimoles per liter expressed as $\overline{X}$ ± SE.

TABLE 25

Three Way ANOVA for Variable Lactate

Source	DF	Sum of Squares	Mean Square	F	Prob F
Group	1	24.6411	24.6411	2.0061	0.1926
Subject(Group)	8	98.2633	12.2829		
Test	1	42.5494	42.5494	5.0801	0.0525
Subject(Group) × Test	8	67.0051	8.3756		
Group × Test	1	4.3263	4.3263	0.5164	0.5018
Subject(Group) × Test	8	67.0051	8.3756		
Sample	7	50.0640	7.1520	10.3995	0.0001
Subject(Group) × Sample	56	38.5123	0.6877		
Group × Sample	7	17.8330	2.5476	3.7044	0.0026
Subject(Group) × Sample	56	38.5123	0.6877		
Test × Sample	7	10.8693	1.5527	4.2764	0.0010
Subject(Group) × Test × Sample	56	20.3335	0.3631		
Group × Test × Sample	7	5.2629	0.7518	2.0706	0.0615
Subject(Group) × Test × Sample	56	20.3334	0.3631		

CHAPTER V

DISCUSSION

Training Effect

The experimental group showed an increase in fitness as evidenced by their 14-percent increase in maximal oxygen uptake (Table 2, page 25). This increase is slightly higher than the 11-12-percent increase in maximal oxygen uptake reported in other women by Flint, et al. (38) and Kilbom (95). Further proof of a training effect was the 11-percent decrease in the submaximal exercise heart rates of the experimental group after training (Table 6, page 30). The 11-percent drop in heart rate for the women agrees with the 8-9-percent decrease reported in women by others (38,95). Similar decreases have been reported in men (21,34,35,112,118). A third indicator of a training effect was the decreased R in the experimental group following training (Table 15, page 40). Similar results have been reported (73,119,120).

No changes were observed in the oxygen uptake during the submaximal work period (Table 12, page 37). Both groups worked at the same submaximal workload (approximately 1.0 liter of oxygen per minute) during the pre- and postexercise test. Other studies have also reported no change in submaximal oxygen uptake following training (21,33,38,119,120,129).

Free Fatty Acids and Glycerol

The lower R values of the experimental group following training in the presence of the same oxygen uptake indicate an increased oxidative

use of fat. This rate appears to be determined by two factors: the concentration of plasma free fatty acids (FFA) and the capacity of the tissue to oxidize fat. During steady state work, the rate of fat oxidation increases linearly with FFA concentration (5,51,54,78,125). Consequently, the availability of the FFA to the mitochondria is probably the rate limiting factor for FFA oxidation at any given work rate (75).

Muscle fibers have an enhanced oxidative capacity after physical training (57). In animal muscle it has been demonstrated that along with the increase in oxidative enzymes there is an increase in the capacity to oxidize FFA (101). Thus, muscles of trained individuals, with their greater capacity for fat oxidation, would be expected to oxidize more fat at the same FFA concentration than those of untrained individuals. The lower R observed after training indicates such an increase in fat oxidation. The lower FFA levels following training may reflect an increased extraction of FFA from the blood with subsequent oxidation. This would further support the observation of a greater rate of fat oxidation.

Closer examination of the results may slightly cloud the picture of relationships just presented. Figure 3, page 43, graphically depicts the results of the pre- and posttest FFA results of the control and experimental groups. It should be noted that the exercise FFA concentration of the pretest experimental group was significantly higher ($P < 0.05$) than the values of the pre- and posttest control group and of the posttest experimental group (Table 20, page 47). Can this significant decrease in FFA levels following training really be explained in terms of the

treatment or was there a source of error? A number of hypotheses may be proposed in order to explain these results. The first (hypothesis) is that the results are true and a definite result of the training program. Secondly, as a result of a small nonhomogeneous population, the means shown in this study may have indicated only extreme subject variability. Thirdly, experimental error in the analysis may explain the results.

Although the sample was small (five subjects per group) care was taken to randomly place the subjects into the two groups. (This was done in hopes of evenly distributing the between subject variability that might be present in nonrandom sampling.) The pre- and posttest resting FFA levels were not significantly different ($P > 0.05$) which indicates that under resting conditions the two groups were homogeneous (Table 19, page 45). Further evidence of homogeneity of the groups is added by the fact that both resting and exercise heart rates, oxygen uptake, respiratory exchange ratio, lactate and glycerol were not significantly different ($P > 0.05$) in the pretest control and pretest experimental groups. Also, all subjects came to the laboratory postabsorptive, having fasted since the previous evening. This helped to control the effect diet may have had on plasma FFA levels.

The observed results of no significant differences ($P > 0.05$) of resting plasma FFA (Table 19, page 45) and significant differences ($P < 0.05$) during exercise (Table 20, page 47) would not be expected from subject variability. If the exercise values were extreme due to subject variability then one might also expect the resting values to be the same. This would mean that both the rest and exercise values would deviate in the same direction. This was not observed in the experiment.

Due to the sex (female) of the subjects variations in metabolic and cardiorespiratory responses to exercise might be expected depending on the phase of the subject's menstrual cycle during the work tests. Each subject was retested four weeks after the initial test to insure that the subject was in the same phase as the pretest. Also, Jurkowski (92) and Fox (39) reported that there were no significant differences between oxygen uptake, heart rate, lactate and respiratory exchange ratio during low intensity exercise and the three phases of the menstrual cycle. The constant R value indicates that the primary source of substrate during exercise did not change even though hormonal changes occurred. This indicates that it was not critical to control for variation in the menstrual phase because of the stable exercise response of the females. The constant R is especially important in that it demonstrates that the cyclical hormones involved in the menstrual cycle have very little effect on substrate utilization.

Experimental error due to technique is a possibility but can probably be excluded. A standard curve, using the same standards, was titrated during each analysis. This allowed for comparisons of all standard curves and examination for any extreme deviations from experiment to experiment. For the most part the standard curves were highly reproducible from day to day. The maximum error possible was 0.05 μmoles per milliliter and this was not enough to negate the results of the present study.

Assuming that the results are due to treatment, the data agree with previous studies on men. They reported significantly lower plasma FFA

levels following training (29,115) or lower levels in trained as compared to untrained subjects (90,91,114). Based on previous research, one might hypothesize that this decrease in plasma FFA would decrease FFA oxidation but the lower R value indicates that the rate of oxidation has increased. The lower venous levels of FFA probably reflect a decreased sympathetic activity as demonstrated by the lower heart rate during submaximal exercise following training. The subjects worked at a lower percent of their maximal oxygen uptake following training (they worked at the same absolute work load) and as a result catecholamine levels might be lower (37,105). The lower blood glycerol and FFA found in this study might reflect a decreased rate of lipolysis by lower catecholamine secretion following training. This would not necessarily negate the possibility of a larger contribution of FFA to oxidative energy metabolism in trained subjects working at the same absolute work load.

The increased rate of fat oxidation at lower plasma FFA levels observed after training [in this study and others (29,115)] may be explained by an increase in the extra- to intracellular gradient of FFA. The trained muscle might be able to decrease its intracellular concentration to a greater extent than untrained muscle and still supply a sufficient amount of fuel for oxidation. As a result of the increased gradient there would be a greater extraction of FFA from the blood.

During the prolonged exercise period there was a progressive increase in plasma FFA concentration and a decrease in R (Figure 2, page 29) indicating a change in the carbon source for oxidation from carbohydrate to fat. Other researchers have reported similar results in

men (23,25,107,115). The observations suggest that the increased plasma FFA concentration during exercise resulted in an increased oxidation of fatty acids and inhibition of carbohydrate utilization. This is supported by other research which has observed a decrease in R long before glycogen stores were depleted (1,25).

The increased levels of glycerol during exercise are the result of lipolysis which produces a sufficient flow of free fatty acids into the blood (70,131). Similar results were reported in this study although the glycerol levels were lower ($P < 0.06$) in the experimental group after training. Whether this glycerol is oxidized by muscle is open to question although current research shows that skeletal muscle has glycerol kinase and glycerol dehydrogenose which are needed for glycerol oxidation (63, 116,128). Hagenfeldt, et al. (63) have reported increases in the turnover and oxidation of glycerol during exercise.

Glycerol is also a very important gluconeogenic precursor taken up by the liver. In this form it contributes as an energy substrate (10,15, 55,104,136). Prolonged exercise increases the rate of formation of glucose from glycerol 8-9 fold (123,124).

The tendency for a decrease ($P < 0.06$) in glycerol during the postexercise test in the experimental group may reflect lower production due to a decrease in lipolysis which is in agreement with the FFA data. Or it might indicate an increased oxidation of glycerol by the skeletal muscle or an increased gluconeogenic action in the liver.

Free Fatty Acids and Lactate

No change in lactate was observed in the experimental and control groups working at 55 percent of their maximal aerobic power. Other data have shown a decrease in lactate following training (7,36,76,93,120,121). This discrepancy may be explained by the low workload at which the subjects worked, not insufficient training. Gollnick (59) has reported that until the work rate reaches 50-60 percent of maximal oxygen uptake, blood lactate concentration remains relatively constant. As Figure 3, page 43, shows, the lactate concentrations remained fairly constant throughout the test although the experimental group was consistently lower after training. Thus, the workload was not high enough to illicit a significant decrease in lactate concentration following training. If the workload was higher, significant differences might have been observed.

The inverse relationship between lactate and plasma FFA level was not evident in this study. Previous work has suggested that the lactate concentration must reach 6-8 μmoles per milliliter in order to decrease plasma FFA levels (16,113). The highest value observed in this study was about 5.9 μmoles per milliliter. Apparently, there is a critical lactate concentration which, when exceeded will inhibit fat mobilization and thus decrease plasma FFA levels. This critical level was not reached in this study because of the light workload (55 percent of maximal aerobic power) of each subject.

CHAPTER VI

SUMMARY AND CONCLUSIONS

The purpose of this study was to investigate the effects of training on free fatty acid metabolism during prolonged work of low intensity in women. Subjects were randomly divided into two groups: 5 in a control group and 5 in an experimental group. A cost of cycling curve and maximal oxygen uptake were determined on each subject on a bicycle ergometer prior to the prolonged work test.

The information obtained from the maximal oxygen uptake test and the cost of cycling was used to select a bicycle ergometer work load of 55 percent of maximal aerobic power for the prolonged work test. Each subject was then catheterized in a vein in the antecubital area and worked at 55 percent of maximal oxygen uptake for 60 minutes on a bicycle ergometer. Heart rate was measured and blood samples were drawn just prior to the start of exercise, every 10 minutes during exercise, and 10 minutes after the cessation of exercise. The blood samples were later analyzed for FFA, glycerol and lactate. Ventilation and gas exchange were measured every 20 minutes during the exercise test. The same work load and sample collection procedures were used for the posttest which was administered four weeks after the pretest.

Following the pretest the control group maintained the same activity schedule they had prior to the pretest. The experimental group began a four week training program following the pretest. They rode a bicycle ergometer three days a week for one hour each day. The workload started

at 300 kilogram-meters per minute and was progressively increased each week to 525 kilogram-meters per minute. At the end of four weeks both groups were retested.

A training effect was observed in the experimental group as evidenced by their 14 percent increase in maximal oxygen uptake, an 11 percent decrease in exercise heart rates at the same absolute work load, and a decreased respiratory exchange ratio (R) following training. No significant changes ($P > 0.05$) in any of these variables were observed in the control group.

Training enhances the oxidative capacity of muscle. The lower R values of the experimental group following training in the presence of the same oxygen uptake suggest an increased oxidation of fat. The lower plasma FFA following training may be due to a decreased sympathetic activity following training (lower exercise heart rate at the same work load). This would result in a decrease in the rate of lipolysis. However, the increase in fat oxidation was probably due to an increased extra- and intracellular FFA concentration gradient with a concomitant increased extraction of FFA from arterial blood. Thus the lower venous FFA levels are observed even though arterial levels may not have changed.

Plasma glycerol levels significantly increased ($P < 0.05$) during the exercise period. There was no significant difference ($P > 0.05$) in the levels between the two groups although the postexercise experimental group had consistently lower values than its pretest levels or the pre- or posttest control. These lower values are in agreement with the FFA data and may reflect a decrease in lipolysis, an increased oxidation of

glycerol by skeletal muscle or an increased gluconeogenic action in the liver.

Lactate's inhibitory effect on FFA mobilization was not observed in this study. A critical lactate concentration was not reached which would inhibit FFA mobilization from adipose tissue. Apparently a lactate concentration of 6-8 μmoles per milliliter is required to inhibit fat mobilization.

Future research in this area should examine free fatty acid metabolism at higher workloads where lactic acid is constantly being produced. This would allow observation of the interrelationships between FFA and lactate. Other substrates, metabolites and hormones should be included in the study to give further insight into the metabolic response to exercise.

Another problem would be to determine the critical lactate concentration which, when exceeded, shuts down FFA mobilization.

The results of this study support the theory that endurance training increases the muscles' ability to oxidize fat. An increased rate of fat oxidation was evidenced by a depressed R and low plasma FFA at the same absolute work load following training.

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APPENDIXES

APPENDIX A

PLASMA FREE FATTY ACID (FFA) DETERMINATION

The mixture of heptane-isopropyl alcohol-water is a convenient two-phase system for extraction of long chain fatty acids. By using suitable proportions of these solvent components, the phases separate without centrifugation. The long chain fatty acids are distributed mainly in the upper, nonpolar phase, whereas the more polar acids remain below. A 3 ml aliquot of the upper phase is then transferred into a 15 ml conical bottomed centrifuge tube, indicator solution added and titrated to its proper end point with a suitable alkali reagent.

Reagents:

1. Isopropyl Alcohol (2-Propanol)
2. n-Heptane
3. H_2SO_4
4. Thymol Blue (Sodium Salt)—Fisher Scientific Co No. T-162
5. Ethanol
6. NaOH
7. Palmitic Acid (Hexadecanoic Acid)—Sigma No. P-0500
8. Phenolphthalein
9. Heparin, Sodium Salt—Sigma No. H-3125

Chemicals should be either Analytical Reagent or otherwise the best available grade.

Daily Working Solutions:

1. Extraction Mixture (for first extraction step)

ml 78% Isopropanol	400	800	1600	2400
ml 20% n-Heptane	100	200	400	600
ml 2% 1 N H_2SO_4	10	20	40	60
ml Final Volume	510	1020	2040	3060

1 N H_2SO_4. Add 28.0 ml of concentrated H_2SO_4 to 500 ml of water and dilute to one (1) liter.

2. Blank Lower Layer (for second extraction step)

Into a 1000 ml separatory funnel add:

50 ml H_2O		100 ml H_2O
250 ml Extraction Mixture		500 ml Extraction Mixture
SHAKE, let stand 5 minutes		SHAKE, let stand 5 minutes
150 ml n-Heptane		300 ml n-Heptane
100 ml H_2O	OR	200 ml H_2O
SHAKE, let stand 5 minutes		SHAKE, let stand 5 minutes
Drain lower layer and store it.		Drain lower layer and store it.

3. Thymol Blue
   Stock: Add 100 mg of Thymol Blue to 100 ml water.
   Working Solution: Add 10 ml of stock to 90 ml of Ethanol.

4. Titration Alkali
   Prepare saturated NaOH and let stand quietly for two weeks or longer to settle out insoluble carbonates. Take 0.05 ml of the clear supernatant up to 50 ml with water.

5. Standards
   Dissolve approximately 50 mg palmitic acid in 250 ml of n-Heptane. Determine the concentration by dividing the actual amount by:

$$\frac{50 \text{ mg Palmitic Acid}}{256.4 \text{ mg Palmitic Acid} \times 0.25 \text{ ml Heptane}} = 780 \text{ µmoles/liter}$$

   Standard stock = 780 moles/l

   a. Add 3 ml of stock to 15 ml of n-Heptane = 130 µmoles/ml
   b. Add 3 ml of stock to 12 ml of n-Heptane = 156 µmoles/ml
   c. Add 3 ml of stock to 9 ml of n-Heptane = 195 µmoles/ml
   d. Add 6 ml of stock to 6 ml of n-Heptane = 390 µmoles/ml

6. Gas Washing Solution
   Add 1 drop of Phenolphthalein (1% in ethanol) to 100 ml of 1N NaOH (4 gm/100 ml).

Sample Collection

1. Heparin (10 U/ml blood) is added to a conical bottomed centrifuge tube. Roll the Heparin around the inside of the tube to spread it out and dry at 80°C overnight.

2. Put blood sample in centrifuge tube containing the Heparin (If using syringe, push slowly to prevent hemolysis).

3. Centrifuge tubes at 4000 RPM for 10 minutes at 5°C.

4. Decant plasma off using disposable pipettes. Be careful not to include any red blood cells.

5. Refrigerate or put on ice until analysis.

Analytical Procedure

1. Use acid washed separatory funnels (60 ml). Separatory funnels should have teflon stopcocks and glass stoppers. Set them up on a wrist action shaker and follow the directions below.

2.

	BLANK	STANDARD	SAMPLE
Single Extraction	3 ml H_2O	3 ml H_2O	3 ml Plasma
	15 ml Extr. Mixture	15 ml Extr. Mixture	15 ml Extr. Mixture
	SHAKE 5 minutes	SHAKE 5 minutes	SHAKE 5 minutes
	STAND 5 minutes	STAND 5 minutes	STAND 5 minutes
	9 ml n-Heptane	9 ml Standard	9 ml n-Heptane
	6 ml H_2O	6 ml H_2O	6 ml H_2O
	SHAKE 2 minutes	SHAKE 2 minutes	SHAKE 2 minutes
	STAND 5 minutes	STAND 5 minutes	STAND 5 minutes
	Drain lower layer	Drain lower layer	Drain lower layer
Double Extraction		Add 21 ml Blank lower layer	Add 21 ml Blank lower layer
		SHAKE 2 minutes	SHAKE 2 minutes
		STAND 5 minutes	STAND 5 minutes
		Drain lower layer	Drain lower layer

3. Standards and unknowns should be titrated in triplicate.

4. Fill titration burette with alkali solution.

5. Add 3 ml upper phase of Blank, Standard, or Sample to a 15 ml conical bottomed centrifuge tube.

6. Add 1 ml of Thymol Blue solution to the centrifuge tube.

7. Place tube on titrator assembly.

8. Place tube with nitrogen gas flowing through it to the bottom of the centrifuge tube.

9. Titrate to a mustard yellow (yellow-green).

10. Record micrometer reading.

11. Reset micrometer to zero and take another sample.

Calculations

1. Subtract the blank from each standard and sample.

2. Use formula, draw a curve or fit a line of least squares to the micrometer values to determine the unknown.

   Assume standard = 780 µmoles/l

   FFA concentration (µEq or µM per ml) = 780 µmoles/l = 7.02 µmoles added to each funnel (9 ml)

   Formula: $\mu\text{moles/ml} = \frac{\text{T sample}}{\text{T standard}} \times 7.02\ \mu\text{moles} \times \frac{\text{1ml plasma}}{\text{3ml plasma}}$

Reference

Dole, V. P. and H. Meinertz. Microdetermination of longchain fatty acids in plasma and tissue. J. Biol. Chem. 235:2595-2599, 1960.

APPENDIX B

ENZYMATIC GLYCEROL ANALYSIS

GLYCEROL PROCEDURE FOR TURNER 450 FLUOROMETER

Free glycerol is determined enzymatically by the following series of reactions:

1. $\text{glycerol} + \text{ATP} \xrightleftharpoons{\text{glycerol kinase}} \text{glycerol-1OP} + \text{ADP}$

2. $\text{ADP} + \text{PEP} \xrightleftharpoons{\text{pyruvate kinase}} \text{ATP} + \text{pyruvate}$

3. $\text{pyruvate} + \text{NADH+H}^{+} \xrightleftharpoons{\text{lactate dehydrogenase}} \text{lactate} + \text{NAD}^{+}$

Reagents

1. Glycerol kinase (GK)—Sigma No. G-9630
2. Lactate Dehydrogenase (LDH)—Sigma No. L-2625
3. Pyruvate Kinase (PK)—Sigma No. P-1381
4. Adenosine-5'-triphosphate (ATP)—Sigma No. A-3127
5. Bovine Serum Albumin—Sigma No. A-4503
6. Reduced Nicotinamide Adenine Dinucleotide (NADH)—Sigma No. N-8129
7. Phosphoenolpyruvate (PEP)—Sigma No. P-7252
8. Magnesium Chloride
9. 0.1 M Potassium Phosphate Buffer (KH_2PO_4), Monobasic Potassium Phosphate
10. Heparin, Sodium Salt—Sigma No. H-3125
11. Glycerol Standard Stock
    Add 92.10 ml of Glycerin and make up to 1000 ml (1.0 M).

Chemicals should be either Analytical Reagent or otherwise the best available grade.

Daily Working Solutions

1. Potassium Phosphate Buffer
   KH_2PO_4 - MW = 136.09

   a. 0.1 M KH_2PO_4. Add 13.61 gm of Monobasic Potassium Phosphate to about 800 ml of water.

b. Adjust pH to 7.0 with concentrated NaOH and bring up to 1.0 liter with water.

Assays	50	100	150	200	250	500
ml Buffer	100	200	300	400	500	1000
gm KH_2PO_4	1.36	2.72	4.08	5.44	6.80	13.61
ml H_2O	80	160	240	320	400	800.0
Final volume	100	200	300	400	500	1000

2. Buffer-Enzyme Solution

Add the following to 100 ml of 0.1 M KH_2PO_4 buffer:

a. 16.7 mg $MgCl_2$-$6H_2O$
b. 92.0 mg ATP
c. 7.0 mg PEP
d. 20.0 µl PK
e. 5.0 µl LDH (use 20.0 µl capillary pipette—estimate 1/4 capacity)
f. 2.0 mg NADH (should result in 150 U fluorescence)

Assays	50	75	200	125	150
ml Buffer	100	150	200	250	300
mg $MgCl_2$-$6H_2O$	16.7	25.05	33.4	41.75	50.10
mg ATP	92.0	138.0	184.0	230.0	276.0
mg PEP	7.0	10.5	14.0	17.5	21.0
µl PK	20.0	30.0	40.0	50.0	60.0
µl LDH	5.0	7.5	10.0	12.5	15.0
mg NADH	2.0	3.0	4.0	5.0	6.0

3. Glycerol Kinase Solution

a. To 1.0 ml of 0.1 M KH_2PO_4 buffer add 1 mg bovine serum albumin; gently mix; don't shake.
b. Add 0.2 mg GK to this 1.0 ml, again, don't shake.

Assays	50	75	100	125	150
ml Buffer	2.50	3.75	5.00	6.25	7.50
mg Albumin	2.5	3.75	5.00	6.25	7.50
mg GK	0.5	0.75	1.00	1.25	1.50

4. Working Standards

   a. Add 0.5 ml stock and make up to 100 ml with water = 0.05 µmoles/ml
   b. Add 1.0 ml stock and make up to 100 ml with water = 0.10 µmoles/ml
   c. Add 2.0 ml stock and make up to 100 ml with water = 0.20 µmoles/ml
   d. Add 4.0 ml stock and make up to 100 ml with water = 0.40 µmoles/ml

Sample Collection

1. Heparin (10 U Heparin/ml blood) is added to a test tube. Roll Heparin around inside of tube to spread it out and dry at 80°C overnight.
2. Put blood sample in test tube containing Heparin (If using syringe, push slowly to prevent hemolysis).
3. Centriguge tubes at 4000 RPM for 10 minutes at 5°C.
4. Decant plasma off using a pipette. Be careful not to include any red cells.
5. Refrigerate or put on ice until analysis.

Analytical Procedure

1. Standards and unknowns should be analyzed in triplicate.

2. Add 0.1 ml (100 µl) of Blank (water), Standards, and Samples to fluorometer tubes.

a	Blank	0.1 ml of Water
b	Standard 1	0.1 ml of Standard 1
c	Standard 2	0.1 ml of Standard 2
d	Standard 3	0.1 ml of Standard 3
e	Standard 4	0.1 ml of Standard 4
f	Sample	0.1 ml of Standard 5

3. Add 2.0 ml of Buffer Enzyme Solution to each tube. Mix thoroughly.

4. Incubate for 10 minutes at 30°C and cool to room temperature.

5. Read fluorometer at 360/460 nm with 15 nm slits.

6. Add 50 µl of Glycerol kinase solution to each tube and incubate at 30°C for 10 minutes, cool to room temperature and read fluorescence.

Calculations

1. Subtract the blank from each standard and sample.

2. Use formula, draw a curve or fit a line of least squares to fluorometer values to determine unknown.

Formula

$$\mu \text{ moles/ml} = \frac{\text{fluorescence sample}}{\text{fluorescence standard}} \times \text{standard} \times \frac{1.01 \text{ ml}}{0.1 \text{ ml}} =$$

Reference

Bucolo, G and H. David. Quantitative determination of serum triglycerides by the use of enzymes. Clin. Chem. 19:476-482, 1972.

APPENDIX C

ENZYMATIC LACTIC ACID ANALYSIS

LACTIC ACID PROCEDURE FOR TURNER 450 FLUOROMETER

$$\text{(L+) Lactic acid} + \text{NAD} \underset{\leftarrow}{\overset{\text{LDH}}{\rightleftharpoons}} \text{Pyruvic acid} + \text{NADH}$$

In the presence of LDH, lactic acid and NAD are converted to pyruvic acid and NADH respectively. As this reaction will not go to completion unless the pyruvic acid is removed from the mixture, a hydrazine-glycine buffer is added in order that the hydrazine will complex with the pyruvic acid.

Reagents

1. 70% Perchloric Acid ($HC1O_4$)
2. Hydrazine-Glycine Buffer
   Hydrazine Sulfate—Sigma No. H-3376
   Glycine —Sigma No. G-7126
3. Lactic Dehydrogenase (LDH)—Sigma No. L-2625
4. Nicotinamide Adenine Dinucleotide, Reduced Form—Sigma No. N-8129
5. Lithium Lactate Standard Stock
   Dry 535-540 mg of Lithium Lactate at 80°C overnight. Add 532.5 mg of the Lithium Lactate to 400 ml of water. Add 0.25 ml of concentrated H_2SO_4. Make up to 500 ml in a volumetric flask.
   1.0 ml = 100 µg LA
6. 4% NaF

Daily Working Solutions

1. Deproteinizing Agent—7% Perchloric Acid (0.40 ml/assay).
   Add 6.0 ml of 70% $HC1O_4$ to a flask and add water up to 100 ml. (Refrigerate)

ml 7% $HC1O_4$	100	200	300	400	500	1000
$HC1O_4$	6	12	18	24	30	60
H_2O	94	188	282	376	470	940

2. Hydrazine Glycine Buffer (2 ml/assay)
   a. 0.4 Molar Hydrazine and 0.5 Molar Glycine. Add 3.76 gm Glycine and 5.20 gm Hydrazine Sulfate to about 80 ml of water.
   b. Adjust pH to 9.0 with concentrated NaOH and bring up to 100 ml with water.

Assays	24	37	50	75	100	250	500
ml Buffer	50	75	100	150	200	500	1000
gm Hydrazine Sulfate	2.60	3.90	5.20	7.80	10.40	26.00	52.00
gm Glycine	1.88	2.82	3.76	5.64	7.52	18.80	37.60
ml H_2O	35.0	60.0	80.0	120.0	170.0	400.00	800.00
Final volume	50.0	75.0	100.0	150.0	200.0	500.00	1000.00

3. Lactic Dehydrogenase
   To a given volume of buffer add enough LDH so that the final concentration is 10 U LDH/ml buffer.

Assays	25	37	50	75	100
ml Buffer	50	75	100	150	200
ml LDH (5900 U/ml LDH)	0.085	0.127	0.169	0.254	0.339

4. β-Nicotinamide Adenine Dinucleotide, Reduced Form
   Add enough NAD to a given volume of buffer so that the final concentration is 1 mg NAD/ml buffer.

Assays	25	37	50	75	100
ml Buffer	50	75	100	150	200
mg NAD	50.0	75.0	100.0	150.0	200.0

5. Working Standards
   Add water-H_2SO_4 solution to all volumes of stock to make up correct standards (0.25 ml concentrated H_2SO_4 made up to 500 ml).

   a. Add 2.5 ml of stock and make up to 100 ml = 25 µg/ml
   b. Add 5.0 ml of stock and make up to 100 ml = 50 µg/ml
   c. Add 10.0 ml of stock and make up to 100 ml = 100 µg/ml
   d. Add 15.0 ml of stock and make up to 100 ml = 150 µg/ml
   e. Add 20.0 ml of stock and make up to 100 ml = 200 µg/ml

Sample Collection

1. 0.05 ml of 4% NaF is added to spot plate and dried at 80°C overnight.
2. Put blood sample on spot plate containing the 4% NaF. Mix.
3. Using 50 µl micropipettes, transfer 50 µl of the sample to a cold test tube containing 0.40 ml of 7% $HClO_4$. Mix and either put on ice or refrigerate.
4. Centrifuge at 4000 RPM for 5 minutes at 0-5°C.
5. Decant supernatant into another chilled tube.
6. Refrigerate or put on ice until analysis.

Analytical Procedure

Standards and unknowns should be analyzed in triplicate.
Add 0.1 ml of Blank, Standards, and Samples to fluorometer tubes:

a.	Blank	0.1 ml of 70% $HClO_4$
b.	Standard 1	0.1 ml of Standard 1
c.	Standard 2	0.1 ml of Standard 2
d.	Standard 3	0.1 ml of Standard 3
e.	Standard 4	0.1 ml of Standard 4
f.	Sample	0.1 ml of Sample

Treat all of the above tubes as follows:

a. Add 2.0 ml of buffer enzyme solution to each tube.
b. Mix thoroughly.
c. Incubate in water at 30°C for 30 minutes and cool to room temperature.

Fluorescence Determination

Read fluorescence at 360/460 with 15 nm slits.
Use the blank to correct for all readings.

Calculations*

1. Subtract the blank for each standard and sample.
2. Use formula, make a curve or fit a line of least squares to fluorometer values to determine unknown.

Formula:

$$\text{mg/100 ml blood} = \text{sample} \times \frac{.45}{0.1} \times \frac{1}{0.05} \times \frac{100}{1000} = \text{m moles/l or}$$

$$\text{mEq} = \frac{\text{mg/100 ml}}{9.008}$$

*Due to quenching by the fluorometer, the Standard Curve is curvilinear, not linear. In order to correctly calculate concentrations, a higher order polynomial or a graph of the Standard Curve must be used.

References

Gutmann, I. and A. W. Wahlefeld. L-(+)-Lactate. Determination with lactate dehydrogenase and NAD. IN: Methods of enzymatic analysis. Edited by H. U. Bergmeyer. Second English Edition. Academic Press, Inc., New York, pp. 1464-1468, 1974.

VITA

Donald Richard Bransford was born in Oakland, California, on June 30, 1947. He attended elementary and secondary schools in Colusa, California, and was graduated from Colusa High School in June, 1965. After studying at Sierra College for two years he transferred to Oregon State University in 1967 and received a Bachelor of Science degree in biological sciences from that institution in June of 1970.

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