1970

Fatty Acid Metabolism In Dystrophic Muscle Of Mice (strain 129)

Chao Hsiung Lin

Follow this and additional works at: https://ir.lib.uwo.ca/digitizedtheses

Recommended Citation


This Dissertation is brought to you for free and open access by the Digitized Special Collections at Scholarship@Western. It has been accepted for inclusion in Digitized Theses by an authorized administrator of Scholarship@Western. For more information, please contact tadam@uwo.ca, wlswadmin@uwo.ca.
The author of this thesis has granted The University of Western Ontario a non-exclusive license to reproduce and distribute copies of this thesis to users of Western Libraries. Copyright remains with the author.

Electronic theses and dissertations available in The University of Western Ontario’s institutional repository (Scholarship@Western) are solely for the purpose of private study and research. They may not be copied or reproduced, except as permitted by copyright laws, without written authority of the copyright owner. Any commercial use or publication is strictly prohibited.

The original copyright license attesting to these terms and signed by the author of this thesis may be found in the original print version of the thesis, held by Western Libraries.

The thesis approval page signed by the examining committee may also be found in the original print version of the thesis held in Western Libraries.

Please contact Western Libraries for further information:
E-mail: libadmin@uwo.ca
Telephone: (519) 661-2111 Ext. 84796
Web site: http://www.lib.uwo.ca/
FATTY ACID METABOLISM IN
DYSTROPHIC MUSCLE OF MICE (STRAIN 129)

by

Chao Hsiung Lin
Department of Biochemistry

Submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

Faculty of Graduate Studies
The University of Western Ontario
London, Canada
March, 1970
This investigation was supported by a grant to Dr. A. J. Hudson and Dr. K. P. Strickland and a predoctoral fellowship to the writer from the Muscular Dystrophy Association of Canada. The writer wishes to express his appreciation to this organization for their generous support.
ACKNOWLEDGMENTS

The writer wishes to express his gratitude to his supervisors, Dr. K. P. Strickland and Dr. A. J. Hudson, for their direction and encouragement throughout this investigation, and for their patience in correcting the drafts of this thesis. He is indebted to Dr. H. B. Stewart, professor and head of the Department of Biochemistry for providing the laboratory facilities and to Dr. J. J. Jato for his helpful discussions during the past four years.

The writer is indebted to Miss Karen Ponath for her expert technical assistance and for her help in the preparation of this thesis. He wishes to thank Mrs. Jill Murray for typing this thesis.

The writer takes great pleasure in thanking his wife, Louise, for her forbearance and encouragement during the entire experimental work.
# CONTENTS

<table>
<thead>
<tr>
<th>Acknowledgments</th>
<th>iv</th>
</tr>
</thead>
<tbody>
<tr>
<td>List of Tables</td>
<td>xi</td>
</tr>
<tr>
<td>List of Illustrations</td>
<td>xvi</td>
</tr>
<tr>
<td>List of Appendices</td>
<td>xx</td>
</tr>
<tr>
<td>Glossary</td>
<td>xxii</td>
</tr>
<tr>
<td>Abstract</td>
<td>xxiii</td>
</tr>
</tbody>
</table>

## I. INTRODUCTION

## II. REVIEW OF LITERATURE

- Progressive muscular dystrophy                                                | 4    |
- Microstructure of striated muscle                                             | 5    |
  - A. The muscle fiber                                                         | 5    |
  - B. Myofibrils                                                              | 7    |
- Morphological changes in progressive muscular dystrophy                      | 7    |
- Energy metabolism in skeletal muscle                                          | 10   |
  - A. The nature of the energy source                                          | 10   |
  - B. Mechanism of muscle contraction                                          | 11   |
    (1) Biochemical evidence about actin-myosin interaction                     | 14   |
    (2) Molecular basis of muscle contraction                                   | 16   |
- Biochemical changes in progressive muscular dystrophy                        | 17   |
  - A. In mouse                                                                 | 17   |
    (1) Carbohydrate metabolism                                                 | 18   |
    (2) Lipid metabolism                                                       | 21   |
| (3) | Protein metabolism .................................. | 23 |
| (4) | Nucleic acid and nucleotide metabolism .......... | 25 |
| (5) | Muscle enzymes ...................................... | 27 |
| (6) | Creatine metabolism .................................. | 28 |
| (7) | Others .................................................. | 29 |

B. In man

| (1) | Carbohydrate metabolism .......................... 30 |
|     | (a)  | Glycogen ........................................... 30 |
|     | (b)  | Respiratory enzymes and oxidative phosphorylation | 31 |
|     | (c)  | Glucose ............................................. 34 |
|     | (i)  | Glucose tolerance .................................. 34 |
|     | (ii) | Arterial and venous glucose concentration ................. 35 |
|     | (d)  | Hyperpyruvicaemia ................................... 35 |
|     | (e)  | Ribosuria ........................................... 36 |
| (2) | Lipid metabolism .................................... 36 |
| (3) | Creatine and phosphorus compounds ............... 38 |
| (4) | Protein and amino acid metabolism ............... 41 |
|     | (a)  | Blood and urine .................................... 41 |
|     | (b)  | Muscle ............................................... 41 |

III. METHODS ......................................................... 44

A. Animals .......................................................... 44
B. Chemical preparations of substrates ................. 44
<table>
<thead>
<tr>
<th></th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. Reagents</td>
<td>46</td>
</tr>
<tr>
<td>(1) Radioactive compounds</td>
<td>46</td>
</tr>
<tr>
<td>(2) Other reagents and materials</td>
<td>47</td>
</tr>
<tr>
<td>D. Analytical methods</td>
<td>48</td>
</tr>
<tr>
<td>(1) Protein determination</td>
<td>48</td>
</tr>
<tr>
<td>(2) Nitrogen determination</td>
<td>48</td>
</tr>
<tr>
<td>(3) Determination of radioactivity</td>
<td>49</td>
</tr>
<tr>
<td>(4) Calculation and expression of radioactivity date</td>
<td>50</td>
</tr>
<tr>
<td>E. Incubations</td>
<td>50</td>
</tr>
<tr>
<td>(1) Palmitic acid-$l$-$^{14}$C oxidation by tissues of mice</td>
<td>50</td>
</tr>
<tr>
<td>(a) Muscle homogenate and mitochondria</td>
<td>50</td>
</tr>
<tr>
<td>(b) Homogenates of liver, kidney and brain</td>
<td>53</td>
</tr>
<tr>
<td>(2) Palmitoyl-CoA synthetase activity in skeletal muscle mitochondria and in 9000 $x$ g supernatant fraction</td>
<td>54</td>
</tr>
<tr>
<td>(a) Mitochondria</td>
<td>55</td>
</tr>
<tr>
<td>(b) 9000 $x$ g supernatant</td>
<td>56</td>
</tr>
</tbody>
</table>
(3) Carnitine palmitoyltransferase activity in skeletal muscle mitochondria .......... 56
(4) L-acetyl-l-14C carnitine oxidation by skeletal muscle mitochondria .......... 57
(5) Pyruvate-3-14C oxidation by skeletal muscle mitochondria ...................... 58
(6) Pyruvate-1-14C decarboxylation by skeletal muscle mitochondria .............. 58
(7) Fatty acid synthesis from acetate-2-14C in tissues of mice ....................... 59
(8) In vivo metabolism of palmitate-1-14C by dystrophic mice ....................... 60
   (a) Determination of cholesterol ................ 62
   (b) Determination of fatty acids ................. 62
   (c) Estimation of radioactivity .................. 62

IV. PALMITIC ACID-1-14C OXIDATION BY THE TISSUES OF DYSTROPHIC AND NORMAL MICE .......................................................... 63

A. Palmitate-1-14C oxidation by skeletal muscle homogenate and mitochondria ........ 63
   Results ....................................................................... 64
   (1) Muscle homogenate .............................................. 64
   (2) Mitochondria ....................................................... 69
   Discussion ..................................................................... 73

B. Palmitate-1-14C oxidation by liver, kidney and brain homogenates of dystrophic mice and their littermate controls ................................. 81
Results and discussion ........................................... 81

V. THE ACTIVITY OF PALMITYL-COA SYNTHETASE IN MITOCHONDRIA
AND THE 9000 x g SUPERNATANT OF SKELETAL MUSCLE .......... 86

Results ................................................................. 87
Discussion ............................................................. 99

VI. ACTIVITY OF CARNITINE PALMITYLTTRANSFERASE IN MUSCLE

MITOCHONDRIA OF DYSTROPHIC AND NORMAL MICE ............ 105

Results ................................................................. 105
Discussion ............................................................. 112

VII. ACETYL-1-14C CARNITINE OXIDATION BY SKELETAL MUSCLE

MITOCHONDRIA OF DYSTROPHIC MICE ............................. 116

Results ................................................................. 116
Discussion ............................................................. 118

VIII. DECARBOXYLATION OF PYRUVATE-1-14C AND OXIDATION OF
PYRUVATE-3-14C BY MITOCHONDRIA FROM DYSTROPHIC MUSCLE

OF MICE ............................................................... 123

Results ................................................................. 123
Discussion ............................................................. 131

IX. FATTY ACID SYNTHESIS FROM ACETATE-2-14C BY TISSUES

OF MICE ............................................................... 135

Results ................................................................. 135
Discussion ............................................................. 149

X. PALMITATE-1-14C METABOLISM IN VIVO .......................... 161

Results ................................................................. 161
Discussion ............................................................. 166
XI. DISCUSSION ........................................... 168
   A. Skeletal muscle mitochondria of mice (strain 129) ... 170
   B. Possible involvement of carnitine acetyltransferase in fatty acid oxidation ....................... 173
   C. Dilution of radioactive substrates ...................... 175
   D. Lipid accumulation in dystrophic muscle .......... 176
   E. The primary defect in muscular dystrophy .............. 177

XII. SUMMARY AND CONCLUSIONS ............................... 180

References .................................................. 184
Appendices .................................................. 218
Vita ......................................................... 230
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Muscular enzymes</td>
<td>32</td>
</tr>
<tr>
<td>II. Cofactor requirements for palmitate-1(^{14})C oxidation by 600 x g supernatant from muscle homogenate of mice (strain 129)</td>
<td>65</td>
</tr>
<tr>
<td>III. Palmitate-1(^{14})C oxidation by low speed supernatant of muscle homogenate from dystrophic mice (D) and their littermate controls (C)</td>
<td>68</td>
</tr>
<tr>
<td>IV. Inhibition of palmitate-1(^{14})C oxidation by malonate and fluorocitrate</td>
<td>72</td>
</tr>
<tr>
<td>V. The oxidation of palmitate-1(^{14})C by mitochondria from muscle of dystrophic mice (D) and their littermate controls (C)</td>
<td>75</td>
</tr>
<tr>
<td>VI. Palmitate-1(^{14})C oxidation by homogenates of liver, kidney and brain of dystrophic mice (D) and their normal littermates (C)</td>
<td>82</td>
</tr>
<tr>
<td>VII. Radioactivity in acetone moiety derived from aceto-acetate from palmitate-1(^{14})C oxidation by liver of mice</td>
<td>84</td>
</tr>
</tbody>
</table>
VIII. The stimulation of palmitoylcarnitine-\textsuperscript{14}C formation by carnitine palmitoyltransferase (CPT) in the presence of palmitoyl-CoA formed during incubation by preparations from normal mouse muscle ........................................ 89

IX. Palmitoyl-CoA synthetase activity in the mitochondria and 9000 x g supernatant of skeletal muscle from dystrophic (D) and normal mice (C) ................. 100

X. The effect of Triton-x-100 on the activity of carnitine palmitoyltransferase ....................... 109

XI. The effect of increasing mitochondria on the activity of carnitine palmitoyltransferase .......... 110

XII. Comparison of carnitine palmitoyltransferase activity between dystrophic and normal skeletal muscle mitochondria of mice (strain 129) ............... 111

XIII. Cofactor requirements of acetyl-\textsuperscript{14}C carnitine oxidation by skeletal muscle mitochondria of mice (strain 129) ........................................ 117

XIV. Acetyl-\textsuperscript{14}C carnitine oxidation by skeletal muscle mitochondria from dystrophic (D) and normal mice (C) (strain 129) ........................................ 120
XV. Effect of ATP and ADP on the decarboxylation of pyruvate-1-$^{14}$C by skeletal muscle mitochondria of mice ........................................... 125

XVI. Effect of malonate on the decarboxylation of pyruvate-1-$^{14}$C by mouse skeletal muscle mitochondria .. 127

XVII. Decarboxylation of pyruvate-1-$^{14}$C by skeletal muscle mitochondria of dystrophic mice (D) and their littermate controls (C) (strain 129) in the presence and the absence of DL-carnitine .............. 129

XVIII. Pyruvate-3-$^{14}$C oxidation by mitochondria from muscle of dystrophic mice (D) and their littermate controls (C) ........................................... 130

XIX. Cofactor requirements for fatty acid synthesis from acetate-2-$^{14}$C by high speed supernatant (71,000 x g) of skeletal muscle of mice (strain 129) .. 136

XX. Cofactor requirements for fatty acid synthesis by high speed supernatant preparations from liver, brain and kidney of mice (strain 129) ............... 137

XXI. Effect of bicarbonate and CO$_2$-flushing on fatty acid synthesis from acetate-2-$^{14}$C by the high speed supernatant preparation from skeletal muscle of mice (strain 129) ....................... 139
XXII. Effect of gas phase on fatty acid synthesis from acetate-2-$^{14}$C by high speed supernatant from skeletal muscle homogenate of mice (strain 129) ........ 140

XXIII. Effect of divalent cations on fatty acid synthesis from acetate-2-$^{14}$C by high speed supernatant from skeletal muscle of mice (strain 129) .................. 141

XXIV. A comparison of the effect of isocitrate and citrate on fatty acid synthesis by high speed supernatant (71,000 x g) of tissues of mice (strain 129) .......... 146

XXV. Effect of CoA on fatty acid synthesis from acetate-2-$^{14}$C by high speed supernatant of muscle and liver homogenates of mice (strain 129) .................. 150

XXVI. Incorporation of acetate-2-$^{14}$C into fatty acids by high speed supernatant of tissues of dystrophic mice (D) and their littermate controls (C) .............. 152

XXVII. Cholesterol and fatty acid content of tissues from dystrophic mice and their littermate controls (strain 129) .................................................. 162

XXVIII. Incorporation of palmitate-1-$^{14}$C into the fatty acid fractions of lipids from tissues of dystrophic mice and their littermate controls (strain 129) ...... 163
XXIX. Incorporation of palmitate-$^{14}$C into cholesterol from tissues of dystrophic mice and their littermate controls .......................... 165
<table>
<thead>
<tr>
<th>Figure</th>
<th>LIST OF ILLUSTRATIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Schema of energetics in skeletal muscle .......... 14</td>
</tr>
<tr>
<td>2.</td>
<td>Effect of carnitine concentration on palmitate-(1^{14})C oxidation by 600 x g supernatant of mouse skeletal muscle homogenates ........................................ 66</td>
</tr>
<tr>
<td>3.</td>
<td>Effect of added CoA on palmitate-(1^{14})C oxidation by 600 x g supernatant of mouse skeletal muscle homogenates ........................................ 67</td>
</tr>
<tr>
<td>4.</td>
<td>Time course of palmitate-(1^{14})C oxidation by mouse skeletal muscle mitochondria ...................... 70</td>
</tr>
<tr>
<td>5.</td>
<td>Comparison of the effect of several Krebs cycle intermediates on the oxidation of palmitate-(1^{14})C by mouse skeletal muscle mitochondria ...................... 71</td>
</tr>
<tr>
<td>6.</td>
<td>Effect of concentration of carnitine, ATP, CoA and succinate respectively, on palmitate-(1^{14})C by mouse muscle mitochondria ........................................ 74</td>
</tr>
<tr>
<td>7.</td>
<td>The effect of carnitine palmityltransferase on palmitylcarnitine formation from palmityl-CoA .......... 88</td>
</tr>
</tbody>
</table>
8. Effect of palmitate concentration on palmitoylcarnitine-Me-\(^{14}\)C formation in the assay method for palmitoyl-CoA synthetase activity ......................................................... 90

9. Effect of CoA on palmitoylcarnitine-Me-\(^{14}\)C formation in the assay method for palmitoyl-CoA synthetase activity ......................................................... 91

10. Effect of carnitine on palmitoylcarnitine-Me-\(^{14}\)C formation in the assay method for palmitoyl-CoA synthetase activity ......................................................... 94

11. Effect of Mg\(^{++}\) on palmitoylcarnitine-Me-\(^{14}\)C formation in the assay method for palmitoyl-CoA synthetase activity ......................................................... 95

12. Effect of ATP on palmitoylcarnitine-Me-\(^{14}\)C formation in the assay of palmitoyl-CoA synthetase activity ...................... 97

13. The effect of time on carnitine palmitoyltransferase of mouse skeletal muscle mitochondria using palmitoylcarnitine-Me-\(^{14}\)C as substrate .............................................. 106

14. The effect of CoA on the activity of carnitine palmitoyltransferase of mouse skeletal muscle mitochondria ................................................................. 108
15. The effect of acetyl-1-\textsuperscript{14}C carnitine concentration on its oxidation by mouse skeletal muscle mitochondria ... 119

16. The effect of carnitine on the decarboxylation of pyruvate-1-\textsuperscript{14}C by mouse skeletal muscle mitochondria .................. 124

17. The effect of pyruvate concentration on the decarboxylation of pyruvate-1-\textsuperscript{14}C by the skeletal muscle mitochondria of mice ................. 128

18. The effect of malate on the decarboxylation of pyruvate-1-\textsuperscript{14}C by skeletal muscle mitochondria of mice ... 133

19. Time course of fatty acid synthesis from acetate-2-\textsuperscript{14}C by high speed supernatant from mouse liver homogenates ... 142

20. Time course of fatty acid synthesis from acetate-2-\textsuperscript{14}C by high speed supernatant from skeletal muscle homogenates of mice (strain 129) .................. 143

21. Effect of enzyme concentration of fatty acid synthesis for mouse liver preparation .................. 144

22. Effect of enzyme concentration on fatty acid synthesis from acetate-2-\textsuperscript{14}C by muscle preparation ................. 145
23. Effect of citrate on fatty acid synthesis from acetate-2-\(^{14}\)C in muscle preparation ....................... 147

24. Effect of isocitrate on fatty acid synthesis from acetate-2-\(^{14}\)C in liver preparation .......................... 148

25. Effect of Mn\(^{++}\) ion on the incorporation of acetate-2-\(^{14}\)C into fatty acids by high speed supernatant of muscle homogenate ........................................ 151

26. Scheme showing the pathways of metabolism for fatty acids (e.g. palmitate) in skeletal muscle ................. 169

27. The "one acetyl-CoA pool" versus the "two acetyl-CoA pool" hypothesis (after Fritz) ............................. 174
LIST OF APPENDICES

Appendix

A. Palmitic Acid-1-\(^{14}\)C Oxidation by Low Speed Supernatant from Muscle of Dystrophic Mice (D) and Their Littermate Controls (C) .............................................. 218

B. The Oxidation of Palmitic Acid-1-\(^{14}\)C by Mitochondria from Muscle of Dystrophic Mice (D) and Their Littermate Controls (C) .............................................. 219

C. Activity of Mitochondrial Palmityl-CoA Synthetase of Skeletal Muscle from Normal (C) and Dystrophic Mice (D). 220

D. Palmityl-CoA Synthetase Activity in the 9000 x g Supernatnat of Skeletal Muscle from Dystrophic (D) and Normal Mice (C) .............................................. 221

E. Activity of Mitochondrial Carnitine Palmityltransferase of Skeletal Muscle from Normal (C) and Dystrophic Mice (D) .............................................. 222

F. Acetyl-1-\(^{14}\)C Carnitine Oxidation by Skeletal Muscle Mitochondria from Dystrophic (D) and Normal Mice (C) ... 223
G. Pyruvate-1\(^{14}\)C Decarboxylation by Skeletal Muscle
Mitochondria of Normal (C) and Dystrophic Mice
(Strain 129) in the Presence of DL-Carnitine .......... 224

H. Pyruvate-3\(^{14}\)C Oxidation by Mitochondria from Muscle
of Dystrophic Mice (D) and Their Littermate Controls
(C) .............................................................. 225

I. Incorporation of Acetate-2\(^{14}\)C into Fatty Acids by
High Speed Supernatant of Muscle Homogenate from
Dystrophic Mice (D) and Their Littermate Control (C) ... 226

J. Fatty Acid Synthesis from Acetate-2\(^{14}\)C by High Speed
Supernatant (71,000 x g) of Liver Homogenate from
Dystrophic (D) and Normal Mice (C) ....................... 227

K. Statistical Formulas Used ............................. 228
GLOSSARY

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>adenosine 5'-diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>ATPase</td>
<td>adenosine triphosphatase</td>
</tr>
<tr>
<td>CoA</td>
<td>coenzyme A</td>
</tr>
<tr>
<td>CAT</td>
<td>carnitine acetyltransferase</td>
</tr>
<tr>
<td>CP</td>
<td>creatine phosphate</td>
</tr>
<tr>
<td>CPT</td>
<td>carnitine palmitoyltransferase</td>
</tr>
<tr>
<td>Carnitine-Me-(^{14})C</td>
<td>carnitine with (^{14})C in the carbon of the methyl group.</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine 5'-triphosphate</td>
</tr>
<tr>
<td>g</td>
<td>acceleration due to gravity</td>
</tr>
<tr>
<td>g.</td>
<td>gram</td>
</tr>
<tr>
<td>NADP</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NADPH</td>
<td>reduced NADP</td>
</tr>
<tr>
<td>P</td>
<td>probability of an event due to chance alone</td>
</tr>
<tr>
<td>PMD</td>
<td>progressive muscular dystrophy</td>
</tr>
<tr>
<td>PPO</td>
<td>2,5-diphenyloxazole</td>
</tr>
<tr>
<td>POPOP</td>
<td>1,4-bis-2-(5-phenyloxazolyl)-Benzene</td>
</tr>
<tr>
<td>S.D.</td>
<td>standard deviation</td>
</tr>
<tr>
<td>S.E.M.</td>
<td>standard error of estimation of mean value</td>
</tr>
<tr>
<td>Tris</td>
<td>2-amino-2-hydroxymethylpropane-1,3-diol</td>
</tr>
<tr>
<td>t</td>
<td>Student's t test</td>
</tr>
<tr>
<td>(\mu c)</td>
<td>microcurie</td>
</tr>
<tr>
<td>(\mu g)</td>
<td>microgram</td>
</tr>
</tbody>
</table>
ABSTRACT

Palmitate-\(^{14}\)C oxidation by 600 x g supernatant fraction of mouse skeletal muscle homogenates and mitochondria from normal and dystrophic mice (Bar Harbor strain 129) has been studied. The optimal oxidation of palmitate-\(^{14}\)C by both preparations required the presence of carnitine, ATP and CoA. The mitochondrial preparations showed an absolute requirement for a Krebs cycle intermediate (e.g. succinate) in addition to the above cofactors. Succinate, malate, α-ketoglutarate and oxaloacetate were all equally effective in supporting the oxidation, but isocitrate was less effective. The oxidation of palmitate-\(^{14}\)C by 600 x g supernatant of muscle homogenate as well as by skeletal muscle mitochondria from dystrophic mice was significantly decreased compared with that of the normal littermate controls. In pursuing the mechanism of this decrease in palmitate-\(^{14}\)C oxidation, the activation of palmitate (palmityl-CoA synthetase) was studied using a radioassay method in both the mitochondrial and 9000 x g supernatant fractions. The activation of palmitate in both preparations required ATP, CoA and Mg\(^{++}\). The activity of mitochondrial palmityl-CoA synthetase was no different in normal and dystrophic muscle, whereas the activity of the cytoplasmic enzyme was significantly increased in the dystrophic muscle. Using palmitylcarnitine-Me-\(^{14}\)C as substrate, the activity of carnitine palmitoyltransf erase was measured. For optimal activity of this enzyme the addition of CoA was required. The enzyme was equally
active in both dystrophic and normal muscle mitochondria. This observation appears to eliminate the possibility that the decrease in palmitate-1-\textsuperscript{14}C oxidation in dystrophic muscle mitochondria is due to a defect in the transport of palmitoyl groups through the membrane.

The oxidation of acetyl-1-\textsuperscript{14}C carnitine by skeletal muscle mitochondria of mice was studied. The addition of malate and ADP were essential for the oxidation of acetyl-1-\textsuperscript{14}C carnitine; the addition of CoA had no effect. The oxidation of acetyl-1-\textsuperscript{14}C carnitine was greatly reduced in dystrophic muscle mitochondria (82%). Pyruvate-1-\textsuperscript{14}C decarboxylation and the oxidation of pyruvate-3-\textsuperscript{14}C were also investigated. Decarboxylation of pyruvate-1-\textsuperscript{14}C was stimulated by DL-carnitine. In the presence of carnitine the decarboxylation of pyruvate-1-\textsuperscript{14}C was significantly decreased in dystrophic muscle mitochondria, while in the absence of carnitine pyruvate-1-\textsuperscript{14}C decarboxylation was not significantly decreased, but a decreasing trend was evident. The oxidation of pyruvate-3-\textsuperscript{14}C was significantly decreased in the dystrophic muscle mitochondria.

The \textit{in vitro} incorporation of acetate-2-\textsuperscript{14}C into fatty acids has been studied using high speed supernatant fractions (71,000 x g) of tissues from mice (strain 129). The cofactor requirements for the synthesis of fatty acids from acetate-2-\textsuperscript{14}C for muscle preparations were the same as those for other tissues such as liver. Bicarbonate,
CoA, ATP, NADp$, Mn$^{++}$, citrate and a sulphydryl compound (cysteine) are all essential. The rate of synthesis of fatty acids from acetate-$2^{-14}$C by liver and muscle preparations from dystrophic mice was significantly increased, while the synthesis of fatty acids by kidney and brain preparations from dystrophic mice was significantly decreased.

The in vivo metabolism of palmitate-$1^{-14}$C was studied. It was found that the incorporation of radioactivity from palmitate-$1^{-14}$C into the cholesterol of muscle from dystrophic mice was significantly lower than that of the normal muscle.

It was concluded that the decrease in palmitate-$1^{-14}$C oxidation in dystrophic muscle mitochondria is most likely due to a defect in the utilization of acetyl groups either in the activation and transport into the mitochondria or in one or more of the steps of the Krebs cycle. The increases in fatty acid synthesis and the activity of cytoplasmic palmityl-CoA synthetase, together with the decrease in palmitate-$1^{-14}$C oxidation in the dystrophic muscle may help to explain the accumulation of neutral lipid normally observed in dystrophic muscle.
I. INTRODUCTION

Duchenne progressive muscular dystrophy is an hereditary muscle disease which occurs in childhood and results in the early death of the affected children. Prior to 1955 study of this type of disease was severely limited due to the unavailability of suitable experimental animals. In 1955, Michelson, Russell and Harman described an hereditary muscular dystrophy in an inbred strain of laboratory mice (Jackson Memorial Laboratory, Bar Harbor strain 129), which showed some degree of similarity to the human Duchenne dystrophy both histochemically and biochemically. This strain has since become the experimental animal of choice in many laboratories for the study of progressive muscular dystrophy.

The metabolism of carbohydrate and protein in the dystrophic muscle of mouse has been the subject of extensive study. However, very few studies have been conducted in the metabolism of lipids. Shull and Alfin-Slater (1958) reported that the total lipid and cholesterol were increased only in the muscle of dystrophic mice. Young, Young and Endelman (1959) reported that triglycerides and non-esterified fatty acids were about two times higher in the dystrophic muscle of mice than the control, whereas the phospholipid content was normal. Rabinowitz (1960) reported that fortified tissue homogenates of dystrophic mice revealed enhanced incorporation of acetate-$^{14}$C
into fatty acids and cholesterol. Partial confirmation of these observations was obtained by Strickland, Hudson, Tanaka and Wilensky (1965). Since these observations incriminate disturbances in fatty acid metabolism in dystrophic mouse and since fatty acids are an important source of energy in skeletal muscle, the writer has studied both the catabolism and anabolism of fatty acid in this animal.

In the investigation on catabolism, C_{16} palmitic acid, was selected as a representative fatty acid. Initially experiments were carried out on the oxidation of palmitic-1\(^{14}\)C acid by whole homogenates of skeletal muscle from normal and dystrophic mice. Since the steps of \(\beta\) -oxidation are confined to mitochondria, studies on the oxidation of palmitate-1\(^{14}\)C were then extended to this organelle isolated from normal and affected skeletal muscle. Further aspects of the study on catabolism involved assessment of the activities, in normal and dystrophic muscles, of enzymes concerned with the activation of fatty acid (palmityl-CoA synthetase) and with the transfer of fatty acid into mitochondria (carnitine palmityltransferase). The study on anabolism was not restricted to skeletal muscle but was extended to liver, brain and kidney because of Rabinowitz's (1960) observation of enhanced fatty acid synthesis in these tissues. In the experiments reported here high-speed supernatant fractions containing the full system for fatty acid synthesis (acetyl CoA carboxylase and fatty acid synthetase complex) were obtained and used as enzyme source for the incorporation of acetate-2\(^{14}\)C into fatty acid.
By carrying out this type of study on the metabolism of palmitic acid it was hoped that information would be obtained which would be of help in determining the normal pattern of oxidation and synthesis for this fatty acid in skeletal muscle of mice (strain 129) and that some assessment could be made regarding any abnormalities in the metabolism of fatty acid in the skeletal muscle of dystrophic animals. Certain abnormalities, such as impaired oxidation and enhanced synthesis of fatty acid, if observed, would be consistent with the finding that neutral fat accumulates in the affected skeletal muscles of dystrophic mice.
II. REVIEW OF LITERATURE

Progressive Muscular Dystrophy

The term progressive muscular dystrophy (PMD) describes a group of diseases characterized by muscle weakness and wasting but without pathological changes in the nervous system.

Before Erb (1890) coined the term dystrophia muscularis progressiva, there was an era when confusion existed in regard to the different muscle diseases which we now know as neuromuscular atrophy, poliomyelitis, peripheral neuritis, peroneal muscular atrophy and muscular dystrophy. Charcot (1869) in France proposed that dystrophy resulted from a primary chronic degeneration of the grey anterior column of the medulla. Eulenberg and Cohnheim (1866) in Germany, performed an autopsy on a case with "hypertrophy paralysis" and commented on the presence of a normal central nervous system. Duchenne, in 1868, described a muscle disease that he named "pseudohypertrophy or myosclerotic paralysis". He noted an increase of connective tissue and of fat cells in the affected muscle samples and concluded that the disease was chiefly one of the interstitial muscular tissue. Friedreich (1873) believed that the origin of the disease lay in the muscle.

Erb in 1884 gave the first detailed account of the juvenile form of progressive muscular dystrophy and its relation to pseudohypertrophy of muscle. He also noted that the spinal cord and peripheral
nerves were all normal. By 1890, he had accumulated sufficient evidence to indicate that progressive muscular dystrophy was a primary degenerative disorder of the muscle fibers. Erb indicated that many forms of muscular dystrophy existed, the first type being pseudohypertrophy as originally described by Duchenne, the second, a facioscapulohumeral form and the third type was "juvenile muscular atrophy".

**Microstructure of Striated Muscle**


Striated muscles are made up of many cylindrical multinucleated fibres, each of which has a diameter between 1 to 10 \( \mu \). The fibres may run the whole length of the muscle so that in a fibre of a few centimeters in length there may be several hundred nuclei. A single muscle fibre consists of many myofibrils, alongside of which lie cell nuclei and mitochondria (sarcosomes).

The muscle fibre is invested by an electrical, polarized membrane called the sarcolemma. Examined under the electron microscope, it appears as a thin dark line of about 100 Å thickness which can be resolved into two dense layers, 50-60 Å apart, between which is a trough of lesser density. The sarcolemma is not smooth but is marked here and there by tunnels or cave-like invaginations called the "caveolae intracellulares".
Mitochondria of striated muscle (sarcosomes) have the usual ultrastructural features of mitochondria from other cells. The size, number, shape and distribution of sarcosomes vary from muscle to muscle. Often symmetrically paired bodies of small sarcosomes are found at the Z-disc. Most of the perifibrillar sarcosomes are seen as small short rods, but sometimes longer mitochondria of greater diameter can be seen between the Z-discs. Some lateral branching of mitochondria may occur at the level of the Z-disc (for description of Z-disc see page 7).

The nuclei of muscle fibres are ovoid in shape and have a double membranous structure. They lie at the surface of the fibre immediately under the sarcolemma and are surrounded by a clear zone of protoplasm. In adult muscle, the nuclei are peripherally located, whereas in embryonic muscle they are found in the middle of the fibre. The distribution of nuclei is normally quite regular along the fibre except towards the tendon attachment where they become more numerous and irregular. In the nucleoplasm of human gastrocnemius muscle can be seen fine granular particles and nucleoli as groups of dense particles enveloped by a membrane.

The sarcoplasmic reticulum (SR), which is analogous to the endoplasmic reticulum of other cells, has no distinguishing features in human skeletal muscle. The SR can be found at the extremities of the A bands. It appears to be made up of vesicles interconnected by a fine tubular network.

The myofibrils are the contracting units in a muscle cell. A longitudinal section of a muscle cell shows striation under the light microscope and alternating dark and light bands under the electron microscope. The dark bands are birefringent and anisotropic under polarized light and are called A bands. The light bands are not birefringent and isotropic and are called I bands. In the middle of the I bands are Z-discs (Zwischenscheibe) and within the A bands are less dense bands called H zones (Heller) which do not appear in the fully contracted muscle. Between two Z-discs is the sarcomere which corresponds to the functional unit of a myofilament. In a transverse section of the muscle fibre, thin and thick filaments are arrayed in a regular hexagonal pattern; a thick filament is surrounded by six thin filaments and each thin filament is shared by three thick ones. The thick filament is composed of myosin and the thin filament of actin.

Morphological Changes in Progressive Muscular Dystrophy

An electron microscopic study of human muscular dystrophy (Duchenne type) was first made by van Breemen (1960b). According to van Breemen, the earliest abnormality in human muscular dystrophy is the vacuolization of the sarcoplasmic reticulum. This change was considered by him to reflect the primary lesion in muscular dystrophy.
In normal fibres with intact myofibrils and mitochondria, vacuoles appeared singly or in small groups between the myofibrils whereas in the affected fibres large vacuoles or large groups of vacuoles were usually observed. Following vacuolization, the sarcoplasmic reticulum then underwent disintegration. The mitochondria first decreased in size and increased in density and then they underwent lobulation and disintegration. The debris from these organelles were left in the space between the affected myofibrils. With the onset of the hyalin state of degeneration, mitochondria migrated to the periphery. The striation of myofibrils then became irregular and diffused. This was followed by the loss of the contracture bands.

Pearce (1962) has studied the morphological changes in Duchenne type muscular dystrophy using the electron microscope. The sarcolemmal membrane was generally intact. Occasionally, the membrane was thickened and the two layers fused. Morphologically, the nuclei and nuclear membrane usually appeared normal, but shrunken nuclei with increased granularity were often observed associated with other substantial changes within the fibres. The myofibrils suffered progressive destruction, commencing with the peripherally situated myofilaments. Complete disappearance of the whole segment of the myofilament occurred in some instances. Some areas in the sarcoplasm seemed to be normal, but debris from the disrupted myofilaments or organelles could be found. Vesicles, which usually appeared singly distributed between the myofibrils of the muscle of normal appearance, very often appeared
in clusters in the abnormal fibre. Changes in mitochondria ranged from normal appearance to complete destruction. The changes were consistently great in the internal parts of the fibre. The most common change observed was the separation of the cristae, which always occurred in enlarged mitochondria. The mitochondria adjacent to the defective myofilaments were usually abnormal. The SR was very often altered in the area of the fibre abnormality. Tubules were dilated so as to assume the appearance of cisterns.

More recently, Santa (1969) reported an electron microscopic study of progressive muscular dystrophy. In contrast to the findings of van Breemen (1960b), Santa found that the SR became swollen only in advanced degeneration. The swelling of the SR was not seen in the early stage of the disease. The sarcoplasm was already abnormal while the SR was not affected. The changes in myofilaments were divided into three stages: light, moderate and severe. In the first the disintegration of myofibrils was only seen in some sarcomeres; the others were completely normal. In the second, the myofilaments became tortuous and separated. The length of the sarcomere was variable. The myofilaments lost their band pattern and disruption could be seen starting at the junction of the A and I bands. The thick filaments were spared more than the thin ones. The advanced changes were characterized by the presence of fibrillar and amorphous fragments of Z-discs, bizarre and swollen mitochondria, many vesicles and in some cases the appearance of "lysosome-like" structures. The sarcolemma and nuclei were usually normal even in advanced stages.
The ultrastructural changes in the skeletal muscle of dystrophic mice of strain 129 have been studied by Ross, Pappas and Harman (1960). In general, similar changes to those of human muscular dystrophy (Duchenne type) were observed. The myofibrils degenerated gradually; this loss of myofibrils was typical of dystrophia muscularis in mice. Mitochondria were swollen and possessed a less dense matrix; their internal structures were obscure. The disorganization of myofibrils was independent of the presence of mitochondria as mitochondria still persisted in the fibre after the myofibrils completely disappeared. The nuclei and the sarcolemma were all intact, but some centralization of the nuclei occurred. The SR was swollen and showed obvious "ballooning".

The above structural alterations have been confirmed by Platzer and Chase (1964) with the one exception that the sarcolemma was usually found to be absent even in the mildly affected fibre. They also reported that the abnormal muscle fibres are already present in the newborn mice which develop dystrophy afterwards. They suggest that the initial defect may involve the sarcolemma.

Energy Metabolism in Skeletal Muscle

A. The nature of the energy source

Skeletal muscle can perform voluntary movement and maintain
its muscle tension at the expense of chemical energy stored in the form of adenosine triphosphate (ATP) which is generated during glycolysis as well as during the oxidation of both carbohydrate and fatty acids via the Krebs cycle by virtue of oxidative phosphorylation.

It was long thought that carbohydrate was the primary energy source in muscle and the formation of lactic acid (Fletcher and Hopkin, 1907) from glycogen (Parnas and Wagner, 1914) was once considered to be the only energy-yielding reaction since a quantitative relationship appeared to exist during anaerobic contraction between lactic acid formation and work done (Meyerhof, 1920). This "lactic acid" theory was accepted for many years until Lundsgaard (1929, 1930) showed that muscle could still contract even when lactic acid formation was blocked by iodoacetate. He suggested that energy for this contraction came from creatine phosphate breakdown and that carbohydrate breakdown was necessary to supply energy for the resynthesis of creatine phosphate. At about this time ATP was discovered independently by Lohmann (1929) and by Fiske and Subbarow (1929). It was the discovery of ATP and the findings of Lundsgaard (1930) which permitted Lohmann (1934) to demonstrate successfully that the hydrolysis of creatine phosphate by dialyzed muscle extract required the presence of adenosine diphosphate (ADP) in the system. This reaction, called the Lohmann reaction, is catalyzed by creatine phosphokinase:

\[
\text{Creatine phosphate} + \text{ADP} \rightarrow \text{creatinine} + \text{ATP} \quad (1)
\]

Reaction (1) was considered to be an energy-yielding reaction linked
to contraction and ATP was thought of as the immediate source of energy.

Although ATP was believed to be the immediate source of energy for contraction, biochemists working in this field were unable to demonstrate a net change of ATP concentration after a single twitch of the muscle fibre (Lundsgaard, 1930); (Carlson and Siger, 1960). However, the change was observed after exhaustive fatigue. The failure to demonstrate the breakdown of ATP after contraction was attributed to the fact that ATP was rapidly resynthesized from creatine phosphate during recovery. It was obvious that an inhibitor which would specifically inhibit the activity of creatine phosphokinase was desirable. Davies (1965) found that Sanger's reagent (1-fluoro-2, 4-dinitrobenzene) was a suitable inhibitor. Using this reagent to inhibit creatine phosphokinase, Davies was able to demonstrate for the first time a net breakdown of ATP after a single twitch of a muscle fibre.

For some time the Lohmann reaction was believed to be the only pathway by which creatine phosphate was formed. However, Cori, Abarca, Frenkel, Traverso-Cori (1956) found in skeletal muscle (but not in cardiac muscle) that creatine phosphate could form directly from creatine and 1,3-diphosphoglyceric acid without the involvement of ATP.

Although anaerobic glycolysis does produce limited quantities
of ATP, sustained muscle contraction is ultimately more dependent upon aerobic metabolism. Under aerobic conditions much larger quantities of ATP are formed through oxidative phosphorylation during oxidation of acetyl CoA derived from pyruvate, or lactate or the β-oxidation of fatty acids via the Krebs cycle.

The importance of non-esterified fatty acids (NEFA) as the fuel of muscle was suggested by the low R.Q. values (0.6–0.9) observed in exercising subjects or in isolated muscle. Good evidence is now available which indicates that fatty acids can be utilized by isolated skeletal muscle (Volk, Millington and Weinhouse, 1952; Tepperman, Tepperman and Shulman, 1956) and by cardiac muscle (Cavert and Johnson, 1956). Andres, Cader and Zierler (1956) have shown that the disappearance of glucose does not readily account for the oxygen consumption of the forearm of man and together with other observations they have postulated that lipid may be the major fuel for skeletal muscle. This idea is supported by the findings of Fritz, Davis, Holtrop and Dundee (1958) who observed that fatty acid degradation can provide the necessary energy source during activity (electrical stimulation).

Energy metabolism in skeletal muscle has been simplified into 3 phases: energy liberation, energy conservation and energy utilization (Olson and Piatnek, 1959) as shown in Fig. 1:
B. Mechanism of Muscle Contraction

(1) Biochemical Evidence about Actin-Myosin Interaction

Actin, which was first discovered by Straub (1942), has a molecular weight of 76,000 and can exist in two forms, globular (G-actin) and fibrous (F-actin). The interconversion of the two forms depends on the salt concentration or ionic strength; as the ionic strength of an aqueous solution of G-actin is increased it undergoes polymerization to F-actin. Myosin is the other main contractile protein of muscle. It possesses a specific ATPase activity. Demonstration of the important role of actin and myosin in contraction comes from the studies of Szent-Györgyi (1947), who showed that actomyosin threads
(prepared from a gel) could be caused to contract by dipping them into boiled muscle juice. Contraction could also be demonstrated with actomyosin gel and with glycerinated fibre bundles (Szent-Györgyi, 1953). These fibres not only contracted upon addition of ATP, but also developed the same tension observed in vivo. Interaction of F-actin and myosin is observed when a solution of myosin and F-actin is mixed. On mixing, the sudden rise in viscosity which results indicates that a reaction has taken place. The new substance, actomyosin, behaves as a typical fibrous high polymer (Szent-Gyorgyi, 1947). On the addition of ATP dissociation of actomyosin occurs with simultaneous decrease in viscosity.

Purified myosin, in the presence of concentrations of magnesium and calcium ions similar to those found in muscle during activity, has a relatively low ATPase activity (Mommaert and Green, 1954). However, in the absence of ATP and in the presence of actin under the same conditions, the combination of actin and myosin occurs with a concomitant enhancement of ATPase activity (20-fold or more, Hasselbach, 1952). The activity of ATPase under these conditions approaches that required to account for the known rate of energy release in a muscle (Perry, 1956). The ATPase activity of unpurified actomyosin and of the myofibril can be regulated in vitro by the concentration of calcium ions (Ebashi, 1963). There is good evidence that an analogous process occurs in vivo. When electrical signals are conducted inward from the
sarcolemma, calcium ions are released from the sarcoplasmic reticulum and the contraction of muscle occurs. Relaxation follows with the removal of calcium ions by the sarcoplasmic reticulum through an ATP-dependent active transport system.

For some time the site of action of calcium in contraction was not known. This was clarified by Ebashi, Ebashi and Kodama (1967) who showed that the calcium ions seem to interact or combine with the troponin moiety (the latter is a protein that appears to be attached at or near the cross bridges interacting with the thin actin filaments) rather than directly with actomyosin. Troponin is believed to act as a "safety valve", which prevents the activation of myosin ATPase by actin when calcium is absent, but allows the activation to occur when calcium is bound to troponin.

(2) **Molecular Basis of Muscle Contraction**

Huxley and Hanson (1960) have done extensive studies on muscle contraction using the technique of electron microscopy. Out of this work has come the "sliding-filament" hypothesis (proposed by Huxley and Hanson in 1960). The hypothesis is briefly described as follows: When the myofibril contracts, the evidence indicates that two kinds of filaments slide past each other; the thin filaments (actin) appear to move into the A bands which are equal in length to the thick filaments (myosin) and which remain constant during contraction.
Between the actin and myosin filaments can be seen cross-links or bridges which are thought to be part of the myosin molecule (heavy meromyosin, HMM). It is considered that there are specific sites on the actin filaments to which the bridges of myosin are attached. More recently Huxley (1969) has slightly modified his original "sliding filament" model. The revised model pictures the light meromyosin (LMM, which is a portion of the myosin molecules) bonded into the backbone of the myosin filament. The linear portion of the heavy meromyosin (HMM, which has a globular head with ATPase activity) is pictured as lying parallel to the backbone of the myosin filament in resting muscle. The junctions of LMM and HMM are considered to be flexible. During the contraction, it is suggested that the linear portion of HMM can tilt out from the filament by bending at the flexible LMM and HMM junction. This allows the globular head to become attached to actin over a range of different side spacings between actin and myosin while still maintaining the same orientation. It is postulated that a sliding force is created through an active change in the angle of attachment between the globular head and the actin filament which is brought about in association with the splitting of ATP. For a more detailed account of the mechanism, the reader is referred to the original article (Huxley, 1969).

Biochemical Changes in Progressive Muscular Dystrophy

A. In Mouse
(1) **Carbohydrate Metabolism**

Baker, Tubis and Blahd (1958) and Baker and Huebottier (1964) investigated the turnover of acetate-1\(^{14}\)C and of glucose-U\(^{14}\)C in the whole animal after single injections of radioactive precursors. The rate of \(^{14}\)CO\(_2\) production from both compounds was the same in the normal and dystrophic mice. They suggested that in the dystrophic animal there was no enzyme missing as a result of a genetic defect which interferes with the oxidation of glucose to CO\(_2\). Since muscle contributes only a portion to the overall metabolism of the intact animal, it is quite possible that any abnormality in either glucose or acetate metabolism in the muscle would not be detectable in the above studies.

The intactness of glycolysis and of the Krebs cycle in mice with muscular dystrophy has been shown from the studies of Mayers and Epstein (1962) who examined the glycolytic pathway in the isolated muscle of the mouse by measuring the formation of lactic acid after incubation and evaluated the Krebs cycle by its oxygen consumption. In support of the previous findings Borgman (1962) observed that tissue slices of skeletal muscle from dystrophic mice utilized oxygen at approximately normal rates.

Although overall glycolysis in dystrophic muscle seems to be normal, many enzyme activities have been shown to be changed. Thus hexokinase (McCaman, 1963; Stamp and Lesker, 1967) was slightly increased while
other glycolytic enzymes, such as phosphoglucomutase (Hazzard and Leonard, 1959; Stamp and Lesker, 1967), aldolase (Srivastava and Berlinguet, 1964; Stamp and Lesker, 1967) and lactic acid dehydrogenase (Stamp and Lesker, 1967) were decreased.

Citric acid content in tissues of normal and dystrophic animals was studied by Taussky, Washington, Zubillaga and Milhorat (1962). They found that citric acid was markedly increased in the muscle of vitamin E deficient rabbits. An increase by 138% in citric acid content was also observed in the muscle of dystrophic mice. These results suggest that there might be a partial block in the pathway of the Krebs cycle after citric acid (for example at the cis-aconitase step) in the dystrophic muscle.

McGaughey (1960) reported that the excretion of α-ketoglutarate by dystrophic mice was higher than in the controls. This observation also suggests that there might be a biochemical lesion in the steps of the Krebs cycle. It is interesting to note that α-ketoglutarate in the dystrophic muscle was not detectable while the normal muscle contained 150 mg per gram fat-free, non-collagen nitrogen (Read and Jones, 1966). Further analysis revealed a higher level of α-ketoglutarate dehydrogenase in dystrophic mouse muscle than in its littermate control, which is in agreement with the report of Rosenkrantz (1959).

Since glycogen is one of the important energy sources for muscle,
the enzymes involving its metabolism have received some attention. Leonard (1957) has shown that phosphorylase a and t (total phosphorylase) activities were significantly lower in dystrophic muscle. These results were confirmed both by Tassoni, Mantel and Harman (1964) and by Stamp and Lesker (1967). The activity of glycogen synthetase was also decreased (Stamp and Lesker, 1967).

In general it has been considered that all of the steps of the "Pentose phosphate pathway" were not operative in muscle. However, Rossi, Zatti and Greenbaum (1963) have shown that this pathway, though of minor importance, definitely exists in the muscle of rats. Canal and Frattola (1962) have studied this pathway in mouse dystrophic muscle. They found that the disappearance of both aldopentose and total pentose was enhanced in dystrophic muscle relative to the normal controls. The activity of the first two enzymes in the pentose phosphate pathway (glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase) was higher in dystrophic muscle. Sedoheptulose formation from hexose phosphate was also enhanced in the dystrophic muscle.

Incubation of dystrophic muscle homogenate has been observed to cause an accumulation of a ketone material which was thought to be acetoacetate (Gould and Coleman, 1961) but which now has been identified as methylglyoxal (Coleman, 1965). Methylglyoxal per se was not the compound which accumulated in the reaction mixture but was an artifact resulting from other compounds, probably triosephosphate, under the
conditions for acetoacetate determination (Greenberg and Lester, 1944). In pursuing the mechanism of this accumulation, Coleman has found that the activities of glyceraldehyde-3-phosphate dehydrogenase and \( \alpha \)-glycerophosphate dehydrogenase were markedly decreased in dystrophic muscle and that the decrease in the activity of glyceraldehyde-3-phosphate dehydrogenase was responsible for the accumulation of triosephosphate seen in the homogenates of dystrophic muscle (Coleman, 1965).

Wrogemann and Blanchaer (1967) have studied oxidative phosphorylation in muscle mitochondria of dystrophic mice. The values of the P/O ratio, respiratory control ratio and phosphorylation rate were all at normal levels. This study seems to suggest that the energy-yielding processes are not affected by dystrophy.

(2) **Lipid Metabolism**

Only very few investigations have been carried out on the metabolism of lipids in the dystrophic muscle of mice. It is now known that non-esterified fatty acids can provide energy for muscle activity (Fritz et al., 1958) and that lipids (especially phospholipids) are important components of membrane structures. Because the membrane permeability of muscle cells from dystrophic mice may be increased (Zieler, 1958) relative to that of the normal muscle, studies of lipids in the membrane could be of great importance.
Shull and Alfin-Slater (1958) have reported that the cholesterol content of the dystrophic muscle of mice was higher than that of the normal controls. Young, Young and Edelman (1959) have shown that triglyceride and fatty acid concentrations were increased in the dystrophic muscle of mice. Rabinowitz (1960) found that fortified tissue homogenates of dystrophic mice enhanced the incorporation of acetate-$2^{14}$C into fatty acid and cholesterol. These observations have been confirmed by Strickland, Hudson, Tanaka and Wilensky (1965), but rather large variations were noted. Based upon in vivo studies Kabara (1964a, 1964b, 1964c) found acetate-$2^3$H incorporation into tissue cholesterol to be higher in several tissues of dystrophic mice. The greatest increase was observed in brain. Recently Susheela, Hudson and Walton (1968) have reported that the content of fatty acid not only increased in the muscle but also in other tissues of dystrophic mice with the exception of the adipose where a decrease in fatty acid content was observed.

The above studies clearly seem to indicate that some abnormality in lipid metabolism must exist in muscular dystrophy. Since the nature of the abnormality is not well defined by any of these studies, it is also very evident that there is need to undertake a detailed investigation of lipid metabolism in this disease. Indeed, these are reasons which have prompted the writer to embark upon the investigations to be reported in this thesis.
(3) **Protein Metabolism**

Kruh, Dreyfus, Schapira and Gey (1960) studied glycine-$^{14}$C incorporation into myosin of mice. They found that the incorporation of radioactivity into myosin of dystrophic muscle increased for the first four days and then decreased without reaching any plateau. By contrast, the incorporation of glycine-$^{14}$C into myosin of normal mice showed an increase in incorporation with time until a plateau was reached. The turnover of water-soluble protein in dystrophic muscle as measured by the disappearance of radioactivity in the protein was also accelerated. The observations of increased turnovers of myosin and other protein components in cells of dystrophic muscle has been interpreted to mean that the life span of the dystrophic muscle cell (or a part of it) is shorter than that of the normal cell.

Oppenheimer, Barany and Milhorat (1964) using various physical-chemical methods reported that the molecular weight and the size of myosin from dystrophic muscle was no different from that of the littermate controls; the ultracentrifuge pattern was similar, but light scattering seemed to indicate polydispersity of the molecular parameters of the myosin from dystrophic mice. The ability of myosin to combine with F-actin was not affected by the disease. Smoller and Fineberg (1964) have studied the amino acid composition of myosin from normal and dystrophic muscle and found that myosin from the dystrophic
animal has a higher content of alanine and isoleucine and a lower
content of glycine and histidine. About 25% of the myosin protein
existed in aggregate form while normal myosin remained monomeric
under the same conditions. Moreover, myosin from dystrophic mice
was found to have fewer SH- groups which reacted with sulfhydryl
reagent, although this myosin contained a normal amount of cystine.

Berlinguet and Srivastava (1966) have reported that the overall
activity of the proteolytic enzymes of dystrophic muscle was higher
than that of the normal muscle; the difference in the activity was
most significant in animals of 60-90 days of age.

The activity of cathepsin was increased in dystrophic muscle
based on wet weight, non-collagen protein or dry weight. The role
of cathepsin in protein metabolism is not clear. Its activity is
not only increased in the dystrophic muscle but also in other types
of muscle atrophy, such as observed in rabbits on vitamin E deficiency
(Weinstock, Goldrich and Milhorat, 1955).

Thus, it may be concluded from the evidence cited that the
loss of protein in dystrophic muscle might be caused by acceleration
of catalytic processes rather than a decrease in protein synthesis.
The shorter life span of myosin from dystrophic muscle, increased
proteolytic enzyme and cathepsin activities in the dystrophic muscle
all support this suggestion.
(4) **Nucleic Acid and Nucleotide Metabolism**

Coleman and Asworth (1959) have reported that skeletal muscle from adult mice with muscular dystrophy contained increased amounts of nucleic acids (up to 60% more DNA and 40% more RNA per gram) when compared with muscle from normal littermate controls. The concentration of nucleic acids from other tissues was not different from that of the normal. The incorporation of glycine-1-\(^{14}\)C (incorporated into carbon 4 and 5, nitrogen 7 of purine bases of nucleic acids) into nucleic acids was significantly enhanced in dystrophic muscle, but decreased in the liver. They found the difference between the two groups to be proportional to the severity of the disease (i.e. age of the animals). Using different precursors Girkin, Fitch and Dining (1962) have shown that following the injection of formate-\(^{14}\)C (incorporated into carbons 2 and 8 of the purine bases of nucleic acids) the turnover time of DNA and RNA was not shortened in dystrophic mice. However, the contents of DNA and RNA were increased in dystrophic muscle. Srivastava (1967) studied the incorporation of uridine-2-\(^{14}\)C into RNA in vivo and in vitro and found an increased incorporation at 90 days of age compared with the normal muscle.

The activities of enzymes involved in the metabolism of nucleotides in the dystrophic muscle were either similar to or lower than those of the normal controls. 5'-Adenylic acid deaminase (Pennington, 1961; Kaldor and Gitlin, 1963; McCaman, 1965) and adenylate kinase
(Kaldor and Gitlin, 1963) activities were lower in dystrophic muscle; purine nucleotide phosphorylase and 5'-nucleotidase were similar (McCaman, 1965). On the contrary, Tassoni, Mantel and Harman (1964) found a significant increase in the activity of 5'-nucleotidase. This discrepancy may be due to the different "base-line" of comparison used. This problem of the correct "base-line" to use constantly arises in regard to all studies on muscular dystrophy. A reasonable case can be made for using NCN (Non-collagen nitrogen) since it is considered to reflect the amount of functional protein present (Lilienthal, Zieler, Folk, Buka and Riley, 1949).

Actinomyosin ATPase activity was significantly higher (Tassoni et al., 1964) and ATPase and myokinase activities of myofibrils of dystrophic mice were the same as those of the litter-mate controls.

Zymaris, Epstein, Saifer, Aronson and Volk (1959) have studied the concentration of several nucleotides in the dystrophic muscle. The types of nucleotides found in the dystrophic muscle were no different from those of the normal muscle. The concentration of ATP was lower, whereas the concentrations of AMP, GTP (including UTP), and GDP (including UDP, CTP and IDP) were all higher in the dystrophic muscle. The significance of these findings remains to be ascertained.
(5) **Muscle Enzymes**

A study on muscle enzymes has been reported by McCaman (1963) who showed that the levels of activities of the NADP-linked enzymes, glucose-6-phosphate dehydrogenase, 6-phospho-gluconic dehydrogenase, isocitrate dehydrogenase and glutathione reductase were increased in dystrophic mice relative to those of the littermate controls. In contrast, the activities of α-glycero-phosphate dehydrogenase and lactic acid dehydrogenase which require + NAD as cofactor were decreased.

The most abundant soluble enzyme in muscle is creatine kinase, which catalyzes the reversible reaction:

\[
\text{ATP + Creatine} \rightleftharpoons \text{ADP + Creatine phosphate}
\]

This enzyme has been assayed in the presence of a sulfhydryl compound by Nichol (1964). The activity of enzyme, based on non-collagen protein nitrogen, was significantly decreased in the muscle from young dystrophic mice. Hooton and Watts (1966) have purified this enzyme from muscles of dystrophic and normal mice. They found that the purified enzyme from dystrophic muscle was one half as active as that from normal muscle. The dystrophic muscle enzyme contained one thiol group instead of two as in normal muscle. "Finger printing" techniques showed that one peptide fragment differed when fragments from creatine kinase of dystrophic muscle were compared with those from creatine kinase of normal muscle.
Weinstock, Epstein and Milhorat (1958) reported that, based on non-collagen protein nitrogen, the activity of succinoxidase was not different from that of the normal, but that the activity of cytochrome c oxidase was enhanced.

Srivastava and Berlinguet (1967), on measuring the levels of cholinesterase and monoamine oxidase in dystrophic muscle, found that the activity of both enzymes was significantly increased. These workers suggested that the increase in monoamine oxidase activity might be due to "induction" caused by the increased amount of amines in the dystrophic muscle.

Glutamic acid dehydrogenase in muscle of dystrophic mice was shown to be significantly increased when the results were expressed either in mg wet weight or mg nitrogen (Tsui, 1967). It was observed that, unlike glutamic acid dehydrogenase from normal muscle which is quite constant in activity, the activity of the enzyme showed fluctuations with age, but reached a peak at 16 weeks of age.

(6) **Creatine Metabolism**

The study of creatine metabolism in dystrophic mice has received much attention since creatine, via creatine phosphate, is one of the most important immediate sources used in the generation of ATP. In mice, creatine is normally present in the urine, but in man, creatininuria is a sign of a pathological process. Fitch, Oates
and Dining (1961) injected intraperitoneally creatine-\(^{14}\)C into normal and dystrophic mice and measured the incorporation and retention of \(^{14}\)C in the muscle and other tissues. It was found that the incorporation of creatine-\(^{14}\)C into muscle was faster but that there was less retention of radioactivity in dystrophic muscle after 38 days relative to that of the normal muscle. Calculation of turnover times from these data resulted in significantly shortened times for both body and muscle creatine in dystrophic mice. It was suggested that dystrophic mice have an impaired ability to retain creatine. This assumption is reinforced by a recent study of Fitch (1969) in which he has shown that entry of creatine into the muscle cells is faster in dystrophic muscle than in the normal muscle.

(7) **Others**

Nichol (1964) has reported a lower sulfhydryl and greater disulfide concentration in dystrophic mouse muscle. Schatz and Hollinshead (1967) have shown that the glutathione level in dystrophic muscle was significantly higher in all female animals (17-112 days), but no significant difference was seen in the young animals (17 days). In male animals, both groups contained the same level of glutathione. Therefore, the overall picture shows that the content of glutathione is not affected by dystrophy.

It has been shown that in dystrophic muscle following contraction the relaxation time was longer than that of the normal muscle (Sandow
and Brust, 1958). This has been correlated with the biochemical finding that Ca^{++} uptake by sarcoplasmic reticulum from dystrophic muscle is considerably decreased in comparison with that of the normal preparation (Sreter, Martonosi and Gergely, 1964). This correlation is of considerable interest since it now seems certain that the uptake and release of Ca^{++} by sarcoplasmic reticulum controls the contraction and relaxation of the muscle (Weber, 1966).

B. In Man

(1) Carbohydrate Metabolism

(a) Glycogen

The content of glycogen in the dystrophic muscle of man was found to be variable by many authors, but, in general a decrease in concentration is observed. Collazo, Barbudo and Torres (1936) reported that muscle glycogen in patients with PMD was decreased. This was confirmed by Vignos and Warner (1963) who found muscle glycogen in Duchenne dystrophy (characteristically a disease of children) to be decreased to one third of the normal level. Only a slight decrease was observed in adult muscular dystrophy. It is likely that some of the variability observed results from failure to take adequate precautions to prevent glycogen breakdown during the removal of the muscle.

Ronzoni, Leonard and Landau (1960) have reported a generalized decrease in the enzymes involved in glycogenolysis with the result that
the dystrophic muscle fails to produce the normal amount of lactic acid. This finding is not in agreement with that of Collazo et al. (1936) who showed no difference in lactic acid concentration. Phosphorylase a and phosphorylase b (Ronconi et al., 1960; Dreyfus, Schapira and Schapira, 1954), aldolase (Dreyfus et al., 1954; Shapira, Dreyfus, Schapira and Kruh, 1955; Ronconi et al., 1960), phosphoglucomutase (Schapira et al., 1955) were found to be decreased in dystrophic muscle. Hexokinase was normal and lactic acid dehydrogenase (Ronconi et al., 1960) was increased. Thus, on an overall basis the evidence indicates that glycogenolysis falls.

(b) Respiratory Enzymes and Oxidative Phosphorylation

The activities of respiratory enzymes in dystrophic muscle were decreased when the results were expressed per gram fresh tissue, but this difference disappeared when the activities were expressed per gram muscle protein as shown in Table I. A normal level of muscle succinic dehydrogenase has been reported by Vignos and Lefkowitz (1959) in the dystrophic muscle. This was further substantiated by the finding of DeLaey (1965) that in biopsies of patients with muscular dystrophy the mitochondrial succinic dehydrogenase activity was at the normal level.

Oxidative phosphorylation by skeletal muscle of Duchenne dystrophy has been studied by Ionasescu, Luca and Vuia (1967). It
<table>
<thead>
<tr>
<th></th>
<th>Total Glycogenolysis</th>
<th>Phosphorylase</th>
<th>Mutase</th>
<th>Aldolase</th>
<th>Cytochrome-oxidase</th>
<th>Succinoxidase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of Cases</td>
<td>No. of Cases</td>
<td>No. of Cases</td>
<td>No. of Cases</td>
<td>No. of Cases</td>
<td>No. of Cases</td>
</tr>
<tr>
<td>Rats*</td>
<td>28</td>
<td>1.74±0.1</td>
<td>12</td>
<td>10±1.4</td>
<td>10</td>
<td>5.25±0.26</td>
</tr>
<tr>
<td>Normal Subjects*</td>
<td>5</td>
<td>0.52±0.1</td>
<td>6</td>
<td>4.0±0.4</td>
<td>4</td>
<td>1.95±0.13</td>
</tr>
<tr>
<td>P M D *</td>
<td>6</td>
<td>0.08±0.02</td>
<td>11</td>
<td>1±0.2</td>
<td>4</td>
<td>0.7±0.13</td>
</tr>
<tr>
<td>Normal Subjects**</td>
<td>5</td>
<td>2.85±0.5</td>
<td>6</td>
<td>23±2</td>
<td>4</td>
<td>11±0.7</td>
</tr>
<tr>
<td>P M D **</td>
<td>6</td>
<td>0.75±0.11</td>
<td>11</td>
<td>8.6±1.6</td>
<td>4</td>
<td>4.4±0.65</td>
</tr>
</tbody>
</table>

* Referred to 1 g of fresh tissue.
** Referred to 1 g of true muscle protein.
<table>
<thead>
<tr>
<th></th>
<th>Succino-dehydrogenase</th>
<th>Aconitase</th>
<th>Fumarase</th>
<th>Transaminases</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of Cases</td>
<td></td>
<td>No. of Cases</td>
<td></td>
<td>No. of Cases</td>
<td></td>
</tr>
<tr>
<td>Rats*</td>
<td>5 3.8 ±0.8</td>
<td>5 2.5 ±0.16</td>
<td>4 0.92±0.1</td>
<td>4 1.210±0.032</td>
<td>4 0.234±0.042</td>
<td></td>
</tr>
<tr>
<td>Normal Subjects*</td>
<td>6 1.81±0.4</td>
<td>4 1.96±0.2</td>
<td>4 0.68±0.01</td>
<td>5 0.878±0.090</td>
<td>5 0.396±0.058</td>
<td></td>
</tr>
<tr>
<td>P M D*</td>
<td>6 1.14±0.2</td>
<td>6 0.9±0.06</td>
<td>6 0.26±0.06</td>
<td>3 0.560</td>
<td>3 0.220</td>
<td></td>
</tr>
<tr>
<td>Normal Subjects**</td>
<td>6 9.6±0.26</td>
<td>4 10.1±1.0</td>
<td>4 3.6±0.5</td>
<td>5 4.950</td>
<td>5 2.200</td>
<td></td>
</tr>
<tr>
<td>P M D**</td>
<td>6 14.7±2.7</td>
<td>6 10.1±1.7</td>
<td>6 2.65±0.6</td>
<td>3 6.250</td>
<td>3 2.500</td>
<td></td>
</tr>
<tr>
<td>N.S.* Rats*</td>
<td>0.48</td>
<td>0.78</td>
<td>0.74</td>
<td>0.73</td>
<td>1.70</td>
<td></td>
</tr>
<tr>
<td>N.S. P M D*</td>
<td>0.63</td>
<td>0.50</td>
<td>0.38</td>
<td>0.64</td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td>N.S.* P M D**</td>
<td>1.5</td>
<td>1.0</td>
<td>0.72</td>
<td>1.26</td>
<td>1.14</td>
<td></td>
</tr>
</tbody>
</table>
was found, when using succinate as substrate in the presence of phosphate acceptor that the oxygen uptake by dystrophic muscle was significantly lower than that of the normal. The respiratory control ratio was lower whereas the P/O ratio and phosphate uptake were at the normal level. Olson, Vignos, Woodlock and Perry (1968) have confirmed this finding using glutamate as substrate. They noted that the decrease in glutamate oxidation was proportional to the severity of the disease. In the less severe cases the mitochondria oxidized glutamate at a normal rate whereas in the more severe cases this oxidation was reduced.

(c) **Glucose**

(i) **Glucose Tolerance**

Contradictory results were obtained in the early studies on glucose tolerance in PMD (McCrudden and Sargent, 1916; Shank, Gilder and Hoagland, 1940; Tyler and Perkoff, 1951). An abnormal glucose tolerance was described by Ionasescu and Luca (1963) in 9 out of 14 patients studied. Herschberg, Coirault and Giboudeau (1964), studying 32 cases of Duchenne muscular dystrophy, confirmed the above findings. These authors presumed that this abnormality is due to a marked reduction in muscle mass and to a disturbance in phosphorylation of glucose. The finding of Ionasescu and Luca (1963) that the maximum fall in inorganic phosphate during the glucose tolerance test was significantly higher in normal subjects than in PMD patients is consistent with the above presumption.
(ii) **Arterial and Venous (A-V) Glucose Concentration**

Dreyfus, Schapira and Démos (1958) have determined the arteriovenous difference of glucose in the femoral vessels of control and dystrophic patients. The glucose consumption seemed to be diminished, but the differences were not statistically significant. Ionasescu and Luca (1963) have measured A-V differences in glucose concentration during glucose tolerance tests. It was found that the A-V difference in glucose concentration was significantly decreased in patients with PMD as compared with the controls at 15, 30, 45 and 60 minutes. Although this study suggests that the metabolism of blood glucose may be decreased in the dystrophic patients, it is uncertain whether this is a true decrease since no assessment was made of any changes in blood flow.

(d) **Hyperpyruvicaemia**

Luzzato and Lodigiani (1954) described hyperpyruvicaemia in dystrophics. Luzzato and Ramelli (1959) reported that they also observed hyperpyruvicaemia in the healthy siblings of families of dystrophics. Ionasescu and Luca (1963) failed to confirm this finding; they found a normal level of pyruvic acid in the blood either in the fasting state or during the glucose tolerance test. However, many workers have confirmed hyperpyruvicaemia in patients with PMD. Hyperpyruvicaemia could result from a decreased activity of the Krebs cycle or of the reductive (pyruvic acid to lactic acid) process, or from increased transaminase activity.
Recently, Radu, Kapusi and Stenzel (1968) have reported that coenzyme A activity in dystrophic muscle (Duchenne type) was significantly decreased, whereas the activity in the serum was relatively increased compared with those of normal subjects. The authors suggested that, as in the case of sarcoplasmic enzymes which pass into the serum, more coenzyme A passes through the muscle membrane into the serum of dystrophic patients. As a consequence the initial stage of pyruvate utilization is partially blocked and pyruvate in the blood rises and citric acid decreases.

(e) **Ribosuria**

Ribosuria, the appearance of ribose in the urine, was first reported by Minot, Frand and Dziewiatkowski (1949). The authors claimed that the constant excretion of this ribose (in combined form) in the urine was specific for PMD. However, Mathews and Smith (1953) failed to confirm the presence of ribose in the urine of dystrophic patients. Walton and Latner (1954) carried out a test for ribosuria in 89 PMD patients and found that only 12 were positive. They concluded that the test for ribosuria had no diagnostic value in muscular dystrophy.

(2) **Lipid Metabolism**

Fat accumulation in the muscle is one of the characteristic features of human muscular dystrophy (van Breeman, 1960). This obser-
vation suggests that there might be some disturbance in lipid metabolism. However, very few efforts have been directed toward an understanding of the mechanism of this accumulation. Ronzoni, Wald, Berg and Ramsey (1958) have reported an increase of fat content (on a wet weight basis) in the muscle of dystrophic patients and Pennington, Park and Freeman (1966) have described the fatty acid composition of fat infiltrating into the muscle of a case of muscular dystrophy (Duchenne type). They found that the values obtained fell within the range of those reported for normal adipose tissue. Sudo, Kuroda and Usui (1966) using thin layer chromatographic techniques to separate the lipid fractions of the muscle into cholesterol esters, phospholipids, and triglycerides found an increase in the triglycerides and a decrease in the phospholipids of the muscle from Duchenne muscular dystrophy. Analysis of the fatty acid composition of total lipid indicated that there was a slight decrease in stearic acid and a slight increase in linoleic acid. However, the fatty acid composition of cholesterol esters and of phospholipids showed the general pattern of a decrease in stearic acid and an increase in oleic and linoleic acids in the dystrophic muscle. The fatty acid composition of triglyceride of dystrophic muscle was not different from that of the control muscles.

Takagi, Muto, Takahashi and Nakao (1968) carried out fatty acid analysis of lecithin from the muscle of a PMD patient and found that a marked increase of oleic acid and a decrease in linoleic acid had occurred.
(3) **Creatine and Phosphorus Compounds**

Levene and Kristeller (1909) observed that patients suffering from muscular dystrophy excrete large amounts of creatine in the urine after the ingestion of protein. This finding has been confirmed (Gibson and Martin, 1931) and Beard (1936) has suggested that in the dystrophic patient creatine is not retained in the muscle. Milhorat, Tschner and Thomas (1932) have reported that glycine administration has some beneficial effects on the symptoms of the disease but these only last for a few weeks. Collazo et al. (1936) reported that the creatine phosphate (phosphagen) content in the muscle of dystrophic patients was lowered. This observation was confirmed by Reinhold and Kingsley (1938) who also showed that the creatine concentration of the dystrophic muscle was decreased compared with that of the normal muscle. The latter finding has been confirmed by many workers (Ronzoni et al., 1960; Ronzoni et al., 1958; Vignos and Warner, 1963). In contrast to creatine, creatininine concentration in the urine was correspondingly lowered in dystrophic patients. From the above findings it may be concluded that three abnormalities develop. These are, hypocreatininuria, creatininuria, and a diminished tolerance to creatine. Unfortunately, these features are not specific to PMD since they also occur in other muscle diseases.

In man, most or all of creatine synthesis occurs in the liver (Sandberg, Hecht and Tyler, 1953). It has been shown that glycine
accepts an amidine group from arginine to form guanidoacetic acid
(Bloch and Schoenheimer, 1941), which is then irreversibly methylated
by methionine to give creatine (Brosook and Dubnoff, 1941; Du Vigneaud,
Cohn, Chandler, Schenck and Simmonds, 1941).

Benedict, Kalinsky, Scarrone, Wertheim and Stetten (1955)
have sought to answer the question of whether urinary creatine derives
from muscle creatine or elsewhere. These authors fed $^{15}\text{N}$-glycine to
a patient with PMD. Urinary creatine-$^{15}\text{N}$ and creatinine-$^{15}\text{N}$ were
isolated and analyzed. It was observed that in a few days following
glycine-$^{15}\text{N}$ injection, the atom per cent excess $^{15}\text{N}$ in urinary creatine
was always higher than that of creatinine. On the assumption that
if creatine and creatinine were of the same origin, i.e. from muscle,
then the same specific activity should have been reached ultimately
for both urinary creatine and creatinine. This pattern was not observed.
Since the $^{15}\text{N}$ content of urinary creatine always remained higher than
that of creatinine it was concluded that the creatine of the urine
in muscular dystrophy is freshly synthesized creatine which has
only to a very limited extent mixed with the creatine stored in the
muscle. Therefore, creatininuria in PMD is considered to be due to an
imbalance in creatine metabolism; the liver synthesizes the normal
amount of creatine daily but the uptake by muscle is diminished due
to loss of muscle mass or some other unknown factors.

Reinhold and Kingsley (1938) first reported that the
total acid-soluble phosphorus was lower in the human dystrophic muscle and that the level of ATP was only slightly decreased compared with a much larger decrease in creatine phosphate. These findings led them to the conclusion that the failure of the dystrophic muscle to maintain the normal level of creatine phosphate was not due to the lack of ATP. However, Vignos and Warner (1963) have shown that the level of ATP is definitely decreased in Duchenne muscular dystrophy. The ratio of ATP to ADP is also decreased, being 1.7 in the dystrophic compared to 2.5 in normal individuals.

Creatine phosphokinase activity in human muscular dystrophy was found to be decreased (Ronzoni et al., 1960; Vignos and Lefkowitz, 1959). Jacob, Okabe, Keutel, Ziter, Palmieri and Tyler (1969) isolated and purified this enzyme from the muscle of PMD patients. They found that the physicochemical properties of this enzyme were not different from those observed for the enzyme isolated from normal controls.

In summary, it may be stated that the concentrations of creatine, creatine phosphate, and ATP are all decreased in the dystrophic muscle. The muscle from dystrophic patients is defective in retaining creatine. The creatine excreted in the urine of patients with PMD is that recently synthesized by the liver. The properties of creatine phosphokinase isolated from dystrophic muscle are not different from those of the normal muscle.
(4) **Protein and Amino Acid Metabolism**

Muscle wasting is one of the prominent characteristics of PMD. This may be caused by an impaired protein synthesis or a higher rate of protein catabolism.

(a) **Blood and Urine**

Ames and Risly (1948) were the first to describe an hyperaminoaciduria in juvenile muscular dystrophy (Duchenne type). This was confirmed by Hurly and William (1955), who found greater amounts of taurine and leucine and possibly also of threonine, valine, and arginine. Bladd, Bloom and Drell (1955) reported that not only the PMD patients but also their mothers excreted larger quantities of amino acids. It is probable that the excretion of amino acids in the urine in PMD results from the excess breakdown of protein that is associated with muscle wasting.

The total plasma proteins and the albumin to globulin ratio are reported by most authors to be normal. Löwenthal and van Sande (1956) have shown an increase in the \( \alpha_2 \)-globulin fraction of serum protein by paper electrophoresis. This has since been confirmed (Ionasescu and Luca, 1960; Oppenheimer and Milhorat, 1961).

(b) **Muscle**

In dystrophic muscle, many intracellular components,
including many glycolytic enzymes and creatine phosphokinase, are lost from the muscle tissue into the serum. It is not surprising that the water extractable muscle protein is found to be decreased in PMD (Ronzoni et al., 1960). However, collagenous nitrogen in the dystrophic muscle is increased (Dreyfus et al., 1954).

The electrophoretic separation of muscle proteins by starch gel electrophoresis was carried out by Laurent, Dreyfus and Schapira (1961). Their preliminary results do not show any significant differences between the normal and dystrophic muscle extracts.

Study on myosin was first initiated by Hoagland (1946). He suggested but was unable to demonstrate the existence of a modification in the myosin molecule. Schapira, Joly and Dreyfus (1955) prepared and examined myosin from human muscle using flow birefringence. Two differences were observed in the myosin solution of PMD; the solutions from PMD were more polydisperse, and the length of the most frequent particle was shorter. The polydispersity of the myosin solution from PMD patients was confirmed by Horvath (1958).

Using the fluorescent antibody technique Klatzo, Horvath and King (1958) studied the antigenicity of myosin obtained from PMD. They were able to show the persistence of antigenic myosin even in the advanced stage of PMD.
Myoglobin (or muscle hemoglobin) which functions mainly in the storage of oxygen for utilization in the production of energy by aerobic metabolism has received some study. Perkoff, Brown and Tyler (1957) have found a decrease in myoglobin content of the muscle in progressive dystrophy. This observation is supported by the finding of Perkoff (1964) that the myoglobins isolated from two patients with childhood muscular dystrophy were characterized by a marked decrease in myoglobin A. However, this decrease is not specific since it can also be observed in patients with dermatomyositis and idiopathic myoglobinuria.
III. METHODS

A. Animals

Dystrophic mice (dydy) of strain 129 and heterozygous (Dydy) or homozygous (DyDy) littermate controls were purchased from the Jackson Memorial Laboratory, Bar Harbour, Maine. The animals were pair-fed ad libitum with standard Purina laboratory chow and allowed free access to tap water.

B. Chemical Preparation of Substrates

(1) Albumin-bound Palmitate-1\textsuperscript{14}C

Albumin-bound palmitate-1\textsuperscript{14}C was prepared according to the method of Milstein and Driscoll (1959) with a molar ratio of albumin to palmitate at 0.14 (Fritz and McEwen, 1959).

(2) L-acetyl-1\textsuperscript{14}C Carnitine

L-acetyl-1\textsuperscript{14}C carnitine was synthesized from acetyl-1\textsuperscript{14}C chloride and L-carnitine chloride according to the method of Fraenkel and Friedman (1957) with slight modification. Acetyl-1\textsuperscript{14}C chloride in a sealed ampoule was chilled in an acetone-dry ice mixture before opening. 1 ml freshly distilled acetyl chloride was added. The
diluted acetyl-\textsuperscript{14}C chloride was then transferred with a Pasteur pipette to a test tube containing 100 mg L-carnitine chloride and 0.3 ml glacial acetic acid. The tube was then sealed and heated for 6 hours at 50\textdegree C. The mixture was transferred to a stoppered tube and evaporated under reduced pressure to remove the residual acetyl chloride. 7 ml acetone were added and crystallization started after a small amount of L-acetylcarnitine was added for seeding. After standing overnight at 4\textdegree C, the crystals were collected and washed with 2 ml cold acetone (yield 97 mg). The acetyl-\textsuperscript{14}C carnitine was recrystallized from \textbar-butanol (yield 71 mg). The product gave a positive reaction to the Hestrin reaction (1949), indicating the appearance of an ester bond. On thin layer chromatography with the solvent system chloroform: methanol: conc. NH\textsubscript{3} (50:30:8), only one spot appeared on exposure to iodine vapor which was separated from carnitine and contained radioactivity. The specific activity of the product was 0.108 \mu c per \mu mole. The product was dried overnight under vacuum and over KOH pellets.

(3) \textbf{L-acetylcarnitine}

L-acetylcarnitine was prepared by the method of Fraenkel and Friedman with the modification described above. The product was crystallized and characterized as already described. The purity of the product was 99\% when analyzed according to Hestrin (1949).
(4) **L-palmitylcarnitine-\text{Me-}^{14}\text{C}**

L-palmitylcarnitine-\text{Me-}^{14}\text{C} was prepared enzymatically according to the method of Bremer and Norum (1967). Carnitine palmityl-transferase (CPT) free of palmityl-CoA hydrolase was prepared according to Farstad, Bremer, and Norum (1967). The radioactive purity of palmitylcarnitine-\text{Me-}^{14}\text{C} was checked on silica gel G thin layer chromatography using the solvent system n-butanol:acetic acid:H_2O(4:1:5). The specific activity of the product was 0.18 μc per μmole.

(5) **L-palmitylcarnitine**

L-palmitylcarnitine was synthesized from palmityl chloride and \text{L-carnitine chloride} according to the method of Bremer as modified by Fritz (1968). The purity of the product measured by the method of Hestrin (1949) in the presence of 50% ethanol (final concentration) was 81%.

**C. Reagents**

All buffers and incubation solutions were prepared with glass distilled water. The chemical reagents used were all of the highest purity available commercially.

(1) **Radioactive Compounds**

Sodium acetate-2-\text{\textsuperscript{14}C} (1 mc/41 mg), sodium pyruvate-
3-14C, sodium pyruvate-1-14C (2.89 mc/millimole), and acetyl-1-14C chloride (0.25 mc/6.2 mg) were purchased from New England Nuclear Corporation, Boston, Mass. DL-carnitine-Me-14C hydrochloride (11.65 mc/mM, purity 99%) was obtained from Tracerlab, Waltham, Mass.

(2) Other Reagents and Materials

The following reagents were purchased from the Sigma Chemical Company, St. Louis, Missouri: palmitic acid, DL-carnitine hydrochloride, cytochrome c, sodium succinate, α-ketoglutarate, L-malate, bovine serum albumin, trizma-HCl, trizma-base, standard cholesterol, dinitrophenol, and malonate.

P-L Biochemicals, Inc., Milwaukee, Wisconsin was the source of the following reagents: ADP, ATP (disodium salt), CoA (lithium salt), NADP+ and NADPH.

Citric acid (monohydrate), EDTA, Florisil (50-100 mesh), digitonin, anhydrous ethyl ether and dioxane were obtained from the Fisher Scientific Company, Fairlawn, New Jersey. Palmityl chloride, aniline hydrochloride, 2,4-dinitrophenylhydrazine and cysteine hydrochloride were purchased from Eastman Organic Chemicals, Rochester, N. Y.

Acetyl chloride, KHCO3, and petroleum ether (30°-60°) came from British Drug Houses Ltd., Poole, England.
L-carnitine chloride and oxaloacetate were purchased from the Nutritional Corporation, Cleveland, Ohio. Sucrose, methanol and phosphorus pentoxide were obtained from the McArthur Chemical Co. Ltd., Montreal, while chloroform and n-butanol were from the Mallinckrodt Chemical Works, Ltd., Toronto, and sodium acetate (anhydrous), \( \text{KH}_2\text{PO}_4 \) and \( \text{NaH}_2\text{PO}_4 \) were from Merck and Co. Ltd., Montreal.

Potassium dihydrogen isocitrate was kindly provided by Dr. H. B. Stewart.

D. Analytical Methods

(1) Protein Determination

Protein was determined according to the method of Lowry, Rosebrough, Farr and Randal (1951) using bovine serum albumin as standard. The latter was standardized against ammonium sulfate.

(2) Nitrogen Determination

Nitrogen was determined by the method of Koch and McMeekin (1924). 0.1 ml 10% tissue homogenate was digested with 1 ml 50% \( \text{H}_2\text{SO}_4 \) and 2 to 3 drops of 30% \( \text{H}_2\text{O}_2 \). When the digestion was complete (usually after 40 to 60 minutes), the clear digest was transferred with several washings of distilled water to a 10 ml volumetric flask. A sample of 1 ml was taken for nitrogen determination using Nessler's reagent.
(3) Determination of Radioactivity

A Model 6725 Nuclear Chicago Liquid Scintillation System was used for the determination of radioactivity. Duplicate samples and a $^{14}C$ standard prepared in glass vials were counted for three 10 minute intervals. The average of the three counts was obtained and corrected for quenching by the channels ratio method. The counting efficiency was about 70%. All counts were corrected to 100 per cent efficiency and recorded as disintegrations per minute.

Three different scintillator solutions were used.

The composition of each solution is as follows:

1. Toluene scintillator solution is composed of 1 g PPO and 0.1 g POPOP per liter of scintillation grade toluene. This solution was used for the counting of all the lipid samples.

2. Dioxane scintillator solution is composed of 100 g naphthalene, 10 g PPO and 0.25 g POPOP per liter dioxane. This solution was used for the counting of labelled water soluble materials.

3. Dioxane-methanol scintillator solution is composed of 60 g naphthalene, 4 g PPO and 200 mg POPOP, 100 ml methanol, 20 ml ethylene glycol, and dioxane up to 1 liter. This solution was used for the counting of $^{14}C$-labelled carnitine and palmitoylcarnitine.
(4) Calculation and Expression of Radioactivity Data

The experimental results using radioactive precur-
sors have been expressed in terms of mumoles of radioactive precursor
incorporated or converted in most cases. For these calculations, the
following relationship was used:

\[
X = \frac{S - S_0}{\text{Sp. Act.} \times \text{mg Protein}}
\]

Where
- \(X\) = mumoles precursor incorporated or converted/mg protein
- \(S\) = net disintegrations per minute of incubated sample (dpm)
- \(S_0\) = net disintegrations per minute of the control sample
- \(\text{Sp. Act.}\) = specific activity of the precursor (i.e. dpm/mumole)

In some cases the data have been expressed in dpm/per mg protein,
dpm/per mg nitrogen or dpm/per flask.

E. Incubations

(1) Palmitic Acid-\(1^{14}\)C Oxidation by Tissues of Mice

(a) Muscle Homogenate and Mitochondria

Dystrophic mice and their littermate controls
(ages 60 to 98 days) of both sexes were decapitated. The hind limbs
were cut, skinned and placed in ice-cold isolation medium (0.25 M
sucrose containing 1 mM EDTA). The muscles were carefully dissected
from the hind limbs and visible fat and extraneous tissues were removed
as completely as possible. The muscle was then blotted, weighed and cut
into fine pieces. The resulting muscle was suspended in nine volumes of
isolation medium and homogenized gently in a loose-fitting all glass
Potter-Elvehjem homogenizer for 60 seconds. Homogenization was carried out another two minutes in a tightly fitting homogenizer. The homogenate was centrifuged at 600 x g for 10 minutes at 4°C. The supernatant fraction was carefully pipetted and used for incubation.

For isolation of mitochondria the medium (0.25M sucrose, 10 mM Tris buffer, pH 7.2 and 0.5 mM EDTA) of Bode and Klingenberg (1965) was used. The muscle was suspended in nine volumes of the above medium containing 1.0 mg of Nagarse proteinase (Enzyme Development Corp., New York, N. Y.) per 30 ml. Homogenization was carried out as described above. After centrifugation at 350 x g the supernatant was carefully removed and centrifuged at 9000 x g for 10 minutes. The mitochondrial pellet was washed once (recentrifugation at 11,000 x g for 8 minutes), resuspended in isolation medium and used directly for incubation.

With muscle supernatant the incubation mixture contained 20 mM sodium phosphate buffer (pH 7.4); 1.6 mM ATP; 1.5 mM DL-carnitine HCl; 6.8 μM cytochrome c; 7.5 mM MgCl₂; 50 mM KCl; 50 μM albumin-bound palmitate-1-¹⁴C (8.9 x 10⁴ disintegrations per minute) and 0.7 ml of muscle supernatant in a final volume of 2 ml. The mitochondrial fraction was incubated under similar conditions except that a Krebs cycle intermediate (C₄ dicarboxylic acids, 0.123 mM) and 200-280 μg mitochondrial protein were added in a final volume of 1.0 ml.
With each system the reaction mixture was placed in the main chamber of a Warburg flask. $^{14}\text{CO}_2$ released was trapped by 0.2 ml 10% KOH on folded filter paper in the center well. After preincubation at 37°C for 5 minutes the system was closed and shaking was continued 15 minutes for mitochondria and 30 minutes for muscle supernatant. The reaction was terminated by tipping 0.2 ml 62.5% citric acid from the side arm followed by shaking for 10 minutes. The filter paper together with the washings from the center well were transferred to Conway vessels. $^{14}\text{CO}_2$, released from the KOH by acidification with 6N $\text{H}_2\text{SO}_4$, was trapped in hydroxide of Hyamine (Packard Instrument Company) in the center compartment. The Hyamine solution was diluted to 5.0 ml with methanol and 1.0 ml was diluted with 10 ml toluene scintillator solution in 20 ml scintillation vials for radioactivity measurements.

The respiratory control ratio was measured using the Clark oxygen electrode. The incubation mixture which contained 20 mM sodium phosphate buffer (pH 7.4); 6.8 µM cytochrome c; 7.5 mM MgCl$_2$ and 50 mM KCl was gassed with air for 5 minutes at 30°C. Bovine serum albumin was added to give a concentration of 1 mg/ml. 2 ml of incubation mixture were added to the incubation chamber. After equilibration, 0.05 ml mitochondria (360 µg protein) was injected into the reaction chamber with a Hamilton microsyringe, followed by pyruvate and malate mixture (2 mM pyruvate and 0.5 mM malate) and ADP (200 µM).
The rates of respiration at states 3 and 4 were measured as described by Estabrook (1968). The respiratory control ratio (RCR) was calculated by dividing the rate of respiration at state 3 by that of state 4.

(b) Homogenates of Liver, Kidney and Brain

Homogenates (10%) (w/v) of each tissue were prepared in ice-cold 0.25 M sucrose from mice starved for 18 hours. The incubation mixture contained in a final volume of 2.4 ml: sodium phosphate buffer (pH 7.4), 40 μmoles; ATP, 3.2 μmoles; albumin-bound palmitate-1\(^{14}\)C, 0.3 μmoles containing 5.8 \times 10^5 disintegrations per minute and 1.0 ml of each tissue. Water was added to make 2.4 ml.

The reaction mixture was placed in the main chamber of a Warburg flask. The \(^{14}\)CO\(_2\) released was trapped by 0.2 ml 10% KOH added to the center well fitted with a folded filter paper. After pre-incubation for 5 minutes with shaking at 38°C, the vessels were closed and incubation was continued for 30 minutes. The reaction was terminated by tipping 0.2 ml 62.5% citric acid from the side arm. Shaking was continued for another 10 minutes. The filter paper together with the washings from the center well were transferred to a counting vial. An additional 0.2 ml 10% KOH and a folded filter paper were placed in the center well, and 0.4 ml aniline citrate (prepared by mixing 3.75 g aniline HCl with 6 ml 62.5% citric acid) was added to the side arm. After 4 minutes of equilibration the aniline citrate was tipped into the main chamber and the mixture was incubated for another 60 minutes to decarboxylate
the acetoacetate. The contents of the center well were transferred as above to new counting vials. The vials were dried in a vacuum dessicator over P_2O_5 overnight and the radioactivity was counted in a Nuclear Chicago Scintillation counting System (Model 6725).

The method for determining the distribution of radioactivity in the carbons of acetoacetate derived from palmitate-1-^{14}C was as follows: after the decarboxylation of acetoacetate by aniline citrate, the reaction mixture was deproteinized by 2.5% ZnSO_4 and 0.15 N Ba(OH)_2. Then 0.3 ml of the supernatant was used for acetone determination according to the method of Barkulis and Lehninger (1951) using carbon tetrachloride to extract the 2,4-dinitrophenylhydrazone formed. 1 ml of this extract was used for radioactivity measurements.

(2) Palmityl CoA Synthetase Activity in Skeletal Muscle Mitochondria and in 9000 x g Supernatant Fraction

Palmityl-CoA synthetase activity was measured according to the method of Farstad et al. (1967). The method depends on the coupling of the following reactions under the conditions indicated:

\[ \text{Mg}^{++}, \text{ synthetase} \]

\[ \begin{align*}
\text{Palmitic acid} + \text{CoA} + \text{ATP} & \quad \rightarrow \quad \text{Palmityl CoA} + \text{AMP} + \text{PPi} \\
\text{Palmityl CoA} + \text{Carnitine}^{14}\text{C (in excess)} & \quad \rightarrow \quad \text{Palmitoylcarnitine}^{14}\text{C (quantitatively)} + \text{CoA}
\end{align*} \]
As soon as palmityl CoA is formed, it is converted to palmitylcarnitine-$^{14}$C in the presence of an excess amount of carnitine-$^{14}$C and carnitine palmityltransferase (CPT). Palmitylcarnitine-$^{14}$C formed is then quantitatively extracted by n-butanol and the amount extracted is determined through estimation of the radioactive carnitine incorporated. The amount of palmitylcarnitine determined is a direct measure of the amount of palmityl-CoA formed during the incubation.

(a) **Mitochondria**

The incubation mixture contained in a final volume of 1.1 ml: Tris buffer (pH 7.5), 100 μmoles; ATP, 4.5 μmoles; CoA, 46 μmoles; MgCl$_2$, 8 μmoles; palmitic acid, 1 μmole; mitochondria, 0.4 mg protein; partially purified carnitine palmityltransferase (Farstad et al., 1967), 0.5 mg protein and DL-carnitine-Me-$^{14}$C, 10 μmoles containing 340,000 disintegrations per minute. Preincubation was carried out for 3 minutes at 37°C without ATP and palmitic acid present. Palmitic acid and ATP were then added and the incubation was continued for intervals of 5 and 10 minutes. The reaction was stopped with 0.1 ml concentrated HCl and 0.9 ml H$_2$O was added. The extraction was carried out twice with 1.0 ml n-butanol saturated with water. The pooled extracts containing the palmitylcarnitine-Me-$^{14}$C formed were washed twice with 2.0 ml distilled water. The washed extract was evaporated to dryness and the residue was taken up in 10 ml dioxane-methanol scintillator solution for counting. The radioactivity present was
determined using a Nuclear Chicago Scintillation Counting System (Model 6725). The μmoles of carnitine-\(^{14}\)C present as palmitoylcarnitine were calculated. The activity of the synthetase enzyme is finally expressed as μmoles L-palmitoylcarnitine formed per mg mitochondrial protein.

(b) 9000 x g Supernatant

The 9000 x g supernatant fraction was obtained by first centrifuging 10% homogenate at 350 xg and 4°C for 10 minutes and subsequent centrifuging of the supernatant at 9000 x g for 20 minutes. The supernatant obtained was used directly as the enzyme source. The incubation mixture contained in a final volume of 1.1 ml: Tris buffer (pH 7.5), 100 μmoles; ATP, 19.2 μmoles; CoA, 1.73 μmoles; MgCl₂, 12 μmoles; palmitic acid, 0.5 μmole; supernatant, 0.3 ml (1.6 mg protein); partially purified carnitine palmitoyltransferase, 0.5 mg and DL-carnitine-Me-\(^{14}\)C, 10 μmoles containing 400,000 disintegrations per minute. The procedures for incubation and extraction were exactly the same as those described for the assay of mitochondrial palmityl-CoA synthetase activity.

(3) Carnitine Palmitoyltransferase Activity in Skeletal Muscle Mitochondria

The activity of carnitine palmitoyltransferase was measured in terms of the μmoles carnitine-Me-\(^{14}\)C release per mg
mitochondrial protein using enzymatically synthesized L-palmityl-
carnitine-Me-\textsuperscript{14}C. The reaction mixture contained in a final volume
of 0.6 ml: Tris HCl buffer (pH 7.5), 20 \( \mu \)moles; CoA, 82 \( \mu \)moles;
malonate, 0.24 \( \mu \)mole; dinitrophenol, 0.12 \( \mu \)mole; mitochondria, 0.2 mg
protein and L-palmitylcarnitine-Me-\textsuperscript{14}C, 50 \( \mu \)moles containing 7900
disintegrations per minute. After preincubation for 5 minutes at
37\(^\circ\)C, mitochondria were added to initiate the reaction. At the end
of the incubation 0.5 ml \( n \)-butanol (saturated with \( H_2O \)) was added and
each tube was shaken vigorously for about 15 seconds and centrifuged.
The upper layer (butanol) was taken off by suction and the aqueous
layer was extracted again with 0.5 ml \( n \)-butanol. The butanol layer
was discarded. 0.3 ml of the aqueous solution (containing radioactive
carnitine released during the incubation from palmitylcarnitine-Me-\textsuperscript{14}C)
was used for radioactivity measurements using 10 ml scintillation
solution (dioxane-methanol). From the specific activity of palmityl-
carnitine-\textsuperscript{14}C added and the radioactivity appearing in the aqueous
phase, the amount of carnitine formed was calculated.

(4) \textbf{L-Acetyl-\textsuperscript{14}C Carnitine Oxidation by Skeletal
Muscle Mitochondria}

The reaction mixture contained in a final volume
of 2.0 ml: 20 mM sodium phosphate buffer (pH 7.4); 1.6 mM ADP;
6.8 \( \mu \)M cytochrome c; 7.5 mM MgCl\textsubscript{2}; 50 mM KCl; 1.0 mM acetyl-\textsuperscript{14}C
carnitine containing 4850 disintegrations per minute and L-malate,
61.5 μM. Incubation and liberation of CO₂ were carried out in Warburg vessels as described in Section 1 (palmitic acid-1- C oxidation by tissues of mice). The CO₂ released was trapped by 0.2 ml hydroxide of Hyamine (Packard Instrument Company) placed in the center well fitted with a folded paper. Following incubation, the hydroxide of Hyamine in the center well was transferred to a counting vial with 0.5 ml methanol. 10 ml toluence scintillation solution were added and the radioactivity present was determined.

(5) **Pyruvate-3- C Oxidation by Skeletal Muscle Mitochondria**

For pyruvate-3- C oxidation the incubation mixture and the method of incubation were the same as in Section 1 for palmitic acid-1- C oxidation by skeletal muscle mitochondria with the exception that DL-carnitine was omitted and ADP (1.6 mM) replaced ATP. The pyruvate-3- C concentration was 1.0 mM (radioactivity approx. 60,000 disintegrations per minute).

(6) **Pyruvate-1- C Decarboxylation by Skeletal Muscle Mitochondria**

The reaction mixture contained in a final volume of 2.0 ml: sodium phosphate buffer (pH 7.4), 40 μmoles; KCl, 100 μmoles; MgCl₂, 15 μmoles; malonate, 2 μmoles; DL-carnitine, 4 μmoles; pyruvate-1- C, 2 μmoles, and radioactivity corresponding to 37,600 disintegrations per minute and 0.4 mg mitochondrial protein. The method of incubation
and the trapping of $^{14}$CO$_2$ were the same as in Section 4 (acetyl-1-$^{14}$C carnitine oxidation). In some experiments, pyruvate disappearance during incubation was determined by the method of Davis (1965) using lactic acid dehydrogenase.

(7) Fatty Acid Synthesis from Acetate-2-$^{14}$C in Tissues of Mice

Homogenates of each tissue (liver, brain, kidney and hind leg muscle) were prepared in 4 volumes of ice-cold 0.1 M KH$_2$PO$_4$-KOH buffer (pH 7.5) and centrifuged in the Spinco Model L ultracentrifuge (No. 40 head) at 71,000 x g for 100 minutes. The clear supernatant was carefully pipetted and used as the enzyme source. This method is essentially that of Allmann and Gibson (1965). The incubation system for liver preparation contained in a final volume of 1.1 ml: KH$_2$PO$_4$-KOH buffer (pH 6.5), 60 μmoles; isocitrate (potassium dihydrogen Ls(+)-isocitrate), 11 μmoles; MnCl$_2$, 0.8 μmole; cysteine, 8 μmoles; NADP$^+$, 0.2 μmole; CoA, 4.3 μmole; KHCO$_3$, 20 μmoles; ATP, 3.64 μmoles; enzyme preparation, 10 mg protein and acetate-2-$^{14}$C, 2.2 μmoles containing 1.33 x 10$^6$ disintegrations per minute. The incubation mixture for skeletal muscle, brain and kidney was the same with the exception that citrate, 5.5 μmoles replaced isocitrate and the amount of CoA added was 21 μmole and that of the enzyme preparation 6 mg for muscle and kidney, and 3 mg for brain. The incubation was carried out in Warburg flasks. After flushing with 100% CO$_2$ for 5 minutes, the system was closed and the incubation was continued for
60 minutes. Following incubation, the reaction mixture was transferred with the aid of 95% ethanol (twice with 0.5 ml) and H₂O (0.5 ml) to a centrifuge tube containing 0.5 ml 10 N KOH. The contents of the tube were heated in a boiling water bath for 1 hour. The resultant boiled mixture was extracted twice with 10 ml petroleum ether. The petroleum ether extracts (upper layer) were discarded. 1.0 ml 6 N HCl and 1.0 ml petroleum ether containing 1 mg palmitic acid were added to the lower phase. The latter was extracted twice with 10 ml petroleum ether and once with 5.0 ml petroleum ether. The pooled extracts were washed twice with 15 ml distilled water and the washed extract evaporated to dryness. 2.0 ml heptane were added to dissolve the residue and 1.0 ml was used for radioactivity measurement. The procedure when checked using radioactive palmitic acid results in 98% recovery.

(8) In vivo Metabolism of Palmitate-1-¹⁴C by Dystrophic Mice

The animals were starved for 18 hours prior to the experiment and albumin-bound palmitate-1-¹⁴C was injected intraperitoneally, at a dose of 0.6 μc per gram body weight. One hour after injection, the mice were sacrificed by decapitation and the tissues (brain, liver, kidney and muscle) were removed, dipped into ice-cold normal saline, blotted and weighed. A 10 per cent homogenate was made of each tissue with water using a conical glass homogenizer of the Potter-Elvejehm type. Extraction at room temperature was
carried out by addition of 20 volumes of chloroform-methanol and the samples were centrifuged. The precipitate was extracted twice with 2.0 ml of chloroform-methanol (2:1). The combined extracts were washed once with 7.0 ml of 1.5% NaCl and twice with 6 ml of washing solution which was the upper phase of the mixture of chloroform-methanol and 0.5 M sodium acetate (8:4:3, v/v/v). The washed extracts were evaporated to dryness under low pressure, dissolved in 3 ml of an acetone-ethanol-ether mixture (4:4:1, v/v/v) and saponified at 50°C for 1 hour with 0.3 ml of 50% KOH solution in methanol. After addition of 5.0 ml of water to the saponified extracts, cholesterol was extracted by two successive extractions with 10 ml petroleum ether. The cholesterol extract was evaporated to dryness and was redissolved in 3.0 ml of the mixture of acetone and absolute ethanol (1:1, v/v). To this solution, 5.0 ml of 0.5% digitonin solution in 50% ethanol were added. The resultant mixture was heated at 70°C for 5 minutes and then was left at room temperature overnight to precipitate cholesterol as digitonide. After centrifugation, the digitonide was washed twice with 10 ml of acetone-ether (1:2, v/v) mixture, once with 10 ml of ether and then dried by a stream of air. This dried cholesterol digitonide was used for determination of the total amount present and for radioactivity measurements. Fatty acid was obtained from the saponified extract left after removal of cholesterol by acidification followed by extraction with two portions (5.0 ml) of petroleum ether. The fatty acid extract was evaporated to dryness and used as described below.
(a) **Determination of Cholesterol**

The dried cholesterol digitonide was dissolved in 2.0 ml of glacial acetic acid. A 1.0 ml portion of the solution was mixed with 2.0 ml of ethyl acetate and 15.0 ml of scintillation solution for determination of radioactivity. To the remaining portion of the cholesterol solution, 4.0 ml of acetic amhydride: sulfuric acid (20:1, v/v) mixture was added and cholesterol was estimated as described by Schoenheimer and Sperry (1934).

(b) **Determination of Fatty Acids**

To the fatty acid sample, 2.0 ml of heptane were added. An aliquot of 1.0 ml of this solution was mixed with 10.0 ml scintillation solution for radioactivity measurements. After the rest of the sample was evaporated to dryness, 2.0 ml of methanol and 2.0 ml of 0.001% cresol red indicator in 90% methanol were added and the mixture was titrated with 0.05 N NaOH in 90% methanol. The latter in each set of determinations is standardized against palmitic acid.

(c) **Estimation of Radioactivity**

Samples of the above lipids were dissolved in toluene scintillation solution as described above. Radioactivity measurements were made using a Nuclear Chicago Liquid Scintillation Spectrometer (Model 6725).
IV. PALMITIC ACID-\textsuperscript{14}C OXIDATION BY THE TISSUES OF DYSTROPHIC AND NORMAL MICE

A. Palmitate-\textsuperscript{14}C Oxidation by Skeletal Muscle Homogenate and Mitochondria

Fat accumulation in mouse dystrophic muscle has been confirmed by histochemical and biochemical techniques (Bajusz and Jasmin, 1962; Young et al., 1959). Lipids are not only an important structural element of the muscle cell but fatty acids are a major source of energy and therefore the abnormal accumulation of fat in dystrophic muscle may be of fundamental importance in the pathological process. Only a few reports have appeared in which the mechanism of this accumulation has been investigated. Rabinowitz (1960) reported that several tissues of dystrophic mice, with the exception of muscle, showed significant enhancement in the in vitro incorporation of acetate-\textsuperscript{2\-14}C into cholesterol and fatty acids. A similar investigation in vitro by Strickland et al., (1965) supported this observation. However, at the time this work commenced no study on the fatty acid oxidation by dystrophic muscle preparations had been reported in the literature. The present chapter describes experiments on the oxidation of palmitic acid-\textsuperscript{1-14}C by skeletal muscle homogenate and mitochondrial preparations from normal and dystrophic mice. The methods used are described in the previous chapter.
Results

(1) **Muscle Homogenate**

The cofactor requirements for palmitic acid-\(^{14}\)C oxidation by mouse muscle homogenates are shown in Table II. It can be seen that carnitine is required for the oxidation of palmitate and that the oxidation is actually dependent upon the presence of both carnitine and CoA in the medium. These findings are in agreement with those of Fritz, Kaplan and Yue (1962) for rat heart homogenate preparations. The omission of cytochrome c resulted in a slight decrease in oxidation and almost no oxidation was observed when ATP was excluded from the system.

Fig. 2 shows the concentration curve for carnitine obtained for the supernatant fraction of muscle homogenate; the optimal concentration of carnitine is 1.5 mM. Fig. 3 shows that the optimal concentration of CoA is reached at 10 \(\mu\)M. Some inhibition was noted at a concentration of 20 \(\mu\)M.

Palmitate-\(^{14}\)C oxidation by the low speed (600 x g) supernatant fraction of muscle homogenates from dystrophic and normal controls was investigated using the incubation system described in Table II. The results in Table III show that the oxidation of palmitate-\(^{14}\)C by dystrophic muscle was significantly reduced (80%) compared
TABLE II

Cofactor Requirements for Palmitate-1-\(^{14}\)C Oxidation by 600 x g Supernatant From Muscle Homogenate of Mice (Strain 129)

The complete system contained: sodium phosphate buffer (pH 7.4), 40 \(\mu\)moles; ATP, 3.2 \(\mu\)moles; DL-carnitine, 3.0 \(\mu\)moles; Coenzyme A, 25 \(\mu\)moles; cytochrome c, 13.6 \(\mu\)moles; MgCl\(_2\), 15 \(\mu\)moles; KCl, 100 \(\mu\)moles; albumin-bound palmitate-1-\(^{14}\)C (1.5 \(\times\) 10\(^5\) disintegrations per minute) and 0.7 ml muscle supernatant (6 mg protein) in a final volume of 2.0 ml. Incubation was performed for 30 minutes at 37\(^\circ\)C in a Warburg flask with shaking. Palmitate-1-\(^{14}\)C oxidation was measured as described in the text. The \(^{14}\)CO\(_2\) released is expressed as disintegrations per minute (dpm).

<table>
<thead>
<tr>
<th>Incubation Mixture</th>
<th>(^{14})CO(_2) released (dpm/flask)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete system</td>
<td>14,700</td>
</tr>
<tr>
<td>Less carnitine and CoA</td>
<td>1,095</td>
</tr>
<tr>
<td>Less CoA</td>
<td>10,000</td>
</tr>
<tr>
<td>Less carnitine</td>
<td>3,860</td>
</tr>
<tr>
<td>Less cytochrome c</td>
<td>11,200</td>
</tr>
<tr>
<td>Less ATP</td>
<td>55</td>
</tr>
</tbody>
</table>
Fig. 2  Effect of carnitine concentration of palmitate-
$\text{L-}^{14}\text{C}$ oxidation by 600 x g supernatant of mouse
skeletal muscle homogenates. Incubation conditions
as described in TABLE II.
Fig. 3  Effect of added CoA on palmitate-$^{14}$C oxidation by 600 x g supernatant of mouse skeletal muscle homogenates.
### TABLE III

**Palmitate-1-^14^C Oxidation by Low Speed Supernatant of Muscle**

**Homogenate from Dystrophic Mice (D) and Their Littermate Controls (C)**

Incubation conditions as in TABLE II

<table>
<thead>
<tr>
<th>Number of pairs</th>
<th>$^{14}$CO$_2$ released (dpm/ per mg protein)</th>
<th>Percentage of control (D/C x 100 ± SEM)</th>
<th>$P$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>D</td>
<td>C</td>
</tr>
<tr>
<td>7</td>
<td>965</td>
<td>3522</td>
<td>20.8 ± 7.5</td>
</tr>
</tbody>
</table>
with that of its littermate controls. While some variation occurs, presumably due to differences in pairs of animals, the effect is still very great.

(2) **Mitochondria**

The isolation procedure used gave a mitochondrial yield (mg protein per gram muscle wet weight) of $1.98\pm 0.28$(S.D.) for dystrophic muscle and $3.36\pm 0.35$(S.D.) for normal muscle. The difference observed is highly significant ($P<0.001$) which is in agreement with that of Srivastava (1968). The respiratory control ratio determined by an oxygen electrode is about 2.4–2.5 for the mitochondria from normal skeletal muscle of mouse.

The time course of palmitate-$l^{-14}C$ oxidation (expressed as $^{14}CO_2$ released) by muscle mitochondria is shown in Fig. 4. In most cases a slightly sigmoidal curve was obtained. In all further experiments a 15 minute incubation time was used. Palmitate-$l^{-14}C$ oxidation by muscle mitochondria was shown to be dependent upon the presence of a Krebs cycle intermediate (Fig. 5). It was observed that succinate, L-malate $\alpha$-ketoglutarate and oxaloacetate were all quite effective in supporting the oxidation, but isocitrate was not as effective.

Further evidence on the importance of the Krebs cycle is provided in Table IV. It can be seen that palmitate-$l^{-14}C$ oxidation
Fig. 4 Time course of palmitate-l-\(^{14}\)C oxidation by mouse skeletal muscle mitochondria. The incubation conditions were the same as in TABLE II except that the reaction was stopped at the times indicated.
Fig. 5  Comparisons of the effect of several Krebs cycle intermediates on the oxidation of palmitate-1-\(^{14}\)C by mouse skeletal muscle mitochondria. For each Krebs cycle intermediate the optimum concentration (0.123 mM) was added.
TABLE IV

The Inhibition of Palmitic Acid Oxidation in Skeletal Muscle Mitochondria by Malonate and Fluorocitrate

In incubation mixture as described in TABLE V except that inhibitors and succinate (0.123 mM) were added and the incubation time was 30 minutes.

<table>
<thead>
<tr>
<th>Additions</th>
<th>$^{14}$CO$_2$ released (dpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>52,000</td>
</tr>
<tr>
<td>Malonate (10 mM)</td>
<td>26</td>
</tr>
<tr>
<td>Fluorocitrate (8 mM)</td>
<td>55</td>
</tr>
</tbody>
</table>
to $^{14}$CO$_2$ is almost completely inhibited by malonate or fluorocitrate, which are both inhibitors of the Krebs cycle.

Fig. 6 shows the effect of concentration of DL-carnitine, ATP, CoA, and succinate, respectively, on the oxidation of palmitate by skeletal muscle mitochondria. Carnitine and ATP have an optimal concentration of 1.5 and 1.6 mM respectively. The optimal concentration of CoA is 12.5 $\mu$M. Increase in the CoA concentration above this level caused an inhibitory response. The optimal concentration of succinate is 0.123 mM. An inhibitory effect was also observed when the concentration of succinate was increased. The same optimal concentrations were obtained for CoA and succinate with mitochondria isolated from dystrophic muscle.

Using the established optimal system, a comparison of palmitate-$^{14}$C oxidation by skeletal muscle mitochondria from dystrophic and normal mice was made. Table V shows the results obtained on a pair comparison basis. The oxidation of palmitate by mitochondria of dystrophic muscle is significantly decreased compared with their littermate controls ($P$ values ranged from 0.02 to 0.001).

Discussion

The role of carnitine in fatty acid oxidation was first demonstrated by Fritz (1955) who showed that the rate of palmitic acid oxidation in rat liver slices and homogenates was greatly increased by the
Fig. 6  Effect of concentration of carnitine, ATP, CoA and succinate, respectively on palmitate-\(1^{14}\)C oxidation by mouse muscle mitochondria.


TABLE V

The Oxidation of Palmitate-1-\(^{14}\)C by Mitochondria from
Muscle of Dystrophic Mice (D) and Their Littermate Controls (C)

The incubation conditions were as in TABLE II except that
0.123 mM malate (or succinate) and 200-280 µg mitochondrial
protein were added in a final volume of 1.0 ml. Incubation
time was 15 minutes.

<table>
<thead>
<tr>
<th>C(^4) Dicarboxylic Acid added</th>
<th>Number of Pairs</th>
<th>Oxidation (µ moles (^{14})CO(_2)/mg protein ± SEM)</th>
<th>Pair Comparison D/C x 100</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malate</td>
<td>8</td>
<td>65.11 ± 9.48</td>
<td>94.18 ± 2.47</td>
<td>69.0 ± 10.54</td>
</tr>
<tr>
<td>Succinate</td>
<td>12</td>
<td>50.81 ± 4.21</td>
<td>86.42 ± 5.19</td>
<td>58.7 ± 3.81</td>
</tr>
</tbody>
</table>
addition of a beef heart extract. It is now generally agreed that
carnitine acts as a carrier to transport the palmityl group through
the mitochondrial membrane barrier. An hypothesis that explains the
stimulating effect of carnitine on fatty acid oxidation has been
proposed as follows (Bremer, 1962; Bremer, 1963; Fritz and Yue, 1963).

\[
\begin{align*}
\text{Palmitic acid} + \text{CoA} + \text{ATP} + \text{Mg} & \rightarrow \text{Palmityl CoA} + \text{AMP} + \text{PPI} \quad (1) \\
\text{Palmityl CoA} + \text{Carnitine} & \rightarrow \text{Palmityl carnitine} + \text{CoA} \quad (2)
\end{align*}
\]

Palmitylcarnitine formed through reactions (1) and (2) is then
considered to pass through the mitochondrial barrier. Inside the
barrier, palmityl CoA is reformed before it is oxidized via \( \beta \)
oxidation and the Krebs cycle.

The complete dependence of palmitic acid oxidation by skeletal
muscle preparations of mice on carnitine may be physiologically important
since it has been observed that carnitine concentrations are highest
in muscles which derive a significant portion of their energy supply
from fatty acid oxidation (Fritz et al., 1958; Volk, et al., 1952;
Frederickson and Gordon, 1958). This situation is different from
that of liver mitochondria for which two pathways of fatty acid oxida-
tion have been implicated. Van den Bergh (1965, 1966) reported
that fatty acids were rapidly oxidized by liver mitochondria in the
absence of carnitine and he suggested that the major pathway of fatty
acid oxidation was carnitine independent (1967). However, Yates,
Shepherd and Garland (1966) have shown that under certain specified conditions the oxidation of palmitic acid was dependent on the presence of carnitine.

The mitochondrial localization of enzymes concerned in the activation and ω-oxidation of fatty acids remains controversial. Bode and Klingenberg (1964, 1965) reported that rat skeletal muscle mitochondria were not able to oxidize long chain fatty acids, but were able to do so with acycarnitine as substrates. They claimed that rat skeletal muscle mitochondria lacked the enzyme required for fatty acid activation. However, Peter and Lee (1967) were able to demonstrate the oxidation of palmitic acid in the presence of carnitine and CoA with their skeletal muscle mitochondrial preparations. In this connection, it should be noted that Pande and Mead (1968) have demonstrated the existence of long chain fatty acid activating enzyme in rat skeletal muscle mitochondria and Allmann, Galzigna, McCaman and Green (1966) have shown that outer membrane fraction of beef heart mitochondria derived from phospholipase treatment contains all the enzymes, including the activating enzyme, required for the oxidation of palmitate. The latter is in disagreement with the finding of Beattie (1968) who reported that 95% of the fatty acid oxidizing system in rat liver mitochondria was localized in inner membrane-matrix fraction.

From the foregoing discussion and consistent with the experimental evidence reported here, it seems reasonable to conclude that skeletal muscle mitochondria have the activation enzyme located either just outside or loosely bound to the inner membrane and the enzymes of ω-oxidation located in the inner membrane-matrix region in such a manner as to permit release by phospholipase treatment.
The oxidation of palmitate-1-\(^{14}\)C by mouse skeletal muscle mitochondria was completely dependent on the addition of a Krebs cycle intermediate. Dependence on the addition of malate for octanoate oxidation by brain mitochondria has been shown by Beattie and Basford (1965). These findings are in contrast with the results obtained from mitochondria of rabbit heart muscle (Kako, 1969) and rat skeletal muscle (Bode and Klingenberg, 1965); in the former exogenous malate was not required for palmitylcarnitine oxidation and in the latter the oxidation of fatty acids became dependent on Krebs cycle intermediates only after aging of the mitochondrial preparations.

Succinate at concentrations higher than 0.123 mM slightly inhibited the oxidation of palmitate-1-\(^{14}\)C to \(^{14}\)CO\(_2\). The mechanism of this inhibition is not known but it is possible that higher concentrations of succinate reduces the availability of CoA in the mitochondria due to the formation of succinyl CoA (Bremer, 1968b).

Although small amounts of exogenous CoA were essential for palmitate oxidation both in muscle homogenate and mitochondrial preparations, concentrations beyond the optimum caused an inhibition of palmitate oxidation in the above preparations. This inhibition may be caused by two factors: first, because of the ready reversibility of reaction (2) excess amounts of CoA may decrease the concentration of palmitylcarnitine available at the site of entry into the mitochondria. Second, CoA may have an effect on the fatty acid synthetase
reaction since CoA decreased the disappearance of palmitate-$l^{-14}C$
from the free fatty acid fraction in the liver homogenate system
(Ontko, 1964). This explanation is supported by the experiment in
Chapter V which shows that palmityl CoA synthetase was inhibited by
high concentrations of CoA.

Palmitate-$l^{-14}C$ oxidation by 600 x g supernatant of dystrophic
muscle homogenates was greatly decreased (80%) compared with that of
the normal controls. However, no correction has been made for possible
isotope dilution of palmitate-$l^{-14}C$ by the endogenous palmitate.
This effect may be considerable since Young et al., (1959) have shown
that the free fatty acid pool in the dystrophic muscle is about twice
(187%) that of the normal muscle. If correction is made using this
value, then the average decrease in palmitate oxidation by dystrophic
muscle becomes 60%. The evidence obtained with both homogenate and
mitochondrial preparations indicates that palmitate-$l^{-14}C$ oxidation
in dystrophic muscle of mice is significantly decreased. Without
further evidence it may be suggested that this decrease is caused
by a defect(s) in either the transport of the palmityl group through
the mitochondrial barrier, the activation of palmitate (i.e. palmityl
CoA synthetase), or in the handling of acetyl CoA derived from palmitic
acid by the Krebs cycle.

The possibility that the decrease in palmitate-$l^{-14}C$ oxidation
is due to a defect in the handling of acetyl CoA is not supported
by the findings of Mayers and Epstein (1962) who measured the oxygen uptake of the muscle homogenate and found no difference between normal and dystrophic preparations and claimed that the Krebs cycle was intact. Wrogemann and Blanchaer (1967, 1968) have reported that the ADP/O ratio, the respiratory control ratio and phosphorylation rate were all normal in mitochondria from skeletal muscle of dystrophic mice 37-77 days old and from the myocardium and skeletal muscle of 97-124 days old hamsters of the Bio 14.6 strain. However, with the mitochondria from the skeletal muscle of the older hamsters (Wrogemann, Jacobson and Blanchaer, 1969) they were able to show that the oxidation of palmitate, palmitoylcarnitine, pyruvate, and acetylcarnitine were all decreased to the same extent in the dystrophic hamsters. They have ascribed the decrease in oxidation to an advanced state of the disease rather than an expression of a primary defect in the mitochondrion.
B. Palmitate-\(1^{14}\)C Oxidation by Liver, Kidney and Brain Homogenates of Dystrophic Mice and Their Littermate Controls

There is no general agreement as to whether muscular dystrophy is a disease affecting skeletal muscle only or whether other tissues might be affected. With this in mind, the oxidation of palmitate-\(1^{14}\)C was also investigated in other tissues such as liver, kidney and brain.

Results and Discussion

In the preliminary experiments, it was found that carnitine stimulated palmitate-\(1^{14}\)C oxidation in all tissues studied. It was routinely added to the incubation of each tissue system. It was also observed that among the tissues examined only liver could accumulate considerable amounts of labelled acetoacetate. Therefore, radioactivity in the carboxyl group of acetoacetate was reported only for the liver preparations. Table VI shows that there is no significant difference, based on pair comparisons, in the ability of liver, kidney and brain preparations of dystrophic and normal mice to oxidize palmitate-\(1^{14}\)C. The finding for the liver confirmed the suggestion of Gould and Coleman (1961), who measured the acetoacetate concentration after the incubation of liver homogenate with octanoate and found no difference between livers
<table>
<thead>
<tr>
<th>Tissue</th>
<th>No. of Pairs</th>
<th>$^{14}$CO$_2$ released (dpm/mg protein x 10$^{-2}$ ± S.E.M.)</th>
<th>Pair Comparison (D/N x 100 ± S.E.M.)</th>
<th>P</th>
<th>$^{14}$CO$_2$-Acetoacetate (dpm/mg protein x 10$^{-2}$ ± S.E.M.)</th>
<th>Pair Comparison (D/C x 100 ± S.E.M.)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>9</td>
<td>C  194 ± 38</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>103 ± 8.8 N.S.</td>
<td></td>
<td>130 ± 37.5</td>
<td></td>
<td>104 ± 6.5 N.S.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>D  195 ± 45</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>7</td>
<td>C  626 ± 250</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>116 ± 8.1 N.S.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>D  703 ± 322</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>9</td>
<td>C  55.2 ± 12.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>99 ± 7.3 N.S.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>D  53.7 ± 9.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

N.S. - Not significantly different
from normal and dystrophic mice. No difference was also found in $^{14}$CO$_2$ derived from acetoacetate in the liver preparations. However, a large variation was observed in all tissue preparations. The ability of the tissue homogenates to oxidize palmitate-$l^{14}$C was the highest in kidney, followed by liver and brain.

Table VII shows that following the decarboxylation to acetone of the labelled acetoacetate formed during incubation of liver homogenates with palmitate-$l^{14}$C, no radioactivity could be found in the hydrazone of the acetone moiety. This indicates that the radioactivity in acetoacetate derived from palmitate-$l^{14}$C oxidation was concentrated in the carboxyl groups. This finding supports a mechanism of non-random condensation of the C$_2$ moiety derived from the oxidation of fatty acids as suggested by Wakil and Bressler (1962). According to their suggestion, the formation of $\text{CH}_3\text{C}^{14}\text{H}_2\text{CH}_{13}^{14}\text{COOH}$ from palmitate-$l^{14}$C can be illustrated as follows:

\[
\begin{align*}
\text{CH}_3\text{C}^{14}\text{H}_2\text{CH}_{13}^{14}\text{CO} & \sim \text{S-CoA} \quad \text{3-oxidation} \quad \text{enzyme-SH(thiolase)} \quad \text{CH}_3\text{C}^{14}\text{H}_2\text{S-enzyme} \\
& + 7 \text{CH}_3^{14}\text{H}_2 \sim \text{S-CoA}
\end{align*}
\]

\[
\begin{align*}
\text{CH}_3\text{C}^{14}\text{H}_2\text{CH}_{13}^{14}\text{CO} & \sim \text{S-enzyme} + \text{CH}_3\text{C}^{14}\text{H}_2\text{S-CoA} \quad \text{CH}_3\text{C}^{14}\text{H}_2\text{CH}_{13}^{14}\text{CO} \sim \text{S-CoA}
\end{align*}
\]

However, Chaikoff, Goldman, Brown, Dauben and Gee (1952) using liver slices have reported that with palmitate-$l^{14}$C or palmitate-$5^{14}$C as
TABLE VII

Radioactivity in Acetone Moiety Derived from Acetoacetate Formed from Palmitate-1-14C Oxidation

3 ml of supernatant obtained following centrifugation of deproteinized incubation mixture of liver homogenate were added to 5 ml of 0.1% 2,4-dinitrophenylhydrazine in 2 N HCl and 10 ml CCl4. After vigorous shaking in a shaking machine 1 ml CCl4 extract was used for radioactivity measurement.

<table>
<thead>
<tr>
<th>Warburg vessel</th>
<th>dpm/ml CCl4 extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>17</td>
</tr>
<tr>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td>3</td>
<td>18</td>
</tr>
<tr>
<td>Blank</td>
<td>17</td>
</tr>
</tbody>
</table>
substrates the radioactivity in acetoacetate is evenly distributed in the carboxyl and carbonyl groups of the molecule.

It is known that liver is unique among the animal tissues in accumulating ketone bodies from fatty acids. The liver produces acetoacetate very actively from acetoacetyl-CoA but has a very low ability to activate acetoacetate to acetoacetyl-CoA due to the lack of the enzyme, acetoacetic thiophorase (McCann, 1957). In the extrahepatic tissues such as muscle and kidney, the acetoacetate can be reactivated to acetoacetyl-CoA and utilized. Therefore, in these tissues there is no significant accumulation of acetoacetate.
V. THE ACTIVITY OF PALMITYL CoA SYNTHETASE IN MITOCHONDRIA AND THE 9000 x g SUPERNATANT OF SKELETAL MUSCLE

In the previous Chapter, results were presented which showed that palmitate-1$^{14}$C oxidation by skeletal muscle of dystrophic mice is significantly lower than that of the normal littermate controls. To assess whether the decrease in oxidation observed might be related to an impairment in the activation of palmitate, the activity of palmityl-CoA synthetase in both mitochondria and 9000 x g supernatant (non-mitochondrial fraction) have been investigated. Palmityl-CoA synthetase catalyzes the formation of palmityl-CoA from palmitic acid. This activation step is essential before transfer of palmitate into the mitochondria can occur via the action of carnitine palmityltransferase. The results obtained are presented in this Chapter.

As described in the Methods section palmityl-CoA synthetase activity is measured by coupling two reactions. In the first reaction palmityl-CoA synthetase catalyzes palmityl-CoA formation. The palmityl-CoA formed is then converted quantitatively to palmitylcarnitine-Me-$^{14}$C in the presence of excess carnitine-Me-$^{14}$C and carnitine palmityltransferase. The activity of palmityl-CoA synthetase is expressed in terms of the carnitine-Me-$^{14}$C converted into palmitylcarnitine which is directly proportional to the palmityl-CoA formed.
Results

In order to test whether the added carnitine palmitoyltransferase is sufficient to convert the palmitoyl-CoA formed during the incubation into palmitoylcarnitine-Me-\(^{14}\)C, an incubation was performed at 37\(^{\circ}\)C for 10 minutes with varying amounts of palmitoyl-CoA and a fixed amount of carnitine palmitoyltransferase. As shown in Fig. 7 a linear response of palmitoylcarnitine-Me-\(^{14}\)C formation was obtained with increasing amounts of palmitoyl-CoA.

Table VIII shows that added carnitine palmitoyltransferase (450-550 \(\mu\)g) slightly stimulated (31\%) the formation of palmitoylcarnitine-Me-\(^{14}\)C in the mitochondria; the stimulation was more pronounced with the 9000 x g supernatant fraction (72\% and 86\%). A 50\% stimulation has been reported for the sonicated rat liver mitochondria (Van Tol, De Jong and Hülsmann, 1969b). In order to ensure an optimal system, carnitine palmitoyltransferase was routinely added to the reaction mixture.

The formation of palmitoylcarnitine-Me-\(^{14}\)C is dependent upon the addition of palmitic acid. The optimal concentrations of palmitic acid were 0.91 mM for mitochondria and 0.46 mM for the 9000 x g supernatant respectively (Fig. 8). The effect of increasing CoA concentrations on the formation of palmitoylcarnitine-Me-\(^{14}\)C is shown in Fig. 9. The optimal concentrations were 45 \(\mu\)M for the mitochondria.
Fig. 7 The effect of carnitine palmitoyltransferase on palmitoylcarnitine formation from palmitoyl-CoA. The incubation mixture contained in a final volume of 1.0 ml: Tris buffer (pH 7.5), 50 μmoles; DL-carnitine-Me-\(^{14}\)C, 10 μmoles (containing 196,000 dpm); palmitoylcarnitine transferase and palmitoyl-CoA. The incubation time was 10 minutes at 37°C. The palmitoylcarnitine-Me-\(^{14}\)C formed was extracted with n-butanol and its radioactivity was measured as described in the METHODS.
TABLE VIII

The Stimulation of Palmitoylcarnitine-Me-$^{14}$C Formation by Carnitine Palmitoyltransferase (CPT) in the Presence of Palmityl-CoA formed during Incubation by Preparations from Normal Mouse Muscles

The incubation mixture contained in a final volume of 1.1 ml: (a) mitochondria—Tris buffer (pH 7.5), 100 μmoles; ATP, 4.5 μmoles; CoA, 46 μmoles; MgCl₂, 8 μmoles; palmitic acid, 1 μmole; partially purified carnitine palmitoyltransferase, 0.5 mg protein; DL-carnitine-Me-$^{14}$C, 10 μmoles containing 340,000 dpm and 0.4 mg mitochondrial protein. (b) 9000 x g supernatant—Tris buffer (pH 7.5), 100 μmoles; ATP, 19.2 μmoles; CoA, 1.73 μmoles; MgCl₂, 12 μmoles; palmitic acid, 0.5 μmole; partially purified carnitine palmitoyltransferase, 0.5 mg; DL-carnitine-Me-$^{14}$C, 10 μmoles containing 400,000 disintegrations and 0.3 ml supernatant (1.6 mg protein).

<table>
<thead>
<tr>
<th>Preparations</th>
<th>CPT added</th>
<th>Palmitoylcarnitine-Me-$^{14}$C formed (μmoles per mg protein)</th>
<th>Percentage stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>18.1</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>23.7</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>16.4</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>21.8</td>
<td></td>
</tr>
<tr>
<td>9000 x g supernatant</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>3.59</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>6.18</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>3.96</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>7.40</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 8 Effect of palmitate concentration on palmitylcarnitine-Me$^{14}$C formation in the assay method for palmityl CoA synthetase activity. •—•, Mitochondria. x—x, 9000 x g supernatant fraction. Incubation conditions were the same as in Table VIII.
Fig. 9  Effect of CoA on palmitylcarnitine-\textsuperscript{14}C formation in the assay method for palmityl-CoA synthetase activity.

A. Mitochondria  B. 9000 x g Supernatant
Palmitylcarnitine - Me\(^{14}\)C formed (\(\mu\) moles per mg protein) vs CoA (mM)
and 1.27 mM for the 9000 x g supernatant. An inhibitory effect was seen in both preparations when the CoA concentrations exceed the optimum. A similar effect of CoA on the palmityl-CoA synthetase of rat liver mitochondria and microsomes has been demonstrated by Farstad (1967). The effect of DL-carnitine-Me-\textsuperscript{14}C on the formation of palmityl-carnitine-Me-\textsuperscript{14}C in mitochondrial preparations is shown in Fig. 10. The optimal concentration was 9.1 mM.

Both Mg\textsuperscript{++} and ATP are absolutely required for the activation of palmitic acid in mitochondria and 9000 x g supernatant. Fig. 11 and Fig. 12 show that almost no formation of palmitylcarnitine-Me-\textsuperscript{14}C can be detected if either Mg\textsuperscript{++} or ATP is excluded from the incubation mixture. The optimal concentrations of ATP observed for mitochondria and 9000 x g supernatant were 5.5 and 17.6 mM respectively and the corresponding values for Mg\textsuperscript{++} are 7.3 and 11 mM. The formation of palmitylcarnitine-Me-\textsuperscript{14}C by 9000 x g supernatant was slightly inhibited by ATP and Mg\textsuperscript{++} at concentrations beyond their optima. Similar inhibitions by ATP and Mg\textsuperscript{++} have been reported by Farstad (1967) for mitochondrial and microsomal palmityl CoA synthetase activity measured in the presence of a supernatant factor.

A comparison of the activities of mitochondrial and 9000 x g supernatant palmityl CoA synthetases from skeletal muscle of mice was made for both dystrophic and normal littermate controls using the optimum conditions established. No significant difference for
Fig. 10  Effect of carnitine on palmitylcarnitine-
Me-\(^{14}\)C formation in the assay of palmityl-
CoA synthetase activity. Incubation conditions
were as in Table VIII.
Fig. 11 Effect of Mg$^{++}$ on palmitylcarnitine–Me$^{14}$C formation in the assay of palmityl–CoA synthetase activity. A. Mitochondria B. 9000 x g supernatant. Incubation conditions were as in Table VIII.
Fig. 12  Effect of ATP on palmitylcarnitine-\textsuperscript{14}C formation in the assay of palmityl-CoA synthetase activity.  A. Mitochondria  
B. 9000 x g supernatant.
mitochondrial palmityl-CoA synthetase activity was found after 5 or 10 minute incubations (Table IX). However, a significant increase in specific activity of this enzyme was observed in the 9000 x g supernatant fraction of dystrophic muscle homogenate at three incubation times, namely 2.5, 5 and 10 minutes (Table IX). The specific activity ratio (D/C x 100) between dystrophic and normal preparations was found to decrease from 139 to 112 per cent as the incubation time increased. After 10 minutes incubation, the difference between dystrophic and normal muscle was small, but still significant based on pair comparison. This change in activity ratio with incubation time may be caused by the higher ATPase activity present in the dystrophic muscle preparations (Sreter, Ikemoto and Gergely, 1967).

Discussion

Ever since Lipmann and Tuttle (1945) developed their assay method for acyl phosphate based on the formation of hydroxamic acid, this method has been applied by many workers (Creasey, 1962; Pande and Mead, 1968) to determine the activity of fatty acyl CoA synthetase. Farstad, et al., (1967) have pointed out that this method has the disadvantage that hydroxylamine is an inhibitor of palmityl-CoA synthetase, at least in purified enzyme preparations (Massaro and Lennarz, 1965). For this reason they developed the simple but sensitive radioassay method which has been described and used in the present study.
TABLE IX

**Palmitoyl-CoA Synthetase Activity in the Mitochondria and 9000 x g Supernatant of Skeletal Muscle from Dystrophic (D) and Normal Mice (C)**

The incubation conditions were the same as in Table VIII.

<table>
<thead>
<tr>
<th>Number of pairs</th>
<th>Incubation time (min)</th>
<th>Palmitoyl-CoA synthetase activity (mmoles palmitoylcarnitine-Me-14C formed per mg protein ± SEM)</th>
<th>Pair comparison</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>104 ± 6.1</td>
<td>$P &lt; 0.4$</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>193 ± 8.4</td>
<td>$P &lt; 0.4$</td>
</tr>
<tr>
<td>9000 x g</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>supernatant</td>
<td>2.5</td>
<td>14.7 ± 1.63</td>
<td>$P &lt; 0.05$</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>35.6 ± 2.27</td>
<td>$P &lt; 0.05$</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>61.8 ± 3.69</td>
<td>$P &lt; 0.05$</td>
</tr>
</tbody>
</table>
Three activation systems for fatty acids in the mitochondria have been described. These are: (a) GTP-dependent acyl-CoA synthetase, specific for long and short chain fatty acids, which is reported to occur in the mitochondria of beef liver (Rossi and Gibson, 1964), rat liver (Galzigna, Rossi, Sartorelli and Gibson, 1967) and rat kidney (Rossi, Alezandre and Sartorelli, 1968). (b) a system present in liver mitochondria which requires "endogenous" ATP for activation of the fatty acids and the oxidation of the latter is not stimulated by the addition of carnitine (Van den Bergh, 1965). (c) an acyl CoA synthetase, which is thought to occur on the surface of the mitochondria, dependent on "exogenous" ATP and CoA for activation and is linked to carnitine for its transport and oxidation. The latter two systems are specific for long chain fatty acids. The palmitoyl-CoA synthetase from mouse skeletal muscle mitochondria seems to fulfill the requirements for the last system (c) described, since it was observed that the activation reaction is dependent on the presence of both ATP and CoA, and oxidation of the acyl-CoA formed requires the participation of carnitine.

The rate of formation of palmitoyl-CoA under the optimal conditions described, as calculated from the radioactivity present in the palmitoyl carnitine-Me-$^{14}$C formed during incubation, averages 20 mmoles per mg protein per minute for mitochondria and 5.8 mmoles for the 9000 x g supernatant fraction. The value for mitochondria is higher than those reported for rat liver mitochondria (Yates, Shepherd and Garland,
1966; Farstad et al., 1967). The reason for this higher rate of palmitate activation in the present study is not known; it may be due to the differences in species of animals and tissues used or, as seems more likely (Van Tol and Hülsmann, 1969) the higher rate results because the optimal system has been used in regard to the required cofactors, such as CoA, ATP and Mg++. The existence of fatty acid activation systems has been demonstrated in the mitochondria of beef liver, rat liver, rat kidney and brain (Beattie and Basford, 1966). There is disagreement regarding the presence of this enzyme system in heart and skeletal muscle mitochondria. However, Pande and Mead (1968), in comparing the relative activity of the ATP-dependent palmitate activating enzyme in the mitochondrial rich fraction from various tissues, found the highest activity in the epididymal fat pad and liver, followed by heart, skeletal muscle and brain preparations. The present findings confirm the presence of an ATP-dependent fatty acid activation enzyme in skeletal muscle mitochondria.

The stimulation of palmityl carnitine-Me-14C formation from palmityl-CoA formed during the incubation by the addition of carnitine palmityltransferase was 32% for the mitochondria and 80% for the 9000 x g supernatant preparation. This response is what might be expected if carnitine palmityl transferase is a mitochondrial enzyme. However, in the absence of added carnitine palmityltransferase, some radioactivity
did appear in the palmitylcarnitine fraction obtained when 9000 x g supernatant was used. This evidence suggests that some carnitine palmityltransferase activity is also present in the cytoplasm of the muscle cells. A recent report by Van Tol and Hülsmann (1969a) showed that this enzyme was also present in the cytoplasm of rat liver cells. However, the possibility that this enzyme activity arises from mitochondrial contamination of the 9000 x g supernatant due to incomplete sedimentation can not be excluded.

Recently, Farstad (1967) has reported that a supernatant factor, probably an enzyme kinase, is required for the maximal rate of palmityl-CoA formation from palmitate either with mitochondrial or microsomal preparations. This supernatant factor is present in various tissues of rat and also in organs from animals of other species.

Several reports in the literature suggested that the GTP-activation system is about as active as the ATP-activation system (Galzigna et al., 1967; Van den Bergh, 1965). It is possible that both systems are equally active in vivo. However, this view is not supported by the in vitro study of Van Tol et al., (1969b) who evaluated the relative activity of these two systems using the radioassay method of Farstad et al., (1967) and found that the specific activity of the ATP-system extracted from sonicated liver mitochondria was 2.9 μmoles/mg protein per hour and that of the GTP-dependent reaction was only 0.1 μmole/mg protein per hour.
Palmitoyl-CoA plays a dual role in cellular metabolism; it is utilized extramitochondrially in the formation of glycerides and within the mitochondria it undergoes oxidation. It is quite possible that the palmitoyl-CoA synthetase of mitochondria is mainly responsible for the conversion of palmitic acid to palmitoyl-CoA for its utilization by the enzymes of $\beta$-oxidation. The palmitoyl-CoA synthetase present in the 9000 x g supernatant fraction, on the other hand, may function primarily in the formation of palmitoyl-CoA for glyceride biosynthesis (for example synthesis of triglyceride and phosphoglycerides). In the muscular dystrophy of mice and man, the triglyceride concentration of dystrophic muscle is higher than that of the normal. This may result either from enhanced biosynthesis of triglyceride in dystrophic muscle or from decreased utilization or a combination of both. The finding that the initial rate of palmitoyl-CoA synthesis in the 9000 x g supernatant fraction was faster in the dystrophic preparations than that of the normal supports the assumption that the biosynthesis of triglyceride is accelerated in the dystrophic condition.

The finding that the activity of palmitoyl-CoA synthetase in the mitochondria from dystrophic and normal mouse skeletal muscle is not different excludes the possibility that the decrease in palmitic acid oxidation in dystrophic muscle mitochondria observed in the previous Chapter occurs because of a decrease in the activity of mitochondrial palmitoyl-CoA synthetase.
VI. ACTIVITY OF CARNITINE PALMITYLTRANSFERASE IN MUSCLE MITOCHONDRIA OF DYSTROPHIC AND NORMAL MICE

The results in Chapter V have shown that the decrease in palmitic acid-\(^{14}\)C oxidation in the mitochondria of dystrophic muscle was not due to a defect in the activation of palmitate to palmityl-CoA. It seems logical to go one step further and determine the activity of carnitine palmityltransferase in the mitochondria preparations, since it has been shown that carnitine palmityltransferase is the key enzyme in the transfer of activated palmitic acid to the site of fatty acid oxidation (Bremer, 1962; 1963; Fritz and Yue, 1963). It has also been demonstrated that this enzyme is localized most likely in the inner membrane fraction of the liver mitochondria (Norum and Bremer, 1967). Therefore, it was considered that studies of this enzyme in the dystrophic muscle mitochondria might help to elucidate the mechanism of the defect in palmitate oxidation observed in dystrophic muscle.

Result

Using palmitylcarnitine-Me-\(^{14}\)C as the substrate the time course of carnitine palmityltransferase activity is shown in Fig. 13. A nonlinear response was obtained in all attempts, even when short time incubations (30 and 90 seconds) were employed. The cause of this inhibition is not known, but probably is due to product inhibition by the palmityl-CoA that is formed during incubation (Bremer and Norum, 1967).
Fig. 13  The effect of time on carnitine palmitoyltransferase of mouse skeletal muscle mitochondria using palmityl-carnitine-Me$^{14}$C as substrate. The incubation mixture contained a final volume of 0.6 ml: Tris buffer (pH 7.5), 20 μmoles; CoA, 82 μmoles; malonate, 0.24 μmole; dinitrophenol, 0.12 μmole; L-palmityl-carnitine$^{14}$C, 50 μmoles containing 7900 dpm and mitochondria (0.2 mg protein).
The effect of CoA on the activity of the enzyme is shown in Fig. 14. The optimum concentration of CoA was 0.14 mM. In the absence of CoA, the activity of carnitine palmityltransferase was only 10% of that when CoA was present.

Since carnitine palmityltransferase is a membrane bound enzyme, the effect of Triton-X-100 on its activity was investigated. Table X shows that Triton-X-100 at concentrations of 0.1% and 0.2% enhanced the activity of the enzyme by about 66%. The effect of Triton is probably related to its detergent effect which disrupts the mitochondria and exposes more of the active sites of the enzyme to the substrate. A large stimulation by Triton has been reported for the carnitine acetyltransferase of liver mitochondria (Barker, Fincham and Hardwick, 1968).

Table XI shows the effect of increasing amounts of mitochondria on the activity of carnitine palmityltransferase. When the amount of mitochondria was doubled, the release of carnitine-\textsuperscript{14}C from palmitylcarnitine-\textsuperscript{14}C was also doubled.

A comparison between mitochondrial carnitine palmityltransferase activity of dystrophic and normal muscle was made. Due to the non-linear response in the time course, the two incubation times, 30 and 90 seconds were chosen. It can be seen from Table XII that no difference in the activity of carnitine palmityltransferase between dystrophic and normal preparations was found at both incubation times.
Fig. 14  The effect of CoA on the activity of carnitine palmitoyltransferase of mouse skeletal muscle mitochondria. Incubation mixture as described in Fig. 13. Incubation time was 90 seconds.
TABLE X

The Effect of Triton-X-100 on the Activity of Carnitine Palmitoyltransferase

The incubation conditions were the same as those described in Fig. 13. The incubation time was 90 seconds.

<table>
<thead>
<tr>
<th>Concentration of Triton-X-100</th>
<th>mmolles carnitine formed/ per sample/90 seconds</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.3</td>
</tr>
<tr>
<td>0.1%</td>
<td>6.4</td>
</tr>
<tr>
<td>0.2%</td>
<td>6.5</td>
</tr>
</tbody>
</table>
TABLE XI

The Effect of Increasing Mitochondria on the Activity of Carnitine Palmitoyltransferase

The incubation conditions were the same as those described in Fig. 13.

<table>
<thead>
<tr>
<th>Mitochondrial protein (mg)</th>
<th>Carnitine-$^{14}$C released (dpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.184</td>
<td>424</td>
</tr>
<tr>
<td>0.368</td>
<td>876</td>
</tr>
</tbody>
</table>
TABLE XII

Comparison of Carnitine Palmitoyltransferase Activity Between Dystrophic (D) and Normal (C) Skeletal Muscle Mitochondria of Mice (Strain 129)

Incubation conditions were the same as those described in Fig. 13. D= Dystrophic and C = Normal Littermate Controls.

Statistical evaluation based on pair comparison test.

<table>
<thead>
<tr>
<th>Number of Pairs</th>
<th>Incubation Time (seconds)</th>
<th>Carnitine palmitoyltransferase activity (mumoles carnitine formed/mg protein)</th>
<th>Pair Comparison</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>D</td>
<td>C</td>
</tr>
<tr>
<td>7</td>
<td>30</td>
<td>12.6 ± 0.87</td>
<td>12.6 ± 0.98</td>
</tr>
<tr>
<td>7</td>
<td>90</td>
<td>23.9 ± 1.10</td>
<td>24.6 ± 1.51</td>
</tr>
</tbody>
</table>
Discussion

The method used for the assay of mitochondrial carnitine palmityltransferase in the present study was first described by Bremer and Norum (1967). This method permits assay of the enzyme in either direction; thus in one direction the formation of labelled palmitylcarnitine from palmityl-CoA is measured in the presence of excess labelled carnitine, or in the other direction the release of labelled carnitine from palmitylcarnitine-Me$^{14}$C is measured in the presence of excess CoA. The latter method has been applied by Entman and Bressler (1967) to study the activity of carnitine palmityltransferase in myocardial homogenates. This method has been used in the present study. The reason for choosing this assay method is that the formation of labelled carnitine from palmitylcarnitine-Me$^{14}$C is free of interference by the palmityl CoA hydrolase present in mitochondria (Fritz and Yue, 1963).

There is no general agreement about the distribution of carnitine palmityltransferase in the mitochondria. Allmann et al., (1966) reported that the enzyme is in the outer membrane of beef heart mitochondria, while Norum and Bremer (1967) reported that the enzyme is most likely located in the inner membrane of rat liver mitochondria. No information is available about the localization of carnitine palmityltransferase in skeletal muscle mitochondria, but the requirement of CoA for the activity of the enzyme observed in the present study suggests that the enzyme may be localized at the outside of the inner
membrane or in the intermembrane space, or in the outer membrane since CoA is not able to penetrate into the inner membrane (Tubbs and Garland, 1968).

The inner membrane of liver mitochondria is not permeable to either carnitine or CoA. Both compounds behave in the same manner as sucrose which can permeate 60% of the volume of the mitochondria (Yates and Garland, 1968).

While studying the carnitine palmityltransferase in the mitochondria using palmityl-CoA and carnitine as the substrates, Yates and Garland (1966) identified two types of carnitine palmityltransferase, the "latent" and "overt" enzymes. The "latent" enzyme is associated with the inner membrane of the mitochondria and the "overt" soluble enzyme is located outside this membrane either between the inner and outer membrane or in the outer membrane. Taking into consideration the permeable space that exists for added CoA in the liver mitochondria and the fact that CoA is required for the activity of carnitine palmityltransferase (using palmitylcarnitine-Me-¹⁴C as substrate), it may be concluded that only the part of the total mitochondrial carnitine palmityltransferase corresponding to the "overt" enzyme was measured in the present study. This suggestion is consonant with the finding that Triton can stimulate the activity of mitochondrial carnitine palmityltransferase.

The oxidation of palmitylcarnitine can occur rapidly in the
absence of CoA and the endogenous CoA has been shown to be acylated during the incubation (Alexandre, Rossi, Sartorelli and Siliprandi (1969). However, this phenomenon has not been observed in the present study. It was always observed that the activity of carnitine palmitoyltransferase was dependent on the added CoA when palmitylcarnitine-\textsuperscript{14}C was used as substrate. In the absence of added CoA the activity of carnitine palmitoyltransferase was only 10% of that observed in its presence. This result seems to suggest that the "overt", soluble enzyme is more predominant in the mouse skeletal muscle mitochondria. A three-fold stimulation by added CoA has been shown by Allmann et al., (1966) with beef heart mitochondria.

It has been suggested that carnitine palmitoyltransferase catalyzes the rate limiting step of fatty acid oxidation in rat liver mitochondria (Sheperd, Yates and Garland, 1966). This may be true if fatty acid oxidation is carnitine-dependent, such as in skeletal and heart muscle mitochondria where the carnitine independent pathway for fatty acid oxidation has not yet been demonstrated. In this connection it is interesting to note that Stevenson (1968) has demonstrated that the oxidation of palmitate in the presence of malate by mitochondria isolated from the flight muscle of the southern army moth Prodenia eridania, was carnitine independent and no activity of carnitine palmitoyltransferase could be demonstrated in the isolated mitochondria. However, ATP-dependent fatty acyl-CoA synthetase was present in these mitochondria.
The fact that the activity of carnitine palmitoyltransferase is not significantly different between dystrophic and normal skeletal muscle mitochondria rules out the possibility that a decreased activity of this enzyme is responsible for the decreased fatty acid oxidation observed to occur in mitochondria from dystrophic muscle.
VII. ACETYL-$l^{-14}C$ CARNITINE OXIDATION BY SKELETAL MUSCLE MITOCHONDRIA OF DYSTROPHIC MICE

In the previous Chapters evidence has been presented that the observed decrease in palmitic acid-$l^{-14}C$ oxidation by skeletal muscle mitochondria of dystrophic mice was not caused by any decrease in the activity of palmityl-CoA synthetase or carnitine palmityltransferase. Other possible sites where the defect might occur are in the steps of $\beta$-oxidation or in the Krebs cycle where the acetyl groups derived from oxidation (as acetyl-CoA) are subsequently oxidized. To help decide where the defect occurs a study was undertaken of the oxidation of acetyl-$l^{-14}C$ carnitine by mitochondria isolated from normal and dystrophic skeletal muscle of mice (strain 129). The results of these experiments are reported in this Chapter.

Results

Table XIII shows the cofactor requirements for acetyl-$l^{-14}C$ carnitine oxidation by skeletal muscle mitochondria of mice. It can be seen that ADP and a small amount of a Krebs cycle intermediate (i.e. malate) are required. This is in agreement with the report of Bremer (1962). ATP can replace ADP, but gives less activity. The addition of carnitine to the reaction mixture decreased the oxidation and the omission of CoA from the complete system caused a slight
TABLE XIII

Cofactor Requirements for Acetyl-1-\(^{14}\)C Carnitine Oxidation
by Skeletal Muscle Mitochondria of Mice (Strain 129)

The complete system contained sodium phosphate buffer (pH 7.4), 40 \(\mu\)moles; ADP, 3.2 \(\mu\)moles; cytochrome c, 13.6 \(\mu\)moles; MgCl\(_2\), 15 \(\mu\)moles; KCl, 100 \(\mu\)moles; acetyl-1-\(^{14}\)C carnitine (4850 dpm), 2 \(\mu\)moles and malate, 133 \(\mu\)moles. Total volume was 2 ml. The incubation time was 15 minutes at 37\(^{\circ}\)C. The method for the measurement of \(^{14}\)CO\(_2\) released is described in the METHODS.

<table>
<thead>
<tr>
<th>Incubation Mixture</th>
<th>(\mu)moles (^{14})CO(_2) released/mg mitochondrial protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete system</td>
<td>562</td>
</tr>
<tr>
<td>less malate</td>
<td>17</td>
</tr>
<tr>
<td>less ADP</td>
<td>73</td>
</tr>
<tr>
<td>less ADP, plus ATP</td>
<td>505</td>
</tr>
<tr>
<td>plus carnitine (3 (\mu)moles)</td>
<td>410</td>
</tr>
<tr>
<td>less ADP, plus carnitine and ATP</td>
<td>304</td>
</tr>
<tr>
<td>plus CoA (25 (\mu)moles)</td>
<td>535</td>
</tr>
</tbody>
</table>
stimulation. The optimal concentration of acetyl-l-\(^{14}\)C carnitine was 1.0 mM (Fig. 15).

A comparison of the ability of dystrophic and normal muscle mitochondria to oxidize acetyl-l-\(^{14}\)C carnitine using the above complete system shows that the oxidation of acetyl-l-\(^{14}\)C carnitine was decreased by 80% in the dystrophic mitochondria (Table XIV).

**Discussion**

The ideal substrate to use in the present study to test the intactness of the Krebs cycle would be acetyl-l-\(^{14}\)C CoA. However, it has been shown by Fritz and Yue (1964) and by Erfle and Sauer (1966) that acetyl-CoA is not oxidizable by heart or skeletal muscle in the absence of carnitine because acetyl-CoA is not permeable to the mitochondrial membrane barrier. Therefore, acetyl-l-\(^{14}\)C carnitine was used in the present experiment. For this substrate to be oxidized by the enzymes of the Krebs cycle it must first be converted to acetyl-CoA in the mitochondria by carnitine acetyltransferase according to the following reaction (Friedman and Fraenkel, 1955; Fritz, Schultz and Srere, 1963):

\[
\text{Acetyl-carnitine} + \text{CoA} \rightleftharpoons \text{Acetyl-CoA} + \text{Carnitine}
\]

In this study it seems logical to assume that the rate of acetyl-l-\(^{14}\)C
Fig. 15 The effect of acetyl-1^-14C carnitine concentration on its oxidation by mouse skeletal muscle mitochondria. Incubation conditions are the same as those described in TABLE XIV.
TABLE XIV

**Acetyl-l-¹⁴C Carnitine Oxidation by Skeletal Muscle Mitochondria**

*from Dystrophic (D) and Normal Mice (C) (Strain 129)*

Incubation conditions as for the complete system described in Table XIII. D = dystrophic; C = normal littermate controls. Statistical evaluation based on pair comparison test.

<table>
<thead>
<tr>
<th>Number of Pairs</th>
<th>Acetyl-l-¹⁴C carnitine oxidation (μmoles oxidized per mg mitochondrial protein)</th>
<th>Pair Comparison</th>
</tr>
</thead>
<tbody>
<tr>
<td>D</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>92 ± 30.9</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>498 ± 28.7</td>
<td>$P \leq 0.001$</td>
</tr>
</tbody>
</table>
carnitine oxidation to CO₂ is determined by the rate of formation of acetyl-CoA and its subsequent oxidation by the Krebs cycle enzymes. The significant reduction of acetyl-1⁴C carnitine oxidation observed in muscle mitochondria of dystrophic mice could, therefore, result either from a decreased activity of carnitine acetyltransferase or a decrease in function of the Krebs cycle or a combination of both. It is interesting to note that Dr. Jato (1969) in this laboratory has shown that the activity of extractable carnitine acetyltransferase from dystrophic muscle mitochondria was only 65% of the normal value. However, it seems unlikely that the large decrease (80%) in the oxidation of acetyl-1⁴C carnitine observed in dystrophic muscle mitochondria could be explained solely by the slight decrease in activity of carnitine acetyltransferase. It seems probable that the operation of the Krebs cycle is also decreased in dystrophic muscle mitochondria.

Although CoA is one of the substrates for carnitine acetyltransferase, it was not required in the present system (Table XIII). Bremer (1962) has made a similar observation with mitochondria from rat liver, heart and kidney using acetylcarnitine as substrate. This suggests that there is within the mitochondria a pool of CoA which can be utilized by the enzyme. This finding gives indirect support to the view that the localization of carnitine acetyltransferase and carnitine palmitoyltransferase in skeletal muscle mitochondria of mice may be different, because it has been shown in Chapter VI
that exogenous CoA is required for the activity of mitochondrial carnitine palmityltransferase. In view of the permeable space to exogenous CoA described by Tubbs and Garland (1968), it is possible that carnitine acetyltransferase may be localized on the inner side of the inner membrane of skeletal muscle mitochondria.

The concept of compartmentalization of CoA in the mitochondria of skeletal muscle raises another possible explanation for the observed reduction of acetyl group oxidation. Mitochondrial CoA content in the dystrophic muscle may be lower than that of the normal muscle and the formation of acetyl-CoA from acetylcarnitine might be lowered. Therefore, the oxidation of the acetyl group would be reduced even though the activities of the Krebs cycle enzymes were at their normal levels. It is interesting that the CoA content in the muscle from dystrophic patients (Duchenne type) was found to be significantly lower than that of normal subjects (Radu et al., 1968).
VIII. DECARBOXYLATION OF PYRUVATE-1$^{14}$C AND OXIDATION OF PYRUVATE-3$^{14}$C BY MITOCHONDRIA FROM DYSTROPHIC MUSCLE OF MICE (STRAIN 129)

Pyruvate, the end product of glycolysis, plays an important role in the energy production of mammalian tissues. In Chapter VII, the oxidation of acetyl-1$^{14}$C carnitine by mitochondria of dystrophic mice was shown to be greatly reduced. Although a decrease in activity of carnitine acetyltransferase could partially account for the impairment in oxidation of acetyl-1$^{14}$C carnitine it could not account for the entire reduction. It was therefore concluded that some impairment exists in the operation of the Krebs cycle. To assess this possibility, both the decarboxylation of pyruvate-1$^{14}$C and the oxidation of pyruvate-3$^{14}$C were investigated. Since labelled pyruvate is unstable in aqueous solution (Von Korff, 1964), this substrate was prepared fresh for each experiment.

Results

Pyruvate-1$^{14}$C decarboxylation was greatly stimulated by the addition of carnitine to the incubation medium as shown in Fig. 16. The maximum stimulation was obtained at a concentration of 2mM. The degree of stimulation varied from one preparation to another, but usually came within the range 200 to 400%. Table XV shows that neither ATP nor ADP are required for pyruvate decarboxylation; the
Fig. 16 The effect of carnitine on the decarboxylation of pyruvate-1$^{14}$C by mouse skeletal muscle mitochondria. The reaction mixture in a final volume of 2 ml contained: sodium phosphate buffer (pH 7.3); 40 µmoles; KCl, 100 µmoles; MgCl$_2$, 15 µmoles; ATP, 3.2 µmoles; thiamine pyrophosphate, 8 µmoles; malonate, 2 µmoles; pyruvate-1$^{14}$C (37,600 dpm), 2 µmoles and mitochondria (398 µg protein). Incubation time was 15 minutes.
TABLE XV

The Effect of ATP and ADP on the Decarboxylation of Pyruvate-1$^{14}$C by Skeletal Muscle Mitochondria of Mice

The complete system, in a final volume of 2.0 ml, contained: sodium phosphate buffer (pH 7.3), 40 μmoles; KCl, 100 μmoles; MgCl$_2$, 15 μmoles; malonate, 4 μmoles; DL-carnitine, 4 μmoles; mercaptoethanol, 1 μmole; CoA, 0.2 μmole; pyruvate-1$^{14}$C, 2 μmoles and 383 μg mitochondrial protein. The amount of ATP and ADP when added was 3.2 μmoles.

<table>
<thead>
<tr>
<th>Incubation mixture</th>
<th>μmoles pyruvate-1$^{14}$C decarboxylated per mg mitochondrial protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete system</td>
<td>1212</td>
</tr>
<tr>
<td>Plus ATP, less mercaptoethanol</td>
<td>1000</td>
</tr>
<tr>
<td>Plus ADP, less mercaptoethanol</td>
<td>1015</td>
</tr>
<tr>
<td>Plus ATP</td>
<td>1213</td>
</tr>
<tr>
<td>Plus ADP</td>
<td>1256</td>
</tr>
</tbody>
</table>
addition of mercaptoethanol caused a slight stimulation. In Table XVI it can be seen that malonate, an inhibitor of the Krebs cycle, did not inhibit pyruvate decarboxylation; instead it caused a slight stimulation both in the absence or in the presence of carnitine. Fig. 17 shows the effect of pyruvate concentration on its decarboxylation. The optimum pyruvate concentration was 1 mM.

Since added ATP, ADP and TPP did not stimulate pyruvate decarboxylation, they were not included in the reaction mixture that was used for making the comparison between the capacity of dystrophic and normal mitochondria to decarboxylate pyruvate. In the presence of carnitine the decarboxylation of pyruvate-$1^{-14}$C to $^{14}$CO$_2$ is significantly decreased in the dystrophic mitochondria ($P < 0.005$). However, in the absence of carnitine the decrease is not significant ($0.1 > P > 0.05$) although a decreasing trend is evident in the five pairs studied (Table XVII).

A study of pyruvate-$3^{-14}$C oxidation by dystrophic muscle mitochondria has been carried out using an incubation medium similar to that used in the study of palmitate-$1^{-14}$C oxidation with the exception that carnitine was omitted and ATP was replaced by ADP. As can be seen in Table XVIII pyruvate-$3^{-14}$C oxidation to $^{14}$CO$_2$ was significantly decreased in mitochondria obtained from dystrophic muscle of mice compared to their normal littermate controls either in the presence of succinate or malate.
### TABLE XVI

**Effect of Malonate on the Decarboxylation of Pyruvate-1-$^{14}$C by Mouse Skeletal Muscle Mitochondria**

The complete system contained: sodium phosphate buffer (pH 7.3), 40 μmoles; KCl, 100 μmoles; MgCl$_2$, 15 μmoles; malonate, 2 μmoles; ATP, 3.2 μmoles; CoA, 0.1 μmole; thiamine pyrophosphate, 8 μmoles; pyruvate-1-$^{14}$C, 2 μmoles and mitochondrial protein, 400 μg. The final volume was 2.0 ml. The amount of DL-carnitine (when added) was 2 μmoles.

<table>
<thead>
<tr>
<th>Incubation mixture</th>
<th>μmoles pyruvate-1-$^{14}$C decarboxylated per mg mitochondrial protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete system</td>
<td>221</td>
</tr>
<tr>
<td>less malonate</td>
<td>193</td>
</tr>
<tr>
<td>plus carnitine</td>
<td>960</td>
</tr>
<tr>
<td>plus carnitine, less malonate</td>
<td>935</td>
</tr>
</tbody>
</table>
Fig. 17  The effect of pyruvate concentration on the decarboxylation of pyruvate-$l-^{14}$C by the skeletal muscle mitochondria of mice. The incubation mixture and conditions were the same as in Fig. 16 with the exception that ATP and thiamine pyrophosphate were not included and 1 umole mercaptoethanol was added.
TABLE XVII

Decarboxylation of Pyruvate-1$^{14}$C by Skeletal Muscle

Mitochondria of Dystrophic Mice (D) and their
Littermate Controls (C) (Strain 129)
in the Presence and Absence of DL-carnitine

<table>
<thead>
<tr>
<th>Number of Pairs</th>
<th>Carnitine Added</th>
<th>$\mu$moles pyruvate-1$^{14}$C decarboxylated per mg mitochondrial protein $\pm$ S.E.M.</th>
<th>Pair Comparison</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>D $\quad$ C $\quad$</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>+</td>
<td>302 $\pm$ 47 $\quad$ 590 $\pm$ 43</td>
<td>$P &lt; 0.05$</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>144 $\pm$ 39 $\quad$ 270 $\pm$ 14</td>
<td>$0.05 &lt; P &lt; 0.1$</td>
</tr>
</tbody>
</table>
TABLE XVIII

Pyruvate-3-¹⁴C Oxidation by Mitochondria from Muscle of Dystrophic Mice (D) and Their Littermate Controls (C)

The incubation conditions were the same as those described in Table V with the exception that ADP (1.6 mM) replaced ATP and DL-carnitine was omitted from the system.

<table>
<thead>
<tr>
<th>C₄ Dicarboxylic Acid Added</th>
<th>Number of Pairs</th>
<th>Pyruvate Oxidation (μm moles $^{14}$CO₂/ mg protein)</th>
<th>Pair Comparison D/C x 100</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malate</td>
<td>9</td>
<td>212.2±28.2</td>
<td>358.9±23.10</td>
<td>61.7±7.2</td>
</tr>
<tr>
<td>Succinate</td>
<td>7</td>
<td>194.0±21.20</td>
<td>332.1±11.83</td>
<td>60.5±8.4</td>
</tr>
</tbody>
</table>
Discussion

The observed stimulation by carnitine of the decarboxylation of pyruvate-$^{14}$C is in agreement with that of Davis (1965) who observed that carnitine stimulated the decarboxylation of pyruvate-$^{14}$C without affecting the Krebs cycle. Erfle and Sauer (1966) have reported a similar carnitine effect on the decarboxylation of pyruvate-$^{14}$C with rat skeletal mitochondria but not with rat liver mitochondria. Bremer (1962) suggested that carnitine stimulated oxidation of pyruvate can be accounted for largely by the formation of acetyl carnitine. This suggestion gains some support from the work of Hulsmann, Siliprandi and Ciman (1964) who reported that the inhibition of oxoglutarate oxidation by pyruvate or acetoacetate was relieved by the addition of carnitine. They attributed the carnitine effect to the regeneration of CoA from acetyl-CoA by carnitine acetyltransferase in the presence of carnitine. Therefore, the effect of carnitine on the decarboxylation of pyruvate-$^{14}$C might be explained by this mechanism. However, Garland and Randle (1964) have demonstrated that acetyl-CoA is a competitive inhibitor with respect to the CoA of pig heart pyruvate dehydrogenase and have proposed that the acetyl-CoA to CoA ratio in the system controlled the activity of the enzyme. As the acetyl CoA to CoA ratio is increased in the system due to the absence of a sparker (i.e. malate), the stimulation by carnitine of decarboxylation could be the result of the removal of the inhibition
of pyruvate dehydrogenase by acetyl-CoA. If this is correct then any compound which can remove acetyl-CoA from the incubation mixture should stimulate pyruvate dehydrogenase. This interpretation is substantiated by Fig. 18 in which it can be seen that malate can further stimulate the decarboxylation of pyruvate even in the presence of an optimum concentration of carnitine. Under these conditions the disappearance of pyruvate was correspondingly increased.

In the absence of carnitine the ability of dystrophic muscle mitochondria to decarboxylate pyruvate was not significantly different from that of the normal controls, although a decreasing trend was evident, whereas in the presence of carnitine the decrease in dystrophic mitochondria was highly significant. It is possible that the decrease simply reflects the decreased activity of carnitine acetyltransferase in the dystrophic muscle mitochondria rather than that of pyruvate dehydrogenase.

The fact that pyruvate-3-\(^{14}\)C oxidation by muscle mitochondria of dystrophic mice is decreased compared with their normal controls is consistent with the suggestion that the operation of the Krebs cycle is decreased in these mitochondria.

Muscle is an organ that contains a high concentration of carnitine, which is the substrate for both carnitine acetyltransferase and carnitine palmityltransferase. The fact that pyruvate decarboxylation is stimulated
Fig. 18  The effect of malate on the decarboxylation of pyruvate-\textsuperscript{14}C by skeletal muscle mitochondria of mice. The incubation conditions were the same as the complete system in Table XV with the exception that malonate was excluded and ADP was added. 1 mM pyruvate-\textsuperscript{14}C (97,000 dpm) was used. ●—●, pyruvate-\textsuperscript{14}C decarboxylation; ×—×, pyruvate disappearance.
by carnitine suggests that carnitine may also play a role in the metabolism of pyruvate in the muscle. The findings by Erfle and Sauer (1966) that the decarboxylation of pyruvate-1-¹⁴C by skeletal muscle mitochondria of rat is stimulated by carnitine and that carnitine stimulation could not be observed in the liver mitochondria, strongly support this concept.
IX. FATTY ACID SYNTHESIS FROM ACETATE-$^{14}$C BY TISSUES OF MICE

In the previous Chapters it has been shown that the decrease in palmitate-$^{14}$C oxidation by dystrophic muscle in vitro is most likely due to a decrease in the enzyme activity in one or more steps of the Krebs cycle. However, fat accumulation in dystrophic muscle may result not only from a decrease in oxidation of fatty acids but also from enhanced lipogenesis. In order to explain the build-up of an intermediate one must have knowledge of both the catabolic as well as the anabolic processes. The studies presented up to this point deal only with catabolic aspects.

This Chapter now describes experiments carried out on the synthesis of fatty acid (i.e. an anabolic pathway) in tissues of normal and dystrophic mice (strain 129). In the investigation acetate-$^{14}$C was used as the precursor of fatty acids.

Results

The cofactor requirements for fatty acid synthesis from acetate-$^{14}$C by the tissues of mice are shown in Tables XIX and XX. It can be seen that citrate or isocitrate was required for optimum fatty acid synthesis by all of the tissues. NADP$^+$ was essential for the synthesis of fatty acid by liver and muscle preparations and NADPH can replace NADP in the muscle preparation without affecting
TABLE XIX

Cofactor Requirements for Fatty Acid Synthesis from
Acetate-2-\textsuperscript{14}C by High Speed Supernatant (71,000 x g)
of Skeletal Muscle of Mice (Strain 129)

The incubation mixture contained in a final volume of
1.1 ml: KH\textsubscript{2}PO\textsubscript{4}-KOH buffer (pH 6.5), 60 μmoles; citrate,
5.5 μmoles; MnCl\textsubscript{2}, 0.8 μmole; cysteine, 8 μmoles; NADP\textsuperscript{+},
0.2 μmole; CoA, 21 μmoles; KHCO\textsubscript{3}, 20 μmoles; ATP, 3.64
μmoles; acetate-2-\textsuperscript{14}C, 2.2 μmoles containing 1.33 x 10\textsuperscript{6}
dpm and enzyme preparation (6 mg protein). The reaction
mixture was gassed for 5 minutes with 100% CO\textsubscript{2} and incubated
for another 60 minutes at 37°C. In the complete system 2.84
μmoles of acetate-2-\textsuperscript{14}C were incorporated per mg protein.

<table>
<thead>
<tr>
<th>Incubation mixture</th>
<th>Percent of complete system</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete system</td>
<td>100</td>
</tr>
<tr>
<td>Less ATP</td>
<td>0</td>
</tr>
<tr>
<td>Less NADPH</td>
<td>1.5</td>
</tr>
<tr>
<td>Less NADPH plus NADPH</td>
<td>99</td>
</tr>
<tr>
<td>Less citrate</td>
<td>5.7</td>
</tr>
<tr>
<td>Less Mn\textsuperscript{2+}</td>
<td>9</td>
</tr>
<tr>
<td>Less KHCO\textsubscript{3}</td>
<td>67</td>
</tr>
</tbody>
</table>
TABLE XX

Cofactor Requirements for Fatty Acid Synthesis by
High Speed Supernatant Preparations from Liver,
Brain and Kidney of Mice (Strain 129)

The incubation conditions were the same as in Table XIX
for brain and kidney preparations. For liver, the incuba-
tion conditions were the same with the exception that
isocitrate (11 \( \mu \)moles) replaced citrate, the CoA concen-
tration was 4.3 \( \mu \)moles and the enzyme protein was 10 mg.
The incorporation of acetate-2-\( ^{14} \)C (\( \mu \)moles per mg protein)
into fatty acids for each of the complete systems of liver,
brain and kidney preparations were 23.6, 2.7 and 3.0
respectively.

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Incubation mixture</th>
<th>Percent of complete system</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>Complete system</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Less isocitrate</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>Less NADP(^+)</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>NADP(^+) (0.1 ( \mu )mole)</td>
<td>122</td>
</tr>
<tr>
<td></td>
<td>NADP(^+) (0.4 ( \mu )mole)</td>
<td>92.5</td>
</tr>
<tr>
<td>Brain</td>
<td>Complete system</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Less citrate</td>
<td>27</td>
</tr>
<tr>
<td>Kidney</td>
<td>Complete system</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Less citrate</td>
<td>12</td>
</tr>
</tbody>
</table>
the acetate-2-$^{14}$C incorporation into fatty acids. The omission of KHCO$_3$ decreased the incorporation by 18% (Table XXI) in the muscle preparation. The effect of changes in the gas phase on fatty acid synthesis is shown in Table XXII. In all of the tissue preparations studied CO$_2$, as the gas phase, was much better than N$_2$ or air. For the requirement of divalent cations in the muscle preparation Mn$^{++}$ was superior to Mg$^{++}$ in supporting fatty acid synthesis. The addition of Mg$^{++}$ to the complete system caused an inhibition in the incorporation of acetate-2-$^{14}$C (Table XXIII).

Fig. 19 and 20 show the time courses for acetate-2-$^{14}$C incorporation into fatty acids for liver and muscle preparations. The incorporation was linear for 60 minutes under the present conditions for liver, but for the muscle preparation a slight lag was seen in the first 15 minutes. The incorporation of acetate-2-$^{14}$C was dependent directly upon the amount of supernatant fraction added for each of the above preparations (Fig. 21 and 22).

In the muscle, brain and kidney preparations the stimulation of fatty acid synthesis by citrate (Tris citrate or sodium citrate) was more than that by isocitrate, while for the liver preparation isocitrate was superior to citrate (Table XXIV). The optimum concentration of citrate for the muscle preparation was 5 mM and that of isocitrate for liver was 10 mM (Fig. 23 and 24).
TABLE XXI

Effect of Bicarbonate and CO₂-flushing on Fatty Acid Synthesis from Acetate-2⁻¹⁴C by the High Speed Supernatant Preparation from Skeletal Muscle of Mice (Strain 129)

The incubation conditions were the same as those of the complete system of Table XIX. Incorporation of acetate-2⁻¹⁴C into fatty acids was 7.0 mmol per mg protein.

<table>
<thead>
<tr>
<th>Bicarbonate (μmoles)</th>
<th>CO₂-flushing</th>
<th>Fatty acid synthesis (percent of complete system)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 (complete system)</td>
<td>+</td>
<td>100</td>
</tr>
<tr>
<td>0</td>
<td>+</td>
<td>82</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>92</td>
</tr>
<tr>
<td>40</td>
<td>+</td>
<td>92</td>
</tr>
<tr>
<td>20</td>
<td>-</td>
<td>8</td>
</tr>
<tr>
<td>40</td>
<td>-</td>
<td>2.5</td>
</tr>
</tbody>
</table>
TABLE XXII

Effect of Gas Phase on Fatty Acid Synthesis from Acetate-2-$^{14}$C by High Speed Supernatant from Skeletal Muscle Homogenate of Mice (Strain 129)

Incubation conditions as in Table XIX and Table XX.

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Gas Phase</th>
<th>Fatty Acid Synthesis (dpm in fatty acids)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle</td>
<td>CO₂</td>
<td>1970</td>
</tr>
<tr>
<td></td>
<td>Air</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>N₂</td>
<td>60</td>
</tr>
<tr>
<td>Liver</td>
<td>CO₂</td>
<td>20,500</td>
</tr>
<tr>
<td></td>
<td>Air</td>
<td>960</td>
</tr>
<tr>
<td></td>
<td>N₂</td>
<td>964</td>
</tr>
<tr>
<td>Brain</td>
<td>CO₂</td>
<td>1,660</td>
</tr>
<tr>
<td></td>
<td>Air</td>
<td>675</td>
</tr>
<tr>
<td>Kidney</td>
<td>CO₂</td>
<td>1,765</td>
</tr>
<tr>
<td></td>
<td>Air</td>
<td>129</td>
</tr>
</tbody>
</table>
### TABLE XXIII

**Effect of Divalent Cations on Fatty Acid Synthesis from Acetate-2-$^{14}$C by High Speed Supernatant from Skeletal Muscle of Mice (Strain 129)**

Incubation conditions as in Table XIX. For complete system, the incorporation of acetate-2-$^{14}$C into fatty acid was 7.0 µmoles.

<table>
<thead>
<tr>
<th>Divalent cations</th>
<th>Fatty Acid Synthesis (percent of complete system)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mn$^{++}$ (complete system)</td>
<td>100</td>
</tr>
<tr>
<td>-</td>
<td>2.2</td>
</tr>
<tr>
<td>Mg$^{++}$ (5 µmoles)</td>
<td>48</td>
</tr>
<tr>
<td>Mg$^{++}$ (12 µmoles)</td>
<td>40</td>
</tr>
<tr>
<td>Mn$^{++}$ plus Mg$^{++}$ (5 µmoles)</td>
<td>85</td>
</tr>
<tr>
<td>Mn$^{++}$ plus Mg$^{++}$ (12 µmoles)</td>
<td>60</td>
</tr>
</tbody>
</table>
Fig. 19  Time course of fatty acid synthesis from acetate-2-\textsuperscript{14}C by high speed supernatant from mouse liver homogenate. Incubation conditions as in Table XX with the exception that the concentration of CoA was 8.5 \( \mu \text{M} \).
Fig. 20 Time course of fatty acid synthesis from acetate-2-\textsuperscript{14}C by high speed supernatant from skeletal muscle homogenates of mice (strain 129). Incubation conditions as in Table XIX.
Fig. 21  Effect of enzyme concentrations on fatty acid synthesis for mouse liver preparation. Incubation conditions as in Table XX.
Fig. 22. Effect of enzyme concentration on the fatty acid synthesis from acetate-2-$^{14}$C by muscle preparation. Incubation conditions as in Table XIX.
<table>
<thead>
<tr>
<th>Tissue</th>
<th>Addition</th>
<th>Fatty acid synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>--</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>Isocitrate (11 μmoles)</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>Citrate (11 μmoles)</td>
<td>17</td>
</tr>
<tr>
<td>Muscle</td>
<td>--</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>Isocitrate (5.5 μmoles)</td>
<td>1.94</td>
</tr>
<tr>
<td></td>
<td>Citrate (5.5 μmoles)</td>
<td>3.7</td>
</tr>
<tr>
<td>Brain</td>
<td>--</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>Isocitrate (5.5 μmoles)</td>
<td>1.74</td>
</tr>
<tr>
<td></td>
<td>Citrate (5.5 μmoles)</td>
<td>3.0</td>
</tr>
<tr>
<td>Kidney</td>
<td>--</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>Isocitrate (5.5 μmoles)</td>
<td>1.58</td>
</tr>
<tr>
<td></td>
<td>Citrate (5.5 μmoles)</td>
<td>2.0</td>
</tr>
</tbody>
</table>
Fig. 23 Effect of citrate on fatty acid synthesis from acetate-2-\(^{14}\)C in muscle preparation. Incubation conditions as in Table XIX.
Fig. 24 Effect of isocitrate on fatty acid synthesis from acetate-2-\textsuperscript{14}C in liver preparation.

Incubation conditions as in Table XX.
CoA, which is required for the activation of acetate, stimulates the acetate-2-\(^{14}\)C incorporation into fatty acids in both muscle and liver preparations. The stimulation was more pronounced in the muscle than in the liver preparation in which only a slight effect of CoA was seen (Table XXV). The optimum CoA concentrations for liver and muscle preparations were 0.43 \(\mu M\) and 2 \(\mu M\) respectively. Concentrations beyond the optimum produced an inhibitory effect in both tissues. The effect of \(Mn^{++}\) concentrations on fatty acid synthesis from acetate-2-\(^{14}\)C is shown in Fig. 25. The optimum concentration in each instance was 0.77 \(mM\) for \(Mn^{++}\).

With the complete system the synthesis of fatty acids from acetate-2-\(^{14}\)C by the tissues from dystrophic and normal mice is compared as shown in Table XXVI. Based on pair comparison it was found that liver and muscle preparations from dystrophic mice showed a significantly greater ability to synthesize fatty acids from acetate-2-\(^{14}\)C than their normal littermates. However, the reverse was seen in brain and kidney; these two preparations from dystrophic mice showed significantly lower activity in acetate-2-\(^{14}\)C incorporation into fatty acids.

**Discussion**

Fatty acid synthesizing systems have been shown to be distributed widely in nature; systems have been demonstrated in plants, micro-
TABLE XXV

Effect of CoA on Fatty Acid Synthesis from Acetate-2-\textsuperscript{14}C by High Speed Supernatant of Muscle and Liver Homogenates of Mice (Strain 129)

Incubation conditions as in Tables XIX and XX for the muscle and liver preparations. Fatty acid synthesis is expressed as μmoles acetate-2-\textsuperscript{14}C incorporated per mg protein.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>CoA Concentration (μM)</th>
<th>Fatty Acid Synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle</td>
<td>0</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>3.8</td>
</tr>
<tr>
<td>Liver</td>
<td>0</td>
<td>31.7</td>
</tr>
<tr>
<td></td>
<td>0.43</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>4.3</td>
<td>34.7</td>
</tr>
<tr>
<td></td>
<td>8.5</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>11.7</td>
</tr>
</tbody>
</table>
Fig. 25 Effect of Mn$^{++}$ ion on the incorporation of acetate-2-$^{14}$C into fatty acids by high speed supernatant of muscle homogenate. Incubation conditions as in Table XIX.
TABLE XXVI

Incorporation of Acetate-2-$^{14}$C into Fatty Acids by
High Speed Supernatant of Tissues of Dystrophic Mice (D)
and Their Littermate Controls (C)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Mouse</th>
<th>No.</th>
<th>Acetate-2-$^{14}$C Incorporated into Fatty Acid (μmoles/mg protein)</th>
<th>Pair Comparison</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>D</td>
<td>8</td>
<td>41.74 ± 2.95</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>8</td>
<td>32.27 ± 1.66</td>
<td></td>
</tr>
<tr>
<td>Muscle</td>
<td>D</td>
<td>14</td>
<td>4.24 ± 0.56</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>14</td>
<td>3.16 ± 0.21</td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>D</td>
<td>11</td>
<td>1.08 ± 0.17</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>11</td>
<td>1.78 ± 0.24</td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>D</td>
<td>11</td>
<td>0.52 ± 0.13</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>11</td>
<td>1.21 ± 0.20</td>
<td></td>
</tr>
</tbody>
</table>
organisms and various animal tissues such as liver (Korchak and Masoro, 1962), adipose tissue (Martin and Vagelos, 1962), brain (Brady, 1960), kidney and skeletal muscle (Rabinowitz, 1960). The enzymes of adipose tissue and liver have been separated into two fractions; one fraction contains the acetyl-CoA carboxylase necessary for the synthesis of malonyl-CoA and the second fraction contains the fatty acid synthetase multienzyme complex. It is now well established that the de novo synthesis of fatty acid (i.e. palmitate) occurs extramitochondrially according to the following reaction:

\[
\text{Acetyl-CoA} + \text{ATP} + \text{CO}_2 \rightarrow \text{Malonyl-CoA} + \text{ADP} + \text{Pi} \quad (1)
\]

\[
\text{Acetyl-CoA} + 7 \text{Malonyl-CoA} + 14 \text{NADPH} + 14 \text{H}^+ \rightarrow \text{Palmitate} + 8 \text{CoA} + 7 \text{H}_2\text{O} + 7 \text{CO}_2 \quad (2)
\]

Acetyl-CoA carboxylase catalyzes reaction (1) and the fatty acid synthetase multienzyme complex catalyzes, in a series of steps, the overall conversion described in reaction (2).

The cofactor requirements for fatty acid synthesis from acetate-2-^{14}C by skeletal muscle in the present study are the same as those for other tissues. Bicarbonate, CoA, ATP, NADP^+, Mn^{++}, citrate and a sulfhydryl compound (cysteine) are all essential for the active incorporation of acetate-2-^{14}C. To the author's knowledge the system described in this thesis is the first cell-free system for fatty acid synthesis from acetate to have been demonstrated for skeletal muscle.
It has been observed that fatty acid biosynthesis from acetate is markedly stimulated by Krebs cycle intermediates, especially citrate and isocitrate (Brady, Mamoong and Stadtman, 1956; Abraham, Mathes and Charkoff, 1961; Holdworth and Neville, 1963). Different suggestions have been offered as to the mechanism of this stimulation. It has been suggested that isocitrate or citrate serves as a source of CO₂ (Gibson, Titchener and Wakiil, 1958) and NADPH (Porter, Wakiil, Tietz, Jacob and Gibson, 1957). Bortz, Abraham, Chaikoff and Dozier (1962) have shown that citrate, isocitrate and aconitate all stimulated fatty acid synthesis from acetate by human liver homogenates. Glucose-6-phosphate plus glucose-6-phosphate dehydrogenase could not meet the requirement of isocitrate. Therefore, it was concluded that the stimulation by citrate or isocitrate could not be explained simply on the basis of supplying NADPH for fatty acid synthesis. The effect of citrate has been established by Martin and Vagelos (1962) to be concerned with stimulation of the carboxylation of acetyl-CoA to form malonyl-CoA; the molecular weight of acetyl-CoA carboxylase and the specific activity of this enzyme were simultaneously increased by citrate. More recent studies by Greenspan and Lowenstein (1967) and by Swanson, Curry and Ankar (1968) have indicated that the activation of acetyl-CoA carboxylase can be achieved in the absence of citrate; Mg³⁺ alone can activate the enzyme but a lag period is seen before full activation is reached. This activation by Mg³⁺ is inhibited by the addition of ATP to the incubation medium (Greenspan
and Lowenstein, 1967; Scorpio and Masoro, 1968). Since the carboxylation of acetyl-CoA to malonyl-CoA is considered to be one of the key control points of fatty acid synthesis (Korchak and Masoro, 1962) and since the change in concentration of citrate in the liver may not be related to fatty acid synthesis (Spencer and Lowenstein, 1967), and moreover, the activation of acetyl-CoA carboxylase can be achieved without citrate, it seems reasonable to conclude that citrate may not be physiologically important in the control of fatty acid synthesis in the liver.

In the present experiments, either citrate or isocitrate was required for maximal incorporation of acetate-$^{14}$C into fatty acids in all of the tissues tested. Isocitrate was superior to citrate in supporting fatty acid synthesis in the liver which is in agreement with the results of Porter et al., (1957). However, with muscle, brain and kidney preparations citrate was the more effective.

Although isocitrate or citrate is required for active fatty acid synthesis from acetate, concentration beyond the optimum usually caused inhibition in both the liver and muscle systems. This type of inhibition by isocitrate has been shown by Porter et al., (1957). Bhaduri (1962) and Doering and Tarver (1965) have reported that citrate at the higher concentration also caused inhibition of acetate-$^{14}$C incorporation into fatty acids in a manner similar to that shown for muscle preparations in the present study. It thus appears
probable that at low concentrations of citrate acetyl-CoA carboxylase is stimulated, whereas at higher concentrations of citrate, either the carboxylase activity is inhibited or the overall synthesis is impaired through direct or indirect influence on the synthetase complex. An indirect effect could be manifested if citrate served as carbon source for fatty acid synthesis after being cleaved to acetyl-CoA by citrate cleavage enzyme. This would cause the radioactive acetyl-CoA pool to be diluted with the result that less incorporation of radioactivity into fatty acids would occur.

CoA is required for maximal fatty acid synthesis from acetate by muscle preparations; but in liver preparations the CoA requirement is less evident. In the latter system where the incorporation of labelled acetate into fatty acids was quite active it would appear that the endogenous CoA is sufficient to meet the requirement for CoA. The inhibitory effects observed in both preparations with CoA concentrations beyond the optimum have been reported previously by Brady et al., (1956) and by Jacob (1963).

In the studies reported on fatty acid synthesis from acetate, most workers have used Mg\(^{++}\) as the divalent cation. While studying the changes in the level of the fatty acid synthesizing enzymes during starvation using particle-free rat liver supernatant, Korchak and Masoro (1962) consistently obtained higher rates of \(^{14}\)C incorporation with Mn\(^{++}\) than with Mg\(^{++}\). They found Mn\(^{++}\) was specifically required
for the activation of acetyl-CoA carboxylase. Replacement of Mn$^{++}$ with Mg$^{++}$ resulted in much less activity. It was also found in the present study that Mn$^{++}$ was superior to Mg$^{++}$ in supporting the $^{14}$C incorporation into fatty acids. The addition of Mg$^{++}$ along with Mn$^{++}$ inhibited the effect of Mn$^{++}$ (Table XXIII). Consistent with this finding is the work of Matsuhashi, Matsuhashi and Lynen (1964) who found that maximum activity of highly purified rat liver acetyl-CoA carboxylase requires Mn$^{++}$ and that this requirement cannot be replaced by increasing the concentration of Mg$^{++}$ alone.

Gassing the incubation mixture with 100% CO$_2$ was found to stimulate markedly the acetate-2-$^{14}$C incorporation into fatty acids in all of the tissues studied (Table XXII). A four to eight fold increase in the incorporation of acetate into fatty acids by the yeast extract in the presence of CO$_2$ has been reported by Klein (1957). Doering and Tarver (1965) have shown that fatty acid synthesis from acetate-$^{14}$C by liver high speed supernatant fraction was dependent upon the partial pressure of CO$_2$ in the incubation system. The effect of CO$_2$ flushing in the present study is most likely related to the CO$_2$ requirement for the carboxylation of acetyl-CoA to form malonyl-CoA. However, it is somewhat puzzling why the incubation mixture which contains 20 umoles KHCO$_3$ still shows such a great requirement for CO$_2$-flushing to obtain maximum acetate-2-$^{14}$C incorporation. To explain this observation, the following suggestions can be made:
(1) \( CO_2 \)-flushing has a stimulatory effect because the concentration of \( KHCO_3 \) used may be below the optimum. (2) \( CO_2 \)-flushing may change the pH of the medium to the optimal pH for fatty acid synthesis and (3) \( CO_2 \) is a better molecular species than \( HCO_3^- \) in the mechanism of carboxylation of acetyl-CoA and hence the system becomes directly dependent on the \( CO_2 \) tension. The first explanation may be eliminated since flushing with \( CO_2 \) is still required to obtain good incorporation of acetate-\( ^{14}C \) even when the concentration of \( KHCO_3 \) is doubled (Table XXI). The second possibility is very unlikely since the lowering of the pH of the medium after \( CO_2 \)-flushing is about 0.10–0.15 pH units, which is not great enough to cause the difference observed in acetate-\( ^{14}C \) incorporation. This leaves the third suggestion as the likely explanation. It is, nevertheless, at variance with the current views which indicate that the molecular species involved in fatty acid synthesis from acetate is bicarbonate ion and not \( CO_2 \) (Longmore, Hastings and Harrison, 1964). However, the observations that \( CO_2 \)-flushing alone gives 82% stimulation and that the optimal system shows a great dependence on \( CO_2 \)-flushing even in the presence of high bicarbonate ion concentration raise the question of whether the mechanism of carboxylation of acetyl-CoA is understood well enough to exclude the direct participation of \( CO_2 \). Without further evidence it is concluded that complete explanation of the effect of \( CO_2 \)-flushing lies in a fuller understanding of the mechanism of carboxylation of acetyl-CoA by the acetyl-CoA carboxylase of the tissues studied.
Since a relatively large variation was encountered in the incorporation of acetate-2-\(^{14}\)C into fatty acids by muscle preparations (for normal mice the range was 2.0 to 7.0 \(\mu\)moles), the comparisons between dystrophic and normal mice were always made on the pair comparison basis. On this basis activity of the fatty acid synthetase of dystrophic muscle was shown to be increased \((P \leq 0.01, \text{ Table XXVI})\). Few reasons can be offered for this enhanced activity apart from the obvious one that dystrophic muscle in its atrophy retains more synthetase activity per mg of protein than normal. It should, however, be noted that Canal and Frattola (1962) have reported that in dystrophic muscle of mice the "Pentose phosphate pathway" was greatly increased and that McCaman (1963) has shown that the activity of NADP-linked enzymes were in general increased in the dystrophic muscle. These data suggest that the rate of formation of NADPH may be increased. Whether this can explain any part of the significant enhancement of fatty acid synthesis from labelled acetate \(\text{in vitro}\) is uncertain and can only be answered by further investigation. It has been reported by Coleman (1965) that in mouse dystrophic muscle triose phosphate accumulates because of decreased activity of glyceraldehyde-3-phosphate dehydrogenase. Howard and Lowenstein (1965) have reported that glycerophosphate stimulates fatty acid synthesis. If this situation occurs \(\text{in vivo}\), one could speculate that the accumulated triose phosphate could permit more \(\alpha\)-glycerophosphate to be formed and become available for glyceride biosynthesis. The latter could in turn stimulate fatty
acid synthesis by decreasing the available palmityl-CoA, since there is evidence that palmityl-CoA, the end product of fatty acid synthesis, is an inhibitor of its own synthesis (Numa, Bortz and Lynen, 1965).

The finding that acetate-\textsuperscript{14}C incorporation into fatty acids is lower in kidney and brain preparations of dystrophic mice compared with their normal littermates is not in agreement with that of Rabinowitz (1960) who found higher incorporation in these tissues of dystrophic mice. The discrepancy may be caused by the different incubation media and system used. Rabinowitz used 600 x g supernatant fraction while a high speed supernatant (71,000 x g) was used in the present study.
X. PALMITATE-1-14C METABOLISM IN VIVO

In addition to the in vitro studies of palmitic-1-14C oxidation by the tissues of dystrophic and normal mice, the in vivo metabolism of palmitic acid-1-14C was investigated. As will be described the results provide evidence which supports the conclusion that the overall oxidation of palmitate is intact in liver and in kidney of dystrophic mice. However, on the basis of the reduction in synthesis of labelled cholesterol from acetyl-CoA derived from the oxidation of palmitic acid-1-14C in the dystrophic muscle, it was concluded that the oxidation of this fatty acid is significantly impaired in dystrophic muscle compared to its littermate control.

Results

The fatty acid and cholesterol content in tissues from dystrophic and normal mice expressed in terms of μmoles/g and mg/g wet tissue weight are shown in Table XXVII. No difference in the tissue content of fatty acid and cholesterol for brain, liver and kidney was observed, but a large increase in fatty acid and cholesterol was found in the muscle from dystrophic mice.

In Table XXVIII are shown the total radioactivity (counts per min. per g. wet tissue) and specific activity (counts per min. μmole fatty acid) for the fatty acids from different tissues. The greatest
TABLE XXVII

Cholesterol and Fatty Acid Content of Tissues from
Dystrophic Mice and Their Littermate Controls (Strain 129)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Mouse</th>
<th>No.</th>
<th>Cholesterol mg/g. wet wt. ± S.E.M.</th>
<th>P</th>
<th>Fatty Acid µmoles/g. wet wt. ± S.E.M.</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>D</td>
<td>13</td>
<td>12.46 ± 0.61</td>
<td></td>
<td>114.0 ± 3.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>13</td>
<td>12.54 ± 0.67</td>
<td>N.S.</td>
<td>112.6 ± 3.5</td>
<td>N.S.</td>
</tr>
<tr>
<td>Liver</td>
<td>D</td>
<td>13</td>
<td>3.17 ± 0.18</td>
<td></td>
<td>228.0 ± 31.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>13</td>
<td>2.83 ± 0.19</td>
<td>N.S.</td>
<td>199.7 ± 19.1</td>
<td>N.S.</td>
</tr>
<tr>
<td>Kidney</td>
<td>D</td>
<td>13</td>
<td>3.39 ± 0.14</td>
<td></td>
<td>99.4 ± 7.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>13</td>
<td>3.50 ± 0.08</td>
<td>N.S.</td>
<td>96.7 ± 5.7</td>
<td>N.S.</td>
</tr>
<tr>
<td>Muscle</td>
<td>D</td>
<td>13</td>
<td>1.05 ± 0.035</td>
<td>.001</td>
<td>181.9 ± 15.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>13</td>
<td>0.58 ± 0.026</td>
<td>.001</td>
<td>92.8 ± 11.5</td>
<td></td>
</tr>
</tbody>
</table>

P = Probability that there is no effect caused by dystrophy
TABLE XXVIII

Incorporation of Palmitate-$1^{-14}$C into Fatty Acid Fractions of Lipids from Tissues of Dystrophic Mice and Their Littermate Controls (Strain 129)

(Mice were injected intraperitoneally with 0.6 \mu c palmitate-$1^{-14}$C per g. body weight 60 min. prior to sacrifice. Fatty acids were isolated as described in METHODS)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Mouse</th>
<th>No.</th>
<th>Total Activity</th>
<th>Specific Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Counts/min/g. x 10^3 ± S.E.M.</td>
<td>Counts/min/\mu mole F.A. ± S.E.M.</td>
</tr>
<tr>
<td>Brain</td>
<td>D</td>
<td>13</td>
<td>23.0 ± 3.5</td>
<td>208 ± 22</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>13</td>
<td>23.5 ± 2.8</td>
<td>209 ± 14</td>
</tr>
<tr>
<td>Liver</td>
<td>D</td>
<td>13</td>
<td>3,361 ± 320</td>
<td>17,250 ± 2,300</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>13</td>
<td>3,850 ± 199</td>
<td>20,710 ± 1,550</td>
</tr>
<tr>
<td>Kidney</td>
<td>D</td>
<td>13</td>
<td>347 ± 48</td>
<td>4,101 ± 668</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>13</td>
<td>445 ± 33</td>
<td>4,756 ± 452</td>
</tr>
<tr>
<td>Muscle</td>
<td>D</td>
<td>13</td>
<td>131 ± 15.7</td>
<td>792 ± 118</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>13</td>
<td>98 ± 10.4</td>
<td>1,336 ± 266</td>
</tr>
</tbody>
</table>

N.S.
activity was found in liver, followed by kidney, muscle and brain. There were no differences in both total radioactivity and specific activity between normal and dystrophic mice.

In order to determine if the injected palmitic acid–l–$^{14}$C was incorporated into different lipid fractions, total lipids from liver and muscle of dystrophic and normal mice were separated on a Florisil column as described by Carrol (1961) or on silica gel TLC using as solvent system; n-hexane: ether: acetic acid: methanol (90:20:2:3). In the tissues examined most of the radioactivity (70%) was found in the triglyceride fraction and the rest of the radioactivity was recovered in the other lipid fractions including phospholipids and free fatty acids.

Total radioactivity and specific activity (counts per min. per mg cholesterol) for cholesterol of different tissues are shown in Table XXIX. A significant reduction in total radioactivity and specific activity were found for liver, kidney and muscle of dystrophic mice, no difference was found for brain.

The radioactivity appearing in the plasma following the intraperitoneal injection of palmitate–l–$^{14}$C was the same for both dystrophic and normal mice (Jato, 1969) which suggests that the pool of radioactivity available is not different between the two groups of animals.
TABLE XXIX

Incorporation of Palmitate-1-\textsuperscript{14}C into Cholesterol from Tissues of

Dystrophic Mice and Their Littermate Controls

(Details as in Table XXVIII.
Cholesterol was isolated and determined as described in METHODS)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Mouse</th>
<th>No.</th>
<th>Total Activity Counts/min/g. ± S.E.M.</th>
<th>P</th>
<th>Specific Activity Counts/min/mg. Chol. ± S.E.M.</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>D</td>
<td>13</td>
<td>372 ± 42</td>
<td></td>
<td>30.8 ± 3.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>13</td>
<td>397 ± 37</td>
<td>N.S.</td>
<td>30.7 ± 3.5</td>
<td>N.S.</td>
</tr>
<tr>
<td>Liver</td>
<td>D</td>
<td>13</td>
<td>971 ± 111</td>
<td>.01</td>
<td>329 ± 89</td>
<td>.005</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>13</td>
<td>6,228 ± 1,584</td>
<td>.01</td>
<td>2,332 ± 612</td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>D</td>
<td>13</td>
<td>239 ± 32</td>
<td>.001</td>
<td>55.9 ± 9.3</td>
<td>.001</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>13</td>
<td>547 ± 58</td>
<td>.001</td>
<td>139.6 ± 16</td>
<td>.001</td>
</tr>
<tr>
<td>Muscle</td>
<td>D</td>
<td>13</td>
<td>35 ± 6</td>
<td>0.05</td>
<td>36.2 ± 6.5</td>
<td>.005</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>13</td>
<td>63 ± 12</td>
<td></td>
<td>104.9 ± 17.2</td>
<td></td>
</tr>
</tbody>
</table>
Discussion

Following palmitate-1-\(^{14}\)C injection, Jato (1969) has shown that the radioactivity appearing in the respiratory \(\text{CO}_2\) was no different between normal and dystrophic mice. Consistent with this finding is the work of Baker et al., (1958, 1964) who injected labelled acetate and glucose into normal and dystrophic mice and found no difference in the production of \(^{14}\)CO\(_2\). These results suggest that the Krebs cycle is probably intact in liver and kidney. This conclusion is further supported by the in vitro finding in Chapter IV that there was no difference in the ability of liver and kidney to oxidize \(^{14}\)Cpalmitate-1-\(^{14}\)C to \(^{14}\)CO\(_2\).

The reductions observed in the incorporation of radioactivity from palmitate-1-\(^{14}\)C into cholesterol were highly significant for liver (\(P \leq 0.005\)), kidney (\(P \leq 0.001\)) and muscle (\(P \leq 0.05\)) of dystrophic mice. This decrease in incorporation of label from palmitate into cholesterol might suggest a block in the degradation of fatty acid to acetate or the synthetic pathway from acetate to cholesterol is less active in dystrophic mice. The former possibility is apparently eliminated on the basis of the normal production of respiratory \(^{14}\)CO\(_2\) in dystrophic mice.

The low activity in the fatty acids of brain no doubt reflects the difficulty fatty acids have in penetrating into this tissue.
Accordingly, there is little conversion of label into cholesterol. It is possible that some of the label in cholesterol of brain may arise from the redistribution of cholesterol synthesized in other tissues such as liver.

It is interesting to note that the total radioactivity in the fatty acid of dystrophic muscle is greater than that of normal muscle, whereas the total radioactivity in cholesterol of the muscle is significantly more in the normal muscle than in the dystrophic (Table XXIX). This pattern is consistent with the interpretation that, even if the availability of palmitate-1-\(^{14}\)C to dystrophic and normal muscle cells is equal, there is a reduction in the oxidation of palmitate. This reduction could then account for the observed decrease in incorporation of label into cholesterol.
XI. DISCUSSION

It would seem most appropriate to begin this discussion using the scheme which appears in Fig. 26. This scheme describes in summary form the pathways concerned in fatty acid metabolism of skeletal muscle of the mouse (strain 129) and indicates the percentage changes caused by dystrophy of certain of the pathways or enzyme activities involved. In the scheme the two shaded areas represent the muscle cell membrane and the mitochondrial barrier. The latter barrier is impermeable to acetyl-CoA and palmitoyl-CoA and the passage of acetyl and palmitoyl groups requires the participation of carnitine acetyltransferase and carnitine palmitoyltransferase respectively (Fritz and Yue, 1964; Fritz and Yue, 1963). The evidence favors the view that the de novo synthesis of fatty acids (e.g. palmitic acid) occurs in the cytoplasm via the malonyl-CoA pathway. The rate of fatty acid synthesis from acetate-$\text{C}^{14}$ is increased by 46% in dystrophic muscle preparations (71,000 x g supernatant) studied. The activation of palmitic acid (palmitoyl-CoA synthetase) occurs in both the cytoplasm and mitochondria. The activity of the cytoplasmic enzyme is increased by 39% in the dystrophic muscle preparation, whereas the activity of the mitochondrial enzyme in the dystrophic muscle is no different from that of the normal. The activity of carnitine palmitoyltransferase, measured by the release of carnitine-Me-$\text{C}^{14}$ from palmitylcarnitine-Me-$\text{C}^{14}$ during the incubation, was the same in the two groups of animals.
Fatty acid metabolism in skeletal muscle

Fig. 26 Scheme showing the pathways of metabolism for fatty acids (e.g. palmitate) in skeletal muscle.
However, the overall oxidation of palmitate-1-\(^{14}\)C by muscle mitochondria is decreased by 35% in the dystrophic mice. Furthermore, the oxidation of pyruvate-3-\(^{14}\)C was also decreased by the same magnitude in the dystrophic muscle mitochondria, indicating the possibility that a common defect probably exists in the Krebs cycle. This view is further supported by the observation that the oxidation of acetyl-1-\(^{14}\)C carnitine to \(^{14}\)CO\(_2\) is greatly reduced (82%). This large decrease in acetyl-1-\(^{14}\)C carnitine oxidation may, in part, be explained by a decrease in the activity of carnitine acetyltransferase (Jato, 1969). It is concluded from the results obtained that one or more of the steps of the Krebs cycle may be defective in the skeletal muscle mitochondria of dystrophic mice. More detailed discussion of the results obtained for each series of experiments has been presented previously in the appropriate Chapter. A number of specific points now deserve additional general discussion.

A. **Skeletal Muscle Mitochondria Of Mice (Strain 129)**

The method which was used to isolate skeletal muscle mitochondria from mice in this study gave a mitochondrial yield significantly higher for normal than for dystrophic mice. This observation is at variance with that of Wrogemann and Blancheer (1967) who obtained the same yield of mitochondria from both groups of animals. The mitochondrial preparations obtained in the present study showed a
small amount of vesicle contamination under the electron microscope. The respiratory control ratio was about 2.5 which is only one-third of that reported by Wrogemann and Blanckaer (1967). Using the author's method to isolate the mitochondria, Blanckaer (private communication, 1968) has observed that these mitochondrial preparations were partially uncoupled. The different values obtained for the respiratory control ratio of these two mitochondrial preparations probably reflect certain differences in methodology. One important factor may be the different concentrations of Nagarse proteinase used in the isolation medium. It is conceivable that the higher concentration of Nagarse proteinase used by Wrogemann and Blanckaer (1967) may have destroyed the abnormal mitochondria and left behind only the more "normal" mitochondria during the preparation of mitochondria from dystrophic muscle. In addition, the mitochondrial membrane enzymes such as carnitine palmitoyltransferase and palmytyl-CoA synthetase may be inactivated or removed by a high concentration of Nagarse proteinase. This suggestion is supported by the observation that the mitochondria prepared either by the method of Wrogemann and Blanckaer (1967) or by our method in the presence of the same amount of Nagarse proteinase (0.5 mg/1 ml) used by the above authors failed to oxidize palmitic acid-1-14C. However, as described in Chapter IV, the mitochondria that were isolated by the present method using low concentration of Nagarse proteinase (1 mg/30 ml) under optimal conditions were able to oxidize palmitate-1-14C. It is possible that the oxidation of palmitate was due, in part, to
the contamination by residues which may contain enzymes for the
activation of palmitate. Further investigation is required to clarify
the difference between the above two preparations.

In the process of development of muscular dystrophy in mice,
and particularly in the more advanced stages of the disease, the
affected muscle fibers are known to be replaced by fat and fibrous
connective tissues. The affected muscle may also be infiltrated by
invading macrophages. This raises the very real possibility that the
cell population obtained from dystrophic muscle is not as homogenous
as that obtained from normal muscle. It, therefore, is probable that
some of the mitochondria isolated from the dystrophic muscle of mice
arise from cells (e.g. from macrophages and fat cells) other than those
of muscle. If the mitochondria contributed by non-muscle cells possess
either a higher or lower fatty acid oxidation activity per mg of protein
than the mitochondria of muscle cells, then the activities reported for
dystrophic muscle will be either too high or too low, the amount being
proportional to the contribution of mitochondria from cells other than
muscle. It may be concluded that a portion of the defect in oxidation
described for mitochondria isolated from dystrophic muscle is either
due to or being masked by the contributions of mitochondria from cells
of non-muscular origin. It has not been possible to make any correction
for this effect which, it should be pointed out, is a chronic problem
in all studies of this kind on pathological material.
B. Possible Involvement of Carnitine Acetyltransferase in Fatty Acid Oxidation

The physiological role of carnitine acetyltransferase is not known. It has been suggested that this enzyme is responsible for the transfer of acetyl groups generated within the mitochondria to the cytosol, where acetyl groups can be used for fatty acid synthesis (Bressler and Katz, 1965; Fritz, 1965), acetylcarnitine formation (Bremer, 1962), and the generation of acetylcholine (Thomitzek and Strack, 1964).

Very recently Brdiczka, Gerbitz and Pette (1969) have reported that there are two kinds of carnitine acetyltransferase in the mitochondria of liver and kidney, one in the outer membrane compartment and the other in the inner membrane compartment. To explain the role of these two enzymes the authors suggested that two acetyl-CoA pools exist, one inside the inner membrane and the other outside. The inner membrane is a barrier to acetyl-CoA whereas the outer membrane is not. The main function of these two carnitine acetyltransferases is to transfer acetyl-CoA generated in the cytosol across the inner membrane of the mitochondria where the Krebs cycle enzymes are located.

Based on the observations obtained from the studies on gluconeogenesis and ketogenesis, Fritz (1968) has recently proposed the existence of more than one pool of acetyl-CoA. This "two acetyl-CoA pool" hypothesis is shown in Fig. 27. The hypothesis postulates a role for carnitine acetyltransferase which, if valid, would permit
ONE ACETYL CoA POOL HYPOTHESIS

![Diagram of one acetyl-CoA pool hypothesis]

TWO ACETYL CoA POOL HYPOTHESIS

![Diagram of two acetyl-CoA pool hypothesis]

Fig. 27  The "one acetyl-CoA pool" versus "the two acetyl-CoA pool" hypothesis (after Fritz, 1968).
the translocation of acetyl-CoA within the acetyl-CoA pools. In the hypothesis it is contended that carnitine acetyltransferase provides the mechanism whereby acetyl group transfer occurs. In support of this idea is the observation that the oxidation of palmitate-1-\textsuperscript{14}C to \textsuperscript{14}CO\textsubscript{2} was inhibited by (+)-acetylcarnitine, a specific inhibitor of carnitine acetyltransferase, while the incorporation of isotope into the carboxyl groups of acetoacetate was increased (Fritz, 1968). Moreover, the concentration of (+)-acetylcarnitine used in the above experiment did not inhibit mitochondrial respiration in the presence of pyruvate or palmitate nor did it inhibit carnitine palmityltransferase. From this evidence it is possible that carnitine acetyltransferase may be involved in fatty acid oxidation to CO\textsubscript{2}. Thus it is possible that the decrease in palmitate-1-\textsuperscript{14}C oxidation in dystrophic muscle mitochondria may be in part due to the decreased activity of carnitine acetyltransferase.

C. **Dilution of Radioactive Substrates**

Researchers often encounter the problem of "pool size" dilution when studying the metabolism of a substrate. This is particularly true where normal and abnormal tissues are compared as in the case of muscular dystrophy. Unless the size of the substrate pools in both normal and dystrophic muscle is known, or can be determined with its necessary correction factor applied, the conclusion drawn
may well be misleading. In the study presented in Chapter IV on the oxidation of palmitate-$l^{14}C$ by 600 x g supernatant of muscle homogenate, there is little doubt from total and free fatty acid analyses that differences exist in the fatty acid pools of dystrophic and normal muscle preparations and that the dilution of radioactive palmitic acid would be different for the two preparations. From the studies of Young et al. (1959) the endogenous free fatty acid concentration in dystrophic muscle preparation was about two times greater than in normal muscle. Under these circumstances the maximum correction factor would be about 2.0. Before any correction the oxidation of palmitic acid-$l^{14}C$ to $^{14}CO_2$ by dystrophic muscle preparations was about 20% of the normal value. If a maximum dilution correction of two times is applied, this would raise the value to 40% of the normal. In the experiments with mitochondria free fatty acid levels are apt to be low and therefore the dilution of substrate would be minimal.

D. **Lipid Accumulation in Dystrophic Muscle**

As already mentioned, the oxidation of palmitate-$l^{14}C$ and acetyl-$l^{14}C$ carnitine is significantly decreased in the mitochondria from dystrophic muscle. The activity of carnitine acetyltransferase was also decreased in these mitochondria. In contrast, the activities of mitochondrial palmitoyl-CoA synthetase and carnitine palmitoyltransferase were all at normal levels, while de novo synthesis of
fatty acid from acetate and the activity of cytoplasmic palmitoyl-CoA synthetase were significantly increased in dystrophic preparations. Furthermore, triosephosphate has been reported to accumulate during incubation of dystrophic muscle homogenates (Coleman, 1965) and the operation of the "pentose phosphate pathway" in the dystrophic preparation was shown to be increased (Canal and Frattola, 1962). All these changes, if they exist in vivo, would favor an enhancement of the biosynthesis of triglyceride. This pattern is consistent with the accumulation of triglyceride in the muscle of dystrophic animals and man.

A recent report by Susheela et al. (1968) has shown that the free fatty acid content in the tissues of dystrophic mice was in general higher than that in the tissues of their littermate controls with the exception of adipose tissue in which a lower value for dystrophic mice was observed. Since adipose tissue has been considered to be the major site of lipid storage and mobilization, a decrease in free fatty acid content in the adipose tissue of dystrophic mice does suggest that free fatty acid may be over-mobilized from adipose tissue of dystrophic mice to other tissues such as skeletal muscle.

E. The "Primary Defect" in Muscular Dystrophy

Whether the decrease in the oxidation of palmitate-$1^{-14}$C, pyruvate-$3^{-14}$C and acetyl-$1^{-14}$C carnitine and the increase in fatty
acid synthesis from acetate and in the activity of cytoplasmic palmityl-CoA synthetase reflects a primary or secondary defect deserves some discussion. In the context used, the term "primary defect" refers to the defect (in metabolism) which is responsible for development of the muscle weakness and degeneration and the secondary defect refers to the biochemical alterations that follow action of the primary defect. It is difficult to say whether the abnormalities relating to fatty acid metabolism and pyruvate metabolism are primary or secondary. In the course of this study, it was frequently found that fairly large variations occur, especially in the dystrophic mice. For example, the percentage decrease in palmitic acid-$^{14}$C oxidation by skeletal muscle mitochondria from dystrophic mice varied from one pair of animals to another. Although most values fell within a fairly small range (40-60%), a few values, as low as 30% and as high as 100% of the normal level, were observed. These variations seemed, in part, to parallel observed differences in the degree of severity of muscular dystrophy in the dystrophic mice. Unfortunately, the observed differences are rather subjective and at present there are no good criteria to determine accurately the severity of the disease in dystrophic mice. If the defect is primary, one would expect that all of the mitochondria isolated from dystrophic muscle would show a decreased ability to oxidize palmitate-$^{14}$C at the level of normal muscle mitochondria.
However, this has not been observed. For this reason, the author is of the opinion that the above mentioned defects are likely secondary.

It is probably fair to say that the ultimate hope and prime motive of all research in the muscular dystrophy of man and animals is to seek and delineate the primary defect. Once this is done it will be possible to develop more suitable methods of treatment of the disease. Up to date all the investigations conducted, either on biopsy samples from dystrophic patients or on animals, have failed to reveal what can be described as the primary defect. Although a defect in the Krebs cycle of dystrophic muscle which the author has proposed is likely secondary, the possibility does exist that the defect is primary or very closely related to the primary defect. The author, as a working hypothesis, would like to speculate on the implications of the primary defect being related to a mitochondrial abnormality such as that described. If it is considered that a vital component of either the mitochondrial membrane or of the mitochondrial oxidative enzyme systems (e.g. the Krebs cycle enzymes) is affected, then the aerobic generation of ATP will be insufficient to meet the requirements of the overall cellular metabolism and activity (e.g. muscle contraction). A chronic defect of this type could result in the disorganization of the entire cell structure of the kind that is observed in Duchenne muscular dystrophy and the muscular dystrophy of mice (strain 129).
XII. SUMMARY AND CONCLUSION

1. The oxidation of palmitate-$1^{14}$C by skeletal muscle homogenates and mitochondria from dystrophic mice (Bar Harbor Strain 129) and their littermate controls has been studied. The oxidation of palmitate-$1^{14}$C required the presence of ATP, carnitine, CoA and a Krebs cycle intermediate (e.g. succinate). The oxidation of palmitate-$1^{14}$C to $1^{14}$CO$_2$ by muscle homogenates and mitochondria from dystrophic mice was found to be significantly lower than that of the littermate controls.

2. The activities of palmitoyl-CoA synthetase in both skeletal muscle mitochondria and 9000 x g supernatant fraction have been determined. It was found that the activity of mitochondrial palmitoyl-CoA synthetase obtained from the skeletal muscle of dystrophic mice was not different from normal, whereas the activity of palmitoyl-CoA synthetase in the 9000 x g supernatant fraction of muscle from dystrophic mice was significantly higher than that of the littermate controls.

3. The activity of skeletal muscle mitochondrial carnitine palmitoyltransferase was determined using palmitoylcarnitine-Me-$1^{14}$C as substrate. The enzyme activity was dependent upon the addition of CoA to the
incubation system. There was no difference in the activity of this enzyme between dystrophic and normal mice.

4. The oxidation of acetyl-1-\(^{14}\)C carnitine by mouse skeletal muscle mitochondria was studied. The oxidation required the presence of a Krebs cycle intermediate (e.g. malate) and ADP. No CoA is required for the oxidation. The oxidation of acetyl-1-\(^{14}\)C carnitine by skeletal muscle mitochondria from dystrophic mice was greatly decreased (82%) compared with that of their littermate controls.

5. Decarboxylation of pyruvate-1-\(^{14}\)C by mouse skeletal muscle mitochondria was studied. The decarboxylation of pyruvate-1-\(^{14}\)C was stimulated 200-400% by carnitine. In the presence of carnitine, the ability of skeletal muscle mitochondria from dystrophic mice to decarboxylate pyruvate-1-\(^{14}\)C was lower than that of the normal skeletal muscle of mice. In the absence of carnitine, the difference was not significant, but a decreasing trend was evident in the dystrophic mice. The oxidation of pyruvate-3-\(^{14}\)C was also investigated. It was found that pyruvate-3-\(^{14}\)C oxidation was significantly decreased in the skeletal muscle mitochondria of dystrophic mice.

6. Fatty acid synthesis from acetate-2-\(^{14}\)C by high speed supernatant was studied in four tissues of mice. The cofactors required for optimum incorporation of acetate-2-\(^{14}\)C into fatty acids
were ATP, citrate (or isocitrate), cysteine, Mn\(^{++}\), NADP\(^+\) and a CO\(_2\) atmosphere (100%). The synthesis of fatty acid from acetate-2-\(^{14}\)C was significantly increased in the muscle and the liver of dystrophic mice, whereas in brain and kidney preparations of dystrophic mice this synthesis was significantly lowered.

7. Study of the in vivo metabolism of palmitate-1-\(^{14}\)C was also carried out. Except for muscle where significant increases were noted, the cholesterol and fatty acid contents of the liver, kidney and brain were not significantly different between dystrophic and normal mice. When tissues from normal and dystrophic mice were compared, no difference was observed for the incorporation of palmitate-1-\(^{14}\)C into the esterified fatty acid fraction in brain, liver, kidney and skeletal muscle. However, the incorporation of palmitate-1-\(^{14}\)C into cholesterol was significantly decreased in liver, kidney and muscle from dystrophic mice.

8. It was concluded that the decreases in the oxidation of palmitate-1-\(^{14}\)C, acetyl-1-\(^{14}\)C carnitine and pyruvate-3-\(^{14}\)C are most likely due to a defect either in the activation and transport of acetyl groups into the mitochondria or in one or more of the steps of the Krebs cycle. The increase in fatty acid synthesis from acetate-2-\(^{14}\)C and in the activity of cytoplasmic palmityl-CoA synthetase, together with the decrease in palmitate-1-\(^{14}\)C oxidation
in the dystrophic muscle preparations may help to explain the lipid accumulation that is observed in dystrophic muscle of mice and perhaps man.
REFERENCES


Garland, P.B. & Randle, P.J. (1964). Control of pyruvate dehydrogenase in the perfused rat heart by the intracellular concentration of acetyl-CoA. Biochem. J. 91: 6C-7C.


Stevenson, E. (1968). The carnitine-independent oxidation of palmitate plus malate by moth flight muscle mitochondria. 


APPENDIX A

Palmitic Acid-$^{14}$C Oxidation by Low Speed Supernatant from Muscle of Dystrophic Mice (D) and Their Littermate Controls (C)

<table>
<thead>
<tr>
<th>Pair Number</th>
<th>$^{14}$CO$_2$ Released (dpm per mg protein)</th>
<th>Percent of Control (D/C x 100)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D</td>
<td>C</td>
</tr>
<tr>
<td>1</td>
<td>45</td>
<td>1145</td>
</tr>
<tr>
<td>2</td>
<td>62</td>
<td>4953</td>
</tr>
<tr>
<td>3</td>
<td>308</td>
<td>1271</td>
</tr>
<tr>
<td>4</td>
<td>627</td>
<td>4022</td>
</tr>
<tr>
<td>5</td>
<td>980</td>
<td>3555</td>
</tr>
<tr>
<td>6</td>
<td>309</td>
<td>2029</td>
</tr>
<tr>
<td>7</td>
<td>4423</td>
<td>7677</td>
</tr>
</tbody>
</table>
APPENDIX B

The Oxidation of Palmitic Acid-1$^{14}$C by Mitochondria from Muscle of Dystrophic Mice (D) and Their Littermate Controls (C)

<table>
<thead>
<tr>
<th>C$_4$ Dicarboxylic</th>
<th>Number of Pairs</th>
<th>Oxidation (mymoles Palmitate-1$^{14}$C oxidized as $^{14}$CO$_2$ mg protein)</th>
<th>D/C x 100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Succinate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>34.17</td>
<td>80.10</td>
<td>42.6</td>
</tr>
<tr>
<td>2</td>
<td>42.85</td>
<td>74.30</td>
<td>57.6</td>
</tr>
<tr>
<td>3</td>
<td>60.30</td>
<td>102.65</td>
<td>58.8</td>
</tr>
<tr>
<td>4</td>
<td>40.72</td>
<td>74.59</td>
<td>54.5</td>
</tr>
<tr>
<td>5</td>
<td>60.24</td>
<td>102.48</td>
<td>58.8</td>
</tr>
<tr>
<td>6</td>
<td>45.71</td>
<td>90.72</td>
<td>50.4</td>
</tr>
<tr>
<td>7</td>
<td>40.00</td>
<td>58.64</td>
<td>68.2</td>
</tr>
<tr>
<td>8</td>
<td>57.42</td>
<td>100.70</td>
<td>54.</td>
</tr>
<tr>
<td>9</td>
<td>39.57</td>
<td>114.24</td>
<td>37</td>
</tr>
<tr>
<td>10</td>
<td>44.59</td>
<td>102.65</td>
<td>43.5</td>
</tr>
<tr>
<td>11</td>
<td>56.34</td>
<td>69.30</td>
<td>81.2</td>
</tr>
<tr>
<td>12</td>
<td>87.33</td>
<td>66.75</td>
<td>131</td>
</tr>
<tr>
<td>Malate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>50.18</td>
<td>107.20</td>
<td>48.3</td>
</tr>
<tr>
<td>2</td>
<td>75.47</td>
<td>95.79</td>
<td>78</td>
</tr>
<tr>
<td>3</td>
<td>88.93</td>
<td>89.82</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>93.41</td>
<td>90.21</td>
<td>103.5</td>
</tr>
<tr>
<td>5</td>
<td>42.96</td>
<td>83.07</td>
<td>51.7</td>
</tr>
<tr>
<td>6</td>
<td>27.10</td>
<td>91.71</td>
<td>29.4</td>
</tr>
<tr>
<td>7</td>
<td>97.60</td>
<td>104.91</td>
<td>93</td>
</tr>
<tr>
<td>8</td>
<td>45.73</td>
<td>90.74</td>
<td>50.4</td>
</tr>
</tbody>
</table>
APPENDIX C

Activity of Mitochondrial Palmitoyl-CoA Synthetase of Skeletal Muscle from Normal (C) and Dystrophic Mice (D)

<table>
<thead>
<tr>
<th>Pair No.</th>
<th>Palmitoyl-CoA Synthetase (μmoles Palmitoyl-carnitine-Me-14C formed)</th>
<th>D/C x 100</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 min.</td>
<td>10 min.</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>C</td>
</tr>
<tr>
<td>1</td>
<td>127</td>
<td>119.7</td>
</tr>
<tr>
<td>2</td>
<td>101.6</td>
<td>100.3</td>
</tr>
<tr>
<td>3</td>
<td>104.8</td>
<td>112.8</td>
</tr>
<tr>
<td>4</td>
<td>113.3</td>
<td>120.2</td>
</tr>
<tr>
<td>5</td>
<td>87</td>
<td>88</td>
</tr>
<tr>
<td>6</td>
<td>90</td>
<td>104.9</td>
</tr>
</tbody>
</table>
APPENDIX D

Palmityl-CoA Synthetase Activity in the 9000 x g Supernatant of Skeletal Muscle from Dystrophic (D) and Normal Mice (C)

<table>
<thead>
<tr>
<th>Pair Number</th>
<th>Palmityl-CoA Synthetase Activity (mmoles palmitylcarnitine-Me-14C formed per mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D</td>
</tr>
<tr>
<td></td>
<td>2.5' 5.0' 10'</td>
</tr>
<tr>
<td>1</td>
<td>21 44.4 75.6 12.3 28.3</td>
</tr>
<tr>
<td>2</td>
<td>16.6 38 67.4 12.2 28.6</td>
</tr>
<tr>
<td>3</td>
<td>14.2 36.5 58.6 10.1 25.3</td>
</tr>
<tr>
<td>4</td>
<td>14.8 27 - 10.8 21.6</td>
</tr>
<tr>
<td>5</td>
<td>22.2 37.6 54.7 11.7 21.4</td>
</tr>
<tr>
<td>6</td>
<td>- 41.6 77.8 - 39.9</td>
</tr>
<tr>
<td>7</td>
<td>9.6 29</td>
</tr>
<tr>
<td>8</td>
<td>13.6 40.5</td>
</tr>
<tr>
<td>9</td>
<td>10.2 26.1</td>
</tr>
<tr>
<td>10</td>
<td>10.1 -</td>
</tr>
</tbody>
</table>

2.5', 5.0' and 10' represent 2.5, 5.0 and 10 minutes
APPENDIX E

Activity of Mitochondrial Carnitine Palmitoyltransferase of Skeletal Muscle from Normal (C) and Dystrophic Mice (D)

<table>
<thead>
<tr>
<th>Pair number</th>
<th>Carnitine palmitoyltransferase hydrolyzed (μmoles palmitylcarnitine-Me(^{14})C)</th>
<th>D/C x 100</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>30 sec</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D</td>
</tr>
<tr>
<td>30 seconds</td>
<td></td>
<td>90 seconds</td>
</tr>
<tr>
<td>1</td>
<td>11.04</td>
<td>24.10</td>
</tr>
<tr>
<td>2</td>
<td>10.70</td>
<td>21.10</td>
</tr>
<tr>
<td>3</td>
<td>10.60</td>
<td>23.50</td>
</tr>
<tr>
<td>4</td>
<td>12.53</td>
<td>22.15</td>
</tr>
<tr>
<td>5</td>
<td>16.90</td>
<td>29.45</td>
</tr>
<tr>
<td>6</td>
<td>14.42</td>
<td>25.60</td>
</tr>
<tr>
<td>7</td>
<td>12.10</td>
<td>21.40</td>
</tr>
</tbody>
</table>
APPENDIX F

Acetyl-1-$^{14}$C Carnitine Oxidation by Skeletal Muscle

Mitochondria from Dystrophic (D) and Normal Mice (C)

<table>
<thead>
<tr>
<th>No. of Pairs</th>
<th>Acetyl-1-$^{14}$C carnitine oxidized (µmoles per mg mitochondrial protein)</th>
<th>D/C x 100</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D</td>
<td>C</td>
</tr>
<tr>
<td>1</td>
<td>220</td>
<td>581</td>
</tr>
<tr>
<td>2</td>
<td>13.1</td>
<td>498</td>
</tr>
<tr>
<td>3</td>
<td>55</td>
<td>429</td>
</tr>
<tr>
<td>4</td>
<td>68.6</td>
<td>532</td>
</tr>
<tr>
<td>5</td>
<td>235</td>
<td>445</td>
</tr>
<tr>
<td>6</td>
<td>25</td>
<td>613</td>
</tr>
<tr>
<td>7</td>
<td>180</td>
<td>589</td>
</tr>
<tr>
<td>8</td>
<td>22</td>
<td>387</td>
</tr>
<tr>
<td>9</td>
<td>11.4</td>
<td>408</td>
</tr>
</tbody>
</table>
APPENDIX G

Pyruvate-1-\(^{14}\)C Decarboxylation by Skeletal Muscle

Mitochondria of Normal (C) and Dystrophic Mice (Strain 129)
in the Presence of DL-Carnitine

<table>
<thead>
<tr>
<th>No. of Pairs</th>
<th>Pyruvate-1-(^{14})C decarboxylation (m(\mu)moles per mg mitochondrial protein)</th>
<th>D/C x 100</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>D</strong></td>
<td><strong>C</strong></td>
</tr>
<tr>
<td>1</td>
<td>281</td>
<td>592</td>
</tr>
<tr>
<td>2</td>
<td>444</td>
<td>618</td>
</tr>
<tr>
<td>3</td>
<td>223</td>
<td>550</td>
</tr>
<tr>
<td>4</td>
<td>415</td>
<td>570</td>
</tr>
<tr>
<td>5</td>
<td>95</td>
<td>824</td>
</tr>
<tr>
<td>6</td>
<td>181</td>
<td>461</td>
</tr>
<tr>
<td>7</td>
<td>430</td>
<td>450</td>
</tr>
<tr>
<td>8</td>
<td>470</td>
<td>760</td>
</tr>
</tbody>
</table>
APPENDIX H

Pyruvate-3-\(^{14}\)C Oxidation by Mitochondria from Muscle of Dystrophic Mice (D) and their Littermate Controls (C)

<table>
<thead>
<tr>
<th>Pair number</th>
<th>Pyruvate-3-(^{14})C oxidation (mmoles oxidized per mg protein)</th>
<th>D/C x 100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Succinate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>288.5</td>
<td>285.2</td>
</tr>
<tr>
<td>2</td>
<td>146.6</td>
<td>217.5</td>
</tr>
<tr>
<td>3</td>
<td>216.48</td>
<td>499.5</td>
</tr>
<tr>
<td>4</td>
<td>209.80</td>
<td>426.5</td>
</tr>
<tr>
<td>5</td>
<td>113.65</td>
<td>342.4</td>
</tr>
<tr>
<td>6</td>
<td>182.72</td>
<td>265.96</td>
</tr>
<tr>
<td>7</td>
<td>199</td>
<td>288</td>
</tr>
<tr>
<td>Malate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>131.47</td>
<td>361</td>
</tr>
<tr>
<td>9</td>
<td>195.65</td>
<td>320</td>
</tr>
<tr>
<td>10</td>
<td>201.9</td>
<td>272.5</td>
</tr>
<tr>
<td>11</td>
<td>283</td>
<td>464.2</td>
</tr>
<tr>
<td>12</td>
<td>332</td>
<td>365</td>
</tr>
<tr>
<td>13</td>
<td>336.26</td>
<td>372.9</td>
</tr>
<tr>
<td>14</td>
<td>161.9</td>
<td>254.9</td>
</tr>
<tr>
<td>15</td>
<td>113.4</td>
<td>418</td>
</tr>
<tr>
<td>16</td>
<td>154.1</td>
<td>300.8</td>
</tr>
</tbody>
</table>
## APPENDIX I

**Incorporation of Acetate-2-$^{14}$C into Fatty Acids by High Speed Supernatant of Muscle Homogenate from Dystrophic Mice (D) and their Littermate Control (C)**

<table>
<thead>
<tr>
<th>Fatty acid synthesis (mMoles acetate-2-$^{14}$C incorporated per mg protein)</th>
<th>D/C x 100</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pair Numbers</strong></td>
<td><strong>D</strong></td>
</tr>
<tr>
<td>1</td>
<td>4.82</td>
</tr>
<tr>
<td>2</td>
<td>3.70</td>
</tr>
<tr>
<td>3</td>
<td>5.08</td>
</tr>
<tr>
<td>4</td>
<td>3.03</td>
</tr>
<tr>
<td>5</td>
<td>2.86</td>
</tr>
<tr>
<td>6</td>
<td>2.76</td>
</tr>
<tr>
<td>7</td>
<td>2.56</td>
</tr>
<tr>
<td>8</td>
<td>7.05</td>
</tr>
<tr>
<td>9</td>
<td>4.98</td>
</tr>
<tr>
<td>10</td>
<td>3.50</td>
</tr>
<tr>
<td>11</td>
<td>5.61</td>
</tr>
<tr>
<td>12</td>
<td>3.62</td>
</tr>
<tr>
<td>13</td>
<td>3.23</td>
</tr>
<tr>
<td>14</td>
<td>6.56</td>
</tr>
</tbody>
</table>
APPENDIX J

Fatty Acid Synthesis from Acetate-2-$^{14}$C by High Speed Supernatant (71,000 x g) of Liver Homogenate from Dystrophic (D) and Normal Mice (C)

<table>
<thead>
<tr>
<th>Pair number</th>
<th>D</th>
<th>C</th>
<th>D/C x 100</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>40.52</td>
<td>27.3</td>
<td>148</td>
</tr>
<tr>
<td>2</td>
<td>29.83</td>
<td>34.46</td>
<td>86.6</td>
</tr>
<tr>
<td>3</td>
<td>53.02</td>
<td>36.3</td>
<td>153</td>
</tr>
<tr>
<td>4</td>
<td>32.10</td>
<td>32.66</td>
<td>98.3</td>
</tr>
<tr>
<td>5</td>
<td>52.27</td>
<td>32.0</td>
<td>163.5</td>
</tr>
<tr>
<td>6</td>
<td>39.57</td>
<td>38.9</td>
<td>101.7</td>
</tr>
<tr>
<td>7</td>
<td>38.9</td>
<td>31.88</td>
<td>122</td>
</tr>
<tr>
<td>8</td>
<td>47.7</td>
<td>24.59</td>
<td>194</td>
</tr>
</tbody>
</table>
APPENDIX K

Statistical Formulas Used:

1. The Normal Distribution
   If there are $n$ observations $X_1 \ldots X_n$ then the average
   \[ \bar{X} = \frac{1}{n} \sum_{i=1}^{n} X, \]
   the standard deviation (S.D.)
   \[ \text{S.D.} = \sqrt{\frac{\sum (X - \bar{X})^2}{n - 1}} \]
   and
   the standard error of estimate of the mean value (S.E.M.)
   \[ \text{S.E.M.} = \frac{\text{S.D.}}{\sqrt{n}} \]

2. Paired data comparison
   If there are $n$ observations from normal mice and their dystrophic
   littermates, only the difference within each littermate is
   considered. If one designates $X_1 \ldots X_n$ as data obtained
   from normal mice and $Y_1 \ldots Y_n$ from dystrophic mice, then:
   the difference between each pair $D = X - Y$ and the average
   difference of all observations, $\bar{D} = \frac{\sum D}{n}$.
   The standard deviation $\hat{\sigma_D}$
   \[ \hat{\sigma_D} = \sqrt{\frac{\sum D^2}{n-1} - \frac{(\sum D)^2}{n(n-1)}} \]
   and
   the standard error of estimate of mean value
   \[ \hat{\sigma_{\bar{D}}} = \frac{\hat{\sigma_D}}{\sqrt{n}} \]
   \[ \hat{\sigma_{\bar{X}}} = \frac{\hat{\sigma_{\bar{D}}}}{\sqrt{n}} \]
Calculation of "p" values are based on the Student "t" test (for n-1 degrees of freedom). This test assesses whether the average difference is significantly different from zero.