THE METABOLISM OF 3-DEOXY-3-FLUORO-D-GLUCOSE BY THE FAT BODY AND FLIGHT MUSCLE OF LOCUSTA MIGRATORIA.

MOSES YAWO. AGBANYO

University of Windsor

Follow this and additional works at: https://scholar.uwindsor.ca/etd

Recommended Citation

This online database contains the full-text of PhD dissertations and Masters' theses of University of Windsor students from 1954 forward. These documents are made available for personal study and research purposes only, in accordance with the Canadian Copyright Act and the Creative Commons license—CC BY-NC-ND (Attribution, Non-Commercial, No Derivative Works). Under this license, works must always be attributed to the copyright holder (original author), cannot be used for any commercial purposes, and may not be altered. Any other use would require the permission of the copyright holder. Students may inquire about withdrawing their dissertation and/or thesis from this database. For additional inquiries, please contact the repository administrator via email (scholarship@uwindsor.ca) or by telephone at 519-253-3000ext. 3208.
The quality of this microfiche is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us a poor photocopy.

Previously copyrighted materials (journal articles, published tests, etc.) are not filmed.

Reproduction in full or in part of this film is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30. Please read the authorization forms which accompany this thesis.

This dissertation has been microfilmed exactly as received.

La qualité de cette microfiche dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S’il manque des pages, veuillez communiquer avec l’université qui a conféré le grade.

La qualité d’impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l’aide d’un ruban usé ou si l’université nous a fait parvenir une photocopie de mauvaise qualité.

Les documents qui font déjà l’objet d’un droit d’auteur (articles de revue, examens publiés, etc.) ne sont pas microfilmés.

La reproduction, même partielle, de ce microfilm est soumise à la Loi canadienne sur le droit d’auteur, SRC 1970, c. C-30. Veuillez prendre connaissance des formules d’autorisation qui accompagnent cette thèse.

La thèse a été microfilmée telle que nous l’avons reçue.
THE METABOLISM OF
3-DEOXY-3-FLUORO-D-GlUCOSE
BY THE FAT BODY AND FLIGHT
MUSCLE OF LOCUSTA MIGRATORIA

by

© MOSES YAWO AGBANYO

A Dissertation
Submitted to the Faculty of Graduate Studies through the
Department of Chemistry in Partial Fulfillment
of the Requirements for the Degree of
Doctor of Philosophy at the
University of Windsor

Windsor, Ontario, Canada

1983
ABSTRACT

THE METABOLISM OF 3-DEOXY-3-FLUORO-D-GLUCOSE

BY THE FAT BODY AND FLIGHT MUSCLE

OF LOCUSTA MIGRATORIA

by

Moses Yawo Agbanyo

The metabolism of 3-deoxy-3-fluoro-D-glucose (3FG) by flight muscle and fat body homogenates of locusts was investigated by initially examining the extent of oxygen consumption with 3FG as substrate. 3FG was not only found to be metabolised by both homogenates at a much slower rate (compared to glucose), but also inhibited oxygen consumption from glucose.

The presence of aldose reductase (EC.1.1.1.21, alditol: NADP+ 1-oxidoreductase), and sorbitol dehydrogenase EC.1.1.1.14, alditol:NAD+ 5-oxidoreductase) enzymes have been shown to be present in the flight muscle of locusts, with the flight muscle enzymes exhibiting a higher affinity for their respective natural substrates compared to the fat body enzymes.

Both tissues have been shown to be capable, not only of glycolysing 3FG, but also of synthesising glycogen and trehalose from 3FG, which was evident in the radiochromato-
graphic, quantitative and $^{19}$F-NMR studies.

With glycolysis, 3FG was found to be metabolised as far down as the triose sugars with significant amounts of detritiation and defluorination.

Finally, the irreversible toxic effects of 3FG is postulated to be via fluoride release (from a metabolite of 3FG) concomitant to alkylation of triosephosphate isomerase (E.C.5.3.1.1), leading to the reversal of glycolysis, hence, the accumulation of fructose. Also implicated to compound the toxic effects of 3FG is the possible alkylation of the enzyme, trehalase (E.C.3.2.1.21), which also led to the accumulation of fluorotrehalose.
DEDICATION

To my family
ACKNOWLEDGEMENTS

I would deeply like to express my sincere thanks to my supervisor, Dr. N. F. Taylor, for giving me the opportunity to expand my horizon and interest in biochemistry, as well as for his guidance throughout my entire program.

I would also like to express my deep appreciation to Drs. R. J. Thibert and K. E. Taylor for their helpful comments and suggestions during the various phases of this work, especially during the absence of my supervisor.

I thank the Ghana Government for their partial financial support throughout my program.

My appreciation also extends to my fellow graduate students and the staff of the Chemistry Department at the University of Windsor, all of whom through their small or large contributions enabled the successful completion of this work.

Special thanks to Ms. A. Gray and Ms. S. Brant for their contribution to the successful completion of this work.

To my family, my sincere thanks for waiting so patiently during this endeavour.

Finally, I would like to thank Ms. W. A. Shaw for her patience and moral support throughout the successful completion of this work.
TABLE OF CONTENTS

ABSTRACT........................................................................................................... ii
DEDICATION........................................................................................................... iv
ACKNOWLEDGEMENTS........................................................................................... v
LIST OF FIGURES................................................................................................... ix
LIST OF TABLES.................................................................................................... xii
LIST OF ABBREVIATIONS....................................................................................... xiv

CHAPTER

I  INTRODUCTION................................................................................................. 1

II  MATERIALS AND METHODS............................................................................. 47
  Reagents.............................................................................................................. 47
  Equipment.......................................................................................................... 48
  Synthesis of 3-Deoxy-3-fluoroglucose................................................................. 48
  Tritiated JFG Preparation...................................................................................... 51
  Locust Rearing Conditions................................................................................... 51
  Preparation of Fat Body and Flight Muscle Homogenates................................. 51
  Respirometric Studies........................................................................................... 52
  Locust Fluoride Measurements............................................................................ 53
  Assay of $^3H_2O$ from Fat Body and Flight Muscle Homogenates...................... 53
  Isolation of Phosphorylated and Non-phosphorylated Sugars.............................. 57
  Separation of Phosphorylated Metabolites......................................................... 58
  Detection of Phosphorylated Metabolites.......................................................... 59
  Separation of Non-phosphorylated Metabolites.................................................. 59
| DETECTION OF NON-PHOSPHORYLATED METABOLITES | 60 |
| LIQUID SCINTILLATION COUNTING | 61 |
| PREPARATION OF CHROMATOGRAPHIC SAMPLES FOR LIQUID SCINTILLATION COUNTING | 61 |
| ESTIMATION OF GLYCOCEN | 67 |
| ESTIMATION OF TREHALOSE USING THE ENZYME TREALASE | 67 |
| ESTIMATION OF FRUCTOSE ELUTED FROM PAPER CHROMATOGRAMS | 71 |
| ACID HYDROLYSIS OF FLUOROTREHALOSE AND FLUORGYCOGEN ELUTED FROM PAPER CHROMATOGRAMS | 74 |
| PREPARATION OF SAMPLES FOR GEL ELECTROPHORESIS | 74 |
| SEPARATION OF ALKylATED PROTEINS BY ELECTROPHORESIS | 78 |
| LOCATION OF PROTEIN BANDS AFTER ELECTROPHORESIS | 79 |
| PREPARATION OF GELS FOR LIQUID SCINTILLATION COUNTING | 80 |
| PREPARATION OF GLYCOCEN AND TREHALOSE FRACTIONS FOR FOURIER TRANSFORM NMR | 80 |
| III RESULTS AND DISCUSSIONS | 84 |
| IV SUMMARY AND CONCLUSIONS | 152 |
| A. RESPIROMETRIC STUDIES | 152 |
| B. ENZYMATIC INVESTIGATION | 152 |
| C. RADIOCHROMATOGRAPHIC STUDIES | 153 |
APPENDICES

I. LEAST SQUARES' METHOD ........................................ 155

IIA. RADIOCHROMATOGRAM OF FLIGHT MUSCLE
     HOMOGENATES INCUBATED WITH D-[U-14C] GLUCOSE. 156

IIB. RADIOCHROMATOGRAM OF FLIGHT MUSCLE
     HOMOGENATES INCUBATED WITH D-[3-3H]3FG .......... 156

IIIA. RADIOCHROMATOGRAM OF FAT BODY
     HOMOGENATES INCUBATED WITH D-[U-14C] GLUCOSE. 158

IIIB. RADIOCHROMATOGRAM OF FAT BODY
     HOMOGENATES INCUBATED WITH D-[3-3H]3FG .......... 158

IVA. RADIOCHROMATOGRAM OF FAT BODY HOMOGENATES
     PRE-INCUBATED WITH GLUCOSE OR 3FG 'CHASED'
     WITH D-[U-14C] GLUCOSE .................................. 160

IVB. RADIOCHROMATOGRAM OF FLIGHT MUSCLE
     HOMOGENATES PRE-INCUBATED WITH GLUCOSE,
     OR 3FG, 'CHASED' WITH D-[U-14C] GLUCOSE .......... 160

VA. CHROMATOGRAM OF PHOSPHORYLATED
     METABOLITES FROM FAT BODY AND FLIGHT
     MUSCLE HOMOGENATES INCUBATED WITH 3FG .......... 162

VB. CHROMATOGRAM OF PHOSPHORYLATED
     METABOLITES FROM FAT BODY AND FLIGHT
     MUSCLE HOMOGENATES INCUBATED WITH GLUCOSE .... 162

VI. CHROMATOGRAM OF PHOSPHORYLATED
     METABOLITES FROM FAT BODY AND FLIGHT
     MUSCLE HOMOGENATES PRE-INCUBATED WITH
     3FG 'CHASED' WITH GLUCOSE ........................... 164

REFERENCES...................................................... 166

VITA AUCTORIS................................................ 173
**LIST OF FIGURES**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Biosynthetic Pathway for Trehalose</td>
<td>13</td>
</tr>
<tr>
<td>2.</td>
<td>Recycling Scheme for Trehalose</td>
<td>19</td>
</tr>
<tr>
<td>3.</td>
<td>NAD⁺ Recycling Scheme under Anaerobic Conditions</td>
<td>26</td>
</tr>
<tr>
<td>4.</td>
<td>Carbohydrate Metabolism in Insects</td>
<td>31</td>
</tr>
<tr>
<td>5.</td>
<td>Proline Oxidation by Insects</td>
<td>35</td>
</tr>
<tr>
<td>6.</td>
<td>Sorbitol Shunt in Locust Fat Body</td>
<td>44</td>
</tr>
<tr>
<td>7.</td>
<td>Calibration Curve for Fluoride</td>
<td>55</td>
</tr>
<tr>
<td>8.</td>
<td>Quench Correction Curve for $[^3H]$-Samples</td>
<td>63</td>
</tr>
<tr>
<td>9.</td>
<td>Quench Correction Curve for $[^14C]$-Samples</td>
<td>65</td>
</tr>
<tr>
<td>10.</td>
<td>Calibration Curve for Maltose</td>
<td>69</td>
</tr>
<tr>
<td>11.</td>
<td>Calibration Curve for Fructose</td>
<td>73</td>
</tr>
<tr>
<td>12.</td>
<td>Calibration Curve for Proteins</td>
<td>77</td>
</tr>
<tr>
<td>13.</td>
<td>Calibration Curve for Molecular Weight Determinations</td>
<td>82</td>
</tr>
<tr>
<td>14.</td>
<td>Respirometric Studies using Fat Body Homogenates</td>
<td>86</td>
</tr>
<tr>
<td>15.</td>
<td>Respirometric Studies using Flight Muscle Homogenates</td>
<td>88</td>
</tr>
<tr>
<td>16.</td>
<td>Inhibition Studies using Fat Body Homogenates</td>
<td>91</td>
</tr>
<tr>
<td>17.</td>
<td>Inhibition Studies using Flight Muscle Homogenates</td>
<td>93</td>
</tr>
<tr>
<td>18a.</td>
<td>Rate Curve for Locust Flight Muscle Aldose Reductase</td>
<td>96</td>
</tr>
<tr>
<td>18b.</td>
<td>Double Reciprocal Plot for Locust Flight Muscle Aldose Reductase</td>
<td>96</td>
</tr>
</tbody>
</table>
Figure

19a. Rate Curve for Locust Flight Muscle Sorbitol Dehydrogenase ........................................... 99
19b. Double Reciprocal Plot of Locust Flight Muscle Sorbitol Dehydrogenase ......................... 99
20a. Rate Curve for Locust Fat Body Sorbitol Dehydrogenase .................................................. 101
20b. Double Reciprocal Plot for Locust Fat Body Sorbitol Dehydrogenase .......................... 101
21. Radiochromatographic Scans of Flight Muscle Homogenates Incubated with D-[3-3H]3PG or D-[U-14C]Glucose ............................................................... 105
22. Radiochromatographic Scans of Fat Body Homogenates Incubated with D-[3-3H]3PG or D- [U-14C] Glucose ................................................................. 107
23a. Distribution of Radioactivity from Radiochromatogram of Flight Muscle Homogenates Pre-incubated with D-[U-14C] Glucose Over Different Time Periods .................................................. 111
23b. Distribution of Radioactivity from Radiochromatogram of Flight Muscle Homogenates Pre-incubated with D-[3-3H] 3PG Over Different Time Periods ........................................................................ 111
24a. Distribution of Radioactivity from Radiochromatogram of Fat Body Homogenates Pre- incubated with D-[U-14C]Glucose Over Different Time Periods .................................................. 115
24b. Distribution of Radioactivity from Radiochromatogram of Fat Body Homogenates Pre-Incubated with D-[3-3H]3PG Over Different Time Periods ........................................................................ 115
25a. Acid Hydrolysis of Glycogen Fraction of Radiochromatograms from Fat Body Homogenates Pre-incubated with D-[3-3H]3PG ............................................. 124
25b. Acid Hydrolysis of Glycogen Fraction of Radiochromatograms from Flight Muscle Homogenates Pre-incubated with D-[3-3H]3PG ...................... 124
26. $^{19}$F-NMR of Fluoroglycogen Isolated from Fat Body Incubates.................. 127

27a. Acid Hydrolysis of Trehalose Fraction of Radiochromatogram from Fat Body Homogenates Pre-incubated with D-[3-H]3FG............. 131

27b. Acid Hydrolysis of Trehalose Fraction of Radiochromatogram from Flight Muscle Homogenates Pre-incubated with D-[3-H]3FG............. 131

28. $^{19}$F-NMR of Fluorotrehalose Isolated from Flight Muscle Incubates.................. 133

29. Distribution of Radioactivity in Flight Muscle Proteins................................. 145

30. Proposed Metabolism of D-[3-H]3FG in Locust Flight Muscle and Fat Body............ 150
# LIST OF TABLES:

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>Distribution of Radioactivity from Radiochromatogram of Fat Body Homogenates</td>
<td>117</td>
</tr>
<tr>
<td></td>
<td>Pre-incubated with 3FG or Glucose over Different Time Periods 'Chased' with</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D-[U-14C] Glucose</td>
<td></td>
</tr>
<tr>
<td>1b</td>
<td>Distribution of Radioactivity from Radiochromatogram of Flight Muscle Homogenates</td>
<td>118</td>
</tr>
<tr>
<td></td>
<td>Pre-incubated with 3FG or Glucose Over Different Time Periods 'Chased' with</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D-[U-14C] Glucose</td>
<td></td>
</tr>
<tr>
<td>2a</td>
<td>Estimation of Glycogen Fraction From Radiochromatogram of Fat Body Homogenates</td>
<td>122</td>
</tr>
<tr>
<td></td>
<td>Pre-incubated with D-[U-14C] Glucose or D-[5-3H] 3FG</td>
<td></td>
</tr>
<tr>
<td>2b</td>
<td>Estimation of Glycogen Fraction From Radiochromatogram of Flight Muscle Homogenates</td>
<td>122</td>
</tr>
<tr>
<td></td>
<td>Pre-incubated with D-[U-14C] Glucose or D-[5-3H] 3FG</td>
<td></td>
</tr>
<tr>
<td>3a</td>
<td>Estimation of Trehalose Fraction of Radiochromatogram from Fat Body Homogenates</td>
<td>128</td>
</tr>
<tr>
<td></td>
<td>Pre-incubated with D-[U-14C] Glucose or D-[5-3H] 3FG</td>
<td></td>
</tr>
<tr>
<td>3b</td>
<td>Estimation of Trehalose Fraction of Radiochromatogram from Flight Muscle Homogenates</td>
<td>128</td>
</tr>
<tr>
<td></td>
<td>Pre-incubated with D-[U-14C] Glucose or D-[5-3H] 3FG with Trehalase</td>
<td></td>
</tr>
<tr>
<td>4a</td>
<td>Estimation of Fructose Fraction from Radiochromatogram of Fat Body Homogenates</td>
<td>136</td>
</tr>
<tr>
<td></td>
<td>Pre-incubated with D-[U-14C] Glucose or D-[5-3H] 3FG</td>
<td></td>
</tr>
<tr>
<td>4b</td>
<td>Estimation of Fructose Fraction from Radiochromatogram of Flight Muscle Homogenates</td>
<td>136</td>
</tr>
<tr>
<td></td>
<td>Pre-incubated with D-[U-14C] Glucose or D-[5-3H] 3FG</td>
<td></td>
</tr>
<tr>
<td>5a</td>
<td>Fluoride Measurements from Fat Body Homogenates Incubated with 3FG</td>
<td>138</td>
</tr>
<tr>
<td>5b</td>
<td>Fluoride Measurements from Flight Muscle Homogenates Incubated with 3FG</td>
<td>138</td>
</tr>
<tr>
<td>Table</td>
<td>Page</td>
<td></td>
</tr>
<tr>
<td>-------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>6a. $^3$H$_2$O Released from Either D-[$^3$-H]3FG or D-$^3$H]Glucose with Flight Muscle Homogenate</td>
<td>140</td>
<td></td>
</tr>
<tr>
<td>6b. $^3$H$_2$O Released from Either D-[$^3$-H]3FG or D-$^3$H]Glucose with Fat Body Homogenate</td>
<td>140</td>
<td></td>
</tr>
<tr>
<td>7. Effect of 3FG and Fluoride Ion on $^3$H$_2$O Released from D-[$^3$-H]Glucose by Flight Muscle Homogenates</td>
<td>142</td>
<td></td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

ATP adenosine triphosphate.
AMP adenosine monophosphate.
EDTA ethylenediaminetetraacetic acid.
FDP fructose-1,6-diphosphate.
F1P fructose-1-phosphate.
F6P fructose-6-phosphate.
GDH glycerol phosphate dehydrogenase.
G1P glucose-1-phosphate.
G3P glyceraldehyde-3-phosphate.
G6P glucose-6-phosphate.
NAD+ nicotinamide adenine dinucleotide.
NADH nicotinamide adenine dinucleotide (reduced form).
NADP+ nicotinamide adenine dinucleotide phosphate.
NADPH nicotinamide adenine dinucleotide phosphate (reduced form).
T6P trehalose-6-phosphate.
UDP uridine diphosphate.
UTP uridine triphosphate.
3FG 3-deoxy-3-fluoro-D-glucose.
3FF 3-deoxy-3-fluorofructose.
3FGL 3-deoxy-3-fluoro-D-glucitol.
CHAPTER I

INTRODUCTION

Locusts occur in all warmer regions of the world. The desert locust, *Schistocerca gregaria*, occurs from west Africa to India, and extends north to Iran and south to Kenya. The migratory locust, *Locusta migratoria* is even more widespread, extending through Africa eastwards to Japan, the Philippines and Australia. By virtue of their widespread importance and sporadic outbreaks, it has become necessary, even as early as 720 AD that a study of their behaviour be undertaken (1). Thus one can see that locusts were a problem to earlier civilizations as they are in this era of modern technology. Up to date, no complete eradication measures have been found.

The increased interest in insect biochemistry is most probably due to the realization that insects can be useful organisms acting as model systems for the experimental study of biochemical principles. The main biochemical processes in insects are being revealed as essentially the same as in other forms of life but, as so often observed in comparative biochemistry, there are interesting variations on the central theme.
Another major reason for the increased interest in insect biochemistry is an economic one, related to production of insecticides in which constant research for new products is essential, due to development of resistant insect strains. On this premise, one would hope that an increased knowledge in insect biochemistry and physiology would facilitate a rational approach to the development of new lethal agents which would interfere with the normal biochemical processes in insects. The aim, in this case, must be selective toxicity in which the agent is insect specific while man, other animals and if possible, beneficial insects and plants are unaffected.

Several difficulties are encountered when assessing metabolic fuel supplies for flight in insects. Because of their small size, it is difficult to obtain tissues in substantial quantities for experimental work. Consequently, many earlier workers resorted to the use of whole insect homogenates which normally gave a general overview of insect metabolism. Difficulties also arise due to a wide variety of insect species that have been used, since it is likely that differences in metabolism are insect specific, sex and age specific. As such, generalizations drawn from these experiments may be misleading. In this respect, the proper choice of the appropriate insect species, sex and age can lead to findings of a particular biochemical mechanism
which has been developed or modified to a considerable extent and the study of such a system may be most informative about general biochemical mechanisms, and thus lead to a correlation between biochemical specialization and physiological functions.

Prior to a consideration of the metabolism of 3-deoxy-3-fluoro-D-glucose (3FG) in the flight muscle and fat body of Locusta migratoria, it is proposed to briefly review carbohydrate metabolism in insects in general.

Carbohydrates, lipids and in some insects, certain amino acids are utilized as respiratory fuels to supply energy for flight (2,3). The flight pattern in insects reflects changes in the utilization of oxidizable substrates. Insects generally fly at high speeds within the first five to ten minutes of flight with the speed gradually decreasing over a period of about twentyfive minutes before attaining a 'cruising' speed. During the initial high speed flight, carbohydrates are the preferred fuel whereas lipid is utilized during the low speed or 'cruising' period (4).

The flight muscle of insects contain its own respiratory fuels, which can be readily mobilized to meet the immediate energy requirements of the flight muscle. For example, glucose (up to 10mg/g wet weight muscle) and trehalose (up to 7.2mg/g wet weight muscle) have been
reported in the flight muscle of *L. migratoria* (5) while
Walker and Bailey (6) have demonstrated the presence of
(4.5 mg percent wet weight flight muscle) lipid in the
male desert locust. The above results all point to the
fact that the reserved fuels in the flight muscle are
just sufficient to initiate flight but not enough to sus-
tain their prolonged flight.

During recent years, increasing attention has been
paid to the possibility that the insect fat body may be a
site of intermediary metabolism in addition to its dis-
tinctive role of serving as a depot for storage of carbo-
hydrates, fat and proteins.

The anatomical position of the fat body is such that
it occupies a central position around the gut and exposed
to a maximum area of the haemolymph. The location makes
it ideal for exchange of metabolites with the haemolymph,
which in turn, effects the transportation and distribution
of the absorbed or reserved fuels to tissues (especially
the flight muscle) for utilization. The fat body is also
an organ involved with blood protein biosynthesis, syn-
thesis of purines and pteridines, a site where carbohydrates
are converted to fats, and finally, a site where detoxifi-
cation processes take place via formation of glucosides with
UDP-glucuronic acid (7, 8). In this respect, the fat body
can be considered as an organ similar to the mammalian 'liver
and adipose tissue combined.

Glycogen forms an important carbohydrate reserve in the fat body, flight muscle and the midgut cells. The quantity varies according to sex, stage in life history, the past nutritional level, and most importantly on the demands on energy requirements.

The large deposits of glycogen in the flight muscles as well as the depletion of these reserves during flight indicates that glycogen provides the major vehicle for storage of flight energy which is rapidly mobilized to meet the metabolic requirements of the flight muscle. In a continuous forced flight, the different loci of carbohydrate stores including those of the fat body and gut are used (8).

Trivelloni (7) on incubating the fat body of Schistocerca cancellata with radiolabelled UDP-D-[U-^{14}C] glucose found that part of the radioactivity was converted to glycogen and on treatment of the glycogen with $\beta$-amylase, all the radioactivity was liberated as maltose. Coupled with this fact, he also demonstrated the presence of glycogen synthetase (UDP-glucose-glycogen transglucosidase) in tissue homogenates of locust and tested for glucose-6-phosphate as a potential activator but found that it rather caused some slight inhibition of glycogen synthesis. Chemical characterization of isolated native glycogen from
Phormia regina flight muscle indicated that the branched chains are quite short and only about twenty five-percent of the total glucose residues was released by glycogen phosphorylase.

The accrued evidence thus shows that, insect fat body as well as flight muscle are capable of synthesising glycogen from commoner sugars such as glucose, fructose, sucrose, maltose (although maltose is used up at a much slower rate than glucose), as well as glycogenic amino acids (7-9). Thus in insect tissues, glycogenesis proceeds via the same pathway as that demonstrated in mammalian tissues as outlined below.

(i) Glucose-1-phosphate + UTP $\rightleftharpoons$ UDP-glucose + PPI

(ii) UDP-glucose + glycogen(n) $\rightarrow$ UDP + glycogen(n+1)

The utilisation of stored glycogen is dependent upon its hydrolysis by the enzyme, glycogen phosphorylase. Purification from flight muscles of Phormia regina of glycogen phosphorylase revealed that, the enzyme exists in the 'a' and 'b' forms. The molecular weights of both forms have been determined and found to be 100,000 each. Their amino acid composition as well as their kinetic properties have also been reported (10). The locust flight muscle phosphorylase has not yet been characterised despite the demonstration of its presence in the flight muscle (11).
Although not required for activity, *Phormia regina* flight muscle phosphorylase 'a' has been found to be stimulated by AMP at saturation levels of inorganic phosphate. The $K_m$ for AMP was found to be 0.6 M and lowering either inorganic phosphate or glycogen concentration has been found to decrease the enzymes activity for AMP (10). Unlike the 'a' form of the enzyme, phosphorylase 'b' has been found to have an absolute requirement for AMP. Furthermore, levels of AMP, 100-fold greater than those which stimulate phosphorylase 'a' are needed to stimulate the 'b' form of the enzyme. Increasing AMP concentrations has been found to decrease the apparent $K_m$ values for inorganic phosphate as well as glycogen (10). On the other hand, increasing glycogen or inorganic phosphate concentrations have been found to lower the affinity of phosphorylase 'b' for AMP. Childress and Sactor (10) also found that ATP is a potent inhibitor of phosphorylase 'b' but not of phosphorylase 'a'. The $K_i$ has been reported to be approximately 2mM. At high AMP concentrations the inhibition by ATP has been found to be reduced, suggesting competitive inhibition with respect to AMP. This view was strengthened by the observations that ATP increased the apparent $K_m$ values for both glycogen and inorganic phosphate.

The conversion of phosphorylase 'b' to phosphorylase 'a' is catalyzed by phosphorylase 'b' kinase. The kinase
has been reported to be localized with phosphorylase 'a' phosphatase, phosphorylases 'a' and 'b', and glycogen in the post-mitochondrial supernatant of the blowfly flight muscle (9) and has been reported to be stimulated by physiological concentrations of calcium \((10^{-6} \text{ M})\). In this regard, one can liken it to mammalian phosphorylase 'b' kinase.

In the blowfly, *Phormia regina*, flight muscle phosphorylase 'b' kinase has been found to be stimulated by high concentrations of inorganic phosphate \((12,13)\) with an apparent \(K_m\) of \(20\text{ mM}\). Maximum activation of the insect phosphorylase kinase has been found to require magnesium ions in addition to inorganic phosphate with the apparent \(K_m\) for magnesium ions being \(3\text{ mM}\). The enhancement in activity could probably involve the conversion of a 'non-active' phosphorylase kinase to an 'active' form. It, however, seems to differ from the conversion of the 'non-activated' to the 'activated' form of the enzyme from rabbit skeletal muscle in that the blowfly activation by inorganic phosphate of the phosphorylase kinase has an immeasurably high \(K_m\) for phosphorylase 'b' at neutral pH and the insect enzyme shows no appreciable change in relative activities at pH 6.8 and pH 8.2 \((13)\), and also the calcium dependent proteolysis has been found to be prevented by the presence of EGTA or EDTA. Further contrasting the mammalian phosphorylase 'b' kinase
from the blowfly enzyme was the observation that the activation of the insect muscle phosphorylase 'b' kinase does not depend on ATP, nor is the activation dependent on cyclic AMP (13). Thus there is no evidence which suggests the presence in the blowfly flight muscle of a protein kinase that phosphorylates and dephosphorylates phosphorylase 'b' kinase as observed in mammalian system although corpus cardiacum extracts have been reported to induce glycogenolysis in the fat body of insects.

In the fat body of the silkworm, Stevenson and Wyatt (14) showed the presence of glycogen phosphorylase with an optimal activity at pH 7. The Km for G1P was found to be 25mM and that the enzyme was activated by 5'AMP and independent of sulfhydryl groups. They also showed that the enzyme, as in the flight muscle (15) exists in two forms and AMP dependent activity in locust fat body has been shown by Hess and Pearse (16).

The conversion of phosphorylase 'a' to phosphorylase 'b' is catalyzed by phosphorylase 'a' phosphatase. This phosphatase has been found to be very active in blowfly flight muscle but its activity is inhibited by sodium chloride as is true for the mammalian system.

Apart from glycogen, another principal carbohydrate which plays a central role in insect carbohydrate metabolism is trehalose (\(\alpha-D\)-glucopyranosyl-\(\alpha-D\)-glucopyranoside).
This disaccharide is non-reducing by virtue of the fact that the anomeric carbon atoms of both glucose moieties are bound in an α-1:1-glucosidic linkage.

Horie (17) noted that during starvation of *Bombyx mori*, fat body glycogen fell rapidly and haemolymph trehalose levels declined only after some hours when glycogen level was minimal. This observation was confirmed by Saito (18) who noted that elevation of haemolymph trehalose levels by injection led to an initial rapid decline of trehalose, later establishing a steady state somewhat above normal levels within three hours. The initial rapid decline of trehalose was interpreted as a rapid conversion of haemolymph trehalose into glycogen in the fat body and to a lesser extent, in the flight muscle.

The conversion of glucose to trehalose in insects is very rapid. About 90% of the glucose absorbed through the gut is converted into haemolymph. Fat body and flight muscle trehalose within ten minutes leaving the actual haemolymph concentration of glucose very low (9, 19, 20). As high as 1500 mg% has been reportedly isolated in crystalline form from locust haemolymph (21). Being a biomolecule that
greatly supports the energetics of flight, trehalose concentration has been reported in the locust to decrease very rapidly within thirty seconds of flight and then at a much slower rate during the remainder of the flight. This discontinuity of trehalose utilization after thirty seconds has been interpreted to mean that there are two different pools of this sugar. The pool metabolized rapidly within thirty seconds is considered to be muscle trehalose and the second pool is derived from the fat body.

Candy and Kilby (22, 23) first demonstrated that the fat body was the major site of trehalose synthesis. Cell free extracts from the fat body fortified with ATP and UDP-glucose was shown to convert D-[U-14C]glucose to trehalose (23). The pathway which was elucidated by autoradiography from the reaction mixture at different times showed the disappearance of glucose in fifteen minutes giving rise to G1P which reacted with UDP-glucose to form trehalose-6-phosphate (T6P). A specific phosphatase has been implicated in the hydrolysis of T6P to free trehalose. The scheme shown in Figure 1 is similar to the one reported by Çabib and Leloir (24).

Trehalose-6-phosphatase has been purified on DEAE column with the enzyme showing activity at an optimum pH of 7, requiring Mg$^{2+}$ but inactive towards a range of monosaccharides and disaccharide as well as nucleoside
FIGURE 1

BIOSYNTHETIC PATHWAY FOR TREHALOSE

Biochemical pathway leading to the synthesis of trehalose in insect tissues. The enzymes involved in this synthesis are:

1. Hexokinase.
2. Phosphoglucomutase.
3. UDPG pyrophosphorylase.
4. T6P synthetase
5. Trehalose-6-phosphatase.

\[ \text{Activation} \]

\[ \text{Inhibition} \]
phosphates tested (25).

Other sugars found to be able to be converted into trehalose in the fat body include mannose and fructose. The fat body, however, appears not to be the only site of trehalose synthesis. Other tissues that have been implicated are leg muscles, flight muscles and head tissues fortified with ATP (26).

The most interesting characteristic of the synthetase is that it is inhibited by free trehalose and exhibits other features interpretable as allosteric effects (27). The inhibition of T6P synthetase by free trehalose could be important in the natural regulation of trehalose levels and thus maintaining the homeostasis of haemolymph trehalose levels. This inhibition does not seem to be a simple inhibition since the immediate end product of the enzyme, T6P does not accumulate because of the high activity of trehalose phosphatase. However, in vitro, trehalose synthetase can be inhibited by trehalose-6-phosphate.

The metabolic fate of trehalose is dependent upon its hydrolysis to glucose by the enzyme trehalase.

\[
\text{Trehalose} \xrightarrow{\text{trehalase}} 2 \text{glucose residues}
\]

The marked decrease in the concentration of trehalose in the thorax at the onset of flight is coincidental to the rapid increase in the concentration of glucose. These opposite
changes, observed at the time when there has been an enhancement of glycolysis, suggests that the cleavage of trehalose has been greatly facilitated. However, the elevation of glucose is transient, returning to basal levels in thirty seconds.

No other enzyme has been established for the metabolism of trehalose. Generally trehalase is found as isozymes. They may be found in different tissues of an insect species or in different tissues of the same insect. There are two types of trehalases normally found in the same insect and are very distinct. However, both are very specific for trehalose and are not general α-glucosidases.

The soluble trehalase can be found in the haemolymph and gut and has an optimum pH between 5.0-5.7, a Km of 0.75mM with no activators reported (28). The muscle trehalase, however, has been reported to be associated with microsomal fractions. Contrarily, other workers have associated it with the mitochondria, reporting its pH optimum to be 6-6.5 and Km of 3.6mM (29; 30). Thoracic muscle trehalase from cockroaches, locusts and moths have been found to be activated by various physical treatments that disrupt the lipoprotein structure. For example, freezing and thawing, detergent as well as phospholipase A treatments all led to an enhancement of activity in muscle trehalase (28; 29).

Exhaustive snake venom treatment was found to release
the muscle trehalase as a homogeneous protein with molecular
weight of about 65,000-80,000, and the molecular weight of
Blaberus discoidalis, gut trehalase have been found to have
a value of about 70,000 (28, 29).

The presence of intestinal trehalase appears at first
enigmatic, since trehalose is only rarely an appreciable
component of the insect diet. The presence, however, can be
interpreted as a necessary corollary to the mode of sugar
absorption in insects to prevent loss of trehalose by dif-
fusion.

The observation that glucose is rapidly absorbed from
the gut is attributed to non-specific physical diffusion
rather than active or facilitated transport across the insect
gut wall, since the transport step has been reported not to
be affected by cyanide poisoning (19, 31, 32). The concen-
tration of glucose in locust haemolymph is normally very
low, about 24 mg %. Thus absorption across the gut wall
seems to be maintained by the rapid conversion of glucose
to trehalose in the haemolymph, fat body and flight muscle,
generating a concentration gradient which enables glucose
to be absorbed (32, 33) As such, since trehalose diffuses
freely and reversibly across the gut wall, intestinal tre-
halase tends to hydrolyse the trehalose into glucose to
generate the concentration gradient needed for reabsorption
of glucose to be resynthesized into trehalose in the
haemolymph, fat body, and flight muscle.

If this hypothesis holds, then insect gut trehalase could be acting to prevent loss of trehalose into feces, and thus implying a cycle (Figure 2).

Since the ultimate product of trehalose hydrolysis is glucose, the normal case would be the phosphorylation by a kinase. Hexokinase activity has been demonstrated in the flight muscle of the housefly (34), locust flight muscle homogenates (35) and also in the fat body (36). This enzyme has been reported to be non-specific (35) and that from the locust flight muscle catalyzes the phosphorylation of D-glucose, fructose, D-mannose and D-glucosamine but not D-galactose, L-sorbose, D-ribose, D-arabinose or N-acetyl-D-glucosamine. The enzyme has been found to be saturated by glucose at a lower concentration (Km 2.8mM) than by fructose (Km 5.5mM). The optimum pH has also been reported to be pH 7.9 and above (35). Phosphorylation of glucose has been found to be strongly inhibited by fructose and mannose but not by galactose.

The end products of the non-specific hexokinase action, G6P and F6P, have been found to be completely inhibitory at 5 mM G6P and F6P concentrations in flight muscle homogenates. In the honey bee, however, ADP has been found to be a competitive inhibitor, with respect to ATP of honey bee hexokinase and has a Ki value of $9 \times 10^{-4}$ M and G6P, a non-competitive
FIGURE 2

RECYCLING SCHEME FOR TREHALOSE

Hypothetical cycle depicting the function of insect gut trehalase preventing loss of trehalose into feces.
inhibitor with respect to glucose with a $K_i$ value of 10mM being estimated (37). In rat heart, the inhibition by G6P is relieved by Pi (38). This reversal of inhibition has not been demonstrated in insect flight muscle (39).

The major pathway for the catabolism of carbohydrates in insect tissues is the classical Embden-Meyerhof scheme which is very dependent on the dietary source. For example, insects feeding on diets rich in mannose, fructose, or galactose have enzyme systems which feed these precursors into the glycolytic pathway.

The pentose phosphate pathway, although prominent in some insect tissues (including the fat body and flight muscle) during the life cycle of the insect, probably contributes very little to the energetics of flight (40,41). Supporting this view on the relative insignificance of this shunt (pentose phosphate pathway) in the flight muscle is the finding of Vogell et al. (42) that the activities of glucose-6-phosphate dehydrogenase in the locust flight muscle is only 0.1% of those of other glycolytic enzymes.

The mechanism whereby phosphofructokinase reaction is facilitated from a slowly metabolising resting muscle to an intensely active working muscle had been suggested from earlier studies with mammalian enzyme in vitro. Later this was confirmed with insect preparations, in vitro, and from examination of changes in concentration of the adenine
nucleotides, arginine phosphate and Pi in *Phormia regina* flight muscle in situ at the onset of flight. Parks and Lardy [43] discovered that, although ATP is a substrate for phosphofructokinase, an excess of ATP was inhibitory. Crabtree *et al.* found that this inhibition by ATP may be overcome by either ADP, AMP, Pi and cyclic AMP, fructose-1, 6-diphosphate or, more effectively, by a combination of these activators. In this respect insect flight muscle phosphofructokinase resembles the mammalian enzyme in that it is inhibited by excess ATP and that this inhibition is reversed by AMP, cyclic AMP and Pi [45].

However, differences between the mammalian and insect enzymes are evident. Fructose-1,6-diphosphate, an activator of the mammalian enzyme, has been found to inhibit locust flight muscle phosphofructokinase, and citrate, a potent inhibitor of the mammalian enzyme, does not affect the locust enzyme [45].

Evidence for glycolytic enzymes in the fat body came from the observation of the presence of phosphorylase, phosphoglucomutase in the silkworm fat body and both enzymes are inhibited by fluoride. Shigematsu [46], using silkworm fat body preparations, demonstrated the phosphorylation of glucose and fructose to their respective sugar phosphates. Glucokinase activity was found to be inhibited by fructose and fructokinase activity was inhibited by the simultaneous
presence of glucose. The enzyme thus appears to be the nonspecific hexokinase observed in locust flight muscle (35).

Several different types of fructose-1,6-diphosphate aldolases are present in insects (47). The enzyme from the flight muscle of Phormia regina has properties resembling the class 1A isoenzyme characteristic of rabbit muscle. These include the similarity in electrophoretic mobility, and 44-fold greater reaction rate with fructose-1,6-diphosphate as substrate that with fructose-1-phosphate as substrate. Digestion of the enzyme with carboxypeptidase was found to decrease the activity towards fructose-1,6-diphosphate with little activity towards fructose-1-phosphate (47,48). The flight muscle enzyme has a molecular weight of about 160,000 and is probably composed of four subunits each of 40,000 daltons.

The presence of glyceraldehyde-3-phosphate dehydrogenase has been demonstrated in the fat body of the locust (49). With glyceraldehyde-3-phosphate as substrate, the housefly triose phosphate isomerase has been shown to exhibit Michaelis type of kinetics except for inhibition at high substrate concentrations (50). With dihydroxyacetone phosphate as a substrate, a sigmoidal curve was observed. The existence of an allosteric site on the enzyme is suggested by the non-competitive inhibition at low inorganic phosphate concentrations and by the competitive inhibition, presumably
at the catalytic site at high inorganic phosphate concentration (50). The molecular weight of triose phosphate isomerase enzyme from the flight muscle of the housefly has been estimated to be around 60,000 (51).

The end products of glycolysis in insects are pyruvate and \( \text{sn}- \)glycerol-3-phosphate. A very interesting feature of many insects is the high \( \text{sn}- \)glycerol-3-phosphate dehydrogenase (GDH) activity which is often associated with the low lactate dehydrogenase (LDH) activity.

Locust fat body has been reported to be able to convert dihydroxyacetone phosphate into \( \text{sn}- \)glycerol phosphate with an activity of about 3 mm for every one gram of fresh weight tissue but pyruvate was reduced to lactate only about 1% of this amount. At the same time, locust flight muscle \( \text{sn}- \)glycerol-3-phosphate dehydrogenase has been reported to have an enzyme activity of 167 units but only two units of the lactate dehydrogenase activity, while the leg muscle has been reported to have thirty-three units of the \( \text{sn}- \)glycerol-3-phosphate dehydrogenase activity and 117 units of lactate dehydrogenase activity (49). Thus, there seems to be two types of \( \text{sn}- \)glycerol-3-phosphate dehydrogenase present in insect tissue as in vertebrate.

One of the enzymes is found in the soluble fraction and is NAD-dependent (GDH I) and the other, in the particulate fraction and cytochrome linked (GDH II). A comparative study
of both enzymes activities in flight and leg muscles as well as fat body in locusts by Delbrück et al. (49), revealed that the fat body enzymes resembled the flight muscle enzyme in their GDH:LDH ratio, while leg muscle differed by having LDH activity several times higher than the GDH and thus much more resembled the pattern in vertebrate skeletal muscle.

Under anaerobic conditions, vertebrate muscle is dependent on regeneration of reduced NAD produced at the glyceraldehyde-3-phosphate dehydrogenase step of glycolysis by coupling with NAD$^+$-dependent reduction of pyruvate to lactate as illustrated in (Figure 3).

However, in those insects tissues in which LDH activity is low or virtually absent, the regeneration could be achieved by reduction of dihydroxyacetone phosphate to sn-glycerol phosphate in the presence of DGHT depicted in the scheme shown below:

\[
\text{Glyceraldehyde-3-phosphate} \xrightarrow{\text{NAD}^+} \text{sn-Glycerol phosphate} \\
\text{3-Phosphoglycerate} \xleftarrow{\text{NADH}} \text{Dihydroxyacetone phosphate}
\]

The fate of α-glycerol phosphate in flight muscle is a rapid reoxidation back to dihydroxyacetone phosphate by active mitochondrial enzyme (GDH II), a reaction in which
FIGURE 3

NAD$^+$ RECYCLING SCHEME UNDER ANAEROBIC CONDITIONS

This figure depicts how NAD$^+$ is recycled to drive glycolysis towards its end product, lactate, under anaerobic conditions in mammalian tissues as well as in insects with high lactate dehydrogenase activity. The enzymes involved in this cycle are:

1. Aldolase.
2. Triose phosphate isomerase.
3. Glyceraldehyde-3-phosphate dehydrogenase.
4. Lactate dehydrogenase.
the equilibrium favours dihydroxyacetone phosphate production and which is coupled with phosphorylation with the P/O ratio reported by Sactor and Cochran to be about 1.0 - 1.7 (52). The mitochondrial scheme is illustrated below.

Despite this unique adaptation, the ultimate oxidation of carbohydrates to carbon dioxide and water, though a slow process, proceeds via pyruvate and the tricarboxylic acid cycle.

Bailey and Walker (53) studied the regulation of pyruvate kinase activity in the flight muscle of the locust, *Schistocerca gregaria*, and found that maximal activity was obtained with concentrations of ADP and phosphoenolpyruvate of 1.0 and 0.1mM, respectively. Higher concentrations of ADP were found to be slightly inhibitory. Fructose diphosphate which activates potently by lowering the apparent Km for phosphoenolpyruvate in the fat body of locusts had no effect on flight muscle enzyme, thus making it biochemically akin to the two enzymes found in rat liver and muscle.
A metabolic process apparently peculiar to insects and other arthropods is the bulk conversion of carbohydrates to glycerol and sorbitol. An NADP-dependent dehydrogenase's activity has been reported by Faulkner in the fat body, blood and other tissues of the silkworm which reduces a number of hydroxyaldehydes and carbonyl compounds to their corresponding alcohols (54).

Among the compounds listed to be reduced are glyceraldehyde, glucuronic acid, and galacturonic acid. Recently, with fat body preparations from Locusta migratoria, 3-deoxy-3-fluoro-D-glucose has been found to be reduced to 3-deoxy-3-fluoro-D-glycitol (55). However, the natural substrate of this enzyme (aldose reductase; EC. 1. 1. 1. 21), is not yet known but, this enzyme could be serving as an alternate key enzyme for the utilisation of glucose via glycolysis.

Ecologically, the accumulation of glycerol and sorbitol appears to be connected with resistance to cold by lowering the freezing point. The biochemical pathway of glycerol production is presumably via reduction from the triose sugar level of glycolysis and the appropriate enzymes have been demonstrated in insects (56, 52) but the precise sequence of reduction and dephosphorylation has not been firmly established.

For glycerol, the most likely route is the reduction of dihydroxyacetone phosphate to sn-glycerol-3-phosphate (α-glycerol phosphate). Since sn-glycerol-3-phosphate dehydrogenase is
very active in insect tissues, there could be a specific phosphatase which would hydrolyse the sn-glycerol-3-phosphate produced to glycerol. A preliminary evidence of such a phosphatase has been reported in the Cecropia fat body (57).

Another pathway whereby glycerol could be produced is via dihydroxyacetone phosphate through sn-glycerol phosphate to the synthesis of triglycerides. With the action of specific lipases, these triglycerides can be selectively hydrolysed leading to the production of glycerol. The glycerol produced could again be re-esterified to produce triglycerides and thus implying a cycle. It is, thus, of great interest in this connection, that exposure of wax moth larvae, Galleria mellonella to cold conditions results in a lowered sn-glycerol phosphate levels (58). Thus the possible interconversion of dietary sugars which are eventually glycolyzed are shown in Figure 4.

The acetyl CoA derived from glycolysis can be oxidized to carbon dioxide and water by the tricarboxylic acid cycle (TCA), the major oxidation pathway found in a wide range of bacterial, animal and plant cells. All the enzymes of the tricarboxylic acid cycle have been demonstrated in acetone powders, extracts, mitochondrial preparations and homogenates prepared from whole Predonia eridania larvae (59). The only two insect tissues which have been examined in any great
A schematic diagram of glycolysis and the glycerol phosphate shunt in the flight muscle of insects. The diagram also depicts the use and interconversion of other dietary carbohydrates as sources of energy.

The enzymes involved are:

1. Glucokinase.
2. Phosphohexose isomerase.
3. Phosphofructokinase.
4. Aldolase.
5. Triosephosphate isomerase.
6. sn-Glycerolphosphate dehydrogenase (Cytosolic)
7. Glyceraldehyde-3-phosphate dehydrogenase.
8. Phosphoglycerate kinase
10. Enolase.
11. Pyruvate kinase.
12. Lactate dehydrogenase.
13. sn-Glycerolphosphate dehydrogenase (mitochondrial).
15. Phosphomannose isomerase.
17. Fructokinase.
18. Phosphorylase.
19. Trehalase.
20. Aldose reductase.
21. Sorbitol dehydrogenase.
FIGURE 4

SORBITOL (20) \rightarrow GLUCOSE \rightarrow TREHALOSE \rightarrow GLYCOGEN

(19)

ATP \rightarrow ADP \rightarrow G_{6}P \rightarrow G_{1}P

(16)

ATP \rightarrow ADP \rightarrow FRUCTOSE \rightarrow F_{6}P \rightarrow MANNOSE-6-P \rightarrow ADP

(15)

(14)

ATP \rightarrow ADP \rightarrow FDP \rightarrow MANNOSE

(13)

(12)

G_{3}P \rightarrow DHA-P \rightarrow Sn-G_{3}P

(11)

ATP \rightarrow ADP \rightarrow 3PGA \rightarrow 2PGA \rightarrow PEP

(10)

(9)

ADP \rightarrow ATP \rightarrow 1,3 DIPHOSPHOGlycerate

(6)

(5)

F_{1} \rightarrow NADH + H+ \rightarrow NAD

(4)

(3)

(2)

(1)

MITOCHONDRIAL MEMBRANE

DHA-P \rightarrow Sn-G_{3}P

FPH \rightarrow FP
detail are the flight muscle and the fat body. Since the flight muscle is a much more active tissue, it has always been the choice of most workers. However, as noted by Kilby (60), although all the enzymes of the tricarboxylic acid cycle have not been demonstrated in the fat body of any single insect species, the findings of various workers on fat bodies of different insect species is indicative of the operation of the cycle in the fat body.

Other confirmatory results of the presence of these enzymes in the fat body came from:

(a) Zebe (61), using locust fat body homogenates showed the presence of citrate synthase and found that the activity of this enzyme was only two percent of that of the flight muscle. Clements (62) also demonstrated the production of $^{14}$CO$_2$ from radiolabelled [2-$^{14}$C] acetate in locusts and found that the $^{14}$CO$_2$ liberation was 95% inhibited by fluoroacetate. This latter compound is known in the rat liver to be converted to fluorocitrate which inhibits aconitase and irreversibly inhibits citrate transport across the mitochondrial membrane. Citrate synthase from locust flight muscle has been reported to be 100 times more active than that of the rat leg muscle (63).

(b) Hearfield and Kilby (64), demonstrated the presence of isocitrate dehydrogenase as well as fumarase in fat body homogenates of locusts.
(c) Bellamy (65) as well as Young (66) demonstrated the presence of α-ketoglutarate dehydrogenase in the locust fat body.

(d) Sactor and Thomas (67) demonstrated the presence of succinate dehydrogenase complex in all the tissues of the American cockroach examined. They found that this enzyme is inactivated on treatment with fat soluble solvents such as cold ether, acetone and ethyl acetate. This finding suggests that the enzyme is a lipoprotein and needs the lipid component to exhibit its activity. Similar conclusions were arrived at by Shigematsu using the fat body of Bombyx mori pupae (46).

In connection with the TCA cycle, insect flight muscle mitochondria including those of locusts are capable of oxidizing a variety of amino acids, mainly proline via Δ''-pyrroline-5-carboxylate to glutamate at a high rate (68). See Figure 5.

The significance of this pathway could be due to provision of the tricarboxylic acid cycle intermediates needed for maximal rate of oxidation of pyruvate at the onset of flight (10) since lactate dehydrogenase activity is normally very low or absent in insect tissues (8).

Transamination of glutamate with pyruvate gives rise to alanine and α-ketoglutarate. Mitochondrial α-ketoglutarate is further metabolized via the tricarboxylic acid cycle to
FIGURE 5
PROLINE OXIDATION BY INSECTS.

Oxidation of proline by flight muscles of some insects (e.g., tsetse fly) which generates an additional oxaloacetate needed to speed up the tricarboxylic acid cycle, consequently generating the much needed energy at the onset of flight. The enzymes involved are:

1. Proline oxidase.
2. Pyrroline carboxylic acid dehydrogenase.
4. Oxaloacetate decarboxylase.
FIGURE 5

Proline → $\Delta'-\text{Pyrroline-5-Carboxylate}$

(1) FP → FPH

(2) $\Delta'-\text{Pyrroline-5-Carboxylate}$ → NAD → NADH

Glutamate → Pyruvate

(3) Pyruvate → Alanine

(4) Oxaloacetate → Ketoglutarate

$\Delta'$ - Pyrroline-5-Carboxylate
form oxaloacetate which combines with acetyl CoA to form citrate which then enters the tricarboxylic acid cycle, generating reducing equivalents which are channeled through the electron transport system to yield ATP (52). A complete explanation for the unique role of proline in providing precursors of oxaloacetate has yet to be formulated.

Mitochondrial oxidation of substrates by enzymes of the tricarboxylic acid cycle is usually coupled to the reduction of coenzymes. Mitochondria also contain the respiratory chain and oxidative phosphorylation system whereby reduced coenzymes generated during catabolism are re-oxidized in a process that couples the re-oxidation of the coenzymes with the phosphorylation of ADP to ATP.

The respiratory chain in insect flight muscle mitochondria is comprised of a system of pyridine nucleotide-linked dehydrogenases, flavoproteins, non-haem iron proteins, quinones and cytochromes all of which catalyze electron transport. The spectral identification, chemical characteristics and concentrations of the components in flight muscle mitochondria, as well as the sequence of the carriers in electron transport have been described elsewhere (13, 40, 60) and will not be elaborated on here. However, there are interesting differences in the pattern of the cytochrome system of the fat body and somatic muscle which are noteworthy.
Cytochromes a + a₃, b and c have been shown by Shappirio and Williams (69) in both tissues of Plastysamia cecropia, but adult fat body in addition, contained relatively high concentrations of b₅. Another notable difference was that while the pattern and the relative concentrations of the cytochromes remained relatively constant in the muscle during development, the pattern in the pupal fat body differed from the larval and adult tissue by the complete disappearance of cytochromes b and c and the relatively low concentrations of cytochromes a + a₃ and b₅. Thus, the very low respiration of tissues during diapause, and their remarkable insensitivity to poisons such as cyanide and carbon monoxide (in contrast to their sensitivity during the larval and adult stages) has been interpreted in terms of the differing cytochrome patterns (69).

At least four enzymes are crucial for gluconeogenesis: Glucose-6-phosphatase, fructose-1,6-diphosphatase, phosphoenolpyruvate carboxykinase and pyruvate carboxylase. Except for the report of phosphoenolpyruvate carboxykinase’s activity in the flight muscle of Locusta migratoria, most of the other three enzymes have been little investigated (70). Pyruvate carboxylase has been reported to be present in all the insect flight muscles that have been examined (71), however, there exists the possibility that the function of this mitochondrial enzyme in insect flight muscle could be
providing oxaloacetate, necessary for the large increase in the tricarboxylic acid cycle activity which occurs at the onset of flight. Fructose diphosphatase, on the other hand, despite its absence in insects like wasps and moths, has been found to be moderately active in the flight muscle of locusts (72). On the contrary, in the flight muscle of the bumble-bees fructose diphosphatase activity has been found to be very high, comparable to phosphofructokinase activity (71,73). Moreover, the bumble-bee phosphatase is unaffected by AMP, which is an important specific effector of gluconeogenesis in the liver. This observation by Newsholme et al. (71) and later by Clark et al. (73) led to the suggestion that both phosphofructokinase and fructose-1,6-diphosphatase are simultaneously active, catalyzing a cycle between fructose-6-phosphate and fructose-1,6-diphosphate leading to continuous hydrolysis of ATP, to release energy in the form of heat. In vivo studies by Bloxam et al. (74) Clark et al. (73) using bumble-bee flight muscle revealed that in flight, glucose was metabolized exclusively through glycolysis and no evidence of cycling was observed. In the resting bumble-bee exposed to low temperatures, however, the pattern of glucose metabolism was altered so that substrate cycling was high and glycolysis decreased. Moreover, the rate of substrate cycling in the flight muscle of a resting bumble-
bee has been found to be inversely related to the ambient temperature. The lower the temperature, the higher the rate of substrate cycling. In addition, Clark et al. (73) have shown that calcium ions inhibit fructose-diphosphatase at concentrations that are without effect on phosphofructokinase. The latter investigators have additionally shown that this inhibition of fructose-1,6-diphosphatase was reversed when calcium ions are chelated with excess of ethanedioxybis-(ethylamine)-tetraacetic acid (EDTA) and proposed that the rate of substrate cycling may be regulated by changes in sarcoplasmic calcium ion concentration associated with the contractile process.

This substrate cycling has been proposed to be the reason why bumble bees are capable of flying and collect food under cold weather conditions whereas honey bees, which have only minimal fructose diphosphatase activity do fly in inclement weather (71).

Experiments conducted tend to suggest that glycerol can enter the glycolytic pathway at the triose sugar levels via the enzymes glycerol kinase and sn-glycerol phosphate dehydrogenase. These two enzymes have been identified in insect tissues (40).

There have been suggestions that insect tissues have the capability to convert lipids to carbohydrates but such a conversion has not yet been unequivocally demonstrated (75).
Spanning an span of about four decades, considerable work has been done on fluorinated compounds which are of biological and biochemical interest. Of biochemical interest is the classical example of the use of 5-fluoro-2'-deoxyuridylate as a probe to obtain information regarding the stereochemistry of the native enzyme's mechanism (75). Apart from providing valuable information regarding the general mechanism of folate enzymes, and in particular, thymidylate synthetase, these fluorouracil analogues are currently used as anti-tumour and anti-viral agents (76) (77).

The only early work reported regarding the use of a fluorocarbohydrate in insects involves fluoroacetate poisoning. This compound has been reported to inhibit aconitase in locust fat body (61): It is now well known that fluoroacetate, which by itself is non toxic, is converted to a toxic metabolite, fluorocitrate, which inhibits aconitase. The toxic isomer of fluorocitrate has been identified by Dummel and Kun to belong to the erythro pair of enantiomers, (−)erythrofluorocitric acid (78, 79). Using sheep liver mitoplast, Kun was able to demonstrate that fluorocitrate inhibits aconitase and irreversibly inhibits citrate transport across mitochondrial membrane (80). Several examples of 'suicide' inhibitors using a carbon-fluorine bond have been reviewed (81,82).
Previous biochemical studies on fluorocarbohydrates by Taylor's group were concerned primarily with enzyme specificity (83) microbial metabolism and transport (84-86) and carbohydrate transport (87-89).

Despite the fact that 3FG elicited many physiological and biochemical effects in rats, it was found not to be toxic even after heavy doses of intraperitoneal injections (81). Some fluorinated compounds were shown to be accumulated in the testes and brain but the majority of the 3FG was found to be rapidly excreted into urine.

With regards to insects, it was found out that 3FG had an LD$_{50}$ of 4.8 mg/g locust when 3FG was administered to two closely related species of locusts, Locusta migratoria and Schistocerca gregaria and the time of the death was reported to be between three to four days. However, the same problem of excretion of substantial amounts of 3FG was encountered as in rats despite the fact that these insect species have a greater water retention ability (90).

Toxicity in whole locusts was reported to be evidenced by a slow loss of motor activity concomitant to death. These symptoms tend to suggest accumulation of toxic metabolite(s) reminiscent of 'lethal synthesis' observed in fluorocitrate poisoning (76). Qualitative estimation of neutral carbohydrates in three major tissues, namely, the haemolymph, fat body, and flight muscle revealed the dis-
appearance of 3FG from these tissues with the appearance of a new metabolite in the haemolymph and fat body identified as 3-fluoro-3-deoxy-glucitol (3FGL). The appearance of 3FGL is suggestive of the fact that 3FG is capable of being metabolized via 3FGL to 3-deoxy-3-fluorofructose which can subsequently be phosphorylated and thus enter glycolysis (Figure 6).

Using partially purified fat body preparations, the presence of the aldose reductase and sorbitol dehydrogenase (EC 1.1.1.21) have been shown to be present in the fat body. However, as mentioned earlier, the precise mechanism as to how 3FGL and for that matter sorbitol is phosphorylated to enable these substrates to enter into the glycolytic pathway is still obscure, despite the demonstration of the presence of a non-specific hexokinase in insect flight muscle and fat body (46, 49).

Since 3FGL acts as an inhibitor for locust fat body sorbitol dehydrogenase with a Ki of 82 mM, and as a substrate for the same enzyme at higher concentrations of substrate (Km 500 mM), it was thought that 3FGL could possibly not be the toxic metabolite causing death in whole adult locusts. Attempts to pinpoint the toxic metabolite or target enzyme led to a series of experiments culminating in the following findings in whole male insects, Locusta migratoria:

(a) That the pentose phosphate pathway contributed very
FIGURE 6
SORBITOL SHUNT IN LOCUST FAT BODY*

This illustrates the proposed pathway for the metabolism of 3PG through 3PGL to 3FF.
The enzymes involved are:

1. Aldose reductase.
2. Sorbitol dehydrogenase.
3. Hexokinase.

*This figure was adapted from Romachin, A. (55)
FIGURE 6

(1) \[ \text{NADPH} \rightarrow \text{NADP} \]

(2) \[ \text{NAD} \rightarrow \text{NADH} \]

(3) \[ \text{GLYCOLYSIS} \]
little to the energetics of 3FG metabolism and as such, is not inhibited by virtue of 3FG metabolism. Similar conclusions were arrived at by Chefurka (40) and Young (41) using glucose.

(b) That the tricarboxylic acid cycle was not inhibited after 3FG administration.

(c) That the glycolytic pathway was inhibited by as much as 50% after 3FG administration and the onset of inhibition was observed to be faster when 3FG was administered at shorter time intervals between 3FG and radiolabel injections.

(d) That 3FG was metabolized as far as the triose phosphate sugars of the glycolytic pathway since small but significant amount of deitritiation was observed when insects were injected with D-[3-3H]-3-deoxy-3-fluoro-D-glucose.

(e) That there was significant defluorination of 3FG which could possibly be inhibiting some fluoride sensitive enzymes in the glycolytic pathway, for example, enolase. This was further substantiated by the fact that whole locusts were very sensitive to very low inorganic fluoride levels (when injected) resulting in death.

(f) Finally, a phosphorylated, fluorinated unidentified metabolite was isolated from whole insects after 3FG treatment.
From the above findings, it appears that somewhere along the glycolytic pathway, a metabolite of 3FG was establishing a bona fide block resulting in the death of whole insects. However, the effect of 3FG on glycogenesis, glycogenolysis, electron transport system as well as fat metabolism has not been investigated (55).

The main objective of this dissertation is to establish where a metabolite of 3FG is acting in the glycolytic pathway using homogenates of locust flight muscle and fat body in view of the fact that the use of whole insects (despite the fact that they produce valuable information) provides only a general overview of the insects metabolism of 3FG.
CHAPTER II

MATERIALS AND METHODS

Reagents

All reagents were of analytical grade meeting ACS specifications unless otherwise stated. All aqueous solutions were prepared using distilled deionized water. The following reagents were used: Glucose-1-phosphate, glucose-6-phosphate, fructose-1-phosphate, fructose-6-phosphate, fructose-1,6-diphosphate, glyceraldehyde-3-phosphate (diethylacetal), UDP-glucose, UTP, α-amylase (1,4-α-D-glucan glucohydrolase; EC.2.1.1), NAD⁺, NADPH and ATP were all purchased from Sigma (St. Louis, Missouri). D-glucose, D-fructose, glycogen, trehalose, sorbitol, sucrose, maltose, Amberlite IR-120 (H⁺), Amberlite IR-45(OH) were purchased from BDH (Toronto, Ontario). Dowex AG 50WX12(H⁺), AG1WX8 (formate) and SDS polyacrylamide gel electrophoresis kit were purchased from Bio-Rad (Richmond, California). D-[3-3H] glucose (2-5 Ci/mmol) and D-[U-14C] glucose (2-4 mCi/mmol) were purchased from Amersham (Oakville, Ontario). Whatman No. 1 chromatography paper (46 cm x 57 cm) was purchased from Fisher (Toronto, Ontario).
Equipment

All pH measurements were carried out on a Metrohm model E-510 using Fisher combination electrode, and all fluoride ion measurements were carried out on the same equipment using fluoride specific electrode purchased from Orion Research (Cambridge, Ma). All respirometric work were carried out on Gilson Differential Respirometer (Gilson Medical Electronics, Middleton, Wisconsin). All densitometric scans of gels were carried out on Ortec Model 4310 and all a radiochromatographic scans were carried out on Packard Model 7220. All centrifugations were carried out on Sorvall Model RC2-B and spectrophotometric measurements were carried out on Beckman UV-VIS ACTA MVI. Liquid scintillation counting was done on Beckman LS 75000 with an built-in microprocessor and all tissue homogenizations were carried out in a polyclon tissue homogenizer type PT 10/35.

Synthesis of 3-Deoxy-3-Fluoroglucoe (3FG)

Crystalline, twice recrystallized 3FG was prepared basically as reported (91) with two modifications. The modification steps are:

1. OXIDATION STEP

Synthesis of 1,2:5,6-Di-O-isopropylidene-α-D-Ribo-3-hexulofuranose (92).

165g (0.72 mole) of potassium or sodium metaperiodate,
18g of potassium or sodium carbonate are added to 500 mL water in a 3-L flask. To this continuously stirred solution is added 2g of ruthenium dioxide (50-60% hydrated) to generate the 'active' form which was yellowish in colour. To this solution is added 125g (0.48 mole) of alcohol-free chloroform (550 mL) solution of pre-dried 1,2:5,6-di-O-isopropylidene-α-D-glucofuranose.

Using a heavy duty stirrer, the reaction mixture was stirred for 15 h at room temperature by which time thin-layer chromatography indicated the conversion of the starting material to the hydrated ketone. (If conversion is incomplete, additional small amounts of potassium or sodium carbonate and metaperiodate are added and the reaction allowed to proceed for another 2 to 3 h. However, prolonged exposure to oxidant could lead to overoxidation).

The oxidation was terminated by the addition of 50 mL of isopropanol and stirring the reaction for a further 10 min. This allows the regeneration of the inactive ruthenium. The reaction mixture was then filtered under vacuum and the residue washed with two 50-mL portions of chloroform. The aqueous layer was then separated from the organic layer with the latter layer washed with three 200-mL portions of dichloromethane. The combined organic layer was then dried over reduced pressure to yield the hydrated or anhydrous ketone which could be crystalline or syrup.
Dissolution of the crystalline mass in minimal amount of warm ether and with the addition of an equal volume of petroleum ether (30°C - 60°C) gives a pure crystalline hydrated ketone of 86% yield. In the case of syrups, the product was washed with cold petroleum ether and used directly in the next step.

2. FLUORINATION STEP

3-Fluoro-1,2:5,6-di-O-isopropylidene-α-D-glucofuranose (93).

A mixture of anhydrous potassium fluoride (140g) and acetamide (700g) is heated internally to about 150°C (to drive off any moisture) with continuous stirring.

To this, 80g of pre-dried 1,2:5,6-di-O-isopropylidene-3-O-toluene-p-sulfonyl-α-D-allofuranose was added and the temperature of the reaction mixture raised to 210°C (internal temperature) for 1 h, by which time thin-layer chromatography indicated that the reaction has gone to completion. The reaction was then allowed to cool to about 95°C then poured into a saturated solution of sodium bicarbonate (1.2 L). The bicarbonate solution is then allowed to cool to room temperature then filtered off from the insoluble tar. The tar and the filtrate were then extracted with ether (2x200 and 4x400-mL, respectively). The combined ethereal extracts were then washed with water (2x300-mL), dried over anhydrous sodium sulfate, and then evaporated to dryness, resulting in a yellow syrup which was then purified
by column chromatography on silica gel 60 (70-230 mesh) with the proportion of compound to silica gel being 1:50 (w/w) using benzene:ether (9:1 v/v) as eluent.

Tritiated 3FG Preparation

D-[3-3H]3FG was prepared synthetically by the procedure developed by Lopes and Taylor (89) with a specific activity of 17.1 mCi/mg. The purity was checked by its melting point and thin-layer chromatography on 20 cm x 20 cm silica gel plates (0.1 mm thickness) with plastic support. The solvent system used was ethyl acetate:ethanol (4:1 v/v).

Locust Rearing Conditions

Locusts were reared under crowded conditions in a temperature and humidity controlled environment room, kept at 30°C and 30% humidity. Diet consisted of washed local field grass and store bought carrot tops in the winter. Only healthy adult male locusts (about two weeks after final ecdysis) were used. They were normally starved for two and a half days (a period during which they were fed with only water) before being sacrificed.

Preparation of Fat Body and Flight Muscle Homogenates

Adult male locusts (about 14 days old after final ecdysis) were starved for about three days within which time they were given water. They were then stunned at -10°C for 15 min after which they were kept under ice in
the process of dissection. The dissected tissues were rinsed three times in 50 mM ice-cold Tris buffer (pH 7.5) and homogenized at a high speed with polytron homogenizer for a total of 1 min with intermittent cooling. The homogenates were then spun at 12,000 x g for 10 min at 4°C and the resulting supernatant passed through three layers of cheese cloth to remove the upper lipid layer (in the case of fat body homogenates) obtained after centrifugation. Protein concentrations of the pre-centrifuged pre-sieved supernatates were determined by Lowry's method (94). These supernatates (referred to as homogenates throughout this text) were used for all studies unless otherwise stated.

Respirometric Studies

The oxidation of exogenous substrates by flight muscle and fat body homogenates was followed manometrically using a Gilson Differential Respirometer with 15-mL calibrated reaction flasks. Each reaction flask contained different concentrations of substrates in the sidearm with 0.2-mL of 20% potassium hydroxide and a folded paper wick placed in the centre well to absorb any carbon dioxide produced. The main compartment of the reaction flasks contained buffered homogenates, and the following cofactors: ATP, MgCl₂, and NAD⁺. The reactions, after preincubation at 37°C for about 45 min, were started by tipping the contents of the
side-arm into the main compartment and the amount of oxygen consumed was monitored as described in the instruction manual. Controls containing no substrate were set alongside the test samples.

**Locust Fluoride Measurements**

Flight muscle and fat body homogenates were incubated in 50 mM Tris buffer with ATP, NAD, MgCl₂ and various concentrations of 3FG. Two controls, containing only homogenates or 3FG were run alongside with test samples. The reactions were all carried out in 'nalgene' plastics covered with cotton wool to prevent evaporation. The reactions were carried out at 37°C in a shaker-water bath and at different time intervals, fluoride measurements were taken using 'Metrohm' meter equipped with a fluoride specific electrode. The fluoride standards were prepared in TISAB* and the fluoride concentrations at different time intervals were determined from a semi-log plot of millivolt potential versus fluoride concentrations as shown in Figure 7.

**Assay of H₂⁰ from Fat Body and Flight Muscle Homogenates**

The simple micro-procedure of assay of H₂⁰ released from tissues was used as reported by Clark (95). The procedure involves the use of plastic micropipet tips (designed to fit Oxford brand automatic micropipets) were plugged at the small end using pre-rinsed fine glass wool. Each pipet

*TISAB is the trade name of a buffer purchased from Orion.
FIGURE 7

CALIBRATION CURVE FOR FLUORIDE

Semilog plot of millivolt potential versus log of fluoride ion concentration used to estimate the concentration of fluoride released from 3FG (see APPENDIX I).
tip was filled with about 0.5g of wet Dowex AG1x8 resin in the borate form. This was prepared by washing Dowex AG1x8 (200-400 mesh) resin in the chloride form with 2M potassium borate, pH 9.0, until the effluent was chloride free. A subsequent wash with distilled deionized water was necessary to bring the pH down to 7.5. 100-μL samples containing 3H2O were loaded onto each column and washed through by the application of 3 x 0.3-mL aliquots of distilled deionized water. The effluents were collected directly in 25-mL scintillation vials and was dispersed with Beckman Ready-Solv. These borate columns have been known to retard glucose, 3PG, fructose, and other metabolites summarized by Clark (95).

Homogenates were pre-incubated with cold glucose or 3PG with the appropriate cofactors at 37°C in a metabolic incubator. At different time intervals D-[U-14C] glucose of specific activity 118 μCi/μg was added. Three hours after adding the radioactive glucose the reactions were stopped by the addition of 0.6 mL of ice-cold perchloric acid (2M). The precipitated proteins were then centrifuged at 12,000 x g for 10 min at 4°C and the resulting supernatant neutralized with ice-cold 2M KOH (about 0.6 mL was required). The neutralized samples were then stored at 4°C to afford the settlement of the potassium perchlorate complex. The neutralized samples were then centrifuged
again at 12,000 x g at 4°C and 100 μL of the supernatant was applied onto the borate column and eluted.

Isolation of Phosphorylated and Non-phosphorylated Sugars

Flight muscle and fat body homogenates prepared in 50 mM Tris buffer, pH 7.5 were incubated with 256 mM (7.0 x 10^5 dpm) of D-(U-14C)glucose or 256 mM (8.6 x 10^6 dpm) of D-(3-3H)3FG. The reaction mixtures also contained 40 mM NAD^+, 40 mM ATP and 20 mM MgCl₂, in a total volume of 5.0 mL. The reactions were covered with loose cotton wool to prevent evaporation and shaken on a shaker-water bath at 37°C. The reactions were stopped at different time intervals by the addition of 0.6 mL of 2M ice cold perchloric acid. The precipitated proteins were then centrifuged at 12,000 x g for 15 min at 4°C. The resulting supernatants were then neutralised with 2M of ice-cold potassium hydroxide and allowed to stand at 4°C overnight. The resulting potassium perchlorate complex was then removed by centrifugation at 12,000 x g for 15 min at 4°C. The resulting neutralised supernatants were then lyophilised and reconstituted in 1 mL of distilled deionised water, stored at -20°C until ready for use.

In a similar experiment, flight muscle and fat body homogenates were pre-incubated with 'cold' 3FG for 6, 12, and 24 h. After these times, 20 mM of D-(U-14C)glucose (6.0 x 10^3 dpm) were added to the reaction mixtures. 4 h after adding the 'hot' glucose to the reaction mixtures, the proteins
in the reactions were precipitated by the addition of 0.5 mL of 2M ice-cold perchloric acid and processed as outlined earlier.

**Separation of Phosphorylated Metabolites.**

The method of separation was an adaptation of that of Runekles and Krotkov (96). Sheets of Whatman No. 1 filter paper (24 cm x 27 cm) were pre-washed in 2M HCL, followed by 8-hydroxyquinoline solution (15 g/2L). This was followed by several rinses of glass distilled deionised water, and given a final wash with 95% ethanol to prevent their wrinkling on drying. The same type of resolution was achieved when the papers were pre-treated with 1N formic acid followed by 0.5% EDTA (brought to pH 8.5 with NaOH) as reported by Eggleston and Hems (97). Standards (5 μL of 20 mM) and 20 μL of samples were spotted. The chromatograms were run three times at room temperature by the ascending method for 2.5h each time to a pre-marked solvent front with about 3h air-drying intervals. The solvent system comprised of: ethylene glycol (monomethyl ether): pyridine: acetic acid: water (80:40:10:10 v/v). The chromatographic tanks were pre-saturated with the above solvent system for 4h prior to running the chromatograms.
Detection of Phosphorylated Metabolites

The location of the phosphate areas was achieved by using the modified method of Wade and Morgan (98). The method involves dipping the chromatograms into a solution of ferric chloride (150 mg FeCl$_3$.6H$_2$O, 3 mL of 0.3N HCL, and 97 mL acetone), air drying the chromatograms, then dipping them in an acetone solution of 1.25% sulfosalicylic acid, then allowing the chromatograms to air-dry. Phosphate spots appear white against a reddish-pink background. Occasionally, the molybdate spray technique of Hanes and Isherwood was used (99).

Separation Of Non-phosphorylated Metabolites

The method employed in the separation was that described by Gordon et al. (100), except for the descending chromatography used in this case.

Sheets of Whatman No. 1 filter paper (46 cm x 57 cm) were serrated at the end opposite point of application of samples. Standards (5 µL of 20 mM) and 20 µL of samples were spotted on the chromatogram. The chromatograms were developed for 16h at room temperature using 100 mL of the developing solvent: methyl ethyl ketone: boric acid saturated water: acetic acid(90:10:10 v/v), after presaturating the tank for 2h with the developing solvent. After 16h period, the chromatograms were air-dried and sprayed three times with methanol, allowing the chromatogram to air-dry after each spraying in the fume hood.
Methanol spraying afforded the boric acid impregnated in the paper to be removed as volatile methyl borate.

Attempts to separate non-phosphorylated metabolites of very close mobilities with other solvent systems did not yield fruitful results.

The other solvent systems tried were:

(a) On silica gel 60
   (i) Ethyl acetate: acetic acid: water (3:3:1 v/v)
   (ii) Ethyl acetate: pyridine: water (9:5:4 v/v)

(b) On Whatman No.1 filter paper
   (i) n-butanol: 95% ethanol: diethylamine (80:10:20 v/v)
   (ii) ethyl acetate: pyridine: water (9:5:4 v/v)
   (iii) Water saturated phenol adjusted to pH 4.4 with glacial acetic acid to which ethylenediamine tetraacetic acid has been added to a final concentration of 1 mM.

Detection of Non-phosphorylated Metabolites

After spraying the chromatograms with methanol and air drying them in the fume hood, the spots on the chromatogram were detected by the method of Gordon et al. (100). The chromatograms were first dipped in a solution of periodic acid (2.28 g H₃IO₆ in 100 mL water; dilute 2 mL of this with 18 mL of acetone before using), air-dried, then finally dipped in a solution of benzidine (184 mg
benzidine, 0.6 mL glacial acetic acid, 4.4 mL water and 95 mL acetone). The spots appear in different shades of colour depending on type of sugar.

Strips of these chromatograms or chromatograms run on silica gel plates were cut lengthwise and radioscanned to locate regions of radioactivity.

**Liquid Scintillation Counting**

All counting was done to a statistical accuracy of 1% using a Beckman LS 7500 liquid scintillation counter with a built-in microprocessor. The H-number concept of quench correction (see manufacturer's manual for the theoretical discussion of this concept) was used to obtain the quench correction curves for both $^3$H (Figure 8) and $^{14}$C (Figure 9) using standards purchased from Nuclear Chicago. Attenuator and discriminator settings of each channel were determined as per operating manual.

**Preparation of Chromatographic Samples for Liquid Scintillation Counting**

(a) **Paper Chromatography**

These samples were spotted in duplicates on Whatman No. 1 filter paper. After developing the chromatogram by the descending method as described earlier, one half
FIGURE 8

QUENCH CORRECTION CURVE FOR $^{3}\text{H}$-SAMPLES
($\delta$ EFFICIENCY VERSUS $\text{H}$-NUMBER)

$^{3}\text{H}$-toluene quenched standards containing $4.55 \times 10^5$ dpm were used to construct the curve.
FIGURE 9

QUENCH CORRECTION CURVE FOR $^{14}\text{C}$-SAMPLES
(% EFFICIENCY VERSUS H-NUMBER)

$^{14}\text{C}$-toluene standards containing $1.9 \times 10^5$ dpm were used to construct the quench curve.
of the chromatogram was stained to enable the detection of sugar spots, which were matched with the unstained spots. The spots corresponding to the stained half were cut and placed into scintillation vials. 1 mL of distilled deionised water was added to the vials for 40 min (to afford elution of the samples) after which 10 mL of cocktail (Beckman Ready Solv) were added and the vials were counted three times. Controls, (containing no spotted samples) were also treated in the same manner and counted. Earlier attempts to dissolve the cut out papers proved unsuccessful.

(b) Thin-layer Chromatography on Silica Gel Plates

The plates, on plastic supports, were treated in a similar manner as was done for the paper chromatograms, except that in this case, the spots were snipped out, eluted in 1 mL distilled deionised water before counting.
**Estimation of Glycogen**

80 μL of sample and standard were run by descending chromatography, for 16 h after which time the glycogen spot was cut and eluted in 20 mL of water. The eluted samples were evaporated to dryness under reduced pressure at 30°C and reconstituted in 1 mL of 10 mM of phosphate buffered saline buffer, pH 7.0. 300-μL aliquots from each reconstituted sample were then used in glycogen estimation by the α-amylase method reported by Rick and Stegbauer (101).

The analysis involved treatment of samples with α-amylase (of specific activity 1000 U/mg) for 10 min at room temperature after which the reactions were stopped by the addition of 1 mL of colour reagent (3',5'-dinitrosalicylic acid reagent). The reaction mixtures were then boiled for 10 min, cooled under tap water and made up to 5 mL.

Two controls, one containing pre-boiled enzyme and a water blank were run alongside.

The optical densities were then taken at 546 nm and the concentration of maltose units produced by the α-amylase action determined from a maltose standard curve (Figure 10).

**Estimation of Trehalose using the Enzyme Trehalase**

After chromatographing 80 μL of samples (in duplicates) on Whatman No. 1 filter paper by descending chromatography, the spot corresponding to trehalose was cut, eluted in 15 mL
FIGURE 10

CALIBRATION CURVE FOR MALTOSE

Standard curve used to estimate the concentration of glycogen as maltose units produced as a result of α-amylase treatment (see APPENDIX I).
distilled deionized water, evaporated to dryness at 30°C under reduced pressure and reconstituted in 1 mL of distilled deionized water. 300 μL of this sample was then used for hydrolysis using crude enzyme preparations obtained from *Dictyostelium discoideum* (NC_4-H). The hydrolytic procedure was adapted from Chan and Cotter (102) except that hydrolysis was allowed to proceed for 3 h at room temperature with continuous shaking. The reactions were stopped by boiling for 10 min, cooled and centrifuged on a bench centrifuge for 15 min. 1-mL aliquot of this supernatant was used for the quantitative and qualitative analysis of glucose residues released by the enzyme trehalase. The quantitative estimation of the glucose released employed an improved micromethod developed by Capaldi and Taylor (103) where 3-methyl-2-benzothiazolinone hydrazone (MBTH) was used as a chromogen (due to its high extinction coefficient) in a glucose oxidase-peroxidase reaction. To 1 mL of sample, 200 μL of solution containing 0.85 mM MBTH and 0.83 mM formaldehyde was added and properly mixed. To this, was added a 50-μL enzyme solution containing 0.5U of peroxidase and 5U of glucose oxidase. The reaction was allowed to proceed for 2.5 h after which the reaction was stopped by the addition of 500 μL of acetone. The absorbance of the colour developed from the reaction was read at 670 nm.
and using the extinction coefficient (55,000) one was able to calculate the concentration of glucose released by the enzyme.

A control of pre-boiled enzyme was run alongside the test samples and a reagent blank without any substrate, served in zeroing the spectrophotometer.

**Estimation of Fructose Eluted from Paper Chromatograms**

Fructose spots were eluted from chromatograms as outlined for trehalose. The method used for the quantitative analysis of fructose was that reported by Roe (104). To 100 μL of test samples, 200 μL of a solution of 50 mg resorcinol in 50 mL of 95% ethanol were added. To this was added 600 μL of a solution of 50 % (v/v) hydrochloric acid, mixed well and the tubes covered with marbles to prevent evaporation. The reaction mixtures were then incubated at 80°C for exactly 8 min, cooled under cold running water and the absorbances read against a water blank containing the hydrochloric acid and resorcinol at 530 nm. Control samples containing all reagents except for the incubation period and time, were run alongside the test samples. Fructose concentrations were determined from a standard curve prepared as shown in Figure 11.
FIGURE 11

CALIBRATION CURVE FOR FRUCTOSE

Standard curve used to estimate the concentration of fructose from spots eluted from radiochromatograms (see APPENDIX I).
FIGURE 11

OD530nm.

[Fructose] µg

0 20 40 60 80 100
Acid Hydrolysis of Fluorotrehalose and Fluoroglycogen Eluted from Paper Chromatograms

Glycogen and trehalose spots (80 µL each) were eluted from paper chromatograms as outlined earlier. The eluted samples were hydrolyzed with 1 mL of 1M hydrochloric acid for 1 h in boiling water bath. The hydrolyzed materials were then neutralized with 1M sodium hydroxide (about 1 mL), passed through Amberlite resin IR-120 (H⁺) to remove the sodium ions and then through Amberlite resin IR-45 (OH⁻) to remove the chloride ions. The collected samples were then evaporated to dryness under reduced pressure at 30°C and then reconstituted in 500 µL of distilled deionised water. 25 µL of hydrolyzed and unhydrolyzed samples were then spotted alongside 20 µL of glucose, glycogen and trehalose standards. The radioactivity in both the hydrolyzed and unhydrolyzed samples were determined by cutting out unstained portions of the chromatograms, adding 1 mL of distilled deionized water and then the cocktail. Liquid scintillation counting was carried out as reported earlier.

Preparation of Samples for Gel Electrophoresis

Flight muscle and fat body homogenates prepared in 50 mM Tris buffer, pH 7.5 as described earlier were incubated at 37°C in a water bath with constant shaking. The reaction mixtures contained 15 mM NAD⁺, 15 mM ATP, 7.5 mM
MgCl₂ and 80 mM ³H-3FG with a dpm of 5.4 x 10⁷. The reaction volume was 5.0 mL and the reaction flasks were covered with cotton wool to prevent evaporation. The reactions were allowed to incubate for periods of 6 h, 12 h and 24 h after which time they were stopped by immersion in liquid nitrogen.

For the fat body, due to lack of substrate, only a 24-hour incubation period was made.

The frozen samples were then warmed up to about 2°C and then poured into dialysis bags of exclusion volume of 10,000 (in terms of molecular weights). The samples were then dialyzed against 25 mM sodium phosphate buffer, pH 7.2 at 4°C. The buffer was changed every 30 min for the first 3 h and thereafter every 6 h for 30 h. Each time the buffer was changed 100 microliters of the buffer was counted for radioactivity by liquid scintillation counting until the counts were about twice the background (i.e., 60 cpm). The samples were then transferred into test tubes and 100-µL aliquots of each sample was used for protein determination by the Lowry’s method (94). A standard curve prepared using bovine serum albumin enabled the concentration of protein in each sample to be assessed (Figure 12). The samples were then capped, frozen until needed for electrophoresis.
FIGURE 12

CALIBRATION CURVE FOR PROTEINS

Standard curve used to determine the concentration of proteins used throughout this study (see APPENDIX I).
Separation of Alkylated Proteins by Electrophoresis

All solutions used in the preparation as well as running of the 10% gels was that reported by Osborne and Weber (105) except that the pH of the gel and electrode buffers were of pH 7.2 instead of pH 7. Clean siliconized tubes of 6 mm in diameter and 16 cm in length were used in the preparation and running of the gels. The height of the gels in these tubes were 14 cm and any gel of uneven surface was discarded.

200 µg of protein samples were vortexed with a solution of 100 µL of 1% sodium dodecyl sulfate (SDS) and 1% β-mercaptoethanol. To this, 100 µL of 50% sucrose solution and 30 µL of 0.05% bromophenol blue (tracking dye, (BPB)) were added and vortexed. The mixture was then heated in boiling water for 8 min after which the tubes were cooled under cold running water to room temperature.

In the heating process, marbles were used to cover the test tubes to minimize evaporation. After cooling to room temperature, about 100 µL of protein samples were first layered onto the gels, the tubes slotted into the electrophoretic chamber and then layered with gel buffer using a Pasteur pipette. The lower and upper chambers of the electrophoresis unit were filled with the electrode buffer (100 mM sodium phosphate buffer pH 7.2 containing 1% SDS). The samples were run at constant current of 8 milliamperes.
per gel with the positive electrode in the lower chamber. The electrophoretic runs were carried out for about 7 h by which time the tracking dye had moved almost 13 cm the length of the tube. The gels were removed from the tubes by squirting water from a syringe between the gel and the glass wall. The distance moved by the tracking dye was marked by pricking the gel with a needle pre-dipped in drafting ink. The gels were then placed in large test tubes (2.5 cm in internal diameter and 20 cm in height) for staining and destaining.

Location of Protein Bands After Electrophoresis

The gels were stained for protein with Coomassie blue. The staining procedure used was that of Fairbanks et al. (106). Individual gels were first placed in about 50 mL of 25% isopropyl alcohol and 10% acetic acid containing 0.03% Coomassie blue and gently shaken overnight at 30°C. This was followed by a 50 mL solution of 10% isopropyl alcohol and 10% acetic acid containing 0.003% Coomassie blue for 12 h.

The third step involved a 50 mL solution of 10% acetic acid containing 0.001% Coomassie blue for 24 h. The last step involved shaking with 50 mL of 10% acetic acid until background was clear. The bands were either scanned using a densitometer or their relative mobilities
to the dye front measured (i.e., distance moved by protein band/distance moved by tracking dye). The molecular weights of the protein bands were estimated by running SDS treated standards alongside test samples. From the mobilities of the standards and their molecular weights the molecular weights of the bands in the samples were estimated (Figure 13).

Preparation of Gels for Liquid Scintillation Counting

After locating the protein bands, the individual bands were cut directly into scintillation vials. To the cut gels were added 100 μL of 30% hydrogen peroxide. The vials were then tightly capped and the gels digested in a water bath at 55°C overnight. After the digestion period, the vials were water cooled to room temperature, 10 mL of scintillation cocktail added and counted. A control sample, not pre-incubated with 3H-3FG was run alongside the test samples which also served as a background count.

Preparation of Glycogen and Trehalose Fractions for Fourier Transform NMR

After the separation of non-phosphorylated metabolites as described earlier, glycogen and trehalose spots were cut and eluted in 15 mL of distilled deionized water. The samples were then evaporated to dryness under reduced
FIGURE 13
CALIBRATION CURVE FOR MOLECULAR WEIGHT DETERMINATIONS

Plot of log of molecular weights versus relative mobility used in estimating the molecular weights of proteins run by SDS gel electrophoresis (see APPENDIX I). Relative mobility is expressed as distance moved by protein divided by distance moved by marker dye.

The proteins (purchased from Sigma) used in the construction of this curve are:

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine serum albumin</td>
<td>66,000</td>
</tr>
<tr>
<td>Egg albumin</td>
<td>45,000</td>
</tr>
<tr>
<td>Trypsinogen</td>
<td>24,000</td>
</tr>
<tr>
<td>β-Lactoglobulin</td>
<td>&gt; 18,000</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>&gt; 14,000</td>
</tr>
</tbody>
</table>
pressure at 30°C, reconstituted in 500 μL of distilled deionized water and then transferred into an NMR tube (17.5 cm in height x 0.5 cm external diameter).
CHAPTER III

RESULTS AND DISCUSSIONS

The use of fat body and flight muscle homogenates afforded their respiration with glucose and 3PG (as substrates) as well as the effect of 3PG on glucose metabolism with these tissue homogenates to be studied.

With micromolar concentrations of glucose or 3PG, fat body homogenates have been found to be capable of utilizing 3PG and glucose. However, the extent of utilization of 3PG was found to be about 48% lower compared to glucose (Figure 14). Two noteworthy observations made with fat body homogenates were: (a) After forty minutes, the extent of respiration using 3PG as substrate remained constant whereas glucose incubated homogenates continuously increased. (b) After ninety minutes, respirometric values subtracted from the endogenous were negative.

These observations which were only peculiar to fat body homogenates were not observed for flight muscle homogenates (Figure 15). One possible explanation could be the utilization of 3PG via biosynthetic routes compared to degradative pathway. Another possible explanation could be the derivation of a metabolite which could possibly
FIGURE 14

RESPIROMETRIC STUDIES USING
FAT BODY HOMOGENATES.

Gilson conditions: 37°C, total volume, 3.0 mL, gas phase, air. Each flask contained 20 μM NAD, 20 μM ATP, 10 μM MgCl₂, 7 mg fat body and 50 mM Tris buffer pH 7.5 to 2.8 mL in the main well, 0.2 mL 20% KOH in the centre well. Reactions were pre-incubated for 45 min before tipping 0.2 mL substrate from side-arm to start the reactions.

○—○ 100 μM glucose. ▲—▲, 100 μM 3FG.

All values have been corrected for endogenous respiration.
Amount of O₂ Consumed (μl/mg prot) vs. Time (min)
FIGURE 15

RESPIROMETRIC STUDIES USING FLIGHT MUSCLE HOMOGENATES

Gilson conditions: 37°C, total volume 3.0 mL, gas phase air. Each flask contained 8 mg protein, 2 mM NAD, 2 mM ATP, 1 mM MgCl₂ and 50 mM Tris buffer pH 7.5 to 7.8 mL in the main well, 0.2 mL 20% KOH in the centre well. Reaction mixtures were equilibrated for 45 min before tipping 0.2 mL substrate from side-arm to start the reactions.

\[ \downarrow, 15 \text{ mM 3PG} \quad \circ--\circ, 15 \text{ mM glucose.} \]

All values have been corrected for endogenous respiration.
be acting to partially inhibit the oxidation of 3FG.

Contrary to the micromolar concentrations of substrate used to obtain measurable amounts of oxygen consumed for fat body homogenates, flight muscle tissue homogenates have been found to require millimolar concentration of substrates (especially 3FG). Figure 15 illustrates the uptake of oxygen resulting from incubation of flight muscle tissue homogenates with glucose or 3FG. While flight muscle tissue homogenates metabolize both glucose and 3FG to a greater extent compared to fat body tissue homogenates (Figure 14), the amount of oxygen consumed using 3FG as substrate has been found to be 33% lower compared to glucose. However, this depression is 23% higher than fat body tissue homogenates.

Examination of the effect of 3FG on glucose oxidation revealed that increasing concentrations of 3FG decreased the amount of oxygen consumed from glucose using both fat body tissue homogenates (Figure 16) and flight muscle tissue homogenates (Figure 17). However, two major differences were noted between the two tissue homogenates. Whereas fat body homogenates required micromolar concentrations of substrates for the effect of 3FG on oxygen consumption to be observed, flight muscle homogenates required millimolar concentration ranges. Secondly, the initial rates of oxygen uptake by fat body homogenates was the same for about one hour before
FIGURE 16

INHIBITION STUDIES USING FAT BODY HOMOGENATES

Gilson conditions: 37°C, total volume, 3.0 mL, gas phase air. Each flask contained 20 μM ATP, 20 μM NAD⁺, 10 μM MgCl₂, 7 mg fat body homogenate and 50 mM Tris buffer pH 7.5 to 7.6 mL in the main well, 0.2 mL KOH in the centre well. The reactions were pre-incubated at 37°C for 30 min, 25 μM, 50 μM and 100 μM 3FG (0.2mL) was now added to flasks 1, 2 and 3, respectively, and incubation continued for 2 h followed by the addition of 100 μM (0.3 mL) glucose from the side-arm. To flasks 4 and 5, 0.2 mL (100 μM) 3FG and glucose were added, respectively.

O—O, 100 μM glucose; Δ—Δ, 100 μM 3FG; □—□, 25 μM 3FG + 100 μM glucose; ●—●, 50 μM 3FG + 100 μM glucose; ▲—▲, 100 μM 3FG + 100 μM glucose.

All values have been corrected for endogenous respiration.
FIGURE 17

INHIBITION STUDIES USING FLIGHT MUSCLE HOMOGENATES

Gilson conditions: 37°C, total volume 3.0 mL, gas phase air. Each flask contained 8 mM NAD, 8 mM ATP, 4 mM MgCl₂, 8 mg protein and 50 mM Tris buffer pH 7.5 to 2.6 mL in the main well, 0.2 mL 20% KOH in the centre well, 17 mM, 34 mM and 67 mM 3FG (0.2 mL) was now added to flasks 1, 2 and 3, respectively, and incubation continued for 2 h followed by the addition of 0.2 mL (67 mM) glucose from side-arm. To flasks 4 and 5, 0.2 mL (67 mM) 3FG and glucose (67 mM) were added, respectively.

O—O, 67 mM glucose; Δ—Δ, 67 mM 3FG; •—•, 17 mM 3FG + 67 mM glucose; ○—○, 34 mM 3FG + 67 mM glucose, ▼—▼, 67 mM 3FG + 67 mM glucose.

All values have been corrected for endogenous respiration.
Figure 17

Amount of O₂ Consumed (μL/mg prot.) vs Time (min)
differences in the rates and extents could be noticed, while flight muscle homogenates demonstrated differences in rates and extents throughout the time period that the measurements were made.

The apparent reason why such differences existed between the fat body and flight muscle homogenates could be due to the relative differences in the activities of both tissue homogenates. In this case, the fat body has been found to metabolise its substrates at a faster rate compared to the flight muscle tissue homogenates.

The demonstration of the presence of aldose reductase and sorbitol dehydrogenase in the fat body of the locust (55) prompted us to find out the possibility of the presence of these two enzymes in the flight muscle.

Figures 18a and 18b show, respectively, the rate curve and the double reciprocal plot of aldose reductase from homogenates of locust flight muscle. With the flight muscle enzyme, the apparent $K_m$ was found to be 30 mM with an apparent $V_{max}$ of 0.004 $\mu$M NADP/min-mg, at pH of 7.5 and a reaction temperature of 30°C. This apparent $K_m$ value is similar (in terms of millimolar concentration ranges (70 mM) obtained for bovine lens enzyme, using glucose as substrate (107). However, no comparison could be made with the locust fat body enzyme due to problems which have been reported to be encountered with the stability of the fat body enzyme (55).
FIGURE 18a

RATE CURVE FOR LOCUST FLIGHT MUSCLE
ALDOSE REDUCTASE

Reaction conditions:

NADPH = 0.1 mM
Substrate = glucose
Temperature = 30°C
pH = 7.5 (50mM Tris)
Homogenate = 5.0 mg protein
Total volume = 2.5 mL

FIGURE 18b

DOUBLE RECIPROCAL PLOT FOR LOCUST FLIGHT MUSCLE ALDOSE REDUCTASE

Apparent $K_m = 0.03$ M
Apparent $V_{max} = 0.004$ μM NADP produced/min-mg
As shown by Faulkner (54), this enzyme is known to reduce a number of hydroxyaldehydes and ketones to their corresponding alcohols; however, kinetic parameters could not be obtained using 3FG as substrate due to the limited supply of 3FG and secondly, the study of this enzyme was not one of the prime objectives of this dissertation.

Figures 19a and 19b show the kinetic parameters obtained for locust flight muscle sorbitol dehydrogenase. With sorbitol as substrate, the apparent Km obtained for the flight muscle enzyme was 13 mM with an apparent Vmax of 0.018 µM NADH/min-mg. This apparent Km value was five-fold lower than the 60 mM value obtained for the fat body enzyme (55). However, the apparent Vmax are the same. This observation suggests that the flight muscle enzyme has a higher affinity for sorbitol compared to the fat body enzyme. However, one cannot preclude the possibility of the fact that the differences in pH's and temperatures at which the fat body and flight muscle enzymes were assayed could have made the difference. This led to the reassaying of the fat body enzyme under the same conditions as the flight muscle enzyme. At pH of 8.5 and a reaction temperature of 30°C, the fat body enzyme had an apparent Km of 20 mM and an apparent Vmax of 0.08 µM NADH produced/min-mg (Figures 20a and 20b). The apparent Km value of 20 mM was found to be lower than reported earlier but the apparent
FIGURE 19a

RATE CURVE FOR LOCUST FLIGHT MUSCLE SORBITOL DEHYDROGENASE

Reaction conditions:

NAD = 1 mM
Substrate = sorbitol
Temperature = 30°C
pH = 8.5 (50 mM Tris)
Homogenate = 5.0 mg protein
Total volume = 2.5 mL

FIGURE 19b

DOUBLE RECIPROCAL PLOT FOR LOCUST FLIGHT MUSCLE SORBITOL DEHYDROGENASE

Apparent $K_m = 0.013$ M
Apparent $V_{max} = 0.018$ $\mu$M NADH produced/ min-mg.
FIGURE 20a

RATE CURVE FOR LOCUST FAT BODY SORBITOL DEHYDROGENASE

Reaction conditions:

- NAD = 1 mM
- Substrate = sorbitol
- Temperature = 30°C
- pH = 8.5 (50mM Tris)
- Homogenate = 8.0 mg protein
- Total volume = 2.3 mL

FIGURE 20b

DOUBLE RECIPROCAL PLOT OF LOCUST FAT BODY SORBITOL DEHYDROGENASE

Apparent $K_m = 0.02$ M

Apparent $V_{max} = 0.08$ μM NADH produced/min-mg.
Vmax was found to be higher. This observation seemed to suggest that the reaction conditions (i.e., pH and temperature) were the main factors leading to higher apparent Km value reported earlier for the fat body enzyme (55). The optimum pH for the assay of this enzyme has been reported to be between pH's of 8-10 (101).

To our knowledge, this is the first time the presence of sorbitol dehydrogenase enzyme has been demonstrated in locust flight muscle. The precise sequence of sorbitol production in vivo is not yet known. Whether glucose is first phosphorylated by a kinase and later reduced before dephosphorylation or that there is no phosphorylation/dephosphorylation involved is still unknown. The physiological importance of the presence of the sorbitol shunt in insects is also still unknown despite suggestions that accumulation of sorbitol and glycerol could be ecologically connected with resistance to cold by the lowering of freezing point (56, 57).

After several unsuccessful attempts to chromatographically isolate and identify metabolite(s) causing death (in whole locusts) using fat body and flight muscle tissue homogenates as a result of 'cold' 3FG administration, the availability of synthetic D-[3-3H]3FG and D-[U-14C] glucose became a matter of convenience enabling informative deductions to be made when the latter substrates were used as tracers.
Figure 21 represents paper radiochromatographic scans of flight muscle tissue homogenates incubated with either \( \text{D-}[\text{U}^{14}\text{C}] \) glucose or \( \text{D-}[3-\text{H}]3\text{FG} \). A very striking qualitative observation made was the difference in the pattern of the distribution of radioactivity in metabolites derived from flight muscle homogenates incubated with \( \text{D-}[\text{U}^{14}\text{C}] \) glucose and homogenates incubated with \( \text{D-}[3-\text{H}]3\text{FG} \). With \( \text{D-}[3-\text{H}]3\text{FG} \) incubated homogenates, there was a build-up of a high molecular weight compound (later identified to be glycogen) within the first six hours of incubation, later breaking down by the twelfth-hour incubation period with appearance of a new product and by the twenty-fourth hour, the high molecular weight compound completely disappeared with increasing amounts of the new product (later identified to be fructose). On the other hand, a comparison of the distribution of radioactivity from flight muscle homogenates incubated with \( \text{D-}[\text{U}^{14}\text{C}] \) glucose showed many and different radioactive peaks indicative of different metabolites within the first twelve hours which finally disappeared by the twenty-fourth hour incubation period.

Similar radiochromatographic results were obtained for fat body homogenates incubated with either \( \text{D-}[\text{U}^{14}\text{C}] \) glucose or \( \text{D-}[3-\text{H}]3\text{FG} \) (Figure 22).

As a result of problems encountered with resolution, the inability of the radiochromatographic scanner to
FIGURE 21

RADIOCHROMATOGRAPHIC SCANS OF FLIGHT MUSCLE HOMOGENATES INCUBATED WITH D-[3-3H]3FG OR D-[U-14C]GLUCOSE

Reaction conditions: 12 mg homogenate, 256 mM (8.6 x 10^6 dpm) 3FG or 256 mM (7.0 x 10^5 dpm) glucose, 40 mM ATP, 40 mM NAD, 20 mM MgCl₂ at 37°C. Incubation times, 6, 12 and 24h. Non-phosphorylated radioactive carbohydrates were isolated as described in MATERIALS AND METHODS then submitted to descending paper chromatography for 16h at room temperature (20 µL samples were applied to chromatogram). The unstained radiochromatograms were cut into strips and scanned for radioactivity. Solvent: Methyl ethyl ketone: Boric acid saturated water: Acetic acid (90:10:10 v/v).

———, D-[3-3H]3FG; ————, D-[U-14C]glucose.
FIGURE 22.

RADIOCHROMATOGRAPHIC SCANS OF FAT BODY HOMOGENATES INCUBATED WITH D-[3-3H]3FG OR D-[U-14C]GLUCOSE

Reaction conditions: 10 mg homogenate, 256 mM (8.6 x 10^6 dpm) or 256 mM (7.0 x 10^5 dpm) glucose, 40 mM ATP, 40 mM NAD, 20 mM MgCl₂ at 37°C. Incubation times, 6, 12 and 24h. Non-phosphorylated radioactive carbohydrates were isolated as described in MATERIALS AND METHODS then submitted to descending paper chromatography for 16h at room temperature. The unstained radiochromatograms were cut into strips and scanned for radioactivity (20 μL samples were applied to chromatogram). Solvent: Methyl ethyl ketone; Boric acid saturated water; Acetic acid (90:10:10 v/v).

-------, D-[3-3H]3FG; --------, D-[U-14C]glucose.
-accommodate long strips of paper radiochromatograms (57 cm).
and most importantly, the very low detection efficiency
especially of $^3$H (3-4%) of the scanner, one turned to the
use of liquid scintillation counting. This was achieved
by co-chromatographing standard neutral carbohydrates along
with duplicate isolated neutral radioactive sugars,
staining half of the chromatogram and unstained spots from
the other half were matched, cut, eluted and counted.
APPENDICES IIA, IIB, IIIA and IIIB illustrate typical
radiochromatograms obtained from which samples were cut,
eluted and counted.

Based on the peaks observed for the radiochromatographic
scans (Figures 21 and 22) as well as the spots observed on
the radiochromatograms (APPENDICES IIA, IIB, IIIA, IIIB),
all subsequent radiochromatographic studies were limited to
spots which had migrated up to 3FG (Rg 2.40). From the
radiochromatograms obtained, it was found that there
were four major spots corresponding to glycogen, trehalose,
glucose and fructose which were common to flight muscle and
fat body homogenates incubated with either D-[U-$^{14}$C] glucose
or D-[3-$^3$H]3FG. However, two important differences were
noted when comparing radiochromatograms obtained as a result
of D-[U-$^{14}$C] glucose incubation to those obtained for
D[3-$^3$H]3FG incubates.

*Rg denotes distance moved by carbohydrate relative to glucose.
1. An extra spot corresponding to an Rg of 0.56 was present for both homogenates incubated with a D-[U-\(^{14}\)C] glucose (APPENDICES IIA and IIIA) which was absent for homogenates incubated with D-[3-\(^{3}\)H]3FG (APPENDICES ITB, IIIB). This extra spot was chromatographically identified to be a phosphorylated amino sugar.

2. The Rg value for trehalose from D-[U-\(^{14}\)C] glucose treated homogenates was 0.27 compared to 0.31 obtained for D-[3-\(^{3}\)H]3FG incubates.

Figures 23a and 23b illustrate the time profile distribution of radioactivity (obtained by liquid scintillation counting from eluted radiochromatogram) into glycogen, trehalose, glucose, fructose and 3FG fractions after incubating flight muscle tissue homogenates with either D-[U-\(^{14}\)C] glucose (Figure 23a) or D-[3-\(^{3}\)H]3FG (Figure 23b).

With flight muscle homogenates, the dominant presence of a phosphorylated amino sugar within the first six hours of incubation with D-[U-\(^{14}\)C] glucose could be observed which was completely utilized by the twenty-fourth hour (Figure 23a). As mentioned earlier, no phosphorylated amino sugar was obtained for D-[3-\(^{3}\)H]3FG incubates (Figure 23b). On this premise, one could argue that 3FG or a metabolite of 3FG had blocked the synthesis of the phosphorylated amino sugar, which are considered to be very important biochemical precursors of chitin synthesis. However, since
FIGURE 23a

DISTRIBUTION OF RADIOACTIVITY FROM RADIOCHROMATOGRAM OF
FLIGHT MUSCLE HOMOGENATES PRE-INCUBATED WITH
D-[U-14C]GLUCOSE OVER DIFFERENT TIME PERIODS

<table>
<thead>
<tr>
<th>Rg value</th>
<th>Sugar</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00</td>
<td>Glucose</td>
</tr>
<tr>
<td>0.27</td>
<td>Trehalose</td>
</tr>
<tr>
<td>1.52</td>
<td>Fructose</td>
</tr>
<tr>
<td>0.06</td>
<td>Glycogen</td>
</tr>
<tr>
<td>0.56</td>
<td>Amino sugar (?)</td>
</tr>
</tbody>
</table>

Spots observed for radiochromatogram (APPENDIX IIA) were cut from duplicate runs, eluted and the dpm's of each spot determined through liquid scintillation counting.

FIGURE 23b

DISTRIBUTION OF RADIOACTIVITY FROM RADIOCHROMATOGRAM OF
FLIGHT MUSCLE HOMOGENATES PRE-INCUBATED WITH
D-[3-3H]3FG OVER DIFFERENT TIME PERIODS

<table>
<thead>
<tr>
<th>Rg value</th>
<th>Sugar</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00</td>
<td>Glucose</td>
</tr>
<tr>
<td>0.31</td>
<td>Trehalose</td>
</tr>
<tr>
<td>1.52</td>
<td>Fructose</td>
</tr>
<tr>
<td>0.06</td>
<td>Glycogen</td>
</tr>
<tr>
<td>2.40</td>
<td>3FG</td>
</tr>
</tbody>
</table>

Spots observed for radiochromatogram (APPENDIX IIB) were cut out from duplicate runs, eluted and the dpm's for each spot determined through liquid scintillation counting.
this was an in vitro study, one does not know whether 3FG would exert the same biochemical effect in vivo.

A comparison of the glycogen fractions shows a similar trend in pattern of radioactive distribution when homogenates are incubated with either D-[U-^{14}C] glucose or D-[3-^{3}H]3FG, in the sense that these fractions increased within the first six hours of incubation period, remaining almost constant within the next six hours and finally declined over the last six hours.

With trehalose fractions however, the time profile distribution of radioactivity was different when comparing D-[U-^{14}C] glucose incubates exhibited an initial sharp increase within the first six hours after which there was a decline, suggesting the utilization of trehalose. On the contrary, the fraction from D-[3-^{3}H]3FG continuously increased for the twenty-four hours incubation period with the peak radioactivity obtained at the twenty-fourth hour incubation period suggesting a non or very slow utilization of trehalose derived from D-[3-^{3}H]3FG compared to D-[U-^{14}C] glucose.

Another major observation made with regards to the distribution of radioactivity was with the fructose fractions. Not only was there a marginal increase in the amount of radioactivity incorporated into the fructose fraction when flight muscle homogenates were incubated with D-[U-^{14}C] glucose but also the amount incorporated in this
fraction was the lowest throughout the incubation period (Figure 23a). Contrary to this, D-[3\(^{3}H\)]3FG incubates had the highest amount of radioactivity incorporated into the fructose fraction throughout the incubation period (Figure 23b). This latter observation was also manifested with the radiochromatographic scans (Figure 21) as well as on the radiochromatograms (APPENDIX IIB) where the fructose spot started to appear after twelve hours of incubation.

All the points discussed pertaining to the flight, muscle incubates apply to fat body incubates (Figures 24a and 24b), except for the differences in the peaks of radioactivities in the different metabolites which appeared faster for the flight muscle tissue homogenates compared to the fat body tissue. Secondly, the amount of radioactivity remaining at the twenty-fourth hour incubation period for all metabolites were higher for the fat body incubates compared to flight muscle. The latter two observations again suggests the differences in the relative activity of both tissue homogenates.

From the results obtained for the radiochromatograms, radiochromatographic scans as well as the distribution of radioactivities (determined by liquid scintillation counting) in the aforementioned metabolites, it appeared 3FG was capable of being incorporated into glycogen, a major carbohydrate reserve stored to meet the energetics
FIGURE 24a

DISTRIBUTION OF RADIOACTIVITY FROM RADIOCHROMATOGRAM OF FAT BODY HOMOGENATES PRE-INCUBATED WITH D-[U-14C]GLUCOSE OVER DIFFERENT TIME PERIODS

<table>
<thead>
<tr>
<th>Rg value</th>
<th>Sugar</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00</td>
<td>Glucose</td>
</tr>
<tr>
<td>0.27</td>
<td>Trehalose</td>
</tr>
<tr>
<td>1.52</td>
<td>Fructose</td>
</tr>
<tr>
<td>0.06</td>
<td>Glycogen</td>
</tr>
<tr>
<td>0.56</td>
<td>Amino sugar (?)</td>
</tr>
</tbody>
</table>

Spots observed for radiochromatogram (APPENDIX IIIA) were cut from duplicate runs, eluted and the dpm's for each spot determined through liquid scintillation counting.

FIGURE 24b

DISTRIBUTION OF RADIOACTIVITY FROM RADIOCHROMATOGRAM OF FAT BODY HOMOGENATES PRE-INCUBATED WITH D-[3-3H]3FG OVER DIFFERENT TIME PERIODS

<table>
<thead>
<tr>
<th>Rg value</th>
<th>Sugar</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00</td>
<td>Glucose</td>
</tr>
<tr>
<td>0.31</td>
<td>Trehalose</td>
</tr>
<tr>
<td>1.52</td>
<td>Fructose</td>
</tr>
<tr>
<td>0.06</td>
<td>Glycogen</td>
</tr>
<tr>
<td>2.40</td>
<td>3FG</td>
</tr>
</tbody>
</table>

Spots observed for radiochromatogram (APPENDIX IIIB) were cut from duplicate runs, eluted and the dpm's of each spot determined by liquid scintillation counting.
of flight, as well as trehalose, the principal insect blood sugar. Secondly, 3FG could be inhibiting glycolysis further down the glycolytic pathway resulting in the reversal of glycolysis leading to the accumulation of fructose and trehalose. To test the latter presumption, fat body and flight muscle tissue homogenates were pre-incubated with either 'cold' 3FG or glucose for different time periods after which D-[U-14C] glucose was added. The rationale behind this was that, should 3FG be inhibiting glycolysis further down the glycolytic pathway, then one should be able to observe the accumulation of neutral radioactive carbohydrates, namely, glucose, fructose, trehalose and glycogen in substantial amounts.

Tables 1a and 1b illustrate the distributions of radioactivity (from D-[U-14C] glucose) into glycogen, trehalose, glucose and fructose fractions as a result of pre-incubating fat body (Table 1a) and flight muscle tissue homogenates (Table 1b) with either 'cold' glucose or 3FG over different time periods before 'chasing' with uniformly labelled glucose.

A comparison of glycogen fractions for fat body tissue homogenates (Table 1a) pre-incubated with glucose or 3FG for six, twelve and twenty-four hours before 'chasing' revealed that about five times the radioactivity was incorporated into glucose compared to 3FG pre-incubates
TABLE 1a

DISTRIBUTION OF RADIOACTIVITY FROM RADIOCHROMATOGRAM OF FAT BODY HOMOGENATES PRE-INCUBATED WITH 3FG OR GLUCOSE OVER DIFFERENT TIME PERIODS 'CHASED' WITH D-[U-14C]-GLUCOSE

Reaction conditions: As outlined in the legend for APPENDIX IVA, spots from duplicate radiochromatograms were cut, eluted and the dpm's of each spot determined through liquid scintillation counting.

<table>
<thead>
<tr>
<th>Rg values</th>
<th>6-Hour pre-incubation with</th>
<th>12-Hour pre-incubation with</th>
<th>24-Hour pre-incubation with</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose (DPM)</td>
<td>3FG (DPM)</td>
<td>Glucose (DPM)</td>
</tr>
<tr>
<td>0.06 (Glycogen)</td>
<td>15,402</td>
<td>2,921</td>
<td>5,595</td>
</tr>
<tr>
<td>0.27 (Trehalose)</td>
<td>5,109</td>
<td>9,321</td>
<td>6,002</td>
</tr>
<tr>
<td>1.00 (Glucose)</td>
<td>1,377</td>
<td>1,929</td>
<td>1,512</td>
</tr>
<tr>
<td>1.52 (Fructose)</td>
<td>331</td>
<td>558</td>
<td>824</td>
</tr>
</tbody>
</table>
TABLE 1b

DISTRIBUTION OF RADIOACTIVITY FROM RADIOCHROMATOGRAM OF FLIGHT MUSCLE HOMOGENATES PRE-INCUBATED WITH 3FG OR GLUCOSE OVER DIFFERENT TIME PERIODS 'CHASED' WITH [D- U-14C] GLUCOSE

Reaction conditions: As outlined in the legend for APPENDIX IVB. Spots were eluted from duplicate radiochromatograms and counted by liquid scintillation counting.

<table>
<thead>
<tr>
<th>Rg value</th>
<th>6-Hour pre-incubation with</th>
<th>12-Hour pre-incubation with</th>
<th>24-Hour pre-incubation with</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose (DPM)</td>
<td>3FG (DPM)</td>
<td>Glucose (DPM)</td>
</tr>
<tr>
<td>0.06 (Glycogen)</td>
<td>13,926</td>
<td>8,951</td>
<td>794</td>
</tr>
<tr>
<td>0.27 (Trehalose)</td>
<td>3,052</td>
<td>8,888</td>
<td>602</td>
</tr>
<tr>
<td>1.00 (Glucose)</td>
<td>1,324</td>
<td>2,412</td>
<td>384</td>
</tr>
<tr>
<td>1.52 (Fructose)</td>
<td>268</td>
<td>323</td>
<td>130</td>
</tr>
</tbody>
</table>
for the sixth-hour pre-incubation period and a two times as much (again in glucose pre-incubates) in the twelfth-hour pre-incubation period. However, with the twenty-fourth hour pre-incubation period, this trend was reversed by as much as five fold when most of the radioactivity was found to be incorporated into 3PG rather than glucose pre-incubates. Similar results were obtained for glycogen fractions of flight muscle pre-incubates except that the reversal of trend occurred by the twelfth-hour pre-incubation period (Table 1b) as opposed to the twenty-fourth hour obtained for fat body pre-incubates.

A comparison of the trehalose fractions of fat body pre-incubates (Table 1a) showed about the same percentage increase in the amount of radioactivity incorporated into 3PG pre-incubates over glucose pre-incubates within the first twelve hours of incubation but the greatest increase, which was thirteen times as much, was obtained for the twenty-fourth hour pre-incubates. Similar results were obtained for trehalose fractions of flight muscle pre-incubates (Table 1b) except that the greatest increase was observed for the twelfth-hour pre-incubates as compared to the twenty-fourth hour increase obtained for fat body pre-incubates.

Comparing the amount of radioactivity remaining in the glucose fraction for fat body pre-incubates (Table 1a), 3PG pre-incubates showed about one and half times more glucose
remaining within the sixth hour incubation period, remaining about the same for both glucose and 3FG pre-incubates over the twelfth-hour pre-incubation period and increasing by three times by the twenty-fourth hour incubation period for 3FG pre-incubates. With flight muscle pre-incubates, however, (Table 1b), similar results were obtained but the percentage of radiolabelled glucose remaining was highest for the twelfth-hour 3FG pre-incubates compared to glucose pre-incubates, and remaining the same for the twenty-fourth hour pre-incubates.

The fructose fractions of fat body pre-incubates showed the same trend (percentage wise) throughout the incubation period. However, by the twenty-fourth pre-incubation period, four times as much fructose accumulated for 3FG pre-incubates compared to glucose pre-incubates. With flight muscle pre-incubate (Table 1b) the highest percentage accumulation of fructose was obtained with twelfth-hour 3FG pre-incubates compared to the same hour glucose pre-incubates.

Generally, for fat body and flight muscle pre-incubates, it was apparent that 3FG inhibited glycolysis but the onset of inhibition was faster for flight muscle pre-incubates (twelve hours) compared to fat body pre-incubates (twenty-four hours), which led to the accumulation of trehalose, glycogen and fructose. The relatively lower percentages obtained for the twenty-fourth hour 3FG pre-incubates of flight muscles (Table 1b) compared to the fat body could be
due to the fact that the added D-[U-14C] glucose could rapidly have been metabolized via alternate metabolic routes since a metabolite of 3FG was exerting its toxic effect on the glycolytic pathway.

To further affirm the conviction that glycogen accumulated with the first six hours of incubation of homogenates with D-[3-3H]3FG, the glycogen fraction was eluted from radiochromatograms and quantitated enzymatically using α-amylase and the results compared with the same fraction eluted from radiochromatograms of D-[U-14C] glucose incubates for the same time periods (pooled 6 and 12 h samples).

Table 2a illustrates the amount of maltose released from glycogen when fat body homogenates were incubated with either D-[3-3H]3FG or D-[U-14C] glucose. There was as much as an eight-fold maltose production from D-[3-3H]3FG incubates compared to D-[U-14C] glucose. Similar results were obtained for flight muscle incubates (Table 2b) except that the amount of maltose released was six times higher for D-[3-3H]3FG incubates (compared to D-[U-14C] glucose) but lower when compared to fat body incubates.

To demonstrate unequivocally that 3FG was indeed being incorporated into the glycogen fraction, two experiments were carried out. The first of the two experiments involved a complete acid hydrolysis of the glycogen fraction derived from D-[3-3H]3FG incubates. Figure 25a illustrates the distribu-
TABLE 2a

ESTIMATION OF GLYCOGEN FRACTION FROM RADIOCHROMATOGRAM OF FAT BODY HOMOGENATES PRE-INCUBATED WITH D-[U-14C] GLUCOSE OR D-[3-3H] 3FG

Pooled glycogen fractions eluted from radiochromatograms of fat body homogenates pre-incubated with D-[U-14C] glucose (APPENDIX IIIA) or D-[3-3H] 3FG (APPENDIX IIIB) for 6 and 12 hours were enzymatically analysed with α-amylase as reported MATERIALS AND METHODS.

<table>
<thead>
<tr>
<th>Samples eluted from radiochromatogram after pre-incubation with</th>
<th>Glycogen fraction treated with α-amylase (μmoles maltose/mL sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3FG, Glucose</td>
<td>7,340, 940</td>
</tr>
</tbody>
</table>

TABLE 2b

ESTIMATION OF GLYCOGEN FRACTION FROM RADIOCHROMATOGRAM OF FLIGHT MUSCLE HOMOGENATES PRE-INCUBATED WITH D-[U-14C] GLUCOSE OR D-[3-3H] 3FG

Pooled glycogen fractions eluted from radiochromatograms of flight muscle homogenates pre-incubated with D-[U-14C] glucose (APPENDIX II A) or D-[3-3H] 3FG (APPENDIX II B) for 6 and 12 hours were enzymatically analyzed with α-amylase as reported in MATERIALS AND METHODS.

<table>
<thead>
<tr>
<th>Samples eluted from radiochromatograms after pre-incubation with</th>
<th>Glycogen fraction treated with α-amylase (μmoles maltose/mL sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3FG, Glucose</td>
<td>2,500, 440</td>
</tr>
</tbody>
</table>
FIGURE 25a

ACID HYDROLYSIS OF GLYCOCEN FRACTION OF RADIOCHROMATOGRAM
FROM FAT BODY HOMOGENATES PRE-INCUBATED WITH D-[3-3H] 3FG

Glycogen fraction eluted from radiochromatograms of fat body homogenates pre-incubated with D-[3-3H] 3FG (APPENDIX-IIIB) for 6 and 12 hours were acid hydrolysed as reported in MATERIALS AND METHODS. Hydrolysed and unhydrolysed (spotted in duplicates) samples were then submitted to descending paper chromatography for 16 hours at room temperature with glycogen, glucose, trehalose and 3FG as markers. Elution solvent: Methyl ethyl ketone; Boric acid saturated water; Acetic acid (90:10:10 v/v).
Glycogen, trehalose and glucose spots were cut but spots beyond glucose were cut into 1 cm x 2 cm pieces and counted by liquid scintillation counting.

•••, unhydrolysed glycogen; →, unhydrolysed glycogen

Rf values: Glycogen, 0.06; 3FG, 2.40.

FIGURE 25b

ACID HYDROLYSIS OF GLYCOCEN FRACTION OF RADIOCHROMATOGRAM
FLIGHT MUSCLE HOMOGENATES PRE-INCUBATED WITH D-[3-3H] 3FG

Samples were treated the same as reported in the legend of Figure 25a except that fractions were eluted from radiochromatograms of flight muscle incubates (APPENDIX-IIIB).

•••, unhydrolysed glycogen; →; hydrolysed glycogen

Rf values: Glycogen, 0.06; 3FG, 2.40

**This fraction was a 6 and 12 hour pooled fraction.**
tion of radioactivity in glycogen fractions before and after acid hydrolysis with fat body incubates. Prior to the acid hydrolysis, all the radioactivity was found to be in the glycogen fraction but upon hydrolysis, all the radioactivity was liberated as D-[3-3H]3FG. Similar results were obtained for flight muscle incubates when the isolated glycogen fraction was acid hydrolyzed and analyzed chromatographically (Figure 25b).

The second experiment was to carry out an 19F-NMR on the isolated glycogen fraction. Figure 26 illustrates the 19F-NMR obtained for the isolated glycogen fraction from fat body incubates. The chemical shift values obtained indicated that the fluorine remained on the third carbon atom of D-[3-3H]3FG when the values are compared with 3FG alone as well as a number of fluorocarbohydrates with fluorine on carbon atom three studied by Kent et al. (108).

Enzymatic quantitative estimation of trehalose fractions derived from D-[3-3H]3FG or D-[U-14C] glucose after 24 hours of incubation revealed a twenty-five fold increase in trehalose synthesized from D-[3-3H]3FG compared to D[U-14C] glucose when fat body homogenates were incubated with equimolar concentrations of either of the above mentioned substrates (Table 3a). Similar results were obtained for flight muscle incubates (Table 3b) where one observed a twenty-one fold increase in trehalose derived from D-[3-3H]3FG over
FIGURE 26

$^{19}$F-NMR OF FLUOROGYCOGEN ISOLATED
FROM FAT BODY INCUBATES

Conditions used for the scan:

- Sweep width = 50,000 Hz
- Offset = 32,000 Hz
- FW = 60,000
- SI = 8
- AQ = 0.0819
- DW = 10
- Line broadening = 60 Hz
- Number of scans = 16,257
- Reference = TFA (External)
- Chemical shift = 121.8 ppm.
**TABLE 3a**

**ESTIMATION OF Trehalose Fraction of Radiochromatogram from Fat Body Homogenates Pre-incubated with D-[U-[14C]] Glucose or D-[3-3H] 3FG with Trehalase**

Trehalose fractions eluted from radiochromatograms of fat body homogenates pre-incubated with D-[U-[14C]] glucose (APPENDIX IIIA) or D-[3-3H] 3FG (APPENDIX IIIB) for 24 hours were analysed with trehalase as reported in MATERIALS AND METHODS.

<table>
<thead>
<tr>
<th>Samples eluted from radiochromatogram after pre-incubation with</th>
<th>Trehalose fraction treated with trehalase (nmoles glucose/mL sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3FG</td>
<td>565</td>
</tr>
<tr>
<td>Glucose</td>
<td>22</td>
</tr>
</tbody>
</table>

**TABLE 3b**

**ESTIMATION OF Trehalose Fraction of Radiochromatogram from Flight Muscle Homogenates Pre-incubated with D-[U-[14C]] Glucose or D-[3-3H] 3FG with Trehalase**

Trehalose fractions eluted from radiochromatograms of flight muscle homogenates pre-incubated with D-[U-[14C]] glucose (APPENDIX IIA) or D-[3-3H] 3FG (APPENDIX IIB) for 24 hours were analysed with trehalase as reported in MATERIALS AND METHODS.

<table>
<thead>
<tr>
<th>Samples eluted from radiochromatogram after pre-incubation with</th>
<th>Trehalose fraction treated with trehalase (nmoles glucose/mL sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3FG</td>
<td>263</td>
</tr>
<tr>
<td>Glucose</td>
<td>12</td>
</tr>
</tbody>
</table>
that derived from D-[U-\(^{14}\)C] glucose. However, the quantities derived from the flight muscle homogenates was about half of those synthesized by fat body homogenates. The above observations suggest that whereas trehalose derived from 3FG was unable to be metabolized or slowly metabolized, that derived from glucose was metabolized much more rapidly. These quantitative results were supported by the qualitative observation that the amount of radioactivity in trehalose fraction increased continually with the highest observed by the twenty-fourth hour when both homogenates were incubated with D-[3-\(^{3}\)H]3FG (Figures 23b and 24b) whereas the amount of radioactivity remaining was dramatically reduced by the twenty-fourth hour when both flight muscle and fat body homogenates were incubated with D-[U-\(^{14}\)C] glucose (Figures 23a and 23b). Confirmation of the incorporation of radiolabelled 3FG into trehalose came from acid hydrolysis as well as \(^{19}\)F-NMR studies of trehalose fractions derived from 3FG. Figure 27a shows the radiochromatographic scans of unhydrolyzed and acid hydrolyzed trehalose fractions derived from D-[3-\(^{3}\)H]3FG when fat body homogenates were incubated with the latter substrate for twenty-four hours. Upon hydrolysis, almost all the radioactivity was found to be liberated in the D-[3-\(^{3}\)H]3FG fraction. Similar results were obtained for flight muscle tissue homogenates (Figure 27b). Figure 28 illustrates the \(^{19}\)F-NMR obtained
FIGURE 27a

ACID HYDROLYSIS OF TREHALOSE FRACTION OF RADIOCHROMATOGRAM FROM FAT BODY HOMOGENATES PRE-INCUBATED WITH D-[3-3H] 3FG

Trehalose fractions eluted from radiochromatograms of fat body homogenates pre-incubated with D-[3-3H] 3FG (APPENDIX X IIB) for 24 hours were acid hydrolysed as reported in MATERIALS AND METHODS. Samples of unhydrolysed and hydrolysed trehalose as well as glycogen, glucose, 3FG and trehalose standards were then submitted to descending paper chromatography for 16 hours at room temperature. Elution solvent: Methyl ethyl ketone: Boric acid saturated water: Acetic acid (90:10:10 v/v). Glycogen, trehalose, and glucose spots were cut but spots beyond glucose were cut into 1 cm x 2 cm pieces and counted by liquid scintillation counting.

- - - - Unhydrolysed trehalose; - - - , Hydrolysed trehalose.

Rg values: Trehalose, 0.31; 3FG, 2.40.

FIGURE 27b

ACID HYDROLYSIS OF TREHALOSE FRACTION OF RADIOCHROMATOGRAM FROM FLIGHT MUSCLE HOMOGENATES PRE-INCUBATED WITH D-[3-3H] 3FG

Samples were acid hydrolysed in the same manner described for fat body (Figure 27a), except that the fractions were eluted from radiochromatograms of flight muscle homogenates pre-incubated with D- 3-3H 3FG (APPENDIX IIB) for 24 hours.

- - - - Unhydrolysed trehalose; - - - , Hydrolysed trehalose.

Rg values: Trehalose, 0.31; 3FG, 2.40.
FIGURE 28

$^{19}$F-NMR OF FLUOROCTREHALOSE ISOLATED FROM FLIGHT MUSCLE INCUBATES

Conditions for scans:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sweep width</td>
<td>= 50,000 Hz</td>
</tr>
<tr>
<td>Offset</td>
<td>= 32,000 Hz</td>
</tr>
<tr>
<td>FW</td>
<td>= 60,000</td>
</tr>
<tr>
<td>SI</td>
<td>= 8</td>
</tr>
<tr>
<td>DW</td>
<td>= 10</td>
</tr>
<tr>
<td>AQ</td>
<td>= 0.0819</td>
</tr>
<tr>
<td>Line broadening</td>
<td>= 60 Hz</td>
</tr>
<tr>
<td>Number of scans</td>
<td>= 59,438</td>
</tr>
<tr>
<td>Reference</td>
<td>= TFA(External)</td>
</tr>
<tr>
<td>Chemical shifts</td>
<td>A = 122.3 ppm, B = 133.3 ppm</td>
</tr>
</tbody>
</table>
for trehalose fraction after incubation of flight muscle homogenates for twenty four hours. Unlike the glycogen fraction, two different chemical shifts were obtained suggesting that there are two fluorine atoms in different environments. A comparison of the chemical shifts obtained with those reported by Kent et al. (108) showed that the fluorine remained on the third carbon atom.

The incorporation of 3FG into both glycogen and trehalose appeared at first enigmatic since 3FG has been reported to be a poor substrate for yeast hexokinase (109), however, the effect of 3FG on locust hexokinase is yet to be examined. With the unavailability of 3FG-1-P it has not yet been possible to examine the effect of this substrate on locust UDPG pyrophosphorylase, a key enzyme in the synthesis of both fluoroglycogen and fluorotrehalose. However, it can be assumed that both hexokinase as well as UDPG pyrophosphorylase would be capable of catalyzing 3FG and 3FG-1-P, respectively. The only evidence to substantiate this assertion came from Kerly and Leaback (35) and Shigematsu (36) who demonstrated the non-specificity of locust flight muscle as well as fat body hexokinases. Thus, one can speculate that 3FG may be phosphorylated by these non-specific hexokinases in locust fat body and flight muscle with isomerization of 3FG-6-P to 3FG-1-P, which can then act as a substrate for UDPG pyrophosphorylase
enzyme leading to the synthesis of both trehalose and glycogen.

Table 4a illustrates the quantitative estimation of fructose fraction when fat body homogenates were incubated for twelve and twenty-four hours with either D-[U-14C] glucose or D-[3-3H]3PG. The fructose concentration obtained from D-[3-3H]3PG was found to be twenty times higher compared to D-[4-14C] glucose incubates. Similar results were obtained for flight muscle incubates (Table 4b). This observation fitted into the earlier observation that, the amount of radioactivity remaining in the fructose fractions of D-[3-3H]3PG incubates by the twelfth and twenty-fourth hours were much higher than D-[U-14C] glucose incubates (Figures 23a, 23b, 24a and 24b).

The above quantitative observation could be rationalized on the basis of the fact that the fructose fractions derived from D-[U-14C] glucose incubates were glycolyzed by the twelfth and twenty-fourth hours whereas the fractions derived from D-[3-3H]3PG were either being metabolized at a much slower rate or that a metabolite of 3PG and/or inorganic fluoride released from 3PG as a consequence of certain enzymic reactions, could have been inhibiting some glycolytic enzyme(s) leading to gluconeogenesis, hence the accumulation of fructose.

The accumulation of fructose was at first suspected to
### TABLE 4a

**ESTIMATION OF FRUCTOSE FRACTION FROM RADIOCHROMATOGRAM OF FAT BODY HOMOGENATES PRE-INCUBATED WITH D-[U-14C] GLUCOSE OR D-[3-3H] 3FG**

Fructose fractions eluted from radiochromatograms of fat body homogenates pre-incubated with D-[U-14C] glucose (APPENDIX IIIA), or D- 3-3H 3FG (APPENDIX IIIB) for 12 and 24 hours were colorimetrically determined according to the method of Roe (104).

<table>
<thead>
<tr>
<th>Samples eluted from radiochromatogram after pre-incubation with</th>
<th>Fruuctose concentration (µg fructose/mL sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3FG Glucose</td>
<td>1310 67</td>
</tr>
</tbody>
</table>

### TABLE 4b

**ESTIMATION OF FRUCTOSE FRACTION FROM RADIOCHROMATOGRAM OF FLIGHT MUSCLE HOMOGENATES PRE-INCUBATED WITH D-[U-14C] GLUCOSE OR D-[3-3H] 3FG**

Fructose fractions eluted from radiochromatograms of flight muscle homogenates pre-incubated with D-[U-14C] glucose (APPENDIX IIIA) or D-[3-3H] 3FG (APPENDIX IIIB) for 12 and 24 hours were colorimetrically determined according to the method of Roe (104).

<table>
<thead>
<tr>
<th>Samples eluted from radiochromatogram after pre-incubation with</th>
<th>Fruuctose concentration (µg fructose/mL sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3FG Glucose</td>
<td>1,009 50</td>
</tr>
</tbody>
</table>

* These are 12 and 24 hour pooled samples.
be due to release of fluoride from 3FG which could possibly be inhibiting enolase, an enzyme, well known to be sensitive to micromolar levels of fluoride ion from rabbit muscle (110).

Table 5a depicts the amount of fluoride when fat body homogenates were incubated with either 3.25 mM or 6.5 mL 3FG. From the results, independent of the concentration of 3FG used, the concentration of fluoride ion remained constant (i.e., 10.8 μM) after 24-hours incubation period (Table 5b). This observation is significant in light of the fact that rabbit muscle enolase is inhibited by micromolar levels of fluoride ion (110). It must, however, be noted that in terms of percentages, the amount released was less than 1% after 24-hours incubation period, a yield, which is comparatively lower than the 3.2% reported for whole locusts (55). In this respect one can assume that the micromolar levels of fluoride ion released could possibly be contributing to the inhibition of glycolysis partially leading to a back up of fructose, however, the potential inhibitory effects of fluoride on other Mg2+ dependent enzymes could not be discounted.

The stereospecific exchange of tritium from the C-3 position of D-[3-3H] glucose with solvent had been previously used by Rongstad and Katz (111) as a measure of the rate of F6P phosphorylation as well as for the estimation of
TABLE 5a

FLUORIDE MEASUREMENTS FROM FAT BODY
HOMOGENATES INCUBATED WITH 3FG

8 mg fat body homogenates were incubated with either 3.25 mM or 6.5 mM 3FG in the presence of 2 mM NAD, 2 mM ATP and 1 mM MgCl2 at 30°C in 50 mM Tris buffer pH 7.5 Fluoride concentrations were measured as outlined in MATERIALS AND METHODS:

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Incubate + 3.25 mM 3FG</th>
<th>Incubate + 6.5 mM 3FG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µM F⁻</td>
<td>µM F⁻</td>
</tr>
<tr>
<td>12</td>
<td>4.85</td>
<td>4.85</td>
</tr>
<tr>
<td>24</td>
<td>10.80</td>
<td>10.80</td>
</tr>
</tbody>
</table>

TABLE 5b

FLUORIDE MEASUREMENTS FROM FLIGHT MUSCLE
HOMOGENATES INCUBATED WITH 3FG

10 mg flight muscle homogenates were incubated under the same conditions as outlined for fat body homogenates (Table 5a):

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Incubate + 3.25 mM 3FG</th>
<th>Incubate + 6.25 mM 3FG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µM F⁻</td>
<td>µM F⁻</td>
</tr>
<tr>
<td>12</td>
<td>9.0</td>
<td>13.0</td>
</tr>
<tr>
<td>24</td>
<td>20.6</td>
<td>20.6</td>
</tr>
</tbody>
</table>
substrate cycling via fructose-1,6-diphosphatase and phosphofructokinase. We had hoped to use the yield of \( ^3 \text{H}_2 \text{O} \) from D-[3-\(^3\)H]3PG as a measure of the extent of 3PG metabolism when compared with identical experiments using D-[3-\(^3\)H] glucose. It was assumed that an appreciable loss of tritium from D-[3-\(^3\)H]3PG was a good indication that 3PG was glycolyzed at least as far as the triose sugars.

Table 6a summarizes the results of \( ^3 \text{H}_2 \text{O} \) released from D-[3-\(^3\)H] glucose and D-[3-\(^3\)H]3PG for flight muscle incubates and Table 6b summarizes the results for fat body incubates.

Control experiments indicated that neither D-[3-\(^3\)H] glucose nor D-[3-\(^3\)H]3PG were contaminated with \( ^3 \text{H}_2 \text{O} \) or that \( ^3 \text{H}_2 \text{O} \) was released from these sugars upon incubation for 24 hours. It seemed likely, therefore, that the slow rate of \( ^3 \text{H}_2 \text{O} \) release in the case of 3PG was due to a kinetic factor, and that 3PG was metabolized slowly as far as triose phosphate isomerase. Respirometric results previously discussed complement the \( ^3 \text{H}_2 \text{O} \) release results.

Based on the known mechanisms and stereochemistry of aldolase (112) and triose phosphate isomerase (113), it was thought that either of these two enzymes or both could have been a potential target for irreversible inactivation by a fluorometabolite. Coupled with these facts, Silverman et al. have shown that 1-fluoro-3-hydroxyacetone phosphate was an irreversible inhibitor of rabbit muscle triosephosphate
TABLE 6a

$^{3}$H$_2$O RELEASED FROM EITHER D-[3-$^{3}$H]3FG OR D-[3-$^{3}$H] GLUCOSE
WITH FLIGHT MUSCLE HOMOGENATE

Flight muscle homogenates (12 mg) were incubated either
100 mM D-[3-$^{3}$H] glucose (containing 12.9 x 10$^6$ dpm) or
100 mM D-[3-$^{3}$H]3FG (containing 63 x 10$^6$ dpm). The reaction
mixture also contained 40 mM ATP, 40 mM NAD, 25 mM MgCl$_2$
in a total volume of 3.5 mL. $^{3}$H$_2$O was determined as out-
lined in MATERIALS AND METHODS.

<table>
<thead>
<tr>
<th>Incubation time (hours)</th>
<th>% $^{3}$H$_2$O released from D-(3-$^{3}$H) glucose</th>
<th>% $^{3}$H$_2$O released from D-(3-$^{3}$H)3FG</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>13.9</td>
<td>4.2</td>
</tr>
<tr>
<td>24</td>
<td>48.4</td>
<td>16.3</td>
</tr>
</tbody>
</table>

TABLE 6b

$^{3}$H$_2$O RELEASED FROM EITHER D-[3-$^{3}$H]3FG OR D-[3-$^{3}$H] GLUCOSE
WITH FAT BODY HOMOGENATE

The reaction mixture contained the same concentration of
substrates and cofactors as outlined for flight muscles
(Table 6a) except that the concentration of protein was 10 mg.

<table>
<thead>
<tr>
<th>Incubation time (hours)</th>
<th>% $^{3}$H$_2$O released from D$_2$(3-$^{3}$H) glucose</th>
<th>% $^{3}$H$_2$O released from D-(3-$^{3}$H)3FG</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>8.5</td>
<td>2.3</td>
</tr>
<tr>
<td>24</td>
<td>34.8</td>
<td>9.6</td>
</tr>
</tbody>
</table>
isomerase (114). With the foregoing knowledge, we decided to examine the effect of 3FG and NaF on $^3$H$_2$O release from D-[3-3H] glucose. The rationale behind this was to test whether 3FG was inhibiting glycolysis at the level of the triose sugars. Results of Table 7 show that 3FG incubates released $^3$H$_2$O 2-3 times compared to glucose and glucose + NaF incubates suggesting the possibility of some inhibition (by alkylation) of some enzyme(s) by a metabolite of 3FG at the triose sugars. However, one cannot discount the possibility that the added 'hot' glucose was being metabolized via other metabolic pathways, especially the synthesis of trehalose or glycogen which was evident in the 'chase' experiment (Tables 1a and 1b) discussed earlier. Due to nonavailability of 3FG, the effect of the latter substrate on $^3$H$_2$O release from D-[3-3H] glucose on fat body incubates could not be examined; but one can presume, on the basis preceding parallel results obtained so far for both flight muscle and fat body homogenates that similar results would be expected.

The relatively high percentage $^3$H$_2$O released from D-[3-3H] glucose with 20 $\mu$M NaF + glucose incubates could be explained on the basis of the fact that the fluoride ion present could have been inhibiting phosphatases with very little or no effect on enolase as such, the percentage $^3$H$_2$O release was almost equal to glucose pre-incubates. This
TABLE 7
EFFECT OF 3FG AND FLUORIDE ION ON $^3$H$_2$O RELEASED FROM D-[3-$^3$H]-GLUCOSE BY FLIGHT MUSCLE HOMOGENATES

Flight muscle homogenates (13 mg) were pre-incubated with 200 mM glucose 3FG or 200 mM glucose + 20 μM NaF in a total volume of 4.0 mL. The reaction mixture also contained 40 mM of Na$_2$HPO$_4$ and ATP, respectively, and 20 mM MgCl$_2$. The reactions were pre-incubated for 20 hours after which time the reactions were followed by a 6-hour incubation with 50 mM D-[3-$^3$H] glucose of specific activity of 118nCi/μmole.

<table>
<thead>
<tr>
<th>Pre-incubation time (hours)</th>
<th>% $^3$H$_2$O release from D-[3-$^3$H]-glucose after pre-incubation with glucose</th>
<th>% $^3$H$_2$O release from D-[3-$^3$H]-glucose after pre-incubation with 3FG</th>
<th>% $^3$H$_2$O release from D-[3-$^3$H]-glucose after pre-incubation with NaF glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 hrs.</td>
<td>*40.7</td>
<td>*14.2</td>
<td>*36.8</td>
</tr>
</tbody>
</table>

*These percentages were calculated by taking into account the new specific activity at the time of the addition of the 'hot' glucose.
assumption was made based on preliminary experiments where flight muscle homogenates were incubated with 20 μM NaF alone for 20 hours before following with 'hot' glucose and it was found that the $^3$H$_2$O yield was only 17.8%. Thus, the presence of glucose (in the case of glucose + NaF incubates) could have been overcoming any inhibitory effects that NaF might have on enolase. However, one cannot preclude the fact that enolase, as well, might have been inhibited leading to reversal of glycolysis, hence the relatively high $^3$H$_2$O release.

Preliminary investigation into the possible alkylation of protein(s) (using D-[3-$^3$H]3FG) revealed that four major protein bands were alkylated when flight muscle homogenates were incubated with D-[3-$^3$H]3FG. (Figure 29). A similar experiment carried out on muscle homogenates with D-[3-$^3$H] glucose did not reveal any protein bands being alkylated. The four major flight muscle proteins found to be alkylated when homogenates were run on an SDS gel electrophoresis had molecular weights of about 10,000, 25,000, 53,000 and 72,000, respectively. Correlation of these molecular weights with literature values revealed that muscle trehalase of molecular weight 70,000 (28, 29) as well as triose phosphate isomerase with molecular weight of 50,000 - 60,000 (51) could be the potential target enzymes being inhibited by the fluorometabolites of 3FG. It was not possible to
FIGURE 29
DISTRIBUTION OF RADIOACTIVITY IN FLIGHT MUSCLE PROTEINS

Flight muscle homogenates were incubated with D-(3-³H)-3FG (6.3 x 10⁶ dpm) for 24h. Proteins were processed for SDS gel electrophoresis as outlined in MATERIALS AND METHODS.
extend similar studies to fat body homogenates because of limited supply of 3FG. It must be pointed out, however, that the latter experiment involving alkylation was only a preliminary study. It would therefore be suggested that the two enzymes mentioned (muscle trehalase and triose phosphate isomerase), could be further investigated in detail in the future. The convincing evidences which support the inhibition of trehalase came from radiochromatographic studies discussed earlier (Figures 23b and 24b), in which an increased amount of fluorotrehalose was obtained after a twenty-four hour incubation with 3FG, as well as the 'chase' experiment (Tables 1a and 1b) where we obtained an increase in trehalose concentration when homogenates were precubated with 3FG before 'chasing' with uniformly labelled glucose. The 'chase' experiment strongly suggested that, with fluorotrehalose inhibiting trehalase, trehalose derived from uniformly labelled glucose could not be utilised. The use of asymmetric fluoro-analogues of trehalose for the study of the mechanism of action of trehalases as well as their possible usefulness as bactericides, fungicides and insecticides have been reported (115). Of interest, was the observation that cockchafer trehalase was found to be inhibited by the 6-fluoro-galactosyl glucoside analogue of trehalose (115). In this respect, it would be of interest to know the effect of the 3-fluoro-analogue of trehalose on insect
as well as other trehalases. The mechanism by which 3FG gets incorporated into trehalose is not yet known. Whether 3FG is the donor or acceptor molecule or both is a subject that needs to be further investigated.

A second possible enzyme believed to be alkylated was triphosphoisomerase. The molecular weight of this enzyme from the flight muscle of the housefly has been estimated to be around 60,000 (51). Coupled with this fact was the observation that 1-fluoro-3-hydroxyacetone phosphate was found to be a slow weak irreversible inhibitor of the rabbit muscle enzyme (114). A similar slow irreversible inhibition with the locust enzyme would explain the much more slower rate of $^3$H$_2$O released from D-[3-$^3$H]3FG (Tables 6a and 6b) as well as the eventual accumulation of fructose and trehalose (Figures 23b and 24b). As mentioned earlier, since the results of the electrophoresis are preliminary, a detailed examination of the fluorinated analogues of the true substrates on the purified enzymes (triose phosphate isomerase and trehalose) would serve the best purpose to enlighten us more about the two enzymes which are believed to be alkylated by metabolites derived from 3FG.

Based upon the results obtained, it is possible to rationalize that 3FG is not only being metabolized via the glycolytic pathway, but also capable of being incorporated into glycogen and trehaloses, the principal carbohydrate
reserves found in insects. This is not too surprising since both carbohydrates share a common biosynthetic route. However, it appears insect trehalase is incapable of metabolizing trehalose derived from 3FG.

From the proposed metabolism of 3FG in both fat body and flight muscle of locusts (Figure 30), it is strongly believed that the most attractive target site for defluorination, detrinitation, and alkylation is the triose phosphate isomerase enzyme, leading to accumulation of fructose and trehalose. One cannot, however, preclude the possibility that defluorination and detrinitation could be occurring on separate enzymes.

Based on work done with whole insects (55), we had initially hoped to isolate phosphorylated fluorinated sugar(s) but met with no success. Incubation of fat body or flight muscle homogenates with 3FG chromatographically revealed only one spot with an Rf value corresponding to inorganic phosphate. This observation was in sharp contrast using glucose as substrate (APPENDICES VA and VB) where several sugar phosphate spots were identified. Similarly, pre-incubation of homogenates with 3FG prior to 'chasing' with glucose yielded only inorganic phosphate spot. However, there is chromatographic evidence of the presence of endogenous GIP before the start of reactions (APPENDIX VI). This observation tends to suggest that the presence
FIGURE 30
PROPOSED METABOLISM OF D-[\(\text{H}^{3}\)]\(^{3}\)FG IN LOCUST FLIGHT
MUSCLE AND FAT BODY

This figure illustrates the metabolic fate of 3FG consequently leading to death in locusts.

The enzymes believed to be involved are:

1. Glucokinase
2. Phosphoglucomutase
3. UDPG-pyrophosphorylase
4. Glycogen synthetase
5. Trehalose synthetase
6. Aldose reductase
7. Sorbitol dehydrogenase
8. Aldolase
9. Triphosphoisomerase
10. Fructose -1,6-diphosphatase

* Tritium on hydrogen
• Alkylation of triose phosphate isomerase (?)
■ Alkylation of trehalase (?)
of 3FG or the inhibition of an enzyme by 3FG somehow triggers an increase in the activity of phosphatases leading to hydrolysis of phosphates from their respective sugars over the time periods that the reactions were carried out. This observation also explains why neutral rather than phosphorylated sugars accumulated. This contrast between tissue homogenates and whole insect work could have possibly been due to insect haemolymph, which was completely washed off before the present studies were carried out.
CHAPTER IV

SUMMARY AND CONCLUSIONS

The summary and conclusions of this work can be divided into three major sections.

A. RESPIROMETRIC STUDIES

Results of the respirometric studies indicated that locust flight muscle and fat body were capable of metabolising 3FG but the extent of utilisation of 3FG was comparatively lower than glucose. Secondly, 3FG was capable of inhibiting the respiration of glucose. It must, however, be stressed that there is an absolute need for starving the insects for about three days prior to any respirometric experiment due to the large reserves of carbohydrates (mainly glycogen) stored by these tissues, leading to very high endogenous respiration rates. A similar observation was made by Kerly and Leaback (35).

B. ENZYMATIC INVESTIGATION

Locust flight muscle has been found to contain aldose reductase as well as sorbitol dehydrogenase activity which suggests the presence of an alternative glycolytic shunt which has previously been demonstrated in the fat body (55). These enzymes from the flight muscle seem to have a higher affinity for their natural substrates compared to the fat.
body enzymes. However, the effect of the fluoro-analogues of the natural substrates was not investigated due to their unavailability. It would thus be of great interest, in the future, to investigate these fluoro-analogues on the flight muscle enzymes.

C. RADIOCHROMATOGRAPHIC STUDIES

Results from these experiments revealed that 3FG was capable of being incorporated into glycogen and trehalose with the fluorine still remaining on C-3 of the 3FG molecule. Not only was 3FG incorporated into glycogen and trehalose, but was metabolized as far as the triose sugars with de- tritiation and defluorination. The resulting fluoride lost could possibly be inhibiting enolase as well as other fluoride sensitive enzymes leading to the accumulation of fructose. Quantitative estimation of trehalose, glycogen as well as fructose derived from 3FG was higher than those derived from glucose. This build-up of carbohydrates observed stemmed from the inhibition of certain key enzymes thereby upsetting the metabolic machinery of the locust.

The key enzymes suspected are the triose phosphate isomerase and trehalase. These two enzymes have previously been demonstrated to be inhibited by the fluoro-analogues of their true substrates (114, 115). However a complete confirmation of the inhibition of these enzymes needs to be
examined in greater detail using purified enzymes. Finally, it appears the insect fat body and flight muscle share the same metabolic routes in metabolizing 3FG.
APPENDIX I

*LEAST SQUARES METHOD

All linear plots were analysed by the least squares method. It was assumed that the set of data points fit the equation \( y = mx + c \), where 'x' and 'y' are the dependent and independent variables, 'c' being the 'y' intercept and 'm', the slope of the resulting line. This method chooses an 'm' and 'c' so that the average sum of the squares of the difference between the experimental 'y' values and the calculated 'y' values are at a minimum.

The equations used to calculate 'c' and 'm' are:

\[
c = \frac{\sum_{n=1}^{k} x^2 \cdot \sum_{n=1}^{k} y_n - \sum_{n=1}^{k} x_n \sum_{n=1}^{k} (x_n y_n)}{k \sum_{n=1}^{k} x_n^2 - (\sum_{n=1}^{k} x_n)^2}
\]

\[
m = \frac{\sum_{n=1}^{k} (x_n y_n) - \sum_{n=1}^{k} x_n \sum_{n=1}^{k} y_n}{z}
\]

* A programmable calculator was used to do these calculations.
APPENDIX IIA

RADIOCHROMATOGRAM OF FLIGHT MUSCLE HOMOGENATES INCUBATED WITH D-[^14C]GLUCOSE

Reaction conditions: 20 mg homogenate, 256 mM (7.0 x 10^5 dpm) D-[^14C]glucose, 40 mM NAD, 20 mM MgCl_2, and 37°C incubation temperature. Non-phosphorylated radioactive sugars were isolated as outlined in MATERIALS AND METHODS then submitted to descending paper chromatography for 16h. Solvent: Methyl ethyl ketone; Boric acid saturated water: Acetic acid (90:10:10 v/v). Amount of sample applied 20 μL.

M1, M2 and M3 represent 6, 12 and 24h incubates, respectively.

Rg values: GLU, 1.0; FRU, 1.52; SORB, 1.67, TRE, 0.27

APPENDIX IIB

RADIOCHROMATOGRAM OF FLIGHT MUSCLE HOMOGENATES INCUBATED WITH D-[^3H]3FG

Reaction conditions: Same as legend for APPENDIX IIA except 256 mM (8.6 x 10^5 dpm) 3FG was used.

M0, endogenous carbohydrate; M1, M2 and M3 represent 6, 12 and 24h incubates, respectively.

Rg values: GLU, 1.0; FRU, 1.52; 3FG, 2.40; 3FGL, 3.03.

GLU = glucose, FRU = fructose, SORB = sorbitol, TRE = trehalose.
### APPENDIX IIA

<table>
<thead>
<tr>
<th>TRE</th>
<th>SORB</th>
<th>FRU</th>
<th>GLU</th>
<th>M3</th>
<th>M2</th>
<th>M1</th>
</tr>
</thead>
</table>

### APPENDIX IIB

<table>
<thead>
<tr>
<th>3FGL</th>
<th>FRU</th>
<th>3FG</th>
<th>GLU</th>
<th>M3</th>
<th>M2</th>
<th>M1</th>
<th>M0</th>
</tr>
</thead>
</table>
APPENDIX IIIA

RADIOCHROMATOGRAM OF FAT BODY HOMOGENATES INCUBATED WITH D-[U-14C]GLUCOSE

Reaction conditions: 15 mg homogenate, 256 mM (7.0 x 10^5 dpm) D-[U-14C]glucose, 40 mM ATP, 40 mM NAD, 20 mM MgCl2 and 37°C reaction temperature. Non-phosphorylated radioactive sugars were isolated as outlined in MATERIALS AND METHODS, then submitted to descending paper chromatography for 16h. Solvent: Methyl ethyl ketone: Boric acid saturated water: Acetic acid (90:10:10 v/v). Amount of sample applied, 20 µL. B1, B2 and B3 represent 6, 12 and 24h incubates, respectively.

Rf values: GLU, 1.0; FRU, 1.52; SORB, 1.67; TRE, 0.27.

APPENDIX IIIB

RADIOCHROMATOGRAM OF FAT BODY HOMOGENATES INCUBATED WITH D-[^3H]3FG

Reaction conditions: Same as outlined in the legend for APPENDIX IIIA except for 256 mM (8.6 x 10^6 dpm) 3FG which was used.

B0, endogenous carbohydrate; B1, B2 and B3 represent 6, 12 and 24h incubates, respectively.

Rf values: GLU, 1.0; FRU, 1.52; 3FG, 2.4; 3FGL, 3.03

GLU = glucose; FRU = fructose; SORB = sorbitol, TRE = trehalose.
APPENDIX IVA

RADIOCHROMATOGRAM OF FAT BODY HOMOGENATES PRE-INCUBATED WITH GLUCOSE OR 3FG 'CHASED' WITH D-[U-14C]GLUCOSE

Reaction conditions: 15 mg homogenate, 256 mM glucose or 3FG, 40 mM ATP, 40 mM NAD, 120 mM MgCl₂, 37°C incubation temperature. Reactions were pre-incubated for 6, 12 and 24h followed by 4h incubation with 50 mM (60 x 10⁵ dpm) D-[U-14C]glucose. Non-phosphorylated carbohydrates were isolated as described in MATERIALS AND METHODS then submitted to descending paper chromatography for 16h. Solvent: Methyl ethyl ketone: Boric acid saturated water: Acetic acid (90:10:10 v/v).

B1GLU, B2GLU, B3GLU represent 6, 12 and 24h pre-incubates with glucose; B1, M2 and B3 represent 6, 12 and 24h pre-incubates with 3FG.

APPENDIX IVB

RADIOCHROMATOGRAM OF FLIGHT MUSCLE HOMOGENATES PRE-INCUBATED WITH GLUCOSE OR 3FG 'CHASED' WITH D-[U-14C]GLUCOSE

Reaction conditions, sample preparation as well as the chromatographic run were all carried out as outlined for fat body homogenates (APPENDIX IVA) except that 20 mg homogenates were used.

M1GLU, M2GLU, M3GLU are 6, 12 and 24h pre-incubates with glucose; M1, M2 and M3 are 6, 12 and 24h pre-incubates with 3FG.

Rg values: GLY = 0.06; GLU = 1.00; TRE = 0.27; FRU = 1.52; 3FG = 2.40

GLY = glycogen; TRE = trehalose; GLU = glucose; FRU = fructose
### APPENDIX IVA

<table>
<thead>
<tr>
<th>3FG</th>
<th>FRU</th>
<th>GLU</th>
<th>TRE</th>
<th>GLY</th>
</tr>
</thead>
<tbody>
<tr>
<td>☺</td>
<td>☺</td>
<td>☺</td>
<td>☺</td>
<td>☺</td>
</tr>
</tbody>
</table>

### APPENDIX IVB

<table>
<thead>
<tr>
<th>3FG'</th>
<th>FRU</th>
<th>GLU</th>
<th>TRE'</th>
<th>GLY</th>
</tr>
</thead>
<tbody>
<tr>
<td>☺</td>
<td>☺</td>
<td>☺</td>
<td>☺</td>
<td>☺</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>GM3</th>
<th>GM2</th>
<th>GM1</th>
</tr>
</thead>
<tbody>
<tr>
<td>☺</td>
<td>☺</td>
<td>☺</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>M3GLU</th>
<th>M2GLU</th>
<th>M1GLU</th>
</tr>
</thead>
<tbody>
<tr>
<td>☺</td>
<td>☺</td>
<td>☺</td>
</tr>
</tbody>
</table>
APPENDIX VA

CHROMATOGRAM OF PHOSPHORYLATED METABOLITES FROM FAT BODY AND FLIGHT MUSCLE HOMOGENATES INCUBATED WITH 3FG

Reaction conditions: Fat body homogenate, 12 mg; flight muscle homogenate, 15 mg. Homogenates were incubated at 37°C with 256 mM 3FG, 40 mM NAD, 40 mM ATP, 20 mM MgCl₂. Isolation, chromatography as well as detection of phosphorylated metabolites were carried out as outlined in MATERIALS AND METHODS.

B₁, B₂ and B₃ represent fat body homogenates incubated for 6, 12 and 24 h. M₁, M₂ and M₃ represent flight muscle homogenates incubated for 6, 12 and 24 h, respectively.

APPENDIX VB

CHROMATOGRAM OF PHOSPHORYLATED METABOLITES FROM FAT BODY AND FLIGHT MUSCLE HOMOGENATES INCUBATED WITH GLUCOSE.

Reaction conditions are the same as for 3FG incubates except that 256 mM glucose was used in this case.

B₁, B₂ and B₃ represent fat body homogenates incubated for 6, 12 and 24 h; and M₁, M₂, M₃ represent flight muscle homogenates incubated for 6, 12 and 24 h, respectively.

Rf values: GlP, 0.51; G6P, 0.55; FDP, 0.23; Pi, 0.71.
APPENDIX VI

CHROMATOGRAM OF PHOSPHORYLATED METABOLITES FROM FAT BODY AND FLIGHT MUSCLE HOMOGENATES PRE-INCUBATED WITH 3PG 'CHASED' WITH GLUCOSE.

Reaction conditions: Fat body homogenate, 12 mg; flight muscle homogenate, 15 mg. Incubation temperature 37°C, with 256 mM 3PG, 40 mM ATP, 40 mM NAD, 20 mM MgCl₂. Reactions were pre-incubated for 6, 12 and 24-h followed by a 2h incubation with 20 mM glucose. Isolation, chromatography, as well as detection of phosphorylated metabolites was carried out as outlined in MATERIALS AND METHODS.

B0 and M0 are endogenous sugar phosphates.
B1, B2 and B3: 6, 12 and 24h fat body pre-incubates.
M1, M2 and M3: 6, 12 and 24h flight muscle pre-incubates.
REFERENCES


63. Zebe, E., Biochem. Z., (1960), 332, 328-332


89. Lopes, D. P., Ph.D. Dissertation, University of Windsor, Windsor, Ontario, Canada.


VITA AUCTORIS

Name: Moses Y. Agbanyo
Place of Birth: Hohoe, Ghana
Date of Birth: February 19, 1954
            University of Ghana
            1977-present. Ph.D. Biochemistry
            University of Windsor
            Windsor, Ont. CANADA
         Postgraduate Scholarship
         1977-present. Ghana Government Scholarship
Societies: 1979-present. Canadian Biochemical Society (Student member)
Working Experience: 1976. Teaching Assistant
                   University of Ghana Medical School