AN ABSTRACT OF THE THESIS OF

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Title CATABOLIC PATHWAYS OF CARBOHYDRATE IN THE INTERMOLT CRAYFISH, PACIFASTACUS LENIUSCULUS

Abstract approved (Major professor)

The radiorespirometric method was used to study the catabolism of glucose in intermolt crayfish. Rapid and extensive conversion of C-3(4) of glucose to \( \text{C}^{14}\text{O}_2 \) indicated that a significant portion of the administered glucose was routed into the glycolysis-Krebs cycle sequence. High yields of C-6 of glucose in the respiratory \( \text{CO}_2 \) suggested the participation of the glucuronate pathway.

Some pentose phosphate pathway activity was suggested by the fact that C-1 of glucose was converted to \( \text{CO}_2 \) at a slightly higher rate than C-2 in the earlier portions of the time-course experiments.

Further evidence for participation of the latter two pathways was obtained by administering glucuronate-6-\( \text{C}^{14} \) and gluconate-1-\( \text{C}^{14} \).

Cumulative (70 hours) \( \text{C}^{14}\text{O}_2 \) yields of 70 percent and 50 percent were obtained respectively, indicating extensive conversion of these substrates to respiratory \( \text{CO}_2 \).
CATABOLIC PATHWAYS OF CARBOHYDRATE IN THE INTERMOLT CRAYFISH, PASIFASTACUS LENIUSCULUS

by

ROBERT LOUIS PUYEAR

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Typed by Nancy Kerley
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Thanks also are extended to my wife, Shirley, for her patience during the many nights she spent alone, while I collected data.
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CATABOLIC PATHWAYS OF CARBOHYDRATE IN THE INTERMOLT CRAYFISH, PACIFASTACUS LENIUSCULUS

INTRODUCTION

Since the detection by Claude Bernard of glycogen in the hepatopancreas of the lobster there have been numerous studies of intermediary carbohydrate metabolism in crustaceans. Recent reviews on the subject include those of Vonk (29, p. 291-311) and Scheer (26, p. 213-227). The substances utilized appear to be similar to those in most other organisms. Morgulis (22, p. lii-liv) was the first to report on the high levels of reducing substances in the blood of crustaceans. The nature of the reducing material was partially clarified by Kleinholz and Little (14, p. 218) who observed that the total reducing substances in the blood of the crab Libinia emarginata were divisible into fermentable and non-fermentable components. McWhinnie and Scheer (19) observed in the crab Hemigrapsus nudus that blood glucose represented a small fraction of the total reducing substances, similar to the fermentable fraction described by Kleinholz and Little. Recently, McWhinnie and Saller (18, p. 110) have isolated glucose, glucose-6-phosphate and maltose oligosaccharides from the blood of the crayfish Orconectes virilis. Meenakshi and Scheer (20, p. 30-41) have extended these observations to the crabs Hemigrapsus nudus and Cancer magister.
Although the amount of glucose in the blood is not large, there is evidence that it is readily catabolized. Hu (11, p. 385) and Meenakshi and Scheer (20, p. 30-41) observed that injection of carbon-14 labeled glucose into *Hemigrapsus nudus* resulted in recovery of radioactivity in the respiratory CO$_2$. Similar findings have been reported for *Cancer magister* (2).

Pathways by which carbohydrates may be catabolized have been studied in tissues from a variety of crustaceans. Hu (11, p. 385), working with *Hemigrapsus nudus*, demonstrated the presence of glucose-6-phosphate, triose phosphate and phosphopyruvic acid, intermediates in the Embden-Meyerhof-Parnas (EMP) pathway. McWhinnie and Kirchenberg (17, p. 117), using metabolic inhibitors, provide evidence for more than one pathway for glucose catabolism in crayfish hepatopancreas tissue. Recently, McWhinnie and Corkill (15, p. 81) have observed a shift in hexose monophosphate shunt activity occurring during the intermolt cycle in crayfish.

Studies on the operation of the Krebs cycle in crustacean tissues have provided somewhat contradictory findings. Ball and Meyerhof (1, p. 483) demonstrated the presence of succinic dehydrogenase in lobster heart but not in striated muscle of the lobster *Homarus americanus*. Scheer *et al.* (27, p. 327) and Kermack *et al.* (13) were unable to stimulate oxygen consumption of tissues of either *Panulirus* or *Homarus* with a number of Krebs cycle intermediates.
Recently, however, Hochachka et al. (10, p. 1043), using hepatopancreas homogenates of Homarus americanus, were able to stimulate oxygen consumption with various intermediates of the Krebs cycle, pentose phosphate pathway and EMP pathway. McWhinnie and Kirchenberg (17, p. 117) were able to stimulate oxygen consumption of crayfish hepatopancreas homogenates with succinate, but not with glucose or hexokinase. Munday and Thompson (23, p. 277) "sparked" pyruvic acid oxidation with a trace of fumarate, in mitochondrial preparations of the hepatopancreas of the crab, Carcinus maenas, thus demonstrating Krebs cycle activity in this system.

The studies on homogenates and tissue fragments show that the metabolic pathways common to other biological systems are at least partly operational in crustaceans. The extent of participation of the various pathways by which glucose is metabolized is not, however, completely understood. In the present study the carbohydrate metabolism of intact, intermolt crayfish has been studied using the radiorespirometric method (34, p. 1869). This method permits one to follow the yields of the respiratory $^{14}\text{CO}_2$ at intervals of time after various specifically labelled radioactive compounds have been administered to the animals. From analysis of the interval and cumulative recovery data for $^{14}\text{O}_2$, one may estimate the extent of participation of known metabolic pathways. The original plans called for a comparison of animals from different stages of the
molt cycle, with respect to metabolic pathway participation. A great deal more time than anticipated was spent developing a procedure which provided satisfactory reproducibility in these animals. It was decided, consequently, to confine the study to a characterization of catabolic pathways in intermolt crayfish.
METHODS AND MATERIALS

Male crayfish, identified as *Pacifastacus leniusculus*, were obtained from ponds and streams in the vicinity of Corvallis, Oregon. These animals were maintained in the laboratory for at least one week before they were used for experimental purposes. They were fed beef liver every two or three days, but were fasted 24 hours prior to the beginning of the experiment. Experimental animals weighing between 40 and 50 grams were used.

Animals used in this study were in the intermolt stage of the molt cycle. Stage in the cycle was crudely estimated by grasping the carapace and applying pressure with the fingers. If the carapace did not yield to a fair amount of pressure the animal was classified as intermolt. Coloration was also used to help establish the intermolt condition. Postmolt animals generally appear brighter red than late intermolt and premolt animals. These two criteria worked best for animals examined during the late spring, summer, and fall. During the late winter and early spring these two characteristics were more unreliable. After the winter molt, however, the carapace was quite firm and the general appearance of the animals was brownish-red to red. Following an experiment, the animals were sacrificed and examined to see if there was any gastrolith formation on the anterio-lateral edge of the cardiac stomach. The absence of
a gastrolith indicates that the animal is in the intermolt stage of the molt cycle (16, p. 4). This last method was considered to be positive evidence for intermolt animals, while the previous two described would have to be considered only as approximate since they deal with two subjective characteristics.

**CO₂ Fixation**

A preliminary experiment was conducted to see if significant CO₂ fixation occurred in this animal. The procedure used was as follows. Two crayfish were placed in a 250 mm desiccator containing one liter of filtered tap water and one millicurie (mc) of NaHCO₃¹⁴O₃. The animals were kept in this mixture for 48 hours. One animal was removed and placed in Bouin's fixative solution for future autoradiography while the other animal was removed and analyzed for incorporation of activity into various tissues. After the tissues were removed, they were placed in 1 N acetic acid for ten minutes to remove any superficial activity not actually incorporated. They were then removed from the acetic acid, rinsed in distilled water and blotted with filter paper. A 50 mg sample was then placed on an aluminum planchet and counted with a gas-flow Geiger-Muller counter.
Chromatographic Analysis

In a few cases, crayfish were injected with a high level of uniformly labelled glucose (8-10 microcurie; μc) and muscle tissues examined for distribution of label into amino acids. A few of these animals were fixed in Bouin's solution for future autoradiographic analysis. Abdominal muscle tissue from the remaining animals was placed in Carius tubes containing three ml of 6 N HCl. The tubes were sealed while being cooled in a dry ice acetone bath. Hydrolysis was carried out in an autoclave at 15 pounds pressure for 24 hours. After the hydrolysis was completed the contents were placed in small beakers. Acid was removed by placing in a vacuum desiccator which contained NaOH and P₂O₅ in crystaline form. After the samples were evaporated to dryness, two ml of water was added to each beaker and they were again dried under the same conditions. This process was carried out three more times. The charred material which resulted from the initial hydrolysis was removed by placing a small quantity of water and charcoal in each beaker and heating. The residue was removed by filtering the solution through a sintered glass filter and the clear filtrate was again evaporated to dryness. One ml of distilled water was added to the contents of each beaker. The samples were then stored in a freezer for future chromatographic analysis.
Single dimensional strip paper chromatography was used for the separation of amino acids present in the protein hydrolysates of crayfish muscle tissue. Amino acid standards were chromatographed at the same time to identify the amino acids from the protein hydrolysate. The solvent system used was 2-butanol-3 percent ammonia (25, p. 502). The paper strips were cut into equal sections (2.5 x 4 cm) and placed in five dram liquid scintillation counter vials which had been modified for counting paper strips of this size. The vials were filled with 15 ml of phosphor- solution and counted in an automatic liquid scintillation counter (33).

Procedure in Radiorespirometric Experiments

Substrate administration is one of the major problems encountered in designing experiments of this type, since it is important that the respective substrate solution be introduced into the animal body quantitatively, and diffuse readily throughout the entire metabolically active tissue of the animal. With this objective in mind, several approaches were undertaken to derive the best and yet simplest procedure whereby the substrate could be administered. The first site chosen for injection was at the base of the fifth walking leg since there is a blood sinus located at the base of this leg. This site was abandoned after it was found that injections into the heart or pericardial cavity gave results with better reproducibility. Relatively
unsuccessful attempts were made to reduce the amount of blood loss at the point of injection by applying vaseline or glue. The problem was partially solved by leaving the needle inserted for three minutes after all the substrate had been delivered. This allowed time for most of the substrate to be circulated from the point of injection, thus reducing the loss of substrate when the needle was withdrawn.

The following is an account of the procedure finally adapted for the injection of substrate into crayfish.

Crayfish were weighed and marked 24-36 hours prior to injection. At the time of injection, the crayfish were removed from the holding tank and thoroughly dried. They were held immobile in a small "v" shaped trough, with the abdomen extending over the end. The abdomen was then bent downward and the articular membrane between the cephalothorax and abdomen dried well with tissue wipes. The trough was then positioned beneath the injection device, consisting of an inverted microscope attached by the base to a frame so that it was suspended above the crayfish. The syringe was mounted to the racking mechanism of a microscope with two clips which permitted removal of the syringe for filling. The syringe was driven by the coarse adjustment knob of the microscope. The substrate solutions, 0.4 ml in volume, were injected through a 3/8 inch number 30 needle. Only about 1/8 to 3/16 inch of the needle was inserted through the previously dried articular membrane. The
needle was inserted at such an angle that it was driven toward the heart.

Following the injection of substrate the animals were placed in cylindrical chambers (2-1/4 x 7 inches) with 200 ml of water. The chambers were held at an angle of about 20 degrees from the horizontal on a rack in a refrigerator maintained at 17±1 degrees centigrade. A stream of CO$_2$-free air was bubbled through the water in each chamber at a rate of 150 ml per minute. The CO$_2$ evolved by the animals was swept out of the chamber in the stream of air and ultimately into a CO$_2$ absorbing trap equipped with a sintered glass disc. Twenty ml of 0.5 N CO$_2$-free NaOH was used as the trapping reagent. Samples were taken every three hours up to 30 hours and every six hours thereafter. The trapped respiratory CO$_2$ was precipitated as BaCO$_3$ with 1 N CO$_2$-free NH$_4$Cl-BaCl$_2$ solution. The resulting BaC$^{14}$O$_3$ precipitates were mounted on aluminum planchets by means of the centrifugation technique and counted with a gas-flow Geiger-Muller counter equipped with a thin mylar window (0.5 mg/cm$^2$). The samples were counted for a sufficient time so that the relative standard deviation of the counting data was less than two percent. All samples were corrected for background and self absorption in the conventional manner. The radiorespirometric experiments were generally terminated 70 hours after the time of substrate injection.
Substrates Used

The radioactivity of the substrate administered in the radio-respirometric experiments was about 0.5 µc (specific activity about one mc/mM) with the exception of glucose-3(4)-C\textsubscript{14}, which was administered with an activity of about 0.2 µc. Glucose-3(4)-C\textsubscript{14} was obtained from liver glycogen isolated from rats injected with \textit{NaH}C\textsubscript{14}O\textsubscript{3}, according to the method of Wood \textit{et al.} (35). The remaining radiochemicals used in this study were obtained from various commercial sources. All substrate activity was determined by the persulfate combustion method described by Katz \textit{et al.} (12). In all experiments excepting those with glutamic acid, and acetate the amount of substrate administered was adjusted, with appropriate amounts of glucose, so that the total chemical level was 2.5 mg per animal. The chemical level in the acetate experiments was 2/3 that for glucose. In the glutamic acid experiments each animal was injected with 0.375 mg of the L-form of the acid since preliminary work demonstrated that the D-form of the acid probably could not be used by these intermolt crayfish. It was found that injection of more than 0.375 mg of L-glutamic acid per animal was frequently fatal. In all cases the unlabelled glutamic acid was injected with an insignificant amount (specific activity greater than one mc/mM), with respect to chemical level, of C-14 specifically labelled
DL-glutamic acid. To facilitate solubilization of glutamic acid, the pH was adjusted to 7.4. All substrates were dissolved in van Harreveld's solution with the final pH set at 7.4.
RESULTS

Table 1 summarizes the results obtained after two crayfish were placed in water containing one mc of NaHCO\(_3\). Relatively little label was incorporated into the tissues analyzed. Further analysis by autoradiography of crayfish preserved in Bouin's was not feasible due to the low incorporation of C-14 label.

During the early phase of this research an attempt was made to determine the distribution of radioactivity after injection of relatively high amounts of labelled glucose. Autoradiographic plates were obtained from a few crayfish injected with 8-10 µc of uniformly labelled glucose. They were prepared for autoradiography by sectioning the entire crayfish down the midventral line and placing on non-screen X-ray film in the dark. Resolution was poor in these crude preparations but it was observed that more of the activity was concentrated in the cephalothoracic region, with less in the abdominal musculature. Chromatographic analysis of the protein hydrolysate from abdominal muscle was carried out 72 hours after injection with 10 µc of glucose uniformly labelled (G-U-C\(_{14}\)). The results are shown in Table 2. The information presented here is preliminary but it may be noted that only the amino acids identified as aspartate through lysine, alanine and leucine appear to contain counts much above background. It is quite possible that only one or two of the
first group of amino acid mentioned above contains all the activity. This would have to be determined upon further analysis.

The optimal substrate level for the radiorespirometric experiments was determined by performing a series of tests in which varying amounts of glucose; namely 100, 50, 10, and 2.5 mg were administered together with 0.5 \( \mu \)c of G-U-C\( ^{14} \). The results of these experiments are presented in Figure 1. From these curves of specific activity (S. A.; cpm/mg) it is seen that the recovery of C\( ^{14} \)O\(_2\) from G-U-C\( ^{14} \) is dependent upon the amount of glucose administered. It may be seen that the peak S. A. appears earlier with the smaller the amount of glucose administered. The rapidity and degree of uniformity of recovery of C\( ^{14} \)O\(_2\) respired by the crayfish was greatest at 2.5 mg and, consequently, this was the glucose level chosen for subsequent experimentation.

Following the establishment of optimal substrate level further radiorespirometric experiments were performed with specifically labelled glucose. The recovery of respiratory C\( ^{14} \)O\(_2\) from crayfish metabolizing these substrates is presented in Figure 2. Two curves are given, one representing the percent recovery of the total label administered per hour (percent interval recovery) and the other the cumulative percent recovery of the total label administered (percent cumulative recovery). It may be noted that during the early phase (up to 30 hours) glucose-3(4)-C\( ^{14} \) is converted to respiratory CO\(_2\).
more extensively than glucose labelled in any other position. It is also apparent from the percent interval recovery curves that glucose-1-$^{14}$C is converted to CO$_2$ at a slightly higher rate than glucose-2-$^{14}$C during the first ten hours. The C-6 of glucose is converted to respiratory CO$_2$ at a greater rate than either C-1 or C-2. In fact, at the end of the experiments the cumulative yield of C$^{14}$O$_2$ from C-6 is greater than the yield from any other carbon atom of glucose tested.

Experiments were carried out using gluconate-1-$^{14}$C and glucuronate-6-$^{14}$C to test the hypothesis that the pentose phosphate pathway and glucuronic acid pathways may be operative in intact intermolt crayfish. The results are shown in Figure 3. It can be seen that the C-6 of glucuronate is extensively converted to CO$_2$, giving rise to a cumulative yield amounting to approximately 70 percent. Similarly, C-1 of gluconate, a key intermediate in the pentose phosphate pathway, is also found to be readily decarboxylated to give rise to a cumulative yield amounting to approximately 50 percent at the end of the experiment.

The operation of the Krebs cycle was studied by using specifically labelled acetate and DL-glutamate as test substrates. Figure 4 shows the results obtained in experiments where crayfish were injected with either C-1 or C-2 acetate. The C-1 label of acetate appears more extensively in the respiratory CO$_2$ than C-2. Figure 5
represents the data obtained from crayfish metabolizing specifically labelled DL-glutamate. The yields of respiratory $^{14}\text{C}O_2$ derived from individual carbon atoms of glutamic acid are in the order of $C-1 > C-2 = C-5 > C-3(4)$. The fact that the readily decarboxylated C-1 of glutamate gives rise to a cumulative yield of less than 50 percent leads one to believe that only L-glutamate was utilized by these animals since values greater than 50 percent would indicate that D-glutamate could also be metabolized. In one experiment a crayfish was injected with 1 $\mu$C of DL-glutamate-1-$^{14}$C along with 6.4 mg of D-glutamate, and a second with the same amount of labelled DL-glutamate plus 1.25 mg of L-glutamate. The radio-respirometric data are shown in Figure 6 as specific activity curves. The results indicate that the amount of $^{14}\text{C}O_2$ relative to $\text{CO}_2$ respired by the crayfish injected with 6.4 mg of D-glutamate is almost three times greater than the crayfish injected with 1.25 mg of L-glutamate. In Figure 7 we see that the cumulative recoveries in both cases was 43 and 40 percent respectively. This indicates that only the L-glutamic acid was utilized.
Table 1. Tissue samples taken from a crayfish after incubation in one mc of NaHCO$_3$ for 48 hours. Each tissue was soaked ten minutes in 1 N acetic acid before counting.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Wet Weight of Tissue (mg)</th>
<th>CPM</th>
<th>CPM Corrected to 50 mg Wet Weight</th>
</tr>
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<tbody>
<tr>
<td>Hypodermis</td>
<td>56.3</td>
<td>169</td>
<td>151</td>
</tr>
<tr>
<td>Heart</td>
<td>58.5</td>
<td>67</td>
<td>57</td>
</tr>
<tr>
<td>Digestive gland</td>
<td>61.2</td>
<td>65</td>
<td>53</td>
</tr>
<tr>
<td>Abdominal muscle</td>
<td>54.8</td>
<td>37</td>
<td>34</td>
</tr>
<tr>
<td>Green gland</td>
<td>33.3</td>
<td>129</td>
<td>194</td>
</tr>
<tr>
<td>Gills</td>
<td>52.3</td>
<td>257</td>
<td>246</td>
</tr>
<tr>
<td>Chitin*</td>
<td>25.2</td>
<td>853</td>
<td>1692</td>
</tr>
<tr>
<td>Chitin</td>
<td>25.2</td>
<td>15</td>
<td>29</td>
</tr>
</tbody>
</table>

*NOT soaked in 1 N acetic acid.
Table 2. Distribution of label in various amino acids after injection of 10 µc of glucose-U-C\(^{14}\). One tenth of the initial 43 mg (dry weight) of abdominal muscle was spotted on the paper.

<table>
<thead>
<tr>
<th>Amino Acid Isolated as a Group or Individually</th>
<th>CPM Incorporated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>813</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>8</td>
</tr>
<tr>
<td>Cysteic</td>
<td>56</td>
</tr>
<tr>
<td>Lysine</td>
<td>161</td>
</tr>
<tr>
<td>Glycine</td>
<td>32</td>
</tr>
<tr>
<td>Histidine</td>
<td>44</td>
</tr>
<tr>
<td>Serine</td>
<td>813</td>
</tr>
<tr>
<td>Threonine</td>
<td>8</td>
</tr>
<tr>
<td>Alanine</td>
<td>56</td>
</tr>
<tr>
<td>Proline</td>
<td>30</td>
</tr>
<tr>
<td>Valine</td>
<td>139</td>
</tr>
<tr>
<td>Methionine</td>
<td>68</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>30</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>139</td>
</tr>
<tr>
<td>Leucine</td>
<td>68</td>
</tr>
<tr>
<td>Tail after leucine</td>
<td>139</td>
</tr>
</tbody>
</table>
Figure 1. Radiorespirometric patterns for crayfish utilizing glucose-U-C\textsuperscript{14} plus 100 mg glucose, X—X (two animals); 50 mg glucose, ■—■ (seven animals); 10 mg glucose, o—o (three animals); 2.5 mg glucose, •—• (five animals).
Figure 2. Radiorespirometric patterns for crayfish utilizing 2.5 mg glucose plus glucose-1-$^{14}C$, $X-X$ (three animals); glucose-2-$^{14}C$, $---$ (six animals); glucose-3(4)-$^{14}C$, $-$ (two animals); glucose-6-$^{14}C$, $-$ (five animals).
Figure 3. Radiorespirometric patterns for crayfish utilizing glucuronate-6-C\(_{14}\), •—• (six animals) and gluconate-1-C\(_{14}\), X——X (two animals).
Figure 4. Radiorespirometric patterns for crayfish utilizing acetate-1-C$_{14}$, •—• (two animals) and acetate-2-C$_{14}$, X—X (two animals).
Figure 5. Radiorespirometric patterns for crayfish utilizing DL-glutamate-1-C₁⁴, •—• (three animals); DL-glutamate-2-C₁⁴, o—o (five animals); DL-glutamate-3(4)-C₁⁴, X—X (three animals); DL-glutamate-5-C₁⁴, X——X (five animals).
Figure 6. Radiorespirometric patterns for crayfish utilizing DL-glutamate-1-C\(^{14}\) plus 6.4 mg D-glutamate, •—• (one animal) or 1.25 mg L-glutamate, X——X (one animal).
Figure 7. Radiorespirometric patterns for crayfish utilizing DL-glutamate-1-C\textsuperscript{14} plus 6.4 mg D-glutamate, \(\bullet\)\(\bullet\) (one animal) or 1.25 mg L-glutamate, \(\times\times\) (one animal).
DISCUSSION

The primary oxidative pathway in animal systems is the Krebs cycle of reactions. Although numerous studies on crustacean homogenates and tissue preparations have shown at least parts of the cycle to be operative, the radiorespirometric methods used in the present study has not, to our knowledge, been used previously. By using acetate or glutamate, with specific carbons labelled, and comparing the $^{14}O_2$ recovery following injection of these substrates, it is possible to determine if these substrates are metabolized via the Krebs cycle. Figure 4 shows the results with acetate as the substrate. It may be noted that C-1 of acetate is preferentially converted to CO$_2$ over that of C-2. This observation is similar to that found in tomato fruit (5) and alligators (3). These authors report that this type of recovery is typical when the Krebs cycle is operative. The results of the glutamate experiments are shown in Figure 5. The cumulative recoveries of respiratory $^{14}O_2$ derived from individual carbon atoms of glutamate are in the order of C-1 > C-2 = C-5 > C-3(4). The relative yields of these carbon atoms are in line with that observed in yeast (30, p. 283) and with that called for by the metabolism of glutamic acid via the following sequence:
Glutamic acid may be decarboxylated by glutamic decarboxylase, with release of CO₂ from the C-1 position. However, this enzyme has not been demonstrated in crustaceans (15). The evidence does suggest, therefore, that the normal Krebs cycle is operative in intermolt crayfish.

The results indicate that only L-glutamate is utilized by crayfish. In no instance was more than 50 percent of the activity recovered from the injected C-1 of glutamate. Recovery of more than 50 percent of the label would indicate that the D-form could also be utilized. Further evidence to support this hypothesis is the fact that a second peak did not appear in the percent interval recovery curve (Figure 7) when 1.25 mg of L-glutamate plus 1 µ DL-glutamate-1-C¹⁴ was administered. A second peak is indicative of the utilization of DL-glutamate-1-C¹⁴, as has been demonstrated in Micrococcus radiodurans (24). The S.A. curves in Figure 6 show that when 6.4 mg of D-glutamate is used as a "carrier" a greater portion of the CO₂ respired by the animal contains C-14 than does the crayfish injected with 1.25 mg L-glutamate. This also indicates that D-glutamate is not utilized to a significant degree; otherwise the S.A. for the crayfish utilizing L-glutamate would be greater since the
chemical level of L-glutamate injected was less. It is concluded
that little, if any, D-glutamate is utilized by crayfish.

The ability to fix CO$_2$ into the intermediates of the Krebs
cycle has been demonstrated in at least one crustacean preparation,
the digestive gland of the blue crab Callinectes sapidus (9). Al-
though only a single experiment was performed in the present study,
the results (Table 1) clearly show that crayfish do not fix CO$_2$ to any
significant degree. Since the integument may only be slightly per-
meable to NaHCO$_{14}$O$_3$, it is possible that injection of NaHCO$_{14}$O$_3$,
rather than immersion of the animal in a solution of the substrate,
would result in a much greater incorporation of C-14.

Results obtained from the preliminary radiorespirometric
experiments indicated that 2.5 mg of glucose constituted an optimal
substrate level. Injection of greater quantities of glucose diluted
the amount of glucose-U-C$^{14}$ present resulting in a reduction in the
specific activity of the respiratory CO$_2$. These same observations
have been previously reported in rats (31). In rats, however, 500
mg of glucose constituted the optimal substrate level (31). One
might explain the great difference in optimal substrate level between
these two animals on the basis of great differences in metabolic
rate and blood glucose values. For example, a 200 gm male rat
consumes 1,333 ml O$_2$/kg/hr while a 35 gm male crayfish consumes
only 55 ml O$_2$/kg/hr (7; 28, p. 258). Blood glucose in the rat varies
from 56-76 mg per 100 ml, while in crayfish the values range from 1-20 mg per 100 ml of blood (18, p. 112; 28, p. 53). Thus one might assume that crayfish will utilize less glucose than the rat and consequently the optimal glucose level for substrate injection would be considerably less than that observed in rats.

Crude autoradiographic analysis of crayfish injected with G-U-C\textsuperscript{14} showed that injected substrate was distributed throughout the crayfish. This observation is pertinent to this type of study since distribution to all tissues of the crayfish is important from the standpoint of knowing whether the entire tissue mass is involved in the metabolism of the injected substrate.

Comparison of the percent interval and cumulative recovery curves of specifically labelled glucose substrates (Figure 2) seems to indicate that glucose is metabolized via three pathways. The extensive conversion of C-3(4) of glucose to CO\textsubscript{2} during the first 30 hours of an experiment reflects the extensive operation of the glycolytic-pyruvate decarboxylation sequence (32). Also, the fact that alanine incorporated a significant amount of activity after injection of G-U-C\textsuperscript{14} (Table 2) would suggest that it was formed by reversible transamination with pyruvate, the final product of the glycolytic pathway (21, p. 257). The C-6 carbon of glucose is converted to respiratory CO\textsubscript{2} at a higher rate and more extensively than either C-1 or C-2. In fact, at the end of the experiments, one finds that
the cumulative yield of \( \text{CO}_2 \) from C-6 of glucose is greater than the yield from any of the other carbon atoms of glucose tested. Such an observation could be explained on the basis that there exists, in crayfish, an active mechanism for triose recombination resulting in the randomization of C-6 of glucose to the C-1 position. However, it is more likely that the high yield of C-6 of glucose in the respiratory \( \text{CO}_2 \) reflects the operation of a mechanism involving the preferential and direct conversion of C-6 to \( \text{CO}_2 \). Such a pathway has been reported by Eisenberg, et al. (6). The mechanism, in essence, involves the conversion of glucose to glucuronic acid, followed by the decarboxylation of C-6 of the original glucose. During the early phase of the experiments (up to ten hours) when the substrate is being rapidly utilized, it may also be noted that C-1 of glucose is converted to \( \text{CO}_2 \) at a slightly higher rate than the C-2, as determined by comparing the areas below each percent interval recovery curve. This is indicative of the operation of the pentose phosphate pathway, since the classical glycolysis-Krebs cycle sequence calls for the preferential conversion of C-2 and C-5 of glucose to \( \text{CO}_2 \) over that of C-1 and C-6.

To examine further the operation of glucuronic acid and pentose phosphate pathways key intermediates in each metabolic pathway were injected into crayfish. Both of these intermediates, glucuronate-6-\(^{14}\text{C} \) and gluconate-1-\(^{14}\text{C} \), contributed significant amounts
of activity to the respiratory CO$_2$ (69 and 50 percent, respectively, at the end of the experiment). There are two possible ways in which these compounds could be metabolized: (1) they could be converted back to glucose with the label present in the same respective position and then the newly formed glucose metabolized; or (2) each substrate could be metabolized via its own specific pathway. The latter explanation, however, appears most likely since it has been found experimentally that the conversion of gluconate to glucose is essentially impossible since the equilibrium greatly favors the formation of gluconate (8, p. 91). Further, the conversion of glucose to glucuronate is essentially irreversible (4; 6). Thus the recovery of labelled carbon from gluconate-6-C$^{14}$ and gluconate-1-C$^{14}$ appears to be possible only if the glucuronic acid and pentose phosphate pathways are operative.

It appears, therefore, that in intermolt crayfish, three major catabolic pathways are operative for the utilization of glucose. They are: (1) the glycolytic pathway, (2) the glucuronic acid pathway and (3) the pentose phosphate pathway. In the absence of a reliable method for quantitatively estimating three concurrent glucose pathways in biological systems, it is not possible to accurately estimate the relative contribution of each of these pathways. Nevertheless, it can be estimated on the basis of the cumulative C$^{14}$O$_2$ yields from C-3(4) (55 percent), from C-6 (60 percent), from C-1 (40
percent) and from C-2 (48 percent) of glucose at the end of the experiments, that the pentose phosphate pathway participates less than the glycolytic and glucuronate pathways in glucose catabolism in intermolt crayfish.

The detection of the glycolytic pathway and pentose phosphate pathway agree with previous results obtained with tissue preparations from various crustaceans (15; 17; 23; 10). The operation of the glucuronate pathway in crustaceans has not, to our knowledge, been reported and it will be of much interest to evaluate the exact role played by this pathway in these animals.
SUMMARY

1. The radiorespirometric method was used to study the
catabolic pathways of carbohydrates in the intact intermolt crayfish,
Pacifastacus leniusculus.

2. The terminal respiratory mechanism was studied using
specifically labelled acetate and DL-glutamate. The experimental
results are in accord with the operation of the Krebs cycle in this
organism.

3. Glucose appears to be catabolized via three concurrent
pathways. The rapid and extensive conversion of C-3(4) of glucose
to CO$_2$ suggests that a large portion of the administered glucose is
routed into the glycolysis-pyruvate decarboxylation sequence. Like-
wise, the high yield of C-6 of glucose in the respiratory CO$_2$ im-
plies the operation of the glucuronate pathway. The pentose phos-
phate pathway appears to be present since C-1 of glucose was con-
verted to CO$_2$ at a slightly higher rate than that of C-2 in the earlier
portions of the time-course experiments.

4. Further evidence for the operation of the glucuronate path-
way and pentose phosphate pathway was obtained by administering
glucuronate-6-C$^{14}$ and gluconate-1-C$^{14}$. Extensive conversion of
these intermediates to C$^{14}$O$_2$ was noted.


