FIXATION AND DISTRIBUTION OF C\textsuperscript{14}O\textsubscript{2} IN BRUCELLA ABORTUS\textsuperscript{1}

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The requirement of many Brucella abortus strains for a pCO\textsubscript{2} of approximately 0.03 atmosphere for growth seems to differ both quantitatively and qualitatively from the CO\textsubscript{2} requirement of other heterotrophic organisms. In some microorganisms, CO\textsubscript{2} can be replaced by certain tricarboxylic acids (Lwoff and Monod, 1946; Ajl and Werkman, 1948), amino acids (Lyman et al., 1947; Abelson et al., 1952a, b; McLean and Purdie, 1952), or purines and pyrimidines (Tuttle and Scherp, 1952; Griffin and Racker, 1956). However, attempts to replace the requirement for CO\textsubscript{2} of B. abortus strains with carboxylic acids, amino acids, purines and pyrimidines, cellular hydrolyzates, and culture filtrates have been unsuccessful (Gerhardt and Wilson, 1950; Ruwet, 1951).

In the investigation of the biochemical basis for the CO\textsubscript{2} requirement of B. abortus strains, Marr and Wilson (1951) have used C\textsuperscript{14}O\textsubscript{2}. They studied the incorporation of C\textsuperscript{14}O\textsubscript{2} into the amino acids of B. abortus strain 6232, a strain which requires an increased pCO\textsubscript{2} in air for growth, and found that the major product of C\textsuperscript{14}O\textsubscript{2} fixation in this strain was glycine. C\textsuperscript{14}O\textsubscript{2} was fixed, in lesser amounts, into aspartic acid, glutamic acid, alanine, and threonine. C\textsuperscript{14}O\textsubscript{2} fixation into the nucleic acids of B. abortus strain 6232 occurs only in the pyrimidines; fixation of C\textsuperscript{14}O\textsubscript{2} into purines seems to be quantitatively insignificant (Newton et al., 1954). When given a choice of pyrimidine precursors, strain 6232 preferred to synthesize pyrimidines from CO\textsubscript{2} (Newton and Wilson, 1954). In comparative experiments, a mutant strain 6232 capable of growth in air was found to fix C\textsuperscript{14}O\textsubscript{2} into nucleic acids in a manner identical to the parent strain 6232.

With the exception of the experiments of Newton and Wilson (1954), C\textsuperscript{14}O\textsubscript{2} fixation has been studied primarily with strains of B. abortus which require an increased pCO\textsubscript{2} for growth. In the present investigation, a comparison has been made of C\textsuperscript{14}O\textsubscript{2} fixation by strains of B. abortus differing in their requirement for an increased pCO\textsubscript{2} for growth and differing also in their virulence for guinea pigs.

EXPERIMENTAL METHODS

Five strains of Brucella abortus were used in these experiments. The moderately virulent strain 6232 NS and the highly virulent strain 6232S' Mika require an increased pCO\textsubscript{2} in air for growth (CO\textsubscript{2}-dependent strains). The highly virulent strains 6232C PA and 2308 and the essentially avirulent strain 11 are mutant strains which grow in air alone (CO\textsubscript{2}-independent strains). The 50 per cent infectious doses of the five B. abortus strains have been reported previously (Tepper and Wilson, 1958). The cultures were grown on Albimi brucella agar slants at 37 C. The CO\textsubscript{2}-dependent strains were incubated in an atmosphere of 10 per cent CO\textsubscript{2} in air and the CO\textsubscript{2}-independent strains were incubated in air under comparable conditions. Cells were harvested at 17 to 19 hr, washed, and resuspended in Albimi brucella broth and used in equivalent concentration as measured turbidimetrically. The suspensions which were used contained 200 to 300 \mu g bacterial nitrogen per ml. These cell suspensions have been found to resemble resting cell suspensions or possibly cultures in the lag phase of growth. No significant increases in cell numbers could be detected after the incubation of these heavy cell suspensions under conditions identical to those used for exposures to C\textsuperscript{14}O\textsubscript{2}. The B. abortus cells were exposed to 0.1 atm C\textsuperscript{14}O\textsubscript{2} in 25 ml Warburg vessels with siamese side arms for generating C\textsuperscript{14}O\textsubscript{2} from BaC\textsuperscript{14}O\textsubscript{2}. Standard manometric equipment was used with the bath

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temperature at 34 C. At the end of an exposure the cells were recovered by centrifugation and washed with distilled water. The cells were chemically fractionated by the modification of the method of Schneider (1945) used by Roberts et al. (1955). By this procedure, the cells were fractionated into a cold trichloroacetic acid (TCA)-soluble fraction (metabolic intermediates), alcohol- and alcohol-ether soluble fractions (lipides, phospholipides, and possibly ethanolsoluble protein), a hot TCA-soluble fraction (nucleic acids) and residual protein. In these experiments the alcohol-soluble and alcoholether-soluble fractions were pooled. These fractions were diluted and assayed for total radioactivity.

The cellular protein was hydrolyzed with 6 n HCl in sealed pyrex test tubes at 121 C for 3 hr. The hydrolyzates were filtered to remove the humin and taken to dryness in vacuo repeatedly to remove the HCl. The residue was dissolved in 25 ml of water and aliquots were used for nitrogen analyses and ion-exchange chromatography. The amino acids of the protein hydrolyzate were separated chromatographically on 2.5 by 55 cm columns of Dowex 50 by the method of Wall (1953). The photometric ninhydrin method of Moore and Stein (1948) was used for the quantitative determination of the amino acids. Fractions containing the separated amino acids were concentrated in vacuo to remove the HCl. The identities of the amino acids eluted from the column were checked by paper chromatography using 82 per cent aqueous phenol as a solvent. Aliquots of known concentrations of the amino acids were counted for radioactivity.

The C\textsuperscript{14} content of the carboxyl carbon of arginine was determined by the method of Van Slyke et al. (1941) as modified by Frank and Loomis (1949). The amino acid was decarboxylated with ninhydrin and the liberated CO\textsubscript{2} was collected in NaOH and precipitated as BaCO\textsubscript{3}. The guanidine carbon was removed by arginase prepared according to the method of Hunter and Downs (1944). The resultant urea was treated with urease liberating CO\textsubscript{2} which, after acidification, was distilled into cold alkali and precipitated with BaCl\textsubscript{2}. Products of the degradation were assayed for radioactivity as BaCO\textsubscript{3}.

All radioactivity measurements were made with a flowing gas Geiger counter. Samples in dilute solution were counted at infinite thinness. C\textsuperscript{14}O\textsubscript{2} was collected as BaC\textsuperscript{14}O\textsubscript{3}, washed several times in absolute methanol, and plated as a slurry in cupped planchets. The appropriate correction for self-absorption was made for the thick BaC\textsuperscript{14}O\textsubscript{3} samples.

RESULTS

Fickation of C\textsuperscript{14}O\textsubscript{2} into amino acids. A comparison was made of the C\textsuperscript{14}O\textsubscript{2} assimilated into the amino acids of the moderately virulent, CO\textsubscript{2}-dependent strain 6232 NS and the avirulent, CO\textsubscript{2}-independent strain 11. Cells of both strains of B. abortus were exposed to C\textsuperscript{14}O\textsubscript{2} for 3 hr after which the amino acids of the cells were isolated and assayed for radioactivity. The data in table 1 show a general similarity in the specific activities of the amino acids of the two physiologically different strains. The major portion of the C\textsuperscript{14}O\textsubscript{2} fixed into the amino acids of both strains was found in glycine. The total C\textsuperscript{14} incorporated into the amino acids was determined and the data are presented relative to the total C\textsuperscript{14} activity of glycine (table 1). The concentrations of the individual amino acids in the protein of B. abortus have been reported previously by Tepper and Wilson (1958). The data in table 1 show a similarity in the total C\textsuperscript{14}O\textsubscript{2} assimilated by the two B. abortus strains.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
Amino Acid & Specific Activity (cpm/\textmu mole) & Relative Total C\textsuperscript{14} Incorporation & \\
\hline
 & Strain 6232 NS & Strain 11 & Strain 6232 NS & Strain 11 \\
\hline
Glycine & 1830 & 1440 & 1.0000 & 1.0000 \\
Arginine & 330 & 280 & 0.1010 & 0.1100 \\
Aspartic acid & 170 & 150 & 0.0798 & 0.0885 \\
Glutamic acid & 65 & 63 & 0.0084 & 0.0423 \\
Threonine & 68 & 49 & 0.0171 & 0.0154 \\
Lysine & 62 & 58 & 0.0182 & 0.0192 \\
Alanine & 44 & 45 & 0.0273 & 0.0385 \\
Leucine & 35 & 33 & 0.0131 & 0.0154 \\
Serine & 30 & 22 & 0.0073 & 0.0071 \\
Valine & 15 & 14 & 0.0040 & 0.0049 \\
Tyrosine & 14 & 10 & 0.0025 & 0.0023 \\
Histidine & 7 & 5 & 0.0011 & 0.0010 \\
\hline
\end{tabular}
\caption{Distribution of C\textsuperscript{14} in amino acids of strains 6232 NS and 11 of Brucella abortus*}
\end{table}

* Cells exposed to 0.1 atm C\textsuperscript{14}O\textsubscript{2} for 3 hr. Specific activity of C\textsuperscript{14}O\textsubscript{2} 1.2 \times 10^7 cpm/\textmu mole.

\textdagger Amino acid concentrations from data of Tepper and Wilson (1958).
TABLE 2

Distribution of C\textsuperscript{14} in strains of Brucella abortus exposed to C\textsuperscript{14}O\textsubscript{2} for 5 min and 3 hr\textsuperscript{*}

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Percentage of Radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B. abortus strain</td>
</tr>
<tr>
<td></td>
<td>6232CPA</td>
</tr>
<tr>
<td></td>
<td>5 min</td>
</tr>
<tr>
<td>Cold-TCA soluble</td>
<td>83.1</td>
</tr>
<tr>
<td>Alcohol + alcohol-ether soluble</td>
<td>5.5</td>
</tr>
<tr>
<td>Hot-TCA soluble</td>
<td>3.5</td>
</tr>
<tr>
<td>Residual protein</td>
<td>7.9</td>
</tr>
</tbody>
</table>

\textsuperscript{*} Cells exposed to 0.1 atm C\textsuperscript{14}O\textsubscript{2}. Specific activity of C\textsuperscript{14}O\textsubscript{2}, 1.2 \times 10\textsuperscript{7} cpm/\mu mole.

TABLE 3

Fixation of C\textsuperscript{14}O\textsubscript{2} by strains of Brucella abortus in a 5 min exposure\textsuperscript{*}

<table>
<thead>
<tr>
<th>B. abortus strain</th>
<th>Specific Activity of Cells (cpm/mg bacterial N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6232C PA</td>
<td>4060</td>
</tr>
<tr>
<td>2308</td>
<td>3620</td>
</tr>
<tr>
<td>11</td>
<td>3530</td>
</tr>
<tr>
<td>6232 NS</td>
<td>4520</td>
</tr>
<tr>
<td>6232S' Mika</td>
<td>4220</td>
</tr>
</tbody>
</table>

\textsuperscript{*} Cells exposed to 0.1 atm C\textsuperscript{14}O\textsubscript{2}. Specific activity of C\textsuperscript{14}O\textsubscript{2}, 1.2 \times 10\textsuperscript{7} cpm/\mu mole.

The data presented in table 1 are in general agreement with the findings of Marr and Wilson (1951). However, with the ion-exchange technique for the separation of amino acids, additional amino acids were found to contain C\textsuperscript{14} activity. Among these, arginine was found to have a high specific activity which was second only to that of glycine. Degradation of the arginine isolated from the B. abortus protein showed that 74 per cent of the total activity was in the guanidine carbon. This observation suggests that the ornithine cycle is operative in B. abortus cells.

Distribution of C\textsuperscript{14}O\textsubscript{2} among chemical fractions of cells. In the first series of experiments, cells of the five B. abortus strains were exposed to C\textsuperscript{14}O\textsubscript{2} for 3 hr and then the C\textsuperscript{14} content of chemical fractions of the cells was determined. Table 2 shows that there is no real difference in the distribution of the C\textsuperscript{14}O\textsubscript{2} assimilated by the CO\textsubscript{2}-dependent and CO\textsubscript{2}-independent B. abortus strains. To preclude the possibility that C\textsuperscript{14}O\textsubscript{2} fixation by the CO\textsubscript{2}-independent strains might occur via an adaptive mechanism, a shorter exposure period was used. A 5 min exposure to C\textsuperscript{14}O\textsubscript{2} was found to be the shortest period in which the pathogenic microorganism could be handled with safety and with the accuracy necessary for the analysis of C\textsuperscript{14}O\textsubscript{2} incorporation. In a 5 min exposure to C\textsuperscript{14}O\textsubscript{2} the five B. abortus strains fixed approximately the same amount of C\textsuperscript{14}O\textsubscript{2} (table 3). There was no observable difference in the distribution of the C\textsuperscript{14} in the chemical fractions of these cells (table 2) which suggests that the mechanism of CO\textsubscript{2} assimilation is constitutive for both CO\textsubscript{2}-dependent and CO\textsubscript{2}-independent B. abortus strains. It should be pointed out, however, that there is a difference in the distribution of C\textsuperscript{14} from the C\textsuperscript{14}O\textsubscript{2} in the 5 min and 3 hr exposures (table 2). The 5 min exposure results in a higher per cent of C\textsuperscript{14}O\textsubscript{2} fixed into the cold TCA-soluble fraction and a much lower per cent incorporation into the protein fraction.

The protein fractions of the five strains of B. abortus were analyzed further. The most active amino acid in the protein hydrolyzates was determined. In all the hydrolyzates tested, including those of cells exposed to C\textsuperscript{14}O\textsubscript{2} for 5 min, glycine was found to contain the major portion of the C\textsuperscript{14} activity.

**DISCUSSION**

In a variety of microorganisms and animal and plant tissues the major products of C\textsuperscript{14}O\textsubscript{2} fixation into amino acids have been found to be arginine, aspartic acid, and glutamic acid. Data have been presented here, confirming the results of Marr and Wilson (1951), that B. abortus also fixes C\textsuperscript{14}O\textsubscript{2} into aspartic and glutamic acids. In addition, we have presented data here to show that C\textsuperscript{14}O\textsubscript{2} is incorporated into arginine in greater...
amounts than in aspartic or glutamic acids. It seems, therefore, that, with the exception of the large amount of C14O2 fixed into glycine, the fixation of CO2 into the amino acids of B. abortus strains is similar to that demonstrated in other biological systems.

The major difference between C14O2 fixation by B. abortus and other microorganisms appears to be the preponderance of the C14O2 which is incorporated into glycine. This reaction in B. abortus is strongly dependent on a high pC14O2. Marr (1952) has shown that at a pC14O2 of 0.005 atm much less C14O2 is incorporated into glycine in comparison with other amino acids. In the experiments presented in this paper exposures to 0.1 atm C14O2 were used. In experiments of this type over 90 per cent of the activity in the glycine molecule is recovered in the carboxyl carbon (Marr and Wilson, 1951). In comparable experiments, Clostridium cylindrosporum (Barker and Elsdon, 1947) and C. kluyveri (Tomlinson, 1954) have been found to fix relatively large amounts of C14O2 into glycine. The carboxyl group of the glycine isolated from these clostridia contained most, if not all, of the C14 incorporated into the molecule. It seems, therefore, that the fixation of large amounts of CO2 into glycine is not unique to B. abortus.

Two possible mechanisms by which C14O2 is incorporated into the glycine molecule have been proposed. The first is based on the observation of Marr and Wilson (1951), that resting cells of B. abortus incorporate C14O2 by an exchange of C14O2 with the carboxyl group of glycine. Marr (1952) suggested that this reaction might represent the initial step in the formation of an active one carbon unit from glycine. The second mechanism for the incorporation of C14O2 into glycine was proposed by Wiame and Bourgeois (1955). They suggest that the enzyme isocitrate, demonstrated in Pseudomonas aeruginosa, which splits isocitrate into succinate and glyoxalate (Campbell et al., 1953; Smith and Gunsalus, 1954) exists in B. abortus. C14O2 incorporated into tricarboxylic acid cycle intermediates could then be found in glycine via the splitting of isocitrate and the subsequent amination of the resultant glyoxalate. The complete tricarboxylic acid cycle is not required to make glycine by these reactions (Saz and Hillary, 1956). In the fixation of C14O2, the predominant labeling in isocitrate would be in the carboxyl groups. The splitting of this labeled isocitrate would give glyoxalate and subsequently glycine predominantly labeled in the carboxyl group. The specific labeling of the carboxyl group of glycine by B. abortus would then support this isocitratase hypothesis.

The incorporation of large amounts of CO2 into glycine by B. abortus does not appear to be a reflection of the CO2 requirement or the virulence of the organism. Glycine will not substitute for CO2 for the growth of CO2-dependent strains of B. abortus (Ruwet, 1951; Marr, 1952). No differences were found in the incorporation of CO2 or glycine in the nucleic acids of a CO2-dependent and a CO2-independent strain of B. abortus (Newton and Wilson, 1954). In the present study, data have been presented which show that glycine contains the major portion of the C14O2 fixed by five strains of B. abortus which differ in virulence for guinea pigs as well as in their requirement for an increased pCO2 for growth. The fixation of large amounts of CO2 into glycine appears to be a peculiarity of all B. abortus strains.

Evidence has also been presented to show that the total incorporation of C14O2 and the distribution of the C14 in chemical fractions of CO2-dependent and CO2-independent B. abortus strains are similar. These findings suggest that the assimilation of CO2 by these strains occurs via the same enzymatic pathway and that this pathway involves constitutive enzymes in both CO2-dependent and CO2-independent strains.

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SUMMARY

The assimilation of C14O2 by five strains of Brucella abortus differing in their requirement for an increased pCO2 in air for growth and also in their virulence was investigated. No differences could be found in the distribution of C14 fixed into the amino acids of a virulent CO2-dependent strain and an avirulent, CO2-independent strain of B. abortus. The total incorporation of C14O2 by the five physiologically different strains of B. abortus was found to be approximately the same. The percentage distribution of the C14 in chemical fractions of the B. abortus cells was similar for all
strains tested. In all strains the major portion of the C\textsuperscript{14} fixed into cellular protein was found in glycine. The data indicate that the ability of *B. abortus* cells to fix CO\textsubscript{2} cannot be correlated with either the virulence of the organism or its requirement for an increased pCO\textsubscript{2} for growth.

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