THE PREPARATION OF C$^{14}$-LABELED BIOTIN AND A STUDY OF ITS STABILITY DURING CARBON DIOXIDE FIXATION

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Several lines of evidence indicate that biotin is involved in the biological fixation of carbon dioxide. Lardy, Potter, and Elvehjem (1) have demonstrated that the biotin requirement of Lactobacillus arabinosus is partially replaceable by oxalacetate and that the growth stimulation of this organism by carbon dioxide is evident only when biotin is present. From microbiological studies with antimetabolites, Shive and Rogers (2) have concluded that biotin is effective in the formation of $\alpha$-keto-glutarate in Escherichia coli, and in the formation of oxalacetate in L. arabinosus. Lichstein and Umbreit (3) have suggested that biotin is a coenzyme in the oxalacetate decarboxylase enzyme system in E. coli. Ochoa and his coworkers (4) found a decreased activity of the carbon dioxide-fixing "malic" enzyme in livers from biotin-deficient turkeys, whereas livers from folic acid-deficient turkeys were normal in this respect. On the other hand, these workers were unable to identify biotin as a constituent of the purified enzyme. In biotin-deficient rats, Robertson and Lardy (5) have reported a decreased fixation of labeled carbon dioxide in aspartic acid and arginine.

While the above studies implicate biotin as an important factor in carbon dioxide fixation, the mechanism involved remains obscure. Based on the structure of biotin and its known chemical reactions, it was early postulated (6) that the vitamin might enter into biological carbon dioxide-transferring mechanisms by virtue of an opening and closing of the ureido ring system. The carbon dioxide thus incorporated into the biotin molecule would then presumably be transferred to pyruvic acid or other substrate concerned in the fixation of carbon dioxide.
Such a mechanism is amenable to investigation with radioactive carbon. Demonstration that the ureido carbon of biotin is biologically labile would offer strong evidence for the mode of action of biotin in carbon dioxide fixation. Conversely, demonstration of its stability would afford a convenient method of labeling biotin with isotopic carbon for further investigations of its metabolic role. Accordingly, we have synthesized biotin containing C\textsuperscript{14} in the ureido carbon atom, and have studied its fate during carbon dioxide fixation in *L. arabinosus*.

The synthesis of the C\textsuperscript{14}-labeled biotin was accomplished by utilization of the previously described reaction of phosgene with the diaminocarboxylic acid derived from biotin (7). Radioactive phosgene was prepared from C\textsuperscript{14}-containing carbon monoxide, which in turn was conveniently prepared by the dehydration of radioactive formic acid obtained by the reduction of bicarbonate (8). Because of the relatively low requirements of living cells for biotin, the radioactive biotin was synthesized with the highest possible specific activity of C\textsuperscript{14} in the ureido carbon atom.

In a preliminary experiment with the C\textsuperscript{14}-labeled biotin, aspartic acid was isolated from *L. arabinosus* cells which had been grown in an aspartic acid-free medium in the presence of the labeled vitamin, since much of the carbon dioxide fixed by this microorganism appears in the synthesized aspartic acid (9). No detectable radioactivity was present in the isolated aspartic acid or in the gas phase above the culture.

Because of the extremely high dilution of radioactivity inherent in this type of experiment if transfer of C\textsuperscript{14} did occur, a different approach was devised to eliminate the necessity of detecting radioactivity of a low order. It was also desirable to ascertain whether C\textsuperscript{14} might be transferred to substances other than aspartic acid.

As described in the experimental section of this paper, *L. arabinosus* cells were grown in an aspartic acid-free medium in the presence of a known amount of radiobiotin. The cells and medium were then separated and the amount of radiobiotin in each was determined by an isotope dilution technique. In the case of the medium, this was accomplished simply by the addition of a conveniently large amount of non-isotopic biotin to the medium and reisolation of the biotin and purification to constant specific radioactivity. In the case of the cells, non-isotopic biotin was added prior to acid hydrolysis and reisolated from the hydrolyzed cells.

Quantitative estimation of the radioactivity present in the isolated biotin samples was accomplished by comparison in the Geiger-Müller counter with a radiobiotin standard prepared by dilution of the highly active radiobiotin with non-isotopic biotin. This procedure eliminated the necessity of conversion of the samples to barium carbonate, with the resultant decrease in specific activity.
Results

The results obtained are given in Table I, in terms of total counts and also, by calculation from the radioactivities, in terms of biotin. For comparison, Table I also lists the biotin contents of the cells and medium obtained by microbiological assay with *L. arabinosus* and with *Saccharomyces cerevisiae*.

It can be seen that 93 per cent of the C\(^{14}\) originally present in the radiobiotin is recovered at the end of the growth period as radiobiotin. Furthermore, the *L. arabinosus* assay values for the biotin contents of the cells and medium are in good agreement with the biotin contents calculated from the radioactivity data. These results demonstrate that no appreciable exchange of the C\(^{14}\)-labeled ureido carbon atom of biotin with non-isotopic carbon has occurred. The conditions of the growth experiment are such that carbon dioxide fixation involving biotin must be actively occurring, since in the absence of aspartic acid in the medium *L. arabinosus* synthesizes the aspartic acid needed for its growth from oxalacetate, which in turn is formed by biotin-controlled carbon dioxide fixation (10, 1). That fixation was indeed occurring under the conditions of the present experiment was confirmed by the demonstration of the formation of C\(^{14}\)-labeled aspartic acid by the cells of a control culture grown under similar conditions, with non-labeled biotin and in the presence of radioactive carbon dioxide.

It seems logical to expect that during active growth an appreciable fraction of the cellular biotin would be participating in the enzymatic reactions concerned with carbon dioxide fixation and that consequently a readily detectable portion of the C\(^{14}\) of the radiobiotin should have been replaced with non-radioactive carbon if the mechanism under investigation were correct. The results obtained, therefore, are a strong indication that the ureido carbon atom of biotin is not directly involved in the

<table>
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<tr>
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<th>Radiobiotin analyses</th>
<th><em>L. arabinosus</em></th>
<th><em>S. cerevisiae</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>counts per min.</td>
<td>(\gamma) radiobiotin*</td>
<td>(\gamma) biotin</td>
</tr>
<tr>
<td>Cells</td>
<td>1955</td>
<td>1.36</td>
<td>1.32</td>
</tr>
<tr>
<td>Medium</td>
<td>7850</td>
<td>5.48</td>
<td>5.53</td>
</tr>
<tr>
<td>Total</td>
<td>9805</td>
<td>6.84</td>
<td>6.85</td>
</tr>
</tbody>
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* Calculated from comparison with the standard radiobiotin preparation containing 3.66 \(\gamma\) of radiobiotin and possessing 5250 counts per minute.
transfer of carbon dioxide during carbon dioxide fixation in *L. arabinosus*. Studies on biotin-deficient rats injected with radiobiotin suggest that essentially the same conclusion with regard to biotin and carbon dioxide fixation can be drawn for the mammal (unpublished observations).

The assays with *S. cerevisiae* demonstrate that a considerable amount of *L. arabinosus*-inactive, yeast-active material is produced by *L. arabinosus* during growth. The major portion (85 per cent) of this material is present in the medium at the end of the growth period. If it is derived exclusively or in large part from biotin, it must possess a biological activity markedly higher than that of biotin, in view of the high recovery of radiobiotin. That it is not readily converted to biotin was shown by the fact that *L. arabinosus* assay values were not increased after hydrolysis of the medium.

**EXPERIMENTAL**

*Synthesis of C$^{14}$-Labeled Biotin*—The radioactive barium carbonate used as the starting material was purchased from the Clinton Laboratories of the Monsanto Chemical Company, on allocation from the United States Atomic Energy Commission. Approximately 1 mc. (101.2 mg.) was converted to potassium formate by the procedure previously described (8). The formate solution was evaporated to dryness in a 20 X 200 mm. glass tube equipped with a ground glass joint. A glass cup containing 2 ml. of concentrated sulfuric acid was placed in the tube and the tube was closed by means of a head equipped with suitable inlet and outlet tubes which could be sealed by means of stop-cocks. After evacuation of the system with an oil pump, the acid and formate were mixed by tipping the apparatus, and the mixture was warmed to 70° for a few minutes to complete the liberation of carbon monoxide. A slight excess (12 ml.) of dry chlorine gas was drawn into the system from a gas burette, and the gas mixture was irradiated with a 100 watt internal reflector lamp for 30 minutes at a distance of 12 inches.

50 mg. of the diaminocarboxylic acid sulfate derived from *d*-biotin (11) and 1.5 ml. of 1.7 N NaOH solution were placed in a second, similar glass apparatus. The inlet tube of this system was attached to a tube containing a small amount of mercuric sulfide, for the removal of unchanged chlorine. The apparatus was flushed with nitrogen and was connected to the apparatus containing the phosgene. A stream of nitrogen was used to carry the phosgene through the tube of mercuric sulfide into the vessel containing the diamino acid, where it was frozen out by means of a liquid nitrogen bath. After 1.5 hours, the flow of nitrogen was stopped and the vessel containing the frozen phosgene and diamino acid was evacuated briefly by the use of an oil pump, and was sealed by means of
stop-cocks. After the contents had warmed to room temperature the apparatus was shaken for 1 hour. The solution was then acidified and the liberated carbon dioxide was collected in a scrubber containing alkali. The d-biotin crystals which separated from the acidified solution were collected and recrystallized from water. The yield was 10.4 mg., micro melting point 226–230°. This material was used in the growth experiments.

The mother liquors from the first crystallization were made alkaline and treated with non-isotopic phosgene in excess. From this reaction 25 mg. of biotin possessing a lower order of radioactivity were obtained.

For radioactivity analyses, samples of the biotin were oxidized to carbon dioxide by the wet combustion method of Van Slyke and Folch (12). Analyses were carried out on barium carbonate pads as previously described (8). From a total of $1.27 \times 10^8$ counts per minute in the barium carbonate used as starting material, $1.28 \times 10^7$ counts per minute were obtained in the 10.4 mg. of biotin, and $5.74 \times 10^6$ counts per minute in the 25 mg. sample of biotin obtained from the mother liquors. The carbon dioxide recovered from the last step of the synthesis contained $7.1 \times 10^7$ counts per minute. Therefore approximately 70 per cent of the original radioactivity was accounted for in these fractions. No attempt was made in this work to improve the yield in the last step of the synthesis.

Growth of L. arabinosus with Radiobiotin—A culture of L. arabinosus 17-5 which had been carried in dextrose-yeast-agar stab cultures (13) was grown for 20 hours at 37° in 10 ml. of basal medium (14) containing 0.0002 γ of biotin. The cells were separated by centrifugation, washed with 10 ml. of saline, and resuspended in 20 ml. of saline. Two 450 ml. portions of aspartic acid-free medium (15), each containing 3.66 γ of the radioactive biotin, were sterilized in 3 liter Fernbach flasks and then inoculated with 1 ml. portions of the saline suspension. The flasks were incubated for 28 hours at 37°.

At the end of the growth period, the cells were separated by centrifugation, washed three times with cold water, and dried from the frozen state. The weight of the dried cells was 327 mg., and the volume of the combined medium and washings was 960 ml. A control culture without added radiobiotin showed no growth.

Microbiological assays on the medium and cells were carried out by the L. arabinosus method of Snell and Wright (13) and the yeast method of Snell, Eakin, and Williams (16), with modifications in the media (14). For assay the cells were hydrolyzed with 2 N sulfuric acid for 2 hours. The results are given in Table I. Hydrolysis of the medium under the same conditions did not increase the biotin values over those obtained with unhydrolyzed medium.
Isolation of Radiobiotin from Medium—To 500 ml. of medium were added 150 mg. of non-isotopic d-biotin (Merck), which was dissolved by warming. The biotin was reisolated by adsorption on 5 gm. of charcoal and elution from the charcoal with two 50 ml. portions of a 1:6:3 concentrated ammonia-ethanol-water mixture. The eluate was evaporated to dryness and the residue was dissolved in a small volume of water. Acidification with HCl yielded crystals of biotin. For further purification the biotin was converted to the methyl ester by treatment with diazomethane, and the ester was sublimed in vacuo and then crystallized from a methanol-ether mixture. Saponification of the ester yielded 94 mg. of crystalline biotin.

Isolation of Radiobiotin from L. arabinosus—To 250 mg. of dried L. arabinosus cells were added 150 mg. of non-isotopic d-biotin and the mixture was autoclaved in a sealed vessel with 12 ml. of 2 N sulfuric acid for 2 hours. Incorporated in the system was a side arm containing NaOH solution to trap liberated carbon dioxide. The amount of radioactivity in the carbon dioxide liberated was markedly decreased by the addition of the non-isotopic biotin prior to hydrolysis, compared to hydrolysis without added biotin. The hydrolysate was adjusted to pH 5 with NaOH and the biotin was removed by adsorption on charcoal and was purified by the same procedure that was used in the case of the medium. The yield of crystalline biotin was 97 mg.

Radioactivity Analyses—Comparisons of the specific activities of the purified biotin samples from the cells and medium were made with a standard prepared by crystallizing 150 mg. of non-isotopic d-biotin in the presence of 3.66 g of radiobiotin. The samples were counted directly as pads of biotin crystals; these were prepared by dissolving 50 mg. aliquots in 5 ml. of water with a few drops of 4 N KOH, and then acidifying with HCl. The precipitated samples were collected on filter paper disks in the same apparatus used for the preparation of barium carbonate pads. That the samples contained no appreciable amounts of radioactive impurities was indicated by the identical specific activities shown by these pads and pads prepared from the corresponding purified methyl esters.

The biotin pads were counted by means of a mica window Geiger-Müller tube and associated scaling circuit. The standard biotin preparation (43.6 mg.), the biotin from the cells (45.5 mg.), and that from the medium (45.2 mg.) gave 307, 88, and 240 counts per minute above background, respectively. The values given in Table I are those obtained after correction to zero self-absorption. The carbon dioxide liberated during the hydrolysis of the L. arabinosus cells was counted as barium carbonate and was found to contain a negligible amount of radioactivity.
Appreciation is expressed to Miss Mary Lloyd and Mrs. Susan Wing for assistance in carrying out the microbiological assays reported in this paper.

SUMMARY

Biotin labeled with C\textsuperscript{14} in the ureido carbon atom has been synthesized from radioactive phosgene and the diamino acid derived from biotin.

The stability of the ureido ring system of biotin during the biotin-controlled fixation of carbon dioxide by \textit{Lactobacillus arabinosus} was studied. Growth of this organism in the presence of the labeled biotin, under conditions requiring the participation of biotin in carbon dioxide fixation, did not result in any detectable replacement of the C\textsuperscript{14} of the radiobiotin with non-radioactive carbon.

It is concluded that the mechanism whereby biotin promotes carbon dioxide fixation does not involve a transfer of the ureido carbonyl group of the biotin molecule.

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