ON THE SOURCES OF CARBON FOR THE INDUCED BIOSYNTHESIS OF ALPHA-AMYLASE IN PSEUDOMONAS SACCHAROPHILA

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Recent experiments by Rotman and Spiegelman (1954) and by Hogness et al. (1955) have indicated that virtually all of the carbon and sulfur for the induced biosynthesis of β-galactosidase comes from the medium rather than from any pre-existing compounds within the cell, using exponentially growing cells of Escherichia coli. These experiments also proved that the proteins of E. coli do not break down to their constituent amino acids at a rate even remotely approximating the rate of synthesis of β-galactosidase. Furthermore, the experiments of Hogness et al. demonstrated that this enzyme is stable whether or not the cells are synthesizing more β-galactosidase during growth. On the other hand, the experiments of Sher and Mallette (1954) indicate that lysine decarboxylase of E. coli is not stable in the absence of its inducer.

With regard to the protein stability of certain yeasts, Spiegelman et al. (1955) summarized the situation as follows: "Here, one can provide convincing evidence that there do exist components in the cell which can break down into their constituent amino acids." In addition, the studies of Halvorson and Spiegelman (1952, 1953a, 1953b) on maltase synthesis in resting cells of yeasts strongly support the contention that internal free amino acids contribute to the formation of this enzyme.

The finding in this laboratory (Markovitz and Klein, 1955) that Pseudomonas saccharophila can produce an extracellular α-amylase in resting cellular suspensions, as well as during growth, and the development of procedures for the purification of this enzyme (Markovitz, et al., 1955), presented the possibility of studying the problem of precursors for the induced biosynthesis of amylase in this organism. It seemed particularly desirable to obtain information pertaining to enzyme formation in resting cells for the following reasons. An organism that can produce an enzyme in the absence of an external supply of nitrogen must contain nitrogenous precursors within the cell. These might comprise high molecular weight proteins (perhaps specific precursors), or smaller molecules like amino acids and peptides, or both. The contribution of endogenous precursors to newly formed protein would be more easily detected in resting cells induced to form a single enzyme than in growing cells. This follows since a much larger share of internal precursors may be expected to go into the synthesis of the one protein when it is the only one being synthesized as compared to the situation in which the cell is synthesizing all the proteins necessary for growth. Under the latter conditions one might not be able to detect the incorporation of the precursors into any one particular protein.

MATERIALS AND METHODS

Information pertaining to the organism used, preparation of media, amylase purification, and

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4 In the case of the amylase of P. saccharophila, the likelihood of a direct conversion of specific protein precursor to active enzyme has been ruled out in previous experiments (Markovitz and Klein, 1955).

5 Upon induction of amylase synthesis by starch, P. saccharophila also produces increased levels of maltase and of an internal amylase (Thayer, 1953). In addition, it is not unlikely that several enzymes involved in the formation of these hydrolases are also increased in activity. Nevertheless, it is evident that the number of proteins synthesized under these conditions is quite small compared to that occurring in growing cells.
amylase assay has been presented (Markovitz and Klein, 1955; Markovitz, Klein and Fischer, 1955). In the present work all amylase assays were done using the reducing sugar method of Noetling and Bernfeld (1948). Amylase activity is expressed as mg of maltose produced per 3 min at 37 C at pH 5.5 with the substrate at a final concentration of 5 mg per ml.

Radioactive glucose, sucrose and starch were prepared by the methods of Putman et al. (1948). The sugars were separated and purified by paper chromatography according to Putman and Haasid (1952).

For the separation of amino acids and peptides, 2 solvent systems were employed. The first of these was acetic acid: n-butanol: water (1:4:5), which was also used for the chromatography of sugars. The second system, described by Redfield (1953), consisted of tert-butanol: methyl ethyl ketone: water: diethylamine (40:40:20:4). All chromatography was carried out using descending solvent assemblies.

For the preparation of extracts for the chromatography of free amino acids and peptides, cells were harvested from the growth medium, washed twice with distilled water, and resuspended to a concentration of about 50 mg dry wt per ml. This suspension was then boiled for 20 min as described by Gale (1947). After cooling, the suspension was centrifuged and the supernatant removed for analysis. Chromatograms of extracts prepared in this manner were identical with those obtained from extracts of cells disrupted by sonic disintegration for 20 min in a Raytheon 10-KC sonic oscillator. Gale (1947) also presented similar results regarding the free amino acids of Streptococcus faecalis. For routine purposes in the present work, amino acid pools were prepared by boiling the cells, rather than by sonic disintegration, because of the ease and simplicity of the former method.

In order to remove heat stable proteins and other interfering ions from boiled extracts of P. saccharophila, the solution to be chromatographed was first treated with "Dowex 50" resin according to the procedure described by Thompson (1952).

Chromatograms (figure 1) of these extracts revealed the presence of unidentified spots. One of these, a peptide, was heated at 110 C in an autoclave for 18 hr in 6 N HCl in sealed glass tubes. After hydrolysis the HCl was evaporated off on a boiling water bath by blowing air over the surface of the solution. Water was then added and the evaporation process repeated. The residue was finally dissolved in 5 N NH₄OH and subjected to chromatography.

All compounds to be assayed for radioactivity were converted to BaCO₃ and distributed evenly on filter paper as previously described (Markovitz et al., 1955). The counts were corrected for background and for self absorption using a curve that was determined for the counter being used.

RESULTS

Growing cell experiment. In order to determine whether pre-existing intracellular carbon compounds contribute to the formation of α-amylase in growing cells, P. saccharophila was grown in a medium containing uniformly labeled C¹⁴-glucose. During this time, a 10-fold increase
in cell mass occurred, which insured the assimilation of considerable glucose and resulted in the uniform labeling of the cells. The cells were then centrifuged, washed twice in a solution containing phosphate buffer (pH 6.6) plus the growth salts without ammonium chloride, and resuspended in the complete growth medium without a carbon source. A sample was then removed for the determination of the initial radioactivity of the cells. Enough maltose was added to give a concentration of 0.1 per cent, and the suspension was aerated at 30 C until the maltose was completely utilized and growth ceased. During this time optical density measurements indicated an increase by a factor of 2.0. At the end of the experiment the cells were removed by centrifugation and samples were taken for determination of their specific radioactivity. The \( \alpha \)-amylase in the supernatant at the end of the experiment was concentrated by starch column adsorption and elution. To this fraction was added a known amount of carrier \( \alpha \)-amylase (i.e., non-radioactive) at the same stage of purity. The enzyme was then further purified and eventually crystallized according to the methods of Markovitz et al. (1955). The specific enzymatic activity and the specific radioactivity of the crystalline amylase were then determined. The pertinent data, presented in table 1, show that the ratio of the specific radioactivity of the amylase to that of the cells at the beginning of the experiment multiplied by 100 is 5.4. The ratio of the specific radioactivity of the amylase to that of the cells at the end of the experiment multiplied by 100 is 15.6. The results, therefore, indicate that a small but significant fraction of the amylase carbon is supplied by the cell during growth. Since some enzyme is produced by \( P. \) saccharophila even in the absence of external inducers, it is possible that the radioactivity found in the enzyme is due solely to “constitutively” produced enzyme.

**Resting cell experiments.** Carbon\(^{14}\)-labeled cells with starch as an inducer. Uniformly labeled non-induced cells were obtained by growing the organisms in the synthetic medium with uniformly labeled carbon\(^{14}\)-sucrose. This resulted, as before, in a 10-fold increase in mass. A resting cell suspension was prepared and a sample was taken for the determination of the initial radioactivity of the cell carbon. Starch was then added to a final concentration of 0.2 per cent and the cells were aerated on a shaker at 30 C for a period of 4 hr. After this, the cells were separated from the supernatant by centrifugation, and a sample of the cells, after washing, was assayed for specific radioactivity of the cellular carbon. The amylase produced was adsorbed and eluted from a starch column, and the partially purified enzyme was then diluted with carrier enzyme at the same stage of purification. At the stage of crystallization the amylase was subjected to analyses by ultracentrifugation and electrophoresis (Markovitz et al., 1955), and found to be homogeneous with respect to molecular size and charge. The specific radioactivity and the specific enzymatic activity of the preparation were then determined. The results of this experiment, recorded in table 2, show that the amylase carbon is 65 per cent as radioactive as the initial cellular carbon and 84 per cent as radioactive as the final carbon. It must be concluded therefore that the major portion of \( \alpha \)-amylase in resting cells of \( P. \) saccharophila comes from carbon compounds con-

### TABLE 1

**Utilization of intracellular carbon for the synthesis of alpha-amylase in growing cellular suspensions**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Specific Radioactivity (cpm/mg C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells before induction</td>
<td>84,500</td>
</tr>
<tr>
<td></td>
<td>89,100</td>
</tr>
<tr>
<td>Cells after induction</td>
<td>30,000</td>
</tr>
<tr>
<td></td>
<td>30,300</td>
</tr>
<tr>
<td>Crystalline alpha-amyIase*</td>
<td>4,700</td>
</tr>
</tbody>
</table>

* The specific enzymatic activity of this preparation, which was crystalline, was 1310. The specific radioactivity of the preparation was corrected for carrier enzyme by multiplying the observed specific radioactivity by the following ratio:

\[
\frac{E_{\text{f}} + E}{E_{\text{f}}} = 21.6
\]

where \( E_{\text{f}} \) = Total activity of the enzyme obtained from the radioactive cells. \( E \) = Total activity of the carrier enzyme.

* About 5 per cent as much \( \alpha \)-amylase is produced by resting cell suspensions in the absence of any external carbon source as would be produced in the presence of maltose or starch.
The specific enzymatic activity of this preparation was 1600. The specific radioactivity of the preparation was corrected for carrier enzyme by multiplying the observed specific radioactivity by the following ratio:

$$\frac{E^+ \text{ plus } E} {E^+} = 29.6$$

See table 1 for definition of symbols.

tained within the cell before the enzyme is synthesized.

Carbon$^{14}$-labeled cells with maltose as an inducer. An experiment similar to the previous one was performed with the exception that maltose was used as an inducer of the α-amylase. The pertinent data, reported in table 3, show that the amylase carbon is 64 per cent as radioactive as the initial cellular carbon and 68 per cent as radioactive as the final carbon. The results obtained in this experiment check well with those of the previous and succeeding experiments. The difference in the ratio of the radioactivity of the enzyme to that of the cells after induction in this experiment and the preceding one is the result of greater assimilation of non-radioactive carbon in the latter.

This preparation has a specific enzymatic activity of 685, which represents only 41.5 per cent of the maximal specific enzymatic activity of the α-amylase observed in other experiments (Markovitz et al., 1955). Since no other criteria of purity were examined with this preparation, a valid criticism of this experiment is that the enzyme has not been shown to be pure. However, it was subjected to the usual purification procedures and was assayed at the stage of crystallization, at which stage all other preparations tested were shown to be pure. In this respect, it may be noted (Fischer and Stein, 1954) that the crystalline α-amylase of Bacillus subtilis shows great variation in specific enzymatic activity, according to the procedures utilized in the activity determinations and, more specifically, according to the manner in which the enzyme is handled prior to or during the recrystallization process.

Non-labeled cells with carbon$^{14}$-labeled starch as an inducer. An experiment identical with the first resting cell experiment was performed—except that the cells used were nonradioactive and the inducer, starch, was labeled with carbon$^{14}$. The radioactivity of the starch, α-amylase, and cells after the induction was determined. At the stage of crystallization the amylase was subjected to analyses in the ultracentrifuge and the electrophoresis apparatus and the enzyme proved to be homogeneous with respect to molecular size and charge. The results of this experiment, reported in table 4, show that the amylase carbon is only 38.8 per cent as radioactive as the starch carbon. Thus 61.2 per cent of the carbon for the induced biosynthesis of the amylase in the resting cellular suspension was obtained from endogenous carbon compounds. The results of this experiment are in agreement with the two preceding ones and demonstrate that the major source of carbon for the induced biosynthesis of α-amylase in resting...
CARBON SOURCES FOR AMYLASE BIOSYNTHESIS

TABLE 4
Utilization of inducer carbon for the synthesis of α-amylase in resting cellular suspensions

<table>
<thead>
<tr>
<th>Sample</th>
<th>Specific Radioactivity (cpm/mg C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starch</td>
<td>36,200</td>
</tr>
<tr>
<td></td>
<td>39,200</td>
</tr>
<tr>
<td>Alpha-amylase*</td>
<td>13,800</td>
</tr>
<tr>
<td></td>
<td>15,600</td>
</tr>
<tr>
<td>Cells after induction</td>
<td>8,800</td>
</tr>
<tr>
<td></td>
<td>7,400</td>
</tr>
</tbody>
</table>

* The specific enzymatic activity of this preparation was 1700. The specific radioactivity of the preparation was corrected for carrier enzyme by multiplying the observed specific radioactivity by the following ratio:

\[
\frac{E \, \text{† plus} \, E}{E \, \text{†}} = 35.2
\]

See table 1 for definition of symbols.

Cellular suspensions of P. saccharophila is present within the cell before enzyme synthesis is initiated.

Free amino acid and peptide pool. No evidence concerning the nature of the intracellular compounds used for enzyme synthesis was provided by the preceding experiments. These substances could be non-nitrogenous carbon compounds, amino acids, peptides, proteins, or any combination of these compounds. Although early reports (Taylor, 1947) indicated that free amino acids are lacking in the gram negative bacteria, more recent results have shown that at least E. coli contains them (Proom and Woiwod, 1949).

Preliminary experiments to test for the presence of free amino acids in P. saccharophila demonstrated that these are present either in boiled extracts or in extracts prepared by sonic disintegration of thoroughly washed cells. The results in such experiment are presented in figure 1. Chromatograms showed the presence of 16 ninhydrin-positive spots, 11 of which have been identified as amino acids and 1 of which was identified as a peptide. The latter, spot #4, was identifiable before development with ninhydrin by heating the chromatogram at 100 C for 5 min and subsequent examination with an ultraviolet lamp, since it absorbed in the ultraviolet range. This spot was cut out and eluted from 6 identical chromatograms, after which the eluate was hydrolyzed and chromatographed. A large amount of threonine, some glycine, and 2 other unidentified spots were obtained.

Total nitrogen determinations were performed on known weights of cells and on the free amino acid and peptide pool obtained from known quantities of cells. It was found that freshly harvested cells in the early stationary phase contain low molecular weight nitrogenous compounds equivalent to 2.5 per cent of the total cellular nitrogen. This is approximately 20 times as much nitrogen as would be needed for the elaboration of α-amylase in resting cells. These data by no means prove the participation of free amino acids and peptides in the synthesis of amylase, but they support this possibility. The work of Turba and Esser (1953) is also in accord with this view.

DISCUSSION

The experiment with growing cells indicates that a small but significant fraction of the α-amylase carbon is derived from carbon compounds present within the cell before synthesis is induced. In comparison, the results of Rotman and Spiegelman (1954) proved that less than 1 per cent of the carbon for the induced biosynthesis of β-galactosidase in E. coli comes from the carbon of the cell. Similar results were obtained by Hogness et al. (1955) using S35-labeled cells. In the latter experiments, the organisms were originally grown with a limiting quantity of S35 and were not harvested until an hour after growth had ceased due to the depletion of S35 from the medium. Hogness et al. (1955) point out that this specific starvation causes all the sulfur to be incorporated into the trichloroacetic acid (TCA)-insoluble fraction of the cell, while exponentially growing cells contain a TCA-soluble fraction—accounting for 25 per cent of the total sulfur. While most of this TCA-soluble sulfur is accounted for as glutathione (Hogness et al., 1955), it should be pointed out that this fraction may contain free amino acids. The specific starvation procedure would thus minimize any contribu-
tion of this TCA-soluble fraction toward the synthesis of the β-galactosidase. Similarly, in the experiments of Rotman and Spiegelman (1954) the inducer was added to the radioactive cells only after logarithmic growth in a non-labeled medium was assured. This interval appears to have been of the order of 10 min. If one postulates that enzymes are synthesized to some extent from endogenous free amino acids in their system, then the period allowed by these authors for the cells to reach exponential growth at the expense of a non-labeled carbon source might be sufficient to deplete the amino acid pool of its original radioactivity.

Resting cell experiments prove conclusively that the major portion of the carbon for the induced biosynthesis of α-amylase comes from pre-existing carbon compounds of the cell. Under the conditions of these experiments, approximately 65 per cent of the carbon for the induced biosynthesis of the enzyme comes from carbon compounds present in the cell prior to the induction, and approximately 35 per cent of the carbon comes from the substrate-inducer carbon.

**SUMMARY**

In a growing cell experiment, using isotopically labeled cells, it was shown that a small but significant portion of the α-amylase was synthesized from pre-existing carbon compounds of the cell. With C14-labeled cells it was shown that in resting cells about 65 per cent of the carbon for the induced biosynthesis of α-amylase comes from carbon compounds of the cell. Using C14-labeled starch it was shown that, in resting cells, approximately 39 per cent of the enzyme carbon comes from the substrate.

Free amino acids and a peptide were demonstrated in extracts of *Pseudomonas saccharophila*.

**REFERENCES**


