THE METABOLISM OF OXYBIOTIN IN YEAST*

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The demonstration of the marked biological activity of oxybiotin (1-4), the oxygen analogue of biotin, has focused attention upon the mode of action of this compound. It became of particular interest to determine whether oxybiotin possesses intrinsic activity or whether its biological potency is due to its conversion into biotin. The evidence in the literature is conflicting on this point. Hofmann and Winnick (5), employing a differential assay procedure based upon the selective destruction of biotin activity by dilute potassium permanganate, have demonstrated that Saccharomyces cerevisiae and Rhizobium trifolii utilize oxybiotin as such and do not convert it into biotin. On the other hand, Rubin et al. (6) have presented data from balance studies on S. cerevisiae, which indicates that O-heterobiotin1 was converted into biotin or some other compound which possessed more activity for S. cerevisiae than did oxybiotin.

The experiments described in this paper were designed to clarify this controversial point. The yeast balance studies of Rubin et al. (6) were repeated under conditions identical with those employed by these authors. In addition, three differential assay procedures for the determination of oxybiotin in the presence of biotin have been utilized to ascertain whether cells of Saccharomyces cerevisiae grown in the presence of oxybiotin contain biotin. These four independent experimental approaches led to the same conclusion; namely, that Saccharomyces cerevisiae does not convert oxybiotin into biotin.

Balance Studies

These experiments were conducted in an effort to repeat the observations of Rubin et al. (6). Saccharomyces cerevisiae was grown in the presence of dl-oxybiotin and the dl-oxybiotin content of the harvested cells and medium was determined by S. cerevisiae assay (8). Since for yeast dl-oxybiotin is only 25 per cent as active as d-biotin, any conversion of oxybiotin into biotin during growth would lead to an increase in total oxybiotin equivalents and an apparent recovery of added oxybiotin ex-

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1 O-Heterobiotin has been shown to be identical with oxybiotin (7).

2 Since in the present studies we are dealing with mixtures of biotin and oxybiotin,
ceeding 100 per cent. Such high recoveries were reported by Rubin et al. and served as a basis for their conclusion that oxybiotin was converted into biotin or a vitamer of similar activity.

Saccharomyces cerevisiae was grown in 250 ml. Erlenmeyer flasks containing 40 ml. of the Snell medium (9). Varying amounts, i.e. 10, 25, 50, or 100 millimicrograms, of dl-oxybiotin were added to each flask. In other experiments, 2 and 8 millimicrograms of d-biotin were employed. Distilled water was added to a final volume of 47 ml. After autoclaving for 10 minutes, the contents were cooled and seeded with 1 ml. of a suspension of Saccharomyces cerevisiae 139 prepared by suspending cells from a 24 hour culture grown on Difco wort agar in sufficient sterile distilled water to give a concentration of 0.55 mg. of dry yeast per ml. This amount of dry yeast contains 0.60 millimicrogram of d-biotin, which is equivalent to 2.4 millimicrograms of dl-oxybiotin in yeast growth-promoting activity. Since this amount of oxybiotin activity represents a considerable proportion of the total oxybiotin added in the 10 millimicrogram balance studies, experiments with this level of oxybiotin were also performed in which the inoculum consisted of 0.01 mg. of dry yeast. The biotin content of this quantity of yeast is negligible. All experiments were conducted in duplicate.

After incubation at 30° for 18 hours, the cells were harvested by centrifugation. The supernatant medium was autoclaved for 15 minutes and assayed without further treatment. The cells were autoclaved for 1 hour with hydrochloric acid. In order to determine the optimum extraction procedure, the effects of varying amounts and concentrations of acid were studied. For the 10 millimicrogram studies, 10 ml. of 2 N hydrochloric acid yielded maximum extraction values. In the remainder of the balance studies, the maximum yield of growth factor was obtained when 5 ml. of 6 N hydrochloric acid were employed. These conditions were utilized to obtain the results reported in the present paper. In our early experiments, the cells were hydrolyzed with sulfuric acid. The same results in the balance studies were obtained with both acids.

The acid hydrolysates were adjusted to pH 4.0 with sodium hydroxide, diluted to a concentration of approximately 0.2 millimicrogram of oxybiotin equivalents per ml., and filtered. Both the clear filtrates and the

it has been found desirable to express all activities in terms of oxybiotin equivalents. These equivalents are obtained by referring growth activity to an oxybiotin standard curve.

8 Obtained from the American Type Culture Collection.

4 The yeast concentration was determined in an Evelyn photocolorimeter from a calibration curve relating turbidity reading to dry weight of yeast. The dry matter content of these cells was 23 per cent by weight.
autoclaved medium were then assayed for their oxybiotin activity by the *Saccharomyces cerevisiae* method of Hertz (8). In those balance studies in which biotin was added, the biotin content was calculated from a biotin standard curve. Satisfactory recoveries of biotin and oxybiotin were obtained when these compounds were added to the harvested yeast cells before acid hydrolysis.

The results of the oxybiotin balance studies are given in Table I. The percentage recovery of added oxybiotin was less than 100 and demonstrates that oxybiotin is not converted into biotin or any other compound with more activity for *Saccharomyces cerevisiae*. In two balance experi-

### Table I

**Balance Studies with Saccharomyces cerevisiae**

<table>
<thead>
<tr>
<th>Oxybiotin added to medium</th>
<th>No. of experiments</th>
<th>Type of inoculum</th>
<th>Oxybiotin equivalents found in cells*</th>
<th>Recovery of added oxybiotin</th>
</tr>
</thead>
<tbody>
<tr>
<td>millimicrograms</td>
<td></td>
<td></td>
<td>millimicrograms</td>
<td>millimicrogram</td>
</tr>
<tr>
<td>10</td>
<td>7</td>
<td>Low†</td>
<td>8.7 (6.0–10.0)</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>13</td>
<td>High‡</td>
<td>8.4 (7.3–9.6)</td>
<td>1</td>
</tr>
<tr>
<td>25</td>
<td>4</td>
<td>&quot;</td>
<td>21.4 (18.5–23.3)</td>
<td>1</td>
</tr>
<tr>
<td>50</td>
<td>4</td>
<td>&quot;</td>
<td>43.3 (40–46)</td>
<td>1</td>
</tr>
<tr>
<td>100</td>
<td>4</td>
<td>&quot;</td>
<td>83.3 (80–86)</td>
<td>1</td>
</tr>
</tbody>
</table>

* Average values are given with ranges in parentheses. In the high inoculum experiments these are corrected values and are the oxybiotin equivalents observed minus the oxybiotin equivalents (2.4 millimicrograms) furnished by the biotin present in the inoculum.

† Flasks seeded with 0.01 mg. of dry yeast.

‡ Flasks seeded with 0.55 mg. of dry yeast.

It appeared desirable to confirm the results of the balance studies by demonstrating the absence of biotin in cells of *Saccharomyces cerevisiae*. The assays were conducted in 50 ml. Erlenmeyer flasks at 30°. It should be pointed out that the hydrolysates contain both d-biotin (from the inoculum) and dl-oxybiotin. Since with *Saccharomyces cerevisiae* the growth curves of these two compounds are different in shape (2), different activity ratios at various portions of the curves are observed. However, the shapes of the growth curves are identical up to 1 millimicrogram of oxybiotin equivalents per flask. Suitable assays can, therefore, be obtained if the amount of oxybiotin equivalents added per assay flask is kept below 1 millimicrogram.

*Differential Assay Procedures*

The assays were conducted in 50 ml. Erlenmeyer flasks at 30°.
grown on oxybiotin. Methods permitting the differentiation of biotin and oxybiotin were necessary for this purpose. Three such differential assay procedures have been devised in our laboratories and were applied to the acid hydrolysates of cells of *Saccharomyces cerevisiae* grown in the presence of varying amounts of oxybiotin. The methods employed in growing the cells and obtaining the acid hydrolysates have been described in the previous section. Each flask was seeded with 0.55 mg. of dry yeast. It was necessary to combine the harvested cells from several flasks and hydrolyze them with proportionally increased amounts of 2 N or 6 N hydrochloric acid in order to obtain enough material for analysis.

**Permanganate Method (β)**—This method is based upon the fact that biotin is converted by dilute potassium permanganate into biotin sulfone, which is inactive for *Saccharomyces cerevisiae* in the amounts present under our assay conditions. In contrast, this treatment causes only a slight destruction of oxybiotin. Therefore, any growth activity for *Saccharomyces cerevisiae* remaining after treatment of a mixture of biotin and oxybiotin with permanganate is due to the oxybiotin present.

The hydrochloric acid hydrolysates prepared from the harvested yeast cells were adjusted to pH 1.0 with sodium hydroxide, diluted to a concentration of approximately 2 millimicrograms of oxybiotin equivalents per ml., and filtered. 3 ml. of the filtrate were treated with 6 ml. of 0.01 N potassium permanganate and allowed to stand at room temperature for 5 minutes. After decolorization of the excess permanganate with 0.05 N sodium bisulfite, the solution was adjusted to pH 4.0 with sodium hydroxide, and diluted to 25 ml. with distilled water. The growth activity for *Saccharomyces cerevisiae* was then determined (8). The same procedure was applied to 3 ml. of a 0.1 N hydrochloric acid solution containing 10 millimicrograms of dl-oxybiotin. The growth curve obtained with this permanganate-treated oxybiotin solution was used as the reference standard. This treatment of oxybiotin with permanganate resulted in a 10 per cent loss in activity. When a solution of oxybiotin was added to the

7 More extensive experience with this differential assay (5) has demonstrated that the acid employed markedly influences the stability of oxybiotin towards potassium permanganate. Whereas oxybiotin is only slightly inactivated in 0.1 N hydrochloric acid solution, it is completely inactivated when 0.1 N sulfuric acid is employed. No explanation can be offered at the present time for this behavior of the oxybiotin molecule. It is interesting to note, however, that the stability of oxybiotin in the presence of a yeast hydrolysate is independent of the acid employed in the preparation of the hydrolysate. The same results were obtained when either hydrochloric acid or sulfuric acid was used to hydrolyze the yeast cells and the hydrolysate treated with potassium permanganate. A 90 per cent recovery of the oxybiotin added to a sulfuric acid yeast hydrolysate was obtained following permanganate treatment.
original yeast filtrate and the mixture treated with permanganate, as described above, a 90 per cent recovery of the added oxybiotin was obtained. Added biotin was completely destroyed under these conditions.

In order to determine the oxybiotin equivalency previous to permanganate inactivation, 3 ml. of the yeast filtrate were treated as shown, with the exception that the permanganate and bisulfite additions were omitted.

Table II

Effect of Permanganate and Raney's Nickel Catalyst upon Growth Activity of Hydrolysates Prepared from Saccharomyces cerevisiae Grown in Presence of Oxybiotin

The values are given in millimicrograms.

<table>
<thead>
<tr>
<th>Oxybiotin added to medium</th>
<th>Oxybiotin equivalents in cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before treatment*</td>
</tr>
<tr>
<td>10</td>
<td>8.0</td>
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<tr>
<td></td>
<td>9.1</td>
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<td></td>
<td>6.8</td>
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<td></td>
<td>7.3</td>
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<td>9.5</td>
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<td></td>
<td>7.5</td>
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<tr>
<td>50</td>
<td>42</td>
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<td>46</td>
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<tr>
<td></td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>82</td>
</tr>
</tbody>
</table>

* Oxybiotin equivalents observed minus the oxybiotin equivalents furnished by the biotin present in the inoculum. In separate experiments, it was demonstrated that the biotin in the inoculum was completely inactivated by both permanganate and Raney's nickel catalyst.

In this case the oxybiotin equivalency was determined by reference to a non-potassium permanganate-treated oxybiotin standard.

The results obtained are shown in Table II. It can be seen that the growth activity present in cells of Saccharomyces cerevisiae grown on oxybiotin is not significantly affected by permanganate treatment. The absence of biotin in these cells is, therefore, demonstrated.

Raney's Nickel Method—This differential assay procedure is described in the preceding paper (10) and is dependent upon the fact that treatment with Raney's nickel, while without effect upon oxybiotin, converts biotin
into desthiobiotin, which is inactive for *Lactobacillus arabinosus*. *dl*-Oxybiotin is 50 per cent as active as *d*-biotin for *Lactobacillus arabinosus*.

The growth activity of the acid hydrolysates of *Saccharomyces cerevisiae* was determined by *Lactobacillus arabinosus* assay both before and after treatment with Raney’s nickel and was expressed as oxybiotin equivalents. Since biotin is inactivated by Raney’s nickel, any activity remaining after such treatment must, perforce, be due to the oxybiotin present. Biotin added to the acid hydrolysates was completely inactivated by treatment with Raney’s nickel, while added oxybiotin was unaffected.

The results of these studies are likewise given in Table II. Since the growth activity of the extracts from cells of *Saccharomyces cerevisiae* grown in the presence of oxybiotin was not affected by Raney’s nickel catalyst, it can be concluded that these extracts do not contain any biotin. On the other hand, the growth activity of extracts from cells grown in the presence of biotin was completely inactivated by Raney’s nickel catalyst.

**Differential Growth Inhibition by γ-(3,4-Ureylenecyclohexyl)-butyric Acid**—We have previously reported (11) that the activity of *dl*-oxybiotin for *Lactobacillus arabinosus* can be completely inhibited by amounts of γ-(3,4-ureylenecyclohexyl)-butyric acid which have only a slight effect upon the activity of equivalent amounts of *d*-biotin. This selective inhibition has been utilized as a basis for the differential assay of biotin and oxybiotin. In the presence of γ-(3,4-ureylenecyclohexyl)-butyric acid the growth-promoting effect of mixtures of biotin and oxybiotin is due only to the biotin present. The decrease in activity observed when such mixtures are assayed in the presence of the inhibitor is therefore due to the oxybiotin content. Complete growth inhibition is indicative of the absence of biotin in a suspected mixture.

Acid hydrolysates of *Saccharomyces cerevisiae* cells grown in the presence of 10, 50, or 100 millimicrograms of oxybiotin were adjusted to pH 6.8 with sodium hydroxide, filtered, and diluted to a concentration of approximately 0.4 millimicrogram of oxybiotin equivalents per ml. (determined by *Lactobacillus arabinosus* assay with an oxybiotin standard curve). The activities of aliquots of these extracts containing from 0.5 to 2.0 millimicrograms of oxybiotin equivalents were then determined in the presence of 150 γ of γ-(3,4-ureylenecyclohexyl)-butyric acid. Complete inhibition of growth at all levels was noted in the extracts prepared from cells grown on 50 or 100 millimicrograms of oxybiotin. In these aliquots the oxybiotin equivalents furnished by the biotin in the inoculum were below detectable limits. The slight growth activity remaining in the extracts from the 10 millimicrogram studies could be attributed to the biotin furnished by the inoculum which represents 10 per cent of the total growth-promoting activity of the extracts for *Lactobacillus arabinosus*. 


The inability of these extracts to support the growth of *Lactobacillus arabinosus* in the presence of γ-(3,4-ureylene-cyclohexyl)-butyric acid again demonstrates the absence of biotin.

**DISCUSSION**

The balance experiments described in this paper demonstrate clearly that oxybiotin is not converted into biotin during the growth of *Saccharomyces cerevisiae* 139. These results are in direct contrast to those obtained by Rubin et al. in identical experiments. The data presented by these authors indicate complete conversion of oxybiotin into biotin at a low level of added oxybiotin. We can offer no explanation for this extreme variance in results. The failure of Rubin et al. to take into account the biotin furnished by the inoculum would lead to an apparent recovery of approximately 120 per cent in the 10 millimicrogram balance studies. This figure, however, falls far short of the 200 per cent recovery observed by these workers.

The analyses of yeast hydrolysates by three distinct differential assay methods have further demonstrated the absence of biotin in cells of *Saccharomyces cerevisiae* grown on oxybiotin. These results are in accord with those obtained in our balance experiments and confirm the work of Hofmann and Winnick (5). These data provide unequivocal proof for the absence of a measurable equilibrium state between biotin and oxybiotin in *Saccharomyces cerevisiae* 139. The biological activity of oxybiotin for this yeast is, therefore, an intrinsic property of the molecule.

**SUMMARY AND CONCLUSIONS**

1. In balance experiments with *Saccharomyces cerevisiae* 139 grown in the presence of varying amounts of *dl*-oxybiotin approximately 100 per cent of the added oxybiotin was recovered.

2. Three independent differential assay methods have been utilized to demonstrate the absence of biotin in cells of *Saccharomyces cerevisiae* grown on oxybiotin.

3. *Saccharomyces cerevisiae* 139 does not convert oxybiotin into biotin and the biological activity of oxybiotin, the oxygen analogue of biotin, is an inherent property of the molecule.

We are indebted to Dr. J. P. English of the American Cyanamid Company, Stamford, Connecticut, for a generous supply of γ-(3, 4-ureylene-cyclohexyl)-butyric acid. The technical assistance of Miss Jean DeWoody and Miss Margaretta Taylor is gratefully acknowledged.
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