On the Osmotic Behaviour of *Saccharomyces cerevisiae* as affected by Biotin Deficiency

BY A. H. ROSE

*Department of Bacteriology, King's College, University of Durham, Newcastle upon Tyne*

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**SUMMARY**

The effect of biotin deficiency on the osmotic behaviour of *Saccharomyces cerevisiae* was studied by following the changes in the extinction of dilute suspensions of the yeast in phosphate buffer containing different concentrations of NaCl. The extinction of suspensions of biotin-optimal yeast increased as the NaCl concentration was increased to 1.5 M, but with yeast grown in unsupplemented biotin-deficient medium the readings decreased with increasing NaCl concentration. Yeast grown in biotin-deficient media supplemented with aspartate, or with oleate + aspartate, showed the same type of osmotic behaviour as yeast grown in unsupplemented biotin-deficient medium. But with yeast grown in biotin-deficient medium supplemented with oleate alone, the extinction changes in buffer containing up to 0.7 M NaCl resembled those of suspensions of biotin-optimal yeast. These differences in osmotic behaviour were not due to breakage of the osmotic barrier by the osmotic pressure differences across the cytoplasmic membrane, but to an increased permeability of the membrane to Cl⁻. This was accompanied by an increased permeability to $H_2PO_4^-$ and to higher molecular weight solutes, including bovine plasma albumin. The increase in permeability of biotin-deficient yeast was not as great as that of biotin-optimal yeast in which the osmotic barrier had been broken with 5 % (v/v) n-butanol.

**INTRODUCTION**

The principal known metabolic role of biotin is in the transfer of carbon dioxide in certain carboxylation and transcarboxylation reactions (Lynen, Knappe, Lorch, Jütting & Ringelmann, 1959; Ochoa & Kaziro, 1961). Since the process of CO₂ transfer is of fundamental importance in the metabolism of living cells, any impairment in this process will bring about a major disturbance in cell metabolism. One effect of biotin deficiency in micro-organisms is an impairment in the metabolic processes leading to synthesis of nucleic acids and protein (Ahmad, Rose & Garg, 1961), adenosine triphosphate (ATP; Katsuki, 1959a, b) and nicotinamide adenine nucleotides (Rose, 1960a, b). These effects would appear to be due largely to the decreased synthesis of oxaloacetate and aspartate under conditions of biotin deficiency (Shive & Rogers, 1947; Stokes, Larsen & Gunness, 1947) since, with *Saccharomyces cerevisiae*, they can be annulled by growing the yeast in a biotin-deficient medium containing aspartic acid (Ahmad & Rose, 1962a). Biotin is also essential for the synthesis of fatty acids through its role in the carboxylation of acetyl-CoA (Wakil, 1961); this explains the biotin-sparing action of certain un-
saturated fatty acids for growth of micro-organisms (Williams & Fieger, 1946; Ahmad & Rose, 1962a).

Membranes in micro-organisms are composed predominantly of lipoprotein (Gilby, Few & McQuillen, 1958; Weibull & Bergström, 1958). Since biotin is essential for the synthesis of both major components of these membranes, it is conceivable that membrane structure may be altered in biotin-deficient organisms. The cytoplasmic membrane constitutes the osmotic barrier in micro-organisms, and it follows that any alteration in membrane structure might result in changes in osmotic behaviour. The present paper reports studies on the effect of biotin deficiency on the osmotic behaviour of a strain of Saccharomyces cerevisiae.

METHODS

Organism. The strain of Saccharomyces cerevisiae (Fleischmann) used was that described previously (Ahmad et al. 1961). It was maintained on slopes of malt wort agar: 10% (w/v) spray-dried malt extract (‘Muntona’, Munton and Fison Ltd., Stowmarket, Suffolk) + 2% (w/v) agar. Cultures were stored at 3°.

Experimental cultures. The glucose + salts + vitamins medium (pH 4-5) of Rose & Nickerson (1956) was used. Portions (100 ml.) of medium, containing either an optimal (8.0 × 10^{-10}M) or a suboptimal (0.4 × 10^{-10}M) concentration of D-biotin were dispensed into 350 ml. conical flasks. In some experiments, biotin-deficient medium was supplemented with L-aspartic acid (2.0 × 10^{-3}M) and/or oleic acid (100 µg./ml.). These biotin-sparing compounds were shown to be free from biotin by using the screening procedure described by Ahmad & Rose (1962a). Gas chromatographic analysis of the sample of oleic acid used, which was that used in previous studies (Ahmad & Rose, 1962a), showed it to contain 76% (w/v) oleic acid, the principal contaminant being the trans isomer of oleic acid, elaidic acid (22%, w/v). Oleic acid when used in this paper refers therefore to this mixture. Flasks containing medium were plugged and sterilized by autoclaving momentarily at 115°. An inoculum was prepared by suspending sufficient material from a slope culture of the yeast into 6 ml. m/15 KH₂PO₄ (pH 4-5) to give the equivalent of 0-32–0-38 mg. dry wt. yeast/ml. (Rose, 1960b). The organisms in this suspension were washed three times with successive portions of m/15 KH₂PO₄ and suspended in 6 ml. of this buffer. Two drops of the washed suspension were added to each flask. Cultures were incubated statically at 25°. Growth was measured turbidimetrically as described by Ahmad et al. (1961), extinction readings being related to dry weight of yeast by a calibration curve.

Yeast suspensions. After growth had been measured, the yeast was separated from culture fluid by centrifugation and the crops washed three times in 0.1M-acetate buffer (pH 4.5; Walpole, 1914) or, when the suspension was to be used for extinction studies, in 0.01 M-phosphate buffer (pH 6.8; Gomori, 1955). The extinction of dilute suspensions of the yeast was measured in 1 cm. glass cells in the Hilger ‘Spekker’ absorptiometer (Model H 760) with neutral green-grey filters and a water blank.

Thick suspension technique. Chloride-, phosphate-, and protein-permeable volumes of the yeast were determined by using the thick suspension technique described by Conway & Downey (1950) and Mitchell & Moyle (1956). Portions of
Osmotic behaviour of yeast

washed yeast sufficient to give a packed cell volume of about 1 ml. were centrifuged to constant volume at about 8000g in 10 ml. graduated tapered centrifuge tubes which had been calibrated; this took about 40 min. The packed cell volume in each centrifuge tube was noted and the supernatant fluid discarded. By using a fine glass rod the pad of packed organisms was quickly suspended in a standard solution of NaCl (0.1M), KH₂PO₄ (0.15M) or crystalline bovine plasma albumin (L. Light and Co. Ltd., Colnbrook, Herts; 0.15%, w/v) in acetate buffer, exactly equal to the volume of packed organisms. The suspensions were then recentrifuged to constant volume. The supernatant fluids were removed and, after being diluted ten times, analysed for dilution of the probing solute as described below. Each determination was carried out in triplicate and included a control tube in which the pad of organisms was suspended in an equal volume of acetate buffer. The results show the space penetrated by the solute expressed as % (v/v) of the total wet volume of the yeast pad.

Analytical methods. Chloride was estimated by titration against 0.01 N-AgNO₃ in a total volume of 1 ml. containing dichlorofluorescein as an indicator (Vogel, 1948). Phosphate was estimated by the method of Fiske & SubbaRow (1925) as described by Umbreit, Burris & Stauffer (1957), with KH₂PO₄ (A.R.) as a standard. Phosphate contents of supernatant fluids from suspensions of the yeast are expressed as µg. P/ml. Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951), with crystalline bovine plasma albumin as a standard. Ultraviolet (u.v.)-absorbing substances in supernatant fluids from yeast suspensions were estimated by measuring the extinction at 260 μm in 1 cm. cuvettes in the S.P. 500 Unicam quartz spectrophotometer (Unicam, Cambridge), with a blank of the appropriate extracting solution.

RESULTS

Effect of osmotic pressure on the extinction of dilute suspensions of biotin-optimal and biotin-deficient yeast

Orskov (1945) first reported that cells of Saccharomyces cerevisiae swell and shrink according to the osmotic pressure of the surrounding fluid. This change in volume can conveniently be observed by measuring the extinction of a dilute suspension of yeast in solutions of different osmotic pressure. Biotin-optimal yeast from 40 hr. cultures was washed twice with 0.01M-phosphate buffer (pH 6.8) and suspended at a concentration equivalent to about 0.2 mg. dry weight/ml. in phosphate buffer containing different concentrations of various solutes. After standing at room temperature (18–21°C) for 1 hr. the extinctions of these suspensions were measured. Preliminary experiments showed that the changes in extinction, expressed as a percentage of the original reading, were greatest in buffer containing NaCl up to 1.5M (Fig. 1). There was a slight decline in the extinction of the suspension as the NaCl concentration was raised to 0.25M, but higher concentrations up to 1.5M caused an increase in the extinction reading. There was no further change in extinction when the NaCl concentration was increased above 1.5M. The changes in extinction of suspensions of biotin-optimal yeast in different concentrations of NaCl were completely reversible as was shown by removing the yeast from the NaCl solution by centrifugation and resuspending in an equal volume of phosphate buffer.
Treatment with aqueous butanol is known to break the osmotic barrier in many micro-organisms (Mitchell & Moyle, 1956; Ahmad et al. 1961). When biotin-optimal yeast from 40 hr. cultures was suspended in a solution of 5% (v/v) n-butanol in acetate buffer (pH 4-5) to a concentration equivalent to about 0-2 mg. dry wt. yeast/ml. before being suspended in phosphate buffer containing different NaCl concentrations, the extinction reading of the suspension declined as the NaCl concentration was increased to 1·0 M; an increase in NaCl concentration to 3·0 M did not cause any further change in extinction reading (Fig. 1).

When yeast grown for 120 hr. in medium containing a suboptimal concentration of biotin was suspended in phosphate buffer containing different concentrations of NaCl, the extinction reading of the suspensions gradually declined as the NaCl concentration was raised to 3·0 M (Fig. 2). Butanol-treated biotin-deficient yeast behaved in an almost identical manner (Fig. 2).

Yeast grown for 120 hr. in biotin-deficient medium containing aspartic acid or aspartic acid + oleic acid when suspended in solutions containing different concentrations of NaCl gave results almost identical with those obtained with yeast grown in unsupplemented biotin-deficient medium. But suspensions of yeast grown in biotin-deficient medium + oleic acid alone showed a slight increase in extinction in the presence of 0·4–0·7 M NaCl, although higher concentrations caused a decrease in extinction (Fig. 3). The response of yeast grown in oleate-supplemented biotin-deficient medium after treatment with aqueous butanol was similar to that recorded for yeast grown in unsupplemented biotin-deficient medium (Fig. 2). The changes in extinction of the yeast suspensions containing NaCl occurred rapidly on mixing and could be detected after the suspensions had been standing at room temperature for only 5 min. No further change in extinction reading was observed when the suspensions were maintained at room temperature for up to 3 hr.

Effect of biotin deficiency on leakage of cell constituents from yeast suspended in solutions of NaCl

One possible explanation for the differences in behaviour between biotin-deficient yeast and biotin-optimal yeast when suspended in buffer containing NaCl...
was that the osmotic barrier of the deficient yeast was broken by the osmotic pressure differences set up across the cytoplasmic membrane. To test this hypothesis, yeast which had been washed twice in acetate buffer was suspended to a concentration equivalent to 5 mg. dry wt./ml. in acetate buffer containing 0.5, 1.0 or 3.0 M NaCl; control suspensions in NaCl-free acetate buffer were also prepared. The suspensions were allowed to stand at room temperature for 1 hr. and were then centrifuged to remove the yeast. The cell-free supernatant fluids were then analysed for the presence of u.v.-absorbing substances, inorganic phosphate and protein (Table 1).

Table 1. Effect of NaCl concentration on the leakage of ultraviolet-absorbing substances, protein and inorganic phosphate from biotin-optimal and biotin-deficient Saccharomyces cerevisiae

Yeast grown in media containing an optimal concentration of biotin (BO) or a sub-optimal concentration (BD) with or without L-aspartic acid (2×10^-3 M; BD + Asp), oleic acid (100 µg/ml; BD + OL) or a mixture of these biotin-sparing compounds (BD + Asp + OL) was washed twice with acetate buffer (pH 4.5) and suspended to a concentration of 5 mg. dry wt. equivalent/ml. in acetate buffer either unsupplemented or containing NaCl up to 3.0 M or 3% (v/v) n-butanol. After the suspensions had stood at room temperature for 1 hr., the yeast was removed by centrifugation and the supernatant fluids analysed for u.v.-absorbing substances, protein and inorganic phosphate.

<table>
<thead>
<tr>
<th>Contents of supernatant fluids</th>
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<tr>
<td>u.v.-absorbing substances (extinction in 1 cm. cuvette at 260 mµ)</td>
</tr>
<tr>
<td>Protein (µg./ml.)</td>
</tr>
<tr>
<td>Inorganic phosphate (µg. P/ml.)</td>
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</table>

<table>
<thead>
<tr>
<th>Medium for yeast growth</th>
<th>Age of culture (hr.)</th>
<th>None</th>
<th>0.5 M NaCl</th>
<th>1.0 M NaCl</th>
<th>3.0 M NaCl</th>
<th>5% butanol</th>
</tr>
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<tbody>
<tr>
<td>BO</td>
<td>40</td>
<td>0.05</td>
<td>0.07</td>
<td>0.06</td>
<td>0.09</td>
<td>2.48</td>
</tr>
<tr>
<td>BD</td>
<td>120</td>
<td>0.17</td>
<td>0.23</td>
<td>0.25</td>
<td>0.16</td>
<td>7.30</td>
</tr>
<tr>
<td>BD + OL</td>
<td>120</td>
<td>0.16</td>
<td>0.21</td>
<td>0.25</td>
<td>0.21</td>
<td>6.60</td>
</tr>
<tr>
<td>BD + Asp</td>
<td>120</td>
<td>0.08</td>
<td>0.21</td>
<td>0.27</td>
<td>0.02</td>
<td>1.54</td>
</tr>
<tr>
<td>BD + OL + Asp</td>
<td>72</td>
<td>0.09</td>
<td>0.18</td>
<td>0.28</td>
<td>0.17</td>
<td>1.91</td>
</tr>
<tr>
<td>BO</td>
<td>40</td>
<td>0.0</td>
<td>15.7</td>
<td>9.3</td>
<td>9.3</td>
<td>164</td>
</tr>
<tr>
<td>BD</td>
<td>120</td>
<td>0.0</td>
<td>23.6</td>
<td>15.7</td>
<td>11.1</td>
<td>254</td>
</tr>
<tr>
<td>BD + OL</td>
<td>120</td>
<td>0.0</td>
<td>2.1</td>
<td>2.1</td>
<td>0.0</td>
<td>286</td>
</tr>
<tr>
<td>BD + Asp</td>
<td>120</td>
<td>0.0</td>
<td>17.8</td>
<td>16.8</td>
<td>14.8</td>
<td>97</td>
</tr>
<tr>
<td>BD + OL + Asp</td>
<td>72</td>
<td>0.0</td>
<td>10.0</td>
<td>18.7</td>
<td>6.4</td>
<td>154</td>
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</table>

There was a detectable leakage of u.v.-absorbing substances from each type of yeast and this increased slightly in buffer containing NaCl. However, the amounts of these substances in the suspending fluid were small when compared with the amounts liberated in acetate buffer containing 5% (v/v) n-butanol, a treatment which is known to break the osmotic barrier of the yeast cell (Admad et al. 1961). The relatively large amounts of u.v.-absorbing substances released from biotin-
deficient yeast grown in the absence of aspartic acid following treatment with aqueous butanol probably include breakdown products of RNA (Rose, 1962). The presence of NaCl in the buffer caused a small leakage of protein although this was not detectable in NaCl-free buffer. Except with biotin-optimal yeast and yeast grown in biotin-deficient medium + aspartic acid + oleic acid, there was no detectable leakage of inorganic phosphate. These results suggested that the slight leakage of cell constituents in buffer containing NaCl up to 3·0 M was not the result of a breakage of the osmotic barrier of the yeast. This was further supported by the observation that the amounts of u.v.-absorbing substances and protein appearing in the extracellular fluid were not in all instances proportional to the concentration of NaCl.

**Effect of biotin deficiency on the permeability of yeast to chloride, phosphate and protein**

An alternative explanation of the differences in osmotic behaviour between biotin-optimal and biotin-deficient yeast was that the deficient yeast was abnormally permeable to Cl⁻ so that no appreciable osmotic pressure difference was created across the cytoplasmic membrane. This hypothesis was tested by determining the extent to which Cl⁻ was able to penetrate yeast grown under various conditions of biotin deficiency, using the thick suspension technique. The results of these experiments are given in Table 2. The value for the chloride-permeable volume obtained

<table>
<thead>
<tr>
<th>Yeast</th>
<th>BO treated with</th>
<th>BD + OL 5%</th>
<th>Probing solute</th>
<th>Conc.</th>
<th>volume of yeast pad</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>BO 10⁻⁵ M</td>
<td>31·4</td>
<td>KD</td>
<td>44·5</td>
<td>48·2</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>BO 15⁻⁵ M</td>
<td>32·0</td>
<td>KD + OL</td>
<td>33·4</td>
<td>48·1</td>
</tr>
<tr>
<td>Bovine plasma albumin</td>
<td>(w/v)</td>
<td>35·9</td>
<td>KD + Asp</td>
<td>42·8</td>
<td>71·4</td>
</tr>
</tbody>
</table>

for biotin-optimal yeast agrees with that reported by Conway & Downey (1950) and is accounted for by the intercellular space between close-packed spheres (about 26 %) and an outer region of the yeast cell which is equivalent to the space occupied
by the cell wall (Conway & Downey, 1950). The chloride ion is incapable of penetrating the cytoplasmic membrane in biotin-optimal yeast. It is, however, able to penetrate the membrane in yeast grown in unsupplemented biotin-deficient medium (Table 2), although the chloride-penetrable volume of this yeast is still well below that of biotin-optimal yeast made completely permeable to Cl\(^-\) by treatment with aqueous butanol. Yeast grown in biotin-deficient medium supplemented with aspartate or aspartate + oleate was slightly more permeable to Cl\(^-\) than yeast grown in unsupplemented biotin-deficient medium. But the value obtained for yeast grown in biotin-deficient medium supplemented with only oleic acid shows that, although this was slightly greater than the value for biotin-optimal yeast, it was well below that for other biotin-deficient yeasts.

The data reported by Ahmad & Rose (1962a) showed that one possible reason for the inability of biotin-deficient yeast to grow as well as biotin-optimal yeast in media containing the biotin-sparing compounds aspartic acid + oleic acid was the apparent inability of this yeast to accumulate phosphate from the medium. In view of the marked differences in permeability to Cl\(^-\) as between biotin-optimal and biotin-deficient yeast, it was of interest to examine the effect of biotin deficiency on the phosphate permeability of the yeast. The data in Table 2 show that the changes in permeability to \(\text{H}_2\text{PO}_4^-\) brought about by biotin deficiency were closely similar to the changes in permeability to Cl\(^-\).

The increased permeability shown by biotin-deficient yeast extended to higher molecular weight solutes. The data in Table 2 give the results obtained with crystalline bovine plasma albumin, which had the highest molecular weight of the solutes tested (minimum mol. wt. 69,000; Scatchard, Batchelder & Brown, 1946), and show that biotin deficiency had qualitatively the same effect on the permeability of the yeast to this solute as to Cl\(^-\) and \(\text{H}_2\text{PO}_4^-\). Although the small increase in temperature during centrifugation of the thick suspensions did not affect the chloride- and phosphate-permeable volumes of the yeast, it was necessary to use refrigeration (0\(^\circ\)) during centrifugation of thick suspensions of the yeast in bovine plasma albumin; otherwise the values obtained for the protein-permeable volumes were extremely high and sometimes exceeded 100\%. The protein is presumably adsorbed on to the yeast cell, the adsorption being greater at higher temperatures. Surface adsorption could explain the somewhat higher values reported for the protein-permeable volumes of the yeast as compared with the corresponding chloride- and phosphate-permeable volume (Table 2).

**DISCUSSION**

The inability of yeast grown in unsupplemented biotin-deficient medium to show the same type of response as biotin-optimal yeast to changes in the NaCl concentration of the suspending fluid is probably attributable largely, if not entirely, to the increased permeability of the deficient yeast to Cl\(^-\), with the result that no appreciable osmotic pressure difference is set up across the cytoplasmic membrane. This increased permeability of biotin-deficient yeast extends to other low molecular weight solutes (e.g. \(\text{H}_2\text{PO}_4^-\)) and also to solutes of molecular weight of the order of that of bovine plasma albumin (about 69,000). But biotin-deficient yeast is not completely permeable to these solutes, possibly because the membranes surrounding
certain organelles in the yeast are not as freely permeable as the cytoplasmic membrane. There are a few reports associating biotin deficiency with altered permeability. Traub & Lichstein (1956) suggested that the biotin-sparing action of oleic acid is due to its ability to allow the more efficient entry of biotin into Lactobacillus arabinosus. More recently, Shiio, Otsuka & Takahashi (1962) claimed that the copious excretion of glutamic acid by biotin-deficient Brevibacterium flavum is due largely to the increased permeability of the cytoplasmic membrane in the biotin-deficient bacterium. Neither of these reports, however, comments on the extent of the permeability changes caused by biotin deficiency or on the nature of the metabolic lesions leading to these changes.

With the data available, it is only possible to speculate about the reason for the increased permeability of yeast grown in unsupplemented biotin-deficient medium. A possible clue is provided by the close similarity in response to changes in the NaCl concentration of the suspending fluid shown by yeast grown in unsupplemented biotin-deficient medium and butanol-treated biotin-optimal yeast. Aqueous butanol is thought to break the osmotic barrier of cells by extracting certain lipid constituents of the cytoplasmic membrane (Hunter, 1961). The cytoplasmic membrane in biotin-deficient yeast may therefore be abnormally permeable because of a deficiency of certain lipid constituents; biotin is known to be concerned in the synthesis of fatty acids (Wakil, 1961). This hypothesis is supported by the finding that the osmotic behaviour of yeast grown in biotin-deficient medium + oleic acid closely approximated to that shown by biotin-optimal yeast. Also the chloride- and phosphate-permeable volumes of yeast grown in biotin-deficient medium + oleic acid approached the values obtained with biotin-optimal yeast. Thus it would seem that the biotin-sparing action of oleic acid and other fatty acids (Williams & Fieger, 1946; Ahmad & Rose, 1962a) is based at least in part on the ability of these acids to become incorporated into the lipids of the cytoplasmic membrane.

The diminished synthesis of nucleic acids and protein caused by growing the yeast in unsupplemented biotin-deficient medium is restored when the yeast is grown in a biotin-deficient medium + aspartate (Ahmad & Rose, 1962a). But, although protein is a major constituent of cytoplasmic membranes, this restoration of protein synthesis does not restore the permeability properties of the yeast. Instead, it renders the yeast slightly more permeable to small and large molecular weight solutes. Ahmad & Rose (1962a) reported that growth of the yeast in a biotin-deficient medium + aspartate + oleate was greater than in biotin-deficient medium supplemented with either of these biotin-sparing compounds alone. Moreover, there was, in this medium, a partial restoration of the lag and exponential phases of growth which characterize growth of the yeast in biotin-optimal medium. But yeast grown in this medium is still abnormally permeable to low and high molecular weight solutes, the ability of oleate to restore the permeability properties of the organism apparently being nullified when aspartate is included in the medium. This may be because one or more of the contaminating fatty acids in the sample of oleic acid used in this study is essential for the restoration of membrane structure and, following the increased growth in this medium, the concentration of this fatty acid in the medium may have become limiting. It is interesting to note too that the activities of certain enzymes in yeast grown in biotin-deficient medium supplemented with aspartate + oleate resemble those of yeast grown in biotin-
deficient medium supplemented with asparate alone, rather than those in yeast
grown in biotin-deficient medium containing only oleate (Ahmad & Rose, 1961b).

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