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A Magnesium Yeast and its Properties

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This is one of three papers describing some of the chief properties of baker's yeast in which almost all the K^+ ion of the normal yeast cells has been replaced by the NH_4^+ ion (Conway & Breen, 1945), the Na^+ ion (Conway & Moore, 1954), or, as here, by the Mg^{2+} ion. The ammonium yeast was produced by fermentation in the presence of ammonium chloride and the sodium yeast by a series of fermentations in sodium citrate. In these ammonium and sodium yeasts 98% of the normal potassium content was replaced by NH_4^+ or Na^+ ions. The yeast cells were undamaged by these procedures, being readily restored to the functional condition of normal potassium yeast by fermentation for an hour or so in the presence of potassium chloride solution.

In the process of forming the magnesium yeast, by fermentation in magnesium acetate, large amounts of magnesium were accumulated through the activity of the cation-carrier (or physiological potassium-carrier) in the yeast-cell wall (Conway & Beary, 1958). Almost no concomitant loss of potassium occurred from the yeast cells as it did during the formation of sodium yeast, so that cells very rich in magnesium but with an almost normal potassium content resulted. It appeared, therefore, that this procedure would not produce magnesium yeast similar to the ammonium yeast and the sodium yeast. The difficulty in producing such a magnesium yeast was overcome by passing first from a normal potassium yeast to a sodium yeast and then fermenting this repeatedly in the presence of magnesium acetate. In this way the high sodium content of the sodium yeast was replaced by magnesium.

Some of the principal properties of this magnesium yeast have been studied, as well as those of the magnesium-rich yeast with normal potassium content, obtained by repeated fermentations in the presence of magnesium acetate.

METHODS

Preparation of sodium yeast. This was carried out by the method of Conway & Moore (1954) from baker's yeast (supplied by the Irish Yeast Co.) which was used throughout after being washed twice.

Preparation of magnesium yeast with minimal potassium content. The sodium yeast was suspended (1:20, w/v) in a solution containing (final concentrations) 0.2M-magnesium acetate, 5% (w/v) glucose and 25 mM-tris-HCl buffer, pH 7.2, at room temperature (18°). The suspending fluid was changed after centrifuging, every 2 hr. throughout the day. This procedure was repeated the next day. The magnesium, potassium and sodium contents of this yeast were determined at regular intervals, and the procedure was continued until no further appreciable change occurred.

Preparation of a magnesium-rich yeast with normal potassium content. This was prepared as described by Conway & Beary (1958), at room temperature, and the samples were bubbled with oxygen during the preparation. The pH of the suspension mixture was maintained at 7.2 by the addition of tris buffer (final concentration 25 mM), the pH being adjusted with 0.1 N-HCl.

Sodium and potassium determinations. Both were determined with the Beckman flame photometer. A sample of twice-washed yeast was boiled in 10–20 vol. of water, centrifuged, and the supernatant diluted as required to obtain a convenient reading on the photometer. The standards used for comparison were in the range 1.25–5.0 mg./l. for both sodium and potassium.

Magnesium determinations. These were carried out by the titan-yellow method, all samples being first wet-ashed (Young & Gill, 1951).

Growth experiments. The composition of the growth medium for the magnesium yeast was: glucose (2.6 g.), $(\text{NH}_4)_2\text{HPO}_4$ (0.38 g.), $(\text{NH}_4)_2\text{SO}_4$ (0.4 g.), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.1 g.), thiamine (1 mg.), *p*-aminobenzoic acid (1 mg.), riboflavin (2 mg.), nicotinic acid (2 mg.), calcium pantothenate (2 mg.), pyridoxine hydrochloride (4 mg.), inositol (0.5 mg.) and biotin, (4 μg .) dissolved in water (final vol. 100 ml.). Where the growth medium was intended to contain K^+ ions, the equivalent amount of $\text{KH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (0.57 g.) was substituted for the $(\text{NH}_4)_2\text{HPO}_4$ (0.38 g.).

In each growth experiment 0.1 ml. of a 1:100 (w/v) suspension of the yeast was added to 100 ml. of sterile growth medium in a 500 ml. conical flask closed with sterile wool and incubated at 27° with constant shaking. At suitable intervals small samples were removed and the turbidity was measured in an EEL spectrophotometer. Comparisons were made with freshly washed yeast by an exactly similar procedure and the levels of growth expressed simply as extinctions on the spectrophotometer. Such extinctions, read directly from the instrument, are proportional to the numbers of yeast cells in a given volume of the fluid examined. An extinction of 6.0 read directly from the instrument by using a 2 cm. stratum depth and wavelength 420 $\text{m}\mu$ corresponds to approx. 16×10^4 normal-shaped yeast cells/ml. Aseptic procedures were used throughout, and both penicillin and streptomycin were incorporated in the growth medium to prevent bacterial growth: 10 000 i.u. of streptomycin (as the sulphate, supplied by the Glaxo Laboratories Ltd.) and 10 000 i.u. of penicillin (Glaxo Laboratories Ltd.) were added for every 100 ml. of medium.

Fermentation experiments. The microdiffusion procedure described by Conway (1957) was followed: 1 ml. of 1:100 (w/v) suspension of yeast in water plus 1 ml. of 5% (w/v) glucose were placed in the outer chamber of a Conway unit (no. 1 type), and the CO_2 evolved was absorbed in 1 ml. of 0.1N-NaOH, containing a mixed phenolphthalein and thymolphthalein indicator (Conway, 1957), placed in the central chamber. After about 30 min. the absorption of CO_2 in the central chamber becomes linear with time, and the slope of the line gives a measure of the fermentation rate. At various times after 30 min., 0.5 ml. of saturated BaCl_2 was added and back-titrated with 0.05N-HCl. Blanks in which the glucose was omitted were incubated for the same time. All units were set up in duplicate or in triplicate.

Oxygen consumption. This was measured by the Warburg manometric method at 30°, 3 ml. of a 1:100 (w/v) suspension of yeast being used; the central chamber contained 10N-NaOH. The suspending medium contained tris buffer (25 mM), the pH of which was adjusted to 7.2 with N-HCl, and the final glucose concentration was 2.5% (w/v).

RESULTS

Properties of magnesium yeast

Table 1 summarizes the results obtained in seven sets of experiments, in which sodium yeast was first produced in the manner described by Conway & Moore (1954) and then magnesium yeast formed

from it by repeated fermentations in solutions containing glucose and magnesium acetate.

A yeast containing as little as 1.4 m-equiv. of K^+ ion/kg. could be obtained by this process. The most representative potassium figure in the seven experiments was 3.0 m-equiv. of K^+ ion/kg. associated with 2.6 m-equiv. of Na^+ ion/kg. and 292 m-equiv. of Mg^{2+} ion/kg. The progressive displacement of sodium in a sodium yeast is shown in Table 2. The gain in magnesium is very much greater than the loss of sodium. Thus after five fermentations of 2 hr. duration, about 151 m-equiv. of Na^+ ion/kg. has been lost, but 378 m-equiv. of Mg^{2+} ion/kg. has been accumulated.

Fermentation of magnesium yeast. The fermentation produced by the magnesium yeast was compared with that of fresh yeast and of magnesium yeast in the presence of K^+ ions. The procedure was conducted as described in the Methods section and the results are summarized in Fig. 1. Though the magnesium yeast can ferment quite well, the fermenting power has been reduced to about 20% of that of the fresh yeast. Also, the addition of 0.1 ml. of M-potassium chloride has a very marked stimulatory effect on the fermentation.

Table 1. *Sodium, potassium and magnesium contents of magnesium yeast prepared from sodium yeast*

The preparation of the magnesium yeast is described in the Methods section.

Na^+ ion (m-equiv./kg.)	K^+ ion (m-equiv./kg.)	Mg^{2+} ion (m-equiv./kg.)
3.2	2.5	78.3
14.8	9.2	412.0
3.0	1.4	189.6
2.5	2.5	200.0
2.6	3.0	292.0
3.4	5.8	236.0
3.5	5.0	216.0

Table 2. *Time-course of the replacement of sodium in a sodium yeast by magnesium over five 2-hr. fermentations*

The suspending fluid contained (final concentrations) 0.2M-magnesium acetate, 5% (w/v) glucose and 25 mM-tris-HCl buffer at pH 7.2.

Time (hr.)	Na^+ ion content of yeast (m-equiv./kg.)	K^+ ion content of yeast (m-equiv./kg.)	Mg^{2+} ion content of yeast (m-equiv./kg.)
0	166.0	9.2	33.6
2	134.0	9.1	136.8
4	104.0	9.0	203.4
8	26.0	9.0	332.0
10	14.8	9.0	412.0

Fig. 1. Fermentation rates of magnesium yeast ($\times$), fresh yeast (Δ) measured by the microdiffusion method for CO_2 evolution and of magnesium yeast in the presence of K^+ ions ($\circ$), 0.1 ml. of M-KCl being added to the outer chambers of the microdiffusion units.

Growth of magnesium yeast. The growth of magnesium yeast, of fresh yeast and of magnesium yeast in the presence of K^+ ions is shown in Fig. 2. For about 3 days almost no growth of the magnesium yeast occurred; after this a slow growth occurred which ceased after about 8 days. The level then reached, from comparison of the amounts of growth by the optical method described in the Methods section, would amount to only 35% of that of the fresh yeast. Owing, however, to the difference in shape of the cells compared with fresh yeast, the growth curve of the magnesium yeast in Fig. 2 (no K^+ ions being added to the external fluid) is not strictly comparable with the other two curves. The effect of the substitution of potassium dihydrogen phosphate for ammonium dihydrogen phosphate in the growth medium for the magnesium yeast was considerable and the final level of growth was the same as that of the fresh yeast.

The type of cells appearing during the slow growth of the magnesium yeast was quite abnormal. The cells were very elongated and often

associated in chains. Fig. 3(a) shows a striking example of this chain formation and may be contrasted with Fig. 3(b), where potassium dihydrogen phosphate has been added to the growth medium of the magnesium yeast. Fig. 3(c) shows the appearance of growing fresh yeast cells.

Oxygen uptake of magnesium yeast. This was only 28% of that of fresh washed yeast after 60 min. (Fig. 4).

Properties of a magnesium-rich yeast having a normal potassium content

A high concentration of Mg^{2+} ions can be accumulated by allowing fermentation in 5% glucose in the presence of 0.2 M-magnesium acetate buffered by tris buffer to pH 7.0. After fermentation for 36 hr. about 200 m-equiv. of Mg^{2+} ion/kg. can be accumulated (Conway & Beary, 1958).

Fermentation of magnesium-rich yeast with normal potassium. The very high content of magnesium in this type of yeast (about 200 m-equiv. of Mg^{2+} ion/kg.) had but a small inhibitory effect on fermentation when compared with a potassium yeast similarly prepared. For this preparation potassium acetate was substituted in equimolecular amount for magnesium acetate in the external fluid. The rate of fermentation has been decreased by only 11% by the high magnesium content (Fig. 5).

Growth experiments. The high magnesium content of the magnesium-rich yeast has no appreciable influence on the growth rate. The limit of growth is attained by both the magnesium-rich and the fresh yeast at the same time.

Oxygen uptake. In Table 3 the average oxygen uptake of the magnesium-rich yeast is 89% of that of a potassium yeast similarly treated and 91% of the oxygen uptake of a fresh yeast. Thus it appears that the very high magnesium content of yeast with a normal potassium content has little inhibitory effect on fermentation or oxygen uptake and there is no apparent effect on the growth rate.

DISCUSSION

The magnesium yeast containing minimal amounts of K^+ ions shows a markedly low growth rate, small oxygen uptake and low fermenting power, whereas in the magnesium-rich yeast having a normal potassium content these properties are almost the same as in fresh yeast. The deficiencies in magnesium yeast arise, therefore, from the absence of K^+ ions and not from the presence of a high content of magnesium. This is also shown by the fact that when the magnesium yeast is allowed to ferment with potassium chloride in the external fluid it returns to normal, although

Fig. 2. Growth rates of magnesium yeast (x), of fresh yeast (Δ), and of magnesium yeast in the presence of K⁺ ions (O), expressed in extinctions as described in the Methods section.

Fig. 3. (a), Cells grown from a magnesium-yeast sample, as described in the Methods section; (b), cells grown from a magnesium-yeast sample with K⁺ ions introduced into the growth medium; (c), cells grown from a sample of fresh yeast (magnification about × 1100).

Fig. 4. Uptake of oxygen by magnesium yeast (Δ) and fresh yeast ($\circ$) measured in Warburg manometers at 30° .

Fig. 5. Fermentation rates measured by a microdiffusion method for CO_2 evolution. Δ , Normal fresh yeast; $\bullet$, potassium yeast; $\circ$, magnesium-rich yeast with normal potassium content.

Table 3. *Oxygen consumption of yeasts*

The preparation of the different yeasts is described in the Methods section. Oxygen consumption is expressed as m-moles/hr./kg. of centrifuged yeast.

Expt. no.	Magnesium-rich yeast with normal potassium content	Potassium yeast	Fresh yeast
1	185	166	99
2	147	194	177
3	140	181	106
4	180	197	90
5	151	165	176
Mean	161	181	130

no appreciable magnesium is lost or excreted from the cells.

In the formation of a magnesium yeast with minimal potassium content as described here, the general procedure by which a sodium yeast was formed would not work, for although large amounts of Mg^{2+} ions could be introduced into the cells by fermenting in the presence of 0.2M-magnesium acetate, a corresponding amount of K^+ ion does not emerge from the cells; also, when large amounts of Mg^{2+} ions are accumulated by the physiological potassium-carrier, no appreciable amounts of Mg^{2+} ions are excreted on suspension later in water or in 0.1M-potassium chloride (Conway & Beary, 1958).

The reason why no K^+ ions come out from the yeast cells during the preparation of a magnesium-rich yeast, as they do in the formation of a sodium yeast, appears to be that the conditions and the presence of the high magnesium concentration in the cells render the yeast-cell membrane impermeable to the free passage of K^+ ions. When, however, K^+ ions have been replaced by Na^+ ions, these can be excreted by the 'sodium pump'. Thus it has been possible to prepare a magnesium-rich yeast in which the inorganic-cation content is almost entirely magnesium.

In the preparation of magnesium yeasts (as here described), the large magnesium uptake occurs with an external pH higher than 6.0, and in the absence of appreciable amounts of other cations, K^+ ions are especially inhibitory (Conway & Beary, 1958). The uptake is different from that studied by Rothstein (1954, 1955) and others, when the external pH was 3.5–4.5. Such transport is not inhibited by K^+ ions, and may be properly termed uptake by the physiological magnesium-carrier.

SUMMARY

1. A method is described for the preparation of a magnesium yeast as one of a series in which almost all the potassium content of the yeast is replaced by another cation. In the preparation of this magnesium yeast it was necessary first to prepare a sodium yeast and then to replace the sodium in the cells by a series of fermentations in the presence of 0.2M-magnesium acetate.

2. The fermentation rate of the magnesium yeast is decreased to about 20% of that of fresh yeast, and the oxygen uptake to about 28%. The growth rate is completely inhibited for about 3–4 days; it then proceeds very slowly. Abnormal elongated cells are found which have a marked tendency to form long chains.

3. The introduction of potassium chloride (final concentration about 0.1M) in the external fluid rapidly restores these properties of the

magnesium yeast to normal or nearly normal values.

4. A magnesium-rich yeast with normal potassium content was prepared by fermenting directly in 0.2M-magnesium acetate. A large amount of magnesium can be taken up in this way under suitable conditions. The magnesium uptake can exceed 400 m-equiv./kg. of yeast, and no appreciable amount of potassium is lost in the process. The properties of this yeast do not appreciably differ from those of fresh washed yeast.

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Incorporation of ^{18}O from Air into Hydroxyproline by Chick Embryo

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Since the suggestion by Stetten (1949) that most of the hydroxyproline in animal tissues arises from proline rather than from free hydroxyproline, the mechanism of the conversion has been studied by many workers. Although many results support Stetten's findings (Smith & Jackson, 1957; Green & Lowther, 1959), experiments in cell-free systems have so far been unsuccessful, and the mechanism of the hydroxylation of proline to hydroxyproline remains unknown.

It has been confirmed that the hydroxylation of phenylalanine (Mitoma, 1956), steroids (Hayano, Saito & Dorfman, 1958) and some other aromatic compounds (Saito, Hayaishi, Rothberg & Senoh, 1957; Posner, Mitoma, Rothberg & Udenfriend, 1961) are catalysed by oxygenases, i.e. the oxygen atoms of hydroxyl groups are derived directly from atmospheric oxygen. On the other hand, hydroxylations of some heterocyclic compounds such as nicotinic acid are not oxygenation reactions and the oxygen atoms of the hydroxyl groups are derived from water (Hunt, Hughes & Lowenstein, 1958).

This paper presents experimental evidence that the hydroxyl group of hydroxyproline is derived from atmospheric oxygen.

MATERIALS AND METHODS

Materials. The H_2^{18}O was purchased from Research and Development, Weizmann Institute of Science, Rehovoth, Israel; ^{18}O -enriched oxygen gas was prepared from H_2^{18}O by electrolysis.

Incubation of chick embryo with ^{18}O -enriched air. Eight 13-day-old eggs and a balloon (2 l.) filled with ^{18}O -enriched

oxygen were placed in a vessel (9.3 l.). Aq. 10% (w/v) sodium hydroxide (5 ml.) was also placed in the vessel to remove expiratory carbon dioxide. The vessel was flushed with nitrogen for 2 min. and the balloon broken by needle puncture through a rubber stopper. The vessel was incubated for 24 hr. at 37°.

Hydrolysis of embryos. After incubation, embryos were collected and homogenized in ethanol (150 ml.). The precipitate was collected by centrifuging, dried *in vacuo* and hydrolysed by refluxing with 6N-hydrochloric acid (200 ml.) for 24 hr.

Isolation of hydroxyproline. The hydrolysate was evaporated to dryness and the residue dissolved in water (40 ml.). Activated charcoal (2 g.) was added and the mixture stirred for 1 hr. Charcoal was removed by filtration and washed with water (5 ml.), and the filtrate and the washings were combined. Sodium nitrite (8 g. in 15 ml. of water) was added to destroy amino acids, and, after the solution had been left for 1 hr. at room temperature, concentrated hydrochloric acid (60 ml.) was added, and the mixture then refluxed for 1 hr. Most of the sodium chloride separated after concentration, and was washed with 6N-hydrochloric acid. The solution and the washings were combined and evaporated *in vacuo*. The residue, dissolved in water (50 ml.), was passed through an Amberlite IR-120 (H^+ form) column (2 cm.² × 20 cm.) which retained the imino acids together with other cations. The imino acids were eluted with 2N-ammonium hydroxide, the eluate was evaporated and the residue dissolved in water (10 ml.). The solution was passed through another Amberlite IR-120 (H^+ form) column (0.8 cm.² × 40 cm.). The column was washed with water (50 ml.) and with 0.5N-hydrochloric acid (50 ml.), and the hydroxyproline was eluted with 1.5N-hydrochloric acid. The eluate was evaporated to dryness *in vacuo*. The hydroxyproline hydrochloride so obtained was passed through a third column (2 cm.² × 5 cm.) of Amberlite IR-120 (H^+ form) to free the imino acid from hydrochloric acid. The hydroxyproline was eluted with 2N-ammonium hydroxide. The eluate was evaporated, the residue was dis-