Lipid Formation in *Cryptococcus terricolus*

II. The Effect of Ion Variation on Growth and Lipid Formation

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The following medium was found satisfactory for maximum growth and lipid production in *Cryptococcus terricolus*: glucose 40.0 g, urea 0.6 g, KH_2PO_4 0.5 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.0 g, $\text{Fe(III)-citrate} \cdot 3\text{H}_2\text{O}$ 0.1 g, 0.2 mg of zinc as $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001 mg of copper as $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, and 0.001 mg of manganese as $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ to one litre of redistilled water. Finally, 50 mg of ethylenediamine tetraacetic acid (EDTA) was added per litre. This medium permits the production of approximately 34.5 g of yeast dry matter containing 21 g of lipids per 100 g of assimilated glucose. This yield has been found to remain constant during a large number of transfers in the same medium.

The following ions have to be added to the medium in order to obtain maximum growth: potassium, magnesium, phosphate, sulphate, iron, zinc, and copper. Supraoptimum doses of potassium, zinc, and copper had a markedly inhibiting effect on the growth of the organism. Addition of sodium, calcium, manganese, molybdenum, cobalt, and chlorine seemed to have no effect on the growth.

The lipid content of the cells was influenced by variations in the different elements in the same manner as the growth. However, contrary to the results obtained in the absence of the other ions, a lack of phosphate or sulphate led to an increased lipid content in the cells. This lipid production may perhaps be compared with the degenerative lipid production found with aging in different organisms. More phosphate and magnesium were required in the medium for producing maximum lipid content, than for producing a maximum amount of yeast dry matter. Manganese, introduced into the medium in a concentration of 0.001 p.p.m., or more, increased the lipid content of the cells. The other elements, which did not affect the growth, had no effect on the lipid content either.

The lipid coefficient generally followed the variations in the lipid content. With increasing magnesium supply, however, it looked as if the effectivity of the conversion of glucose into lipids decreased.

The protein content of the yeast was found to be fairly constant, viz. 10–15 % of the dry weight.

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The effects of certain variations in the composition of the medium and the incubation conditions on the growth of *Cryptococcus terricolus* were investigated in earlier experiments¹. The growth and lipid production of this yeast have, moreover, been found to be closely related². Consequently, it was thought that maximum lipid production should be dependent on optimum growth conditions. However, there is no reason to assume that the *Rhodotorula* medium³, without beer wort addition, employed as basal medium in earlier investigations², should be the best nutrient solution for *C. terricolus*. The amounts of lipids produced by this yeast were found to be independent of the quantities of nitrogen supplied². These results indicated that the lipid production dominates quantitatively in the life activities of this organism, also under conditions found to be unfavourable to lipid production in other "fat" yeast species. It would, therefore, be of general interest to investigate the effect of variations in the composition of the medium on the lipid production.

It is a well-known fact that lipid formation can be brought about in other "fat" yeasts, if the amounts of certain nutrient substances, other than nitrogen, be decreased below critical levels. Reductions in the phosphate^{4,5}, sulphate, and iron⁶ concentrations have been shown to increase the lipid production of various yeasts. With the close relationship between yeast growth and lipid production in *C. terricolus*, it is essential to obtain a balanced nutrient solution that will result in the development of the largest number of cells containing a maximum amount of lipids. With the earlier results in mind, it was hoped that both aims could be attained during one growth phase in a single, unaltered medium.

EXPERIMENTAL

Strain No. 1 of *Cryptococcus terricolus* was used in this investigation. Details concerning treatment of stock cultures, inoculation, and methods of growing the organism have been described earlier¹. The same paper also contains descriptions of methods used for growth determination, nitrogen and lipid analyses, and determination of sugar consumption.

The *Rhodotorula* medium³, without addition of beer wort, was employed at the beginning of this investigation. When it was found¹ that urea gave a more rapid growth than did $(\text{NH}_4)_2\text{SO}_4$, the medium composition was changed as follows: glucose 40.0 g, urea 0.6 g, KH_2PO_4 1.0 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.0 g, NaCl 0.5 g, $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ 0.5 g, and $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ 0.005 g to one litre of distilled water (medium A). These and the other chemicals used were of analytical grade. Urea was autoclaved separately and added to the medium under sterile conditions. This medium was employed only in the first experiments to find the effects of simultaneous variations in the potassium and phosphate contents, supplied as KH_2PO_4 , and in the magnesium and sulphate contents, supplied as $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. Later on, when each individual ion concentration of interest was varied, it was found more advantageous to work with media prepared by the use of redistilled water. This modification of the composition of the original medium caused a marked decrease in the yeast yield. The decrease could be approximately compensated for by adding 1 ml (per litre nutrient solution) of a trace metal solution containing 39.3 mg of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 360.3 mg of $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, and 879.7 mg of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ per litre of redistilled water. The final medium contained 0.01 p.p.m. of copper, 0.2 p.p.m. of zinc, and 0.1 p.p.m. of manganese (medium B).

In testing the effect of variations in the iron concentration on the growth of the organism, it was found more practical to use ferric citrate instead of ferric chloride, as a source of this element (medium C). A subsequent modification was obtained by omitting sodium chloride and calcium chloride from this medium (medium C₁).

Fig. 1. Effect of different concentrations of KH_2PO_4 on growth, lipid and protein content, and lipid coefficient of *Cryptococcus terricolus*, Medium A.

- — Per cent of maximum growth
- — Protein content in per cent of dry matter
- — Lipid content in per cent of dry matter
- △ — Lipid coefficient

For further investigations on the effects of additions of different trace metals on the organism, ethylenediamine tetraacetic acid (EDTA) was supplied to the medium to prevent possible precipitation or formation of non-assimilable complexes. This was especially necessary when metal ions were present in rather large quantities. Medium C₁, without additions of copper, zinc, and manganese, but with 50 mg of EDTA per litre, was called medium C₂.

Variations in the concentration of a single ion can be obtained only by changing the supply of a compound containing the desired element. It is of course necessary that the concentrations of the other essential ions present in this compound are kept at a constant level. This is obtained by adding equimolecular amounts of compounds containing the ion to be held constant in combinations with known, non-essential components. The exact methods are described in connection with each particular experiment.

To obtain reproducible results when investigating the effect of micro-elements, it was necessary to cultivate the inoculum twice in media, to which no addition of the actual component had been made, before inoculation of the experimental series. All series were run at least in duplicate, and all experiments repeated.

RESULTS

The contents of glucose and urea in the nutrient solution were kept constant during this investigation. Glucose has been shown to be a satisfactory energy source in the concentration used here¹, and urea has been found to provide an optimum nitrogen source for both growth and lipid production². The other components introduced into the solution at different stages of this investigation, included, in addition to the macro- and micro-elements proved to be generally necessary for growth in most micro-organisms, some components exerting more irregular or undefined effects.

Fig. 2. Effect of different concentrations of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ on growth, lipid and protein content, and lipid coefficient of *Cryptococcus terricolus*. Medium A. Symbols as in Fig. 1.

Fig. 3. Effect of different concentrations of Na^+ on growth, lipid and protein content, and lipid coefficient of *Cryptococcus terricolus*. Medium B. Symbols as in Fig. 1.

The optimum concentrations of KH_2PO_4 and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ were determined in a series of preliminary experiments. The concentration of KH_2PO_4 was varied from 8.0 g to 0.0 g per litre of nutrient solution (Fig. 1). As would be expected, the growth decreased rapidly as the amount of KH_2PO_4 approached zero. The protein contents of the cells were independent of the KH_2PO_4 concentration, while the lipid content increased with decreasing supply. This last result is probably connected with the stagnation of the growth. The lipid coefficient (g of newly formed lipids/100 g of assimilated sugar) increased with decreasing KH_2PO_4 supply, evidently as a result of a more economic glucose consumption. In practice, the most effective lipid production was obtained when the content of KH_2PO_4 was kept between 1.0 and 0.5 g per litre. The dotted part of the lipid coefficient curve indicates that a certain lipid formation took place even after the stagnation of the growth and the sugar assimilation. The concentration of KH_2PO_4 in the media employed in the subsequent experiments was kept at 1.0 g per litre.

Variations in the $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ content of the medium showed that the maximum yeast production was obtained at 1.0 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ per litre (Fig. 2). Further additions seemed to have no effect on the growth, while a reduced concentration, as was to be expected, resulted in a considerable decrease in growth. The lipid content was also kept at its maximum at rates exceeding 1.0 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ per litre, while both the lipid content and the effectivity of the lipid production decreased at rates below this level. In addition, it was found that a slight decrease in the protein contents of the yeast cells occurred when the concentration of magnesium sulphate was decreased

Fig. 4. Effect of different concentrations of Fe^{3+} on growth, lipid and protein content, and lipid coefficient of *Cryptococcus terricolus*. Medium C. Symbols as in Fig. 1.

Fig. 5. Effect of different concentrations of Cl^- on growth, lipid and protein content, and lipid coefficient of *Cryptococcus terricolus*. Medium C. Symbols as in Fig. 1.

below 1.0 g per litre. Consequently, the concentration of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ was kept at 1.0 g per litre in the subsequent experiments.

The main interest was now focused on the effect of variations in single nutrient ions. Since it was not possible to vary the ion contents individually, it was necessary first to find elements that had no effect on the organism. These elements could then be combined with the ion to be studied.

The effect of sodium on the organism was tested by means of medium B, in which NaCl had been replaced by various amounts of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$. From Fig. 1 it might be assumed that the supply of additional phosphate, as $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, to the amount already present in medium B, would have no effect on the organism. Later results (Fig. 8) verified this suggestion. The chlorine content was diminished by the same amount in all series, but this element was still present in the calcium, iron, and manganese salts. The content of sodium varied from 2 000 to 0 p.p.m. No effect on growth, lipid content, protein content, or lipid coefficient resulted from the variations in the sodium content within the limits used here (Fig. 3).

Ferric citrate was used to test the effect of different concentrations of iron on the organism. It was first proved that no difference existed between the standard solution B and the same medium with equivalent iron amounts added as ferric citrate. The iron content was then varied between 2 000 and 0 p.p.m. The growth of the organism was greatly affected by changes in the iron content, a maximum yeast yield being obtained at an addition of 20 p.p.m. (Fig. 4). It also emerges that the changes in both lipid content and lipid coefficient paralleled the alterations in growth. The protein content,

Fig. 6. Effect of different concentrations of Ca^{++} on growth, lipid and protein content, and lipid coefficient of *Cryptococcus terricolus*. Medium C. Symbols as in Fig. 1.

Fig. 7. Effect of different concentrations of K^{+} on growth, lipid and protein content, and lipid coefficient of *Cryptococcus terricolus*. Medium C_1 . Symbols as in Fig. 1.

however, remained nearly constant, although a slight increase with increasing iron content was perhaps indicated.

The comparatively good growth obtained in media to which no iron had been added (*cf.* Fig. 4) must be due to iron contaminations in the other chemicals, rather than to a growth response in a medium actually iron-free. If a linear dependence is assumed between the iron content and the yeast yield in the lower part of the growth curve, an iron contamination of approximately $90 \mu\text{g}$ per litre can be calculated for medium C. This amount is far below the purity limits for the chemicals used. It should be pointed out here that similar reservations must be made for any possible contamination when micro- or "non-effective" elements are tested.

Medium C contains chlorine in the following components: NaCl , $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, and $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$. The last component was replaced by equivalent amounts of $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$. The original $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ content could be diminished to 1/10 of the original amount without affecting the growth and lipid production of the organism. This reduced amount was replaced by equivalent amounts of calcium in the form of $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$. A nitrogen-containing compound was chosen since an additional supply of this kind is known to be of no effect on either growth or lipid production². A special testing of this amount of $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ indicated no differences from the original medium C. Now the chlorine content could be varied simply by altering the NaCl content. Fig. 5 shows that the growth and the lipid and protein contents of the yeast were independent of the chlorine concentrations employed in this investigation.

The effect of calcium was investigated simply by varying the $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ content of medium C, since it had been proved that the presence of chlorine had no effect on the organism. Just as for chlorine, the calcium content was

Fig. 8. Effect of different concentrations of PO_4^{3-} on growth, lipid and protein content, and lipid coefficient of *Cryptococcus terricolus*. Medium C_1 . Symbols as in Fig. 1.

Fig. 9. Effect of different concentrations of Mg^{++} on growth, lipid and protein content, and lipid coefficient of *Cryptococcus terricolus*. Medium C_1 . Symbols as in Fig. 1.

found to have no influence on the measured activities of the organism (Fig. 6). The results obtained from the variation of the sodium, calcium, and chlorine contents provide no absolute proof of the yeast being completely independent with respect to these elements, but, at any rate, it has been established that further supply, in addition to the amounts possibly added as contaminants in other chemicals, is unnecessary. Because of these findings, both $NaCl$ and $CaCl_2 \cdot 6H_2O$ were omitted from the medium, which resulted in the modified medium C_1 .

It was now possible to investigate the effects of potassium, phosphate, magnesium, and sulphate on the organism. Medium C_1 was used as basal medium. Additional potassium was supplied to the medium as KCl . When the potassium content in the original medium was reduced by decreasing the amount of KH_2PO_4 , equivalent amounts of $NaH_2PO_4 \cdot 2H_2O$ were added to keep the phosphate content at a constant level. The growth of the organism was greatly affected by alterations in the potassium content (Fig. 7). Potassium contents less than 36 p.p.m., or exceeding 1198 p.p.m., depressed the growth. The same influence was also found to be present with respect to the lipid content and the lipid coefficient. The relative protein content, however, increased to a certain extent, when the growth conditions became unfavourable to the organism.

A similar method was used to establish the effect of variations in the phosphate supply on the organism. $NaH_2PO_4 \cdot 2H_2O$ was added to the solution to increase the phosphate content beyond that of medium C_1 . By reducing the KH_2PO_4 content, on the other hand, a reduction in the phosphate content was obtained. KCl was added in sufficient amounts to keep the potassium con-

Fig. 10. Effect of different concentrations of SO_4^{--} on growth, lipid and protein content, and lipid coefficient of *Cryptococcus terricolus*. Medium C_1 . Symbols as in Fig. 1.

Fig. 11. Effect of different concentrations of Zn^{++} on growth, lipid content, and lipid coefficient of *Cryptococcus terricolus*. Medium C_2 . Symbols as in Fig. 1.

centration constant. A rapid decrease in the growth occurred when less than 44 p.p.m. of phosphate was added to the medium (Fig. 8). Larger amounts, however, seemed to have no distinct effect on the growth of the organism. On the other hand, to obtain maximum lipid production, the amount of phosphate must exceed that required for obtaining maximum growth. The lipid content of the cell material decreased by one-fourth when the phosphate supply was reduced from 175 to 44 p.p.m., while the growth remained practically constant. As found in other connections, the stagnation of the growth, in this case due to the absence of phosphate, was accompanied by an abrupt increase in the lipid contents of the cells. The protein content seemed to be unaffected by variations in the amount of phosphate added to the medium.

To study the effect of magnesium on the organism, the amount of this element added to the solution was varied, the sulphate supply being of course kept at a constant level. An increase in the magnesium content was obtained by adding $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$. When the $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ amount was decreased below the original content of medium C_1 , the sulphate supply was kept constant by adding $\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$. About 3 p.p.m. of magnesium was sufficient for maximum growth (Fig. 9). In contrast to the results obtained for potassium, large quantities of magnesium seemed to have no inhibitory effect on the growth. The lipid contents of the cells increased slightly with increasing magnesium content up to 100 p.p.m., while the economic yield of the transformation of glucose into lipids decreased with increasing amount of magnesium. The highest output of lipids, relative to the amount of sugar consumed, was obtained at 100 p.p.m. of magnesium in the nutrient solution. The protein content seemed to be quantitatively unaffected by variations in the magnesium content.

Table 1. Effect of different concentrations of ethylenediamine tetraacetic acid (EDTA) on the growth of *Cryptococcus terricolus*. Medium C₁.

Added EDTA g/l	Yeast yield g/l
5.0	0
0.5	0
0.05	6.95
0	6.07

Variations in the sulphate content of the medium seemed to result in more pronounced effects on the growth than did alterations in the magnesium content (Fig. 10). The sulphate content was increased by adding $\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$ and decreased by reducing the supply of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. In the latter case, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ was added to keep the magnesium content constant. For optimum growth the addition of at least 97 p.p.m. of sulphate was required. Further additions had no effect on the growth. Alterations in the sulphate content of the medium had no quantitative effect on the protein production of the cells. The lipid contents of the cells remained unchanged for additions exceeding 195 p.p.m. of sulphate. Reductions in the sulphate supply led to a decrease in the lipid content, followed by a formation of lipid-rich cells when the sulphate content became so low that the growth was markedly inhibited. Only minor changes in the lipid coefficient were obtained, since the sugar consumption almost paralleled the lipid production of the organism.

The addition of some micro-elements was necessary to obtain maximum growth when the medium was prepared by the use of redistilled water. The effects of additions of copper, zinc, manganese, molybdenum, and cobalt were tested. In recent years, chelating agents, such as EDTA (ethylenediamine

Table 2. The effect produced by excluding different trace metals from a complete medium, on the growth, sugar consumption, and lipid content of *Cryptococcus terricolus*. Medium C₂ to which was added 0.01 p.p.m. of Cu as $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.2 p.p.m. of Zn as $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 p.p.m. of Mn as $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.1 p.p.m. of Mo as $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, and 0.1 p.p.m. of Co as $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$.

Medium	Growth		Sugar consumption		Economic coeff.	Lipid content		Lipid coeff.
	g/l	%	g/l	%		g/l	%	
complete	13.09	95	38.4	96.0	34.1	7.30	55.8	19.0
— Cu	12.40	90	37.4	93.5	33.2	7.11	57.3	19.0
— Zn	6.56	47	16.2	40.5	40.5	3.49	53.2	21.5
— Mn	12.54	91	37.2	93.0	33.7	7.12	56.8	19.1
— Mo	13.56	98	39.2	98.0	34.6	7.70	56.8	19.6
— Co	13.83	100	39.5	98.8	35.0	7.77	56.2	19.7
— all	4.55	33	11.8	29.5	38.6	2.54	55.9	21.5

Table 3. The effect produced by adding different trace metals to medium C₂, on the growth and lipid content of *Cryptococcus terricolus*. When added, the elements were present in the same concentrations as in Table 2.

Addition	Growth		Lipid content	
	g/l	%	g/l	%
None	6.35	46.0	3.77	59.4
+ Cu	8.31	60.2	4.89	58.9
+ Zn	13.29	96.2	7.88	59.3
+ Mn	7.72	55.9	4.48	58.0
+ Cu, Zn	13.39	97.0	8.10	60.5
+ Cu, Mn	7.88	57.1	4.72	59.9
+ Zn, Mn	13.42	97.2	8.00	59.6
+ Cu, Zn, Mn	13.81	100.0	8.11	58.7

tetraacetic acid), have received much attention in similar investigations, because they prevent precipitation and formation of unassimilable complexes.

An addition of 50 mg of EDTA per litre increased growth, while larger amounts inhibited growth completely (Table 1). Medium C₂ was used as a basal medium throughout the subsequent part of the investigation. The addition of micro-elements has been specified in each case.

The effects of various trace metals were tested by using a medium containing all the elements, excluding one element at a time. The composition of the media and the results obtained are given in Table 2.

The growth decreased greatly when zinc was excluded from the medium. The exclusion of copper and manganese seemed to have a slightly growth-decreasing effect, while, on the other hand, molybdenum and cobalt had no effect on the organism. The lipid content in per cent of the dry weights, showed only minor variations, but both the economic coefficient and the lipid coefficient increased when the growth became seriously retarded.

The effects of the three elements, copper, zinc, and manganese were further tested by adding the elements singly, and in different combinations, to the medium. The addition of zinc alone had a marked influence on the growth, whilst copper and manganese added separately, had a more moderate, or a slight effect (Table 3). When zinc was present in the medium, the addition of the other elements resulted in only insignificant effects on the organism. The lipid content in per cent of the dry weights remained almost constant during these experiments.

Since the addition of zinc was so decisive for the development of the organism, the influence of different concentrations of this ion was tested (Fig. 11). 200 p.p.m. of zinc inhibited the growth markedly. To obtain a maximum yeast yield, the concentration had to be decreased to 0.2 p.p.m. With further restrictions in the supply, the yield decreased to approximately 54 % of the maximum yield. The total lipid production was at its maximum at the addition of 0.2 p.p.m. of zinc, but the highest lipid content was obtained at only one-tenth of this concentration. The lipid coefficient showed a maximum between 0.02

Fig. 12. Effect of different concentrations of Cu^{++} on growth, lipid content, and lipid coefficient of *Cryptococcus terricolus*. Medium C_2 + 0.2 p.p.m. of zinc. Symbols as in Fig. 1.

Fig. 13. Effect of different concentrations of Mn^{++} on growth, lipid content, and lipid coefficient of *Cryptococcus terricolus*. Medium C_2 + 0.2 p.p.m. of zinc + 0.001 p.p.m. of copper. Symbols as in Fig. 1.

and 20 p.p.m. of zinc. As a result of these findings, the zinc concentration of the medium was subsequently kept at 0.2 p.p.m.

The influence of variations in the copper content was investigated, by the use of medium C_2 , which contained 0.2 p.p.m. of zinc. The growth was completely inhibited when 100 p.p.m. of copper, or more, was added to the medium (Fig. 12). The growth increased with decreasing copper content, maximum yield being obtained at 0.001 p.p.m. A further decrease in the copper supply led to an insignificant decrease in the yeast yield, thus indicating a slightly stimulatory effect of this element. 0.01 p.p.m. of copper gave maximum lipid content, while one-tenth of this concentration gave maximum lipid coefficient. It was therefore considered justifiable to use additions of 0.001 p.p.m. of copper in the subsequent experiments.

Finally, the effect of manganese was investigated. Medium C_2 , containing 0.2 p.p.m. of zinc and 0.001 p.p.m. of copper, was used. The results differed markedly from those obtained for varying concentrations of copper and zinc (Fig. 13). No effects on the growth, due to manganese, were found within the concentration limits used here. To obtain the highest lipid production, however, the content of manganese had to be at least 0.001 p.p.m. Further additions had no effect on the lipid content or the effectivity of the lipid production. Consequently, 0.001 p.p.m. of manganese was used in the final medium.

DISCUSSION

The fundamental results obtained in this investigation and in earlier ones² on the lipid production of *Cryptococcus terricolus*, are to the effect that growth and lipid production are closely related. This means that, in this yeast, the lipid formation follows the proliferation of the cells, not, as is the case of the other "fat" yeasts, starting after the growth conditions have become unsatisfactory because of restrictions in or a complete assimilation of the supply of a particular nutrient other than the energy- and carbon source. In agreement with this finding, the content of proteins was found throughout to remain at a constant, comparatively low level, only in exceptional cases exceeding 15 % of the dry weight.

However, in spite of the close relationship between the growth and the lipid production in *C. terricolus*, it is of course possible that the optimum conditions for the two processes may be different with respect to the different medium constituents. Since both growth and content of lipids are always determined in the same material, conclusions may be drawn regarding both processes. This is at any rate true of the changes in the amounts of the final products (dry matter, lipids, and proteins).

Both potassium and phosphate are essential to the growth of yeasts. The very low growth of *C. terricolus* found with no addition of KH_2PO_4 is due to the presence of small amounts of these ions in the inoculum, and as contaminants. A comparison of Figs. 7 and 8 with Fig. 1 will show, that the absence of phosphate and potassium decreases the growth, but that the absence of phosphate has the greatest effect. Addition of approximately 44 p.p.m. brought the yeast yield almost to its maximum. In trying to obtain maximum growth in his lipid-forming yeasts, Schulze⁷ discovered definite, upper and lower limits with respect to the amount of phosphate, that could be accumulated by the yeast cells. At higher concentrations, the excess of phosphate was left unassimilated, and did not affect the yeast yield. However, in Schulze's investigations, the lipid content was decreased under such conditions, the same phenomenon being observed by Nielsen and Nilsson⁴, Maas-Förster⁵, and others. Garrido *et al.*⁸ working with lipid-producing fungi, found great variations among the phosphate requirements of different organisms. While *Aspergillus nidulans* produced a maximum amount of lipids when supplied with 4 737 p.p.m. of phosphate as KH_2PO_4 , *Penicillium javanicum* required only 1/10 of this amount. 175 p.p.m. of phosphate or more was found to result in a maximum lipid production in *Cryptococcus terricolus*. At levels from 175 to 44 p.p.m. of phosphate, an intermediate decrease in the lipid content was found.

Phosphate plays an important role in the carbohydrate metabolism, as well as in the synthesis of the fatty acids. Consequently, the development of energy for the growth of the organism seems to proceed at a normal rate under conditions resulting in a decreased lipid production. When phosphate is not supplied to the medium, the increased production of lipids may probably be compared with the degenerative lipid production following the stagnation of growth in many microorganisms. It is further pointed out that a rich supply of phosphate to the organisms used in the above-mentioned investigations^{4,5} led to the production of protein-rich cells. In *C. terricolus*, the protein content remained unaf-

fects by changes in the phosphate addition. This result would be expected if, as has been briefly discussed elsewhere², the low protein content is due to a blocking of the amino acid synthesis of the organism.

The function of potassium in yeast growth is not fully understood, but it has been found to be essential. To obtain maximum growth the presence of 36 p.p.m. of potassium is required (Fig. 7). The same is true of maximum lipid production. Maas-Förster⁵ found that a lack of potassium decreased the production of yeast cells. The composition of the cells, however, remained constant. Garrido *et al.*⁸ obtained similar results regarding the growth of different fungi, finding, however, especially in the case of *Penicillium javanicum* great variations in the lipid content. An addition of 9.7 p.p.m. of potassium to this organism gave a lipid content only about one-third of the maximum value found at an addition of 48.8 p.p.m. A further increase caused the lipid content to decrease by one-third. The large variations found in the growth and lipid production of *P. javanicum* for different potassium concentrations may be compared with those found in *Cryptococcus terricolus* in this investigation.

It is a well known fact that the total intracellular potassium concentration increases in yeast cells with increasing extracellular potassium supply⁹. Due to a decrease in the cell-water content, internal osmotic variations which, in some way, may inhibit the life activities of the cells may occur. Interesting results were obtained by Rothstein and Bruce¹⁰, who found that the sulfhydryl groups of the cell membrane were essential to potassium retention. Since the presence of active coenzyme A plays an important role in the normal development of lipid-forming yeasts, a possible effect on the active sulfhydryl group of coenzyme A should be kept in mind.

1.0 g/l or more of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ is necessary for obtaining maximum growth and maximum lipid production in *C. terricolus*. The effect of increasing $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ concentrations on the lipid production seems to be variable, depending on the actual organism under investigation. Garrido *et al.*⁸ found that the lipid contents of *Aspergillus nidulans* and *Penicillium spinulosum* increased with increasing $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ supply, whereas the reverse was found to be the case with *Penicillium javanicum*. Both magnesium and sulphate are essential to obtain high yields of yeasts. In *Cryptococcus terricolus* magnesium and sulphate are also required to obtain a maximum lipid production. Nielsen and Rojowski⁶ found that a lipid-rich *Rhodotorula gracilis* yeast was obtained with a reduced sulphate content in the medium. The authors suggested that the primary effect was the decrease in the normal protein content of the cells, followed by a simultaneous increase in the lipid content. Fundamental differences have been found between *R. gracilis* and *Cryptococcus terricolus*, the protein content of the latter species always remaining at a low level. As was the case when the phosphate content was varied, an intermediate decrease in the lipid content occurred at sulphate additions between 195 and 24 p.p.m. When sulphate was absent, the increased production of lipids was probably a result of a degenerative lipid synthesis. Consequently, as far as the effects of both magnesium and sulphate are concerned, optimum growth conditions are accompanied by a maximum lipid production, indicating that, in this case also, a high lipid content is a normal property of the cells.

Addition of iron generally enhances the yield of yeasts. The optimum amount varies from one organism to another. Olson and Johnson¹¹ found that amounts ranging from 0.025 to 0.15 p.p.m. of iron were necessary for obtaining maximum growth in four different yeasts. Steinberg and Ordal¹² found the addition of iron to be unnecessary for the attainment of maximum lipid synthesis in *Rhodotorula gracilis* grown in a medium containing unrefined carbohydrates. Nielsen and Rojowski⁶ succeeded in increasing the lipid content of *R. gracilis* by cultivating the organism with a greatly reduced iron supply. Their results also indicated that the yeast protein in this case decreased from 45 to 20 % of the dry weight.

In *Cryptococcus terricolus*, 0.2 p.p.m. of iron is sufficient to produce approximately 90 % of the maximum yield obtained at 20 p.p.m. Larger doses have a slightly inhibitory effect, the tolerance limits being very wide, however. From 0.2 to 2 000 p.p.m. of iron gives 90 % or more of maximum growth yield. The amounts required for maximum lipid production are the same as those for maximum growth. The positive correlation between healthy growth and maximum lipid production was thus once again stated for *C. terricolus*.

The following ions were shown to have no effect on either the growth or the lipid production of *C. terricolus*: sodium, calcium, cobalt, molybdenum, and chlorine. These results are partly in agreement with those found earlier. Olson and Johnson¹¹ found that calcium and cobalt were not necessary for maximum yeast growth. No addition of either molybdenum or chlorine was made. Steinberg and Ordal¹² found the amounts of sodium and calcium present in unrefined carbohydrates to be sufficient to meet the requirements for lipid synthesis in *Rhodotorula gracilis*. In the present investigation, the possible presence, as contaminants, of sufficient quantities of the five elements mentioned should not be overlooked. However, no special additions of compounds containing these elements are required.

Ethylenediamine tetraacetic acid (EDTA) is used to keep the concentration of one or more metal ions below certain critical concentrations, above which undesirable precipitates may form, or undesirable reactions occur. The addition of 50 mg of EDTA to the medium resulted in an improvement in growth (cf. Table 1). Shukla and Prabhu¹³ found that the EDTA effect was due to the prevention of the sugar-salt complex formation, which leads to losses of assimilable sugars in both molasses and synthetic media. More than 500 mg of EDTA per litre of nutrient solution resulted in a total inhibition of the growth. This is probably due to the removal of the free metal ions from the medium, since the equilibrium between free metals, chelating agent, and chelate is strongly shifted towards the metal chelate.

Addition of 50 mg of EDTA per litre was used throughout the investigations of the effects of zinc, copper, and manganese, since no visible precipitation was found to have occurred, even at the highest metal concentrations used. At large metal concentrations, the free metal ions will of course dominate. With a reduced metal supply, an increasing part of the metal ions will be bound in the chelate complexes, but, as the free metal ions are being assimilated, new ions will be set free from the chelates, to an extent depending on the dissociation constants of the different chelate complexes.

The effect of zinc on the growth of the organism is very pronounced. More than 90 % of maximum growth is obtained at 0.2–20 p.p.m. of zinc. The largest lipid production is also found in this interval, although, in this case, also 0.02 p.p.m. must be included in the optimum range. Olson and Johnson¹¹ found that 0.025–0.2 p.p.m. of zinc caused a maximum growth in four different yeasts. Concentrations within the range 300–500 p.p.m. have generally been found to inhibit the growth of the yeasts partially¹⁴. Zinc has to be included in the *Rhodotorula gracilis* medium to obtain maximum lipid production¹⁵. This is also the case with many other lipid-forming microorganisms. To a certain extent, the lipid synthesis seems to be dependent on the addition of zinc.

It is a generally accepted view that, although copper is not essential to yeasts, small concentrations of this metal enhance the growth. Olson and Johnson¹¹ found that *Torulopsis utilis* 3 required 0.003 p.p.m. of copper for maximum growth — an amount that can be compared with the requirements of *Cryptococcus terricolus*. The requirements for maximum growth and total inhibition seem to vary considerably from one species to another. The growth is generally found to be totally inhibited at an addition of 100 p.p.m. or less.

Usually no effect on the growth is encountered at variations of the manganese content. This element was not required in short experiments on the lipid formation in *Torulopsis lipofera*¹⁶. In the present experiments, involving cell propagation, however, the lipid production was decreased to a certain extent when the manganese content was decreased below a certain critical value.

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