The Dissimilation of the Carbohydrate Reserves of a Strain of *Saccharomyces cerevisiae*

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(Received 11 July 1962)

The utilization of storage carbohydrate by yeast has been the subject of a number of conflicting reports. Harden & Rowland (1901), using pressed brewing yeast that had been grown anaerobically, showed that when stored under anaerobic conditions glycogen disappeared from the cell and carbon dioxide and ethanol were liberated. When stored aerobically their yeast produced 20 % more carbon dioxide. Stier & Stannard (1936), using aerobically grown baker's yeast, found that the anaerobic liberation of carbon dioxide was negligible and concluded that yeast is unable to ferment its reserve carbohydrate. Spiegelmann & Nozawa (1945), who studied a number of strains of yeast, reached similar conclusions. Chester (1959*a*), using a brewing yeast grown under substantially anaerobic conditions, reported an endogenous fermentation in suspensions of washed cells under both aerobic and anaerobic conditions, resulting in considerable losses of reserve carbohydrate. Chester (1959*b*), using a respiration-deficient mutant of the brewing yeast, further reported that the rate of endogenous fermentation was greatest under aerobic conditions. Eaton (1960) also observed endogenous fermentation under anaerobic conditions and reported results which indicate the presence of three endogenous carbohydrate substrates, one of which requires the presence of oxygen for utilization.

Ephrussi & Slonimski (1950), Slonimski & Hirsch (1952) and Slonimski (1955) have reported that yeast grown in the absence of oxygen has a very low content of respiratory enzymes, but that, after a period of aeration in the presence of glucose as an energy source, respiratory enzymes are synthesized.

This paper describes the fermentation of the storage carbohydrate that accumulates in a brewing yeast during anaerobic growth. Observations on the dissimilation of the products of the endogenous fermentation and the concurrent respiratory adaptation are also reported. The effect of aerobic and anaerobic growth on the reserve carbohydrate was also examined.

METHODS

Yeast cultures and preparation of suspensions. The yeast used in these experiments was a brewing strain of *Saccharomyces cerevisiae*. It has an absolute requirement for panto-

thenate and biotin. When cultures were grown in stationary flasks in the medium indicated below, all the yeast was at the bottom of the flask at the end of fermentation. The yeast was powdery in appearance when shaken and microscopic examination showed that most of the cells were separate.

Spontaneous respiration-deficient mutants of the brewing yeasts were obtained by plating cultures on agar and staining the colonies with triphenyltetrazolium chloride. The normal colonies were stained red and the smaller mutant colonies remained white (Ogur, St John & Nagai, 1957).

Anaerobic yeast was grown in stationary 250 ml. conical flasks containing 75 ml. of the medium described by Spiegelman & Nozawa (1945). For each experiment a number of flasks were inoculated with 10 ml. of a 48 hr. culture in the same medium. Growth, as measured by determinations of dry weight, continued until about 48 hr. after inoculation, when the concentration of yeast in the medium was about 4.5 mg./ml. During the fermentation there was a steady increase in the total carbohydrate content of the yeast, which reached a maximum (about 500 μ g. of hexose equivalent/mg. dry wt.) at about 48 hr. After incubation at 25° for 48 hr. the specific gravity of the supernatant medium was measured by the drop-gravity method (Williams & Stringer, 1955). A culture was selected in which the specific gravity was closest to 1.002. At this gravity there was about 0.4 % of glucose remaining in the medium.

Aerobic yeast was grown for 48 or 72 hr. in shaken 2 l. flasks containing 150 ml. of the growth medium. The yeast in the aerobic flasks reached a maximum concentration of 3.8 mg. dry wt./ml. after incubation for about 30 hr. The total carbohydrate content of the yeast at this stage was about 210 μ g. of hexose equivalent/mg. dry wt., which remained the same until about 72 hr. The drop-gravity method could not be used as an indication of the state of these aerobic cultures because of ethanol oxidation in the later stages of growth.

The yeast was washed three times by suspension and centrifuging in 67 mM-KH₂PO₄ (pH 4.5) at 15°. The washed yeast was suspended in the same solution and the concentration adjusted to give 5.5–9.0 mg. (dry wt.) of yeast in each manometer flask. A colorimeter calibrated in terms of dry weight facilitated the adjustment of the yeast concentration.

Analytical methods. Carbohydrate loss on harvesting was so rapid in brewing yeast grown under anaerobic conditions that samples for measurement of initial dry weight and initial carbohydrate content had to be taken very rapidly. The samples of suspension for determination of dry weight were centrifuged, the supernatant buffer was poured off and the yeast washed with water at 0–3°. The temperature of the yeast was then rapidly raised to 100° by immersion in a

boiling-water bath. The drying was completed in an oven at 100° for 48 hr.

Initial total carbohydrate of the yeast was determined by adding samples of the suspension to an equal volume of *m*-trichloroacetic acid, diluting to a suitable volume and estimating carbohydrate by the anthrone method with glucose as a standard. Yeast carbohydrate was fractionated by the scheme described by Trevelyan & Harrison (1956). In experiments in which the carbohydrate was fractionated, the total carbohydrate determined on whole cells was corrected for mannan.

Manometric measurements of respiration and fermentation were by the direct Warburg method at 28°, Q_{O_2} and Q_{CO_2} were expressed as μ l. of O_2 or of CO_2 /mg. initial dry wt. of yeast/hr. The first manometric readings were always taken 55–60 min. after harvesting. Each manometer flask contained a total volume of 3 ml. Anaerobiosis was obtained by flushing for 10 min. with N_2 freed from traces of O_2 with vanadous sulphate (Meites & Meites, 1948). Ethanol in the suspending medium was distilled off and determined enzymically (Kaplan & Ciotti, 1957).

Total acetate in the yeast and supernatant was determined by a micro-diffusion technique (Scarbrick, 1955). The contents of the manometer flasks were washed into centrifuge tubes and these were heated in a boiling-water bath for 3 min. The yeast was centrifuged and re-extracted with 3 ml. of water. No loss of acetic acid was detected from standard solutions of acetate treated in the same way. The combined supernatants were neutralized with 4*N*-

KOH, thymol blue being used as indicator, and dried *in vacuo* over conc. H_2SO_4 . The residues were dissolved in 1 ml. of water and the solutions used for the analysis. Paper chromatography did not reveal any volatile acids other than acetate in the extract.

Glucose was estimated by a colorimetric copper-reduction method (Nelson, 1944).

Cytochrome oxidase was determined manometrically with ascorbic acid for the reduction of cytochrome *c* (Hartree, 1955). The cell-free extracts for the oxidase estimation were prepared in a Nossal cell disintegrator (Nossal, 1953). The oxidase preparation was suspended in 67 mM-phosphate buffer (pH 7.1).

RESULTS AND DISCUSSION

Carbohydrate content of yeast. The carbohydrate contents of yeast grown under aerobic and anaerobic conditions are compared in Table 1. The storage carbohydrates, glycogen and trehalose, accounted for about 40 % of the dry weight of anaerobically grown cells from 48 hr. cultures. The concentration of glucose left in the medium when the 48 hr. cultures were harvested was less than 0.5 %. The analysis of the 72 hr. culture indicated that reserve carbohydrate was utilized after all the glucose in the medium had been fermented.

Table 1. *Carbohydrate composition of brewing yeast*

Total carbohydrate determined on whole cells was calculated as follows: total (as hexose) = total (as glucose) + mannan (as mannose) – mannan (as glucose).

Growth conditions	Anaerobic		Aerobic	
	48	72	48	72
Age of culture (hr.)	Percentage of total carbohydrate			
Carbohydrate				
Trehalose	7.6	5.6	1.2	0.9
Glycogen	75.6	67.9	37.5	44.8
Mannan	7.4	12.5	35.8	27.6
Glucan	9.4	14.0	25.5	26.7
Sum of fractions (μ g. of hexose equivalent/mg. dry wt. of yeast)	487	373	206	205
Total carbohydrate (μ g. of hexose equivalent/mg. dry wt. of yeast determined on whole cells)	505	385	214	216

Table 2. *Endogenous fermentation by anaerobically grown yeast*

Each manometric flask contained 3 ml. of yeast suspension. The initial dry weight of 3 ml. of the brewing yeast suspension was 7.78 mg. and the total carbohydrate content was 17.7 μ moles. The corresponding figures for the respiration-deficient yeast were 6.12 mg. and 15 μ moles. Results are expressed in μ moles. The duration of the experiments was 60 min. for (a) the brewing yeast and 150 min. for (b) the respiration-deficient mutant.

Gas in manometer flasks		Carbohydrate decrease		Ethanol release		Release of CO_2		Uptake of O_2	
		Air	N_2	Air	N_2	Air	N_2	Air	N_2
Changes during preparation and equilibration of flasks	(a)	1.8	1.8	3.6	3.6	—	—	—	—
	(b)	3.3	2.9	6.0	5.5	—	—	—	—
Changes during manometric readings	(a)	3.2	1.7	1.7	2.8	9.2	4.0	6.0	—
	(b)	4.7	3.1	6.5	5.2	9.8	6.2	0.6	—

Table 3. Time relations of carbohydrate disappearing and gaseous exchanges with anaerobically grown yeast

Initial dry weight of yeast suspension per manometer flask was 5.97 mg. and the initial carbohydrate content was 2.87 mg. After equilibration of the flasks the carbohydrate content had fallen to 2.49 mg., i.e. a decrease of 2.11 μ moles. Results are expressed in μ moles.

Gas in manometer flasks	Time (hr.) ...	Changes per flask					
		0-1	1-2	2-3	3-4	4-5	Total 0-5
N ₂	Carbohydrate decrease	2.16	0.72	0.67	0.43	0.43	4.41
	Release of CO ₂	3.80	1.60	1.38	1.04	0.79	8.61
Air	Carbohydrate decrease	2.70	1.39	0.78	0.36	0.36	5.6
	Release of CO ₂	7.30	4.70	4.30	6.40	2.76	25.4
	Uptake of O ₂	4.30	5.30	6.30	6.20	2.78	24.88

When the yeast was grown aerobically, the glucose in the growth medium was utilized at a slower rate and after 48 hr. the concentration of glucose remaining was 2.3 % (w/v). This slower utilization of glucose under aerobic conditions indicated that this yeast showed a normal Pasteur effect. All the glucose had been used when the 72 hr. culture was harvested. In aerobically grown cultures less than 10 % of the yeast dry wt. was storage carbohydrate. The amount of trehalose in these aerobic cultures was extremely small.

Manometric data from aerobically grown cells. The rates of oxygen uptake and carbon dioxide release by aerobically grown cells were very small (Q_{O_2} 1.2, Q_{CO_2} 1.6, $Q_{CO_2}^N$ 0.40). These measurements were made for 1 hr. immediately after preparing the suspension, but over 80 % of the gaseous exchange occurred during the first 30 min. of the period of measurement. These results indicated that there was very little utilization of reserve carbohydrate by the yeast grown under aerobic conditions.

Fermentation of reserve carbohydrate in anaerobically grown yeast. Table 2 shows the loss of carbohydrate and the formation of ethanol and carbon dioxide in experiments with suspensions of a brewing yeast and a respiration-deficient mutant of the brewing yeast. The decrease in the reserve carbohydrate in the period between suspending the washed yeast in the buffer solution and starting the manometric readings is included to illustrate the rapid initial rate of fermentation.

Under anaerobic conditions the ethanol released into the suspending medium by both yeasts was 80-100 % of the amount calculated from the Gay-Lussac equation: $C_6H_{12}O_6 \rightarrow 2CH_3 \cdot CH_2 \cdot OH + 2CO_2$. The carbon dioxide released under anaerobic conditions corresponded fairly closely with the calculated amount in both the normal and respiration-deficient yeast.

As the carbohydrate reserves were used, the rate of fermentation slowed (Table 3). It can be seen that the release of carbon dioxide is a measure of carbohydrate fermentation only under anaerobic conditions.

Fig. 1. Changes in respiratory and metabolic quotients with time. Each manometer flask contained 7.0 mg. of anaerobically grown yeast in 3 ml. of 67 mm-KH₂PO₄. ●, Respiratory quotient; □, $Q_{O_2}^{Ar}$; ○, $Q_{CO_2}^{Ar}$.

Under aerobic conditions much more carbohydrate disappeared in the period of the experiments than under anaerobic conditions (Tables 2 and 3). The experiments with the respiration-deficient yeast, in which the small oxygen uptake made any direct oxidation negligible, indicated that the increased carbohydrate breakdown was due to the alcoholic fermentation being stimulated by oxygen.

In experiments with the parent yeast the effect of oxygen was not so clear. The oxygen uptake was much higher than in the respiration-deficient mutant but the high initial R.Q. (Fig. 1) and the

release of ethanol indicated that the fermentation was continuing. Table 2 shows that the ethanol released during the 15 min. equilibration period was as expected from the Gay-Lussac equation, but the increase in ethanol during the subsequent hour was only about 20 % of that expected from carbohydrate which disappeared.

The similar effect of aerobic conditions on the carbohydrate loss in both the normal and respiration-deficient yeast suggests that a similar mechanism was operating in both yeasts. The low ethanol production in the normal yeast under aerobic conditions might have been due to the oxidation of NADH by oxygen. The reduction of some of the acetaldehyde to ethanol might have been prevented. Another possible reason for the low yield of ethanol was that some might have been oxidized during the experiment.

Changes in carbohydrate fractions. Larger-scale experiments carried out under aerobic conditions showed that the carbohydrate loss was due to the disappearance of both trehalose and glycogen from the yeast. Over a period of 5 hr. at 28° the glycogen content fell from 410 to 154 μg . of hexose equivalent/mg. dry wt. (decrease 63 %) and the trehalose content fell from 35.5 to 17 μg . of hexose equivalent/mg. dry wt. (decrease 52 %).

Oxidation of ethanol. Eaton & Klein (1957) showed that when 'young' cells of yeast oxidized glucose, ethanol was first formed and then disappeared, and acetate accumulated before being itself oxidized. Examination of the gaseous exchanges (Fig. 1) measured in the anaerobically grown brewing yeast suggested that the reserve carbohydrate was being degraded by a similar pathway. Over a period of several hours the R.Q. dropped from a high initial level to about 0.7 and subsequently rose to 1.0. The rise to 1.0 coincided with an increased rate of release of carbon dioxide. The low R.Q. would be expected if the ethanol was being respired and the R.Q. of 1.0 suggested that a substrate of the oxidation level of acetate was being respired. To check this, ethanol and acetate were measured in the suspension at intervals during the dissimilation of the reserve material (Fig. 2). After an initial accumulation of ethanol it disappeared completely from the suspending medium. As the ethanol was removed a small amount of acetate accumulated in the suspension. The relatively small acetate accumulation might have been due to the rate of acetate oxidation (or utilization by other pathways) being only slightly less than its formation.

Rate of respiration during dissimilation of reserve carbohydrate. The rate of respiration increased during the metabolism of endogenous reserves (Figs. 1, 2) and then decreased as the ethanol and acetate disappeared from the suspension. The fact

that this decrease in respiration coincided with the fall in ethanol and acetate concentrations was additional evidence that ethanol and acetate were the substrates of the respiration.

To find whether respiratory-enzyme synthesis was involved in the increase in respiration, experiments were carried out with inhibitors of protein synthesis. *p*-Fluorophenylalanine and chloramphenicol partially inhibited this increase in respiration, which suggested that enzyme synthesis was involved (Figs. 3, 4).

To confirm that respiratory-enzyme synthesis did take place, measurements were made of cytochrome-oxidase activity in cell extracts prepared from yeast immediately after harvesting and for 5 hr. after aeration in 67 mM-potassium dihydrogen phosphate (pH 4.5) at 25°. The oxidase activity increased considerably during aeration (activity after harvesting, 40 μl . of oxygen/hr./mg. of protein; final activity was 240 μl . of oxygen/hr./mg. of protein), confirming that cytochrome oxidase had been synthesized.

Dissimilation of acetate. Comparison of the total oxygen uptake during the period of manometric readings with the total carbohydrate breakdown from the time of preparing the suspensions showed that, even when the R.Q. reached 1.0 and the rate of respiration had fallen sharply, the oxygen uptake was only sufficient to account for the complete oxidation of just over 50 % of the carbohydrate; 2.11 μmoles of hexose equivalent of carbohydrate disappeared during equilibration and 5.6 μmoles were broken down during the manometric readings, whereas only 24.88 μmoles of oxygen were taken up (Table 3). The oxygen uptake during the first few minutes after equilibration of the manometer flasks was very small and it was assumed from this

Fig. 2. Changes in concentrations of ethanol and acetate in suspensions in manometer flasks. Each flask contained 8.3 mg. of anaerobically grown yeast in 3 ml. of 67 mM- HK_2PO_4 . $\square$, $Q_{6\text{Ar}}$; $\bullet$, ethanol; $\circ$, acetate.

that the oxygen uptake during equilibration was negligible. The difference between the oxygen uptake and the carbohydrate loss suggested that some of the products of the carbohydrate breakdown were being diverted to other metabolic pathways. To check this, acetate was added to samples of yeast taken at intervals from manometer flasks during the period of respiratory adaptation. The yeast was washed and resuspended in either buffer alone to measure the endogenous uptake of oxygen or in buffer plus acetate to measure the oxidation of acetate (Fig. 5). A sample of yeast taken 5 min. from the start of the experiment showed very little ability to oxidize acetate during the first hour of measurement, but acquired the ability while in the acetate solution. The later samples showed greater oxidative ability in the first hour of measurement. The rate of oxygen uptake decreased in each case to the endogenous value before sufficient oxygen had been taken up to oxidize completely the added acetate.

It is possible that the acetate which was not oxidized was used for the synthesis of reserve material in the cell. This suggestion is supported by the observation of Smedley-MacLean & Hoffert (1923) that aerated suspensions of brewing yeast formed fat at the expense of cell carbohydrate. They also showed that fat formation was greater if ethanol, acetate, lactate or pyruvate was added to the suspension. In the present work fat formation

may be the fate of part of the degradation products of the yeast carbohydrate and of the rest of the acetate in the acetate-oxidation experiments.

Initiation of fermentation of carbohydrate reserves. The rapid disappearance of reserve carbohydrate after harvesting the anaerobically grown cells suggested that an autolytic process had been started by damage to the cells during the washing procedure. However, when the yeast was treated with methylene blue, only 3 % of the cells were stained, indicating that most of the cells were viable. During the experiments there was less than 2 % decrease in viability. These viability measurements were confirmed by plating the washed cells on nutrient agar. No amino acid release was observed and enzyme synthesis continued in the cells. It was concluded from these results that the fermentation of reserve carbohydrate was not due to autolysis.

The carbohydrate breakdown might have been initiated by contact with oxygen during the harvesting procedure. To investigate this, anaerobically grown yeast was shaken in its own growth medium after the flasks had been flushed for 40 min. with either nitrogen or air. The carbohydrate content of the yeast in the flasks containing nitrogen decreased by only 5 % in 3 hr. at 28°, whereas the carbohydrate of the yeast shaken in air decreased by 30 % in about 15 min. (Fig. 6). The oxygen taken up during this period was only sufficient to account for about 10 % of the break-

Fig. 3. Effect of chloramphenicol on endogenous respiration. Each flask contained 6.8 mg. of anaerobically grown yeast in a final volume of 3 ml. of buffer. The chloramphenicol solution in 67 mM- KH_2PO_4 was placed in the main compartments of the flasks. □, Control; ○, 50 µg. of chloramphenicol/ml.; ◐, 500 µg./ml.; ●, 750 µg./ml.

Fig. 4. Effect of *p*-fluorophenylalanine on endogenous respiration. Each flask contained 6.2 mg. of anaerobically grown yeast in a final volume of 3 ml. of buffer. The *p*-fluorophenylalanine solution in 67 mM- KH_2PO_4 was placed in the main compartment of the flasks. □, Control; ●, 1500 µg. of *p*-fluorophenylalanine/ml.

Fig. 5. Acetate oxidation by yeast after metabolism of endogenous reserves for increasing periods of time. Anaerobically grown yeast (7.8 mg.) in phosphate buffer was added to each of several manometer vessels and allowed to metabolize aerobically its reserve material. After 5, 95 and 185 min. the cells were removed, washed once with buffer solution and resuspended in 3 ml. of buffer or in 3 ml. of buffer plus 7.5 μmoles of acetate. Yeast was examined (a) after adaptation for 5 min., (b) after 95 min., (c) after 185 min. ●, Endogenous O₂ uptake; ○, O₂ uptake in the presence of acetate.

Fig. 6. Carbohydrate degradation when anaerobically grown yeast was shaken aerobically and anaerobically in its own growth medium. Cells were grown in 3 ml. of medium in stationary manometer flasks for 72 hr. After flushing with air or N₂ the flasks were shaken at 28° and removed at intervals for analysis. The yeast was washed once with chilled water and 3 ml. of M-trichloroacetic acid added to it. ●, Yeast from aerobic flasks; ○, yeast from anaerobic flasks.

down. It was concluded from these experiments that contact with oxygen during harvesting started the fermentative breakdown of the carbohydrate reserves.

When the experiments already described (Tables 2 and 3) are reconsidered in the light of these conclusions it seems probable that the endogenous fermentation obtained under anaerobic conditions was initiated during the harvesting procedure. This would account for the same amount of carbohydrate disappearing in the aerobic and anaerobic flasks during the gassing and equilibration of the flasks.

Mechanism of oxygen effect on carbohydrate reserves. The mechanism by which oxygen stimulates the disappearance of reserve carbohydrate is obscure. Respiration via the full cytochrome system is not necessary since the effect is shown by mutants deficient in cytochrome oxidase and also by freshly harvested normal cells in which the cytochrome-oxidase content is low. Anaerobically grown respiration-deficient mutants have a small residual endogenous respiration and it seems probable that this small oxygen uptake is linked with the endogenous fermentation since both are increased by increased partial pressure of oxygen (Chester, 1959b). The terminal oxidase involved in this respiration is not known.

When glucose was fermented by a suspension of washed cells of anaerobically grown yeast, the carbon dioxide release in the first 60 min. of the experiment was exactly the same under aerobic and anaerobic conditions and much greater than the endogenous fermentation ($Q_{CO_2}^{N_2}$ and $Q_{CO_2}^{Ar}$ were both 180, compared with the endogenous $Q_{CO_2}^{N_2}$ and $Q_{CO_2}^{Ar}$ values of 16 and 28 respectively). Glucose disappearing from the suspension aerobically was the

Table 4. *Aerobic and anaerobic fermentation of glucose*

Each manometer flask contained 5.9 mg. of anaerobically grown yeast in 67 mM-KH₂PO₄ (pH 4.5). After gassing and equilibration of the flasks, 100 μ moles of glucose dissolved in 0.5 ml. of phosphate buffer were tipped from the side arms. Results are expressed in μ moles. The glucose equivalent to the CO₂ released is calculated on the assumption that the CO₂ was formed from the alcoholic fermentation of the glucose under both aerobic and anaerobic conditions.

Gas in manometer flasks	Time (min.)	Changes per flask			
		Uptake of O ₂	Glucose metabolized	Release of CO ₂	Glucose equivalent to CO ₂
N ₂	30	—	25	23.8	11.9
	60	—	34.4	47.7	23.8
	90	—	46.0	69.2	34.6
Air	30	1.4	25	24.3	12.1
	60	3.5	36	47.5	23.7
	90	6.3	44.5	70.6	35.3

same as anaerobically but was greater than the amount calculated from the carbon dioxide release (Table 4). This discrepancy may have been due to some of the glucose being used for the synthesis of cell carbohydrate, since Stier & Newton (1939) and Fales (1951) have reported accumulation of cell carbohydrate by resting cells in glucose solution.

In the Embden-Meyerhof-Parnas fermentation pathway, glycogen is broken down and phosphorylated to glucose 1-phosphate, which is then converted into glucose 6-phosphate. When glucose is fermented, it is phosphorylated directly to glucose 6-phosphate. The enzymic pathways for the fermentation of glycogen and glucose differ only up to the formation of glucose 6-phosphate. The observation that the fermentation of glucose was not limited by the absence of oxygen suggests that the oxygen effect on the breakdown of endogenous carbohydrate is exerted before the formation of the glucose 6-phosphate.

Another aspect of the effect of oxygen on this yeast is the ready utilization of much of the large quantity of reserve carbohydrate accumulated during anaerobic growth compared with the very sparing use of the smaller reserves laid down during aerobic growth. It seems therefore that oxygen plays a part in the control of the synthesis of the reserves and also suggests that a control mechanism is operating in the aerobically grown yeast which regulates the utilization of the cellular carbohydrate.

SUMMARY

1. When a brewing strain of *Saccharomyces cerevisiae* was grown under substantially anaerobic conditions the carbohydrate reserves, glycogen and trehalose, made up 40 % of the cell dry weight.

2. Contact with oxygen initiated alcoholic fermentation of these carbohydrate reserves. The fermentation continued at a faster rate under aerobic than anaerobic conditions. A complete

cytochrome system was not necessary for this effect of oxygen since it occurred in a respiration-deficient mutant. The yeast fermented glucose at the same rate under both aerobic and anaerobic conditions.

3. In aerobically grown yeast, the carbohydrate reserves made up less than 10 % of the yeast dry weight. The trehalose fraction was very small. Measurement of the endogenous gaseous exchanges indicated that there was very little utilization of the reserve carbohydrate in the yeast grown in this way.

4. The yeast reserves provided a sufficient energy source for the synthesis of respiratory enzymes during the aeration of anaerobically grown resting cells. Evidence is presented which indicates that part of the degradation products of the yeast carbohydrate was oxidized and the rest used for the synthesis of long-term reserves.

I thank the Directors of Arthur Guinness, Son and Co. (Park Royal) Ltd. for permission to publish this paper and Dr T. C. N. Carroll for his interest during this work. I am also indebted to Mrs Ursula M. Hodges for technical assistance.

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Biochem. J. (1963) **86**, 160

Flavin Requirement and Partial Separation of Enzymes Catalysing the Reduction of Folic Acid to Tetrahydrofolic Acid in Liver

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(Received 26 February 1962)

The conversion of folic acid into tetrahydrofolic acid by liver enzymes is mediated by NADPH₂ (Futtermann, 1957). Many of the enzymes reoxidizing reduced nicotinamide dinucleotides are flavo-proteins (Mahler, 1956), and recent observations (Braganca & Kenkare, 1959) have indicated that flavins are also components of the system transforming folic acid into tetrahydrofolic acid in mammalian liver.

The question whether more than one enzyme protein participates in the reduction of folic acid to tetrahydrofolic acid is still controversial. Earlier observations (Futtermann, 1957; Osborn & Huenekens, 1958; Peters & Greenberg, 1958) suggested that the reduction of folic acid to tetrahydrofolic acid in chicken liver is mediated by two different enzymes catalysing the reduction of folic acid and dihydrofolic acid respectively [5,6,7,8-tetrahydrofolate-NAD(P) oxidoreductase, EC 1.5.1.3, and 7,8-dihydrofolate-NADP oxidoreductase, EC 1.5.1.4]. Recently the failure to separate the two component activities in chicken-liver preparations and the similarity in some of the properties of the systems reducing folic acid and dihydrofolic acid have led Zakrzewski & Nichol (1960) to claim that a single enzyme catalyses the reduction of both these substrates.

We now describe methods for the separation of the enzyme system converting folic acid into dihydrofolic acid from that which reduces dihydrofolic acid. Studies on the selective effects of flavins and flavin analogues also conform to the view that the two-stage conversion of folic acid into tetrahydrofolic acid requires two separate

enzymes. One of these steps may be mediated by a flavoprotein in which FMN or FAD can function as the flavin moiety.

MATERIALS AND METHODS

Isocitrate dehydrogenase from pig heart. The procedure of Ochoa (1955) was followed up to the end of the first stage of purification.

Acetone-dried powder of sheep liver. This was prepared according to the method of Kaplan & Lipmann (1948).

Assay of enzymes reducing folic acid and dihydrofolic acid

Diazotizable-amine method. This is described by Futtermann & Silverman (1957). It depends on the fact that solutions of tetrahydrofolic acid are unstable and quantitatively liberate *p*-aminobenzoylglutamic acid in the presence of air. This is estimated, as aromatic amine, by the method of Bratton & Marshall (1939).

Spectrophotometric method. NADP, sodium citrate, phosphate buffer, pH 5.5, and an extract containing isocitrate dehydrogenase were added to a 1 cm. cuvette in a Beckman spectrophotometer, model DU. The total volume was 2.7 ml. The reaction was allowed to proceed at 24° until about 70% of the NADP was reduced (ΔE_{340} approx. 0.26). At this stage 0.3 ml. of a mixture of CaCl₂ and ascorbic acid was added. The CaCl₂ trapped the excess of citrate and so prevented further reduction of NADP; ascorbate prevented the spontaneous decomposition of tetrahydro- and dihydro-folic acids (Futtermann & Silverman, 1957; Futtermann, 1957). During the following 5 min. E_{340} remained constant. The substrate (folic acid or dihydrofolic acid) was then added, when a rapid fall in E_{340} ensued. A blank cuvette was set up containing everything except NADP. The concentrations of the components in the reaction mixture are given in Table 1. Enzyme activities