the folding of a peptide chain within a protein may decrease the reactivity of α- or ε-amino groups by rendering them less accessible; on the other hand, charged groups may be close to a particular amino or imidazole group, and thereby enhance its reactivity. The distinction between 'reactive' and 'unreactive' groups in a protein is best established by a careful investigation, preferably involving degradation of the modified protein and analytical studies on the isolated peptides. In this way, the extent and sites of a reaction can be determined with precision. Methods involving measurement of the disappearance of the reagent (Alexander, 1958), in contradistinction to measurement of the formation of a derivative, provide only circumstantial information on the nature of the reaction.

SUMMARY

1. The rates of reaction of *N*-ethylmaleimide with the α-amino groups of three peptides are reported.

2. Some observations are presented on the isomerism of the addition products of *N*-ethylmaleimide with thiol compounds.

3. A detailed analysis of the products of hydrolysis of these derivatives has been performed. A method for the determination of the degree of reaction of *N*-ethylmaleimide with the thiol groups of proteins is proposed, together with a means of assessing the specificity of the reaction.

4. The results are of significance in the use of *N*-ethylmaleimide as a reagent for the introduction of chemical modifications in proteins.

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Development of Respiration in Yeast Grown Anaerobically on Different Carbon Sources

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This paper describes the aerobic behaviour of yeast, previously grown anaerobically on a number of different carbon sources. The results show that yeast grown anaerobically on galactose retains its respiratory activity whereas the yeast grown similarly on glucose loses it.

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METHODS

Maintenance and growth of yeast. Saccharomyces cerevisiae strain no. 77 of the National Collection of Yeast Cultures (Brewing Industry Research Foundation, Nutfield, Surrey) was used in the present work. The organism was maintained aerobically on agar slopes containing inorganic salts, 2·25% (w/v) of Difco malt extract, 0·05% of Difco yeast extract and 0·5% of sucrose. The yeast was grown for 48 hr. at 30° and subcultured monthly.

Growth of anaerobic cells. The medium for the anaerobic bulk growth of the yeast contained (per l.): glucose, 20 g.;

Difco yeast extract, 10 g.; Oxoid casein hydrolysate (acid), 5 g.; KH_2PO_4, 9 g.; $CaCl_2$, 0·3 g.; $MgSO_4,7H_2O$, 0·5 g.; $(NH_4)_2SO_4$, 6 g.; 70–72% (w/w) sodium lactate, 5 ml.; wheat-germ oil, 0·15 ml.; Tween 80, 5 ml.; ergosterol dissolved in ethanol, 20 mg. in 5 ml. The inoculum of yeast was 0·93 mg. dry wt. of cells/g. of hexose in the medium. Growth was in a wide-based conical stoppered flask (Fernbach flask) with a gas inlet and outlet which was continuously flushed with a slow stream of oxygen-free nitrogen and shaken at 30°. The harvested cells were washed three times with ice-cold water and finally suspended in water at a concentration of 100 mg. wet wt./ml.

Adaptation of cells to respiration. Usually this was done in Warburg vessels at 25°. The vessels contained KH_2PO_4 (66 mM) and usually glucose (33 mM). Casein hydrolysate, when present, was added at 20 mg./vessel. The aqueous suspension of yeast (0·2 ml.; 4 mg. dry wt.) was added to the side arm of the vessel. The final volume was 3·0 ml., and the centre well contained 0·2 ml. of 10N-NaOH and filter paper. The yeast was tipped from the side arm after 10 min. of equilibration at 25° and the subsequent gas changes were measured over several hours. The Q_{O_2} values given are calculated over a 15 min. period at the times indicated. Occasionally the yeast was aerated in bulk in a Fernbach flask and samples were removed periodically for analysis. When carbon dioxide production was to be measured the vessels were gassed with nitrogen and the centre well contained a stick of yellow phosphorus.

Analytical methods. A biuret technique for the determination of protein was designed by Professor M. F. Utter for use with yeast suspensions. To 10 ml. of yeast suspension was added 1 ml. of 10% (w/v) trichloroacetic acid. The denatured material was centrifuged, resuspended in 4 ml. of 10% (w/v) NaOH and the mixture was shaken mechanically for 15 min. After standing at room temperature overnight the undissolved material was removed by centrifuging and 0·38 ml. of 1·0% (w/v) $CuSO_4$ was added to 3·0 ml. of the supernatant. The solution was mixed and after 20 min. the extinction was measured at 560 mμ. Bovine serum albumin was used as a standard.

Ethanol was measured with alcohol dehydrogenase by the method of Bonnichsen & Theorell (1951), and glucose with glucose oxidase (Huggett & Nixon, 1957). Dry weights of the yeast were measured on the twice-washed suspensions by drying overnight at 105°. Since the dry weights of the yeast changed during the adaptation period, the Q_{O_2} values were calculated with an interpolated value of the dry wt. at the appropriate time, assuming a linear change between the measured initial and final dry weights.

Cytochrome *c* oxidase was measured according to the method of Minnaert (1961) and the activity is expressed as the first-order rate constant (Smith, 1955).

Special chemicals. NADH, glucose oxidase, peroxidase and yeast alcohol dehydrogenase were from Boehringer und Soehne G.m.b.H. (Mannhein, Germany); cytochrome *c* (type III) was from Sigma Chemical Co. (St Louis, Mo., U.S.A.); Tween 80 was from L.Light and Co. Ltd.(Colnbrook, Bucks.). Anhydrous D-galactose was obtained from Thomas Kerfoot and Co. Ltd. (Vale of Bardsley, Lancs.). It was free of material estimated as glucose by the glucose-oxidase test of Huggett & Nixon (1957) and it had a specific rotation $[\alpha]_D^{20}$ of 70·0°. Fructose was reagent grade.

RESULTS

Comparison of the anaerobic growth of yeast on glucose, fructose or galactose. Fig. 1 shows the time-course of the anaerobic growth of yeast with the three hexoses. The growth curves with glucose and with fructose were almost identical. With galactose there was a delay of about 14 hr. before growth started. The slope of the exponential phase of growth was slightly less with galactose than with the other two sugars but the final yield of cells was the same with all three sugars. Under aerobic conditions, as Strittmatter (1957) showed, there is almost twice the yield when the cells are grown on galactose compared with the yield with glucose; since the glycolytic energy available from the anaerobic breakdown of the three sugars is almost identical the same growth yield is to be expected. Presumably the prolonged lag period before growth commenced on galactose is a measure of the time required for the adaptive synthesis of the enzymes necessary for the initial steps in metabolizing galactose.

Effect of the physiological age of yeast cells grown anaerobically on galactose on the ability to oxidize glucose. Yeast grown anaerobically on galactose did not lose its ability to oxidize glucose, and the initial Q_{O_2} values with glucose as substrate were about 40 at all stages of growth (Table 1). The abrupt change in the rate of respiration found with yeast grown anaerobically on glucose giving a respiratory 'peak' was not so marked and increased in magnitude as the cells were harvested later in the growth cycle. There was little or no ability to oxidize acetate at any stage of the growth cycle.

Respiration of yeast on different substrates. Table 2 illustrates the range of substrates oxidized by the yeast grown anaerobically on galactose and the

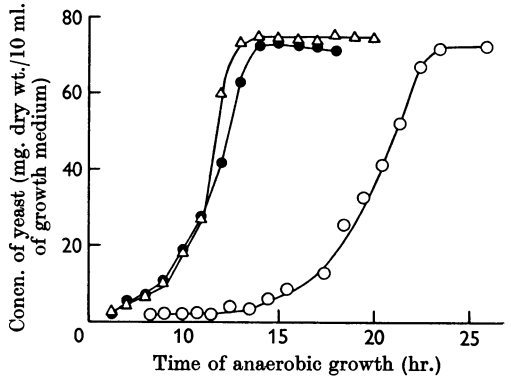

Fig. 1. Increase in weight of yeast with time when grown anaerobically on glucose (●), fructose (△) or galactose (○). Experimental details are given in the text.

change with time of the magnitude of the respiration. In all cases the rate of respiration increased with the time of incubation. The increase in respiration with time was much slower with the sugars as substrate, so that after 2 hr. the oxidation with lactate had already surpassed that with the two sugars; after 3 hr. the oxidations with lactate and pyruvate were faster, and after 4 hr. the oxidations with lactate, pyruvate and ethanol all substantially exceeded the respiration with the hexoses.

Effect of casein hydrolysate on the respiration of yeast grown anaerobically on galactose. When yeast is grown anaerobically on glucose, respiration is lost. The subsequent exposure of the yeast to oxygen results in the development of respiration with a time lag which depends on the composition of the medium and the previous history of the yeast. Tustanoff & Bartley (1964) found that the respiration that developed under their experimental conditions was very low unless a source of amino acids (usually acid-hydrolysed casein) was added to the medium. The effect of acid-hydrolysed casein on the respiration of the yeast cells grown anaerobically on galactose was therefore tested to see whether the anaerobic conditions of growth had in any way limited respiration. With the sugars as substrates casein hydrolysate increased respiration substantially (threefold increase with galactose over 4 hr. and somewhat less with glucose). With pyruvate or ethanol casein hydrolysate had no effect, but with lactate the respiration was increased, and as with the sugars produced the typical respiratory peak observed by Tustanoff & Bartley (1964) with yeast grown anaerobically on glucose and subsequently incubated aerobically. It is possible that the effect of casein hydrolysate in stimulating respiration may be related to the stimulation of glycolysis that casein hydrolysate brings about (see the next section).

Effect of casein hydrolysate on the anaerobic production of carbon dioxide from glucose by yeast grown anaerobically on galactose. This was tested since Tustanoff & Bartley (1964) showed that, with cells grown anaerobically on glucose, casein hydrolysate increased greatly the production of glycolytic carbon dioxide. Similar tests with the yeast grown on galactose gave the same effect (Fig. 2). Whereas the anaerobic production of carbon

Table 1. *Effect of the physiological age of yeast cells grown anaerobically on galactose and their ability to oxidize glucose*

The yeast was grown anaerobically on 5·4% galactose (see Fig. 1) medium at 30°. Samples were removed for testing at the times given. Respiration was measured for 4·5 hr. at 25°. The incubation medium contained glucose (33 mM), KH_2PO_4 (66 mM) and hydrolysed casein (7 mg./ml.). The final volume was 3·0 ml. The amount of yeast used was 4 mg. dry wt.

Time of harvesting (hr.)	Q_{O_2} at 30 min.	Q_{O_2} at 60 min.	Q_{O_2} at 120 min.	Maximum Q_{O_2} achieved	Time of maximum Q_{O_2} (hr.)
12	40	40	40	42	—
14	38	42	52	55	2·5
18	40	40	45	64	3·0
20	42	40	40	65	4·0
22	38	36	46	72	2·5

Table 2. *Effect of casein and substrate on the respiration of yeast grown anaerobically on galactose*

Yeast was grown anaerobically on galactose for 18·5 hr. at 30°. After harvesting and washing, test of respiration was made with or without casein hydrolysate (7 mg./ml.) on the substrates given below. The same amount of carbon was added for each substrate (final concentrations: glucose and galactose, 33 mM; lactate and pyruvate, 66 mM; ethanol, 100 mM). The Q_{O_2} without substrate was less than 2. Respiration was tested at 25°.

	Q_{O_2} at 1 hr.		Q_{O_2} at 2 hr.		Q_{O_2} at 3 hr.		Q_{O_2} at 4 hr.	
Substrate	Without casein hydrolysate	With casein hydrolysate	Without casein hydrolysate	With casein hydrolysate	Without casein hydrolysate	With casein hydrolysate	Without casein hydrolysate	With casein hydrolysate
Galactose*	23	23	22	35	27	72	32	70
Glucose*	23	22	29	37	30	55	31	54
Lactate*	18	19	35	30	46	42	55	58
Pyruvate	18	18	25	25	39	35	46	50
Ethanol	15	20	22	27	31	31	40	44

* Galactose induced a peak of respiration of 82 at 3 hr., glucose one of 63 at 2·75 hr. and lactate one of 62 at 3·75 hr. in the presence of casein hydrolysate.

dioxide without casein hydrolysate showed a steady decline, the production of carbon dioxide in the presence of casein hydrolysate rose to twice the rate after 1·75 hr. and declined to zero after 2·75 hr.

Effect of casein hydrolysate on the aerobic consumption of glucose and production of ethanol by yeast grown anaerobically on galactose. Fig. 3 shows the time-course of utilization of glucose and of production of ethanol. The consumption of glucose under both conditions was linear for the first 2 hr. and was about 30 % faster when casein hydrolysate was added. Under both conditions the time-course of ethanol production followed a sigmoid curve, but more ethanol accumulated when casein hydrolysate was present. The delay in obtaining maximum ethanol accumulation is presumably due to the time taken to build up acetaldehyde concentration which can effectively compete with the electron-transport chain for NADH.

Changes in respiration and cytochrome c-oxidase activity of yeast during oxidation of glucose after anaerobic growth on galactose. Table 3 shows that, when casein hydrolysate was omitted from the adaptive medium, respiration did not increase with time and the cytochrome *c*-oxidase activity declined. When the casein hydrolysate was present, respiration always increased with time and cytochrome *c*-oxidase activity remained constant after

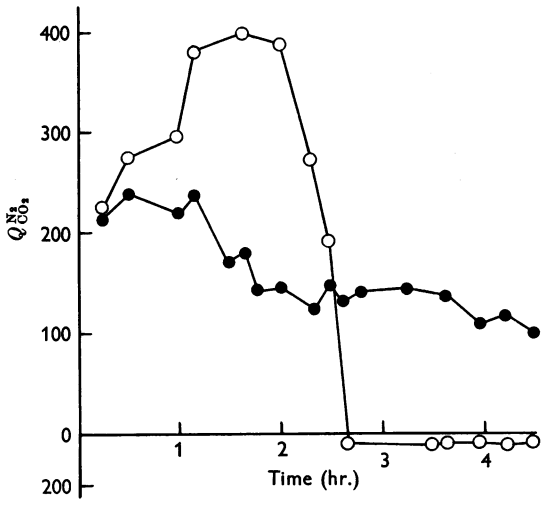

Fig. 2. Effect of casein hydrolysate on the anaerobic production of carbon dioxide from glucose by yeast grown anaerobically on galactose. Experimental details are given in the text. ○, Casein hydrolysate present; ●, casein hydrolysate absent.

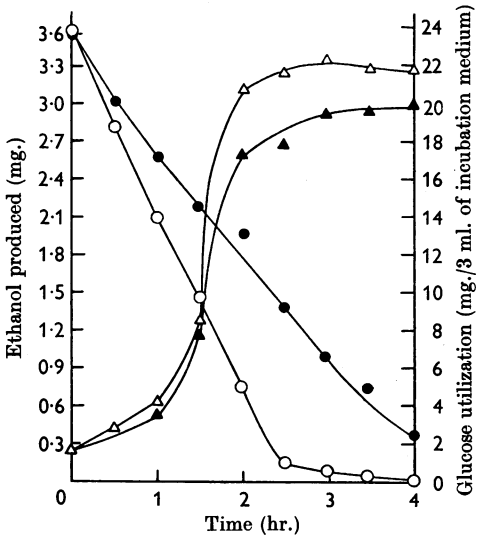

Fig. 3. Effect of casein hydrolysate on the consumption of glucose (○, ●) and the steady-state concentration of ethanol (△, ▲) by yeast grown anaerobically on galactose. Experimental details are given in the text. ○, △, Casein hydrolysate present; ●, ▲, casein hydrolysate absent.

Table 3. *Comparison of the changes in respiration and cytochrome c-oxidase activity of yeast cells during respiratory adaptation*

Cells were grown on 5·4 % galactose anaerobically for the times stated and respiration was measured in the presence of 33 mM-glucose with or without casein hydrolysate (7 mg./ml.). *K* was measured at room temperature (about 25°) and respiration at 25°.

Expt. no.	Time of growth of cells (hr.)	Casein hydrolysate	Initial *K*	*K* at 30 min.	Q_{O_2} at 30 min.	Percentage of Q_{O_2} or *K* at 30 min. At 1 hr.	At 2 hr.	At 3 hr.	At 4 hr.	Time of peak respiration (min.)
1	18·5	+	—	—	18	122	205	305	300	350
2	18·5	+	0·002	0·0023	—	243	227	205	205	222
3	21	+	—	—	21	105	133	186	176	395
4	21	−	—	—	24	108	92	88	96	—
5	21	+	0·0041	0·0046	—	78	83	72	100	72
6	21	−	0·0041	0·0029	—	100	72	33	31	—

the first 30 min. of incubation. There is thus no clear correlation between oxygen uptake and cytochrome *c*-oxidase activity. For example, in the absence of the casein hydrolysate the respiration remained constant in spite of the loss of two-thirds of the cytochrome *c*-oxidase activity. In the cells grown on galactose it appears that cytochrome *c*-oxidase activity never limits respiration. The cytochrome *c*-oxidase activity was greater initially in the yeast grown anaerobically on galactose than the maximum developed on adaptation by the yeast grown anaerobically on glucose (see Tustanoff & Bartley, 1964). From the present experiments and those of Tustanoff & Bartley (1964) with yeast grown on glucose it appears that cytochrome *c* oxidase is an enzyme whose activity can change rapidly during the life of the yeast. It may be suggested that cytochrome *c* oxidase is an enzyme with a high rate of turnover with its rate of synthesis inhibited by the presence of glucose. The inhibition of synthesis by glucose may be overcome in the presence of amino acids, as shown by the present experiments and those of Tustanoff & Bartley (1964).

Comparison of the properties of yeast cells grown anaerobically on fructose or galactose. Yeast grown anaerobically on fructose could develop respiration on glucose and fructose but not on galactose or pyruvate. It thus resembles yeast grown anaerobically on glucose (E. R. Tustanoff & W. Bartley, unpublished work). The cells grown on fructose showed the same changes of respiration with glucose as substrate as cells grown on glucose (see Table 2 of Tustanoff & Bartley, 1964). The 'peak' of respiration occurred at the same time (2½ hr.) as with the cells grown anaerobically on glucose. The cells grown on galactose by contrast could oxidize all three hexoses and pyruvate immediately. The respiration with the sugars increased with time and showed a respiratory 'peak'. The maximum rate of respiration was obtained with galactose as substrate throughout the incubation time. Cells grown on galactose oxidized fructose faster than glucose except for the first hour and during the brief period of the respiratory 'peak'.

DISCUSSION

Changes in cytochrome c-oxidase activity of yeast grown anaerobically on galactose. The cytochrome *c*-oxidase activity of the yeast cells providing the inoculum for growth was K 0·0121 sec.$^{-1}$ (expressed as the first-order rate constant; see Smith, 1955). During anaerobic growth on glucose cytochrome *c*-oxidase activity virtually disappears, but appears again on exposure to air in the presence of small amounts of glucose (Tustanoff & Bartley, 1964). The maximum activity obtained on adaptation was K 0·002 sec.$^{-1}$. When the yeast was grown anaerobically on galactose, cytochrome *c*-oxidase activity also decreased but at the end of growth was still present with K 0·003–0·0045 sec.$^{-1}$. This activity is considerably greater than that appearing in the glucose-grown cells after exposure to air. Since the weight of cells produced anaerobically is the same whether galactose or glucose is utilized for growth, it is probable that the number of generations of cells passed through is the same. For yeast cells grown on glucose this was shown to be five generations by Tustanoff & Bartley (1964). Thus the specific activity of the cytochrome *c*-oxidase would be expected to be only 3 % of that initially if no new enzyme were synthesized. The same conclusion is arrived at if the change in weight of cells with time (Fig. 1) is used for the calculation. Since the specific activity of the cytochrome *c* oxidase at the time of harvesting of the cells grown anaerobically on galactose is about 30 % of that initially, a continued large synthesis of cytochrome *c* oxidase must have occurred.

The rate of growth of yeast anaerobically on galactose is slower than the rate on glucose or fructose (Fig. 1) (approximate doubling-time on glucose 1·1 hr. and on galactose 1·7 hr.). In yeast synthesizing cytochrome *c* oxidase there are, then, similarities to the behaviour of *Escherichia coli* synthesizing β-galactosidase (Mandelstam, 1962), in that the repression varies inversely as the growth rate in the alternative substrate. It appears that glucose is acting as a repressor of cytochrome *c* oxidase and that the presence of oxygen is not necessary to induce the synthesis of the enzyme. This is in agreement with the findings of Tustanoff & Bartley (1964). The decrease in the specific activity of cytochrome *c* oxidase brought about during the anaerobic growth on galactose may be assumed to be a reflexion of the steady-state concentration of glucose (or a repressor derived from glucose) in the cells. It is here assumed that the rate of production of glucose from galactose is comparatively slow.

Substrates of respiration in anaerobically grown yeast. The number of substrates on which anaerobically grown yeast will develop respiration is limited. If the yeast has been grown on glucose, then only glucose (or possibly fructose) serves as a respiratory substrate; ethanol, pyruvate and acetate are not oxidized. Yeast grown anaerobically on galactose can respire on a wider range of substrates. Thus (Table 2) galactose, glucose, lactate, pyruvate and ethanol are all oxidized, though acetate is not. These observations, together with the results of Table 4, suggest that glucose (and fructose) prevents the synthesis of many dehydrogenases and the condensing enzyme as well as cytochrome *c* oxidase.

Table 4. *Comparison of the oxidation of glucose, fructose, galactose and pyruvate by yeast cells grown anaerobically on galactose or fructose*

Cells were grown on 5·4% of the sugar for 12 hr. for fructose and 21 hr. for galactose. Both types of cell were thus at the same physiological age. F, Yeast grown on fructose; G, yeast grown on galactose. The Q_{O_2} without substrate was less than 2. Respiration was measured at 25°.

	Q_{O_2} at 1 hr.		Q_{O_2} at 2 hr.		Q_{O_2} at 3 hr.		Q_{O_2} at 4 hr.		Maximum Q_{O_2}		Time of maximum Q_{O_2} (hr.)	
Substrate	F	G	F	G	F	G	F	G	F	G	F	G
Glucose	<2	20	14	28	28	80	37	43	60	95	2·5	2·75
Fructose	<2	17	8	30	47	88	34	52	47	88	3·0	3·0
Galactose	<2	23	<2	38	<2	106	<2	75	<2	106	—	3·0
Pyruvate	<2	21	<2	23	<2	36	<2	50	<2	50	—	4·0

SUMMARY

1. The weight of cells obtained when yeast is grown anaerobically is the same when any of the three sugars, glucose, fructose and galactose, is used as substrate.

2. Yeast grown anaerobically on galactose retains its ability to respire. The activity of cytochrome *c* oxidase in these cells is about one-third of that of the aerobic inoculum.

3. In non-growing cells, casein hydrolysate added to the medium caused the development of a more rapid respiration with time when galactose or glucose was the substrate but not when lactate, pyruvate or ethanol was the substrate.

4. Casein hydrolysate stimulated the consumption of galactose and the production of carbon dioxide by yeast grown anaerobically on galactose.

5. Yeast grown anaerobically on galactose could respire immediately on glucose, fructose, galactose or pyruvate, but yeast grown anaerobically on fructose oxidized none of these substrates immediately but could adapt to oxidize glucose or fructose.

6. The results are discussed in the light of the repressive action of glucose on the synthesis of oxidative enzymes.

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Variation of Activity of Bacterial Diaminopimelate Decarboxylase under Different Conditions of Growth

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The amino acid $\alpha\epsilon$-diaminopimelic acid is mostly confined in Nature to the mucopeptide component of the cell wall of certain bacteria and blue–green algae, where it is usually present as the *meso*- or the LL-isomer. In some species, diaminopimelic acid is replaced in the mucopeptide by lysine. However, even organisms that lack diaminopimelate may contain enzymes for which it is a substrate. One such enzyme is diaminopimelate decarboxylase (EC 4.1.1.20; *meso*-2,6-diaminopimelate carboxy-lyase), which converts *meso*-diaminopimelic acid into lysine (Dewey & Work, 1952). The enzyme is