matrix has certain advantages. Since adsorption is ruled out, choice of the inert material in the column becomes much simpler. The requirements for the material from which the gradient is formed are that it should be a non-electrolyte, unable to react with the substances being investigated and, in preparative work, easily separable from these substances. In the present work only sucrose and ethanol have been used for the gradients, but for other problems different materials might well be more suitable. The outstanding advantage of the gradient method, due to the relatively small surface area of solid exposed to the samples, has been illustrated in the present work by the experiments with slow-reacting substance A. Thus, whereas the ease with which this material is adsorbed has prevented useful electrophoresis in other zone systems, adequately sharp bands have now been obtained both in sucrose and in ethanol gradients. Thus it is probable that this type of zone electrophoresis will be of increasing usefulness in the separation of biologically active substances which are readily adsorbed on the matrix materials used for zone electrophoresis.

SUMMARY

1. A density-gradient electrophoresis apparatus somewhat modified from that designed by Svensson is described. Experiments have been carried out in gradients made from ethanol and from sucrose.

2. The reproducibility of movement and shape of bands of human albumin and the completeness of separation of this protein from a band of haemoglobin have been studied in the sucrose gradient.

3. The specific activities of the fractions thus obtained from human plasma albumin trace-iodinated with ^{131}I have been found to vary considerably and to show a maximum just preceding the maximum protein concentration as measured by ultraviolet-light absorption.

4. Insulin has been caused to migrate in both the sucrose and the ethanol gradients; in the latter the protein concentration curve possessed a shoulder.

5. Suitable conditions have been found for the electrophoresis of slow-reacting substance A, a substance causing constriction of the guinea-pig ileum.

We are grateful to Dr A. S. McFarlane and Dr S. Cohen for numerous discussions and for preparation of some of the protein samples.

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Assimilation of Carbon Dioxide by Yeasts

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The formation of carbon dioxide during glucose oxidation or fermentation by yeasts is well established, The fixation of carbon dioxide is less well known, though its existence is supported by the following evidence: (1) Carbon dioxide is necessary for the growth of yeasts (Rockwell & Highberger, 1927) and the synthesis of succinic acid by *Saccharomyces cerevisiae* (baker's yeast) (Kleinzeller, 1941). (2) Both carbon dioxide and dicarboxylic acids promote substrate oxidation by baker's yeast (Sperber & Runnström, 1939; Stoppani, 1951) but their actions are not additive. The replacement effect indicates that carbon dioxide is incorporated into dicarboxylic acids, which are the true stimulants of substrate oxidation. (3) During pyruvate oxidation in a medium

containing bicarbonate, baker's yeast fixes carbon dioxide (Runnström & Sperber, 1942). (4) Baker's yeast incorporates $^{11}CO_2$ (Ruben & Kamen, 1940) and $^{14}CO_2$ during glucose oxidation (Liener & Buchanan, 1951). (5) After pyruvate oxidation in the presence of $^{14}CO_2$, baker's-yeast amino acids contain radiocarbon and the labelling pattern indicates that the first fixation product is a C_4 intermediate (Davis, Cheldelin, Christensen & Wang, 1956).

Carbon dioxide is incorporated into cell metabolism by several reactions (cf. Utter & Wood, 1951). The condensation with a C_3 compound is one of the most important and leads to dicarboxylic acids which, through the citric acid cycle, promote cell respiration and liberation of energy (Wiame, 1957) or contribute to the synthesis of amino acids and other essential cell components (Roberts, Cowie, Abelson, Bolton & Britten, 1955; Wiame, 1957). Reactions of the citric acid cycle can be shown to take place in *S. cerevisiae*, either with living cells or cell-free preparations (cf. Wiame, 1957), and many workers claim that the cycle operates as in animal tissues (Martius & Lynen, 1950; Weinhouse & Millington, 1947; Barron, Ardao & Hearon, 1950; etc.). However, important effects dependent on the cycle operation cannot be demonstrated with baker's yeast (Krebs, Gurin & Eggleston, 1952), and on these grounds it is still a subject of controversy (Krebs, 1954) whether in this organism the cycle is the main pathway of substrate oxidation and energy liberation.

The operation of the citric acid cycle implies a flow of carbon through definite positions of the cycle intermediates and further, in aspartic and glutamic acid, two amino acids closely related to the cycle (Roberts *et al.* 1955). When $^{14}CO_2$ is used, the fixation reactions yield dicarboxylic acids (malic or oxaloacetic acid) labelled in C-4 and, accordingly, the aspartic acid formed by transamination of oxaloacetate would be expected to be labelled similarly in C-4. Also the position of ^{14}C in glutamic acid can be predicted as, on passing around the cycle, C-4 of oxaloacetic acid labels α-oxoglutaric (and glutamic) acid in C-1. Finally succinic acid should not contain ^{14}C if the cycle operates in one direction only, but partial equilibration of oxaloacetic with malic, fumaric and succinic acid, or the reverse operation of the cycle (or some of its reactions), may yield succinic acid symmetrically labelled in the carboxyl groups.

The experiments reported below have been planned in order, to establish (i) the mechanism of carbon dioxide fixation by *S. cerevisiae*, (ii) its metabolic role, and (iii) to test the validity of isotope-distribution models based upon the cycle operation in living yeast cells under different environmental conditions. Some complementary experiments have also been carried out with *Saccharomyces carlsbergensis*.

A preliminary account of this work has already appeared (Stoppani, de Favelukes, Conches & Sacerdote, 1957).

EXPERIMENTAL METHODS

Organisms and medium. Commercial preparations of baker's yeast were used in most of the experiments. Their bacteriological purity was checked and found satisfactory. Similar results were obtained with a pure strain of *S. cerevisiae* grown at 22° in a medium containing: malt extract (commercial preparation), 200 g.; Difco yeast extract, 3 g.; KH_2PO_4, 2 g.; $(NH_4)_2SO_4$, 0·5 g.; urea, 1 g. and glucose, 10 g., in 1 l. of tap water, under continuous aeration. The final pH of the medium was about 6·0. *S. carlsbergensis* was cultured from a pure strain in the same medium but without aeration. The yeast cells were washed with water in the centrifuge, suspended in water and starved by aeration at 22° for at least 12 hr. before use. All the operations were carried out under sterile conditions. The final concentration of the yeast suspensions was estimated in a photoelectric colorimeter by comparison with a standard suspension, the dry weight (100°) of which was established.

Enzymes. Carboxylase was prepared according to Green, Herbert & Subrahmanyan (1941) and yeast transaminase, as described by Bigger-Gehring (1955), from yeast acetone-dried powder; 50 mg. of acetone-dried powder was rubbed up with 0·1 M-phosphate buffer (pH 7·8; 1 ml.) and the extract dialysed against 0·02 M-KCl at 1–2° for 6 hr. The suspension was centrifuged and the clear supernatant tested for transaminase activity. Heart-muscle transaminase was the precipitate obtained at pH 4·2 from Cohen's (1940) transaminase preparation, but the grinding up of pig-heart muscle mince was replaced by homogenizing with the Waring Blendor.

Radioactive bicarbonate. This was obtained from The Radiochemical Centre, Amersham, Bucks. The specific activity was 1 mc/0·17 m-mole which under the conditions of radioassay used was equivalent to $1·18 \times 10^8$ counts/min. The radioactive bicarbonate was diluted to 5·8 ml. with water (CO_2-free).

Chemicals. Analytical-grade reagents were used throughout. Pyruvic acid was obtained by continuous ether extraction at pH 1 from sodium pyruvate prepared according to Robertson (1942). Acetaldehyde was distilled from a commercial preparation, dissolved in water and stored at 4°. The strength of the solutions was checked when necessary by the bisulphite method (Elliott, Benoy & Barker, 1935). Antimycin A was obtained from the Wisconsin Alumni Research Foundation (P.O. Box 2059, Madison 1, Wis., U.S.A.).

Manometric methods. Oxygen uptake and production of CO_2 were measured in the Warburg apparatus at the temperature stated in each case. Carbon dioxide was determined by the direct method. Each flask contained a total volume of 2 ml. of fluid.

Incubation techniques. In order to study the kinetics of $^{14}CO_2$ fixation, a closed system (cf. Stoppani, Fuller & Calvin, 1955) was used. The reaction chamber was a 125 ml. separating funnel with the drain tube cut obliquely at 5 mm. below the stopcock. The top orifice was closed by a

rubber bung pierced with a glass tube connected to a 10 ml. graduated glass syringe (volume compensator) lubricated with paraffin oil. Four reaction vessels were fixed on a shaking machine (used for Kahn tests) with the reaction chamber vertically and the volume compensator horizontally placed on the carriage. The substrate and radioactive bicarbonate were mixed successively with the yeast suspension in the reaction vessel. This was immediately closed with the stopper attached to the volume compensator, the plunger of which had been adjusted previously to the 10 ml. mark. Shaking was started and, after the required time, samples (about 1 ml.) of yeast suspension were taken by opening the stopcock of the separating funnel and pushing the plunger of the volume compensator to the corresponding mark. In this way a closed atmosphere was secured throughout the experiment, any losses of radioactive bicarbonate at the moment of taking the yeast samples being avoided. These precautions were necessary as, in most of the experiments, the initial pH of the yeast suspension was sufficiently acid to transform all the bicarbonate into CO_2.

For experiments with pure O_2 or N_2 in the gas phase, each reaction vessel was provided with a flushing system; the gas escaped through a steel needle inserted through the bung. The reagents were quickly added through the needle with a thinner needle attached to a calibrated syringe; in this manner almost air-tight conditions were maintained during the additions. The rest of the procedure was as described in the experiments with air.

The pH values of the incubation mixtures were measured with a glass electrode before the addition of the radioactive bicarbonate; the addition of the bicarbonate to the media of low pH raised the pH by about 0·3–0·4 when acetic acid or pyruvic acid was present and about 1·0 when phosphate buffer was the only electrolyte present. The values given are approximately the ones obtained after the addition of the bicarbonate. At an initial pH above 7·0 the influence of bicarbonate addition was far less important, as the increase was about 0·1–0·2. No significant variation in the yeast metabolism can be expected from differences of external pH in the ranges mentioned as the intracellular pH is largely independent of the medium pH (Krebs *et al.* 1952).

Analytical methods. The yeast samples were added to and thoroughly mixed with 9 ml. of methanol previously weighed in a tared 15 ml. centrifuge tube. The tubes were weighed, spun at 2500 rev./min. and the precipitate was discarded. The supernatants were placed in a vacuum system provided with solid CO_2–ethanol and liquid-air traps, and evaporated at low temperature to 0·4–0·8 ml. volume. The residues were chromatographed with the phenol–water and butanol–propionic acid–water solvent systems described by Benson *et al.* (1950). The chromatograms were radioautographed on X-ray film for 2–3 weeks and the radioactive compounds identified by cochromatography and radioautography with pure specimens.

Glutamic and aspartic acid were determined, after elution from the chromatograms, by the methods of Russell (1944) or Moore & Stein (1948). Eluates from non-radioactive areas were used as controls. Pyruvic acid was estimated according to Friedemann & Haugen (1943).

Preparation of radioactive samples. In order to measure the total ^{14}C fixed by the yeast cells, 300 μl. of the methanolic yeast suspension was evenly distributed on 3·8 cm.^{2} aluminium cups, evaporated to dryness on a turning table under a stream of hot air (Benson, 1949) and counted. When the pH of the incubation medium was high, the methanolic suspensions were acidified in order to remove the labelled CO_2. The thickness of the samples was always less than 0·5 mg./cm.^{2} and corrections for self-absorption were therefore unnecessary. After centrifuging the yeast suspension, 300 μl. of the clear supernatant was plated as above and counted. The activity represented the ^{14}C fixed in the methanol–water-soluble substances. The difference between this and the activity found in the total extracts corresponds to ^{14}C fixed in proteins and other materials insoluble in the methanol–water mixture.

The $BaCO_3$ obtained from total or partial degradation of radioactive compounds was filtered off on tared filter paper (Schleicher and Schull 589), washed twice with 10 ml. of CO_2-free water and 5 ml. of acetone, and dried under a stream of hot air, and placed on the aluminium cups and reweighed in order to calculate the weight of $BaCO_3$.

Degradation of radioactive compounds. Aspartic, glutamic and succinic acid were degraded in order to establish the intramolecular distribution of ^{14}C. The radioactive compounds eluted from the chromatograms, conveniently diluted with the respective carrier, were subjected to combustion with the mixture of Van Slyke, Plazin & Weisiger (1951) in an all-glass apparatus, based on the Lindenbaum, Schubert & Armstrong (1948) model. Six sets of apparatus were installed on a vacuum line provided with a 100–1 mm. Hg vacuum gauge. These modifications enabled the combustion train to be operated smoothly and the results were much more reproducible than with the original device. The CO_2 was precipitated as $BaCO_3$; this was washed twice with CO_2-free water, and filtered and mounted as described above. The C-1 of glutamic acid and the C-1 and C-4 of aspartic acid were quantitatively liberated at pH 2·5 with chloramine-T (Van Slyke, Dillon, MacFadyen & Hamilton, 1941). The operation was performed in Warburg manometers with 0·2 ml. of 5N-NaOH (carbonate-free) in the centre well (without filter paper). After all the CO_2 was fixed, the carbonate was transferred quantitatively to centrifuge tubes with 2 ml. of water, and 60 μmoles of carbonate carrier added and the carbonate precipitated as $BaCO_3$. The $BaCO_3$ was washed twice in the centrifuge with water, filtered off and mounted on paper disks. C-4 of aspartic acid was liberated by transamination of aspartate with α-oxoglutarate and decarboxylation of oxaloacetate with aniline at pH 5·0, a heart-muscle transaminase preparation (Krebs, 1950) being used. The CO_2 evolved was fixed in 0·2 ml. of 5N-NaOH placed in the centre well of the Warburg manometer and precipitated as $BaCO_3$ as in the decarboxylation with chloramine-T. C-5 of glutamic acid and the carboxyl carbons of succinic acid were liberated by the Schmidt reaction (Phares & Long, 1955).

After elution from the chromatograms, portions of the radioactive amino acids were diluted with carrier and subjected to degradation. The yield of CO_2 liberated was determined either by a separate manometric measurement (decarboxylation with chloramine-T or the transaminase–aniline system) or by the weight of the $BaCO_3$ (total combustion and Schmidt reaction), and corrections for losses were introduced when required. As the amount of carrier varied according to the degradation procedure, the activities obtained are not referred to a weight unit; this is un-

necessary because of the separate control of each degradation procedure. With aspartic acid, the activity (*a*) in C-4 was obtained from the CO_2 liberated with the transaminase–aniline system; the activity in C-1 by subtracting *a* from the activity in the CO_2 liberated with chloramine-T (*b*) and the ^{14}C in the amino and methylene carbons by subtracting *b* from the activity (*c*) in the CO_2 obtained after the total combustion. The percentage proportion of *a* is established in relation to *c*. With glutamic acid a similar system of calculation was used.

The specific activity of ^{14}C in the carboxyl groups of succinic acid was obtained directly from the CO_2 liberated with the Schmidt reaction and the specific activity in the methylene carbons by difference between the activity in the carboxyl groups and the total activity. Specific activities are expressed as counts/min./mg. of $BaCO_3$.

Assay of radioactive samples. Activity measurements were carried out with a Geiger–Müller end-window counter connected to a decimal scaler. The activity values were corrected for background, self-absorption and dead time of the counting system and are expressed as counts/min. at infinite thinness. All measurements were standardized with reference to a standard of ^{14}C to which 700 counts/min. was ascribed. Radioactive compounds were also counted directly on the chromatograms. The active areas were located with the aid of the radioautograph and counted with a Scott-type Geiger–Müller tube placed directly on the paper. The neighbouring radioactive areas were covered with masks. Activity values were corrected for background and dead time of the counting system and standardized as above. The number of counts recorded was enough to make the standard error less than 3%.

Expression of results. The ^{14}C fixed by the yeast cells (*R*), expressed as counts/min./mg. of cells (dry wt.), is calculated with equation (1), where 700 is the activity ascribed to the ^{14}C standard;

$$R = 700 \times C_y \times W_m \times V/(0{\cdot}81 \times v \times C_{st} \times W_y \times Y) \quad (1)$$

[C_y, counts/min. of yeast sample; W_m, the weight of the suspension of yeast cells in the 90% (v/v) methanol–water mixture; V, volume of the incubation mixture; 0·81, sp.gr. of the 90% (v/v) methanol–water mixture; v, volume of yeast suspension taken for the assay of radioactivity; C_{st}, activity found with the ^{14}C standard; W_y, weight of the sample of incubation mixture; Y, amount (mg.) of yeast used in the experiment]. It is assumed that the sp.gr. of the incubation mixture is 1·0 and that small variations in the volume of the sample of yeast suspension do not affect significantly the sp.gr. of the 90% (v/v) methanol–water mixture. The ^{14}C fixed in the methanol–water-soluble compounds is calculated in a similar way.

When the radioactivity was counted directly on the chromatograms the relative activity of each substance was calculated with the equation $A_X(\%) = (C_X/C_N) \times 100$, where C_X was the number of counts of substance X and C_N the total activity counted on the chromatogram. The ^{14}C fixed in X by 1 mg. of cells was obtained by multiplying A_X by the total ^{14}C fixed by 1 mg. of cells in the methanol–water-soluble substances.

RESULTS

Fixation of carbon dioxide by Saccharomyces cerevisiae *with acetate or acetaldehyde as substrates*

Fixation and distribution of $^{14}CO_2$. Baker's yeast incorporates carbon dioxide during acetate and acetaldehyde oxidation. The ^{14}C fixed depends on the amount of substrate oxidized (Table 1), which shows that besides possible exchange reactions the $^{14}CO_2$ incorporation is bound to metabolic processes. With acetate (Fig. 1) at the beginning of the experiment the oxygen uptake and carbon dioxide fixation are relatively slow, which corresponds to the 'induction period' of acetate oxidation

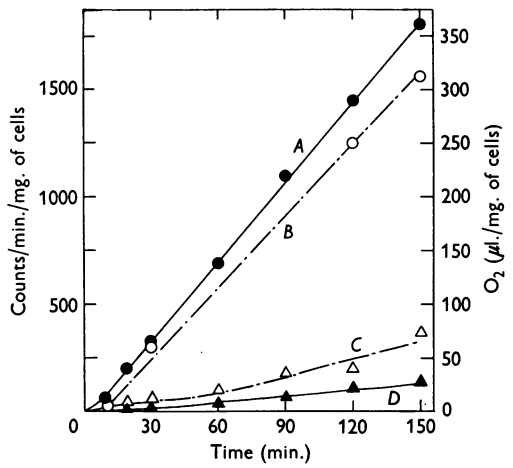

Fig. 1. Fixation of $^{14}CO_2$ and oxygen uptake of *S. cerevisiae* with acetate as substrate. Yeast, 21 mg./ml.; 20 mM-acetic acid; 0·4 mM-phosphate; pH 2·8 in the manometric experiment, 3·1 in the fixation experiment. In the fixation experiment 4·2 μmoles of $NaH^{14}CO_3$ ($3{\cdot}1 \times 10^6$ counts/min.). Total vol.: 2 ml. in the manometric experiment and 10 ml. in the fixation experiment. Temp. 30°. Air was in the gas space. *A*, Oxygen uptake with acetate and *D*, control. *B*, $^{14}CO_2$ fixation with acetate and *C*, control.

Table 1. *Oxygen uptake and* $^{14}CO_2$ *fixation by* Saccharomyces cerevisiae *with acetate and acetaldehyde as substrates*

Experimental conditions as in	Substrate	Q_{O_2}	^{14}C fixed (counts/min./mg. of cells/hr.)
Fig. 1	Acetate	13·8	870
Fig. 1	None	1·1	103
Fig. 2	Acetaldehyde	15·3	900
Fig. 2	None	2·9	90

(Wieland, Probst & Crawford, 1938). With acetaldehyde (Fig. 2) the initial oxygen uptake is faster than the carbon dioxide fixation, which shows the same delay as with acetate. This difference between the rate of oxidation and $^{14}CO_2$ fixation can be attributed to the oxidation of acetaldehyde to acetic acid, which apparently does not involve carbon dioxide fixation. In order to increase the permeability of yeast to acetate, with this substrate the initial pH of the medium was decreased. The strong acidity did not affect qualitatively the fixation reactions as, with acetaldehyde, experiments carried out at low and high pH gave, after sufficient time of incubation, the same isotope distribution (Table 2).

The substances labelled after $^{14}CO_2$ fixation are listed in Table 2. All are soluble in the methanol–water phase because the lack of nitrogen compounds and growth factors in the medium prevented the synthesis of yeast proteins. The radioactive compounds may be grouped as (1) Krebs-cycle intermediates, (2) amino acids, and (3) glycolysis intermediates. The largest reservoirs of ^{14}C are aspartic and glutamic acid and, among the Krebs-cycle intermediates, malic and the tricarboxylic acids (citric, aconitic and *iso*citric acid). Most of the amino acids labelled are related to the Krebs-cycle intermediates. With acetaldehyde as substrate, succinic, fumaric, phosphoglyceric and phosphopyruvic acids fix ^{14}C, which leads to the assumption that the reducing power of acetaldehyde reverses some reactions of the Embden–Meyerhof sequence, and Krebs cycle. A similar but more evident effect will be described with glucose.

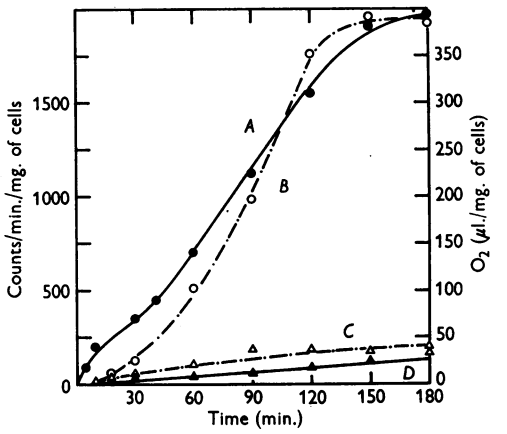

Fig. 2. Fixation of $^{14}CO_2$ and oxygen uptake by *S. cerevisiae* with acetaldehyde as substrate. 20 mM-Acetaldehyde; 0·4 mM-phosphate; pH 2·8 in the manometric experiment, 3·1 in the fixation experiment. Other experimental details are as in Fig. 1. *A*, Oxygen uptake with acetaldehyde and *D*, control. *B*, $^{14}CO_2$ fixation with acetaldehyde and *C*, control.

Table 2. *Distribution* (%) *of* $^{14}CO_2$ *fixed by* Saccharomyces cerevisiae *with acetaldehyde or acetate as substrate*

The reaction mixture contained 210 mg. of yeast cells, 20 mM-acetaldehyde (or acetate), 0·4 mM-phosphate and 4·2 μ-moles of $NaH^{14}CO_3$ ($3·1 \times 10^6$ counts/min.). Incubation was for 120 min. with acetaldehyde and 150 min. with acetate. Temp. 30°. Air was in the gas space. Total ^{14}C fixed (counts/min./mg. of cells): 1510 (with acetaldehyde, pH 3·1), 1700 (with acetaldehyde, pH 7·0) and 1505 (with acetate, pH 3·1). ^{14}C fixed in the methanol–water-soluble substances: 1600 (with acetaldehyde, pH 3·8), 1520 (with acetaldehyde, pH 7·0) and 1400 (with acetate).

	^{14}C fixed (% of total in the methanol–water-soluble substances)		
Substance	Substrate: acetaldehyde (pH 3·8)	Substrate: acetaldehyde (pH 7·0)	Substrate: acetate (pH 3·1)
Krebs-cycle intermediates			
Malic acid	7·5	7·3	5·6
Citric, aconitic and *iso*citric acid	3·7	2·7	3·8
Fumaric acid	1·1	0·9	0
Succinic acid	1·0	1·2	0
Amino acids			
Aspartic acid	58·0	60·2	59·6
Glutamic acid	17·5	17·8	21·9
Alanine	2·3	2·5	2·0
Threonine	0	0	1·7
Glycine	2·5	2·5	1·5
Arginine–ornithine	0·9	0·9	0·8
Glycolysis intermediates			
Phosphoglyceric acid	0·9	0	0
Phosphopyruvic acid	0·9	0·6	0
Other substances	3·7	3·4	3·1

The oxidation of the yeast endogenous substrates brings about carbon dioxide fixation, which is differentiated from the fixation associated with substrate oxidation by the far smaller amounts of ^{14}C incorporated (cf. Figs. 1 and 2) and of compounds labelled (Table 3). Furthermore, aspartic and glutamic acids fix relatively more ^{14}C than during acetate or acetaldehyde oxidation. The distribution pattern varies slightly from one yeast preparation to another and, in some, the tricarboxylic acids, fumaric acid and succinic acid also appear radioactive.

The kinetics of $^{14}CO_2$ fixation with acetate as substrate (similar results were obtained with acetaldehyde) are shown in Fig. 3, where only those compounds presumably related to the fixation reactions (aspartic, malic, glutamic and the tricarboxylic acids) have been plotted. According to the time of incubation, three different distributions of ^{14}C can be described. In the first 30 min. aspartic acid has most of the activity, which decreases continuously. Also the curve of malate shows negative slope, but this may not be significant as the sign of the slope depends upon a single experimental point at a time when the total counts in the cell are very low and the error involved in the determinations is consequently higher. On the other hand, the incorporation of ^{14}C in glutamic acid increases continuously, and after 30 min. nearly reaches its maximum. In the 30–120 min. interval, aspartic acid shows a minimum whereas glutamic, malic and the tricarboxylic acids have maxima. From 120 min. onwards the relative distribution of ^{14}C in aspartic acid increases steadily, whereas decreases occur in glutamic, malic and the citric acid group. If the absolute radioactivity fixed by each compound is plotted against time of incubation (Fig. 4) a close relationship with the percentage-distribution plot is observed and it becomes evident that by the end of the experiment aspartic acid is the largest reservoir of ^{14}C.

The kinetics of $^{14}CO_2$ fixation during the oxidation of endogenous substrates have also been studied. In the percentage-distribution plot (Fig. 5), only aspartic acid has a negative slope and after incubation for 30 min. a steady-state distribution appears. The curves do not overlap, which means a striking and systematic difference from the plots of ^{14}C distribution after substrate oxidation. Accordingly, the absolute distribution of ^{14}C in each compound increases continuously throughout the incubation (Fig. 6).

Table 3. *Distribution* (%) *of* $^{14}CO_2$ *fixed by* Saccharomyces cerevisiae *during the oxidation of endogenous substrates*

Experimental conditions were as described in Table 2. Substrate was omitted. Incubation was for 2 hr. Total ^{14}C fixed (counts/min./mg. of cells): 178; ^{14}C fixed in the methanol–water-soluble substances: 161.

Substance	^{14}C fixed (% of total in the methanol–water-soluble substances)
Krebs-cycle intermediates	
Malic acid	3·6
Amino acids	
Aspartic acid	65·4
Glutamic acid	25·6
Alanine	2·2
Glycine	1·8
Other substances	1·4

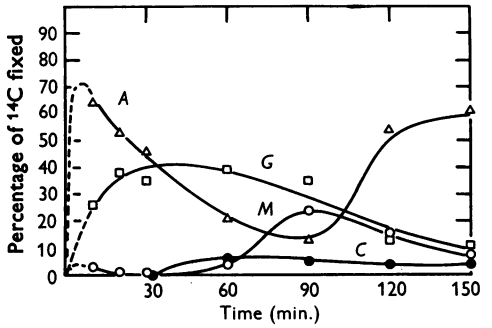

Fig. 3. Kinetics of ^{14}C distribution after $^{14}CO_2$ fixation by *S. cerevisiae* with acetate as substrate. Experimental conditions were the same as in Fig. 1 (fixation experiment). *A*, Aspartic acid; *G*, glutamic acid; *M*, malic acid; *C*, citric, aconitic and *iso*citric acid.

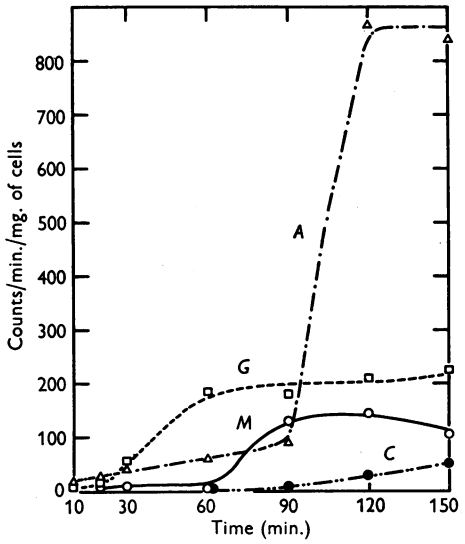

Fig. 4. Absolute distribution of $^{14}CO_2$ fixed by *S. cerevisiae* during acetate oxidation. Details as for Fig. 3.

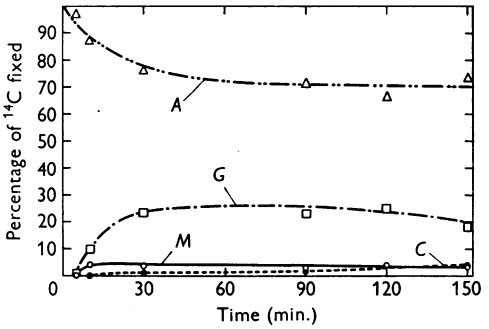

Fig. 5. Kinetics of ^{14}C distribution after $^{14}CO_2$ fixation by *S. cerevisiae* during the oxidation of endogenous substrates. Experimental conditions were the same as in Fig. 2. Acetaldehyde was omitted. *A*, Aspartic acid; *G*, glutamic acid; *M*, malic acid; *C*, citric, aconitic and *iso*citric acid.

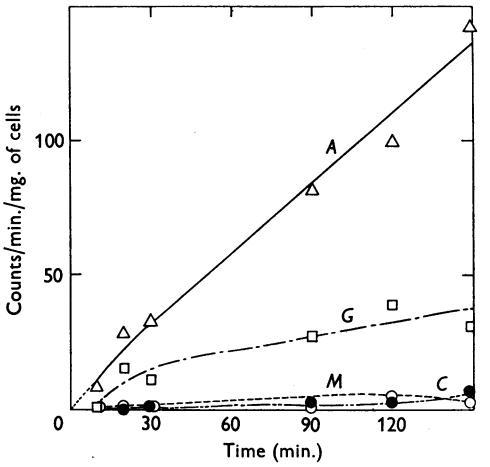

Fig. 6. Absolute distribution of $^{14}CO_2$ fixed by *S. cerevisiae* during the oxidation of endogenous substrates. Details as in Fig. 5.

Fixation of ^{14}C *in aspartic and glutamic acid.* For the understanding of the kinetic experiments summarized above, the specific activity and intramolecular distribution of ^{14}C in aspartic and glutamic acid during the incubation are essential. Both acids are in equilibrium with oxaloacetic and α-oxoglutaric acid, two significant intermediates of carbon dioxide assimilation, and the respective transaminations have been shown in *Saccharomyces fragilis* (Bigger-Gehring, 1955) and confirmed in *S. cerevisiae* (Table 4).

Aspartic and glutamic acid were contained free in the respective proportions of about 3 and 11 μmoles/100 mg. of dry cells of the yeast preparations used (Table 5), which is consistent with previous observations of Halvorson & Spiegelman (1953) and other workers. The pool of these amino

Table 4. *Oxaloacetate–glutamate transaminase in cell-free extracts of* Saccharomyces cerevisiae

The reaction mixture (2 ml.) contained 0·6 ml. of dialysed yeast extract (prepared as described under Methods), 0·1 M-phosphate buffer, pH 7·8, and 0·01 M-amino acid or -keto acid or both. Incubation was for 2 hr. at 30°. The reaction was stopped by addition of 10 ml. of absolute ethanol and boiling for 2 min. in a water bath. The precipitate was discarded after centrifuging and the supernatant evaporated to 2 ml. Amino acids were separated on paper by chromatography with the phenol–water-solvent system and, after elution, estimated by the Moore & Stein (1948) method.

Additions	Amino acid formed (μmoles) Glutamate	Aspartate
None	0	0
L-Glutamate	—	0
L-Aspartate	0	—
α-Oxoglutarate	0·4	—
Oxaloacetate	—	3·4
α-Oxoglutarate + L-aspartate	6·9	—
Oxaloacetate + L-glutamate	—	9·6

Table 5. *Concentration and specific activity of free aspartic and glutamic acids in* Saccharomyces cerevisiae *during* $^{14}CO_2$ *fixation with acetaldehyde as substrate*

Amounts of amino acid and radioactivity were measured in samples of aspartic and glutamic acid eluted from the chromatograms of the experiment on acetaldehyde oxidation (at pH 7·0) summarized in Table 2. Amino acids were measured according to Russell (1944).

Time of acetaldehyde oxidation (min.)	Aspartic acid (μmoles/100 mg. of cells)	Aspartic acid Specific activity (counts/min./μmole)	Glutamic acid (μmoles/100 mg. of cells)	Glutamic acid Specific activity (counts/min./μmole)
10	3·0	210	11·0	13
20	2·0	1180	16·0	83
30	3·0	1300	—	—
60	—	—	42·0	236
90	4·0	1440	—	—
120	18·0	2800	42·0	272
150	—	—	44·0	332
180	25·0	2400	37·0	1160

Table 6. *Distribution of* ^{14}C *in aspartic acid from* Saccharomyces cerevisiae *after fixation of* $^{14}CO_2$ *with acetaldehyde and acetate as substrates*

Radioactive aspartic acid samples eluted from the chromatograms of the experiments quoted in Figs. 1 and 2 were added to 31 μmoles of non-isotopic aspartic acid. Portions were subjected to total combustion, or decarboxylation with chloramine-T and the transaminase–aniline–citrate system. For details see under Methods. No activity was found in $C_{(2)}+C_{(3)}$.

Substrate: acetate			Substrate: acetaldehyde		
Time of substrate oxidation (min.)	Percentage activity in $C_{(4)}$	Percentage activity in $C_{(1)}$	Time of substrate oxidation (min.)	Percentage activity in $C_{(4)}$	Percentage activity in $C_{(1)}$
10	100	0	20	100	0
30	100	0	30	85	15
60	76	24	60	73	27
120	64	36	120	58	42
150	60	40	150	48	52
180	62	38	180	46	54
210	60	40	—	—	—

Table 7. *Distribution of* ^{14}C *in glutamic acid from* Saccharomyces cerevisiae *after fixation of* $^{14}CO_2$ *with acetaldehyde and acetate as substrates*

Samples of radioactive glutamic acid eluted from the chromatograms of the experiments described in Figs. 1 and 2 were added to 25·5 μmoles of non-isotopic glutamic acid. Portions were subjected to total combustion or decarboxylation with chloramine-T.

Time of substrate oxidation (min.)	Percentage activity in $C_{(1)}$: Substrate: acetaldehyde	Percentage activity in $C_{(1)}$: Substrate: acetate
20	47	—
30	48	83
60	58	66
90	77	70
120	72	80
150	77	—
180	66	94
210	—	83

acids increased after substrate oxidation (Table 5), but this effect was not constant and depends probably on the store of amino groups available for transamination. In controls where only endogenous substrates were oxidized, aspartic and glutamic acid remained constant or slightly diminished.

The specific activities of aspartic and glutamic acid increased continuously throughout the $^{14}CO_2$ fixation, which is due to the progressive labelling of each carbon, as well as the distribution of the isotope in different positions of the molecule. The distribution of ^{14}C in aspartic acid is summarized in Table 6. At first ^{14}C entered only in C-4 but subsequently there was a progressive incorporation of ^{14}C into C-1, which, by the end of the experiment, had nearly half the ^{14}C incorporated. The amino and methylene carbons did not fix the isotope. Aspartic acid labelled during the oxidation of endogenous substrates was also degraded. The distribution of ^{14}C was similar to that shown in Table 6 but the ^{14}C fixed in C-1 never exceeded 30 % of the total.

The distribution of ^{14}C in glutamic acid depends somewhat on the substrate oxidized. With acetaldehyde, only 47–48 % of the ^{14}C incorporated labelled C-1 initially although the proportion increased to 77 % at later stages of incubation. With acetate, more ^{14}C entered C-1, and after 180 min. its activity reached 94 % (Table 7).

Fixation of carbon dioxide by Saccharomyces cerevisiae *with pyruvate as substrate*

Pyruvate metabolism in baker's yeast. Baker's yeast can (1) decarboxylate pyruvate into acetaldehyde, as in glucose fermentation (Meyerhof, 1937), (2) dismute pyruvate to carbon dioxide, acetic and lactic acid (Barron *et al.* 1950), and (3) completely oxidize pyruvate with oxygen (Lieben, 1923) through the citric acid cycle (Barron *et al.* 1950; Wang, Christensen & Cheldelin, 1953). Oxidative decarboxylation to acetate should be the first step of pyruvate oxidation (Barron *et al.* 1950) which is associated with phosphate exchange (Stoppani, 1951) as in the animal enzyme preparations. When baker's yeast metabolizes exogenous pyruvate, the third of these metabolic paths is by far the most important, as shown by the lack of consumption of pyruvate in the absence of oxygen (Barron *et al.* 1950) or in the presence of 2:4- and 2:6-dinitrophenols (Table 8). These substances are strong inhibitors of pyruvate oxidation, but in the same concentrations do not inhibit carboxylase either in the purified state or in the intact yeast cell, as shown by their lack of action on glucose fermentation. Exogenous pyruvate is presumably not accessible to carboxylase and therefore cannot be decarboxylated to acetaldehyde.

Fixation and distribution of $^{14}CO_2$. Baker's yeast incorporates carbon dioxide during pyruvate oxidation (Fig. 7). The amount of ^{14}C fixed is proportional to the oxygen consumption and, during the first 60 min. of incubation, also to the uptake of pyruvate. It is noteworthy that after that time pyruvate apparently disappeared from the yeast suspension, although the amount of oxygen consumed was only 40 % of that required for complete combustion. The close connexion of ^{14}C incorporation and oxygen uptake after incubation for 60 min. shows that in the last period of incubation carbon dioxide fixation was bound more to the oxidation of pyruvate products than to the presence of pyruvate itself. With pyruvate, the rate of fixation is faster than with acetate (or acetaldehyde) as substrate (Fig. 8), which is consistent with the lack of an 'induction period' in pyruvate oxidation.

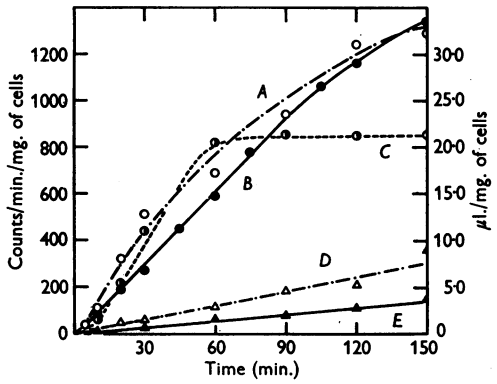

Fig. 7. Fixation of $^{14}CO_2$ and uptake of oxygen and pyruvate by *S. cerevisiae* with pyruvate as substrate. Pyruvic acid 20 mM. Other experimental conditions were as in Fig. 1. *A*, $^{14}CO_2$ fixation with pyruvate and *D*, control; *B*, oxygen uptake with pyruvate and *E*, control; *C*, pyruvate consumption. Pyruvate consumption is expressed in μl. (1 μmole ≡ 22·4 μl.).

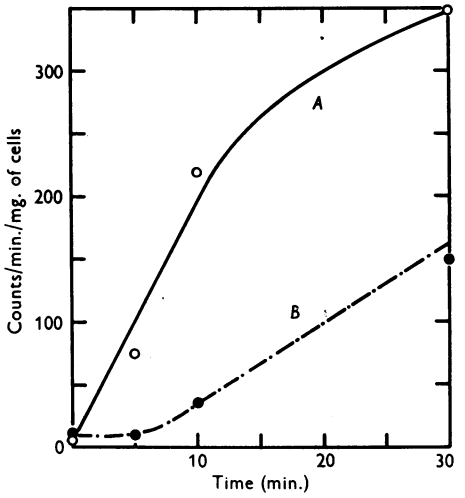

Fig. 8. Comparative rate of $^{14}CO_2$ fixation with pyruvate and acetate as substrates. Yeast: 250 mg.; 5 mM-pyruvic acid (*A*); 5 mM-acetic acid (*B*); pH 3·1; 4·2 μ-moles of $NaH^{14}CO_3$ ($3·1 \times 10^6$ counts/min.); 10 ml. final volume. Temp. 30°. Air was in the gas space.

Table 8. *Effect of dinitrophenols on pyruvate oxidation, glucose fermentation and carboxylase activity*

Expt. *A*: 12·1 mg. of yeast; 0·05 M-fumarate; 0·027 M-pyruvate; pH 4·5; incubation for 90 min. Expt. *B*: 3·3 mg. of yeast; 0·05 M-succinate; 0·02 M-pyruvate; pH 4·5; incubation for 90 min. Expt. *C*: 5·5 mg. of yeast; 0·05 M-phosphate; pH 4·5; 0·014 M-glucose; incubation for 60 min. Expt. *D*: 0·3 ml. of carboxylase; 0·05 M-phosphate; 0·03 M-pyruvate; incubation for 10 min. In Expt. *C*, N_2 was in the gas space; air was used in the other experiments. Temp. 30°. The results are expressed in μmoles. 2:4-DNP and 2:6-DNP, 2:4- and 2:6-dinitrophenol.

Expt.		Control	Inhibitor	Inhibition (%)
	Pyruvate oxidation		2:4-DNP (0·1 mM)	
A	CO_2 formation	16·9	0·7	95·8
A	O_2 uptake	14·9	0·9	94·0
A	Pyruvate consumption	16·8	0·1	99·0
	Pyruvate oxidation		2:6-DNP (0·25 mM)	
B	CO_2 formation	14·0	2·2	84·1
B	O_2 uptake	11·2	2·7	76·0
B	Pyruvate consumption	12·9	4·0	69·0
	Glucose fermentation		2:4-DNP (0·1 mM)	
C	CO_2 formation	33·4	31·6	5·4
	Glucose fermentation		2:6-DNP (0·25 mM)	
C	CO_2 formation	33·4	34·7	−3·9
	Carboxylase activity		2:4-DNP (0·1 mM)	
D	CO_2 formation, pH 4·7	94	78	17·0
D	CO_2 formation, pH 6·5	40	40	0·0
	Carboxylase activity		2:6-DNP (0·25 mM)	
D	CO_2 formation, pH 4·8	43	41	4·6

The distribution of $^{14}CO_2$ fixed with pyruvate as substrate is shown in Table 9. No significant amount of ^{14}C was incorporated into the yeast proteins and the distribution in the methanol–water-soluble substances follows, in general, the same pattern as with the C_2 substrates. However, after incubation for 60 min., the dicarboxylic acids (especially malic acid) were far more radioactive than at the same period of the experiments with acetate or acetaldehyde.

The kinetics of the $^{14}CO_2$ fixation with pyruvate (Fig. 9) resemble those with acetate (Fig. 3) with the exception of (*a*) the first period of ^{14}C distribution, which is far shorter with pyruvate (15 min.) and (*b*) the initial positive slope of malic acid. If the absolute distribution of ^{14}C is calculated and plotted against time, it can be shown that, as with acetate, after fixation for 120–180 min. aspartic and glutamic acid are the main reservoirs of the $^{14}CO_2$ incorporated.

^{14}C fixation in aspartic and glutamic acid. During pyruvate oxidation the pools of aspartic and glutamic acid increase; with glutamic acid this occurs at the beginning and with aspartic acid at the end of the experiment. The specific activity of the amino acids increases as well, the variation being particularly marked with glutamic acid (Table 10). ^{14}C first labels the C-4 position of aspartic acid and subsequently C-1 (Table 11), and after incubation for 2 hr. the activity in C-1 approaches 50% of the total fixed. In glutamic acid, ^{14}C labels exclusively C-1 (Table 12), which constitutes a striking difference from the results obtained with acetate or acetaldehyde.

Table 9. *Distribution* (%) *of* $^{14}CO_2$ *fixed by* Saccharomyces cerevisiae *with pyruvate as substrate*

Experimental conditions were as in Fig. 7. Total ^{14}C fixed (counts/min./mg. of cells): 641 (incubation for 60 min.) and 1410 (180 min.); ^{14}C fixed in the methanol–water-soluble substances: 652 (60 min.) and 1358 (180 min.).

Substance	^{14}C fixed (% of total in the methanol–water-soluble substances) 60 min.	180 min.
Krebs-cycle intermediates		
Malic acid	18·4	5·1
Citric, aconitic and *iso*citric acid	6·4	3·7
Fumaric acid	2·4	0·5
Succinic acid	2·0	0·5
Amino acids		
Aspartic acid	16·1	48·0
Glutamic acid	44·7	30·3
Alanine	1·0	2·5
Threonine	4·2	1·6
Arginine–ornithine	0·0	1·7
Glycolysis intermediates		
Hexose phosphates	1·4	1·1
Phosphoglyceric acid	0·0	0·3
Phosphopyruvic acid	0·0	2·1
Other substances	3·4	2·6

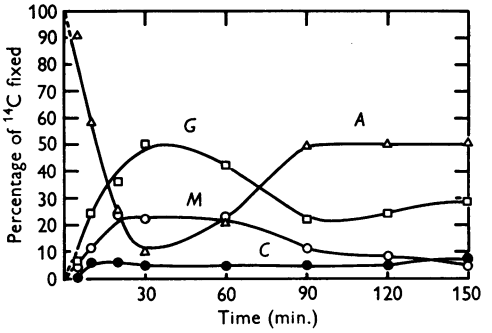

Fig. 9. Kinetics of ^{14}C distribution after $^{14}CO_2$ fixation by *S. cerevisiae* with pyruvate as substrate. Experimental conditions were as in Fig. 7. *A*, Aspartic acid; *G*, glutamic acid; *M*, malic acid; *C*, citric, aconitic and *iso*citric acid.

Table 10. *Concentration and specific activity of free aspartic and glutamic acid in* Saccharomyces cerevisiae *during* $^{14}CO_2$ *fixation with pyruvate as substrate*

Amounts of amino acids and radioactivity were measured in samples of aspartic and glutamic acid eluted from chromatograms of a fixation experiment similar to that described in Fig. 7, except that the amount of yeast was 500 mg. Amino acids were measured according to Russell (1944).

Time of pyruvate oxidation (min.)	Aspartic acid (μmoles/100 mg. of cells)	Aspartic acid Specific activity (counts/min./μmole)	Glutamic acid (μmoles/100 mg. of cells)	Glutamic acid Specific activity (counts/min./μmole)
2	2·0	425	26·0	7
5	—	—	32·0	11
10	2·0	2700	36·0	20
30	36·0	2360	22·0	162
60	39·0	3220	28·0	373
120	32·0	3100	20·0	778

Table 11. *Distribution of* ^{14}C *in aspartic acid from* Saccharomyces cerevisiae *after fixation of* $^{14}CO_2$ *with pyruvate as substrate*

Radioactive aspartic acid samples eluted from chromatograms of Expts. *A* and *B* were added to 31 μmoles of non-isotopic aspartic acid, and portions were degraded as in Table 6. Conditions of fixation experiments: yeast 490 mg. (Expt. *A*) or 250 mg. (Expt. *B*); pyruvate 10 mM (Expt. *A*) or 5 mM (Expt. *B*). Other details were as in Fig. 1.

Time of pyruvate oxidation (min.)	Percentage activity in $C_{(4)}$ Expt. *A*	$C_{(4)}$ Expt. *B*	$C_{(1)}$ Expt. *A*	$C_{(1)}$ Expt. *B*
5	—	97	—	0
10	91	73	9	27
20	86	—	14	—
30	82	57	18	43
60	69	61	31	39
120	77	56	18	44

Table 12. *Distribution of* ^{14}C *in glutamic acid from* Saccharomyces cerevisiae *after fixation of* $^{14}CO_2$ *with pyruvate as substrate*

Fixation experiment: 490 mg. of yeast and 10 mM-pyruvate. Other experimental details were as in Fig. 1. Radioactive glutamic acid was eluted from the chromatograms, diluted with 25·5 μmoles of non-isotopic glutamic acid and portions were subjected to degradation.

Time of pyruvate oxidation (min.)	Percentage activity in $C_{(1)}$
5	98
10	98
20	86
30	92
120	101

Carbon dioxide fixation by Saccharomyces cerevisiae *with glucose as substrate*

Fixation and distribution of $^{14}CO_2$. In the presence of glucose there is a rapid incorporation of carbon dioxide which greatly exceeds the rate of oxygen uptake. However, this is a transient effect and soon the fixation of ^{14}C slows down to become almost negligible. On the other hand, the velocity of glucose oxidation remains constant throughout the experiment (Fig. 10). The fast initial rate of fixation clearly differentiates the fixation with glucose from the fixation with the other substrates.

Glucose, a non-electrolyte, allowed the effect of the medium pH on the rate of $^{14}CO_2$ fixation to be tested (Table 13). After incubation for 2–5 min. the ^{14}C incorporated at pH 2·8 was less than at pH 7·1, notwithstanding the similar rate of glucose oxidation, but in the course of the incubation the pH of the initially acid suspension increased (up to 6·0) and simultaneously the rate of $^{14}CO_2$ incorporation increased, in such a way that by the end of the experiment the activity fixed by the yeast suspensions was nearly the same. The initial difference may possibly be explained by the lower concentration of HCO_3^- ions at the acid pH.

The distribution of fixed $^{14}CO_2$ is shown in Table 14. It must be noted that the activity incorporated into the cell proteins is significant and that at 5 min. incubation there is strong labelling of malic, fumaric and succinic acid but low activity in aspartic and glutamic acid; this is not observed after short periods of incubation with the other substrates. Nevertheless, if an adequate time of oxidation is allowed, glutamic and aspartic acid regain their place as main reservoirs of ^{14}C and the dicarboxylic acids appear with their usual low percentage of isotope. Other amino acids related to the citric acid-cycle intermediates, such as alanine, glycine, threonine and basic amino acids, fix a small amount of ^{14}C and the activity in the glycolytic

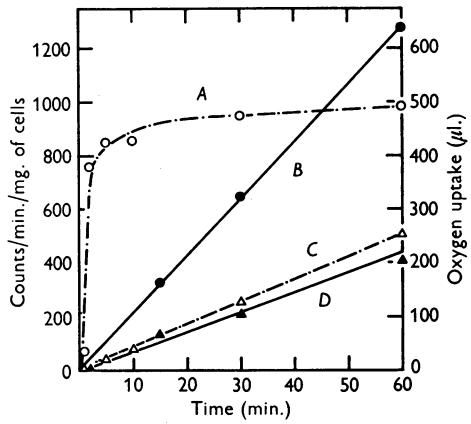

Fig. 10. Fixation of $^{14}CO_2$ and oxygen uptake of *S. cerevisiae* with glucose as substrate. Yeast, 28·5 mg./ml.; 5 mM-glucose and 0·4 mM-phosphate, pH 7·4. Other experimental conditions were as in Fig. 1. *A*, $^{14}CO_2$ fixation with glucose and *C*, control. *B*, Oxygen uptake with glucose and *D*, control.

Table 13. *Effect of initial* pH *on* $^{14}CO_2$ *fixation by* Saccharomyces cerevisiae, *with glucose as substrate*

Yeast, 285 mg.; 0·4 mM-phosphate buffer; 5 mM-glucose. Other experimental conditions were as in Fig. 1 (fixation experiment).

Time of glucose oxidation (min.)	$^{14}CO_2$ fixation (counts/min./mg. of cells) Initial pH 2·8	Initial pH 7·1
2	248	760
5	630	852
30	990	952

Table 14. *Distribution* (%) *of* $^{14}CO_2$ *fixed by* Saccharomyces cerevisiae *with glucose as substrate*

Experimental conditions were as in Fig. 10. Total ^{14}C fixed (counts/min./mg. of cells): 630 (incubation for 5 min.); 832 (10 min.); 994 (30 min.) and 1150 (2 hr.). ^{14}C fixed in the methanol–water-soluble substances: 580 (5 min.) 640 (10 min.) 630 (30 min.) and 850 (2 hr.).

Substance	^{14}C fixed (% of total in methanol–water-soluble substances) 5 min.	10 min.	30 min.	2 hr.
Krebs-cycle intermediates				
Malic acid	31·3	12·5	8·3	3·8
Citric, aconitic and *iso*citric acid	5·7	4·8	2·0	3·5
Fumaric acid	5·0	1·4	1·6	0·5
Succinic acid	2·8	1·4	0·9	0·8
Amino acids				
Aspartic acid	24·5	13·4	38·4	32·7
Glutamic acid	24·4	61·6	43·7	50·8
Alanine	0·7	0·9	1·2	1·5
Glycine	0·0	1·6	0·9	1·2
Threonine	1·4	1·5	0·2	1·1
Arginine–ornithine	0·0	0·0	0·0	0·4
Glycolysis intermediates				
Hexose phosphates	0·0	0·4	0·0	1·1
Phosphoglyceric acid	0·0	0·0	0·0	1·2
Phosphopyruvic acid	0·0	0·5	0·4	0·6
Other substances	4·2	0·0	2·4	0·8

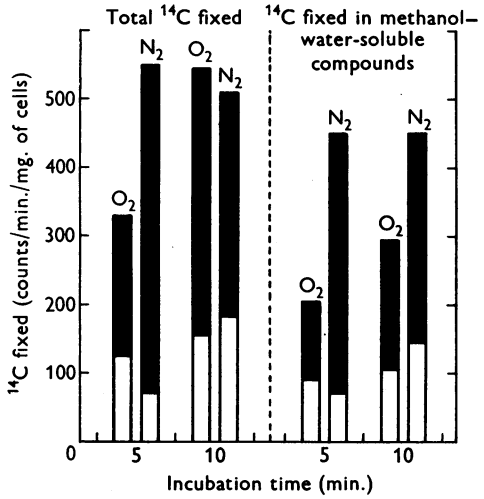

Fig. 11. Aerobic and anaerobic fixation of $^{14}CO_2$ by *S. cerevisiae* with glucose as substrate. Yeast, 22·7 mg./ml.; 5 mM-glucose. Temp. 18°. Other experimental details were as in Fig. 1. Gas exchange with glucose: Q_{O_2}, −16·1; Q_{CO_2}, 15·9 and $Q^{N_2}_{CO_2}$, 19·0; control: Q_{O_2}, −2·6; Q_{CO_2}, 2·6 and $Q^{N_2}_{CO_2}$, 2·3. Shaded areas represent the difference between $^{14}CO_2$ fixed with glucose and the controls.

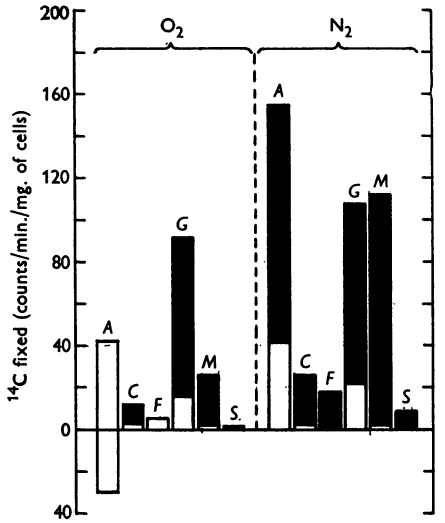

Fig. 12. Action of oxygen on the distribution of $^{14}CO_2$ fixed in methanol–water-soluble substances from *S. cerevisiae* with glucose as substrate. Experimental conditions were as in Fig. 11. Incubation was for 5 min. Shaded areas represent the difference between $^{14}CO_2$ fixed with glucose and the controls (less $^{14}CO_2$ was fixed in aspartic acid when glucose was present). *A*, Aspartic acid; *C*, citric, aconitic and *iso*citric acid; *F*, fumaric acid; *G*, glutamic acid; *M*, malic acid; *S*, succinic acid.

intermediates proves the reversibility of the Embden–Meyerhof sequence of reactions.

The kinetics of $^{14}CO_2$ fixation with glucose are similar to those with pyruvate, as previously shown (Stoppani *et al.* 1957). After 15–30 sec. fixation aspartic acid contains most of the ^{14}C fixed and is the only compound in the distribution plot showing negative slope.

Aerobic and anaerobic fixation of $^{14}CO_2$. Oxygen is not necessary for carbon dioxide fixation in the presence of glucose and, furthermore, after fixation for 5 min. more ^{14}C is incorporated with nitrogen than with oxygen in the gas phase (Fig. 11). The reverse is true in the absence of substrate,

which proves that the anaerobic fixation depends on glycolysis. The distribution of the fixed $^{14}CO_2$ is also affected by the composition of the gas phase. In the first place, oxygen enhances the incorporation of ^{14}C into the cell proteins (Fig. 11). Thus after incubation for 5 min. the percentage of ^{14}C fixed in the cell protein was 40 under oxygen and 21 under nitrogen; after 10 min. the difference was more significant: 51% under oxygen and 6% under nitrogen. Secondly, the distribution of ^{14}C in the methanol–water-soluble substances is also dependent on oxygen, as anaerobically more ^{14}C enters into aspartic, malic, fumaric, succinic and citric acid (Fig. 12).

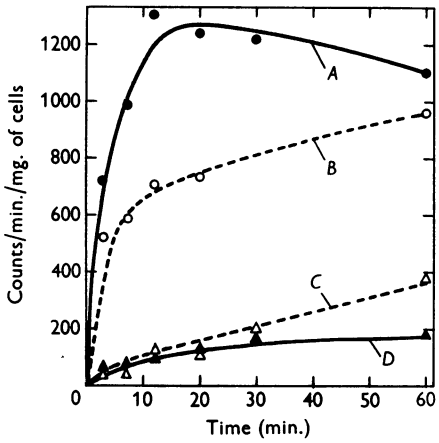

Fig. 13. Action of antimycin A on $^{14}CO_2$ fixation by *S. cerevisiae*. Yeast 12 mg./ml.; 5 mM-glucose; antimycin A, 13 μg./ml.; 4·2 μmoles of $NaH^{14}CO_3$ ($3·1 \times 10^6$ counts/min.). Total volume: 2 ml. in the manometric experiment and 6 ml. in the fixation experiment. Temp. 25°. Air was in the gas space. Gas exchange with glucose: Q_{O_2}, −30·7 and Q_{CO_2}, 37·7; with glucose and antimycin A: Q_{O_2}, −1·5 and Q_{CO_2}, 46·2; with antimycin A: Q_{O_2}, −1·5 and Q_{CO_2}, 2.0; control, Q_{O_2} −2·3 and Q_{CO_2}, 3·1. *A*, $^{14}CO_2$ fixation with glucose and antimycin A; *B*, with glucose; *C*, control without and *D*, with antimycin A.

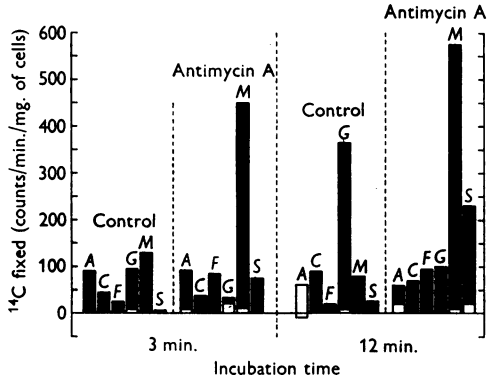

Fig. 14. Action of antimycin A on the distribution of $^{14}CO_2$ fixed by *S. cerevisiae* with glucose as substrate. Details as for Fig. 13 (fixation experiment). The shaded areas represent the difference between the $^{14}CO_2$ fixed with glucose and the controls. *A*, Aspartic acid; *C*, citric, aconitic and *iso*citric acid; *F*, fumaric acid; *G*, glutamic acid; *M*, malic acid; *S*, succinic acid.

Effect of inhibitors. Antimycin A, a selective inhibitor of aerobic cellular oxidation (Ahmad, Schneider & Strong, 1950), brings about changes in $^{14}CO_2$ fixation that resemble those induced by anaerobiosis. With glucose, antimycin A increases the rate of $^{14}CO_2$ fixation, inhibits almost completely the oxygen uptake and promotes evolution of carbon dioxide. On the other hand, in the absence of glucose antimycin A inhibits the $^{14}CO_2$ fixation and oxygen uptake as well (Fig. 13). The distribution of ^{14}C in the methanol–water-soluble substances varies accordingly. With antimycin A there is more labelling of aspartic, malic, fumaric and succinic acid and less of glutamic acid (Fig. 14, 12 min. incubation). Similar changes are observed after incubation for 3 min. except in aspartic acid, the relative labelling of which does not vary.

Two inhibitors of oxidative phosphorylation (2:4-dinitrophenol and sodium azide) and two of glycolysis (fluoride and iodoacetate) have been also tested for their effects on $^{14}CO_2$ fixation. 2:4-Dinitrophenol diminishes the $^{14}CO_2$ incorporation with either glucose or the control but has more effect on the latter (Table 15). As 2:4-dinitrophenol does not interfere with glucose oxidation or fermentation in yeast (Stickland, 1956), it may be assumed that energy-rich phosphate compounds are involved in the fixation reaction. This is confirmed with sodium azide, which strongly inhibits both aerobic and anaerobic fixation of $^{14}CO_2$ (Table 15). The anaerobic inhibition is unexpected, as azide does not inhibit the rate of glucose fermentation and anaerobic phosphorylation by yeast cells (Stickland, 1956) or by yeast extracts (Robertson & Boyer, 1955).

Fixation of ^{14}C *in aspartic and glutamic acid.* The pool and specific activity of glutamic acid varied in the experiments with glucose as with the other substrates. Aspartic acid was not measured. The distribution of ^{14}C in aspartic and glutamic acid is shown in Table 16. ^{14}C is located only in the carboxyl groups of aspartic acid and, at the beginning of the experiment, only in C-4. Subsequently the isotope appears in C-1, which, at the end of the incubation, contained about 40% of the total ^{14}C fixed. In glutamic acid at short time intervals most of the label is located in C-1 but later also in other positions, especially in C-5.

Fixation of ^{14}C *in succinic acid.* Fumaric and succinic acid are labelled during $^{14}CO_2$ fixation. As their isotope distribution is important for the elucidation of the paths of carbon assimilation,

Table 15. *Action of inhibitors on* $^{14}CO_2$ *fixation by* Saccharomyces cerevisiae

Yeast, 14·7 mg.; 0·01 M-glucose; 5·5 μmoles of $NaH^{14}CO_3$ ($1·2 \times 10^6$ counts/min.); 2 ml. of 0·18 M-KH_2PO_4; pH 4·8. Temp. 30°. Corrected fixation: ^{14}C fixed with glucose less control. The percentage inhibition in the experiments with glucose are calculated with the corrected fixation values.

Gas phase	Time of incubation (min.)	Additions	$^{14}CO_2$ fixation (counts/min./mg. of cells) Total	Corrected	Inhibition (%)	CO_2 formation (μl.)
Air	30	Glucose	1830	1010	—	—
Air	30	Glucose + 2:4-dinitrophenol (0·2 mM)	420	360	58·8	—
Air	30	None	820	—	—	—
Air	30	2:4-Dinitrophenol (0·2 mM)	60	—	87·8	—
Air	20	Glucose	2020	1185	—	—
Air	20	Glucose + sodium azide (3·2 mM)	33	29	98·2	—
Air	20	None	835	—	—	—
Air	20	Sodium azide (3·2 mM)	4	—	99·5	—
N_2	20	Glucose	1120	610	—	440
N_2	20	Glucose + sodium azide (3·2 mM)	80	62	89·8	452
N_2	20	None	510	—	—	0
N_2	20	Sodium azide (3·2 mM)	18	—	96·6	30
N_2	30	Glucose	1680	1250	—	290
N_2	30	Glucose + fluoride (5·0 mM)	830	230	81·5	58
N_2	30	Glucose + iodoacetate (0·046 mM)	250	−30	102·2	0
N_2	30	None	430	—	—	—
N_2	30	Fluoride (5·0 mM)	600	—	−39·6	—
N_2	30	Iodoacetate (0·046 mM)	280	—	34·6	—

Table 16. *Distribution of* ^{14}C *in aspartic and glutamic acid from* Saccharamyces cerevisiae *after fixation of* $^{14}CO_2$ *with glucose as substrate*

Samples of radioactive aspartic and glutamic acid eluted from the chromatograms of the experiments summarized in Table 13 (Expt. *A*) or Fig. 10 (fixation Expt. *B*) were added to 31 μmoles of non-isotopic aspartic acid and 25 μmoles of non-isotopic glutamic acid respectively and portions subjected to degradation.

Time of glucose oxidation (min.)	Aspartic acid. Percentage activity in C-4 Expt. *A*	C-4 Expt. *B*	C-1 Expt. *A*	C-1 Expt. *B*	Glutamic acid. Percentage activity in C-1 Expt. *A*	C-1 Expt. *B*	C-5 Expt. *B*
2	94	91	6	9	89	—	—
5	72	77	28	23	81	79	21
10	71	56	29	44	77	78	22
20	79	—	21	—	70	—	—
30	66	60	34	40	71	63	25
60	69	—	31	—	67	59	27
120	61	—	39	—	61	—	—

Table 17. *Distribution of* ^{14}C *in succinic acid formed by* Saccharomyces cerevisiae *in the presence of glucose and antimycin* A

Samples of succinic acid eluted from the chromatograms of the fixation experiment quoted in Fig. 13 were added to 23·3 mg. of non-isotopic succinic acid and portions subjected to combustion or decarboxylation. Specific activities are in counts/min./mg. of $BaCO_3$ and the probable error of the mean is quoted.

Time of glucose oxidation (min.)	Specific activity of succinic acid carbon atoms $^{14}CO_2H$	$^{14}CH_2$	Percentage activity in CO_2H
7	3·5±0·2	−0·1±0·3	103±4·6
12	7·2±0·4	−0·1±0·4	101±4·2
20	6·8±0·5	−0·6±0·6	109±6·2

samples of radioactive succinic acid from the experiment of $^{14}CO_2$ fixation with glucose and antimycin A were degraded. Table 17 shows that only the carboxyl carbons were radioactive.

Fixation of carbon dioxide by Saccharomyces carlsbergensis

The relative deficiency of oxidizing mechanisms does not prevent the assimilation of carbon dioxide by *S. carlsbergensis* (Table 18). Glucose and lack of oxygen stimulate the isotope incorporation, the distribution of which (Table 19) shows the same pattern as that with baker's yeast under anaerobic conditions, namely, a comparatively strong labelling of malic, fumaric and succinic acid and low activity in glutamic acid.

Table 18. *Carbon dioxide fixation by* Saccharomyces carlsbergensis

Yeast, 7·5 mg.; 5 mM-glucose; 1·6 μmoles of $NaH^{14}CO_3$ ($1·2 \times 10^6$ counts/min.). Final volume, 2 ml. Incubation was at 20° for 20 min.

Gas phase	Additions	$^{14}CO_2$ fixation (counts/min./mg. of cells)	CO_2 formation (μl.)	Oxygen uptake (μl.)
N_2	None	950	40	—
N_2	Glucose	2000	160	—
Air	None	350	60	28
Air	Glucose	1600	220	30

Table 19. *Distribution of* $^{14}CO_2$ *fixed by* Saccharomyces carlsbergensis *with glucose as substrate*

Experimental conditions were as in Table 18. ^{14}C fixed in the methanol–water-soluble substances (counts/min./mg. of cells): 1600 (with glucose) and 300 (control). Corrected distribution (%) of ^{14}C: total ^{14}C fixed with glucose less the respective control value, %.

Substance	Total ^{14}C fixed (counts/min./mg. of cells)		Percentage distribution of ^{14}C with glucose	
	With glucose	Control	Found	Corrected
Krebs-cycle intermediates				
Malic acid	510	105	32·0	31·2
Citric, aconitic and *iso*citric acid	48	5	3·0	3·3
Fumaric acid	96	27	6·0	5·3
Succinic acid	152	63	9·5	6·8
Amino acids				
Aspartic acid	480	66	30·0	32·0
Glutamic acid	107	21	6·7	6·6
Alanine	91	9	5·7	6·3
Threonine	40	0	2·5	3·1
Glycolysis intermediates				
Phosphopyruvic acid	26	0	1·6	2·0
Other substances	48	4	3·0	3·4

DISCUSSION

Of the primary reactions for the incorporation of carbon dioxide into cell metabolism (Utter & Wood, 1951) only the formation of dicarboxylic acids by β-carboxylation provides a satisfactory explanation for carbon dioxide assimilation by yeasts. The synthesis of dicarboxylic acids can be performed by (1) reductive carboxylation of pyruvate, which yields malate (Ochoa, Mehler & Kornberg, 1948), (2) carboxylation of phosphoenolpyruvate, which yields oxaloacetate (Utter & Kurahashi, 1954; Bandurski & Lipmann, 1956), and (3) carboxylation of propionate, which yields succinate (Lardy & Adler, 1956). In yeasts, only the second mechanism seems to be valid, even if oxaloacetic acid, on account of instability, does not appear on the chromatograms. In fact, oxaloacetic acid being the primary fixation compound, the curves of ^{14}C distribution in its immediate derivatives, aspartic, malic and citric acid, will at first show a positive slope following the 'mammillary' model of the kinetics of isotope distribution (cf. Whitehouse & Putman, 1953). However, if one of the secondary reactions (e.g. the transamination of oxaloacetic acid) were much faster than the other two, the maximum of the respective curves may be reached before the isotope distribution could be measured and then the curve of aspartic acid would appear as having a negative slope. On the other hand, if carbon dioxide is incorporated through malic or succinic acid the isotope fixed should follow, totally or in part, the sequence succinic, fumaric, malic, oxaloacetic, aspartic (or citric), that is, a 'catenary' model of isotope distribution (cf. Whitehouse & Putman, 1953) and, accordingly, the curve of succinic (or malic) acid would be the first to have a negative slope. In general the experimental data fulfil the requirements for carbon dioxide fixation through oxaloacetic acid, but with acetate or acetaldehyde the curve of malic acid shows a small negative slope. This, however, if significant does not oppose the role of oxaloacetic acid as the first product of carbon dioxide fixation and can be easily explained by the delay in recording ^{14}C fixation and distribution in the experiments with C_2 substrates. The 'induction' period of oxidation

prevents the measurement of ^{14}C distribution at a time interval short enough to permit the plotting of the malic acid curve before it reaches its maximum.

All the experimental evidence supports phosphopyruvic acid as the primary carbon dioxide acceptor. Phosphopyruvic acid is formed in anaerobic glycolysis (Meyerhof, 1937), a process inhibited by iodoacetate and fluoride; by oxidation of Krebs-cycle intermediates (Kalckar, 1939; Leloir & Muñoz, 1944) and by direct phosphorylation of pyruvic acid (Lardy & Ziegler, 1945). An effective carbon dioxide fixation takes place under experimental conditions involving any of the above-mentioned reaction mechanisms. Thus (1) with glucose as substrate the fastest fixation occurs under anaerobic conditions and is inhibited by iodoacetate and fluoride; (2) during acetate or acetaldehyde oxidation the amount of ^{14}C incorporated is proportional to the amount of substrate metabolized, in other words to the extent of the Krebs-cycle operation, and (3) phosphoenolpyruvic acid can be carboxylated by cell-free extracts of baker's yeast, which also contain pyruvic phosphokinase (observations with J. Cannata, unpublished work). This explains why, after a short incubation, the ^{14}C incorporation and oxygen consumption are faster with pyruvate than with acetate, as the first forms directly phosphopyruvate which immediately promotes carbon dioxide fixation and consequently, the synthesis of the oxaloacetate required to set in operation the cycle. With acetate, except for other less efficient mechanisms such as endogenous-substrate oxidation, phosphopyruvate and oxaloacetate must be formed through the Krebs cycle, that is, the same process they are supposed to start. The carboxylation of pyruvate is also consistent with the distribution of ^{14}C in succinic acid formed by living yeast cells from [2-^{14}C]pyruvate (Stoppani, de Favelukes & Conches, 1958).

The location of ^{14}C in aspartic acid fits in with the proposed C_3+C_1 condensation reaction. Thus after short time fixation all the isotope concentrates in C-4 which reflects the isotope concentration of the β-carboxyl group of oxalacetate. Later, also C-1 of aspartic acid incorporates ^{14}C and this is explained by the partial equilibration of oxaloacetic acid with malic acid, and through the latter with the symmetrically labelled fumaric acid. This interpretation is supported by the distribution of ^{14}C in the succinic acid isolated from the experiments of $^{14}CO_2$ fixation in the presence of glucose and antimycin A. Further, it is still possible that aspartic acid equilibrates directly with fumaric acid, as aspartase has been reported in yeast (Haen & Leopold, 1937). The direct relationship of aspartic acid with the first product of $^{14}CO_2$ fixation, its double labelling and the increase of its pool after sufficient substrate oxidation has occurred account for the large amount of ^{14}C stored in aspartic acid and, consequently, for the peculiar pattern of its curve in the distribution plots.

With pyruvate as substrate, the incorporation of ^{14}C in glutamic acid confirms the fixation path predicted, as the label is located in C-1. This is consistent with the distribution of ^{14}C in aspartic acid (and therefore in oxaloacetate), the synthesis of α-oxoglutarate through the reactions of the Krebs cycle and the asymmetrical behaviour of the citric acid molecule (cf. Krebs, 1954). The concentration of ^{14}C in C-1 agrees also with the distribution of ^{14}C found by Wang *et al.* (1953) in glutamic acid formed by *S. cerevisiae* from [2-^{14}C]pyruvate. With glucose as substrate, only in the initial stages of the fixation does ^{14}C concentrate in C-1 of glutamic acid, and later a significant proportion appears in C-5. Also, with acetaldehyde and acetate, a relatively low labelling of C-1 is found, but in this case the abnormal distribution is already evident at the beginning of the experiment, especially with acetaldehyde. Nevertheless, even with these substrates, after prolonged incubation most of the ^{14}C fixed in glutamic acid still appears in C-1, which proves that the reactions leading the isotope to other carbons are of secondary importance. The labelling of C-5 of glutamic acid suggests previous formation of carboxyl-labelled acetate and resembles the synthesis of glutamic acid obtained with some preparations of rat liver (Plaut & Lardy, 1951), hen oviduct (Hendler & Anfinsen, 1954) or *Clostridium kluyveri* (Tomlinson, 1954). Whether similar reactions take place in baker's yeast cannot be ascertained with the data available. Further studies (now in progress) of ^{14}C distribution in the amino acids formed by *S. cerevisiae* from [1-^{14}C]- and [2-^{14}C]-acetate may elucidate the point.

The Krebs-cycle model is the mechanism that better explains the distribution of the $^{14}CO_2$ fixed during substrate oxidation by *S. cerevisiae*, as well as the distribution of ^{14}C in succinic acid formed by this organism from [1-^{14}C]-, [2-^{14}C]-acetate and [2-^{14}C]pyruvate (Stoppani *et al.* 1958). As the nature of the main path of substrate oxidation in baker's yeast has been the subject of a long debate, before closing its discussion it seems worth while to consider other metabolic routes, the possibility of whose existence is indicated by recent studies. Thus the glyoxylate cycle (Kornberg & Krebs, 1957; Kornberg & Madsen, 1957), or the direct oxidation of acetate through glyoxylate, is consistent with the presence of *iso*citratase in baker's yeast (Olson, 1954) and the trapping experiments of Bolcato, de Benard & Leggiero (1957) respectively. However, none of these

mechanisms can account for (1) the strong labelling of glutamic acid after the fixation of $^{14}CO_2$ (Davis *et al.* 1956, and the present study) or the oxidation of [^{14}C]acetate (Labbe, Thomas, Cheldelin, Christensen & Wang, 1952 and our own unpublished observations); (2) the insignificant appearance of glyoxylic acid in the chromatograms of yeast extracts after fixation of $^{14}CO_2$ or the oxidation of [^{14}C]-acetate (unpublished); and (3) the lack of isotope incorporation into glyoxylate by yeast preparations oxidizing [^{14}C]acetate (Krebs *et al.* 1952). This does not rule out glyoxylic acid as an intermediate in yeast metabolism since this compound may well intervene in the synthesis of glycine. In support of this possibility it must be recalled that the glyoxylic acid formed from *iso*citric acid labelled after $^{14}CO_2$ fixation is radioactive and that glycine appears consistently among the amino acids which incorporate ^{14}C.

To appraise the role of carbon dioxide fixation in yeasts it must be considered in connexion with the metabolism of glucose, i.e. their main source of carbon and energy. Glucose is degraded through the Embden–Meyerhof path (Eaton & Klein, 1957) to pyruvate and this is then decarboxylated and oxidized to acetyl-coenzyme A which, in the presence of oxaloacetate, yields citrate. Under aerobic conditions, citrate can be completely oxidized in order to liberate energy, but if nitrogen compounds are available, citrate as well as oxaloacetate is assimilated and provides the carbon skeletons for many amino acids. Both the oxidation and assimilation processes are dependent on carbon dioxide fixation because this reaction is the main supply of the oxaloacetate required to initiate and maintain the Krebs cycle, to replace the carbon drained into the amino acid pools and to replace the oxaloacetate destroyed by a carboxylase present in baker's yeast (observations with J. Cannata, unpublished work) which resembles the oxaloacetate carboxylase from *Micrococcus lysodeikticus* (Krampitz & Werkman, 1941). The relationship between assimilation processes and carbon dioxide fixation is further supported by their similar inhibition with azide and 2:4-dinitrophenol, two selective poisons of carbon assimilation (Clifton, 1946).

Under anaerobic conditions (this case includes *S. carlsbergensis*), the degradation of glucose to pyruvate also follows the Embden–Meyerhof path, but the rate of the reactions depends on the concentration of organic hydrogen acceptors and, among these, the fumarate–succinate system is one of the more powerful oxidants. The efficiency of that system is enhanced by the continuous excretion of succinic acid into the medium (Conway & Brady, 1950) which keeps its intracellular concentration at a low level. In the presence of nitrogen compounds, assimilation reactions are also possible and the carbon of citrate and oxaloacetate is turned into amino acids, consequently diminishing the synthesis of succinic acid (Kleinzeller, 1941). Again, carbon dioxide fixation is essential for oxidation and assimilation reactions as, through furnishing oxaloacetate, it provides C_4 hydrogen acceptors and carbon skeletons for amino acids.

SUMMARY

1. The mechanism of carbon dioxide fixation by the yeasts *Saccharomyces cerevisiae* (baker's yeast) and *Saccharomyces carlsbergensis* has been studied.

2. Baker's yeast assimilates carbon dioxide during the oxidation of acetate, acetaldehyde, pyruvate and glucose, and also during glucose fermentation, but the oxidation of endogenous substrates is far less effective.

3. After incorporation of radioactive carbon dioxide, ^{14}C labels the intermediates of the Krebs cycle, a number of amino acids and compounds related to glycolysis. Aspartic, glutamic, malic and the tricarboxylic acids are the main reservoirs of the ^{14}C fixed. The distribution patterns vary somewhat according to the substrate oxidized.

4. Wide variations in pH of the medium do not significantly affect the fixation and distribution of radioactive carbon dioxide.

5. Oxaloacetate is believed to be the primary product of fixation and its formation to take place by carboxylation of phosphoenolpyruvate.

6. The patterns of ^{14}C distribution and the location of ^{14}C in aspartic, glutamic and succinic acid are, as a rule, consistent with the Krebs-cycle model.

7. In the presence of glucose, carbon dioxide fixation is inhibited by 2:4-dinitrophenol, sodium azide, iodoacetate and fluoride, and increased by antimycin A. The fixation dependent on endogenous substrate oxidation is inhibited by 2:4-dinitrophenol, sodium azide, iodoacetate and antimycin A, and increased by fluoride.

8. With glucose as substrate, aerobic and anaerobic patterns of fixation differ. In the absence of oxygen (or inhibition of oxidizing mechanisms with antimycin A) more ^{14}C enters into malic, fumaric, succinic and aspartic acid. In *S. carlsbergensis*, the distribution of ^{14}C belongs to the anaerobic type.

9. Carbon dioxide fixation is an essential reaction for yeast metabolism as it supplies carbon for biosynthetic processes and promotes aerobic and anaerobic oxidations.

10. Baker's yeast consumes exogenous pyruvate mainly by oxidation.

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