BIOCHEMISTRY OF YEASTS—AN ANALYSIS OF SOME CONDITIONS FOR GROWTH

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INTRODUCTION

In the course of time yeast cells have yielded many of their internal secrets to biochemists trying to explore various aspects of metabolism in general. The classical period of reconnaissance—commemorated by the names of Buchner, Meyerhof, Harden and Young, among others—gave us the gross outline of glycolysis and fermentation. Later on, as the result of an intensive inventory of yeast enzymes, the first organic cofactors were isolated and identified: cozymase and cocarboxylase, or in the nomenclature of later days, TPP and NAD. The schools of von Euler and Warburg paved the way for the discovery of still more cofactors, activators and a host of intermediates of metabolism. In this age of the discovery of a multitude of phosphate esters the concept of high energy phosphate bonds gradually emerged as the result of the clearsighted work of Lipmann and his group. Isotopes, not only ^{32}P, but ^{13}C, ^{14}C, ^{15}N and the rest, came into the picture and gave, in the hands of Schoenheimer, Rittenberg, Harland Wood and Krebs, new data concerning the internal network of metabolic reactions, with the TCA cycle connected to oxidative phosphorylation as the most prominent feature. The last 20 years of yeast research have, in addition, yielded coenzyme A, biotin, and new concepts of lipid metabolism, which have had a feed-back influence on many fields—the exploration of the biosynthesis of aromatic ring structures in *Penicilliae* and moulds in general, among others. Finally, the patterns of inheritance in yeasts—centred around *Neurospora*—have given an outline of the interplay between genes and protein synthesis, a field which has developed explosively in the last few years.

In paying this homage to yeasts and related micro-organisms as most valuable objects for research with far-reaching stimulae for nearly all sectors of biochemistry and medicine, one may perhaps give a thought to the general tendency in current microbiological chemistry which gives more attention to bacteriae and micro-organisms outside the field of yeasts and fungi. Clearly *Escherichia coli* and related organisms have taken over much of the interest from the yeast sector, and one might perhaps get the occasional impression that the biochemistry of yeast has reached its peak of development, and that the remaining problems of general importance cover only a limited area. Such a view—if it exists—is, however, somewhat misleading. The field has much more to give an inquiring mind; there is no lack of problems. Among the questions that arise in connection with growth of yeasts on various substrates there is one especially that needs a closer analysis: the adaptation problem, including the general influence of the substrate on

enzyme formation in the transition stage between the lag phase of a culture and the subsequent phase of active growth. The problems of this sector of yeast biochemistry are hard to penetrate, yet the extensive use of isotopes in combination with enzyme studies has opened up new possibilities for a closer understanding of the sequence of events involved in the stage preceding the actual growth phase. Some examples of an experimental approach in this direction could be mentioned.

Torulopsis utilis (in newer nomenclature renamed *Candida utilis*) is a well-known member of the yeast series which has an unusual capacity for adaptation on a variety of substrates, both with regard to carbon and nitrogen sources as nutritional components. The early work of Fink, Sperber *et al.*[1-4] provided the background for later studies of the metabolism of this organism. Our own group in Stockholm, later in Lund, Sweden, made in 1947–52 a preliminary survey of the utilization in *Torulopsis* of acetate as the sole source of carbon for amino-acid formation during growth[5-8]. The technique was based on the use of doubly ^{13}C- and ^{14}C-labelled acetate as the sole carbon substrate and a subsequent analysis of the ^{13}C–^{14}C-content of all atoms in the structures of amino-acids isolated. The total pattern of isotope incorporation gave clear evidence of the existence of a cyclic process in the enzymic utilization of acetate by *Torulopsis*—the TCA-cycle in short. Subsequent experiments along the same lines with *Neurospora crassa*[9] gave analogous results, indicating that acetate metabolism in both these members of the yeast series follows the same general pathway as in acetate-utilizing *E. coli*[10], with the exception of the lysine biosynthesis[7-11]. A review in 1955[12] covers the situation at that period.

In later experiments we have tried to extend our isotope techniques to cover some wider aspects of the mechanisms involved in growth of *Torulopsis utilis* on simple substrates. The following questions have been investigated:

(*i*) The extent of acetate metabolism in the lag phase.
(*ii*) The differential utilization of acetate carbon atoms in the log phase of growth.
(*iii*) The utilization of acetate carboxyl oxygen.
(*iv*) The metabolism of formate in lag and log phases.
(*v*) The enzymatic situation in lag and log phases.

Details of methods and techniques are given in a paper by Ehrensvärd, Molander and Saluste[13].

Utilization of acetate carbon

Three parallel cultures of *Torulopsis utilis* were grown on substantially the same medium with regard to carbon sources, but with different isotope-labelling (*Table 1*).

The same ^{13}C-content in the acetate–COOH group in the three parallel experiments, *A*, *B*, and *C* enables a certain correlation between these cultures, not withstanding unavoidable biological variations. The ^{14}C content of acetate–CH_3 (*A*), tracer formate (*B*) and tracer acetone (*C*) made some correlation of the differential utilization of acetate carbon possible, in addition to giving some information about the rôle of formate, and its possible precursor material, in the metabolism of the lag and log phases.

The volumes of the three cultures were the same at start: 3000 ml. The

acetate substrate corresponded to 500 mg of carbon per 1000 ml; the temperature was 25°, and aeration 4–5 litres of air per min. Acetate adaptation of pre-cultures was carried out according to a scheme adapted from Sperber[3] and Roine[4] by Baddiley, Ehrensvärd, Johansson, Reio, Saluste and Stjernholm[5]. Samples of respiratory CO_2 were taken at intervals and analysed with conventional techniques for ^{13}C and ^{14}C content. A uniform

Table 1. Substrate composition in media *A*, *B*, and *C* with regard to isotope labellings

	Substrate composition				*Series*		
	Substance	*Amount per 3 l. of medium*	*Atom % excess*	*Radioactivity* (mC)	*A*	*B*	*C*
Acetate:	$CH_3{}^{13}COOH$†	22 g	12·0	—	+	+	+
	$^{14}CH_3COONa$	Tracer (45 mg)	—	3·0	+	—	—
Formate:	$H^{14}COONa$	Tracer (51 mg)	—	3·0	—	+	—
Acetone:	$^{14}CH_3CO^{14}CH_3$	Tracer (36 mg)	—	2·5	—	—	+
Ammonia nitrogen:	$(^{15}NH_4)_2SO_4$	2·0 g	2·0	—	+	+	+

† The substance acetate, given as $CH_3{}^{13}COOH$, was partly neutralized with 1 N NaOH at the start of the experiment. To all media that had not received a certain tracer amount of radioactive formate or acetone a corresponding amount of non-labelled material was added.

^{15}N-labelling of the pre-cultures enabled the possibility of dilution of amino-acids, isolated from hydrolysates, for subsequent degradative analysis and localization of isotope content: ^{13}C and ^{14}C. Samples were withdrawn at intervals for analysis of the acetate content of the medium and the isotope content of the yeast material.

The over-all picture of the experiment, with regard to acetate utilization, is given in *Figures 1–3*.

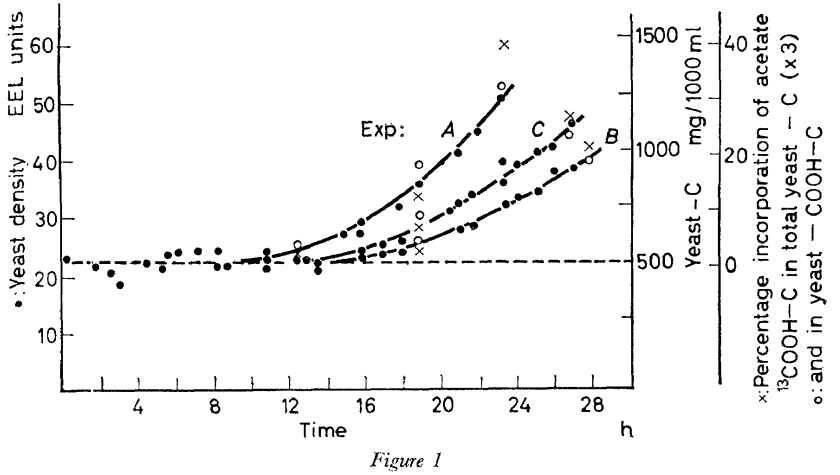

Figure 1

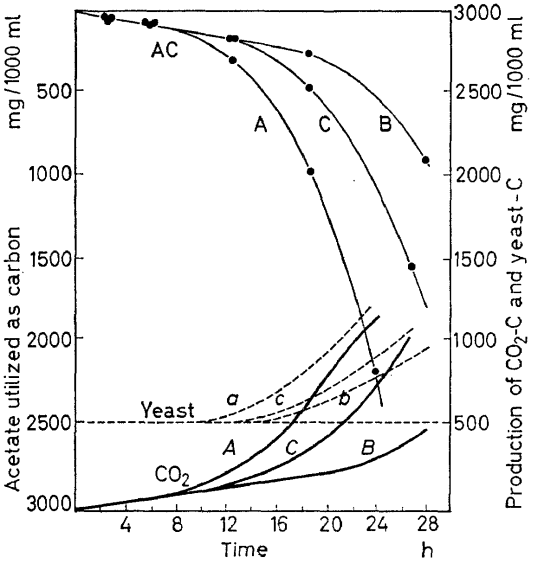

Figure 2

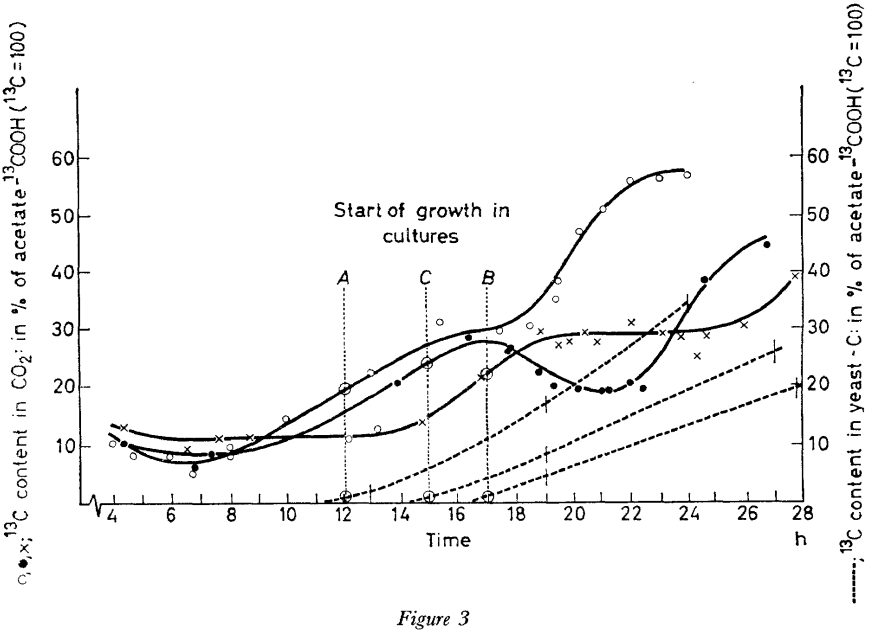

Figure 3

A striking feature is a very extensive lag phase, during which a considerable amount of acetate carbon—indicated by ^{13}C and ^{14}C, in respiratory CO_2—is metabolized, yet without any incorporation in yeast carbon. The general trend in experiments *A*, *B*, and *C* with regard to ^{13}C-output in CO_2 (from

acetate $^{13}COOH$) is the same: at first a fairly constant output, then a marked increase prior to the actual growth period. In the latter period the ratio $^{13}C/^{14}C$, reflecting the metabolism of $^{14}CH_3{}^{13}COOH$ in experiment *A*, is changed from 1 in the lag phase to around 2 (*Figure 4*).

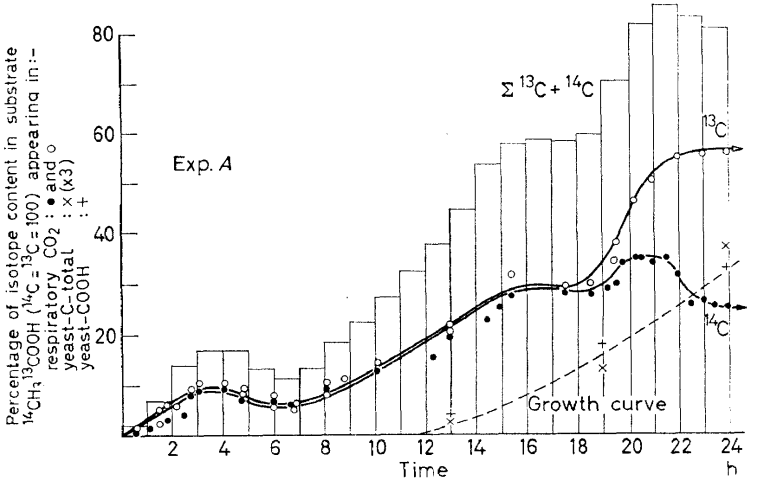

Figure 4

This changed ratio of $^{13}C/^{14}C$ in respiratory CO_2 obviously coincides with the phase of active growth (*A*) where the incorporation of acetate $^{14}CH_3$ into yeast cellular material is more intensive than corresponding incorporation of acetate–$^{13}COOH$-carbon.

The utilization of acetyl carboxyl oxygen

In addition to the experiments *A*, *B*, and *C* outlined, a separate experiment, *D*, was performed, where the substrate was $CH_3{}^{13,14}C^{18}OOH$ in the same general medium as before, as regards inorganic constituents. The synthesis of this acetate follows conventional methods: a certain amount of $CH_3{}^{13,14}C^{18}OOH$ was treated with a minute amount of acetyl chloride, followed by hydrolysis by $H_2{}^{18}O$. The resulting $CH_3{}^{13,14}C^{18}OOH$ had the following isotopic composition:

^{13}C: 0·42 atom per cent excess
^{14}C: 3615 cpm per mg of carbon
^{18}O: 1·67 atom per cent excess.

Estimation of ^{18}O and ^{13}C were made simultaneously by mass spectrometric analysis of CO_2, masses 46 and 45 respectively. The absorption of CO_2 in non-aqueous solution was made according to Ehrensvärd[18] utilizing the formation of an insoluble carbamate or reaction CO_2 with octadecylamine in heptane solution. The experimental conditions for the growth of *Torulopsis* on this medium were the same as described for experiments *A*, *B*, and *C*. The growth curve and the isotope output in respiratory CO_2 is given in *Figure 5*.

As can be seen the values of ^{13}C and ^{14}C—as percentages of the corresponding values in substrate acetate-COOH—keep at the same level throughout the experiment, whereas the ^{18}O-values are about ten times lower. Still, the ^{18}O-output follows, with the factor $\times 10$, the general course of ^{13}C and ^{14}C-output in CO_2, through all the phases in the pre-growth-stages and the period of active growth.

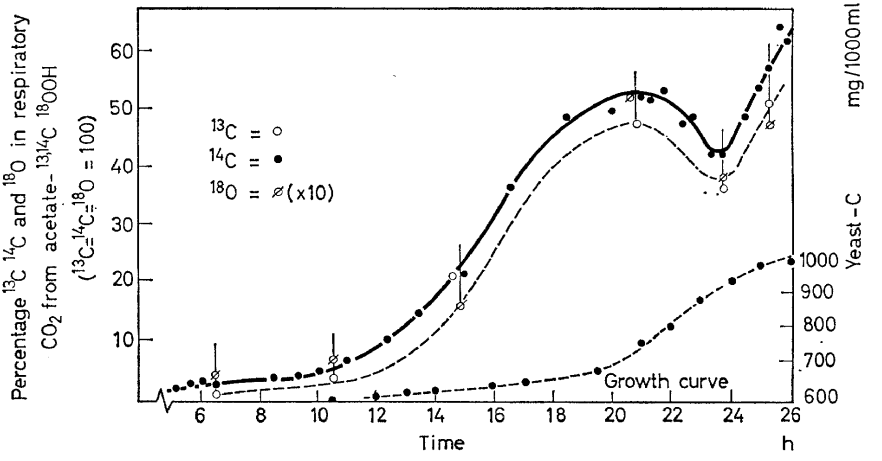

Figure 5

The utilization of formate (experiment *B*)

With regard to acetate utilization in the lag and log phases of growth the experimental data point to a marked metabolism of acetate in the lag phase, yet without any incorporation of acetate into cellular material. In the case of formate (using $^{14}HCOOH$ as tracer) the result is the same in this respect, with the exception that in the later stages of growth the output of ^{14}C, from formate, in respiratory CO_2 becomes less, in contradistinction to the case of acetate isotope carbon, appearing in CO_2, during the same period (*Figure 6*).

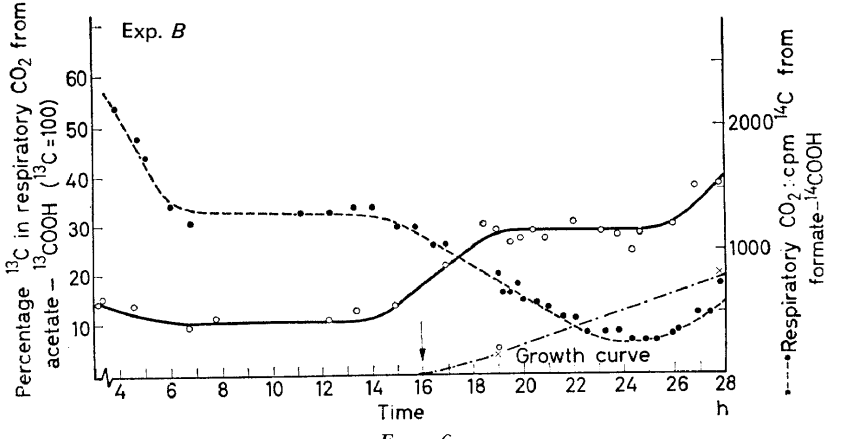

Figure 6

The enzymic background: the rôle of isocitritase

Experiments concerning various enzyme activities during the growth of *Torulopsis*, and the preceding lag phase, have been made. The evidence so far points to a connection between the formation of isocitratase in the late lag phase, (possibly adaptive), preceding the phase of active growth. This is in accordance with the findings of Kornberg and Krebs in *E. coli* with regard to the utilization of acetate *via* the glyoxalate shunt[15–17]. A series of experiments with *Torulopsis* grown on various substrates has given evidence that the isocitratase activity in the yeast cells is low, except in the case of active growth on acetate or ethanol as the sole source of carbon (*Figure 7*).

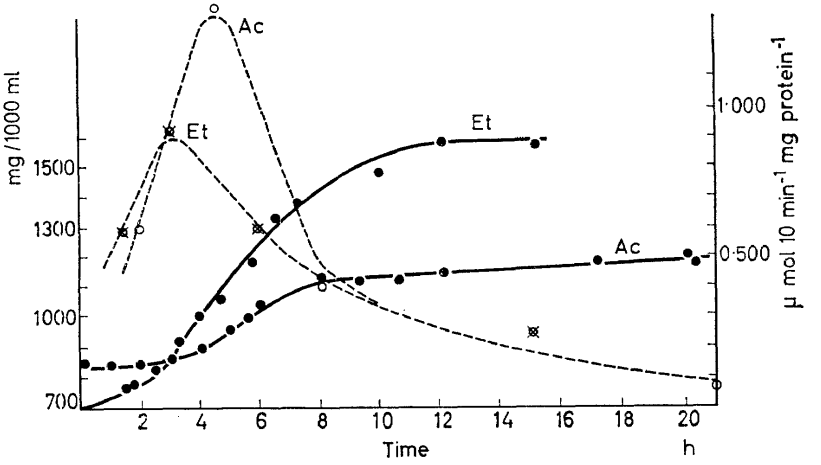

Figure 7 ●: Yeast–C, values are shown along left axis; ○, ⊗: Isocitratase activity, amount of isocitrate split is shown on right axis

DISCUSSION

The above data give a composite picture of the interplay of metabolic reactions during the pre-growth stages and the phase of active growth of *Torulopsis utilis*. Some features of this complex series of events can be discussed.

General aspects of acetate metabolism

It is evident that the metabolism of acetate in the lag phase involves a complete and equal conversion of both carbon atoms into CO_2. Experiment *A*, with $^{14}CH_3{}^{13}COOH$ as the substrate, shows that the $^{13}C/^{14}C$ ratio in respiratory CO_2 remains 1 throughout the lag phase, notwithstanding the relatively high isotope output during this period. Likewise the ^{13}C–^{14}C incorporation into cellular material after 13 hours is neglible. The operation of the acetate oxidation mechanism is thus most likely localized to a cyclic process of the TCA-type, *i.e.* some mitochondriae-like organization of a multi-enzyme system. The main point is, however, that this process functions with 100 per cent efficiency *with no withdrawal of intermediates* liable to be utilized for synthetic purposes, as for instance in the transamination of keto-acids involved in the cycle.

It is interesting to observe how, prior to the onset of active growth, the rate of acetate oxidation is considerably increased, but that the $^{13}C/^{14}C$-ratio is still 1 and that there is no incorporation of acetate carbon into cellular material. Metaphorically speaking, the engine, running for some time at idle speed, is slowly accelerated to higher rev/min values, still with the clutch down. Then the moment comes when the clutch is gently let in and the engine begins to transform the potential energy of the fuel into a specialized kind of work. In the actual case the "clutch" mechanism enables a transition from acetate combustion to acetate combustion and utilization: the synthesis of cellular material. Every indication points towards the synthesis of isocitratase as the key mechanism by which part of the isocitrate is diverted from its pathway towards total oxidation to CO_2 and to the C_2–C_4 split leading to a secondary $C_2 + C_2$ condensation and thence to synthesis in general. Our data concerning isocitratase activity in the lag and log phases (*Figure 7*) fit well with the common view of the glyoxalate pathway[14–17].

Formate metabolism and the distribution of acetate and formate carbon during the growth phase

From series *B* of the experiments (*Figure 6*) it is evident that formate is metabolized at a steady rate throughout the lag phase without any incorporation into cellular material. At the onset of growth the output of formate-^{14}C into respiratory CO_2 decreases considerably; at the same time a considerable incorporation into certain amino-acids takes place (*Table 2*).

Table 2. Distribution of ^{14}C-activity in various amino-acids from A: $^{14}CH_3$ of acetate and B: ^{14}C from tracer formate, both experiment with the same ^{14}C-activity/ml of medium and with the same substrate concentration, *i.e.* acetate, CH_3COONa; ^{14}C-activity in cpm per mg carbon, as $BaCO_3$

Amino-acids	*Carbon atoms*	*Time* (h)	*Series A*	*Time* (h)	*Series B*
Alanine	COOH	13	3	13	0
	$CHNH_2$		10		76
	CH_3		46		
	COOH	19	846	19	0
	$CHNH_2$		1755		3
	CH_3		885		150
	COOH	24	2915	28	38
	$CHNH_2$		7955		40
	CH_3		8655		1020
Serine	COOH			28	120
	$CHNH_2$				360
	CH_2OH				1820000
Glutamic acid	COOH	24	4200	28	49
	$CHNH_2$		12350		373
	CH_2		12975		120
	CH_2		14000		41
	COOH		1125		12
Histidine	Total C			28	291000

The data given in *Table 2* definitely point to a non-interdependence of the acetate and formate metabolism. At least the full oxidation of formate carbon does not seem to pass over the sequence: glycine + C_1 → serine → pyruvate → acetyl-CoA + CO_2 → and *via* the TCA cycle down to CO_2. A comparison between serine and alanine from the formate experiment B gives no comparative features indicating any intracellular transition between serine and pyruvate. Thus, the mechanism of formate oxidation to CO_2 in the lag phase is most likely to be localized to an enzyme system outside the TCA cycle, possibly a plain formate dehydrogenase, splitting formate to H_2O and CO_2. The decrease of output of formate-^{14}C during the growth phase needs further investigation. On the one hand it could be the result of a gradual inhibition of a formate dehydrogenase, or simply the result of a gradually increasing sweeping-up by THFA-controlled formate incorporation into serine and histidine; maybe both of these processes.

A word should be said about the origin of formate carbon from acetate. The addition of tracer acetone, $^{14}CH_3CO^{14}CH_3$ to $CH_3{}^{13}COOH$ in experiment *C* was made in order to investigate the possibility of formate production from aceto-acetate *via* decarboxylation and subsequent dehydrogenation of acetone (*via* acetol) to formate and acetate. The data obtained are not reliable, and do not in any way support this theory. The most probable pathway for formate production from acetate in the log phase would be from glyoxalate from isocitrate *via* decarboxylation. From the point of view of synthesis the possibility has been mentioned[17] that two mechanisms might co-operate:

$$\text{(Isocitrate)} \rightarrow \text{Glyoxylate} \xrightarrow{+\,\text{AcCoA}} \text{Malate} \ldots \rightarrow \text{Pyruvate} + CO_2 \qquad (1)$$

$$\text{(Isocitrate)} \rightarrow \text{Glyoxylate} \downarrow \text{Formate } (+\,CO_2) \rightarrow \text{Serine} \ldots \rightarrow \text{Pyruvate} \qquad (2)$$

Glycine

Our experiments decidedly favour scheme (1).

The utilization of acetate–carboxyl oxygen atoms

The few data from experiment *D* point to the possibility that the labelling of a carboxyl-group with ^{18}O could, in part, fulfil the rôle of an isotope label of the carboxyl carbon itself. Thus the metabolic fate of a three-carbon system: C—C—COOH might, in principle, be followed by using a general labelling of, let us say pyruvate, as $^{14}CH_3{}^{13}COC^{18}OOH$. What is needed is further knowledge of the extent of dilution of the carboxyl-^{18}O on its way towards CO_2. Some experiments with *Penicilliae* and other fungi with regard to the formation of phenolic OH-groups in aromatic structures[18, 19] as derived from acetate–malonate carboxyl-^{18}O definitely point to the fact that in biological systems—at least in yeasts and fungi—there is little exchange between acetate-$C^{18}OOH$ and the medium—$H_2{}^{16}O$ during transformation of acetate into phenolic compounds, for instance orsellinic acid and anthraquinones. The whole subject of the utilization of oxygen isotopes, ^{17}O and ^{18}O, is worth further investigation as far as biological applications

are concerned. As seen from *Figure 5* there is a constant ratio: $^{13,14}C/^{18}O \sim 10$ in respiratory CO_2 from experiment *D*. This ratio defies any explanation at present.

CONCLUSIONS AND SUGGESTIONS

No experiments, however elaborately they utilize present biochemical techniques, give accurate information with regard to a crucial point: the chain of events at the transition stage in the life of living cells, *i.e.* the spontaneous transition between lag phase and log phase of growth. Neither the use of isotopes nor enzymic methods give a clear answer to the problem of what we could formulate as the motivation of the living cell to divide at a certain moment. As seen from the experiments described in the preceding section it is evident that in spite of all precautions three cultures of identical inocculation grown under identical circumstances still show up considerable biological variation. The onset of growth in cultures *A*, *B* and *C* occurs for instance after about 12, 20 and 18 hours. Likewise there is a marked quantitative difference between the cultures in the output of isotopic carbon from acetate-^{13}COOH. A common feature is, however, the fact that prior to the onset of growth, the output of ^{13}C in CO_2 increases considerably, indicating an increased oxidation of acetate, yet with no take-off of material for synthesis. It is most likely that this phase involves an increased oxidative phosphorylation, initiating a DNA and RNA synthesis that will in turn have an immediate impact upon protein synthesis. The data obtained indicate that one of the earliest proteins to be formed must be isocitratase, which holds the key position in the system. As soon as isocitratase is formed the way is paved for the utilization of acetate for synthetic purposes in general: the cell starts to grow and divide.

It is obvious that the experiments described here are still incomplete in many ways. First of all the use of ^{32}P in the medium suggests itself as a tool for studying the turnover of ATP, DNA and RNA-phosphorus during the lag phase, especially with regard to its correlation with the marked rise of the metabolic rate, prior to the onset of the growth phase. In this way it would be possible to observe what is happening in the cell at the stage when the reactions that later will trigger off the growth phase are arranged effectively. It is of the utmost interest from the general biochemical point of view to penetrate the chain of causations behind the series of events leading to the synthesis of the key enzyme, in this case isocitratase. As another suggestion it should be technically possible to isolate isocitratase in pure form and study its specific activity as derived from ^{14}C-acetate in order to determine the exact time when this enzyme is being synthesized from acetate. On the other hand, it is obvious that prior to this moment the isocitratase synthesis (or activation) must occur on the basis of endogenous material.

As has been seen, a study of such a straightforward thing as a yeast growing on a simple medium provokes many questions; the more elaborate such a study is made, utilizing the whole paraphenalia of isotopes and enzyme determination and isolation, the more questions will arise about the main point: what is the first spontaneous event in the lag phase that becomes the trigger mechanism for the complex set of reactions that in the course of time will lead to an organization enabling cell division? We have tried to follow

the chain of events as far back as possible. The experiments here described are by no means conclusive; rather they should be regarded as a challenge to much more refined attempts leading to some insight into what we, metaphorically, could formulate as the motivation of the living cell to start the interplay of reactions leading to cell division. As stated in the introduction, the study of yeast biochemistry has much to give an inquiring mind.

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