PROTEIN SYNTHESIS IN YEAST

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INTRODUCTION

The subject of the synthesis of protein by yeast is one of obvious general interest but is also one of paramount importance in technical brewing. Briefly this importance arises because when a quantity of yeast is introduced into wort, the success of the brewing operation depends upon the prompt production of a sufficient number of cells of adequate fermentative power. In this general approach it is necessary to leave aside, for simplicity, considerations of fine differences in flavours such as those concerned with the production of diacetyl, volatile sulphur compounds and fusel oil components. These nuances will perhaps in due course be related to particular aspects of nitrogen metabolism but with regard to the two main requirements we are clearly concerned with protein synthesis in broader aspects.

In yeast fermentation, the succession of the lag, log and stationary phases is customarily regarded even by biochemists and microbiologists in mechanistic terms. The lag phase would seem to be one in which the metabolism of the cells is becoming geared to their environment, the log phase that in which the necessary numbers of cells of appropriate enzymic content are produced, while the stationary phase is characterized, apart from the occasional death of some of the cells and their balanced replacement, by the action of preformed maltase and glycolytic enzymes in converting glucose, fructose, maltose and maltotriose substantially into ethanol and carbon dioxide. From both the scientific and practical fermentation aspects, therefore, we are concerned with the nature of the gearing process, the course of protein synthesis when the gearing has been achieved, and with the direction of protein synthesis into useful enzyme synthesis at the various stages. This contribution is concerned with recent progress in understanding these events, but to bring this understanding into sharper focus, it may be useful to recall certain other circumstances in relation to yeast fermentations.

THE AGE ASPECTS OF YEAST CULTURES

The elucidation of the development of specific properties in individual cells of, preferably, known cultural history might seem at first to be hampered by the fact that to some extent, at least, a yeast culture is usually heterogeneous in various respects. Thus it has been found[1] by counting the bud-scars on sufficiently large numbers of cells that, for reasons which need not be detailed here, the inoculum in a normal fermentation by *Saccharomyces cerevisiae* usually consists of cells of different ages but an age distribution which is fairly characteristic (*Figure 1*). Thus some 68 per cent consists of newly formed cells, 18 per cent of cells which have borne one daughter, 9 per cent which have borne two and progressively smaller proportions of

older cells. Now it is a simple arithmetic exercise to show that if the cells of these different histories all divide with the same ease and the progeny have the same chance of survival, then after a period of true logarithmic growth the distribution should consist of 50 per cent of new cells, 25 per cent of one-daughter cells, 12·5 per cent of two-daughter and so on along the series. It will be seen, however, that under normal conditions the main bulk of final yeast approximates quite closely in composition to that of the inoculum.
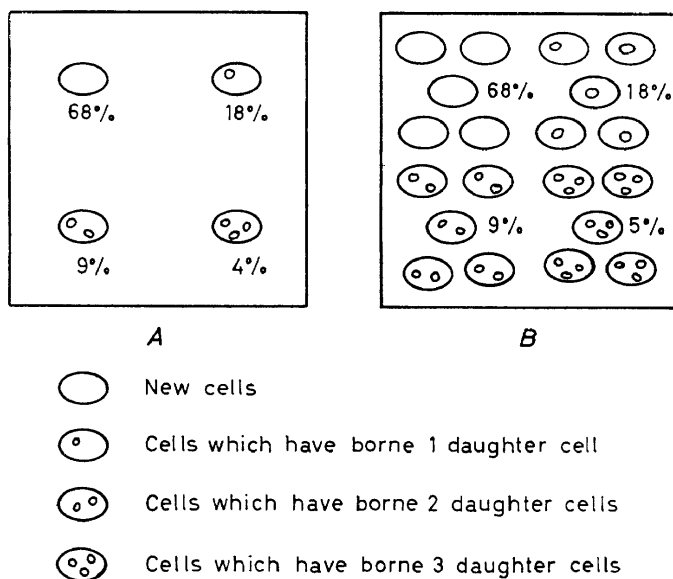

New cells

Cells which have borne 1 daughter cell

Cells which have borne 2 daughter cells

Cells which have borne 3 daughter cells

Figure 1. Distribution of age and relative yeast cell numbers in normal fermentation; (A) Pitching; (B) Completion

To some extent there is inevitably some segregation of older cells by deposition and moreover it must be remembered that using a massive inoculum there is generally only about a five-fold increase in the total amount of yeast, so that despite the use of the term log phase, the yeast never enters into a period of sustained logarithmic multiplication. However complex the conditions are and however remote they may be from the theoretical ideal of logarithmic growth, they are those with which in large-scale yeast fermentations we are concerned. It is, therefore, of interest to find that even when special steps are taken to employ an inoculum consisting as nearly as possible entirely of new cells, the ultimate cell distribution is substantially unchanged even though the distribution at an intermediate point may be rather different (*Figure 2*).

This might suggest that under similar conditions an old cell which had already borne a number of daughter cells became relatively slower to produce new cells than a younger one. Moreover, one might envisage that the progeny of an old cell might be somewhat impaired in fermentative efficiency and might itself be weakened in respect of ability to give rise to further cells. Johnston[2] has therefore formed an estimate of the maximum life-span of
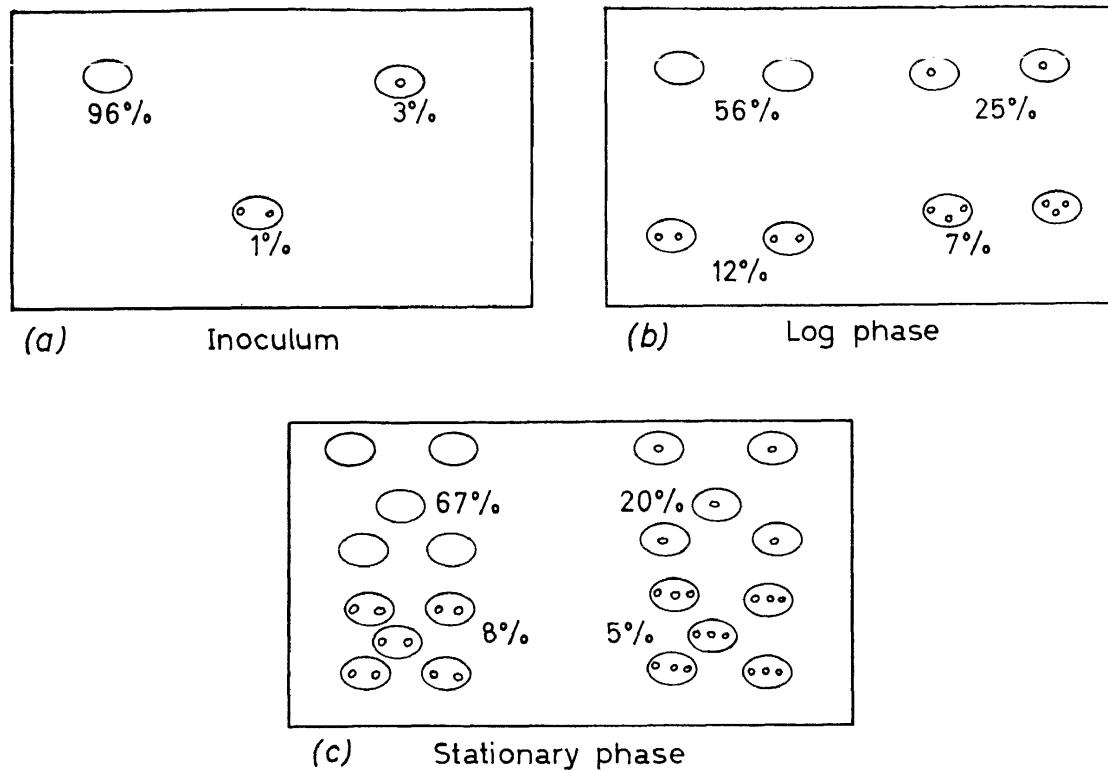
Figure 2. Development of age distribution during fermentation using new yeast cells

large numbers of cells taken at random from a culture of *S. cerevisiae*. Instead of counting bud-scars after extended periods of growth, the method of investigation consisted in culturing the selected cells one at a time under conditions of adequate nutrition, removing daughter cells as they were formed—sometimes for continued observations, but continuing to observe the production of daughters and their generation time by the mother-cell until the death of the latter brought the whole sequence to an end (*Figure 3*).

The results have shown (*Figure 4*) that though in detail they depend on temperature and perhaps other variables, the average yeast cell stands a
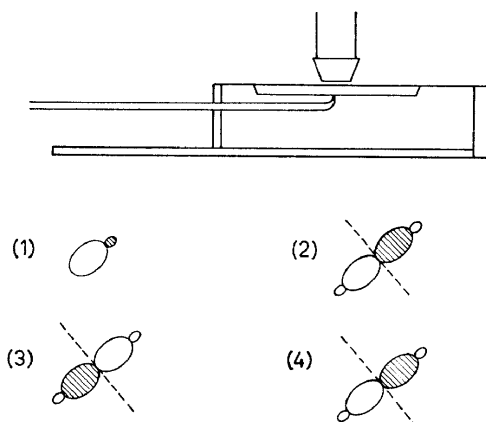

Figure 3. Method used to examine the life span of a yeast cell; (1) Isolated budding cell; (2) 1st bud of selected mother cell; (3,4) 2nd and 3rd bud of the mother cell
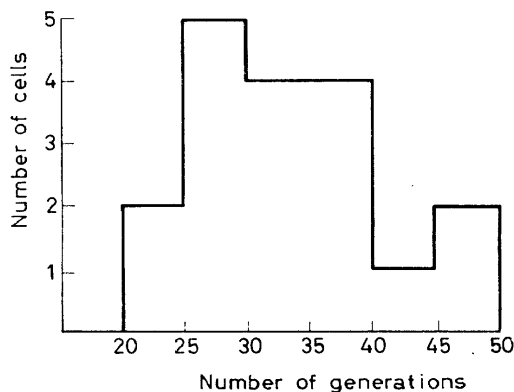

Figure 4. Life span distribution among yeast cells

good chance of being able to produce many more daughter-cells than it is usually called upon to do. Thus a large proportion is capable of producing 20–40 daughters and some as many as 50. What is perhaps more important, however, is that late daughter cells seem not to be distinguished from young ones either in respect of fermentative power or ability to give rise to their

own progeny. In other words, to outward appearances the final death of the mother-cell does not appear to be due to a progressive weakening but to a sudden cessation in the division process as though due to final exhaustion of a limiting metabolite or to what may be best called a "biochemical mistake".

Returning then to normal fermentations in which the amount of growth falls so far short of the maximum, perhaps the most important questions which arise are (a) why the amount of growth should be so curtailed when so far as is known the essential nutrients are far from being exhausted; (b) seeing that the cultures have scarcely entered into any period of regular logarithmic growth before their increase is curtailed, whether there are discernible differences of importance in their fermentative and their metabolic activities over the first few generations which might have a bearing on the fermentative behaviour of the culture as a whole.

In connection with (a), Harris and Wiseman[3] have found that spent media of various kinds used in yeast fermentations contain at least two factors which influence the rate of growth of the yeast. The effects are demonstrated on fresh yeast cultures using added amounts of the factors which, it has to be emphasized, have only been partially separated and not as yet identified. It thus emerges that one has an inhibitory action towards division and is both volatile and dialysable while the other, which is probably associated with a co-factor, is non-dialysable, heat-labile, oxygen-labile and perhaps protein and enzymic in nature. Its function is to promote cell division and as it shows no disulphide reductase activity towards cystine it is probably different from the division-catalysing enzyme reported independently[4]. These findings are, however, sufficient to suggest that under normal yeast fermentation conditions, the curtailment of the incipient logarithmic phase is at least in part due to the development and interplay of inhibitory and auxiliary factors. One may reasonably suppose that this curtailment with presumably its curtailment of the synthesis of structural protein and nuclear material, serves to provide opportunity for the fermentative mechanisms to come more fully into play. It remains, however, to enquire into the second question above, (b), namely, whether it is possible to discern differences of likely importance in cells over their first few generations before we can go with confidence to draw any penetrating conclusions from observations on cultures of different age- or generation-distributions.

SYNCHRONIZED YEAST CULTURES

Faced with a heterogeneity of cells, it is clearly almost impossible to contemplate studying individual organisms. Attention was, therefore, turned to the examination of synchronized cultures. It would be tedious to describe the methods[5, 6] which have been developed to ensure that substantially all the cells under examination are, to judge from microscopic appearance, in the same state of budding and all undergo division for a few generations at least at, for practical purposes, the same time. It is unnecessary to detail the limitations on any conclusions which may be drawn, not the least being that because the conditions, usually of alternate starvation and growth, have the effect of putting a restraint upon those cells which would

otherwise have gone on to produce daughter cells, they may result in an abnormal metabolic pattern in the period when the culture is returning to its normal random growth. It is, however, the best that can be done in present circumstances and the limitations are lessened by the thought that it would be an accountable change in metabolic pattern over the first few generations which might forecast doubt of the usefulness in attempting to examine the comparable metabolism of random cultures. Conversely, regularity in metabolic pattern over the early growth period of the synchronized cultures would suggest that over-all changes in random cultures could be regarded as significant with regard to the culture as a whole and not merely with regard to the changing age-distribution of the component cells.

These aspects of *S. cerevisiae* cultures have been studied, notably by Williamson and Scopes[7] with respect to such features as total protein synthesis (*Figure 5*). It will be seen that while nitrogen uptake is stepwise,
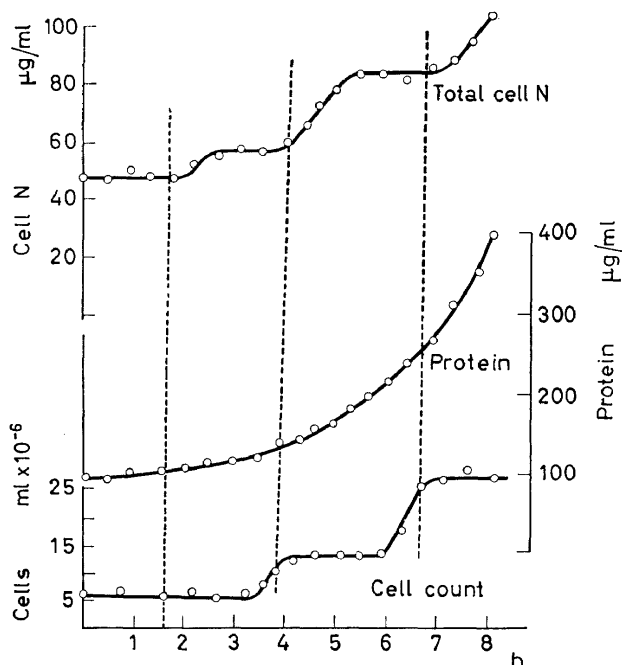

Figure 5. Uptake of nitrogen and protein synthesis in synchronous yeast cells

protein content of the culture increases regularly though respiration shows the stepped pattern (*Figure 6*). Again phosphorus uptake is substantially regular (*Figure 7*) though DNA synthesis is stepwise, being laid down abruptly almost immediately after each cell has given the first sign of producing a bud (*Figure 8*). RNA is also synthesized intermittently though at a different point in the budding cycle, some of these differences being summarized schematically in *Figure 9*. Among the general conclusions from this voluminous work, it seems reasonable to conclude that no progressive changes in metabolic pattern have been discerned over at least
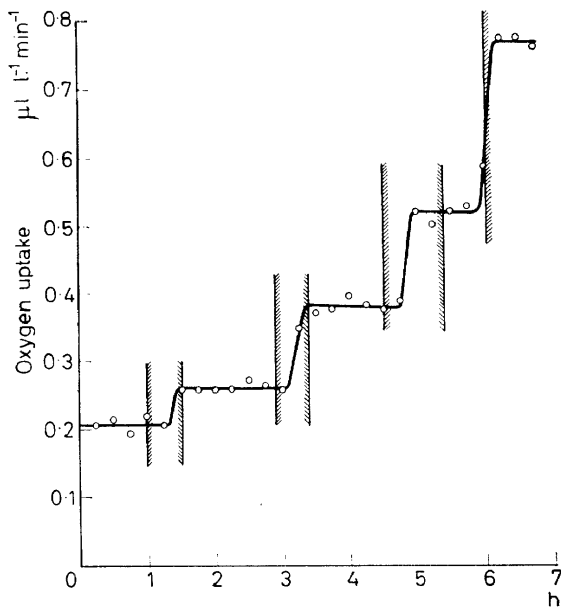
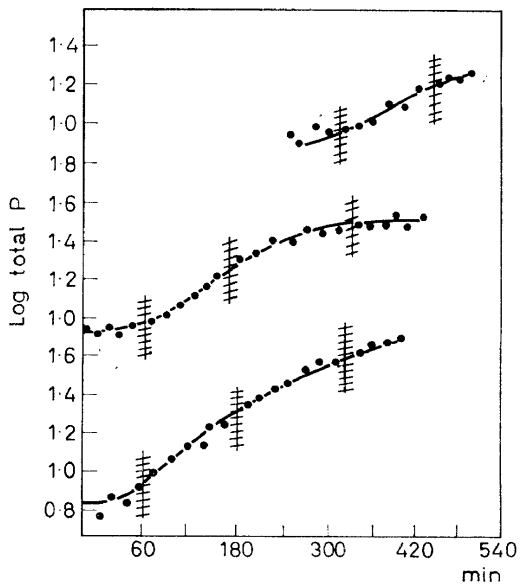*Figure 6.* Respiration of synchronized yeast cells
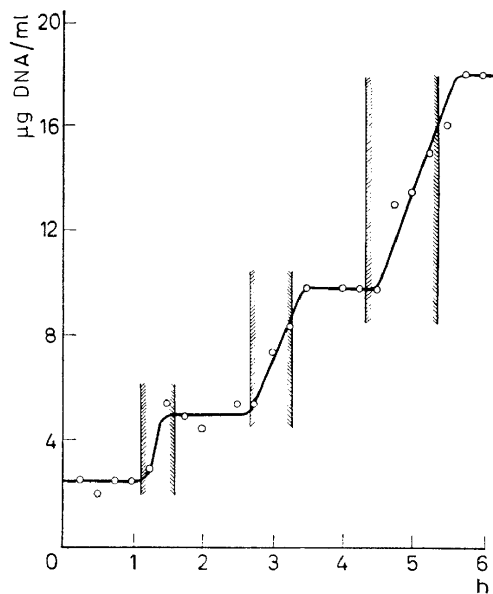

Figure 7. Phosphorus uptake of synchronized yeast cells
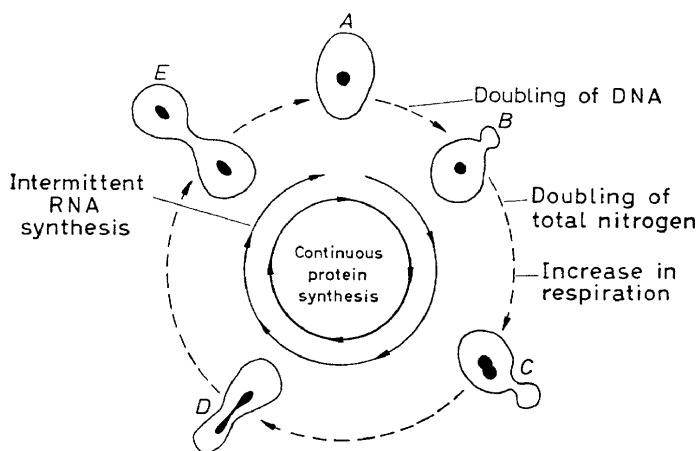
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Figure 8. DNA synthesis in a synchronized yeast cell

Figure 9. Metabolic activities of synchronized yeast cells

A : Parent cell
B : Cell with bud, single nucleus
C : Cell with bud, replicated nucleus

D : Cell with daughter nucleus moving to bud
E : Cell with daughter at onset of final division

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a few initial generations. On the other hand, since for instance total nitrogen uptake is not directly in step with either protein, DNA or RNA synthesis, the cells must provide themselves with a substantial pool of intermediates which can be drawn upon in different ways according to whether they are devoting most of their energy to production of new cells or to maintaining or varying their fermentative abilities.

SOME NITROGENOUS INTERMEDIATES IN YEAST

It has for some time been known that various yeasts contain compounds of a general nature which would fit them into the brief outline given above[8]. Moreover "amino-acid adenylates" had still earlier[9] been implicated as intermediates in protein synthesis by cells generally. Davies and Harris[10] working with yeast carried the story an important step forward by recognizing arginylalanalarginylalamyl-5'-uridylate (*Figure 10*) as an individual
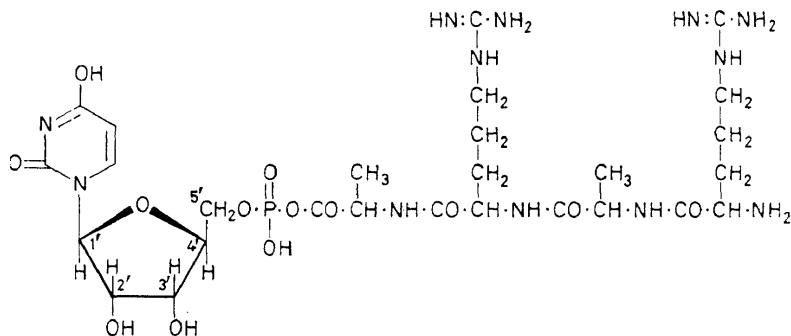

Figure 10. Arginylalanylarginylalanyl-5'-uridylate isolated from yeast

compound present in certain extracts and that this was only one of a large array of peptidyl nucleotidates[11]. The structure shown in *Figure 9* was established by degradative studies (*Figure 11*) and confirmed by synthesis[12] (*Figure 12*).

The importance of these observations lies, however, not so much in this specific compound as (*a*) in demonstrating unequivocally the occurrence of active anhydrides in yeast, (*b*) leading to the qualitative recognition of a large number of similar compounds, (*c*) revealing the occurrence of compounds containing not exclusively uridine but variously each of the purine and pyrimidine bases found in RNA and (*d*) leading to the recognition of a large number of comparable di- and polynucleotides[13-15] (see *Figure 13*). It has thus become clear that the variety of such intermediates is extremely wide and that their theoretically possible variety having regard to the possible combinations among peptides with nucleotides of different sequences, surpasses that of the peptides themselves.

Their significance was underlined by the finding that the amounts of the total of such intermediates changes during growth of a culture, reaching a maximum during the early stages of growth when they are perhaps produced in excess of requirements so that their later, more rapid, turnover reduces their stationary concentration[16]. Moreover, while their precise rôle remains
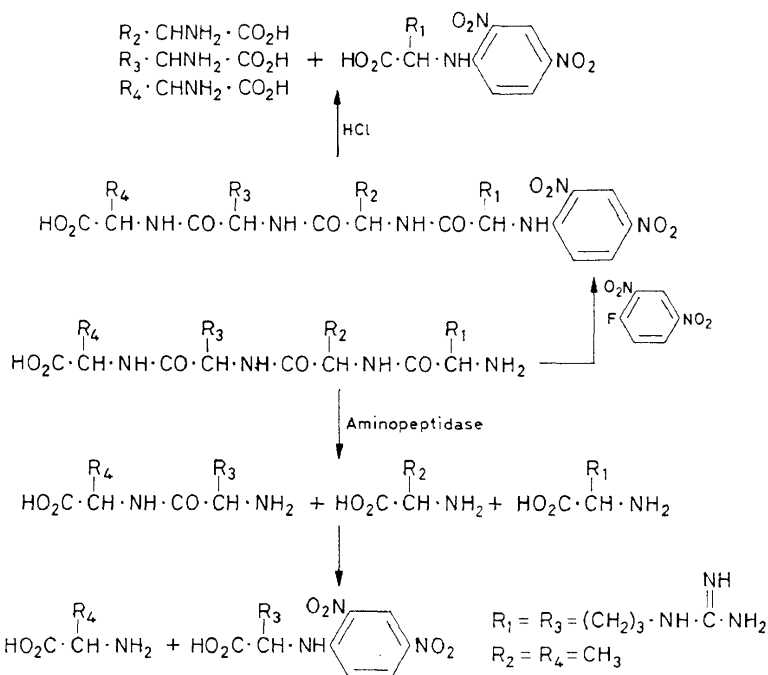
Figure 11. Stepwise degradation of peptide moiety of arginylalanylarginylalanyl-5′-uridylate

where

R = Uracil or adenine
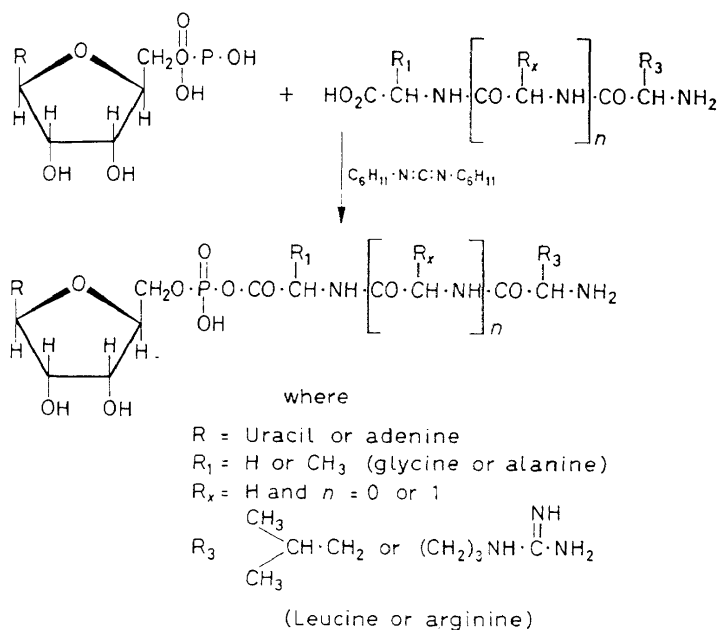R_1 = H or CH_3 (glycine or alanine)
R_x = H and n = 0 or 1

R₃ ... $CH \cdot CH_2$ or $(CH_2)_3 NH \cdot \overset{NH}{\underset{||}{C}} \cdot NH_2$

(Leucine or arginine)

Figure 12. Synthesis of peptidyl nucleotide anhydrides

	Nucleotide peptide			
	A	B	C	D
Amino-acid				
Cystine	1	—	1	3
Arginine	10	7	14	9
Glutamic acid	4	2	2	—
Aspartic acid	1	1	—	—
Valine	1	1	2	2
Leucine	2	1	2	1
Alanine	2	4	3	6
Base				
Uracil	+	+	+	+
Adenine	+	—	+	+

Figure 13. General composition of several nucleotide
peptides occurring in yeast

incompletely elucidated, their general status as intermediates seems certain from, say, the observed incorporation of [^{14}C]-uracil into the nucleotide peptide-anhydride pool[17] and the similar uptake of [^{14}C]-arginine[18, 19]. The view that amino-acids are not merely incorporated into such peptidyl-nucleoticates but that the latter are indeed intermediates in protein synthesis has also been substantiated. It has, of course, always been appreciated that such demonstrations do not necessarily imply that the intermediates are concerned exclusively in the synthetic pathway since they may also be concerned wholly or in part with protein breakdown. At first, therefore, the picture seemed to be almost hopelessly complicated by the finding that the total intermediate pool contains as well an assortment of ribose-esters in which the peptide residue is attached to the 2′- or 3′-position instead of the 5′-phosphate grouping as will be clear from the illustrative structure in *Figure 14*[20].

where R peptide residue

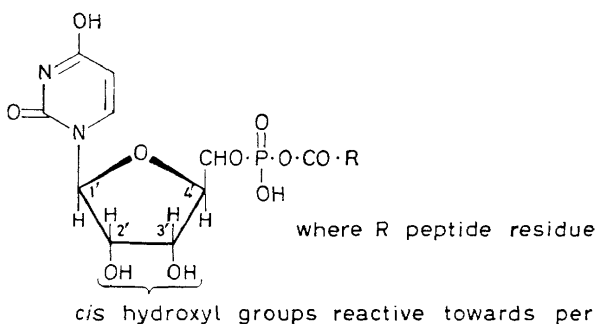*cis* hydroxyl groups reactive towards periodate

Figure 14. 5′-Anhydride nature as compared with 2′- or 3′-peptide ester nature of further
yeast components

The proportions of the esters and anhydrides vary according to the state of the culture in such a way[21, 22] as to suggest that the anhydrides are more particularly concerned in the synthesis of microsomal and perhaps other structural proteins[23]. Still other groups of compounds including acidic as
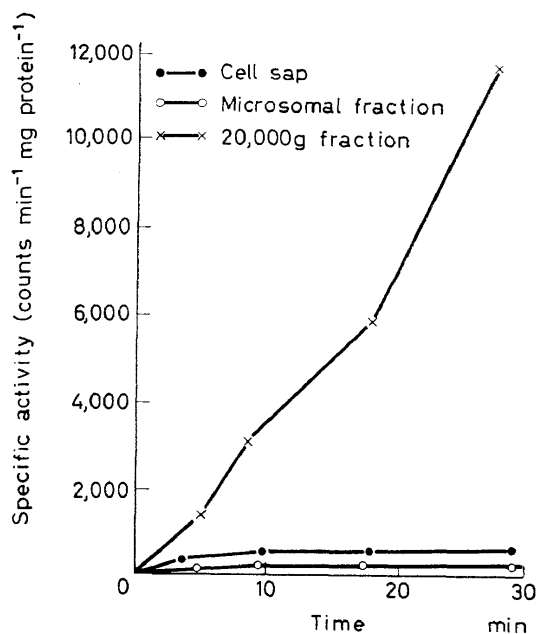
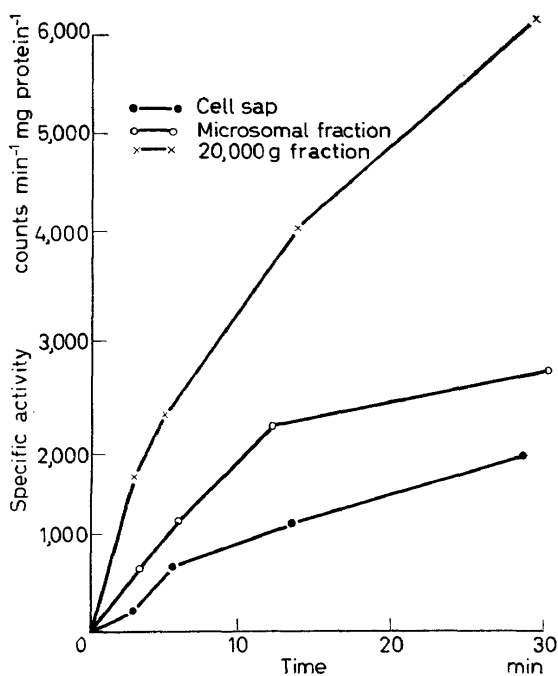631

Figure 15. Uptake of radioactivity by a yeast disintegrate

Figure 16. Course of uptake of radioactivity by whole yeast subsequently disintegrated

distinct from basic peptidylnucleotidates also appear in yeast extracts especially in later phases of growth[24]. Limitations of space preclude a description of other extensions of this work to studies of the uptake of radio-active amino-acids into yeast cell sap and segregated cell particles and of the recognition of enzyme systems capable of giving rise to intermediates of the type described from added free amino-acids. Important aspects of the integration of the process will, however, be apparent from a comparison of *Figures 15* and *16* showing the very different relative uptakes of cell-sap, so-called microsomal and mitochondrial fractions in pre-isolated cell disintegrates and in whole cells disintegrated following the incorporation of radioactive material. It is clear, however, even without this more detailed knowledge, that the chemistry just outlined represents considerable insight into the paths by which proteins and enzymes are synthesized in yeast and promises to afford welcome external control over their development. We turn, therefore, to a quite different series of researches which already reveal how useful and desirable such control is becoming.

ENZYME DEVELOPMENT IN YEAST

Figure 17 recalls diagrammatically a number of well-known facts, the salient of which are as follows:
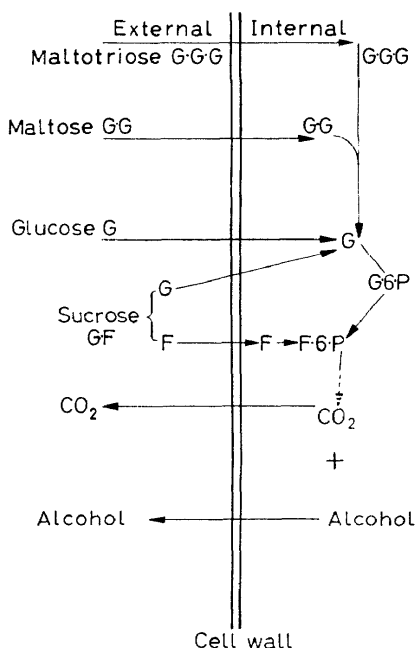

Figure 17. Diagrammatic representation of fermentation by yeast of carbohydrates

(*i*) The fermentation of wort sugars, glucose (G), fructose (F), maltose (G·G) and maltotriose (G·G·G) takes place within the yeast cell. The first two gain entry by what has been termed facilitated diffusion, sucrose being hydrolysed by invertase on or near the surface of the cell.

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(*ii*) Maltose and maltotriose, however, are pumped into the cell *via* specific permease systems and are there broken down by maltase which, at least in some instances, possesses maltotriase activity as well within the same entity[25-27].

(*iii*) Glucose and fructose are fermented through a common pathway, with the ultimate products carbon dioxide and ethanol diffusing out into the external medium.

It is from a research viewpoint fortunate, but in practical respects unfortunate, that both the permease and maltase types of system are inducible. In other words when the cells are kept out of contact with the relevant carbohydrates as in normal yeast storage, both types of systems undergo degeneration. On introducing such cells therefore into a new medium, there follows a lag period (*Figure 18*) during which both systems
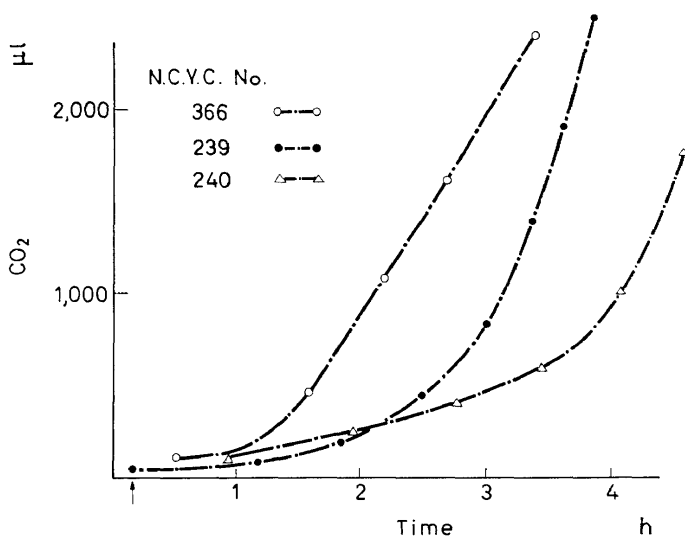

Figure 18. Varying adaptation to maltose utilization by *S. cerevisiae* strains previously re-adapted to maltose

are renewed. Harris and Millin[28] have indeed been able to distinguish the separate synthesis of these systems with respect to maltose, in that the relevant maltose–permease is generated in response to the carbohydrate alone, the nitrogen requirements being necessarily met from within the cell (*Figure 19*). That there are nitrogen requirements can be inferred from the enzymic nature of the permeases while their synthesis as proteins is confirmed by the findings that their appearance is inhibited by the unnatural analogues of amino-acids such as β-2-thienyl- and p-fluorophenyl-alanine in the surrounding medium[25]. Maltose–permease is therefore synthesized by stimulation with maltose which accumulates within the cell though is not utilized, as is seen from *Figure 19*, until maltase is formed following the addition of external nitrogenous nutrients.

The practical and scientific importance of these facts appears when taken in conjunction with the further findings that the capacities of representative
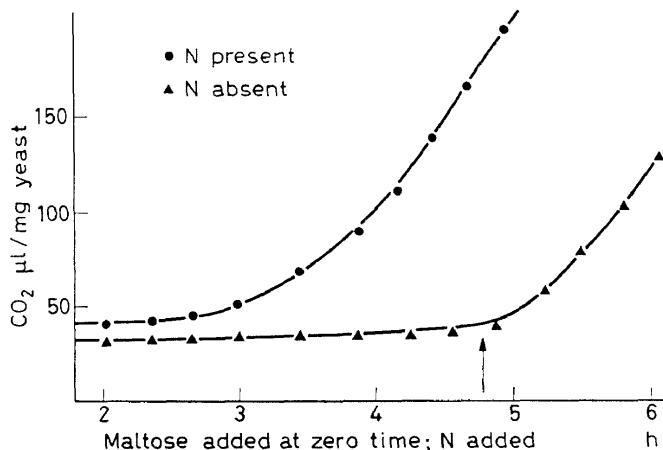
Figure 19. Development of maltose–permease (▲) and maltose–permease (●) together with maltase in *S. Cerevisiae*

strains of *S. cerevisiae* to ferment glucose, maltose and maltotriose appear to be so dependent on the presence of the relevant carbohydrates in adequate concentrations that when these concentrations alter during normal fermentations they vary in a cyclical manner (*Figure 20*)[29].

So whereas the glucose-utilizing ability rises quickly and slowly subsides, the maltose-utilizing ability first falls to a low level, then rises sharply and thereafter drops off slowly. The behaviour of the maltotriose-fermentative power follows a similar pattern though on a different time scale as reflected by the drop in specific gravity. There is little doubt that these changes, which must in part be ascribed in fluctuations in the relevant transport mechanisms, lie behind the empirical observation that in a normal fermentation using a mixture of carbohydrates such as brewers' wort it is the simple sugars which are removed first, followed by the di- and tri-saccharides.

Obviously the efficiency of such a normal fermentation, *i.e.* the speed with which the sugars over-all are removed and the specific gravity reduced, is an integrated function of the rate of formation and subsequent degeneration of at least several enzymes. It would thus become subject to some control if it were possible to influence this formation and degeneration, *e.g.* by promoting the former and discouraging the latter or at least by synchronizing the optimal formation of the relevant enzymes. No means of doing this are so far known but fortunately the knowledge can still be put to useful account in continuous yeast fermentations. This use arose since the behaviour of the yeast in responding to the carbohydrate composition of its environment, suggested that in a simple single-vessel system in which the fresh medium was continuously introduced into a vessel containing the stirred medium and relevant yeast while beer was continuously withdrawn, the physiological state of the yeast would depend on the specific gravity of the spent medium. The specific gravity would in turn be linked with the rate of introduction of the medium and thus necessarily with the rate of production of the fermented medium. This has been confirmed as will be seen from *Figure 21.*

300

100

$Q_{CO_2}^{N_2}$glucose

1·0

0·5

$\dfrac{Q_{CO_2}^{N_2}\text{ maltose}}{Q_{CO_2}^{N_2}\text{ glucose}}$

0·5

$\dfrac{Q_{CO_2}^{N_2}\text{ maltotriose}}{Q_{CO_2}^{N_2}\text{ glucose}}$

10

5

Maltase
(units x 10^2)

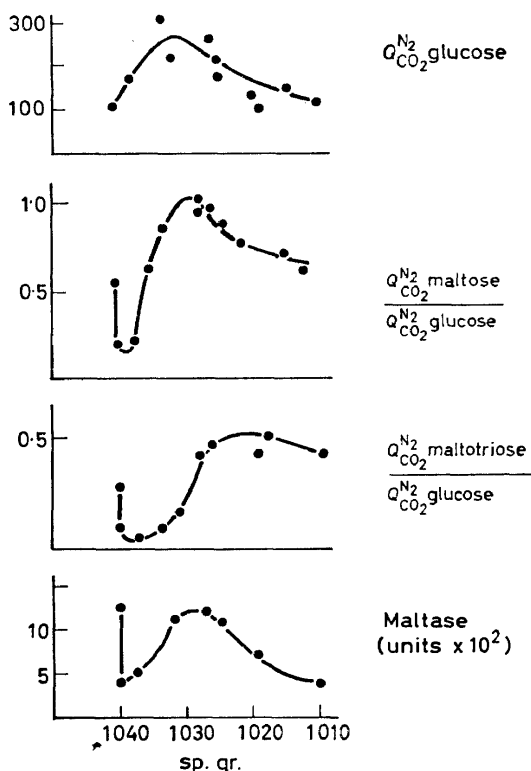1040   1030   1020   1010
sp. gr.

Figure 20. Variation in fermentative ability in *S. cerevisiae* during normal fermentation

Clearly if, for example, the beer is withdrawn at sp.gr. 1·020 the yeast is then in such a condition that its glucose-utilizing ability is nearly maximal while its maltose-metabolizing ability is little more than half what it would have been at a somewhat higher gravity and its maltotriose-utilizing ability considerably less than it would be at a still lower gravity. Ideally the performance would be much improved if, thinking of the removal of three

Initial $Q_{CO_2}^{N_2}$

Specific gravity	Glucose	Maltose	Maltotriose	Maltase
1·032	260	221	19	1·7
1·020	246	120	53	9·3
1·009	201	130	80	4·0

Figure 21. Variation in fermentative ability in *S. cerevisiae* during continuous fermentations of selected final gravity (original gravity 1·040)

sugars, glucose, maltose and maltotriose, one were to arrange for all the glucose to be removed in a first vessel, the partly spent medium freed from yeast passed into a second vessel charged with yeast adapted to removing

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maltose, and the further spent medium again freed from yeast passed into a third vessel adapted with its yeast to removing maltotriose. This has in fact been achieved in principle with considerable improvement in efficiency though probably the maximum still remains to be reached since the relative volume at each stage needs theoretically to be adjusted according to the amount of each sugar to be removed and according to the prevailing rate of its removal. This point represents probably the frontier of knowledge in this respect and the temporary culmination of widely differing lines of research, but the effort in so many diverse disciplines is surely justified by the breadth of the scientific and technical vistas so opened up.

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