DYNAMIC ASPECTS OF THE PEPTIDYL-NUCLEOTIDATE POOL OF BREWER'S YEAST DURING GROWTH

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SUMMARY

Concentrates of peptidyl-nucleotidates were obtained from yeast at various stages of growth by successively extracting it with aqueous ethanol and trichloroacetic acid solution followed by electrophoresis of the extracts. The total amount of these substances increased to a maximum during the onset of logarithmic growth of the yeast and then declined to a low value during the logarithmic phase. As the rate of growth decreased the amount of peptidyl-nucleotidate again increased, only to fall once more as the culture aged.

Resolution of the concentrates of peptidyl-nucleotidates at each stage of growth was achieved by chromatography on paper and the amino acid, purine and pyrimidine composition of the individual fractions determined. The results, taken in conjunction with both the chemical structures of the peptidyl-nucleotidates as already elucidated and the above data on the derived hydroxamates, suggest that the peptidyl-nucleotidates examined participate in the synthesis of nucleoprotein. It appears that they are synthesized by the yeast cell in excess during early growth but are then organized for very rapid turnover during logarithmic growth so that their stationary concentration at any one time at this stage is low. Subsequently, as growth becomes unbalanced the synthesis of certain peptidyl-nucleotidates continues but these compounds are no longer integrated with others and hence accumulate.

INTRODUCTION

In the previous paper[1] it was shown that peptidyl-nucleotidates from yeast are 5'-phosphoanhydrides derived from a mononucleotide or an oligonucleotide on the one hand and a peptide or amino acid on the other. They were derived from a 48 h culture of yeast and it was of interest further to discover whether or not they were present in the cells at earlier stages of growth and how the pool of peptidyl-nucleotidates varied during the life of the culture. The present communication describes attempts to obtain information on these aspects of yeast cell growth.

MATERIAL AND METHODS

Culture of yeast

Saccharomyces cerevisiae (No. 240, British National Collection of Yeast Cultures) was grown for 48 h in shake culture in MYGP. The yeast was centrifuged off and a

Abbreviation: MYGP, maltose extract–yeast extract–glucose–peptone medium.

sample removed for analysis as below. The bulk was immediately inoculated into fresh MYGP in which it was grown with shaking and samples removed as required.

Extraction of yeast

The yeast was centrifuged off at intervals and immediately treated with cold ethanol to inactivate it. It was washed quickly with a mixture (1:1, v/v) of ethanol and ether and then with ether and finally placed in a mixture of ether and solid carbon dioxide to disrupt it. The cell residue was then dried *in vacuo* over phosphorus pentoxide and weighed (Table I).

TABLE I

WEIGHT OF YEAST AND VARIOUS EXTRACTS DURING GROWTH

Growth period (h)	*Weight of yeast (mg)*	*Weight of total extracted material (%)*	*Hydroxamate yielded by extract (μmole tyrosine hydroxamate)*					
			(a) per mg yeast × 10³			*(b) per total culture*		
			Alcohol extract	*TCA extract*	*Total*	*Alcohol extract*	*TCA extract*	*Total*
0.0	190	24.3	5.75	0.67	6.42	1.09	0.127	1.21
1.0	225	27.2	8.75	3.08	11.83	1.968	0.693	2.66
2.0	247	27.5	7.30	2.02	9.32	1.803	0.490	2.30
3.0	291	—	3.70	1.07	4.77	1.076	0.311	1.38
5.0	333	30.7	1.62	0.97	2.59	0.539	0.323	0.86
5.6	390	24.5	1.50	1.17	2.67	0.585	0.456	1.04
6.6	404	21.1	2.38	0.53	2.91	0.961	0.214	1.17
23.6	476	24.8	1.70	0.63	2.33	0.809	0.299	1.10
29.6	439	24.4	3.99	0.81	4.80	1.751	0.355	2.10
49.8	439	22.7	2.10	0.75	2.85	0.921	0.329	—

One fraction of the peptidyl-nucleotidates (a) was extracted from the residues by successive elution with 50% ethanol (3 × 25 ml) for 3 h at room temperature. The extracts were combined and concentrated to dryness at room temperature. The cell debris remaining from the extraction was washed with ether and dried *in vacuo* as above. Similar results were obtained when the ethanol used for killing the original cells was diluted directly to 50% with water and extraction continued with 50% ethanol as above. A second fraction of peptidyl-nucleotidates (b) was obtained from the dried debris (100 mg) by mixing it with ice-cold 10% trichloroacetic acid solution (10 ml), freezing the mixture and keeping it overnight. The mixture was allowed to thaw, the solid rapidly centrifuged off and the supernatant solution immediately extracted with ether to remove the bulk of trichloroacetic acid. The remainder of the acid was removed by continuous extraction with ether for 16 h and the aqueous residue then concentrated to dryness at room temperature.

Concentration of peptidyl-nucleotidates

Fraction a was dissolved in 50% ethanol (2 ml) and fraction b in water (2 ml), the solutions divided into halves and each half submitted to electrophoresis in acetate buffer at pH 4.0 on Whatman No. 3 paper for 16 h at 10–20 V/cm.

From one of each pair of electropherograms, the material migrating towards the cathode was eluted by extraction with cold water (3 × 15 min) and the total extract in each case concentrated *in vacuo* at room temperature (Concentrates I).

Formation and chromatography of hydroxamates

The second member of each pair of electropherograms was treated with the hydroxylamine reagent[2] and the hydroxamates formed were eluted with water. The extracts were concentrated at room temperature to 2 ml. A sample (1 ml) was reacted with the ferric chloride reagent of DE MOSS *et al.*[3,4] (2 ml) in water (7 ml) and the red coloration was immediately measured in a Hilger Uvispek at 540 mμ. N-[2-Amino-3-(*p*-hydroxyphenyl)propionyl] hydroxylamine, prepared from tyrosine methyl ester and hydroxylamine[5], was employed as a reference substance (Table I).

A second sample of the peptidyl-nucleotidates was reacted with hydroxylamine in the same way and the hydroxamates then concentrated by chromatography on

TABLE II

HYDROXAMATES FORMED FROM YEAST EXTRACTS AT DIFFERENT STAGES OF GROWTH

a, alcoholic extract; b, trichloroacetic acid extract.

Growth period (h)	*RF value of hydroxamates with respect to the tyrosine derivative in butanol–acetic acid–water (4:1:1, v/v) on Whatman No. 3 paper*															
	0.64		*0.72*		*0.88*		*0.96*		*1.28*		*1.4*		*1.6*		*2.2*	
	a	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*
0.0	+						+		+	+			+			
1.0	+								+				+	+	+	+
2.0						+			+					+	+	+
3.0													+	+		+
4.0									+				+	+		+
5.0					+				+				+	+		+
5.6						+				+				+	+	
6.6					+										+	+
23.6					+								+	+		+
29.6			+								+		+	+	+	+
47.6						+					+		+	+	+	

TABLE III

DISTRIBUTION OF PEPTIDYL-NUCLEOTIDATES FROM YEAST EXTRACTS AT DIFFERENT STAGES OF GROWTH

a, alcoholic extract; b, trichloroacetic acid extract.

Growth period (h)	*RF values in n-butanol–acetic acid–water (4:1:1, v/v) on Whatman No. 3 paper*																			
	0.022		*0.047*		*0.088*		*0.138*		*0.22*		*0.33*		*0.40*		*0.45*		*0.55*		*0.69*	
	a	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*
0.0		+	+					+	+									+		
1.0	+	+					+	+	+		+		+					+	+	
2.0	+		+			+	+	+	+	+	+	+	+		+			+	+	
3.0		+					+		+	+	+		+					+	+	
4.0	+						+	+	+	+	+		+					+	+	
5.0		+				+	+	+	+	+	+							+	+	
5.6	+	+				+	+	+	+		+		+						+	
6.6		+				+	+	+	+	+	+		+				+	+	+	
23.6	+	+				+	+	+	+		+	+	+		+		+		+	+
29.6	+						+	+	+		+	+	+		+	+			+	
47.6	+					+	+	+	+	+	+	+	+	+	+	+		+	+	

TABLE

COMPOSITION OF PEPTIDYL-NUCLEOTIDATE

a, alcoholic extract;

Amino acids	Zones A a	A b	B a	B b	C a	C b	D a	D b	E a	E b	F a	F b	G a	G b
α-alanine	+	+	+	+++	tr	+	+	+	+			+	+	+
Arginine	+	++	++	tr	+	++	+	tr	+				+	tr
Aspartic acid	+	+	+	tr			+		+			++		
Cystine	tr	++			tr			tr				tr		
Glutamic acid		++	+	+	+		+		+			++	+	
Leucine	+	tr	tr	+				++	tr			tr	+	+
Valine	+	tr	tr	+					tr				+	tr
Purines														
Adenine	+	+	+	+	+	+	+	+					+	
Guanine		+			+									
Hypoxanthine	+		+	+				+						
Xanthine		+												
Pyrimidines														
Cytosine		+			+									
Uracil	+	+	+	+	+	+	+	+	+			+	+	+

Whatman No. 3 paper in ethanol–2 *N* ammonia (9:1, v/v)[6] in which the hydroxamates moved as a broad band ($R_{leucine} = 1.27$) ahead of contaminating amino acids and peptides. The hydroxamates themselves were then resolved further by chromatography on Whatman No. 3 paper in butanol–acetic acid–water (4:1:1, v/v). Most of the hydroxamates had R_F values greater than those of the amino acid derivatives (α-alanine, 0.36; proline, 0.45; valine, 0.69; leucine, 0.88). Individual zones (Table II) were extracted with water, the solutions concentrated and the hydroxamates hydrolyzed by means of 6 *N* hydrochloric acid at 100° overnight. The resulting amino acids were examined by two-dimensional chromatography[6].

Examination of peptidyl-nucleotidate concentrates I

The peptidyl-nucleotidates were separated initially by chromatography in butanol–acetic acid–water (4:1:1, v/v) using Whatman No. 3 paper. The majority had R_F values ranging from 0.088–0.40 (*cf.* Table III) and although this permitted a moderate degree of separation, further resolution was required. Accordingly, they were separated by two-dimensional chromatography using first butanol–acetic acid–water (4:1:1, v/v), and then acetone–30% acetic acid (1:1 or 3:1, v/v). Their positions were revealed by means of the hydroxylamine-ferric chloride reagent[7] (Fig. 1).

In addition to peptidyl-nucleotidates the concentrate was examined for free amino acids[6] and purines and pyrimidines[8].

The peptidyl-nucleotidates, separated from amino acids, peptides, purines and pyrimidines on Whatman No. 3 papers by two-dimensional chromatography as above, were eluted individually with 0.05 *N* hydrochloric acid and the acid extracts hydrolyzed

IV

ZONES IN EXTRACTS OF EAST

b, trichloroacetic acid extract.

(cf. Fig. 1)

H		*J*		*K*		*M*		*O*		*Q*		*S*		*T*	
a	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*
+	+	+	+	+	+	++	+	+		+		++	+		tr
+	+	+	++	+	tr	++	++	+		+		+	tr		+
+						++	tr								
r						tr									
+-	+			+		++	+	+							
r	·			+	tr	++									
ır				+	+	++									
+			+	+	+		+			+					
										+					
							+								
+	+	+	+	+	+	+	+	+		+		+	+		+

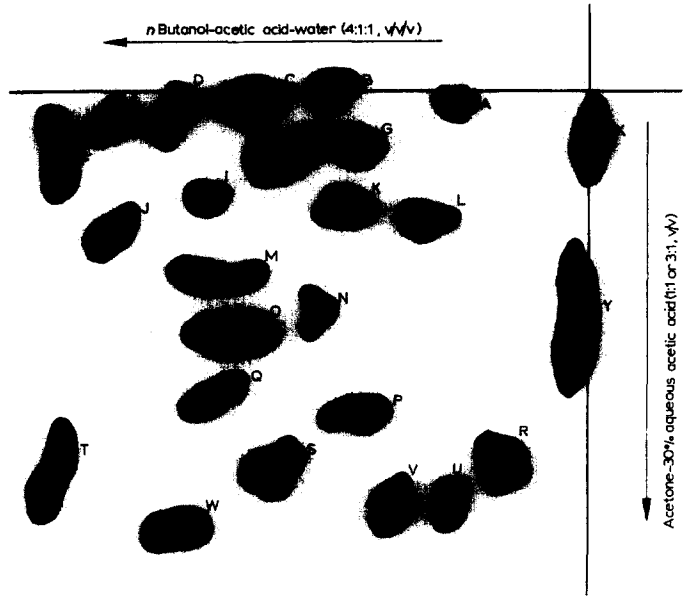

Fig. 1. Diagrammatic representation of chromatogram of compounds reactive towards hydroxylamine. Reference zones; hypoxanthine (P); guanosine, xanthine, xanthosine (R); uracil (S); adenosine (U); adenine, cytosine, inosine (V); thymine (W); adenosine diphosphate, adenosine triphosphate, cytidylic acid (X); and adenylic acid, diphosphopyridine nucleotide, guanylic acid, thymidylic acid, uridine diphosphate glucose, uridylic acid (Y).

with 6 *N* hydrochloric acid as above. The hydrolyzates were freed of hydrochloric acid by distillation, finally repeatedly extracted with ethanol. The acid-free solutions were treated with Amberlite IR120 (acid form, 5 g), the resin removed and amino acids and bases desorbed from the adsorbent by soaking it in 2 *N* ammonia solution for 48 h. The ammoniacal solutions were concentrated to dryness *in vacuo* and the residues individually chromatographed for purines and amino acids in solvent systems already described[6,8] (*cf.* Table IV).

The original effluents from the resin were concentrated *in vacuo* and the concentrates hydrolyzed by means of 72 % perchloric acid[9]. Excess perchloric acid was neutralized by means of potassium hydroxide, potassium perchlorate removed by filtration and the filtrate concentrated and chromatographed for purines and pyrimidines[8] (Table IV).

RESULTS AND DISCUSSION

The variation in total activated material is shown in Table I from which it may be seen that the pool of peptidyl-nucleotidates grows to a maximum 1 h after transfer of the yeast to fresh medium, thereafter declining during active growth of the yeast and subsequently increasing up to about 48 h. After this period autolysis apparently sets in rapidly and the amount of peptidyl-nucleotidate falls abruptly. Similar effects are observed in both the ethanolic and trichloroacetic acid extracts.

Although the rise in reactivity of the extracts towards hydroxylamine was striking, whether the results were calculated on the basis of unit dry weight or total weight of culture (Table I), the possibility existed that the bulk of the increase was due to only one or a few components of the mixture. However, chromatography of the extract (Tables II, III and V) revealed that this was not the case. The chromatographic pattern of materials reacting with hydroxylamine at all stages of growth of the yeast is shown in Fig. 1. It is noteworthy that the bulk of peptidyl-nucleotidates is well separated from most of the common purines and pyrimidines and derived nucleosides and nucleotides, which are located at the periphery of chromatograms prepared with the aid of the new solvent system. The variation in the chromatographic pattern is indicated in Tables II, III and V. From Table V particularly it may be seen that although the pattern varies from time to time, no one compound is predominant at any time. It was of particular interest that certain components or mixtures of components were present during both the early active growth phase and the later phases. It seems reasonable to assume, therefore, that these are intermediates in synthetic processes and that their synthesis continues even during the resting phase of the cell, presumably because they are not integrated further into protein. The accumulation of material reactive towards hydroxylamine after active growth of the yeast had ceased, would then be explicable as the result of the increase in amount of these particular peptidyl-nucleotidates among others.

In view of the above findings and of the isolation of individual peptidyl-nucleotidates of known composition from a 48 h culture of this yeast[1,8], the composition of the zones separated chromatographically (Fig. 1) at various stages of growth of the yeast was determined (Table V; *cf.* Table IV). As in the previously isolated compounds[1,8], the predominant amino acids were arginine and α-alanine and the only other amino acids detected were aspartic acid, cystine, glutamic acid, leucine and valine. The presence of only these amino acids in the active compounds was

TABLE V

VARIATION IN COMPOSITION OF MIXTURE OF PEPTIDYL-NUCLEOTIDATES IN EXTRACTS OF YEAST DURING GROWTH

* Zones were clearly u.v. light absorbing and gave strong reaction with hydroxylamine. + Zones absorbed u.v. light but reaction with hydroxylamine doubtful. a, alcoholic extract; b, trichloroacetic acid extract.

Growth period (h)	*Zones (cf. Fig. 1 and Table IV)*																																			
	A		*B*		*C*		*D*		*E*		*F*		*G*		*H*		*I*		*J*		*K*		*L*		*M*		*N*		*O*		*P*		*Q*		*S*		*T*		*W*	
	a	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*
0.0			*		*	*	*						*	*	*	+					*				+	*	+		+	+	+		+		+					
1.0					*	+	*	+		+				*	*	+				*	+	*			*	*							+				*		
2.0			*	*									*	*	*						*		*			*	+	*	+	+	+				+	+			+	
3.0		*	*	*	*	+	*	+	*	+		*		*	*	*			*	+	+	+				*				+							*	*	
4.0		*	*	*	*		+		+				*	*	+	*										*	+	*	+	+	+		+			*				
5.0		*		+		*	+						*	+	*	*					*				+	*			+	+										
5.6	+	+	+		+	+			+	+			*		*	+	*			*	*	*			+	*	+		+		+				+					
6.6					*	*	*	+					*			+				*	*				+	*	+		+										
23.6	+	*	+	*	*	+	*	+					*	*	*	+	*		+		+	*				+		*	+		+						*		
29.6			+		*	*	*		*				*	+	*	*			*	*	*	*			+	*					+						+	+		
47.6		+	*	*	*	+	*	+				+	*			+	*			*	*						+		+								*			
49.8							*					+			*					*	+					+				+							*			

confirmed by hydrolysis of the hydroxamates freed from contaminating amino acids and peptides by chromatography. Of the purines and pyrimidines the principal representatives were again uracil and adenine but cytosine and guanine were also detected as constituents of the zones A and C (Fig. 1). It is relevant to note that less well defined concentrates with acylating properties from guinea pig liver have recently been found to contain all the bases, including thymine[10], derived from the nucleic acids. This base has not been detected in the activated compounds from yeast, but small amounts of hypoxanthine and xanthine have been found. It is probable that these last compounds are degradation products formed by the action of perchloric acid on the bases originally present during hydrolysis.

The central position of uracil in the active compounds from yeast is quite clear. In view of the participation of this base uniquely in the structure of ribonucleic acid it appears probable that active compounds such as those described here form a link in the well-known relationship between ribonucleic acid or its precursors[11-14] and protein synthesis. This idea is supported by the predominance in the peptidyl-nucleotidates of the above-mentioned amino acids, which are common to nucleoproteins and indeed to ribonucleoprotein particles which participate in protein synthesis, *e.g.*, in rat liver[15]. The common occurrence with uracil of adenine in many of the yeast components suggests that di- or polynucleotides of the type found in the isolated compounds CE_1, TCA_1, TCA_2, D_1 and D_2 from a 48 h culture of the same yeast (*cf.*[1]) are present. Certainly, the first four of the above isolated compounds occupy the zones N, K, A, and B respectively in Fig. 1 of the present communication, these zones in each case being found to contain adenine, uracil and the amino acids expected. Incidentally, the compounds D_3 and D_4 previously described occupy zones P and M (Fig. 1) while arginylalanylarginylalanyl-5'-uridylate[8] is located in zone N. The presence in such peptidyl-nucleotidates of the dinucleotide having the 5',3'-phosphodiester linkage[1] typical of ribonucleic acid lends weight to the suggestion that the activated compounds play a part in elaborating nucleoproteins. That other oligonucleotides may be present also is suggested by the presence together of further bases, *e.g.*, adenine, guanine, cytosine, and uracil in zones A and C.

The important role of uracil in peptidyl-nucleotidates perhaps provides a rationale for the earlier finding[16] that pyrimidine derivatives stimulate protein synthesis, *e.g.*, in particulate fractions from pea seedlings, and the more recent observation[17] that in a uracil-requiring mutant of *E. coli*, induction of β-galactosidase requires the pyrimidine. In connection with the present findings it is of especial interest that OKAZAKI AND OKAZAKI[18] have shown that during unbalanced growth of *Lactobacillus acidophilus*, which is in some respects like that at the end of the logarithmic growth of yeast as above, large increases occur of the pool of uracil ribomononucleotide derivatives. When, however, the supply of uracil was depleted, the synthesis of both ribonucleic acid and protein was inhibited. Possibly, peptidyl-nucleotidates represent various stages in the synthesis not only of protein but of ribonucleic acid also (*cf.* MICHELSON[19]). Evidence that peptides and oligonucleotides, rather than ribonucleic acid itself, take part in protein synthesis at least, was adduced by STEINBERG AND AFINSEN[20] who observed that, in the formation of ovalbumin, ribonucleic acid could be replaced by di- and trinucleotides. Moreover, JORDAN AND INNIS[21] noted as a result of studying the action of the antibiotic vancomycin on the synthesis of protein in *S. aureus* that nucleic acid precursors can replace nucleic acid

in effecting such synthesis. A similar conclusion is reached by GORMAN AND HALVORSON[22] as a result of the finding that the rate of protein synthesis in *Pseudomonas azotogensis* does not necessarily parallel the rate of synthesis of nucleic acid, and by REIS, COOTE AND WORK[23] from the failure of ribonuclease to inhibit incorporation of amino acids into the proteins of liver fractions. It is noteworthy that in some such systems, *e.g.*, the "lubrol-KCl" microsomes[24], incorporation of amino acid into protein occurs independently of the above-mentioned S-ribonucleic acid and in other systems, *e.g.*, extracts of bull spermatozoa[25], incorporation occurred in the absence of nucleic acid but in presence of oligonucleotides. It is perhaps significant in view of the amino acid composition of peptidyl-nucleotidates that the "lubrol-KCl" microsomes contain proteins particularly rich in basic amino acids[26].

Alternatively, the peptidyl-nucleotidates might be formed as a template composed of nucleic acid or oligonucleotide, presumably *via* activated amino acids. The peptidyl-uridylates would then represent the products formed by peeling of a peptide chain from the template bearing the uridylic acid residue at one end and scission of this residue from the nucleotide chain, perhaps by a ribonuclease. The presence of a latent ribonuclease, capable of activation by bases, in ribonucleoprotein particles from various organisms, including yeast[27], has been described by various workers (*cf.* ELSON[28]; ZILLIG, KRONE AND ALBERS[29]). Similarly, the dinucleotide derivatives would be the products of splitting of the penultimate phosphodiester bond of a polynucleotide chain bearing the terminal dinucleotide. Whether or not the function of terminal uridylic acid in this respect is related to the rapid turnover of this terminal nucleotide in ribonucleic acid of thymocyte nuclei[30], in contrast to the stability of cytidylic acid, remains to be elucidated.

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