YEAST PEPTIDYL-NUCLEOTIDATES; ISOLATION OF DINUCLEOTIDE DERIVATIVES INCLUDING ALANYL-(3'-[5'-ADENYLYL])-5'-URIDYLATE

J. W. DAVIES AND G. HARRIS

Brewing Industry Research Foundation, Nutfield, Surrey (Great Britain)

(Received May 23rd, 1960)

SUMMARY

The isolation of individual peptidyl-nucleotidates from ethanolic and trichloroacetic acid extracts of a 48 h culture of brewers' yeast has been accomplished using electrophoresis and chromatography on paper and ion-exchange resins.

Structural study of the individual compounds revealed that they were nucleotide-5'-phosphoanhydrides. Three of those investigated were derived from the mononucleotide, uridine-5'-phosphate, and five from the dinucleotide, 3'-(5'-adenylyl)-uridine-5'-phosphate. Of the latter group, one proved to be alanyl-(3'-[5'-adenylyl])-5'-uridylate, analogous with the amino acid adenylates participating in protein synthesis. Other members of the group have similar structures in which the α-alanine unit is replaced by peptide residues containing some or all of the amino acid residues of α-alanine, arginine, aspartic acid, cystine, glutamic acid, leucine and valine.

INTRODUCTION

Understanding of the course of the biosynthesis of proteins is hampered by lack of detailed knowledge of the intermediates involved, and, indeed, the necessity for such intermediates apart from the activated amino acids is discounted in some versions of the template theory (see *e.g.* DOUNCE[1]; review by HARRIS[2]). However, the presence of intermediates in the synthesis of ovalbumin was deduced by STEINBERG AND ANFINSEN[3], and a number of investigators[4] have found peptides, regarded as precursors of proteins, to be present in extracts of various microorganisms. Recent developments are the reports or inferences that peptide derivatives of nucleotides are present in extracts from a number of sources, including bakers' yeast[5–7], brewers' yeast[8–10], *Candida utilis*[11], *Azotobacter vinelandii*[12], *Chlorella*[6,7], *Staphylococcus aureus*[13], *Streptococcus faecalis*[14], *Escherichia coli*, *Bacillus subtilis*[15,16] and liver cytoplasm[17].

In most instances no pure compounds of this class have been isolated and comparison of the properties reported for the groups of substances isolated by various workers reveals that considerable differences exist. For instance, some of the substances migrate towards the anode[13,14] on electrophoresis in acidic buffer solutions while others move towards the cathode[5–10,17]. The compounds most likely to be intermediates in protein synthesis are those which possess either amino- or carboxyl-

groupings in activated form to facilitate combination into protein. Accordingly, in the case of the substances from brewers' yeast[8–10] and from liver cytoplasm[17] attention has been confined to those which have acylating properties as reflected in their yielding hydroxamic acids on reaction with neutral hydroxylamine[18,19]. It is remarkable that nearly all these compounds migrate towards the cathode when submitted to electrophoresis at pH 4.0 (see ref.10). Of such compounds one was isolated earlier and shown by degradation[9,10] and by synthesis[20] to be arginylalanylarginylalanyl-5'-uridylate (see ref. 21). It was hence closely related chemically to the amino acid-5'-adenylates[22], now generally recognised to be the active form of amino acids participating in protein synthesis in various cells[18,19,23,24] including yeast[25]. It and similar compounds are therefore referred to as peptidyl-nucleotidates as distinct from the less well-defined nucleopeptide preparations.

Of the other nucleotide–peptide complexes, GILBERT AND YEMM[11] proposed that one in *Candida utilis* was derived not from a nucleoside monophosphate, like the compound from brewers' yeast, but from uridine diphosphate. Furthermore, BERNLOHR AND WEBSTER[12] suggested that compounds reactive towards hydroxylamine in extracts of *Azotobacter vinelandii* are derived from poly- rather than mono-nucleotides and polynucleotide-amino acid compounds have been postulated as intermediates for protein[26–28]. Accordingly, further examination of the acylating components in extracts of a brewing yeast has been undertaken to gain insight into the range of their chemical structures. These compounds are unstable, particularly in alkaline solutions, and any step in working them up or chromatographing them which involves the use of alkaline conditions decomposes them. This may account for the fact that some workers have isolated anode-migratory nucleopeptides and that others have failed to recognize acylating properties in their products. For this reason, it remains to be seen whether or not the peptides observed, for example, in *E. coli*[4] were originally attached to nucleotides.

MATERIALS AND METHODS

Culture and extraction of yeast: This was carried out as described earlier[10] with the exception that extraction with hot aqueous ethanol was omitted.

Ion-exchange resins: These were treated for use as outlined in a previous communication[10].

Analytical methods

The determinations of purines, pyrimidines, ribose, phosphate and amino acids were effected largely as before[10]. It was found convenient, however, to separate the purines and pyrimidines on acid-washed Whatman No. 3 papers.

The peptide moiety of the peptidyl-nucleotidates was removed by treating these compounds with 0.3 *N* potassium hydroxide for 16 h at 37°. The resulting solution was neutralized with perchloric acid at 0° and then kept for 16 h at 0° to precipitate potassium perchlorate. This was filtered off, using acid-washed Whatman No. 42 paper, and thoroughly washed with cold water. The combined filtrate and washings were concentrated at room temperature *in vacuo*, further potassium perchlorate removed in the same way and the procedure repeated until the amount of potassium perchlorate separating was negligible. The concentrate was chromatographed overnight

in butanol–acetic acid–water (4:1:1, v/v) on acid-washed Whatman No. 3 paper, the peptide zone located by means of the ninhydrin reagent and eluted by means of water. The extract was concentrated and submitted to electrophoresis on paper[10] to separate nucleotides migrating towards the anode from the peptide, which was then eluted and analyzed for amino acids[10].

The nucleotide portions of the peptidyl-nucleotidates were obtained by treatment of these compounds with neutral hydroxylamine and separation of the products by electrophoresis as before. The nucleotides were eluted and chromatographed in KIRBY's solvent No. II (see ref. 29) and the constituent bases identified by hydrolysis and chromatography[10].

Peptidyl-nucleotidates in solution were estimated by reaction with hydroxylamine and ferric chloride and measurement of the resulting red colour[18,19]. The reaction of peptidyl-nucleotidates with periodate was measured by means of the decrease in the u.v. light absorption of the solution[30].

Isolation of peptidyl-nucleotidates

Cold ethanol extract: The extract was concentrated at room temperature and the residue submitted directly to electrophoresis on Whatman No. 3 paper at pH 4.0. Three components reactive towards hydroxylamine migrated towards the cathode. The major component, of intermediate mobility, was eluted and the extract freed of amino acids and peptide contaminants by passage down a column of Amberlite CG 50. The effluent was loaded on to a column of Dowex-1 (formate) and the peptidyl-nucleotidates eluted in a formic acid gradient (0.05 *M*–4.0 *M*). Several compounds were thereby detected of which the two most reactive towards hydroxylamine were present respectively in the direct effluent and the eluate obtained using 2.0 *M* formic acid (Fig. 1). The former (D_4) behaved as a single compound on chromatography on paper in either of the two solvent systems, butanol–acetic acid–water (4:1:1, v/v) or *n*-propanol–ethyl acetate–water (7:1:2, v/v) containing acetic acid[10]. It was therefore analyzed further (*cf.* Table I).

Cold aqueous ethanol extract: The method used for working up this extract is shown in Scheme I. The peptidyl-nucleotidates desorbed from Dowex-1 (chloride) by acetate buffer largely pass through Dowex-1 (formate) in the presence of excess acetate, but

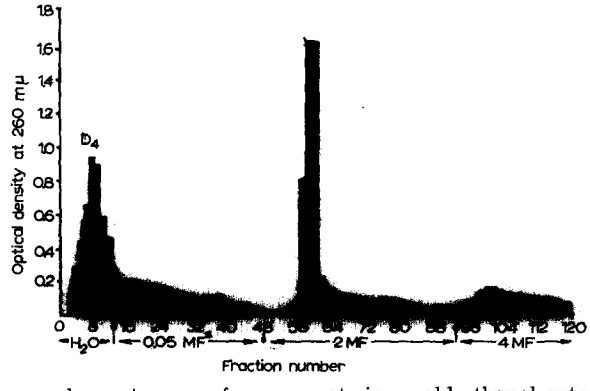


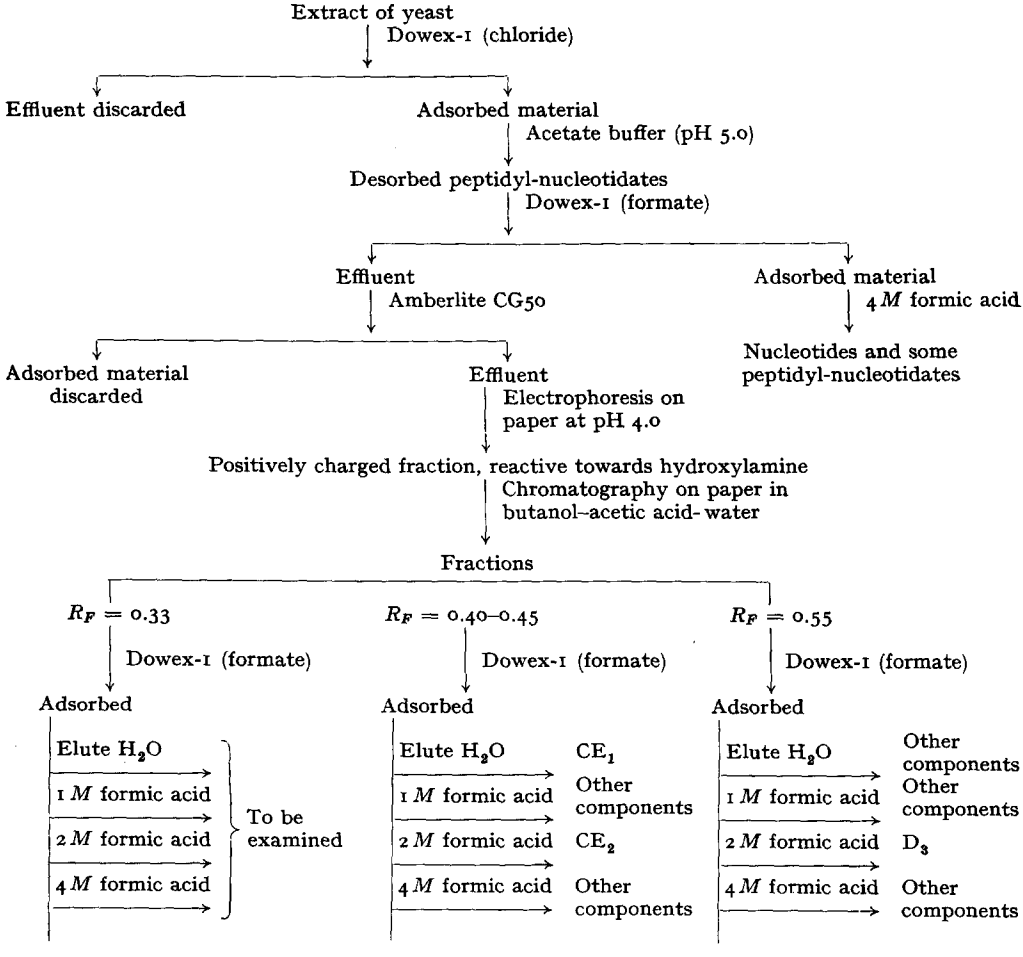
Fig. 1. Ion exchange chromatogram of components in a cold ethanol extract of yeast. Column composed of Dowex-1 (× 4) formate (200–400 mesh). Column dimensions 17 cm × 1.76 cm². Volume of fraction 5 ml. *F = formic acid.

TABLE I

GENERAL ANALYSIS OF PEPTIDYL-NUCLEOTIDATES FROM YEAST

Component analysed	*Alcohol-soluble*				*Soluble only in trichloroacetic acid*			
	CE_1	CE_2	D_3	D_4	TCA_1	TCA_2	D_1	D_2
Adenine	1.0	0.0	0.0	0.0	1.0	1.0	1.0	1.2
Uracil	1.0	1.0	0.97	1.12	1.0	1.0	0.9	1.0
Phosphorus	2.0	1.0	1.0	1.0	2.0	2.0	1.75	2.0
Ribose	2.0	1.0	1.1	1.3	2.0	2.0	2.06	2.54
α-alanine	2.2	4.2	5.05	4.0	3.0	5.8	1.15	6.6
Arginine	10.0	7.0	4.1	2.3	14.0	8.8	0.0	5.6
Aspartic acid	0.7	1.1	0.0	0.0	0.0	0.0	0.0	0.0
Cystine	0.7	0.0	1.4	1.4	0.8	3.2	0.0	2.7
Glutamic acid	3.7	1.6	0.0	0.0	1.8	0.0	0.0	0.0
Leucine	1.9	1.26	6.5	1.6	2.0	1.2	0.0	6.3
Valine	1.0	1.0	6.5	2.4	2.0	2.0	0.0	5.3

Scheme I



after successively treating with Amberlite CG 50 and chromatographing on paper they are more readily adsorbed on the Dowex-1 (formate) (17×2.25 cm^2). Elution first with water and then with formic acid solutions of increasing strengths (0.05 M–4.0 M) gave a range of peptidyl-nucleotidates of which the most reactive towards hydroxylamine were the components CE_1, CE_2, and D_3, which were accordingly studied further (Fig. 2).

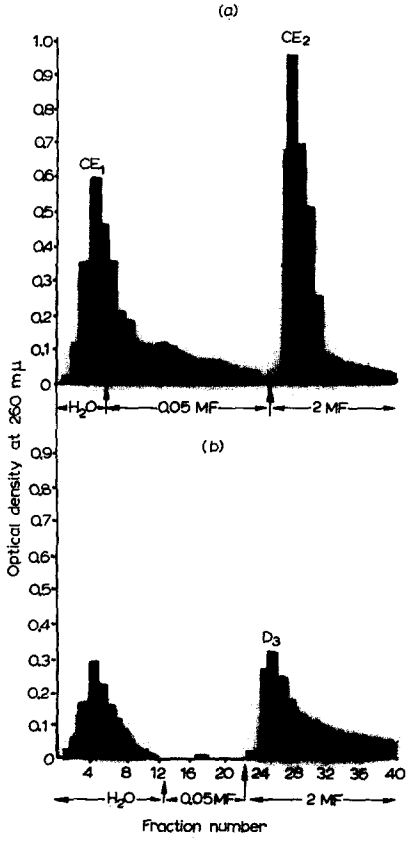


Fig. 2. Ion exchange chromatograms of components in cold aqueous ethanol extracts of yeast. Column composed of Dowex-1 (×4) formate (200–400 mesh). Column dimensions 17 cm × 1.76 cm^2. Volume of fraction 5 ml.

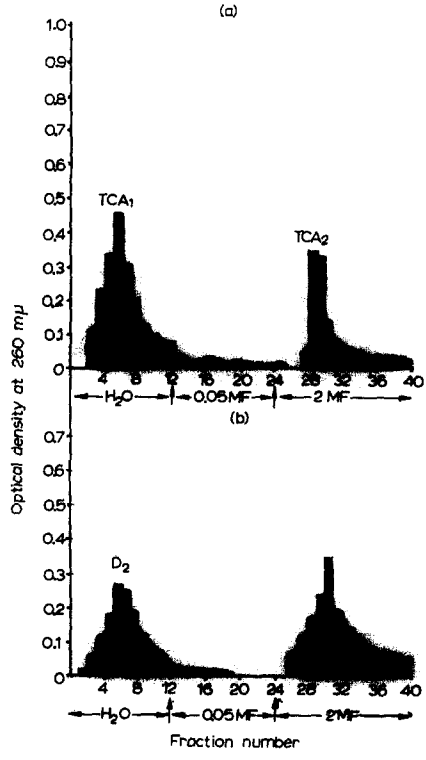


Fig. 3. Ion exchange chromatograms of components in cold trichloroacetic acid extracts of yeast. Column composed of Dowex-1 (×4) formate (200–400 mesh). Column dimensions 17 cm × 1.76 cm^2. Volume of fraction 5 ml.

Trichloroacetic acid extract: This extract was worked up much as in Scheme I with the exception that it was originally passed through Dowex-1 (formate). Although some peptidyl-nucleotidate was adsorbed together with nucleotides, the bulk passed through the resin and was subsequently treated as in Scheme I. On chromatography on Whatman No. 3 paper, the major constituents had R_F values of 0.55 and 0.40–0.45. Subsequent chromatography on Dowex-1 (formate) showed that the former constituent (D_2) was eluted with water, subsidiary compounds only being displaced by formic acid. Similar ion-exchange chromatography of the materials of R_F 0.40–0.45

gave two main components, one (TCA_1) being displaced from the resin by water and the other (TCA_2) being displaced by 2 *M* formic acid (Fig. 3).

In experiments designed to handle larger quantities, chromatography on Whatman No. 3 paper was replaced by chromatography on a cellulose column (91 cm × 12.25 cm^2) in butanol–acetic acid–water (4:1:1, v/v). The effluent was collected in 5 ml fractions and the progress of separation followed by means of the u.v. absorption of the fractions at 260 mμ (Fig. 4).

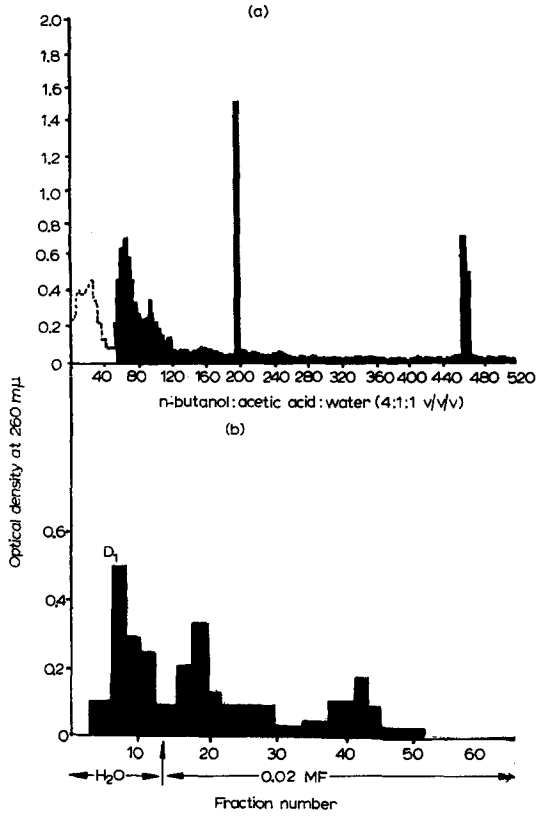


Fig. 4a. Chromatogram of components in cold trichloroacetic acid extract of yeast. Column composed of cellulose. Column dimensions 91 cm × 12.25 cm^2. Volume of fraction 5 ml.
Fig. 4b. Ion exchange chromatogram of pooled fractions 45–80 from (a). Column composed of Dowex-1 (× 4) formate (200–400 mesh). Column dimensions 17 cm × 1.76 cm^2. Volume of fraction 5 ml.

Of the four major "peaks" the first eluted was diluted with water, the organic phase removed and the remaining aqueous phase extracted with ether to remove butanol and acetic acid. The aqueous solution was passed through a column of Dowex-1 (formate). The main component (D_1) passed through the column on washing with water. The remaining compounds present were displaced by means of 0.02 *M* formic acid.

Structural examination of compound D_1

By means of pancreatic ribonuclease: Compound D_1 was treated with pancreatic ribonuclease in acetate buffer solution at pH 5.4. As this pH is well removed from

the optimum (7.5)[51] for the enzyme, a relatively large amount of the enzyme was employed. The mixture was kept for 16 h at 37° after which the reaction was terminated and enzyme removed[32]. The solution was concentrated at room temperature and the product submitted to electrophoresis on paper at pH 4.0. Two components were detected by examination of the dried electropherogram in u.v. light at 254 mμ. One migrated towards the anode and reacted with hydroxylamine to form α-alanine hydroxamate and a nucleotide. Chromatography of the nucleotide in the solvent systems isopropanol–water[33] and ethanol–1 *M* ammonium acetate[33] showed that it had R_F values characteristic of uridine 3',5'-diphosphate, and hydrolysis with formic acid followed by chromatography[10] showed that it contained uracil. The second component separated by electrophoresis above had the chromatographic and u.v. light absorption behaviour of adenosine and, as expected for this compound and in contrast to the uridine diphosphate, consumed periodate.

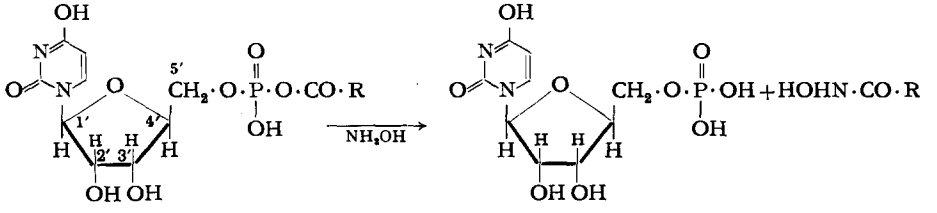
By means of snake venom phosphodiesterase: The material was treated with Russell viper venom in 0.05 *M* tris(hydroxymethyl)aminomethane–maleate buffer solution at pH 7.0 (see ref. 34). The reaction was stopped and enzyme precipitated by adding trichloroacetic acid to a final concentration of 5 %. Precipitation was completed by keeping the mixture for 16 h at 0° and the precipitate centrifuged off at 0° at 5,000 rev./min. The supernatant solution was freed of trichloroacetic acid by extraction with ether and the aqueous phase freeze-dried. The residue was submitted to electrophoresis at pH 4.0 on Whatman No. 3 paper for 4 h and the separated products detected by their u.v. absorption at 254 mμ. One product migrated towards the anode and the second, which not only absorbed u.v. light but also reacted with hydroxylamine to form a hydroxamate and consumed periodate[30], migrated towards the cathode. The first product showed the chromatographic and u.v. absorption properties characteristic of adenosine-5'-phosphate. The second product was treated as above with potassium hydroxide whereupon a compound having the characteristics of uridine-5'-phosphate was formed.

RESULTS AND DISCUSSION

In earlier investigations[10] it was observed that, although some of peptidyl-nucleotidates withstood heating in aqueous ethanolic solution, others were decomposed by this treatment. In the present work, therefore, attention was confined to extracts obtained from yeast using successively cold ethanol, cold aqueous ethanol and cold aqueous trichloroacetic acid. The adsorption on Dowex-1 (chloride) of materials reactive towards hydroxylamine and their selective elution from the resin by means of acetate buffer at pH 5.0 resulted in quantitative recovery of the colour-forming materials[18,19]. This initial concentration procedure probably, therefore, yields a product representative of the compounds present in the original extracts. On the other hand, some loss of peptidyl-nucleotidate was incurred during the later treatment with Amberlite CG 50 (see MATERIALS AND METHODS) but this step in the purification was usefully retained as it ensured the absence of free amino acids or peptides.

Of the large number of acylating compounds detected in the alcoholic and trichloroacetic acid extracts of the yeast, four in each extract were selected for further study as being present in readily workable quantities. The results of the analysis[10] of these compounds are shown in Table I. It is remarkable that, in common with the first peptidyl-nucleotidate isolated from yeast[9,10], all the compounds contain uracil,

and compounds CE_2, D_3 and D_4 contain only this base of those commonly derived from nucleic acids. In these three compounds the uracil was in the form of a uridine-5′-phosphate residue as this nucleotide was liberated on degradation of the original compounds with alkali[10]. The uracil, phosphorus and ribose (Table I) were thus accounted for. Each of the three compounds reacted with neutral hydroxylamine to give not only the nucleotide but also a peptide hydroxamate which behaved as a single unit on chromatography. Moreover, each consumed periodate, presumably as a result of the reaction of the reagent with the 2′,3′-*cis*-hydroxyl groups of the ribose moiety. It follows, therefore, that the compounds CE_2, D_3 and D_4 have structures (I), analogous with that of arginylalanylarginylalanyl-5′-uridylate (I; R = $CH(CH_3)\cdot NH\cdot CO\cdot CH[(CH_2)_3\cdot NH\cdot C(NH_2):NH]\cdot NH\cdot CO(CH_3)\cdot NH\cdot CO\cdot CH[(CH_2)_3\cdot NH\cdot C(NH_2):NH]NH_2$) and react with hydroxylamine thus:



Peptidyl-nucleotidate (I) Uridine-5′-phosphate Peptide hydroxamate

The compounds CE_1, TCA_1, TCA_2, D_1 and D_2 contained adenine in addition to uracil, and on degradation with alkali or neutral hydroxylamine gave not a mixture of the corresponding mononucleotides but a hitherto unknown oligonucleotide. The chromatographic behaviour (R_F-value 0.65–0.68 in solvent II of KIRBY[29]) and molar ratios (Table I) for adenine, uracil, phosphorus and ribose suggested that this oligonucleotide was a dinucleotide (see below). It had u.v. absorption almost identical with that of uridylic acid.

From the point of view of their amino acid composition most of the compounds isolated contained a relatively high proportion of arginine and α-alanine, again resembling arginylalanylarginylalanyl-5′-uridylate and recalling to mind the composition of nucleoprotein. In this connection it is perhaps a curious feature of the whole peptidyl-nucleotidate concentrate from the particular yeast used, that only the amino acids listed in Table I occur to any considerable extent. Comparison of this amino acid pattern with the amino acid composition of whole brewers' yeast (*cf.* EDDY[35]) reveals considerable differences although it is nevertheless true to say that, of the combined amino acids in yeast, α-alanine, arginine, leucine and valine are present in largest amounts. Further, TAYLOR[36] has observed that a 40-h culture of yeast contains a high proportion of extractable arginine but relatively low proportions of tyrosine and histidine.

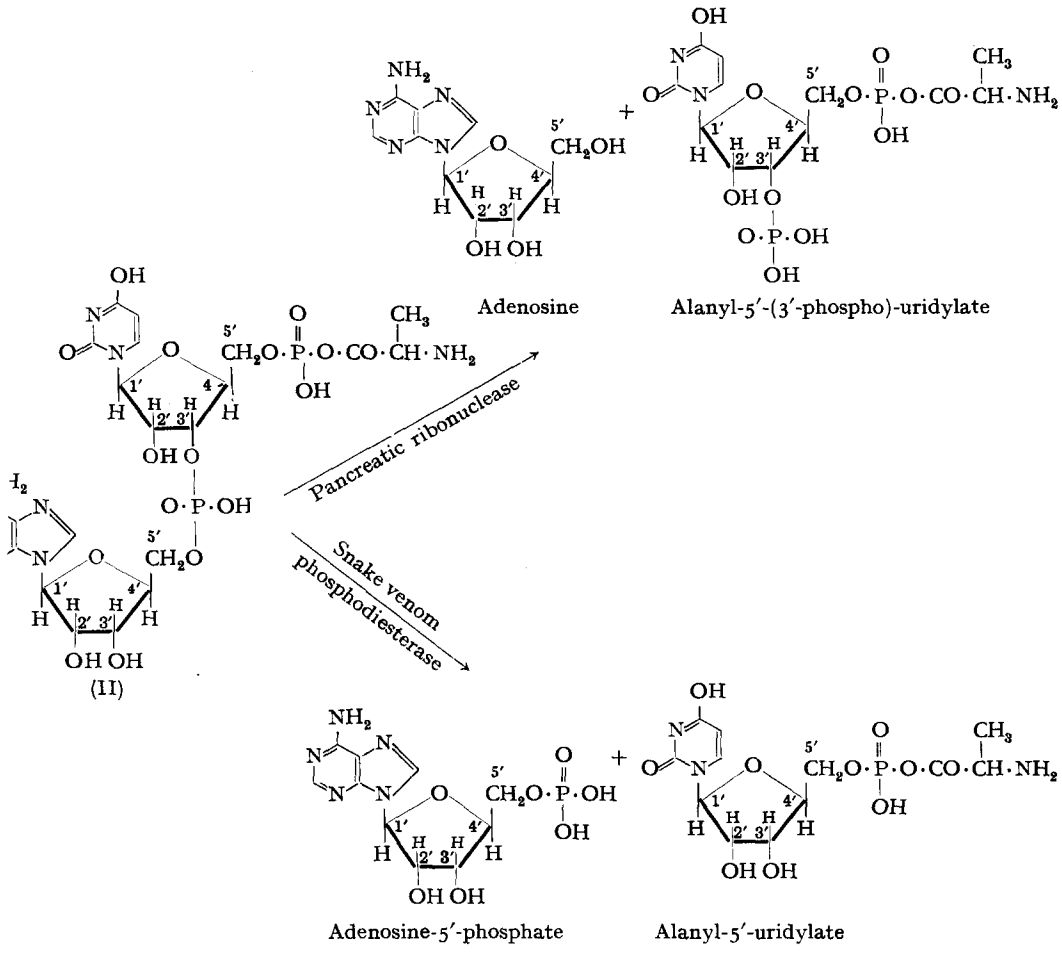
Compound D_1 was of particular interest because it contained only one amino acid, namely α-alanine. As, moreover, it gave the above mentioned oligonucleotide on degradation, it afforded a particularly suitable subject for structural analysis especially in view of its bearing also on the structures of all the compounds CE_1, TCA_1, TCA_2 and D_2. Degradation with alkali gave, in addition to the oligonucleotide, a compound which co-chromatographed with α-alanine while treatment with hydroxylamine

afforded a substance having the chromatographic behaviour of N-[2-amino-2-methyl-acetyl] hydroxylamine. On the basis of this dual chromatographic evidence it is concluded that α-alanine is present as a single amino acid unit in the original compound. In view of the molar ratios between the constituents of compound D_1 (Table I) the oligonucleotide formed on degradation must be a dinucleotide as suggested above.

That one at least of the mononucleotide moieties of the dinucleotide probably bore only a 5′-substituent was shown by the uptake of periodate by the original compound. This mononucleotide was shown to be adenylic acid by hydrolysis of the parent compound by pancreatic ribonuclease at pH 5.4, whereupon adenosine, which reacts with periodate, and a derivative of uridylic acid, which failed so to react, was formed. Under the conditions used the uridylic acid derivative retained the acylating properties of the original molecule. As pancreatic ribonuclease specifically hydrolyzes phosphate linkages at position 5′ of purine nucleotides of, for example, ribonucleic acid, leaving the phosphate grouping linked to the 3′-position of the attached pyrimidine nucleotide[31], it follows that adenylic acid, the purine nucleotide in this case, must have been linked to uridylic acid through its 5′-phosphate grouping. In view of the absence of products of degradation other than adenosine and the above uridylic acid derivative, the *cis*-2′,3′-hydroxyl groups of the adenylic acid residue of the original compound must have been free and responsible for the uptake of periodate.

The uridylic acid derivative must have been linked through its 3′-position to the 5′-adenylic acid residue as the ribonuclease is specific for the hydrolysis of 5′,3′-internucleotide bonds[31]. The lack of reactivity of this part of the molecule towards periodate was thus explained and only positions 2′- and 5′- of the uridylic acid moiety were left as alternative points of attachment of the second phosphate grouping of the dinucleotide. Of these alternatives, the 5′-position was the one in question as degradation of the uridylic acid derivative by means of alkali gave uridine-3′,5′-diphosphate with concomitant liberation of α-alanine. This latter amino acid must have been bound through a phosphoanhydride linkage therefore to either the 3′- or the 5′-position of the uridine diphosphate. By analogy with the peptidyl-nucleotidates above, containing only uridylic acid residues, the latter alternative appears most likely. Furthermore, it is improbable that ribonuclease, a phosphodiesterase, would attack a tri-substituted phosphate grouping as would be necessary were the amino acid bound to the internucleotide phosphate grouping. Confirmation of these ideas was obtained by hydrolyzing compound D_1 with a ribonuclease of different specificity from the pancreatic enzyme, namely the phosphodiesterase from Russell viper venom. This enzyme hydrolyzes, for example, ribonucleic acid to liberate the 5′-nucleotides of both purines and pyrimidines[31]. In conformity, compound D_1 gave rise to 5′-adenylic acid and a uridylic acid derivative which in this case consumed periodate. As this latter derivative still contained the reactive amino acid-phosphoanhydride grouping, it follows that this was in the 5′-position. Consequently, compound D_1 has the structure (II), *i.e.*, alanyl-(3′-[5′-adenylyl])-5′uridylate, which, to our knowledge, is the first activated nucleotide-derivative of a single amino acid isolated from the cell itself as against products of *in vitro* enzymic reaction.

It seems probable by analogy that compounds CE_1, TCA_1, TCA_2 and D_2 are corresponding peptidyl-(3′-[5′-adenylyl])-5′-uridylates and in this event all the compounds from yeast so far examined are anhydrides of either peptides or an amino acid with uridine-5′-phosphate or with the above derived dinucleotide. The dinucleotide



derivatives are particularly interesting in that they provide a chemical bridge between, on the one hand, amino acid adenylates and simple peptide-nucleotidates and, on the other hand, the S-ribonucleic acid–amino acid complexes, which have been proposed to play a part in protein synthesis in various cells[18–26]. Moreover, they are the first isolated representatives of the polynucleotide–amino acid complexes earlier postulated to exist in cell extracts[12,27,28] (see INTRODUCTION).

ACKNOWLEDGEMENTS

The authors thank Dr. A. H. COOK, F.R.S., for his encouragement during the course of these investigations. They also wish to acknowledge a generous gift of Russell viper venom by Dr. A. W. PHILLIPS.

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