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POSSIBLE INTERMEDIATES IN THE BIOSYNTHESIS OF PROTEINS

I. EVIDENCE FOR THE PRESENCE OF NUCLEOTIDE-BOUND CARBOXYL-ACTIVATED PEPTIDES IN BAKER'S YEAST*, **, ***

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INTRODUCTION

Important data with respect to the biosynthesis of proteins have been obtained from studies on the incorporation of labeled amino acids into proteins by KELLER AND ZAMECNIK[2], LITTLEFIELD AND KELLER[3], and a number of other authors. It seems hardly speculative to conclude from these data that the final condensation of protein precursors to cytoplasmic proteins occurs in the microsomal RNP particles.

A number of hypotheses has been proposed[4,5,6] concerning the nature of these precursors and their condensation to proteins[7,8], but to our knowledge no obvious intermediates in the biosynthesis of proteins have ever been isolated. During the course of a study on the enzymic carboxyl group activation of tyrosine[9] in our laboratory it was found that the dialysate of crude extracts of ether–CO_2-frozen baker's yeast yields a fairly high ferric hydroxamate color after incubation with salt-free hydroxylamine and the addition of an acidified $FeCl_3$ solution. In this paper, experimental data are presented indicating the occurrence of carboxyl-activated peptide compounds, both in crude extracts and purified microsomal RNP particles of baker's yeast.

METHODS AND MATERIALS

Salt-free hydroxylamine was prepared as described by BEINERT *et al.*[10]; its concentration was determined colorimetrically[11].

Tyrosine hydroxamate was prepared from methyl tyrosinate and a 10 *M* salt-free hydroxylamine solution in absolute methanol; it was used as a standard for the approximate estimation of

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* A preliminary note on this subject has been presented to the *Koninkl. Ned. Akad. Wetenschap., Proc.*[1].

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*** The following abbreviations will be used: RNA ribonucleic acid, RNP ribonucleoprotein, AMP adenosine monophosphate, ATP adenosine triphosphate, PP inorganic pyrophosphate, TCA trichloroacetic acid.

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the peptide hydroxamates Ferric hydroxamates were determined by measuring the absorption at 530 mμ by means of an "Engel" colorimeter, absorption measurements in the U V were performed with the aid of a Unicam Spectrophotometer SP 600 For the development of the ferric hydroxamate color a solution of 6 6% $FeCl_3$, 0 67 *N* HCl, and 5% TCA was used Redistilled absolute ethanol was used for alcoholic extractions "Nojax" (Viscora) dialysis tubing (diameter 2 4 cm) was purchased from G H Prins & Zn at Amsterdam

For paper chromatography we used Whatman No 1 filter paper; paper electrophoresis experiments were performed on Whatman No 3 paper

In all our experiments we used freshly-obtained pressed baker's yeast—"Koningsgist"—from the Koninklijke Gist- en Spiritusfabriek at Delft According to our information the yeast was harvested and cooled at the end of the exponential growth period and delivered at our laboratory 16–24 hours later For the preparation of the dialysate of crude extracts, about 500 g of yeast were crumbed and frozen for 3–4 hours in ether–CO_2, the frozen yeast was extracted and dialyzed simultaneously against water for 20 hours in about 50 cm of dialysis tubing at 0° C. Subsequently, the dialysate was highly concentrated by freeze-drying, the concentrated solution was stored in a refrigerator at —16° C

Purified preparations of microsomal "80 *S*" RNP particles from the same yeast were obtained according to the method described by SCHACHMAN *et al* [12] They were centrifuged with the aid of a Spinco analytical ultracentrifuge with a preparative head Ultracentrifuge patterns of such preparations at different stages of the isolation procedure are shown in Fig 3

EXPERIMENTS WITH THE CONCENTRATED DIALYSATE OF CRUDE EXTRACTS

The following experimental data have been obtained:

(a) *Paper chromatography*

An almost instantaneous ferric hydroxamate color was obtained upon addition of $FeCl_3$ solution after incubation of the concentrated dialysate with 2*M* salt-free hydroxylamine (pH 7) for 1–10 minutes at room temperature. However, the intensity of this color increased considerably when the reaction was allowed to take place for a few minutes at 100° C or 2–4 hours at 30° C. Some data on the course of hydroxamate formation with time are given in Table I.

TABLE I

COURSE OF HYDROXAMATE FORMATION AT 30° C BY A CONCENTRATED DIALYSATE CONTAINING 2*M* SALT-FREE HYDROXYLAMINE (pH 7)

Time of incubation (minutes)	*μmoles hydroxamate* per mg (dry weight) dialyzed material*
1	0.036
30	0 045
70	0 051
125	0 068
240	0 075

* Tyrosine hydroxamate was used as a standard

As the treatment at 30° C is somewhat milder, we chose it as a routine procedure for preparing the hydroxamate material. After incubation of the concentrated dialysate with hydroxylamine, the reaction mixture was freeze-dried and extracted with a 60% ethanol–40% H_2O mixture. These extracts were subjected to paper chromatography with *n*-butanol–acetic acid–water (90 : 10 : 10) as the solvent; after development with $FeCl_3$ solution usually 5–7 distinguishable ferric hydroxamate spots with R_F values between 0.08 and 0.73 were obtained.

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The R_F values obtained so far are not accurate because it has not yet been possible to carry out the chromatography experiments under well-defined and constant conditions. Therefore, in this paper these values will be mentioned only occasionally.

Moreover, since the hydroxamate reaction is not a very sensitive one, it is quite possible that apart from the 5–7 hydroxamates found by paper chromatography more compounds of the same nature are present in the dialysis liquid.

Furthermore, before adding $FeCl_3$ some of these spots could be detected rather easily by their bright blue and violet fluorescence in the U.V.

The hydroxamic acid-containing spots were eluted with water; half of each eluate was kept as a blank. The other half was hydrolyzed for 6 hours with 6 N HCl. Subsequently, the hydrolysates and their blanks were freeze-dried and run in separate paper chromatograms with the same (BuOH–HAc–H_2O) solvent. The chromatograms of the hydrolysates showed 3–10 ninhydrin-positive spots, the greater number of these spots being obtained from the hydroxamic acids with relatively high (0.45–0.73) R_F values. This finding indicates that the isolated hydroxamates were peptide- rather than amino acyl-hydroxamates.

The blue and violet fluorescence was no longer observed in chromatograms of the hydrolyzed compounds. U.V. extinction measurements in eluates of the hydroxamic acid spots with a relatively high R_F value failed to show any significant amount of nucleotide material. The identification of the amino acids obtained upon hydrolysis of the peptide hydroxamates is in progress.

(b) *Paper electrophoresis*

50% ethanol–50% H_2O extracts of the concentrated dialysate were subjected to paper electrophoresis in 0.02 M citrate buffer solution (pH 3.9) in the cold. The results of two representative experiments are drawn schematically in Figs. 1 and 2. The starting line was taken in the middle of the paper. After 16–18 hours of electrophoresis at 10 V cm^{-1}, a large amount of nucleotide material had migrated in the direction of the anode, as was visible in the U.V. and determined by extinction measurements (Fig. 1). However, a very large and distinct increase in the 260 mμ absorption was found at the other side of the starting line, right at the spot that showed the ferric

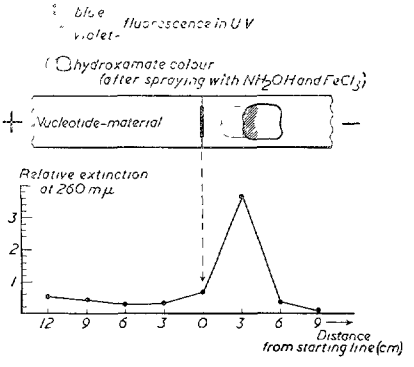

Fig. 1. Schematic outline of an electrophoresis experiment with the concentrated dialysate, showing the coincidence of the hydroxamate-forming material with a peak in the 260 mμ absorption. Electrophoresis at 10 V cm^{-1} for 16 hours in 0.02 M citrate buffer, pH 3.9.

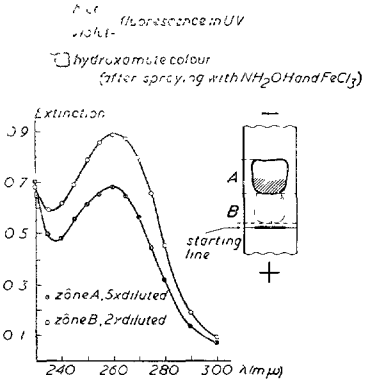

Fig. 2. U.V.-absorption spectra in 0.05 M phosphate buffer (pH 6.8) of the hydroxamate-forming material as obtained after an electrophoresis experiment for 16 hours at 10 V cm^{-1} in 0.02 M citrate buffer, pH 3.9.

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hydroxamate color after spraying with salt-free hydroxylamine, heating to 100° C and subsequent spraying with $FeCl_3$-solution. This is illustrated in Fig. 1. This phenomenon was not observed in a similar experiment with an extract that had been preincubated with hydroxylamine: a distinct increase in the 260 mμ absorption was found at the starting line or slightly nearer to the anode.

Moreover, the typical U.V. absorption spectrum of nucleotides and nucleic acids was found in the eluate of the same (hydroxamate color-yielding) spot, indicating that the hydroxamate-forming compound(s) may contain nucleotide material. This fact was partly confirmed by the finding that about 60% of the hydroxamate-forming material in the dialysate could be adsorbed by norit.

Finally, the blue and violet fluorescence was also observed at the spot containing the hydroxamate-forming compound(s), indicating that peptides are present as such before the incubation with hydroxylamine.

The experiments described above suggest strongly that the concentrated dialysate contains peptides combined with nucleotide(s) by a high energy bond involving the carboxyl group.

PRELIMINARY EXPERIMENTS WITH MICROSOMAL RNP PARTICLES

The finding of carboxyl-activated peptides would gain more interest with respect to the biosynthesis of proteins if it were possible to trace their origin. As the microsomal RNP particles seem to be actively involved in a final step during protein synthesis, they were considered as possible sites of origin of the activated compounds. Consequently, some preliminary experiments with microsomes were carried out in order to obtain experimental support for this idea.

Ultracentrifuge patterns of microsomal preparations of baker's yeast at two different stages of the isolation procedure according to SCHACHMAN *et al.*[12] are shown in Fig. 3. Fig. 3A represents a rather crude preparation, since it was obtained by a single ultracentrifugation. It contains three components, the main one (about 90%) consisting of "80 *S*" microsomal particles. A pattern of the purest preparations is shown in Fig. 3B. It indicates that these preparations may be considered as nearly homogeneous. It was found that both kinds of preparations yield similar hydroxamic acids; it was decided to study the cruder one since it could be prepared faster and in larger amounts.

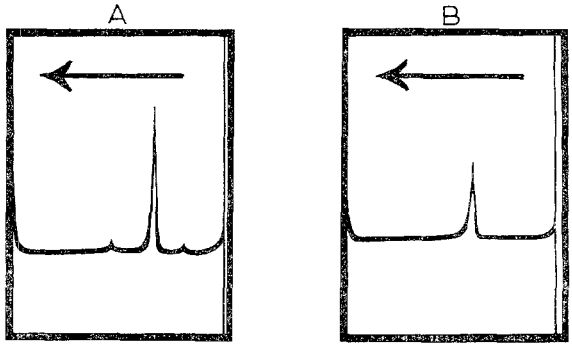

Fig. 3. Ultracentrifuge patterns of two preparations of microsomal RNP particles at different stages of the purification procedure. A. A rather crude preparation (see text) in a solution containing 0.0025 *M* K_2HPO_4, 0.0025 *M* KH_2PO_4, 0.001 *M* $MgSO_4 \cdot H_2O$, $7.5 \cdot 10^{-4}$ *M* $CaCl_2$. Speed 42,040 r.p.m., bar angle 45°, after 13 minutes of sedimentation. B. A final preparation in the same solution. Speed 42,040 r.p.m., bar angle 40°, after 14 minutes of sedimentation.

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Attention should be drawn to the rather peculiar rate of hydroxamate formation by microsomal preparations. The increase in ferric hydroxamate color in a reaction mixture containing microsomes and 2 *M* salt-free hydroxylamine was followed over a long period; the results are shown in Table II.

Regarding the analogy between the very high concentrations of hydroxylamine that have to be used as a trap for activated amino acid compounds in enzymic amino acid activation[17] and the considerable macroscopic changes that were observed in our microsomal preparations during the incubation with hydroxylamine, we are inclined to attribute the slowness of this reaction at least in part to a protective action by the structure of the microsomes. In addition, it should be mentioned that no significant hydroxamate color was obtained after 24 hour's incubation of solutions of 30 mg/ml bovine serum albumin, of 55 mg/ml commercial yeast RNA (B.D.H.) and of 30 mg/ml bovine serum albumin + 55 mg/ml yeast RNA in 2 *M* hydroxylamine under conditions similar to those described in Table II.

TABLE II

RATE OF HYDROXAMATE FORMATION AT 30° C BY A CONCENTRATED SOLUTION OF "80 *S*" MICROSOMAL PARTICLES CONTAINING 2 *M* SALT-FREE HYDROXYLAMINE (pH 7)

Time of incubation (hours)	*Macroscopic changes in the reaction mixture*	*μmoles hydroxamate* formed per mg of microsomes*
1.25	Nearly clear suspension	0.011
2		0.018
4	Increasing viscosity, a precipitate begins to be formed	0.026
7		0.035
12		0.052
24	Very viscous gel with large amounts of white precipitate	0.14
31		0.20
34		0.21
after 3 days	Decreased viscosity, precipitate for the greater part redissolved	—

* Tyrosine hydroxamate was used as a standard.

(a) *Paper chromatography*

After incubation of the microsomes with hydroxylamine, the reaction mixture was freeze-dried and extracted with 60% ethanol–40% H_2O. The experiments carried out with these extracts were essentially the same as described in the preceding section. Usually 3–5 hydroxamate spots were found; they were more distinct than those obtained with the dialysate, but the bright blue fluorescence was observed so far with only one microsomal preparation. Upon acid hydrolysis, the separate hydroxamates yield a number of ninhydrin-positive spots, the highest number (13) being obtained in the hydrolysed eluate of a hydroxamic acid-containing spot with an R_F value of 0.9. The finding of peptide hydroxamates suggests the presence of carboxyl-activated peptides in the "80 *S*" microsomal RNP particles.

(b) *Paper electrophoresis*

It was thought that the activated peptide–nucleotide compounds in the extracts of the ether–CO_2-frozen yeast might be loosened from the microsomes by the freezing procedure. Therefore, a batch of microsomes was frozen for 18 hours in ether–CO_2, and dialyzed for 20 hours in the cold. The results obtained with the freeze-dried dialysate of these microsomes were by no means conclusive. In the first place, the ferric hydroxamate color obtained after incubation of the concentrated dialysate

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with hydroxylamine was far less than could be expected with regard to the data of Table II. After a paper electrophoresis experiment (at pH 3.9) similar to those described on p. 485, two spots were obtained that could be easily detected by a bright yellow and blue fluorescence in the U.V. Both spots were found a little in the direction of the cathode. After spraying with hydroxylamine and $FeCl_3$, a distinct but unusual orange-red color was obtained at the spot that showed a blue fluorescence (a finding that is similar to those described on p. 486). This color changed somewhat with time to the violet-red color of ferric hydroxamates. Again, a peak in the 260 mμ absorption, as well as the typical U.V. spectrum of nucleotide material, were found at the same spot. However, as has been stated above, the very small amount of dialysable hydroxamic acid-forming material and the unusual color of the ferric hydroxamates failed to provide substantial support for the theory of a loosening of the peptide-nucleotide compounds from the microsomes by the freezing procedure.

DISCUSSION

Although one must be very cautious in drawing any conclusion from the data described above, it is tempting to look for a possible relation between the activated peptide compounds and what is known about protein synthesis at present. Amino acid activation catalyzed by soluble enzymes as described for the first time by HOAGLAND *et al.*[13,14] very probably forms the introductory step of cytoplasmic protein synthesis. From the data obtained by HOAGLAND *et al.*[14], NOVELLI *et al.*[15] and BERG[16], it appears that these enzymes catalyze the following reaction:

$$E(nzyme) + ATP + R\ CHNH_2\text{–}COOH \leftrightarrows E\text{–}AMP \sim CO\text{–}CHNH_2\text{–}R + PP$$

The action of different purified enzyme preparations proved to be rather specific with regard to the amino acid substrate[17-20].

However, very little—if anything—is known about what happens next with the activated amino acid compounds. Recent data obtained by HOAGLAND *et al.*[20] indicate that in rat liver a "low molecular weight supernatant RNA" functions as an acceptor or a carrier of the activated amino acids. This RNA is acylated, as follows from the fact that an intermediate hydroxamic acid yielding compound is formed. It might serve to transport the carboxyl-activated amino acids to the microsomes where they are incorporated into proteins. Our findings seem to agree with these data since they indicate the occurrence of nucleotide-bound carboxyl-activated peptides in a microorganism that is capable of protein synthesis.

Moreover, the presence of carboxyl-activated peptides in microsomal RNP particles seems to stress the occurrence of such compounds as intermediates in protein synthesis. With regard to our present knowledge we would suggest the following tentative general scheme for cytoplasmic protein synthesis:

I. Enzymic amino acid activation according to

$$ATP + R\ CHNH_2\text{–}COOH \underset{+E}{\leftrightarrows} E\text{–}AMP \sim CO\text{–}CHNH_2\text{–}R + PP$$

II. Transport of activated amino acids to the microsomes

$$E\text{–}AMP \sim CO\text{–}CHNH_2\text{–}R + Nucl \leftrightarrows Nucl \sim CO\text{–}CHNH_2\text{–}R + E + AMP$$

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III. Condensation of activated amino acids to peptides and proteins:

a. Nucl ~ CO–CHNH$_2$–R → Nucl ~ CO–peptides

b Nucl ~ CO–peptides → Nucl + protein

where E means amino acid-activating enzyme and Nucl supernatant oligo- or polynucleotide material.

With regard to the scheme outlined above, attention may be drawn to the following aspects:

1. It is suggested that oligo- or polynucleotides serve for the transport of activated amino acids to the microsomes. These compounds, together with the microsomal RNA, may provide the necessary information about protein synthesis; speculations about the role of RNA have been made by several authors[4,5,21,22]. For a detailed understanding of this information the nature of the—presumably high energy—bond between the carboxyl group of peptides (and amino acids) and the nucleotides should be known; an amino acyl–phosphate bond has been proposed by two of us a few years ago[5]. Furthermore, it would be interesting to know whether the nucleotide material consisted of a rather constant number of nucleotides or of a growing chain, in other words, to find out whether RNA synthesis starts in the supernatant fraction and occurs parallel to cytoplasmic protein synthesis[13]. A discussion on this subject lies beyond the scope of this paper.

2. From the finding of *peptide*–nucleotide compounds in crude extracts of baker's yeast the question may arise whether the condensation of activated amino acids (reaction IIIa) starts during their transport to the microsomes, or if it is taking place only in these particles. A conclusive answer can only be obtained from additional and detailed data on the nature and the origin of the activated compounds.

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SUMMARY

1 In this paper evidence is presented for the occurrence of dialysable nucleotide-bound carboxyl-activated peptide compounds in extracts of ether–CO_2-frozen fresh pressed baker's yeast

2 Furthermore, some data are given indicating the presence of carboxyl-activated peptides in "80*S*" microsomal RNP particles of the same yeast.

3 The possible relation of the activated peptide compounds to cytoplasmic protein synthesis is discussed

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