Les précultures ont toujours été réalisées en aérobiose. Les cellules destinées à l'ensemencement de la culture ont été prélevées quand la préculture se trouvait en phase exponentielle de croissance et quand la densité optique correspondait à environ 100 unités du photomètre de Coleman (ce qui correspond à environ 2.10^4 cellules/ml).

Les cultures aérobies ont été réalisées dans des récipients[6] d'une capacité de 2 litres et contenant 200 ml de milieu constamment agité à raison de 130 oscillations minutes.

Les cultures anaérobies ont été réalisées dans des récipients entièrement en verre Pyrex (Fig. 4) contenant 200 ml de milieu. L'atmosphère est constituée par de l'azote hautement purifié (azote R contenant moins de 0.005 % d'oxygène). L'air a été éliminé de l'appareil par un barbotage d'azote R sous une pression de 8 cm de Hg, 45 min avant et 45 min après l'ensemencement. En vue d'assurer sa constante homogénéité, la culture a été agitée à raison de 130 oscillations minutes durant toute l'incubation. La courbe de croissance de la levure a été établie grâce à des mesures néphélométriques faites avec le photomètre de Coleman, à l'aide du tube T du récipient de culture.

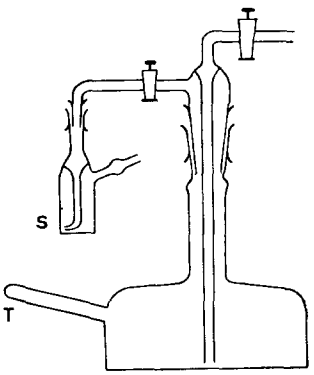

Fig. 4. Récipient utilisé pour les cultures anaérobies. T = tube permettant d'effectuer des mesures néphélométriques directes; S = soupape au mercure permettant le dégagement du gaz carbonique de fermentation.

En tel ou tel point de la période de croissance, les cellules de levure ont été récoltées par centrifugation et lavage dans NaCl à 0.9 %, à une température voisine de 0°, sans qu'aucune précaution spéciale ait été prise pour mettre les cellules de levure à l'abri d'un contact avec l'oxygène.

L'étude spectrale du culot de centrifugation de levure, à l'état réduit, a été faite soit à la température ordinaire soit à la température de l'azote liquide suivant la technique précédemment décrite[6,7].

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Oxygen transfer to AMP in the enzymic synthesis of the hydroxamate of tryptophan*,**

The enzymic synthesis of amino acid hydroxamates from amino acids and hydroxylamine is coupled to the cleavage of ATP into AMP and pyrophosphate[1–3], and it has been assumed that amino acyl adenylates are intermediates in these reactions. Although the formation of these compounds by the enzyme systems has not yet been demonstrated, amino acid adenylates are converted to ATP by these enzymes[4,5]. Results are presented in this paper showing the direct transfer of oxygen from the carboxyl group of tryptophan to the phosphate of AMP in the formation of the hydroxamate of tryptophan by the purified pancreas enzyme of DAVIE *et al.*[3] Suggestive preliminary results had been obtained with a less purified liver enzyme system. These results support the concept of an intermediate in which the carboxyl group of the amino acid is linked to the AMP moiety of ATP through its phosphate group[6].

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TABLE I

^{18}O ANALYSES OF PHOSPHATE FRACTIONS

Tryptophan used	*Atom % excess ^{18}O*	
	Phosphate oxygen	
	From AMP	*From pyrophosphate*
0.00	0.00*	0.00*
1.09	0.25**	0.00*

* Observed 46/44 ratios were very close to the value of 0.00405 for non-isotopic phosphate.

** Observed mass 46/44 ratios of duplicate samples were near 0.0052. The atom % excess was calculated with consideration of carrier added, small amounts of non-labeled, non-AMP phosphate in the fraction, and small amounts of non-labeled tryptophan added with the enzyme.

The results of isotopic analyses of the phosphate fraction formed when ^{18}O-labeled tryptophan was converted to its hydroxamate, coupled to ATP cleavage, are shown in Table I. The values are averages of duplicate analyses, which agreed closely. The trials with non-labeled tryptophan under identical conditions serve as controls for the ^{18}O analyses; such controls guard against possible analytical error from contaminants when rather small amounts of phosphate are used.

The data show clearly that excess ^{18}O from the amino acid carboxyl appeared only in the inorganic phosphate derived from the AMP. The amount found, 0.25 atom % excess, was slightly less than the value of 0.27 atom % excess expected if one oxygen from the amino acid carboxyl had appeared in each AMP molecule formed. The difference is greater than the expected analytical error and might reflect a small amount of hydrolytic cleavage of some intermediate, slight exchanges of oxygen during the isolation, or possibly other experimental errors. The results warrant the conclusion that the major and probably the only reaction pathway was one which would account for a transfer of one oxygen from the amino acid carboxyl to the phosphate group of AMP.

The most plausible explanation of the results is the participation of an intermediate involving a C–O–P linkage between the acyl carbon of the amino acid and the phosphate of the AMP, with the oxygen being furnished by the amino acid. The results are obviously in harmony with the formation of an amino acyl adenylate through a displacement of pyrophosphate from ATP by a carboxyl oxygen, followed by displacement of the AMP moiety through reaction of hydroxylamine with the acyl carbon. A type of reaction sequence ruled out by the findings is one in which a cofactor or group to which pyrophosphate was attached reacted with the amino acid with displacement of the pyrophosphate, followed by reaction with hydroxylamine, and then regeneration of the original pyrophosphate compound by phosphate transfer from ATP. In such a sequence oxygen from the amino acid carboxyl would be expected to appear in the pyrophosphate even though an amino acyl pyrophosphate was not an intermediate in the reaction.

At least two categories of syntheses coupled to ATP cleavage may be recognized. In one, oxygen from a substrate will appear in the inorganic orthophosphate formed; namely in the synthesis of glutamine[7,8], succinyl coenzyme A[9], AMP from inosine-5'-phosphate[10], citrulline[11], and a 3-phosphoglyceryl-enzyme[9,12]. In the second category, substrate oxygen will appear in the phosphate of AMP; namely in the synthesis of acetyl coenzyme A[7], pantothenic acid[13], and the hydroxamate from tryptophan (this paper). Syntheses with oxygen transfer to ADP or to pyrophosphate have not been found and may not exist. Incorporation of water oxygen into inorganic phosphate occurs in S-adenosylmethionine synthesis coupled with cleavage of inorganic phosphate and pyrophosphate from ATP[14]; the source of the oxygen for pyrophosphate formation has not been reported.

Experimental

Preparation and analysis of ^{18}O-labeled tryptophan. 0.01 mole L-tryptophan, 2.0 ml $H_2{}^{18}O$ (> 1.4 atom % excess), and 0.27 ml conc. H_2SO_4 were heated in a sealed tube at 125° for 64 h. Water was added to a vol. of 15 ml, the sample neutralized to pH 7 with ammonia, and the precipitated tryptophan collected. The product was reprecipitated following charcoal decolorization, washed, and dried *in vacuo* over P_2O_5. The product was qualitatively and quantitatively identical to authentic L-tryptophan as tested by the color reaction of SPIES AND CHAMBERS[15]. Analysis by paper chromatography, using a butanol: acetic acid: water (4: 1: 5) solvent with ninhydrin development of the chromatogram[16] gave the characteristic grey spots with an R_F identical to that of authentic L-tryptophan. No other spots appeared on the chromatogram.

For determination of the ^{18}O content, 25.6 mg tryptophan-^{18}O was heated with 0.15 ml 20 % HCl in a sealed tube at 130° for 33 h. The contents of the tube were lyophilized with collection of the H_2O–HCl distillate. The distillate was neutralized with solid tris(hydroxymethyl)aminomethane (to avoid H_2O addition) to bromocresol green, and again lyophilized. The water was

collected and its ^{18}O content determined using the sulfite-bicarbonate procedure described previously[12].

Enzyme incubations. Approx. 20 mg enzyme, obtained by the first four purification steps described in Table IV of the paper by DAVIE *et al.*[3], having a specific activity of 30 units were used. This amount of enzyme was enough to convert 600 μmoles tryptophan to tryptophan hydroxamate in 1 h under the usual experimental conditions[3]. In view of the special purpose of the experiment, such a large excess of enzyme was used in order to bring the reaction to completion. The enzyme was incubated for 60 min at 37° and pH 7.8, in a total volume of 10 ml containing the following additions: tryptophan or tryptophan-^{18}O, 100 μmoles; ATP, 100 μmoles; NH_2OH, 10,000 μmoles; tris(hydroxymethyl)aminomethane, 1000 μmoles; $MgCl_2$, 100 μmoles; crystalline yeast pyrophosphatase, 100 μg. The amount of hydroxamate formed corresponded within the limit of experimental error (± 5%) to the amount of tryptophan added, which indicated that the ATP was completely cleaved. The reaction was terminated by addition of $HClO_4$ and HCl, the protein precipitate removed, the samples brought to approximately pH 4 with KOH, frozen and shipped from Boston to Minneapolis for ^{18}O analyses.

Determination of ^{18}O content of the phosphate fractions. Reaction mixtures were adjusted to pH 8.2 with KOH, allowed to stand several hours in the cold, and the precipitated $KClO_4$ removed by centrifugation. Determinations of AMP by absorptions at 259 mμ and of inorganic P were performed, and the samples separated into two portions for duplicate analyses. A 4-fold excess of barium acetate was added as a 0.9 *M* solution, and the samples were refrigerated. The precipitates were collected and freed of barium by Dowex-50 (H^+), then hydrolyzed with *N* HCl at 100° for 10 min to split any residual pyrophosphate. The inorganic phosphate, derived from the pyrophosphate originally cleaved from the ATP in the enzymic reaction, was precipitated as $MgNH_4PO_4$.

The supernatant solution from the first barium precipitations was treated with 4 vol. 95% ethanol and refrigerated for precipitation of the AMP. The barium was removed as $BaSO_4$ by careful treatment with H_2SO_4. The supernatant solution was adjusted to about 0.25 *M* NH_3–NH_4^+ at pH 9.3 and an excess of Mg^{++} was added. The AMP was hydrolyzed over a several-hour period by addition of alkaline phosphatase[8]. The precipitated $MgNH_4PO_4$ was removed by centrifugation, treated with 5% trichloroacetic acid, and recentrifuged to remove precipitated protein. After determining inorganic P, carrier phosphate was added, and the P reprecipitated as $MgNH_4PO_4$. Determinations of the amount of ^{18}O in the $MgNH_4PO_4$ samples were made as previously described[12].

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