THE INFLUENCE OF TRIS-HCl ON THE BIOSYNTHESIS OF POLYPHENYLALANINE

C. J. REINECKE, *Department of Physiology, Potchefstroom University, Potchefstroom, Tvl*

The requirements for the biosynthesis of polyphenylalanine in the cell-free system of *E. coli* have been the subject of a considerable number of investigations, and are now fairly well established.[10,21,22] Tris-HCl* was used predominantly as buffer in these investigations, as well as in experiments on the biosynthesis of proteins in general. A review of the Tris-HCl concentrations used revealed large differences, as is indicated in Table I. This observation necessitates a study on the effect of Tris-HCl concentrations on the biosynthesis of proteins. In this paper experiments in this regard will be described, using polyphenylalanine synthesis in the *E. coli* cell-free system as model of protein synthesis.

MATERIALS AND METHODS

Buffers

Potassium standard buffer had the following composition: 10 mM Tris-HCl (pH 7·8), 10 mM magnesium acetate, 60 mM potassium chloride and 6 mM β-mercaptoethanol.

Ammonium standard buffer had the same composition but contained 60 mM ammonium chloride instead of potassium chloride.

Preparation of Ribosomes and Soluble Enzymes

Cell-free extracts from *E. coli*, free of endogenous messenger ribonucleic acid (mRNA) activity, were prepared according to the slightly modified procedure of Nirenberg and Matthaei[23] as described by Voorma *et al.*[24] Potassium standard buffer was used in this part of the preparation. The extracts were centrifuged for 3·5 hours at 105,000 × g, yielding a ribosomal sediment and a supernatant fraction. The upper 4/5 part of the supernatant was dialysed at 4°C against 2 litres of ammonium standard buffer for 18 hours, and is referred to as the soluble enzyme fraction. The ribosomal sediment was rinsed once with ammonium standard buffer and then resuspended in 1·0 ml. of this buffer.

Amino-Acid Incorporation

The reaction mixture contains per ml.: 20 μg. polyuridylic acid, 20 μg. transfer ribonucleic acid (tRNA), 0·5 μc [^{14}C]-phenylalanine (spec. act. 12·6 mc/mM), Tris-HCl (pH 7·8) as indicated, magnesium acetate as indicated, 6 μmoles β-mercaptoethanol, 3 μmoles adenosine triphosphate, 2 μmoles phosphoenolpyruvate, 0·2 μmoles guanosine triphosphate, 20 μg. pyruvate kinase (EC 2.7.1.40), 54 μmoles ammonium chloride, 1 mg. ribosomes and 0·3 ml. of the soluble enzyme fraction.

Reaction mixtures of 100 μl. were incubated at 37°C for the times as indicated. The reactions were terminated by the addition of 2·0 ml. 10% trichloroacetic acid. The resulting suspensions were centrifuged at 600 × g, the supernatant was discarded and the precipitates were dissolved in 0·5 ml. 1M sodium hydroxide, incubated at 37°C for 30 minutes and reprecipitated by the addition of 5 ml. 10% trichloroacetic acid. The final precipitates were collected on cellulose acetate filters (pore size 0·45 μ; diameter 2·5 cm.) and washed with five 4-ml. portions of 5% trichloroacetic acid. The dried filters were placed in glass counting vials containing 10 ml. scintillation fluid (1 litre toluene containing 7 G of 2, 5-diphenyloxazol and 0·05 G of 4,4-bis(2-5-phenyl)oxazolyl benzene), and the radio activity was determined in a liquid scintillation counter. The counting efficiency was 70%.

*Tris = tris(hydroxymethyl)amino-methane.

Chemicals

Pyruvate kinase, phosphoenolpyruvate (trisodium salt), adenosine triphosphate (disodium salt) and guanosine triphosphate (sodium salt) were purchased from Seravac Laboratories. A frozen paste of *E. coli* B cells (mid-log phase) and frozen *E. coli* B tRNA were obtained from Miles Laboratories, as well as polyuridylic acid (Ep at 260 mμ: 11·0 × 10^3 at pH 7·0; 280 mμ/260 mμ: 0·224). Radioactive amino acids were obtained from the Radiochemical Centre, Amersham, England.

RESULTS

The Tris-HCl concentration of reaction mixtures used for the study of protein synthesis ranged from 10 mM to 100 mM in various investigations (Table I). The effect of this range of Tris-HCl concentrations on the biosynthesis of polyphenylalanine is illustrated in Fig. 1. Virtually no effect was noticed if the biosynthesis was performed in reaction mixtures containing 10 - 40 mM Tris-HCl. However, higher concentrations (50 - 120 mM) markedly lowered the yield of polyphenylalanine. This effect was also noticed on the slight incorporation of phenylalanine into polypeptides formed under direction of residual endogenous *E. coli* mRNA still present on the ribosomes.

Kinetic experiments performed at 16 mM magnesium acetate revealed that higher Tris-HCl concentrations significantly retarded the polymerization of phenylalanine in the initial phase of synthesis (curves 2, 3, 5 and 6 in Fig. 2). The effect increased with the Tris-HCl concentration. At 20 mM Tris-HCl phenylalanine incorporation began to reach a plateau after about 40 min. incubation (curve 1, Fig. 2), whereas no plateau was reached even after 90 minutes' incubation at 100 mM Tris-HCl (curve 3, Fig. 2). Synthesis at 60 mM Tris-HCl was intermediate between these two extremes (curve 2, Fig. 2).

TABLE I. TRIS-HCl CONCENTRATIONS USED IN VARIOUS INVESTIGATIONS ON POLYPEPTIDE SYNTHESIS

Tris-HCl concentration used (mM)	*mRNA used*	*Reference*
10	poly U*	20
10	R17-RNA	26
43	poly U	8
50	$AUGU_3$	4
50	R17-RNA	2
50	poly U	9
60	MS2-RNA	23
60	poly AUG	5
100	*E. coli* mRNA	11
100	poly AUG	6
100	poly U	1
100	f2-RNA	3

*poly U = polyuridylic acid.

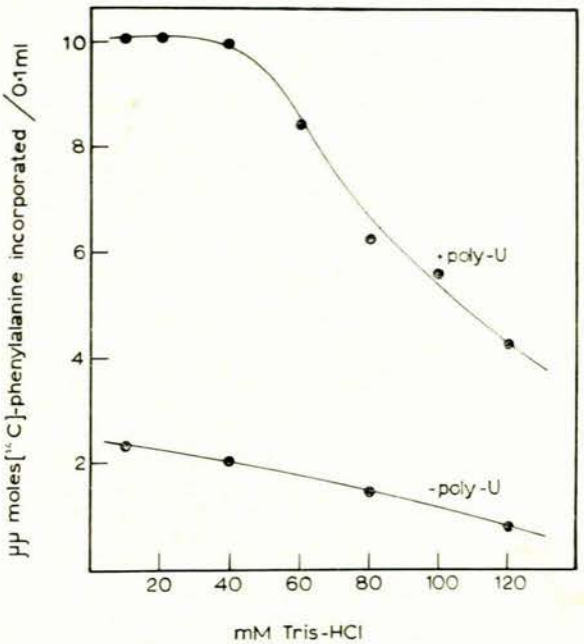

Fig. 1. Influence of Tris-HCl on polyphenylalanine synthesis. The reaction mixture contained all the components as described in the text, except that the Tris-HCl concentration was varied as indicated, and that the magnesium acetate concentration was 16 mM. The mixtures were incubated for 30 minutes at 37°C. Poly U = polyuridylic acid.

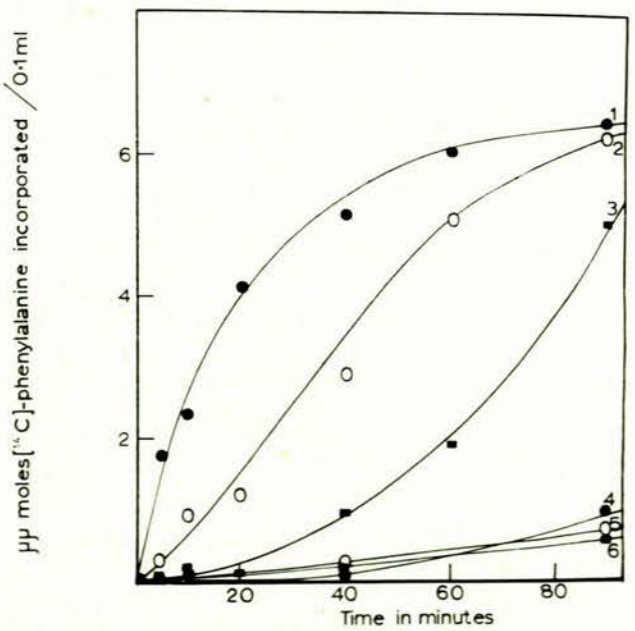

Fig. 2. Kinetics of polyphenylalanine synthesis at various Tris-HCl concentrations. The reaction mixtures contained all the components as described in the text, except that the magnesium acetate concentration was 16 mM, and the time of incubation was varied as indicated. The kinetic experiments were performed at 20 mM (curves 1 and 4), 60 mM (curves 2 and 5) and 100 mM (curves 3 and 6) Tris-HCl. Curves 1, 2 and 3 were obtained in the presence and curves 4, 5 and 6 in the absence of polyuridylic acid.

The lower level of polyphenylalanine synthesis at higher Tris-HCl concentrations was accompanied by a shift in the magnesium concentration necessary for optimal phenylalanine incorporation (Fig. 3). A sharp optimum at 16 mM magnesium acetate was noticed when the Tris-HCl concentration was 20 mM (curve 1, Fig. 3). This optimum

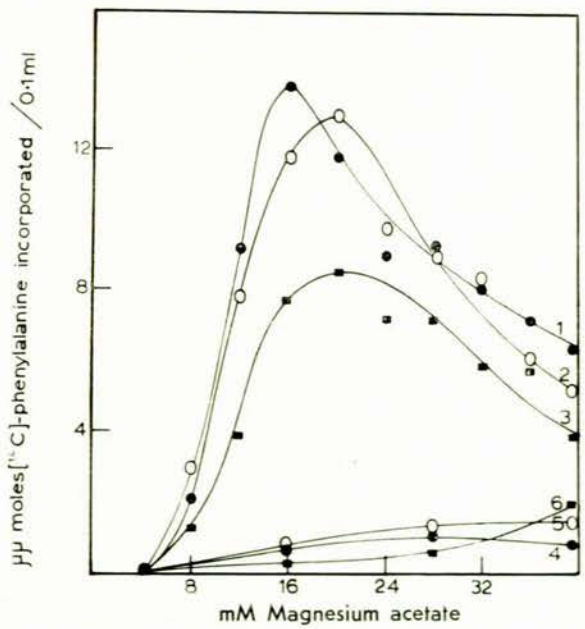

Fig. 3. Influence of the magnesium concentration on polyphenylalanine synthesis at various Tris-HCl concentrations. Reaction mixtures contained all the components as described in the text, except that the magnesium concentration was varied as indicated. The mixtures were incubated at 37°C for 30 minutes. The experiments were performed at 20 mM (curves 1 and 4), 60 mM (curves 2 and 5) and 100 mM (curves 3 and 6) Tris-HCl. Curves 1, 2 and 3 were obtained in the presence and curves 4, 5 and 6 in the absence of polyuridylic acid.

shifted to 20 mM magnesium acetate in the presence of 60 mM Tris-HCl (curve 2, Fig. 3), and became eventually a relatively broad optimum in reaction mixtures containing 100 mM Tris-HCl (curve 3, Fig. 3).

It may be concluded that polyphenylalanine synthesis required longer times of incubation and higher magnesium concentrations for maximal incorporation of phenylalanine into the homopolymer, if high Tris-HCl concentrations were used.

DISCUSSION

The contradictory results obtained by Ohta *et al.*[34] and Nomura and Lowry[35] on the binding of f2-RNA to 70S *E. coli* ribosomes were mainly due to differences in the composition of the reaction mixtures used. This again stressed the important influence that the composition of the reaction mixture may exhibit on the experimental results. In many investigations on the biosynthesis of proteins Tris-HCl has been utilized to maintain the pH of the reaction mixture (Table I). However, the concentrations of Tris-HCl used varied from 10 mM to 100 mM. From experiments described in this paper it appeared that Tris-HCl concentrations exceeding 40 mM significantly influenced amino-acid incorporation into polypeptides. This

influence is noticed both on the kinetics of amino-acid incorporation and on the magnesium concentration required for maximal incorporation.

Kinetic experiments indicated that the influence of Tris-HCl is particularly relevant in the initial phase of synthesis. A considerable lag phase appeared if high Tris-HCl concentrations were used. Apparently the high Tris-HCl concentration inhibited early formation of a chain initiation complex, which could probably only be formed after incubation for some time.

Polyphenylalanine synthesis can only be accomplished at low magnesium concentrations (5 mM) if the reaction mixture is supplied with acetylphenylalanyl-tRNA,[9,12] an artificial chain initiator for polyphenylalanine synthesis.[7] Several authors have shown that polyphenylalanine synthesis in the absence of this chain initiator required high magnesium concentrations for the formation of a chain initiation complex.[12,14,15,18] The higher magnesium concentrations required for optimal phenylalanine incorporation at high Tris-HCl concentrations therefore similarly suggest that the Tris-HCl interfered with the formation of a chain initiation complex, in accordance with the conclusions drawn from the kinetic experiments. A similar situation occurs when polypeptide synthesis is performed with natural mRNA in the presence of purified ribosomes.[17]

An inhibitory effect of high Tris-HCl concentrations on other enzymes has also been reported.[19,25] These reports, together with the results reported in this paper, emphasize the important role that the composition of the reaction mixtures may play in biochemical reactions. In investigations on the initiation of protein synthesis, care should be taken that the Tris-HCl concentration of the reaction mixture never exceeds 40 mM.

SUMMARY

The influence of Tris-HCl on the biosynthesis of polyphenylalanine was studied. It was found that Tris-HCl concentrations exceeding 40 mM depressed phenylalanine incorporation into polypeptides. The effect increased with Tris-HCl concentration. Kinetic experiments indicated that the initial phase of synthesis is significantly inhibited at higher (40 - 100 mM) Tris-HCl concentrations. Under the latter conditions higher magnesium concentrations (20 mM) were required for maximal incorporation than in the presence of lower (10 - 40 mM) Tris-HCl concentrations.

This work was supported in part by financial aid from the CSIR, Pretoria.

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