Formylmethionyl-tRNA Dependence of Amino Acid Incorporation in Extracts of Trimethoprim-Treated Escherichia coli

Abstract. *The growth of* Escherichia coli *cells in rich, thymine-containing medium was stopped by the addition of trimethoprim, an inhibitor of dihydrofolate reductase. In an extract of these cells (supernatant from an extract centrifuged at 30,000*g*) amino acid incorporation at 0.005*M *magnesium-ion concentration, directed by f2 bacteriophage RNA depended strictly on added formylmethionyl-transfer RNA or* N^5*-formyltetrahydrofolate. Hydroxylamine inhibited the amino acid incorporation directed by f2 bacteriophage RNA, when* N^5*-formyltetrahydrofolate had been added, but had no effect when formylmethionyl-transfer RNA had been added.*

Results of studies of amino acid incorporation in cell-free extracts of *Escherichia coli* promoted by various types of mRNA (*1*), for example, endogenous (*2*), viral (f2 and R17) (*3, 4*) and certain synthetic RNA's (random polyAGU) (*5, 6*) are consistent with formylmethionyl-tRNA being the major, if not the only, initiator of proteins synthesized in *E. coli*. This organism contains at least two kinds of methionine-specific tRNA's separable from each other ($tRNA_1$ and $tRNA_2$). Formylation of methionine occurs only after the methionine has been attached to $tRNA_2$ (*7*). The AUG, GUG, and UUG triplets promote binding of methionyl-$tRNA_2$ or formylmethionyl-$tRNA_2$ to ribosomes; AUG also promotes that of methionyl-$tRNA_1$ (*5, 8*).

Dependence of amino acid incorporation on added formylmethionyl-tRNA was demonstrated by using random polyAGU (a copolymer containing the codons for methionyl- and formylmethionyl-$tRNA_2$) as a messenger (*6*). The cell-free *E. coli* system, in which this dependence was determined, was reconstituted from washed ribosomes, discharged tRNA, and the supernatant fraction from high speed centrifugation of an extract of disrupted cells from which most or all of the tRNA was removed. The experiments were performed at low Mg^{++} concentration (*6*).

It was conceivable that if the growth of an *E. coli* culture was stopped by inhibiting formylmethionyl-tRNA formation, then a crude extract (S-30) of such a culture should be dependent on added formylmethionyl-tRNA for amino acid incorporation. Tetrahydrofolate is a precursor of N^{10}FTHF, the formyl donor to methionyl-$tRNA_2$ (*3, 7*).

The growth of a culture was stopped by addition of TMP (*9, 10*), an inhibitor of dihydrofolate reductase, thus presumably blocking the formation of tetrahydrofolate. Much lower concentrations of TMP are required for inhibiting *E. coli* growth than of aminopterin or of most of the other structural analogs of folic acid (*10*). Tetrahydrofolate is also required for the synthesis of essential metabolites other than formylmethionyl-tRNA (*11*). Since we wanted to be sure that all formylmethionyl-tRNA was used up before preparing the cell-free extract, we provided as many as possible of the other essential metabolites by growing the cells in a rich medium supplemented with thymine.

On addition of TMP (50 μg/ml) to a culture, growth (Fig. 1A) and uracil incorporation (Fig. 1D) decreased and stopped within 5 minutes; amino acid incorporation slowed down immediately and stopped after 15 minutes (Fig. 1*C*); and β-galactosidase formation stopped after a delay of about 10 minutes (Fig. 1B).

The observed cessation of uracil incorporation may have been a consequence of the stoppage of protein synthesis. This might be in accordance with the model based on the hypothesis that protein synthesis is required for the synthesis of mRNA (see, for example, *12*). However, further studies are needed to eliminate more trivial explanations.

Preparation of the cell-free system. A culture of *E. coli* K10 S26 (a phosphatase-negative strain from A. Garen) was grown in trypticase soy broth supplemented with thymine (100 μg/ml) on a rotary shaker at 37°C. Growth was followed by measuring absorbancy at 600 mμ. TMP (dissolved in 95 percent ethanol) was added in the early log phase of growth, at an absorbancy of 0.25. Incubation was continued for 30 minutes, and then the culture was cooled to 0°C. The cells were harvested and washed twice, each time with 40 ml of extracting medium per gram of packed cells. The extracting medium consisted of 0.01*M* tris-

Table 1. Formylmethionyl-tRNA or N^5FTHF requirement for amino acid incorporation directed by f2 RNA in an S-30 extract from a culture of *Escherichia coli* treated with TMP. The incubation was performed at 37°C for 30 minutes; the total volume was 0.2 ml. One milliliter of the reaction mixture contained the following components (in μmole unless otherwise indicated): $(NH_4)Cl$, 75; tris-HCl buffer, *p*H 7.8, 50; glutathione, 10; PEP, 5; ATP, 3; GTP, 0.2; and each of the 20 amino acids (labeled or not as indicated), 0.025; also 20 μg of aminopterin; 490 μg of discharged tRNA; and 0.25 ml of inhibited S-30 which had been incubated at 37°C for 15 minutes in a mixture, 1 ml of which contained the following components (in micromoles unless otherwise indicated): $(NH_4)Cl$, 75; tris-HCl buffer, *p*H 7.8, 50; magnesium acetate, 10; glutathione, 10; PEP, 5; ATP, 3; GTP, 0.2; each amino acid except those labeled in the experiment, 0.025; 20 μg of aminopterin; 20 μg of pyruvate kinase; and 0.5 ml of inhibited S-30 (containing about 5 mg of total RNA). The inhibited S-30 was used immediately after the aforesaid incubation in the incorporation experiments. Radioactivity was determined in 0.05-ml or 0.1-ml portons by the filter paper disc method (*24*). RNA was determined by treating the samples in 0.5*N* perchloric acid at 70°C for 20 minutes and measuring the absorbancy of the acid-soluble components of the digest at 260 mμ (*25*). Phenylalanine and threonine incorporation were tested in separate experiments. Amounts of amino acids incorporated are expressed as micromicromoles per milligram of S-30 RNA.

Mg^{++} conc. (*M*)	Messenger		Amino acid incorporation				
			No addition		N^5FTHF *		Formylmethionyl-tRNA †
	Type	Amt. (μg/ml)	Threonine	Phenylalanine	Threonine	Phenylalanine	Threonine
0.005			29 (81) ‡	10	45 (84)	14	14
.005	f2 RNA	100	27 (855)	13	764 (875)	734	723
.005	PolyU	20		3652		3360	
.01			218	74	197	76	235
.01	f2 RNA	100	1703	492	1443	545	1527
.01	PolyU	20		3290		2990	

* 450 μg/ml reaction mixture. † 427 μg/ml reaction mixture. ‡ The values in parentheses were obtained with reaction mixtures containing "noninhibited" S-30.

HCl buffer, *p*H 7.8; 0.06*M* ammonium chloride; 0.01*M* magnesium acetate; 0.006*M* β-mercaptoethanol; and 10 μg of TMP per milliliter. The yield of packed cells was 0.5 to 0.7 g per liter of culture.

All subsequent operations were carried out at 2°C. The cells were suspended in the extracting medium (1 volume of medium per gram of packed cells) and disrupted in the French cell (Aminco, Silver Spring, Maryland) at 12,000 psi (about 800 atm). The resulting extract was incubated with 10 μg of deoxyribonuclease (per milliliter of extract) for 15 minutes at 0°C and centrifuged at 20,000*g* for 20 minutes. The supernatant was centrifuged at 30,000*g* for 30 minutes, and the resulting supernatant was dialyzed against two changes, at 3-hour intervals, of 200 volumes of extracting medium. The dialyzate was centrifuged at 10,000*g* for 10 minutes. This supernatant was divided into small portions and stored in liquid nitrogen. It was designated "inhibited" S-30. "Noninhibited" S-30 was prepared in an identical fashion except that no TMP was added to either the culture or the extracting medium. The f2 bacteriophage RNA was prepared by treating purified virus with phenol (*13*). The tRNA was prepared from a culture of *E. coli* K10 S26 grown in trypticase soy broth at 37°C. The culture was harvested in late log phase of growth and washed as described. The cells were suspended in 0.1*M* tris-HCl buffer, *p*H 7.6, and disrupted in the French cell at 12,000 psi (about 800 atm). The resulting material was mixed immediately with an equal volume of cold 80 percent aqueous phenol and stirred at room temperature for 1 hour. After centrifugation at 12,000*g* for 10 minutes the resulting aqueous phase was aspirated, and the nucleic acids were precipitated from it by the addition of one-tenth volume of 10 percent sodium chloride and 2.5 volumes of 95 percent ethanol. After being kept at 4°C overnight, the precipitate was sedimented by centrifugation, and the pellet was extracted twice with 1*M* sodium chloride at 0°C for 60 minutes. Then 2.5 volumes of 95 percent ethanol were added to the combined extracts. After being kept at 4°C overnight, the precipitate was collected by centrifugation, washed with 66 percent aqueous ethanol, and extracted twice with 0.2*M* tris-HCl buffer, *p*H 9.0. The combined extracts were incubated at 37°C for 2 hours to discharge the aminoacyl-tRNA; they were then dialyzed against 0.4*M* sodium chloride containing 0.02*M* sodium citrate at 4°C for 2 hours and against distilled water for 24 hours. The tRNA was stored frozen in small portions (at a concentration of 4 to 5 mg per milliliter) at −20°C. The yield was 1 to 2 mg of tRNA per gram of packed cells.

The unfractionated tRNA was charged with C^{14}-methionine and formylated, N^5FTHF serving as a source of formate (*6*). The recovery of charged tRNA with a specific activity of 16,300 count min^{-1} mg^{-1} was about 90 percent. Paper electrophoresis of the ribonuclease digest of the charged

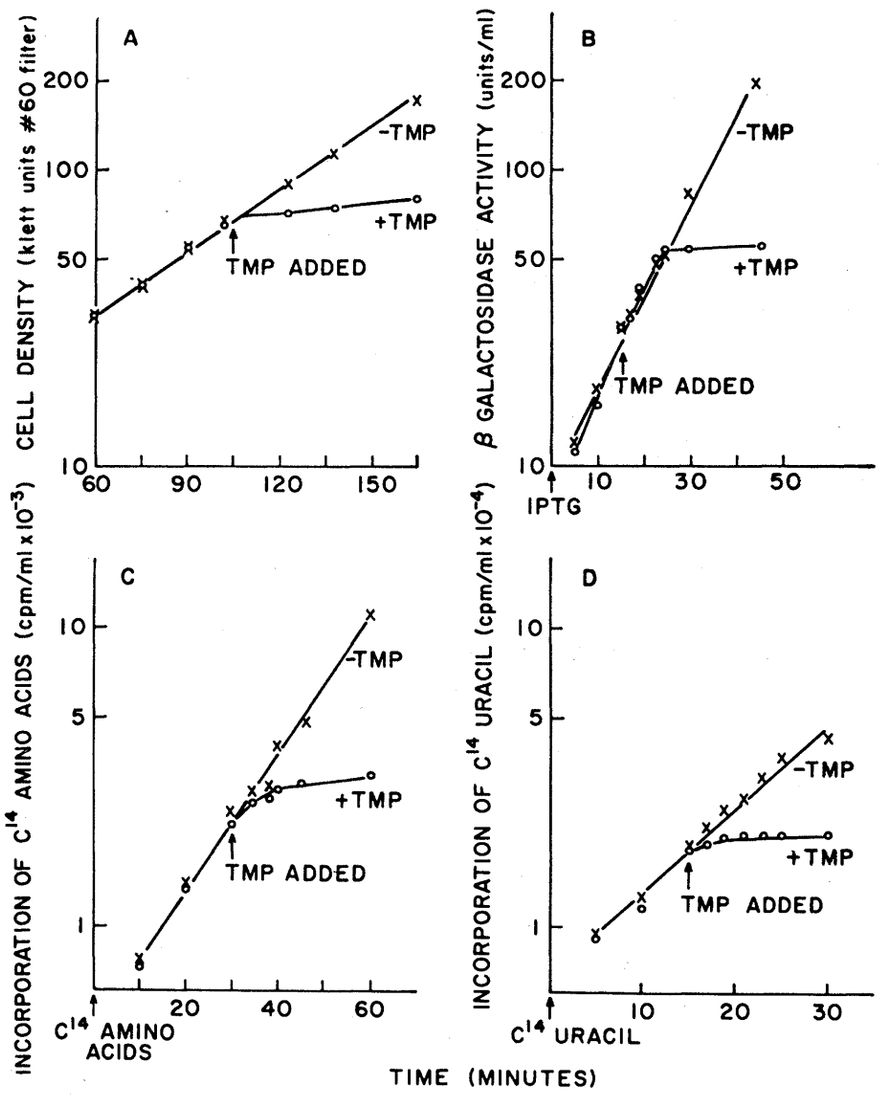

Fig. 1. Kinetics of TMP inhibition of cell growth of β-galactosidase formation, and of incorporation of uracil and amino acids. Cells of *E. coli* K10 S26 were inoculated into 20 ml of trypticase soy broth supplemented with thymine (100 μg/ml) in 125-ml erlenmeyer flasks on a rotary shaker at 37°C. To one of two identical flasks, 50 μg of TMP per milliliter was added at the time indicated by the arrow. (A) Growth was followed by measuring turbidity in the Klett colorimeter (No. 60 filter). (B) $10^{-3}M$ IPTG was added to a pair of cultures in log phase at 50 Klett units 15 minutes before addition of TMP to one of the cultures. β-Galactosidase activity was determined at the times indicated (*23*). (C) C^{14}-L-Phenylalanine (2.25 μc) and C^{14}-L-histidine (2.25 μc) were added to a pair of cultures at 50 Klett units, 30 minutes before addition of TMP to one of the cultures. Radioactivity in the product insoluble in hot acid was determined on 0.1-ml portions with the filter paper disc method at the times indicated (*24*). (D) C^{14}-Uracil (2.0 μc) was added to a pair of cultures, at 50 Klett units, 15 minutes before addition of TMP to one of the cultures. The radioactivity of the product insoluble in cold acid was determined in 0.1-ml portions with the filter paper disc method at the times indicated.

Table 2. Increase in the ratio of histidine to threonine incorporation with the concentration of N^5FTHF in amino acid incorporation promoted by f2 RNA in an S-30 extract from an *E. coli* culture treated with TMP. Amounts of amino acids incorporated are expressed as micromicromoles per milligram of S-30 RNA. The values represent net stimulation of amino acid incorporation promoted by f2 RNA [values of amino acid incorporations with no f2 RNA added (given in parentheses) were subtracted]. Conditions were the same as those described in text and Table 1. To 1 ml of the reaction mixture 5 μM magnesium acetate and 100 μg of f2 RNA were added.

N^5FTHF added (μg/ml)	Threonine	Histidine	Histidine/threonine (ratio)
None	16 (16)	0 (7)	
75	171 (19)	0 (11)	
150	265 (14)	12 (7)	0.05
300	700 (12)	74 (11)	.11
450	1045 (30)	156 (7)	.15
600	876 (42)	172 (11)	.20

and formylated tRNA resulted in two labeled areas, 60 percent corresponding to adenosylmethionine and 40 percent to adenosylformylmethionine. The kinetics of discharging methionine from methionyl- and formylmethionyl-tRNA in 0.1M tris-HCl buffer pH 8.5 at 37°C were biphasic, an indication that there were two methionyl-tRNA components with different decomposition rates (*3*).

The dependence of amino acid incorporation on formylmethionyl-tRNA. In the S-30 extract of TMP-treated *E. coli*, the amino acid incorporation (*13*) directed by f2 RNA required the addition of formylmethionyl-tRNA (Table 1). This requirement was found at low (0.005M) Mg^{++} concentration, but not at high (0.01M) Mg^{++} concentration. The dependence of the formylmethionyl-tRNA requirement for amino acid incorporation directed by f2 RNA on the concentration of Mg^{++} was similar to that found for incorporation directed by polyAGU (*6*). Phenylalanine incorporation directed by polyU was neither dependent on nor affected by added formylmethionyl-tRNA.

In agreement with other studies, N^5FTHF was found to substitute for formylmethionyl-tRNA (*6*) (Table 1). Amino acid incorporation promoted by bacteriophage f2 RNA in an S-30 extract of a culture not treated with TMP did not depend on added formylmethionyl-tRNA (Table 1, values in parentheses).

Several observations indicate that f2 RNA is a polycistronic messenger promoting the formation of bacteriophage coat protein and of other protein or proteins in the cell-free amino-acid incorporating system (*13–15*). The coat protein does not contain histidine; the other protein or proteins do (*13, 14*). Thus, the ratio of histidine incorporation to that of another amino acid presumably present in all proteins coded by f2 RNA is an indication of the ratio of the amount of the other protein or proteins synthesized to that of the coat protein.

The translation of mRNA starts at its 5′-end (*16*). We assumed: (i) that the coat-protein specific cistron was nearer to the 5′-end of the mRNA than the cistron, or cistrons, specifying the histidine-containing protein or proteins; (ii) that the translation was sequential (see *17*); and (iii) that formylmethionyl-tRNA was required for initiating all peptide chains (*3, 18*). Under these circumstances the translation of the histidine-containing protein or proteins would require one (or two) formylmethionyl-tRNA's in addition to those required for that of the coat protein.

The ratio of histidine to threonine incorporation increased several-fold when the amount of N^5FTHF added to the S-30 extract (*19*) was increased (Table 2).

Thus, more formylmethionyl-tRNA was required for the translation of the histidine-containing protein or proteins than for that of the coat protein. However, finding the expected result does not prove the validity of the assumptions.

Hydroxylamine ($10^{-5}M$) has been reported to stop DNA, RNA, and protein synthesis (*20*). From an analysis of the kinetics of the sequence of the steps in the induced synthesis of β-galactosidase it was concluded that $10^{-4}M$ hydroxylamine specifically inhibited peptide-chain initiation (*21*).

We tested the effect of several concentrations of hydroxylamine on the amino acid incorporation in the S-30 extract of cells treated with TMP. The promotion by N^5FTHF of threonine incorporation directed by f2 RNA was inhibited by hydroxylamine. However, in the same system hydroxylamine had no effect on the threonine incorporation promoted by formylmethionyl-tRNA (Table 3).

The inhibitory effect of hydroxylamine was much more pronounced at 0.005M Mg^{++} concentration than at 0.01M Mg^{++} concentration. Histidine incorporation was decreased more than that of threonine; this might, perhaps, be explained by sequential translation of the cistrons as outlined earlier.

Table 3. Inhibition by hydroxylamine of N^5FTHF-dependent threonine incorporation directed by f2 RNA in an S-30 extract from an *E. coli* culture treated with TMP. Conditions were the same as those already described in text and in Table 1. Each reaction mixture contained 100 μg of f2 RNA per milliliter. Reaction mixtures containing all components except f2 RNA were incubated at 37°C for 2 minutes before addition of f2 RNA.

Hydroxylamine (μmole/ml)	Other additions		Threonine incorporated	
	Compound	Amt. (μg/ml)	0.005M Mg^{++}	0.01M Mg^{++}
None	N^5FTHF	450	579	1515
1.25	N^5FTHF	450	601	1475
2.5	N^5FTHF	450	200	1485
5	N^5FTHF	450	107	1519
10	N^5FTHF	450	49	1555
None	Formylmethionyl-tRNA	427	564	1533
2.5	Formylmethionyl-tRNA	427	525	1504

Further studies are needed to elucidate the nature of the step inhibited by hydroxylamine (*22*).

The simple cell-free system prepared from TMP-inhibited cells might be of further use in the study of peptide-chain initiation. It has served here to establish the strict formylmethionyl-tRNA requirement of amino acid incorporation directed by f2 RNA.

It remains to be seen whether TMP or hydroxylamine, or both, might be used in investigating the dependence of RNA synthesis on protein synthesis.

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References and Notes

1. The abbreviations used are as follows: ATP, adenosine-5′-triphosphate; GTP, guanosine-5′-triphosphate; mRNA, messenger RNA; tRNA, transfer RNA; PEP, phosphoenol-pyruvate; TMP, trimethoprim; IPTG, isopropyl-β-D-thiogalactopyranoside; ONPG, orthonitrophenyl-β-D-galactopyranoside; N^5FTHF, N^5-formyltetrahydrofolate; DOC, sodium deoxycholate; tris, trishydroxymethylaminomethane; polyU, polyuridylic acid; polyAGU, a random copolymer of adenylate, guanylate, and uridylate residues.
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Pyruvate Inhibition of Lactate Dehydrogenase Activity in Human Tissue Extracts

Abstract. *Pyruvate inhibition of lactate dehydrogenase activity of crude human tissue extracts has been studied at both 25° and 37°C. The lactate dehydrogenase activity of tissues containing predominantly the slower moving isoenzymes is inhibited significantly less than that of other tissues. These findings are not in accordance with some recently reported.*

Variations in the catalytic response towards pyruvate of human and rabbit lactate dehydrogenase isoenzymes were first described by Plagemann *et al.* (*1*), who showed that the enzyme from both human and rabbit heart muscle was inhibited at much lower concentrations than the enzyme from human or rabbit liver. Similar observations have been described in relation to other species (*2*, *3*). A report (*4*), however, has indicated that the lactate dehydrogenase (LDH) activities of whole human tissue extracts behave more similarly toward increasing concentration of pyruvate and lactate than would be expected from the differences in substrate inhibition of the individual isoenzymes LDH-1 and LDH-5. It was pointed out that even these differences were abolished at 37°C.

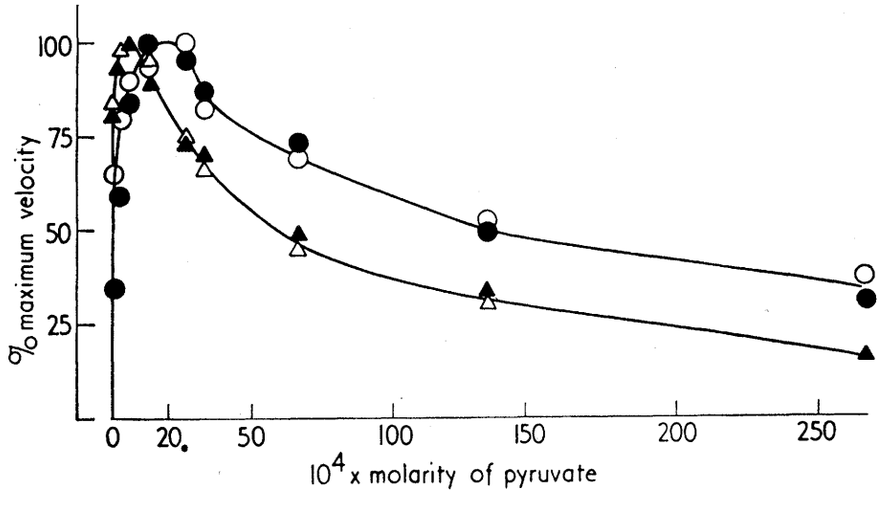

Fig. 1. Pyruvate inhibition of lactate dehydrogenase activity of human tissue extracts at 25° and 37°C (○, liver 25°C; ●, liver 37°C; △, heart 25°C; ▲, heart 37°C).

It has been suggested that the variations in substrate inhibition of the lactate dehydrogenase isoenzymes may be related to their metabolic function (*3*, *5–7*). Those tissues containing a preponderance of LDH-1 usually function under aerobic conditions, whereas those containing a preponderance of LDH-5 function under relatively anaerobic conditions. Both the pyruvate concentration at which the enzyme is most active and the concentration at which the enzyme is strongly inhibited are much lower for LDH-1 than for LDH-5 (*1*, *3*).

Although temperature dependence of the pyruvate inhibition of lactate dehydrogenase from rabbit tissues has been reported (*8*), observations on the lactate dehydrogenase activity of extracts of amphibian tissues suggest that there is little change in the overall shape of each of the substrate inhibition curves in the temperature range 11° to 50°C, although the optimum pyruvate concentration may be increased as much as tenfold (*7*). These observations and our own knowledge of the kinetic properties of human tissue lactate dehydrogenases (*6*) led us to reexamine the pyruvate inhibition of lactate dehydrogenase activity of human tissues to see whether the phenomena reported by Vesell (*4*) could be confirmed.

Using saline extracts of human tissues, we have examined the pyruvate inhibition of lactate dehydrogenase activity at both 25° and 37°C. The tissues studied included liver, skeletal muscle, heart, kidney, erythrocytes, and spleen. Extracts were prepared from such tissues obtained within 24 hours of death by homogenizing with approximately five parts of physiological saline, in an electrically driven ground-glass homogenizer. The homogenates were centrifuged at 100,000*g* for 20 minutes, and the supernatants were assayed spectrophotometrically for LDH activity by measuring the rate of change in optical density at 340 mμ. The reaction mixture contained 1 ml of 0.1*M* tris-HCl