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REGULATION OF PROTEIN SYNTHESIS BY ELONGATION FACTOR I

by

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ABSTRACT

Levels of polypeptide elongation factor 1 (EF-1) in liver cytosol are shown to be directly correlated with elongation rates *in vivo* of temperature acclimated toadfish. The molecular size distribution of fish liver EF-1 is found to be different from other eukaryotes studied, with 80% of total activity distributed about equally between large (400,000-700,000 daltons) and small (50,000-135,000 daltons) forms. The increase in EF-1 activity associated with cold acclimation occurs almost entirely in the small forms of EF-1 (50,000-200,000 daltons).

Levels of EF-1 in rat liver cytosol are three times that of fish liver, although elongation rate *in vivo* is comparable when body temperature difference is taken into account. No change is soluble EF-1 activity occurs with thyroidectomy or treatment with excess thyroid hormone. Ribosome-bound EF-1 activity, however, is depressed in thyroidectomized rats and correlates with observed decreases in elongation rate *in vivo*. The role of EF-1 as the rate-limiting factor in the control of polypeptide chain elongation in liver protein synthesis is assessed.

DEDICATION

This dissertation is dedicated to the memory of my ineffable husband, Philip Plant, Lt. Col. AUS. His encouragement, his understanding, and his devotion sustained me throughout the course of this work until his death in January, 1976.

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I. INTRODUCTION

This thesis presents an investigation of two vertebrate species in which changes in liver protein synthetic rate *in vivo* have been found to occur in response to environmental and hormonal parameters. The systems studied involve metabolic compensation to temperature (temperature acclimation) in a marine fish and the effect of thyroid hormone in the rat. The work concerns the possible correlation of the activity of polypeptide elongation factors with changing elongation rate in protein synthesis.

A. Mechanism of Polypeptide Chain Elongation in Protein Synthesis

Polypeptide chain elongation has been shown to consist of three consecutive steps: binding of the aminoacyl-tRNA to the codon recognition site (A site) on the ribosome-mRNA complex, peptide bond formation, and translocation of the newly synthesized peptidyl-tRNA from the A to P site (Haselkorn and Rothman-Denes, 1973). The binding of the aminoacyl-tRNA to the A site with the concomitant hydrolysis of GTP is catalyzed by elongation factors (EF-Tu-Ts) in prokaryotes and elongation factor 1 (EF-1) in eukaryotes (Haselkorn and Rothman-Denes, 1973).

In *E. coli* aminoacyl-tRNA is bound to the A site from a ternary complex of AA-tRNA-Tu-GTP, GTP is hydrolyzed, and

EF-Tu is released from the ribosomes as an EF-Tu-GDP complex (Weissbach and Brot, 1974). EF-Ts catalyzes the nucleotide exchange of Tu-GDP to Tu-GTP through the formation of an EF-Tu-Ts intermediate; EF-Ts is not required for the reaction of free EF-Tu with either GDP or GTP (Weissbach et al., 1970). Elongation factors EF-Tu and EF-Ts from E. coli are single polypeptide chains of molecular weights 47,000 and 35,000 daltons respectively (Arai et al., 1973).

After the aminoacyl-tRNA is bound to the ribosomal A site, peptidyltransferase catalyzes peptide bond formation between the α-amino group of the aminoacyl-tRNA on the A site and the carboxyl group of the C-terminal amino acid of the peptidyl-tRNA on the P site (Lucas-Lenard and Lipmann, 1971). The peptidyltransferase center is located on the large ribosomal subunit (50S in prokaryotes and 60S in eukaryotes); no soluble factors or GTP are required for peptide bond formation (Haselkorn and Rothman-Denes, 1973).

Translocation with the accompanying GTP hydrolysis is catalyzed by EF-G in prokaryotes and EF-2 in eukaryotes; translocation has been shown to involve not only the transfer of the peptidyl-tRNA from the A to P site but also the removal of the deacylated-tRNA from the P site and the movement of the messenger by one codon (Haselkorn and Rothman-Denes, 1973). EF-G and EF-2 appear to have similar catalytic function in translocation, both enzymes catalyze ribosome-dependent GTP hydrolysis, and both are inhibited by the antibiotic fusidic

acid (Weissbach and Brot, 1974). Crystalline EF-G from E. coli is a single polypeptide of 83,000 daltons (Kaziro et al., 1972). Collins et al. (1971) have shown homogenous EF-2 from rat liver to be a single polypeptide chain of molecular weight 96,500 ± 9,000 daltons.

The EF-Tu-dependent and EF-G-dependent GTP hydrolysis is associated with the GTPase center of the 50S ribosomal subunit (Pongs et al., 1974). Two molecules of GTP are hydrolyzed for each amino acid incorporated during polypeptide chain elongation in E. coli (Cabrer et al., 1976).

Hybrid systems of elongation factors and ribosomes from prokaryotic sources are active in protein synthesis in vitro, as are hybrid systems from different eukaryotic sources (Modolell and Vazquez, 1975). However, the only interchangeability between prokaryotic and eukaryotic systems reported is the substitution of prokaryotic EF-Tu-Ts for EF-1 on eukaryotic ribosomes (Krisko et al., 1969).

B. Elongation Factor 1 of Eukaryotes

In contrast to prokaryotic elongation factors Tu, Ts and G and eukaryotic EF-2, eukaryotic elongation factor 1 can exist in active forms of various molecular weights. For example, G-200 Sephadex chromatography of rat liver EF-1 produced three active peaks of different molecular weights: the largest form eluted with the void volume, while the intermediate and small forms eluted in fractions corresponding

to molecular weights of 300,000 and 100,000 respectively (Schneir and Moldave, 1968). All three forms appeared to have similar functional capacity in the polymerization assay system (Schneir and Moldave, 1968). A more extensive purification of rat liver EF-1 was achieved by a combination of chromatography (Sepharose 6B followed by hydroxylapatite) and isoelectric focusing, but a homogeneous active form was not obtained because of the generation of smaller forms at each step of the purification (Collins et al., 1972). When the large form of rat liver EF-1 from 6B Sepharose chromatography was subjected to sucrose gradient centrifugation, two active species of molecular weights 400,000 and 170,000 daltons were observed (Collins et al., 1972). When rat liver EF-1 was purified 50-fold with a 1% yield from the isoelectric focusing step, it still showed a heterogeneous distribution (60,000-170,000 daltons) of active species on sucrose gradients (Collins et al., 1972).

McKeehan and Hardesty (1969) purified rabbit reticulocyte EF-1 500-fold with a 9% yield by a combination of 4B Sepharose and hydroxylapatite chromatography; the homogeneous enzyme was a single active species of molecular weight 186,000 daltons which could be dissociated under denaturing conditions into inactive subunits of 62,000 daltons. The purified enzyme was functionally similar to rat liver EF-1 by the following criteria: catalyzed both the binding of phe-tRNA to poly(U)-saturated ribosomes and the ribosome-aminoacyl-tRNA-dependent

hydrolysis of GTP and also complemented EF-2 in polypeptide synthesis (Lin et al., 1969). Evidence that the enzymatic binding, GTP hydrolysis and polymerization activities were linked enzymatic functions of EF-1 was provided by heat stability studies. When the enzyme was heated 5 minutes at 50^{o} with GTP, all three activities were essentially destroyed; whereas, when heated in the presence of both GTP and aminoacyl-tRNA, the three activities were only decreased 10-20% (Lin et al., 1969).

Sucrose gradient centrifugation of a calf brain post-mitochondrial supernatant fraction gave a nearly continuous profile of EF-1 activity; active species showed a molecular weight range from 60,000 to more than 1 million daltons (Moon et al., 1973). Both a heavy ($EF\text{-}1_H$) and light ($EF\text{-}1_L$) form were purified 50-fold by a combination of 6B Sepharose and hydroxylapatite chromatography followed by ammonium sulfate fractionation; the recovery of activity was 3% for $EF\text{-}1_L$ and 2% for $EF\text{-}1_H$. $EF\text{-}1_H$ showed a heterogeneous distribution of active species from 250,000 to over 1 million daltons while $EF\text{-}1_L$ gave one major peak at 60,000 daltons (Moon et al., 1973).

When partially purified EF-1 from Krebs II mouse ascites cells was chromatographed on Biogel A-5m, EF-1 activity was found in many peaks corresponding to estimated molecular weights of 90,000, 130,000, 190,000, 230,000 and 280,000 daltons (Drews et al., 1974). The 250,000-280,000 molecular weight

fraction was further purified by hydroxylapatite and sucrose gradient electrophoresis to achieve a total purification of 600-fold with a 5% yield. This highly purified enzyme had an apparent molecular weight of 190,000 daltons from its elution profile on Biogel A-5m, but it gave a single polypeptide chain of 47,000 daltons on SDS polyacrylamide gel electrophoresis. The higher molecular weight forms of EF-1 thus appeared to be aggregates of the 47,000 dalton subunit.

Wheat embryo EF-1_H was not a simple aggregate of the EF-1_L form. Homogeneous wheat embryo EF-1_H (purified 200-fold) was partially converted to EF-1_L by incubation with GTP and phe-tRNA (Lanzani et al., 1974). Bollini et al. (1974) then isolated EF-1_L from the ternary complex. The molecular weights of the two forms as determined by low speed sedimentation equilibrium studies were 187,000 daltons for EF-1_H and 51,000 daltons for EF-1_L (Bollini et al., 1974). When the purified wheat embryo EF-1_H and the EF-1_L prepared from the ternary complex were subjected to SDS polyacrylamide gel electrophoresis, EF-1_H was shown to contain three polypeptides (A, B and C of molecular weights 52,000, 47,000 and 27,000 daltons respectively) while EF-1_L contained only polypeptide A (Bollini et al., 1974).

In the pig liver system, Iwasaki et al. (1973) separated EF-1 into two complementary factors, EF-1α and EF-1β. EF-1β had no binding activity alone and was not labile to GTP-stimulated heat inactivation; EF-1α was shown to catalyze the binding of

phe-tRNA to poly(U)-saturated Artemia ribosomes, to be rapidly inactivated by heat in the presence of GTP, and to be protected from this inactivation by phe-tRNA (Iwasaki et al., 1973). The addition of EF-1β significantly stimulated the activity of EF-1α in both the binding and polymerization assay systems (Iwasaki et al., 1976). EF-1β (purified 50-fold) was shown to consist of two polypeptides of 55,000 and 30,000 daltons by SDS gel electrophoresis (Iwasaki et al., 1976). On the other hand, EF-1α at a 450-fold purification was shown to be a single polypeptide of 53,000 daltons on SDS gels (Iwasaki et al., 1974).

It would therefore appear that pig liver EF-1 has been separated into an unstable binding component comparable in size and function to prokaryotic EF-Tu and a heat stable stimulatory factor possibly comparable to prokaryotic EF-Ts. Similar results have been found with rabbit reticulocyte EF-1. Homogeneous rabbit reticulocyte EF-1 was reacted with GTP and phe-tRNA and subsequently passed through millipore filters; the filtrate was highly unstable, retaining only 15-20% of the initial polymerization activity (Prather et al., 1974). The activity of the filtrate could be restored to 60% of the initial level by the addition of heat inactivated EF-1. SDS gels revealed that the factor in the filtrate had a polypeptide chain weight of 50,000 daltons (Prather et al., 1974).

In addition to differences in polypeptide composition of EF-1 forms in the wheat embryo and pig liver systems discussed above, a difference in the lipid content of high and low molecular weight forms of eukaryotic EF-1 has been found in some systems. In the calf brain system, $EF\text{-}1_H$

contained 30 times more cholesterol than EF-1_L per unit of EF-1 activity (Moon et al., 1973). A comparison of phospholipid and cholesterol content of EF-1_H from calf brain and calf liver at similar specific activities revealed that the calf liver enzyme had about 4 times more phospholipid but only one-half the total cholesterol content of EF-1_H from calf brain (Legocki et al., 1974a). In both cases, however, EF-1_H forms had approximately 10 times the phospholipid content of their respective EF-1_L forms (Legocki et al., 1974a). Moreover, after calf brain EF-1_H was treated with phospholipase AB, sucrose density gradient activity profiles showed that EF-1_H had actually been converted to EF-1_L by the phospholipase treatment (Legocki et al., 1974a). Redfield et al. (1975) were able to reconstitute a substantial amount of calf brain EF-1_H from the reaction of EF-1_L with egg yolk lecithin.

With respect to the function of EF-1_H and EF-1_L in eukaryotic systems, EF-1_L is the form of the enzyme involved in the formation of the ternary complex (aa-tRNA-EF1-GTP). Using a Sephadex G-150 gel filtration procedure, EF-1_H (greater than 150,000 daltons) from calf brain was shown to react with ^{3}H-GTP to form a binary complex but only EF-1_L (60,000 to 80,000 daltons) reacted with both ^{3}H-GTP and ^{14}C-phe-tRNA to form a ternary complex (Moon et al., 1972). Similar results were obtained with wheat embryo EF-1 (Lanzani et al., 1974). Pig liver EF-1 λ (53,000 daltons) was reacted with GTP and phe-tRNA and the ternary complex which was isolated from

G-75 Sephadex contained EF-1α, GTP and phe-tRNA in a molar ratio of 1:1:1 (Nagata et al., 1976).

A difference in reactivity between EF-1_H and EF-1_L has been noted between the amount of GTP bound to EF-1 in a binary complex and the amount of phe-tRNA transferred to the ribosomes per mg EF-1 protein. Bollini et al. (1974) found the ratios of picomoles of phe-tRNA bound to ribosomes to picomoles of GTP bound to EF-1 were 14.6 and about 1.0 for wheat embryo EF-1_H and EF-1_L respectively. In the calf brain system, the ratios of picomoles of GTP bound to EF-1 to picomoles phe-tRNA transferred to ribosomes per mg EF-1 protein were 0.1-0.3 for EF-1_H and about 1.0 for EF-1_L (Moon et al., 1973; Legocki et al., 1974b).

This difference in reactivity between EF-1_H and EF-1_L could be viewed in two ways. EF-1_H may be able to recycle after binding one phe-tRNA molecule to the ribosome and therefore act catalytically in aminoacyl-tRNA binding, whereas EF-1_L acts stoichiometrically. An alternative explanation is that the aminoacyl-tRNA binding activity of EF-1_L is more subject to inactivation than is its GTP binding activity.

In summary, the work of other laboratories has shown eukaryotic EF-1 to be a very complex system indeed. It apparently contains two types of activities paralleling the bacterial elongation factors EF-Tu and EF-Ts, but even qualitative description has proven to be extremely difficult in eukaryotic preparations. The lability of the enzyme,

particularly the lower molecular weight forms, hinders purification procedures. Association of the basic molecule, probably the 50,000 to 60,000 dalton form, into very high molecular weight aggregates is found in preparations from many types of tissue including liver. Lipids found in the high molecular weight forms may have some functional significance as yet unknown. In spite of the difficulties of working with such a complex system, its importance as a possible major regulatory center necessitates its thorough examination.

C. Regulatory Role of Elongation Factors

1. Prokaryotic

Studies on *E. coli* have been carried out by various investigators in an effort to determine the amount of elongation factors present in the cell and whether the synthesis of ribosomes and elongation factors is coordinated.

Leder *et al.* (1969) reported that EF-G comprised 2-3% of the soluble *E. coli* protein. Gordon (1970) measured the amounts of EF-T, EF-G and ribosomes in *E. coli* at different steady state growth rates in various media. Immunochemical assays indicated that approximately one mole of each factor was present per mole of ribosomes. With respect to EF-T, this observation was subsequently restricted to equimolar amounts of EF-Ts and ribosomes when the anti-T serum was found to be specific for EF-Ts (Gordon and Weissbach, 1970).

Furano (1975) assayed the EF-Tu content of *E. coli* by

both radioimmune and GDP binding assays and found that EF-Tu comprised 6% of the S-100 protein. With respect to the EF-Tu/ribosome ratio as a function of growth rate of the cells, at 2 doublings per hour there were 8 EF-Tu molecules per ribosome and at 0.22 doublings per hour there were 14 EF-Tu per ribosome (Furano, 1975). While the ratio of EF-Tu to ribosomes correlated with different growth rates, EF-Tu did not show the equimolar ratio to ribosomes characteristic of both EF-Ts and EF-G (Gordon and Weissbach, 1970).

Similar amounts of EF-Tu were determined by Jacobson and Rosenbusch (1976); EF-Tu made up 5% of the total protein in E. coli, and the ratio of EF-Tu to EF-Ts was approximately 4:1. EF-Tu was identified as a major protein component of the plasma membrane, since 50-80% of the EF-Tu present in the cell could be released by osmotic shock (Jacobson and Rosenbusch, 1976).

The question of the control of the synthesis of elongation factors and ribosomes by the stringest control system (rel gene) has been explored in E. coli. The synthesis of both ribosomal RNA and ribosomal proteins was shown to be under stringest control (Dennis and Nomura, 1974). Furano and Wittel (1976) demonstrated that the synthesis of elongation factors EF-Tu and EF-G as well as stable RNA was subject to stringent control.

Two copies of the structural gene for EF-Tu have been located on the haploid E. coli chromosome (Jaskunas et al., 1975).

One EF-Tu gene was in the same transcriptional unit as the gene for EF-G and some ribosomal proteins; the second EF-Tu gene was in a cluster with genes for ribosomal RNA, ribosomal proteins and the B,B' subunits of RNA polymerase (Jaskunas et al., 1975).

The coordinated synthesis of elongation factors, ribosomal RNA and ribosomal proteins may thus reflect their location on the same transcriptional units on the E. coli chromosome. However, Carpenter and Sells (1973) observed that the synthesis of elongation factors and ribosomes was not coordinated during the first 20 min after a nutritional shift-up of E. coli from acetate to enriched medium, although the increase in elongation factors parallelled the increase in ribosomes at later times. Also, while two copies of the EF-Tu gene have been found, both are in clusters with ribosomal protein genes. Thus, the existence of two copies of the EF-Tu gene does not necessarily account for the large molar excess of EF-Tu over the ribosomes.

The E. coli studies indicate that elongation factors constitute about 10% of the cellular protein. The elongation factors and ribosomes in general appear to be synthesized in a coordinate manner, and no evidence of protein synthetic control by the factors has been obtained. It is, however, not clear to date why the level of EF-Tu is much higher than that of EF-Ts and EF-G.

2. Eukaryotic

Control of protein synthesis at the translational level by eukaryotic EF-1 has been suggested by several *in vitro* studies. EF-1 was found to be the limiting factor *in vitro* in pH 5.2 supernatants from rat brain, liver and kidney by binding and polymerization assays (Girgis and Nicholls, 1972). The addition of partially purified EF-1, but not EF-2, stimulated the binding and polymerization activity of liver and brain pH 5 supernatants 2-3 fold and of a kidney supernatant 5-6 fold (Girgis and Nicholls, 1972). Girgis and Nicholls (1971) had previously demonstrated that pH 5.0 supernatants from kidney of nephrotic rats had twice the EF-1 activity of that from control animals in both binding and polymerization assays. The pH 5.2 supernatants from livers of laser-irradiated rats were also shown to have twice the EF-1 activity as those from control rats in both assays (Nicholls, 1973). Willis and Starr (1971) reported that N-ethylmaleimide-treated pH 5.2 supernatants from spleen of control rats had only 40% of the EF-1 binding activity of that from immunized rats.

Nephrotic kidney, laser-irradiated liver, and spleen during the immune response are examples of tissues undergoing rapid growth which may be accompanied by increased protein synthesis. However, actual measurements of elongation rate *in vivo* were not done in conjuction with measurements of EF-1 activity levels in these studies. Moreover, a certain degree of caution must be exercised in the interpretation of EF-1

activity assays of pH 5 supernatants as representing the amount of cytosol enzyme. Hubert et al. (1974) suggested that the amount of cytosol EF-1 precipitated at pH 5 was influenced by the amount of charged-tRNA available to co-precipitate with EF-1. In this laboratory, serious losses of both rat and toadfish liver EF-1 activities have resulted from precipitation in the pH 5.0-5.2 range.

In an effort to correlate the levels of eukaryotic elongation factors with that of ribosomes under varying metabolic conditions, Gill and Dinius (1973) quantitatively assayed EF-2 from rat tissue homogenates by the diptheria toxin catalyzed ADP-ribosylation reaction. The EF-2 to ribosome ratio was found to be essentially constant in the various organs tested from rats; the average ratio was 1.2 molecules of EF-2 per ribosome (Gill and Dinius, 1973). This EF-2 assay was used by Alexis et al. (1974) who demonstrated that the concentration of both EF-2 and ribosomes decreased about 30% in muscle from rats fed a protein deficient diet for 6-10 days. The ratio of EF-2 to ribosomes, however, remained constant in different nutritional states. It was therefore concluded that the 50% decrease in in vivo protein synthesis found in muscle of rats fed a low protein diet was not due to a deficiency of EF-2 (Alexis et al., 1974).

Smith et al. (1976) have investigated the coordinate synthesis of elongation factors and ribosomes during a metabolic shift-up in protein synthesis in immature chick liver following

estradiol treatment. The amount of EF-2 was determined by the ADP-ribosylation assay and the amount of ribosomes quantitated by an analysis of the amounts of 18S and 28S rRNA; the average ratio of EF-2 to ribosomes was found to be 1:1 (Smith et al., 1976). Activity assays of EF-1 coupled with the above-mentioned measurements of EF-2 and ribosomes indicated that the synthesis of EF-1, EF-2 and ribosomes increased coordinately (Smith et al., 1976).

A correlation between EF-1 activity levels and protein synthetic capacity has been shown during a nutritional shift-down of Vero M_3 cells (Hassell and Engelhardt, 1976). Serum was removed from growing cultures of Vero M_3 cells; at intervals after serum deprivation, the cells were labelled for 60 min with ^{14}C-leucine, and the amount of radioactive protein was measured (Hassell and Engelhardt, 1973). After 10 hours of serum deprivation, the amount of ^{14}C-leucine incorporated into protein was decreased to 35% of the control level. The polysome profiles were similar for control and serum-deprived cells which suggested that the loss of protein synthetic capacity in the latter was due to a decrease in peptide elongation (Hassell and Engelhardt, 1973). EF-1 binding activity of post-ribosomal supernatants from cells deprived of serum for 20 hours was reduced to 42% of the value for control S-100 (Hassell and Engelhardt, 1976).

In summary, a variety of results suggest a possible role for EF-1, but not EF-2, in the control of protein synthesis in eukaryotes. The principal sources of uncertainty are the

absence of elongation rate data *in vivo* for most systems studied and the difficulty in obtaining reliable assays of tissue EF-1 concentrations, particularly in those cell fractions whose preparations are known to cause losses of activity. The present study is concerned with the characterization and quantitative assay of EF-1 in two systems for which the needed *in vivo* data are available.

D. Control of Liver Protein Synthesis in vivo by Elongation Rate

1. Changes in Elongation Rate Associated with Temperature Acclimation in Toadfish

a. Physiology of Temperature Acclimation

Many species of poikilothermic ("cold-blooded") organisms undergo metabolic adaptation in order to compensate for the direct effect of temperature on biochemical reaction rates (Bullock, 1955; Prosser, 1962). In fish, adaptation to lower temperatures can be conveniently assayed by measurements of oxygen consumption. Kanungo and Prosser (1959) reported that goldfish acclimated to 10°C showed a 50% increase in oxygen consumption compared to 30°-acclimated fish when both were measured at 20 or 25°. Salmon acclimated to 6° consumed oxygen 55% faster than 18°-acclimated fish when both were measured at the lower temperature (Peterson and Anderson, 1969). Roberts (1967) found that sunfish acclimated to the temperature range of 10° to 20° increased oxygen consumption sufficiently at the lower temperature to almost completely compensate for the temperature effect on metabolism.

The activity of a number of enzymes has been shown to change in various fish species at different acclimation temperatures (Prosser, 1967). Levels of succinic dehydrogenase activity, an oxidative enzyme, were found to be 1.3-2.2 times higher in muscle from 5°-acclimated goldfish than from 25°-acclimated fish (Hazel, 1972). Freed (1965) reported the activity of cytochrome oxidase, another oxidative enzyme, was 3-5 times greater in muscle from 5°-acclimated goldfish compared to 30°-acclimated fish. The activity of a key glycolytic enzyme, aldolase, was 50% higher in gill from 5°-acclimated carp than 25°-acclimated fish (Ekberg, 1962). The activity of the hexose monophosphate shunt enzyme, 6-phosphogluconic dehydrogenase, was 134% greater in carp gill from 5° compared to 25°-acclimated fish (Ekberg, 1962). The variety of enzyme changes associated with cold acclimation has suggested a role for protein synthesis in the process.

b. Protein Synthesis Studies

Toadfish (Opsanus tau), a marine fish of wide distribution along the Atlantic coast, show typical adaptation to cold, as evidenced by behavior and oxygen consumption (Haschemeyer, 1969a). The increase in metabolic rate could be correlated with an apparent increase in protein synthetic capacity of the liver under the same conditions (Haschemeyer, 1968). Liver protein synthetic capacity was measured in the cold and warm acclimated fish in the following manner: ^{14}C amino acids were injected into an artery of the gill arch; incubation was continued

in vivo at 23°C for 30 minutes; and the amount of the injected ^{14}C amino acids incorporated into total liver protein was determined (Haschemeyer, 1968). The 10°-acclimated fish showed a 75% increase in the incorporation of ^{14}C amino acids into liver protein as compared to the control fish acclimated at 20°-23° when both were measured at 23°C. These studies, however, did not yield true rates of protein synthesis since incorporation was measured at only one incubation time (30 min).

A method has been developed to measure the rate of polypeptide chain assembly or elongation rate in vivo based on a translational control model in which the ratio of incorporation of injected amono acids into soluble protein (S) to that in total protein (T) is analyzed as a function of incubation time (Haschemeyer, 1969b). The salient features of the rapid kinetic technique are as follows: (1) injection of radioactive amino acids by the hepatic portal vein which results in rapid uptake by the liver and facilitates the determination of a zero time; (2) incubation times in vivo of between 1 and 6 minutes to obtain timepoints at one round or less of polypeptide synthesis; (3) rapid homogenization of liver at the desired incubation time to give an incorporation value for the total homogenate (T); (4) centrifugation of the homogenate at 100,000 x g, after sodium deoxycholate treatment, to yield an incorporation value for released chains (S) and for ribosomal bound chains (R). The average polypeptide chain assembly time (t_c) was determined from a plot of S/T vs

incubation time since a linear relationship exists between these parameters up to the time for the completion of one round of protein synthesis.

This technique revealed a change in elongation rate in liver protein synthesis associated with temperature acclimation of toadfish. The average time for the assembly and release of completed polypeptide chains (t_c) was 3.9 minutes in 10°C-acclimated fish and 6.6 minutes in 21°C-acclimated fish when both were measured at 21°C. These values represent elongation rates of 1.7 and 1.0 amino acid residues per second per ribosome in cold- and warm-acclimated fish respectively. Cold-acclimated fish thus show a 70% increase in elongation rate; this change is comparable to the overall increase in liver protein synthesis as measured by amino acid incorporation (Haschemeyer, 1969b).

<u>In vitro</u> studies of protein synthesis in toadfish liver were undertaken to identify the rate-limiting component responsible for the increase of protein synthetic rate in cold acclimation (Haschemeyer, 1969c). The control point did not appear to be the number of active ribosomes since 70% of total RNA in control fish liver was already found in the polysome region of sucrose density gradient profiles. No difference was found in the activity of aminoacyl-tRNA synthetases between the temperature acclimation groups, so the control point did not appear to be at the level of charged transfer RNA. Post-ribosomal supernatants (S-100) prepared from fish acclimated at 10°C and 21°C were tested for stimulation of

polyphenylalanine synthesis in a polymerization assay using poly(U)-saturated toadfish liver ribosomes; the S-100 from 10^{o}-acclimated fish showed an approximate 60% greater activity for polyphenylalanine synthesis over the level of the S-100 from 21^{o}-acclimated fish (Haschemeyer, 1969c). This correlation thus suggested a role for elongation factors in the acclimation process, although the particular factor responsible for the effect was not identified.

2. Changes in Elongation Rate Associated with Thyroid Hormone State in Rat

Studies, parallel to those in toadfish, have been carried out on protein synthetic rates in rat liver in vivo in relation to thyroid state. A number of similarities exist between the effects of thyroid hormone in mammals and of adaptation to cold temperature in fish. In addition to changes of basal metabolic rate, a variety of enzymes are affected by thyroid hormone levels in rat. Upon thyroxine administration liver mitochondrial α-glycerolphosphate dehydrogenase activity increased 10- and 30-fold in intact and thyroidectomized rats, respectively (Ruegamer et al., 1964). The activity of cytoplasmic α-glycerolphosphate dehydrogenase did not change with thyroid state (Colton et al., 1972). Activities of two key enzymes in gluconeogenesis increased upon 3,3',5-triiodothyronine treatment: pyruvate carboxylase increased 6-fold and phosphoenolpyruvate carboxykinase 3-fold (Bottger et al., 1970). Among other gluconeogenic enzymes, glucose-6-phosphatase increased 2-fold upon thyroxine admini-

stration (Freedland, 1965) while fructose 1,6-diphosphatase activity did not change (Colton et al., 1972). Two hexose monophosphate shunt enzymes, glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase, showed about a 2-fold increase in activity in hyperthyroid rats (Freedland, 1965). Increased activity of 4- to 6-fold in hyperthyroid rats was also shown for malic enzyme, a lipogenic ensyme (Freedland, 1965; Colton et al., 1972).

In an effort to relate thyroid hormone action to overall protein synthetic rate in rat liver, polypeptide chain assembly time (t_c) has been determined in rat liver in vivo using an experimental procedure similar to that developed for toadfish. A direct correlation was found between the rate of liver protein synthesis in vivo and the thyroid hormone state of the animals (Mathews et al., 1973). The average time for the assembly and release of completed peptide chains was 1.92 min for thyroidectomized rats, 1.16 min for euthyroid and 0.92 min for T3-treated rats. This represented a decrease in elongation rate of 39% in hypothyroid rats and an increase of 27% in hyperthyroid rats compared to euthyroid controls.

E. Rationale of Thesis

The two systems described above provide evidence of the regulation of protein synthesis in vivo by changes in polypeptide elongation rate. This thesis is concerned with the proposition that elongation factor 1 (EF-1) is the

controlling factor in both of these responses. The following specific questions have been investigated:

1. Are protein synthetic changes that occur with temperature acclimation in toadfish correlated with tissue concentrations of EF-1?

2. How do the properties of toadfish liver EF-1, i.e. molecular weight distribution and activities in binding and polymerization assays, compare with other eukaryotic EF-1's?

3. Are the changes in elongation rate in rat liver in response to thyroid hormone correlated with levels of liver EF-1?

4. How do rat and toadfish liver EF-1 compare qualitatively (characteristics of the binding activity, temperature dependency <u>in vitro</u>) and quantitatively (tissue concentrations relative to elongation rate <u>in vivo</u>)?

II. MATERIALS AND METHODS

A. Animals

Adult toadfish, Opsanus tau, of both sexes, 200-300 g, caught in late spring, were obtained from the supply department at the Marine Biological Laboratory, Woods Hole. The toadfish were maintained during summer in running seawater tanks at ambient seawater temperature (20-22°C) or at 10-11° using a Neslab heat exchanger. Two weeks were allowed for temperature acclimation. Winter toadfish caught in November were maintained during December-January at ambient temperature (7-8°) or were acclimated to 25° in a recirculating system. Killifish were provided for ad lib feeding.

Adult, male, Long-Evans rats, bred from stock kept at Hunter College, were used throughout. Control and T3-treated rats were fed Purina laboratory chow; thyroidectomized rats received the iodine-deficient diet, TD-70283 (General Biochemicals). All the rats were kept at normal building temperature and outdoor light cycles. Tap water was given ad lib. Animals 2 to 2½ months of age were sacrificed at 10-11 a.m. As indicated in particular experiments, the rats were either fed until the time of sacrifice or starved overnight (18 hours). Experimental animals were matched with controls of the same age. At 30 days of age, surgical thyroparathyroidectomy was performed by Dr. Rita W. Mathews according to Mathews et al. (1973). T3-treated normal rats

received injections of 3,3',5-L-triiodothyronine (Calbiochem) at a dosage of 40 µg/100 g of body weight (Mathews et al., 1973). Animals received subcutaneous injections daily for three consecutive days prior to sacrifice. Control rats were given subcutaneous injections of 0.9% NaCl.

B. Preparations

1. S-100 Preparation

Fish were stunned and livers quickly excised and homogenized using a loose-fitting Dounce homogenizer in 2 volumes of cold Medium A (0.25 M sucrose, 0.05 M Tris-HCl (pH 7.4), 0.025 M KCl, 0.01 M $MgCl_2$, 2 mM 2-mercaptoethanol). The homogenates were centrifuged at 15,000 x g for 10 min at 4°C to yield a post-mitochondrial supernatant (S-15) and at 100,000 x g for 90 min to obtain the post-ribosomal supernatant (S-100). Small aliquots were frozen and stored in liquid nitrogen. Rat liver S-100 was prepared similarly except that 3 instead of 2 volumes of Medium A were used for homogenization. The EF-1 activities of both preparations were stable during several years of storage in liquid nitrogen. Protein concentrations as determined by weight (Haschemeyer, 1968) and by the method of Schaffner and Weissmann (1973) were about 13 mg/ml for toadfish S-100 and 26 mg/ml for rat S-100. Since a modification of the Biuret procedure described under Other Determinations gave comparable protein values for rat S-100, it was routinely used for this determination.

2. Rat Liver ^{3}H-phe-tRNA

Rat liver transfer RNA prepared according to Petrissant et al. (1971) was charged in the following mixture: 100 mM Tris-HCl (pH 8.1); 10 mM $MgCl_2$; 0.5 mM EDTA (adjusted to pH 7); 10 mM ATP; 10 mM KCl; 8 mg/ml tRNA; 9 mg/ml rat liver pH 5 enzyme protein (Haschemeyer, 1969c); 20 μM ^{3}H-phenylalanine (2 mCi/μmole). After 20 min at 30°, the charged-tRNA was recovered from the aqueous phase by phenol extraction at 4°C. The aqueous phase was adjusted to 2% potassium acetate (pH 5.0) and treated with 2 volumes of 95% ethanol. The precipitate collected after 18 hr at -20° was dissolved in a solution that was 10 mM potassium acetate buffer (pH 5.6), and 1 mM $MgCl_2$, chromatographed on Sephadex G-25 in the same solution, and stored in liquid nitrogen. The final product had an A_{260}/A_{280} ratio of 2 and a specific activity of 900 cpm/picomole.

3. Rat Liver Ribosomes, Puromycin-treated

Freshly excised livers of overnight starved Long-Evans rats were minced and Dounce homogenized with 3 volumes cold Medium A' (0.25 M sucrose, 0.05 M Tris-HCl (pH 7.5), 0.01 M KCl, 5 mM $MgCl_2$, 2 mM 2-mercaptoethanol). The supernatant obtained after centrifugation at 15,000 x g for 10 min in a Sorvall refrigerated centrifuge was filtered through 4 layers of gauze and brought to 1% sodium deoxycholate. The mixture was stirred for 10 min at 4° and 10 min at 37° for release of elongation factors (McKeehan and Hardesty, 1969) and centrifuged

at 100,000 x g for 15 min for removal of glycogen. The supernatant was clarified by filtration through Whatman No. 1 paper and centrifuged at 100,000 x g for 2 hr. The ribosomal pellets were rinsed, resuspended in Medium A' and treated with 1 mM puromycin (Calbiochem) at 37° for 30 min in order to release endogenous mRNA and peptidyl-tRNA (Siler and Moldave, 1969). The mixture was centrifuged at 18,000 x g for 10 min to remove denatured material, chromatographed on Sephadex G-25 in Medium A' for elimination of puromycin, and stored in liquid nitrogen at a ribosome concentration of 100 A_{260} units/ml. Yield was 1.5-2.0 mg RNA/g liver.

4. Rat and Toadfish Liver Ribosomes, EF-2 Saturated

Post-mitochondrial supernatants (see S-100 preparation) were brought to 0.25% sodium deoxycholate, centrifuged 15 min at 100,000 x g for glycogen removal, and layered over 5 ml aliquots of Medium A containing 2 M sucose. The ribosomes were pelleted through the 2 M sucrose layer by centrifugation at 130,000 x g for 5 hr (4°C) in a Spinco 40 rotor. The tops of the tubes were cut away to avoid supernatant contamination and the ribosomes were rinsed and resuspended in Medium A containing 2 mM 2-mercaptoethanol by gentle Dounce homogenization. Aliquots at a concentration of 50 A_{260} units/ml were stored in liquid nitrogen.

C. Assays

1. ^{3}H-phe-tRNA Binding to Puromycin-treated Rat Ribosomes

The EF-1-dependent binding of aminoacyl-tRNA to ribosomes (McKeehan and Hardesty, 1969) was determined in the following freshly prepared medium: 30 mM Tris-HCl (pH 7.5); 120 mM NH_4Cl; 5 mM $MgCl_2$; 4 mM 2-mercaptoethanol; 25 µg polyuridylic acid (Miles Laboratories); 0.4 mM GTP; 1.6-2.0 A_{260} units (30 pmoles) of puromycin-treated rat liver ribosomes; 18 pmoles rat liver ^{3}H-phe-tRNA (about 16,000 cpm); and enzyme (10-100 µg/ml S-100 protein). The reaction volume was 0.12 ml; under standard assay conditions the reaction was carried out for 5 min at 30°. The reaction was terminated by rapid addition of 3 ml cold buffered salts solution (BSS) containing 80 mM NH_4Cl, 30 mM Tris-HCl (pH 7.5), and 5 mM $MgCl_2$, and the product collected under suction on a nitrocellulose filter (Millipore 0.45 µ pore size), (Nirenberg and Leder, 1964) that had been pre-washed with 0.1 N NaOH and stored in BSS. Filters were washed with an additonal 6-7 ml of BSS and dried at 50° for 30 min. Radioactivity was determined in a Packard scintillation spectrometer with toluene-based scintillation fluid; ^{3}H efficiency on filters was 23%. Preparations were tested for sulfhydryl group inhibition by pre-treatment with N-ethylmaleimide (McKeehan and Hardesty, 1969). Pre-treatment of enzyme preparations with 10 mM N-ethylmaleimide gave maximal inhibition (70-80%) of the EF-2-dependent polymerization assay. Heat inactivation of EF-1 binding activity was carried out following Iwasaki et al. (1973).

2. Polyphenylalanine Synthesis (Polymerization) Using EF-2 Saturated Toadfish Ribosomes

The EF-1-dependent formation of polyphenylalanine from ^{3}H-phe-tRNA in the presence of EF-2-saturated toadfish ribosomes and poly(U) was assayed in the following medium: 40 mM Tris-HCl (pH 7.5); 80 mM NH_4Cl; 4.2 mM $MgCl_2$; 10 mM 2-mercaptoethanol; or 4 mM dithiothreitol; 0.4 mM GTP; 25 µg poly(U); 0.5 A_{260} units of toadfish ribosomes; 16 pmoles rat liver ^{3}H-phe-tRNA; and enzyme protein (40-200 µg/ml toadfish liver S-100 protein). Reaction volume was 0.12 ml. The mixture was brought to temperature (30°), enzyme and phe-tRNA added, and incubation carried out up to 30 min. The radioactivity incorporated from ^{3}H-phe-tRNA into polyphenylalanine was determined by hot trichloroacetic acid precipitation using filter paper discs as described by Haschemeyer (1968). ^{3}H efficiency on the discs was 23%.

3. Polyphenylalanine Synthesis (Polymerization) Using EF-2 Saturated Rat Ribosomes

The rat polymerization assay conditions were as indicated for the toadfish system except that the $MgCl_2$ optimum was 6 mM and 1 A_{260} unit of EF-2 saturated rat liver ribosomes was used per reaction. Rat liver enzyme concentration in the assay was about 30-120 µg/ml of S-100 protein or 50-350 µg/ml of pH 5 supernatant protein.

4. Polyphenylalanine Synthesis (Polymerization) Using Puromycin-treated Rat Ribosomes

The EF-1-dependent formation of polyphenylalanine from ^{3}H-phe-tRNA using puromycin-treated rat liver ribosomes in the presence of saturating amounts of exogenous EF-2 and poly(U) was assayed in the following medium: 50 mM Tris-HCl (pH 7.5); 80 mM NH_4Cl; 5.0 mM $MgCl_2$; 10 mM 2-mercaptoethanol; 0.4 mM GTP; 25 µg poly(U); 0.7 A_{260} units puromycin-treated rat liver ribosomes; 18 pmoles rat liver ^{3}H-phe-tRNA (about 16,000 cpm); saturating amounts of EF-2; and enzyme (20-40 µg/ml rat liver S-100 protein). Reaction mixtures were preincubated 10 min at 30° with saturating levels of partially purified EF-2 (Sutter and Moldave, 1966); enzyme and ^{3}H-phe-tRNA were added to start the polymerization reaction. Incubations were carried out at 30° for up to 15 min in a reaction volume of 0.11 ml. A range of S-100 protein concentrations between 10-50 µg/ml were tested in the polymerization reaction; the assay was linear with protein concentration between 20-40 µg/ml. The reaction was linear with time up to 15 min under these conditions.

The EF-2-dependent polymerization reaction was carried out in a similar system using puromycin-treated rat liver ribosomes and excess EF-1 (high molecular weight form). The latter enzyme and partially purified EF-2 were obtained from the 30-70% ammonium sulfate fraction of toadfish liver S-100 by chromatography on Sepharose 6B in the following medium:

0.05 M Tris-HCl (pH 7.5); 0.25 M KCl; 0.1 mM EDTA; 2 mM 2-mercaptoethanol; and 5% glycerol. The profile of toadfish EF-1 activity from Sepharose 6B was similar to that shown for rat liver EF-1 (Appendix Figure 4) with one important exception: no small form of toadfish EF-1 activity was recovered. Therefore, the partially purified EF-1 and EF-2 recovered from the column were not cross-contaminated and could be used as complementary factors in the polymerization assay with purified ribosomes.

The radioactivity incorporated into polyphenylalanine was determined by hot trichloroacetic acid precipitation using filter paper discs as described by Haschemeyer (1968). ^{3}H efficiency on the discs was 23%.

D. Toadfish EF-1 Purification

Toadfish liver S-100 was brought to pH 6.0 by addition of 1 N acetic acid with stirring for 10 min at 0^{o}. The supernatant recovered after centrifugation at 15,000 x g for 10 min read pH 5.8. It was immediately neutralized to pH 7.5 with 1 N NaOH and passed through Sephadex G-25 equilibrated with enzyme buffer (0.02 M Tris-HCl (pH 7.5); 0.05 M NH_4Cl; 5 mM 2-mercaptoethanol; 0.1 mM EDTA; 25% glycerol). The effluent (180 ml) was applied to a 2.5 x 40 cm CM-Sephadex column equilibrated with the same buffer and subjected to stepwise elution with NH_4Cl solutions following Iwasaki *et al.* (1974). The bulk of the activity eluted at 0.35 M NH_4Cl. For purification of the large form of the enzyme, the

0.35 M NH_4Cl pool was concentrated to one-third its volume in an Amicon ultrafiltration cell (PM 30 membrane) and 6 ml was chromatographed on a 2 x 60 cm Sepharose 6B column equilibrated and eluted with enzyme buffer containing 0.25 M NH_4Cl. Active fractions were pooled and frozen in liquid nitrogen. For purification of the small form, the 0.35 M NH_4Cl eluate was diluted to 0.14 M NH_4Cl (using enzyme buffer containing no NH_4Cl) and loaded on a second CM-Sephadex column. The latter was eluted with an ammonium chloride gradient (0.14 M to 0.50 M NH_4Cl) in enzyme buffer (Iwasaki et al., 1974). Contaminating EF-2 eluted early in the gradient well before EF-1. EF-1 fractions, freed of EF-2, were pooled from the second CM-Sephadex column and chromatographed on Sepharose 6B as described above; the low molecular weight activity eluted with the bulk of the protein. Aliquots frozen in enzyme buffer and stored in liquid nitrogen were stable for several months.

E. Preparation of Antibodies to Purified Large and Small EF-1

Salts, glycerol, and mercaptoethanol were removed from the 70-fold purified large and small toadfish liver EF-1 preparations by passage through a Sephadex G-25 column equilibrated and eluted with 0.05 M potassium phosphate buffer (pH 7.5). A 1.0 ml aliquot of the enzyme solution at 0.5 mg/ml was mixed with an equal volume of methylated serum albumin (Mandell and Hershey, 1960) at 0.5 mg/ml, homogenized with 1 ml of Freund's Complete Adjuvant (Calbiochem), and injected subcutaneously,

intramuscularly and into the footpads of a young, white, female, New Zealand rabbit. After 4 weekly injections, anti-EF-1 activity was found in serum collected from marginal ear veins. The titer was maintained by fortnightly injections; blood was collected 10 days after each injection. The serum was treated with an equal volume of 1% glycerol, 0.15 M NaCl, and then with saturated ammonium sulfate solution to 33% saturation. After 10 min at 0° the γ-globulins were centrifuged down, washed with 50% saturated ammonium sulfate, and dissolved in 1% glycerol-0.15 M NaCl. After overnight dialysis at 4° against the same medium, the protein solution was stored in small aliquots at -70°. Inhibition was tested by incubation for 20 min at 0° at concentrations up to 200 μg immunoglobulin protein/μg enzyme protein, prior to carrying out the standard binding assay. Preparations of γ-globulin from control rabbits had no effect on the assay system.

F. Other Determinations

Toadfish and rat liver homogenates were prepared and analyzed for nucleic acids and protein as described by Haschemeyer (1968). Total lipid analysis of toadfish and rat S-100 was carried out by the method of Getz and Bartley (1961). S-100 protein determinations by the standard Biuret procedure (Gornall et al., 1949) gave erroneously high values due to turbidity caused by the presence of lipid. Satisfactory results were obtained with rat liver S-100 by the following

modification of the Biuret procedure. After the tubes were brought to 4 ml by the standard procedure, 1 ml of n-heptane was added and mixed vigorously with a vortex mixer. The mixture was incubated at 37°C for about 4 min and the heptane layer containing the lipids was aspirated. After a total incubation time of 20 min at 37°C, the tubes were centrifuged 2 min at 600 x g and the residual heptane layer aspirated. The absorbance of the resulting clear solution was determined at 550 nm. Rat liver S-100 protein concentrations were routinely determined by this procedure. It was not satisfactory for fish liver S-100 protein determinations; substantial turbidity remained, even after repeated extractions with heptane, which resulted in erroneously high absorbance values at 550 nm.

III. TOADFISH RESULTS

A. Characteristics of EF-1 Binding Assay

The EF-1-dependent binding assay was carried out as described in Methods using puromycin-treated rat liver ribosomes saturated with poly(U). The dependency on toadfish liver S-100 protein concentration is shown in Figure 1. The reaction was linear with S-100 protein concentration over the range of 10-80 μg/ml. The EF-1-dependent binding of ^{3}H-phe-tRNA to poly(U)-ribosome complexes was linear with time for about 8 min at 30°. Pre-incubation with N-ethylmaleimide (NEM) to inhibit sulfhydryl-requiring proteins, e.g., EF-2, had no effect on the standard 5 min assay at the lower S-100 concentrations normally used. At higher S-100 concentrations and longer assay times, some inhibition was noted, indicating the possible involvement of EF-2 or other NEM-susceptible proteins.

Properties of the standard assay system (5 min at 30°) are summarized in Table 1. The presence of a messenger RNA [poly(U)] and GTP was required, as observed with EF-1 of other organisms. Addition of excess toadfish liver EF-2 or NEM had no effect on ^{3}H-phe-tRNA binding at 5 min. Chromatography of the reaction product after deacylation (Lin et al., 1969) showed less that 5% polymerized phenylalanine. Thus the assay appeared to be specific for EF-1-directed binding at the A site and did not include EF-2-dependent contributions (^{3}H-polyphenylalanine). Background incorporation (minus EF-1)

of the freshly made puromycin-treated rat liver ribosomes was 0.15 pmole increasing to about 0.3 pmole with storage. Without puromycin treatment of the ribosomes, endogenous mRNA background values [minus poly(U) binding] were unacceptably high.

Temperature dependency of the assay was substantial, with Q_{10} about 3 for 10^{o}-20^{o} and about 2 for 20^{o}-30^{o} (Figure 2). It is not clear which step in the binding process is primarily responsible for the temperature effect. In a similar assay pre-incubation of poly(U) with ribosomes or of EF-1 with GTP and ^{3}H-phe-tRNA had little or no effect on subsequent binding (Grasmuk et al., 1974); thus, the effect does not appear to involve rate limitation at these stages. Above 30^{o} a clear decrease of binding activity by toadfish S-100 was observed. Such lability at higher temperatures is characteristic of EF-1 binding activity in all organisms studied, particularly when aminoacyl-tRNA is absent. The present data suggest that the toadfish binding protein may be somewhat less stable above 30^{o} even in the presence of its substrate than the rat enzyme (Figure 6). A similar temperature dependency was observed with purified toadfish EF-1.

B. Partial Purification of Multiple Forms of Toadfish Liver EF-1

Purification of toadfish liver EF-1 was based on the method developed for pig liver by Iwasaki et al. (1973, 1974). A summary of the purification results are presented in Table 2. Acidification of the S-100 to pH 5.2, a step used for elimination of aminoacyl-tRNA synthetases, tRNA's and EF-2 (Iwasaki et al.,

1973), was not acceptable in the toadfish system due to serious losses of EF-1 in the precipitate. At pH 5.0, about 50% of EF-1 activity was precipitated of which very little was recoverable. Sucrose gradient analysis of the pH 5.0 supernatant showed a preferential loss of low molecular weight forms. Losses were reduced by limiting acidification to pH 5.8. Iwasaki et al. (1974) purified a low molecular weight (53,000 daltons) form of pig liver EF-1α 450-fold with a recovery of 26% by an aqueous two-phase system followed by two successive carboxymethyl-Sephadex C-50 chromatography steps. With toadfish liver EF-1, the recoveries of activity were unacceptably low when the enzyme was salted-out of the two-phase system with ammonium sulfate following Iwasaki et al. (1974). However, acidification of the S-100 to pH 5.8 followed by CM-Sephadex chromatography produced a 30-fold purification with a 35% recovery of the activity applied. The molecular weight distribution of EF-1 binding activity from the first CM-Sephadex column was similar to that of the unfractionated S-100 on sucrose density gradients. Large form EF-1 was purified from the CM-Sephadex eluate by Sepharose 6B chromatography. Since small form EF-1 and EF-2 elute in the same region of the Sepharose 6B profile, contaminating EF-2 was removed by a second CM-Sephadex column with gradient elution. The active fractions (freed of EF-2) from the latter column were then chromatographed on Sepharose 6B to separate the residual large form from the small form of the enzyme. The procedure gave a total purifi-

cation of 70-fold for both forms with recoveries of 2% for the large form and 9% for the small form.

In these steps the presence of 5 mM 2-mercaptoethanol and 25% (v/v) glycerol was essential for recovery and stability of the small form. Nagata et al. (1976) similarly observed that pig liver EF-1 α of low molecular weight was very unstable but could be protected by 25% glycerol, whereas the high molecular weight form was stable in buffers without glycerol. Both the large and small forms of toadfish liver EF-1 at a 70-fold purification were stable for months upon storage in 25% glycerol at either -20°C or in liquid nitrogen.

Earlier efforts at purification of toadfish liver EF-1 were severely impeded by the instability of the small form in the absence of 25% glycerol. Following the procedure developed for rat liver EF-1 by Collins et al. (1972), a 30-70% ammonium sulfate fraction of the S-100 was chromatographed on either 4B or 6B Sepharose in buffer containing 5% glycerol and 1 mM 2-mercaptoethanol. Based on large form recovery, toadfish EF-1 was purified about 10-fold by this step, but no small form activity was recovered. Hydroxylapatite chromatography of the large form of EF-1 from Sepharose 6B produced two active peaks at a purification of 30-fold and 60-fold respectively, relative to the S-100 (Nielsen and Haschemeyer, 1974). However, both hydroxylapatite fractions rapidly lost activity during storage in liquid nitrogen. Mixing of fractions did not improve the keeping quality or restore activity, thus these fractions did

not appear to be analogous to the α and β fractions of EF-1 reported by Iwasaki et al. (1973).

C. Comparison of EF-1 Activity of Toadfish Liver S-100 and Partially Purified Large and Small Forms

Sucrose density gradient centrifugation of toadfish liver supernatant (S-100) revealed a major band of EF-1 activity sedimenting at about 4-7S [Figure 3(a)]. EF-2 activity was also found at this position as was the bulk of the S-100 protein. A second broad band of EF-1 activity was observed in the range of 14-22S. With toadfish liver S-100 from 20°-acclimated fish, the EF-1 activity recovered from the gradient (80% of the applied activity) was about equally divided between these large and small forms. In addition, 20% of the activity applied to the gradient was pelleted under these conditions; shorter time runs indicated the activity sedimented at 35-40S, suggesting a molecular weight in excess of 1 million. Molecular weights were estimated by means of the following equation:

$$M = 6.6 \times 10^3 \, \underline{s}^{3/2}$$

This equation expresses the relationship between molecular weight and sedimentation coefficient (in Svedberg units) for spherical particles (Haschemeyer and Haschemeyer, 1973) averaged for several globular proteins (the same degree of hydration was assumed). This calculation, based on seven runs, indicated that the major components of large form EF-1 activity occur at about 700,000 $\pm$ 50,000; 540,000 $\pm$ 40,000; and 420,000 $\pm$ 40,000.

The partially purified large form of the enzyme showed a distribution of sizes [Figure 3(b)] like those observed in the unfractionated supernatant [Figure 3(a)]. Similar analysis of the small form of the enzyme, both in unfractionated S-100 and in the purified preparations [Figure 3(c)], indicated a broad, slowly-sedimenting peak in the range of 50,000 to 135,000 daltons.

Activities of the EF-1 preparations and unfractionated S-100 for formation of polyphenylalanine in a polymerization system containing EF-2-saturated toadfish ribosomes are given in Table 3. With enzyme and ^{3}H-phe-tRNA added at t = 0, a linear rate of incorporation was observed for 30 min at 30°, with linear dependency on amount of added enzyme up to 50% incorporation of the precursor. This system showed a sharp magnesium optimum at 4.2 mM, whereas the monovalent cation optimum was broad (70-140 mM NH_4Cl or KCl). Phosphoenolpyruvate and pyruvate kinase, which were reported to stimulate polymerization activity of rat liver EF-1 (Collins et al., 1972), showed a slight inhibitory effect under optimal conditions, but could reverse the inhibition caused by high magnesium (10 mM) in this system.

The results from the toadfish polymerization system in the presence of EF-2-saturated ribosomes indicate that both large and small toadfish liver EF-1 possess activity to promote the complete protein synthetic reaction, i.e., the formation of polypeptide. The ratios of polymerization to binding activity

are comparable for the purified forms and for the unfractionated S-100 preparations from both toadfish (20^{o}-acclimated fish) and rat (euthyroid). Specific activity in the binding assay was also similar for large and small EF-1 under the standard assay conditions. When rat liver ribosomes containing residual messenger RNA [which show high background values in the binding assay in the absence of poly(U)] were used for binding, however, small form binding activity doubled whereas large form activity was unchanged. When added to the standard assay system in which small and large binding activities are identical, these same ribosomes (0.5 A_{260} units) likewise stimulated small form activity 2-fold. This ribosome preparation showed the normal low level of activity in the absence of added EF-1, thus the effect was apparently not due to contaminating EF-1 binding activity. This suggests the possibility of a ribosome-associated factor capable of stimulating this system.

Binding activity of mixtures of large and small EF-1 was equal to the sum of the separate activities. When large EF-1 was heat-inactivated (Iwasaki et al., 1973) and added to small form EF-1, the binding activity was 20% higher than the sum of the separate assays. Although these experiments suggest a difference in the nature of large and small EF-1, e.g., the possibility that a heat stable factor which serves to stimulate binding activity may exist in the large EF-1 complex, no difference in response to antibody preparations

was noted. Inhibition of both large and small EF-1 binding activity was about 50-70% with antibody to small EF-1 and about 20% for antibody to large EF-1.

D. EF-1 Binding Activity of S-100 from Control and Cold-Acclimated Toadfish

Table 4 summarizes the results of assays of EF-1 binding activity in liver homogenates of summer and winter toadfish. The 70% increase in binding activity in summer cold-acclimated fish compared to summer controls parallels the change in S-100-stimulated polymerization activity previously observed with cold-acclimation (Haschemeyer, 1969c). Winter fish at ambient temperature of 7-8^{o} showed the highest activity (relative to liver weight) in any group although specific activity was reduced as a result of the higher protein content of winter S-100. A single group of ten winter fish acclimated to 25^{o} for 10 days showed one-half the EF-1 activity of winter cold-acclimated fish. Activity per gram liver of this group was similar to that of summer control (20^{o}-22^{o}-acclimated) fish.

Liver weight and composition data for the summer and winter toadfish used in this study are collected in Table 5. About 80% of summer fish were male. Liver to body weight increases over the course of the summer from about 2.5% to 3.3%, concomitant with increasing lipid deposition in liver and gonad development. No significant changes in protein synthetic parameters or in EF-1 binding activity, however, have been

observed during this period, and therefore summer data have been averaged. Similarly, no significant effects of sex on these parameters have been observed. In winter fish, gonad weight of females averaged 8% of body weight; liver to body weight was somewhat greater in females although individual variation was considerable. Protein content of liver was elevated about 20% in winter fish. Average RNA content was about the same as in summer fish and correlated with sex, as it does in late summer and early fall fish (Haschemeyer, 1968). Winter fish showed a slight decrease in liver to body weight ratio (L/BW) with increasing size; values for the larger fish used in most enzyme preparations were in the range of 2.4-2.7%. Nutrition strongly influenced this parameter in summer (e.g., starvation at 20^{o} for 1 week or at 10^{o} for 2-3 weeks reduced L/BW to about 2.2%). Since winter fish were less active in feeding than were summer cold-acclimated fish, an effect on liver size would be expected.

Sucrose density gradient analysis of EF-1 activity in S-100 of winter cold-acclimated fish showed a bimodal distribution of large and small forms [Figure 4(a)]. The proportion of total activity occurring in the low molecular weight species was consistently elevated relative to control preparations assayed with the same ribosome and ^{3}H-phe-tRNA preparations. The distribution of EF-1 activity in the S-100 of winter 7^{o}-acclimated toadfish was 28% large form and 52% small form (based on 80% recovery of applied activity). Summer cold-

acclimated supernatants showed the same distribution of EF-1 activity as winter cold-acclimated preparations. Winter fish acclimated to 25° showed a nearly equal distribution between large and small forms [Figure 4(b)] similar to that seen in summer controls [Figure 3(a)]. The bulk of total protein was recovered in the low molecular weight region in all supernatants examined.

Sedimentation coefficients and molecular weights determined on the basis of globular protein standards indicated a range of 400,000-700,000 daltons for large form activity in all fish groups. Small form activity for winter fish fell in a broad band peaking at about 100,000 daltons, with a leading edge extending into the region of 200,000 daltons.

Table 6 summarizes the distribution of EF-1 binding activity as the per cent of small form (50,000-200,000 daltons) relative to total activity applied to the gradients. These distributions were to some extent dependent on the ribosome preparation used. Several ribosome preparations showed stimulatory activity toward the small form of EF-1. Although this led to a shift in distribution in favor of small form activity in all S-100's, differences between temperature groups were maintained. The data of Table 6 are based on those ribosome preparations that were judged most free of endogenous components. These preparations gave a 40-40 distribution of large and small EF-1 binding activity with summer control supernatants. The distribution is based on 80% recovery of

applied activity. The difference in % small form between cold-acclimated and warm-acclimated preparations was 13 ± 7% (0.95 confidence interval).

Total lipid analyses of the various S-100's were carried out since Legocki et al. (1974a) detected lipid in high molecular weight forms of calf liver EF-1. Although lipid content of toadfish liver S-100 was elevated in winter fish (Table 6), this did not correlate with an increase in activity in the 400,000-700,000 dalton range. Aging of winter preparations led to a loss of activity to about one-half of the original levels, with a concomitant reduction in small form activity on sucrose gradients.

IV. RAT RESULTS

A. ^{3}H-phe-tRNA Binding Assay with Rat Liver EF-1

The time-course of ^{3}H-phe-tRNA binding to puromycin-treated rat liver ribosomes is presented in Figure 5(a). The S-100 from control rat liver was preincubated with 10 mM N-ethylmaleimide (NEM) to inhibit elongation factor 2, a sulfhydryl-requiring protein. At a protein concentration of 40 μg/ml, the reaction was linear with time for about 6 min at 30°C. The reaction rate decreased appreciably with time so that the 15 min value was only twice the 5 min value.

S-100 protein concentration dependency of the binding assay is seen in Figure 5(b). ^{3}H-phe-tRNA binding to the puromycin-treated ribosomes was linear with protein concentration over a range of approximately 10-50 μg/ml at an incubation time of 5 min at 30°C.

Temperature dependency of the binding assay is depicted in Figure 6. A Q_{10} of 2.7 was observed for 10°-20°C and 2.2 for 20°-30°C. Above 30°C the binding activity with rat liver S-100 dropped off markedly; the Q_{10} for 30°-37°C was 1.4. An even more pronounced decrease in the rate of the binding assay above 30°C was previously observed with toadfish liver S-100 (Figure 2).

B. EF-1 Activity Levels in Supernatants from Rats in Different Thyroid States

EF-1 binding activity was measured in NEM-treated S-100 prepared from individual T3-treated and control rats which had been initially matched by weight. The data for control and T3-treated animals are presented in Tables 7 and 8 respectively. No difference was noted between the two groups with respect to liver to body weight ratio or DNA, RNA and protein concentration in the crude homogenates. Protein concentration was 14% higher in the S-100 from control rats. No statistically significant difference in EF-1 binding activity could be seen between high speed supernatants from the two groups. EF-1 activity in the S-100 from T3-treated rats was about 10% higher on the average than that from control animals, but the overlap of standard deviations, calculated from the mean values for individuals, made this difference statistically insignificant.

Since no difference in activity was observed with the post-ribosomal supernatants, 0.5 M KCl microsomal washes were prepared of the microsomal pellets, pooled separately, from the last four T3-treated and control animals. The microsomal salt wash from T3-treated animals had 6% of the activity found in the S-100 and a specific activity in the binding assay of 152 ± 9 (pmoles ^{3}H-phe-tRNA bound/min/mg protein) based on four determinations. The comparable values for the controls were 9% of the S-100 activity and a specific activity of

174 ± 11 based on four determinations. While both microsomal salt washes had a higher specific activity than the comparable S-100, the salt wash from the controls was even higher than that from T3-treated animals. Hence no increase in EF-1 binding activity was found in either S-100 or microsomal wash fractions from T3-treated rats that could be correlated with the 27% increase in liver protein synthetic rate *in vivo* in hyperthyroid animals (Mathews *et al.*, 1973).

The question arose whether a 27% difference in EF-1 binding activity could be reliably detected by the assay when the combination of variations in assay values for a particular S-100 was coupled with the variations among individuals of the same thyroid group. EF-1 binding activity was therefore measured in NEM-treated S-100 from control and thyroidectomized rats. A 39% decrease in *in vivo* liver protein synthetic rate had been found with hypothyroid animals (Mathews *et al.*, 1973). A decrease in binding activity of the same magnitude was expected with S-100 from thyroidectomized rats. The results are presented in Table 9. The binding activity of the S-100 from thyroidectomized rats was not significantly lower than that from controls. S-100 from the thyroidectomized rats was perhaps slightly more active although the overlap of standard deviations indicated no significant difference.

Since no difference in binding activity of the S-100 was observed in 18 hour starved rats regardless of thyroid state, non-starved animals were tested. Young male rats (31 days age, 50-70 g) were selected for both thyroidectomized and control

groups. The growth rate of both groups was monitored carefully by weighing the animals every second day for 30 days. The data from these matched animals is seen in Tables 10 and 11 for control and thyroidectomized animals respectively. The growth rate of the thyroidectomized animals was only 30% that of the controls over the last week. However, the EF-1 binding activity of the S-100 was very similar in the two groups. To preclude some possible artifact in the response of the binding assay to the NEM-treated S-100, all the S-100 fractions from both animal groups were also tested in the polymerization assay with puromycin-treated rat ribosomes and excess EF-2. No significant difference was noted in the EF-1 dependent polymerization of polyphenylalanine between the slow-growing thyroidectomized rats and the normal-growing control rats. The ratio of binding/polymerization activity was approximately 2.3.

No difference in the level of EF-1 activity in the S-100 from T3-treated, thyroidectomized or control rat liver was seen with either the binding or polymerization assays. Yet the rate of polypeptide chain assembly *in vivo* in rat liver had been demonstrated to differ significantly as a function of the thyroid state of the animals (Mathews *et al.*, 1973). In an effort to reconcile this discrepancy, the liver high speed supernatants were assayed using the same animals which had received ^{14}C-leucine injections for the determination of *in vivo* protein synthetic rate. The rats were not starved

in order to have them in a more natural nutritional state. Two important changes in the preparation of the S-100 were necessitated by this procedure: the animals were under ether anesthesia during the injection period and after the injection the livers were homogenized in a Sorvall Omnimixer for 90 seconds at half speed instead of the more gentle Dounce homogenization normally employed.

The S/T data for these control and T3-treated animals were obtained and the average polypeptide chain assembly time (t_c) was calculated according to Mathews et al. (1973). The control animals had a t_c = 1.30 min and the T3-treated rats had a t_c = 1.10 min. These T3-treated rats thus showed a rate of polypeptide chain assembly about 15% faster than the controls. This value was somewhat less than the 27% increase previously found in starved animals (Mathews et al., 1973).

The in vitro results are seen in Table 12. The protein concentration of these S-100 fractions, prepared by the Omnimix homogenization procedure, was about 30% greater than that found with Dounce homogenization. The specific activity of these post-ribosomal supernatants in the binding assay was less than 50% that obtained by the normal procedure which indicated a substantial loss of enzyme activity in the Omnimix homogenization. No statistically significant difference was noted in the level of EF-1 binding activity of the S-100 from these T3-treated and control animals. The variation in the assay was particularly severe with the Omnimix preparations

which were observed to lose activity as a function of time even with liquid nitrogen storage. The combination of initially lower activity plus instability upon storage made the continued use of this procedure impractical.

C. Distribution of EF-1 Activity in S-100 from Thyroidectomized and Control Rats

No difference in the level of EF-1 activity could be demonstrated in the S-100 from the livers of control, T3-treated, and thyroidectomized rats. It was of interest, therefore, to explore whether the molecular weight distribution of soluble EF-1 was influenced by thyroid state.

EF-1 activity profiles from sucrose density gradient centrifugation of rat liver S-100 from a non-growing thyroidectomized rat and from a normally growing control rat are seen in Figure 7(a) and 7(b) respectively. A similar distribution of EF-1 binding activity was observed. The small form of the enzyme comprised 84% of the activity recovered from the gradient and sedimented in a broad band with the bulk of the S-100 protein at 4-7S. Only 16% of the EF-1 activity sedimented as the large form in the range of 14-22S. A pronounced shoulder of activity sedimented at 11S (tube 15) which was the position of the catalase marker (M = 250,000).

The distribution of EF-1 activity on the sucrose gradients was also of interest with respect to the lipid content of the high speed supernatants. It has been reported that the heavy

form of EF-1 from calf liver contains about 10 times more phospholipid than the low molecular weight (light) form (Legocki et al., 1974a). Ms. Shree Dhawale did preliminary determinations of total lipids on rat S-100 according to the procedure of Getz and Bartley (1961). Rat liver S-100 from thyroidectomized, T3-treated and control rats gave values for total lipid of 6.4, 5.3 and 3.7 mg/ml respectively. Thus, while the supernatant from thyroidectomized rats did contain significantly more total lipid than that from control animals, the proportion of EF-1 activity recovered from sucrose density gradients as the large molecular weight species was unchanged (about 15% in both cases).

D. EF-1 Activity Associated with Polysomes from Liver of Thyroidectomized and Control Rats

Since no difference was found in either the amount or the molecular weight distribution of soluble EF-1 activity from animals in different thyroid states, the polysome fraction was examined as a potential source of factors influencing in vivo protein synthetic rate. Polysomes were prepared by treatment of the postnuclear supernatant with 0.5% sodium deoxycholate and subsequent pelleting through 2M sucrose. The polysome pellets were resuspended in Medium A and assayed in the standard binding assay without added factors. In a preliminary investigation (two experiments) the ^{3}H-phe-tRNA binding activity of thyroidectomized rat polysomes compared to

control rat polysomes was found to be reduced approximately 50% and 80%. The addition of a saturating level of low background puromycin-treated ribosomes increased the extent of binding at low concentrations of both control and thyroidectomized polysomes but did not eliminate the relative deficiency in binding activity shown by the polysomes from thyroidectomized animals. When the resuspended polysomes were centrifuged through a 15-30% sucrose gradient for 20 min at 50,000 rpm, EF-1 binding activity was observed throughout the polysome profile coincident with the absorbance at 260 nm. While the polysomes from control rat liver were more active throughout the profile, the pattern was otherwise identical to that of thyroidectomized rat liver. Polyribosome recovery and size distribution were the same in control and thyroidectomized preparations.

V. DISCUSSION

A. EF-1 and Protein Synthetic Rate

1. Toadfish

These results establish a relationship between acclimation temperature and aminoacyl-tRNA binding activity of toadfish liver supernatants (Table 4). The 70% increase in S-100 binding activity of the summer cold-acclimated fish relative to summer controls correlates well with the 60% stimulation of polymerization activity previously reported by Haschemeyer (1969c). S-100 from winter fish acclimated at 7-8^{o} shows only a 33% increase in EF-1 specific activity compared to summer controls because of the higher protein concentration in the winter S-100. However, EF-1 activity level in cold-acclimated winter fish is approximately double that of warm acclimated (25^{o}) winter fish at a similar S-100 protein concentration. Thus, cold acclimation in both winter and summer fish produces a pronounced elevation in EF-1 binding activity for a 10-15^{o} difference in acclimation temperature.

The elevated binding activity in liver preparations from cold-acclimated fish is directly related to the increase in polypeptide chain elongation rate observed *in vivo* in these animals. The activity levels determined *in vitro* are summarized in Table 13 relative to liver ribonucleic acid concentration. This takes into account differences in total quantity of protein synthetic components between winter and summer fish. The

second column of Table 13 presents average polypeptide chain assembly time *in vivo*. Normalization to 20°C was made by use of Arrhenius plots of the t_c data. Comparison of columns 1 and 2 indicates that fish with elevated levels of EF-1 activity exhibit shorter average times for polypeptide chain assembly *in vivo*. If one excludes the unlikely possibility that synthesis is significantly shifted toward smaller proteins, the t_c data provide strong evidence that elongation rate has increased with cold acclimation. This change also would account for the generalized increase of amino acid incorporation into protein characteristic of cold acclimation in toadfish (Haschemeyer 1968, 1969b).

In summer fish the change in liver EF-1 activity closely parallels the apparent *in vivo* elongation rate determined from t_c. Thus, at 20° the t_c data for an average polypeptide chain size of 47,000 daltons yield elongation rates of 74 amino acid residues per minute in 20°-acclimated fish and 117 residues per minute in 10°-acclimated fish. The change associated with cold acclimation therefore represents a 58% increase in elongation rate. This falls within the 0.95 confidence range for the change in EF-1 activity as assayed *in vitro* (55-88%) based on the data of Table 4. In winter fish elongation rate *in vivo* (normalized to 20°) is 105 residues per minute, representing an increase of 42% compared with 20°-acclimated summer fish at the same experimental temperature. The elevation in specific EF-1 binding activity in these fish relative to

summer fish is 33% (0.95 confidence range 20-45%) in good agreement with the *in vivo* data. However, when EF-1 activity is expressed relative to liver RNA content (Table 13), winter fish show a 90% activity increase compared with summer 20° fish. Although an error in RNA determination may account for some of this difference, these results suggest that winter fish may indeed have an apparent excess of EF-1 binding activity relative to the concentration of protein synthetic units.

2. Rat

In contrast to the toadfish liver results, no correlation is shown between EF-1 activity in the S-100 fraction and the *in vivo* rate of protein synthesis in rat liver at different thyroid states. Mathews *et al.* (1973) observed a 27% increase in the rate of liver polypeptide chain assembly *in vivo* in T3-treated compared to control rats. However, no statistically significant difference in the level of EF-1 binding activity in the post-ribosomal supernatants of control and T3-treated animals was observed (Tables 7 and 8). Binding activity also was measured in preparations obtained from animals given radioactive leucine injections for the determination of *in vivo* protein synthetic rate. In this series the rate of polypeptide chain assembly was 15% faster in the T3-treated rats than in the control rats. Average binding activity was 25% higher in the supernatants from the T3-treated compared to control

animals (Table 12). This result, however, is not definitive in view of the severe loss of enzyme activity (greater than 50%) that occurred when livers were subjected to vigorous homogenization, as required for the t_c measurement. This together with subsequent lability during storage produced unacceptably large variation in the results from the binding assay. In any case the effect of excess hormone injections on protein synthetic activity in the euthyroid rat appears to be a small one.

Mathews et al. (1973) found the in vivo rate of liver protein synthesis was decreased by 39% in thyroidectomized rats compared to control animals. The EF-1 binding activity of the S-100 from thyroidectomized animals was not lower than the controls in initial studies (Table 9). No difference is seen in EF-1 binding activity of the S-100 from a larger group of carefully matched and weighed thyroidectomized and control animals (Tables 10 and 11). These supernatants were also tested for EF-1-dependent polyphenylalanine synthesis using puromycin treated ribosomes and excess EF-2. In the polymerization assay, the enzyme activity of the S-100 from the slow-growing thyroidectomized rats is essentially identical to that of the control animals.

3. Comparison of Species

The levels of EF-1 binding activity in toadfish liver relative to rat liver (Table 3) are of interest in relation

to the role of EF-1 in control of elongation rate. The binding activity of rat S-100 (expressed in terms of S-100 protein) is about three times greater than that of control toadfish S-100. The question arises whether this difference in cytosol EF-1 activity level is related to differences in protein synthetic rates. Values of average polypeptide chain assembly time (t_c) determined *in vivo* in liver of toadfish (20°-acclimated) at two temperatures (Haschemeyer and Persell, 1973), rat (Mathews *et al.*, 1973), and mouse (Scornik, 1974) are plotted *vs.* inverse absolute temperature in Figure 8. The linear relationship between log t_c and 1/T suggests that differences in elongation rate among the three species are only temperature related (E_a = 18 Kcal, $Q_{10} \sim 3$). Data obtained in cooled rabbits and in turtles generally support this result for Q_{10} (Green and Anker, 1955). Therefore, rate-controlling components would be expected to occur at similar concentrations in the various species. The results of Table 3, however, indicate that specific binding activity in rat is about three times that of toadfish. The role of EF-1 binding activity in the control of protein synthetic rate therefore may differ in the two species, as will be discussed later.

B. Multiple Active Forms of EF-1

1. Toadfish Liver S-100

EF-1 binding activity from both toadfish and rat liver is found in active forms of different molecular weights as in other eukaryotic systems (cf Introduction). In control toadfish liver S-100 the EF-1 binding activity recovered from sucrose density gradients (80% of the applied activity) is about equally divided between small forms (50,000-135,000 daltons) and large forms (420,000-700,000 daltons) as seen in Figure 3(a). The characteristic activity found at about 200,000 daltons in other species (McKeehan and Hardesty, 1969; Collins et al., 1972) appears to be absent in liver S-100 from warm-acclimated fish.

The data of Table 6 indicate that the increase in liver EF-1 activity associated with cold acclimation is not equally distributed among the various molecular weight classes. In warm-acclimated summer fish, about 40% of activity or 14 units/mg is found in the small class (50,000-200,000 daltons), whereas with cold acclimation this class accounts for 53% of activity or 32 units/mg. The increase in small EF-1 is thus 18 units/mg or about 70% of the total change of activity associated with cold acclimation. Results for the warm- and cold-acclimated winter fish are comparable. In addition to the change in the major peak of EF-1 activity at 50,000-135,000 daltons, a clearly discernible shoulder at 150,000-200,000 daltons is observed in supernatants from cold-acclimated fish.

Although large forms of EF-1 have been typically regarded as aggregates of small forms in other eukaryotes (Moon et al., 1973; Grasmuk et al., 1976), the present results suggest a qualitative difference that may be associated with the control of elongation rate. Evidence for a proteolytic conversion of EF-1 from large to small forms has been obtained in Artemia salina (Twardowski et al., 1976), however in the present case the increase in lower molecular weight forms is not accompanied by a diminution of large forms. Similarly, preliminary experiments indicate no change in the size distribution of EF-1 in liver extracts prepared in the presence of a protease inhibitor, phenylmethylsulfonylfluoride. The possibility remains, however, that an inactive precursor of EF-1 not detected by the assays used here may be the source of the elevated small form activity in cold-acclimated fish. Preparation of a sufficiently specific antibody to toadfish liver EF-1 is needed to distinguish between a precursor activation mechanism and de novo synthesis of EF-1.

2. Rat Liver S-100

With rat liver S-100 multiple active peaks are observed with chromatography on G-200 Sephadex (Appendix Figure 3) as reported by Schneir and Moldave (1968). Two broad peaks of EF-1 activity are recovered from Sepharose 4B chromatography (Appendix Figure 4) as observed by Collins et al. (1972). With gel filtration procedures run in buffer containing only

5% glycerol most of the rat liver EF-1 activity recovered is in large forms because small form activity is not stable under these conditions. However, 70-90% of liver EF-1 activity is recovered in small forms with Sephadex G-200 chromatography in buffer containing 25% glycerol (Nagata et al., 1976b). A similar distribution of EF-1 binding activity from rat liver S-100 is seen with sucrose density gradient centrifugation (Figure 7). Approximately 85% of the activity recovered is in small forms (50,000-200,000 daltons) and only 15% is in large forms (400,000-700,000 daltons). The distribution of cytosol EF-1 binding activity between large and small forms is very similar for thyroidectomized and control rat liver.

3. Partially Purified Large and Small Forms of Toadfish EF-1

The 70-fold purified large form of EF-1 from toadfish liver on sucrose gradient centrifugation shows a heterogeneous distribution of activity with major components at 700,000, 540,000 and 420,000 daltons [Figure 3(b)]. The 70-fold purified small form activity is found in the range of 50,000 to 135,000 daltons [Figure 3(c)]. Inhibition of large form activity by antibody prepared against the small form, and vice versa, indicates the presence of common subunits in the two forms. It appears likely that the large forms are EF-1 aggregates. If subunit molecular weight were 50,000-60,000 as indicated in other eukaryotes, these aggregates appear to

be concentrated at sizes representing 8, 10 and 12 subunits. Mixing experiments suggest the possibility that subunits other than the binding component of EF-1 might be contained in the aggregates as in wheat embryo (Bollini et al., 1974). Small form binding activity is stimulated about 20% over the additive value by the addition of heat inactivated large form. However, this stimulatory effect is small compared to that found in the pig liver system (Iwasaki et al., 1973, 1974, 1976).

C. Conclusions

The toadfish results provide the strongest evidence to date that activity associated with EF-1 is rate-controlling for protein synthesis under certain physiological conditions. In toadfish, EF-1 binding activity has been directly correlated with polypeptide chain elongation rates measured in vivo. On the other hand, no correlation is observed between cytosol EF-1 binding activity of rat liver and changes in elongation rate associated with thyroid status. The question arises as to a possible change in mechanism of control of elongation rate in evolving from fish to mammals. A number of pertinent observations on this point can be made. The data in Figure 8 show that the liver protein synthetic rates in vivo are similar in rat and toadfish when the difference in body temperature is considered. Yet rat liver S-100 has about three times more EF-1 binding activity per mg of protein than does control

toadfish liver S-100 (Table 3). This suggests that EF-1 of rat liver may be in excess and therefore not rate-limiting for protein synthesis. There may be additional EF-1 if membrane-bound forms occur as in E. coli (Jacobson and Rosenbusch, 1976). It is of interest that in the bacterial system EF-Tu (binding activity) has been found in excess relative to the concentrations of EF-Ts and ribosomes (Furano, 1975).

Since soluble EF-1 activity levels appeared to be in excess in rat liver, ribosomal-bound EF-1 activity was investigated as the potential rate-controlling component of protein synthesis in relation to thyroid hormone. In the two preliminary experiments included in this thesis, ^{3}H-phe-tRNA binding to polysomes from liver of thyroidectomized rats was reduced 50% and 80% compared to the values for controls.

These findings have been confirmed by subsequent work in this laboratory (Nielsen et al., 1977a). In addition, the molecular weight distribution of EF-1 released from rat liver polysomes was found to resemble that of soluble EF-1 from 20°-acclimated toadfish [Figure 3(a)] rather than soluble EF-1 from rat (Figure 7). Toadfish ribosome-bound EF-1, on the other hand, did not differ significantly from soluble EF-1 (Nielsen and Haschemeyer, 1975). Differences in molecular weight distribution between soluble and ribosome-bound EF-1 have been observed in wheat embryo (Quintard and Julien, 1975; Lanzani et al., 1975). Lanzani et al. (1975) have also shown differences in activity and

peptide composition between soluble and ribosome-bound EF-1 in the same system. No physiological significance of these observations, however, has yet been shown.

A component of EF-1 other than that responsible for the binding activity could mediate the change in ribosome-bound activity found in thyroidectomized and control rats. This component may be responsible for GTP recycling thus making it analogous to bacterial EF-Ts. The existence and properties of this component in eukaryotic EF-1, however, are still somewhat in doubt. In bacterial systems, EF-Ts catalyzes the exchange of GTP for GDP on EF-Tu (Weissbach et al., 1970). EF-Tu has 100 times greater affinity for GDP than for GTP (Miller and Weissbach, 1970). At high ionic strength, the purified binding component of pig liver EF-1 (EF-1 δ) has slightly greater affinity for GTP than for GDP (Nagata et al., 1976a) and thus no recycling factor appears to be required. At the ionic strength optimal for protein synthesis, however, nucleotide exchange of GTP with EF-1δ-GDP is stimulated by a second factor denoted EF-1β (Nagata et al., 1976c).

On the other hand, no evidence was found for an activity stimulating nucleotide exchange with calf brain EF-1_L (Legocki et al., 1974b). Similarly, Kemper et al. (1976) observed that the addition of phosphoenolpyruvate and pyruvate kinase did not stimulate either the binding or polymerization activity of 100-fold purified rabbit reticulocyte EF-1 (450,000 dalton form). In contrast, in the bacterial system,

Weissbach et al. (1971) had shown the EF-Ts dependency could be replaced up to 60-80% by the direct phosphorylation of Tu-GDP to Tu-GTP by phosphoenolpyruvate and pyruvate kinase. Thus, although evidence for stimulation of binding by an unidentified second component has been obtained in two eukaryotic systems (cf Introduction), no evidence by direct assay is yet available. Stimulation of binding has also been observed in the present studies. The binding activity of the 70-fold purified small form toadfish EF-1 is stimulated about 20% by heat inactivated large form. Also, some rat liver ribosome preparations stimulate the binding activity of toadfish small form EF-1 but not the large form. Investigation of potential catalytic factors in the rat and toadfish liver systems will necessitate the development of a quantitative assay for such a component.

It is possible that a stimulatory component could be related, not to GTP recycling on the binding enzyme, but rather to the release of EF-1 from the ribosomes after aminoacyl-tRNA binding. In the Krebs II ascites system, Nolan et al. (1975) have demonstrated that ^{3}H-EF-1 is released from the ribosomes after phe-tRNA binding only if the binding occurs with GTP and not with nonhydrolyzable GMPPCP. In the yeast system a ribosome-dependent GTPase activity has been isolated which is required in addition to EF-1 and EF-2 for poly(U)-directed polyphenylalanine synthesis (Skogerson and Wakatama, 1976). This possibility may be testable in the

rat and toadfish systems using a GTP hydrolysis assay provided non-specific GTPase activity can be eliminated.

In conclusion, the present research has provided evidence that control of liver protein synthesis through changing elongation rate is exercised by elongation factor 1. In toadfish undergoing metabolic compensation to temperature change, total soluble EF-1 aminoacyl-tRNA binding activity is shown to be proportional to measured elongation rate *in vivo*. In contrast, in the rat, soluble EF-1 is present at high, presumably saturating, levels and does not correlate with changing elongation rate associated with thyroid hormone. In this case, control appears to be exercised by ribosome-bound EF-1. Further study of the latter may indicate whether a possible evolutionary modification in the control of protein synthesis by elongation factor 1 is involved.

TABLE 1

Characteristics of EF-1 binding assay

	pmoles/5 minutes
Complete system	2.0
minus Poly(U)	0.3
minus GTP	0.1
minus EF-1	0.25
Complete + EF-2	2.0
Complete (NEM-treated S-100)	2.0
(NEM-treated S-100 and ribosomes)	1.7

TABLE 2

Purification of EF-1 binding activity
(large and small forms) from toadfish liver

Fraction	Volume ml	Protein mg/ml	Specific Activity units/mg x 10^{-3}	Total Units* x 10^{-3}	Yield %	Purification (fold)
100,000 x g Supernatant	170	18.2	0.040	124	100	1
pH 5.8 Supernatant	180	9.8	0.047	83	67	1.2
0.35 M NH_4Cl Eluate from CM-Sephadex	30	1.2	1.20	43	35	30
Sepharose 6B (large form)	15	0.06	2.70	2.4	2.0	68
CM-Sephadex (Gradient Elution) followed by Sepharose 6B (small form)	40	0.10	2.80	11	9.0	70

* 1 unit = 1 pmole ^{3}H-phe-tRNA bound/min in standard assay, 30°C.

TABLE 3

Comparison of binding and polymerization activities of EF-1 preparations

Preparation	Binding* pmoles/min/mg	Polymerization pmoles/min/mg	Polymerization/ Binding
Toadfish S-100	35 ± 7 (17)	9.5	0.27
EF-1 (large form)	2700	460	0.17
EF-1 (small form)	2800	670	0.24
Rat liver S-100	127 ± 16 (26)	22	0.17

* Results are presented as mean ± S.D. (number of animals). Each preparation was assayed 3-5 times.

TABLE 4

Elongation factor 1 binding activity
in toadfish liver S-100

Season	Acclimation Temperature	Number of Preparations (number of animals)	S-100 Protein mg/ml	Binding Activity*
Summer	20-22°	13 (130)	13.2 ± 0.6	35.0 ± 6.5 (50)
	10-11°	5 (60)	12.9 ± 1.3	60.0 ± 6.5 (25)
Winter	25°	1 (10)	20.0	23.0 ± 1.1 (5)
	8°	5 (70)	20.3 ± 1.5	46.5 ± 5.5 (20)

* pmoles ^{3}H-phe-tRNA bound/min/mg S-100 protein ± S.D. (number of assays)

TABLE 5

Liver weight and composition
in summer and winter toadfish

Season	Sex	Liver/Body Weight (%)	RNA (mg/g liver)	Protein (mg/g liver)
Summer	♂ & ♀	2.9 ± 0.6 (40)	4.1 ± 0.7 (5)	96 ± 10 (8)
Winter	♂	2.6 ± 0.5 (40)	3.5 ± 0.5 (6)	117 ± 15 (6)
	* ♀	3.1 ± 0.5 (30)	5.2 ± 1.0 (10)	110 ± 10 (6)

* Gonad weight = 8.2 ± 2.3% of body weight

TABLE 6

Percentage of EF-1 binding activity* recovered in small forms (50,000-200,000 daltons) from liver post-ribosomal supernatant of summer and winter temperature-acclimated toadfish

Season	Acclimation Temperature	% of Activity in Small Forms	S-100 Lipid mg/ml
Summer	20-22°C	40 ± 7 (5)	4.7 ± 0.2
	10-11°C	53 ± 7 (5)	4.9
Winter	25°C	39	-
	8°C	52 ± 2 (7)	6.7 ± 0.7

* Standard assay conditions 30°C

TABLES 7-12

EF-1 activity assays of S-100 from liver of control, thyroidectomized and T3-treated rats

Specific activity in the binding assay is expressed as pmoles ^{3}H-phe-tRNA bound/min/mg S-100 protein. Polymerization activity is expressed as pmoles ^{3}H-polyphe/min/mg S-100 protein. Results are given as mean $\pm$ standard deviation (number of assays). Mean and standard deviation for the group were determined from mean values for each animal. All S-100 fractions were pre-treated with 10 mM N-ethylmaleimide for the binding assay. S-100 protein concentrations were determined by the modified Biuret procedure described in Methods. DNA, RNA and protein determinations were done on duplicate aliquots of crude homogenate.

TABLE 7

Individual control rats were killed at 64-78 days of age after 18 hour starvation.

Animal No.	Body wt. (grams)	% liver/ body wt.	DNA	RNA	Protein	S-100 protein (mg/ml)	Binding activity
			(mg/g wet tissue)				
1C	192	3.39	-	-	-	34.7	129 ± 22 (4)
7C	184	3.59	3.20	10.7	203	30.3	152 ± 22 (5)
8C	186	3.76	3.18	9.8	200	27.3	128 ± 10 (5)
9C	175	3.09	3.80	10.7	198	31.0	132 ± 12 (5)
13C	203	2.96	3.20	10.0	188	28.7	127 ± 16 (5)
16C	168	3.33	3.95	8.1	240	29.4	137 ± 11 (5)
18C	201	3.98	3.63	6.2	228	31.0	121 ± 9 (5)
19C	218	3.12	3.53	7.6	234	30.1	127 ± 5 (4)
21C	223	3.36	3.29	7.8	223	27.1	86 ± 7 (4)
Average	194±19	3.4±0.3	3.5±0.3	8.9±1.7	214±19	30±2.3	127 ± 18

TABLE 8

Individual T3-treated rats were killed at 64-78 days of age after 18 hour starvation.

Animal No.	Body wt. (grams)	% liver/ body wt.	DNA	RNA	Protein	S-100 protein (mg/ml)	Binding activity
			(mg/g wet tissue)				
3T3	159	3.77	-	-	-	24.5	174 ± 5 (4)
4T3	140	3.79	-	-	-	25.8	120 ± 15 (5)
5T3	181	3.48	3.87	12.0	196	27.9	131 ± 14 (5)
6T3	166	3.80	4.08	11.5	192	26.1	151 ± 16 (5)
12T3	249	3.41	3.60	10.4	190	28.2	145 ± 8 (5)
14T3	207	3.19	3.20	9.7	146	25.2	118 ± 12 (5)
15T3	177	3.95	3.29	9.4	211	28.6	155 ± 13 (5)
17T3	180	4.00	3.35	7.5	193	25.8	130 ± 11 (5)
20T3	197	3.35	4.26	8.4	243	26.4	139 ± 13 (4)
22T3	210	3.52	3.72	7.8	220	26.4	131 ± 5 (4)
Average	187±31	3.60±0.3	3.7±0.4	9.6±1.7	199±28	26.4±1.3	139 ± 17

TABLE 9

Individual control and thyroidectomized rats. The animals were thyroidectomized at 31 days and killed 31 days later after 18-hour starvation.

A. Control animals

B. Thyroidectomized animals

Animal No.	Body wt. (grams)	% liver/ body wt.	DNA (mg/g wet tissue)	RNA (mg/g wet tissue)	Protein (mg/g wet tissue)	S-100 protein (mg/ml)	Binding activity
A. 26C	156	3.6	4.0	7.8	203	27.4	134 ± 18 (10)
27C	198	3.3	3.6	7.3	212	30.0	123 ± 11 (5)
29C	173	3.2	3.8	6.9	192	32.0	126 ± 10 (8)
Average	176±21	3.4±0.2	3.8±0.2	7.3±0.5	202±10	29.8±2.3	128 ± 6
B. 25Tx	111	3.6	4.1	7.6	189	29.1	146 ± 13 (9)
28Tx	136	3.2	3.6	6.6	186	30.0	115 ± 11 (5)
31Tx	120	3.6	3.4	5.5	189	27.9	135 ± 13 (10)
Average	122±13	3.5±0.2	3.7±0.4	6.6±1.1	188±1.5	29.0±1.0	132 ± 16

TABLE 10

Individual control rats (not starved) were killed at 67 days of age. Weight gain per day has been averaged over the last 7 days.

Animal No.	Body wt. (grams)	Growth rate (g/day)	% liver/ body wt.	Protein mg/ml S-100	Binding activity	Polymerization activity
3C	218	4.7	4.0	25.6	130 ± 18 (9)	53 ± 1 (4)
4C	222	4.0	3.8	24.1	148 ± 13 (6)	64 ± 2 (4)
6C	237	5.7	4.4	25.2	112 ± 14 (9)	51 ± 3 (6)
8C	276	5.3	3.8	28.3	119 ± 11 (9)	52 ± 2 (4)
10C	286	8.0	4.2	25.6	-	48 ± 2 (4)
12C	220	5.4	4.0	25.9	-	53 ± 5 (4)
14C	238	4.9	4.1	26.1	-	53 ± 2 (4)
16C	238	5.9	4.2	26.3	-	44 ± 3 (4)
18C	246	7.0	4.1	27.6	-	57 ± 4 (5)
20C	206	6.6	3.9	23.6	-	53 ± 1 (4)
21C	261	7.0	3.9	23.8	-	53 ± 2 (4)
22C	242	7.4	4.3	25.1	-	53 ± 3 (4)
24C	258	6.3	4.2	27.2	-	51 ± 2 (9)
26C	266	9.1	4.4	27.2	-	52 ± 3 (8)
Average	244±23	6.2±1.4	4.1±0.2	25.8±1.4	127 ± 16	53 ± 4

TABLE 11

Individual thyroidectomized rats (not starved). Animals were thyroidectomized at 31 days of age and killed 36 days later. Weight gain per day has been averaged over the last 7 days.

Animal No.	Body wt. (grams)	Growth rate (g/day)	% liver/ body wt.	Protein mg/ml S-100	Binding activity	Polymerization activity
5Tx	204	3.0	3.3	25.9	128 ± 19 (6)	56 ± 6 (4)
7Tx	184	1.1	3.2	25.9	124 ± 17 (8)	61 ± 3 (4)
9Tx	134	-0.1	2.9	26.5	124 ± 20 (9)	55 ± 8 (4)
11Tx	201	2.7	3.5	25.9	-	53 ± 4 (8)
13Tx	155	-0.1	3.1	24.7	-	56 ± 5 (4)
15Tx	161	0.7	3.7	29.4	-	59 ± 3 (5)
17Tx	202	2.9	3.2	27.0	-	54 ± 4 (4)
19Tx	86	-1.7	3.0	28.2	-	52 ± 3 (9)
23Tx	181	5.0	3.3	25.3	-	55 ± 1 (4)
25Tx	194	4.9	4.0	27.0	-	54 ± 6 (8)
27Tx	177	4.7	3.5	28.2	-	53 ± 2 (8)
Average	171±36	2.1±2.3	3.3±0.3	26.7±1.4	125 ± 2	55 ± 3

TABLE 12

Individual control and T3-treated rats were killed at 70 days of age (not starved). Liver homogenates were prepared by Omnimix homogenization.

A. S-100 from control

B. S-100 from T3-treated

Animal No.	Body wt. (grams)	% liver/ body wt.	Protein S-100 mg/ml	Binding activity
A. 2'C	231	3.9	37.8	44 ± 4 (10)
3'C	224	4.2	31.4	43 ± 10 (9)
4'C	201	3.7	32.3	53 ± 12 (15)
5'C	201	4.0	39.8	36 ± 8 (10)
Average	214 ± 16	4.0 ± 0.2	35.3 ± 4.1	44 ± 7
B. 6'T3	222	3.8	33.8	56 ± 5 (10)
7'T3	209	3.9	33.2	49 ± 5 (10)
8'T3	205	3.1	35.5	76 ± 9 (10)
9'T3	238	4.0	35.2	39 ± 6 (10)
10'T3	184	4.0	34.7	68 ± 10 (5)
11'T3	247	3.9	34.8	39 ± 4 (10)
Average	218 ± 23	3.8 ± 0.4	34.5 ± 0.9	55 ± 15

TABLE 13

Correlation of EF-1 binding activity assayed *in vitro* with protein synthetic rates at 20° *in vivo* of temperature acclimated toadfish

Fish Group	EF-1 Activity[c] units/mg liver RNA	$t_c^{20^o}$ minutes	Protein Synthetic Rate *in vivo*[d] mg/g liver/hour
Summer, 20° acclimated	330	5.5[a]	0.62
Summer, 10° acclimated	560	3.5[a]	0.97
Winter, 8° acclimated	640	3.9[b]	0.93

[a] Based on data of Haschemeyer and Persell (1973) and Persell (1976)

[b] Based on data of Nielsen *et al.* (1977b)

[c] 1 unit = 1 pmole ^{3}H-phe-tRNA bound/min, 30°C

[d] Calculated from polypeptide chain synthesis time using 47,000 for average chain molecular weight and assuming all ribosomes to be active. Ribosome concentration calculated from liver RNA (Table 5) taking 80% as ribosomal RNA and molecular weight of total ribosomal RNA as 2.7×10^6

FIGURE 1

Dependency of the binding assay on toadfish liver S-100 (20^{o}-acclimated fish) protein concentration at t = 5 min (standard assay) and at t = 15 min. Preincubation of the S-100 with 10 mM N-ethylmaleimide gave 80% inhibition of the EF-2-dependent polymerization assay. Non-enzymatic binding values (120 cpm at 5 min and 300 cpm at 15 min) were subtracted from the plotted results. Incubation temperature was 30^{o}. Pre-incubation of the S-100 with 10 mM N-ethylmaleimide (○, □); without pre-incubation (●, ■).

FIGURE 1

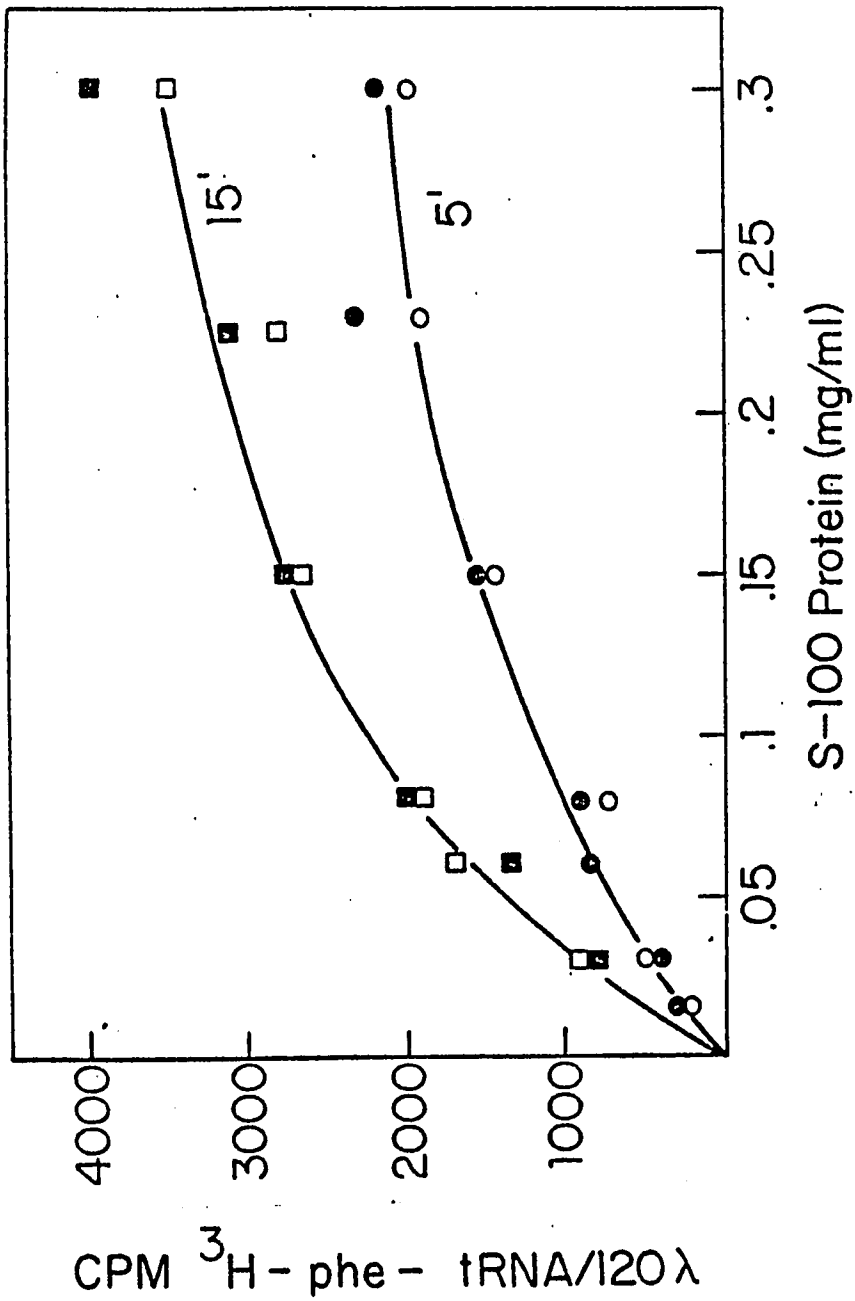

15'
5'
.05
.1
.15
.2
.25
.3
S-100 Protein (mg/ml)
1000
2000
3000
4000
CPM ^{3}H - phe - tRNA/120λ

FIGURE 2

Temperature dependency of binding of ^{3}H-phe-tRNA in the standard poly(U)-rat liver ribosome system (5 min incubation time) with toadfish liver S-100 (20^{o}-acclimated fish) protein at 30 μg/ml. CPM bound ^{3}H-phe-tRNA is given on a logarithmic scale. Standard error bars are based on 5 determinations.

FIGURE 2

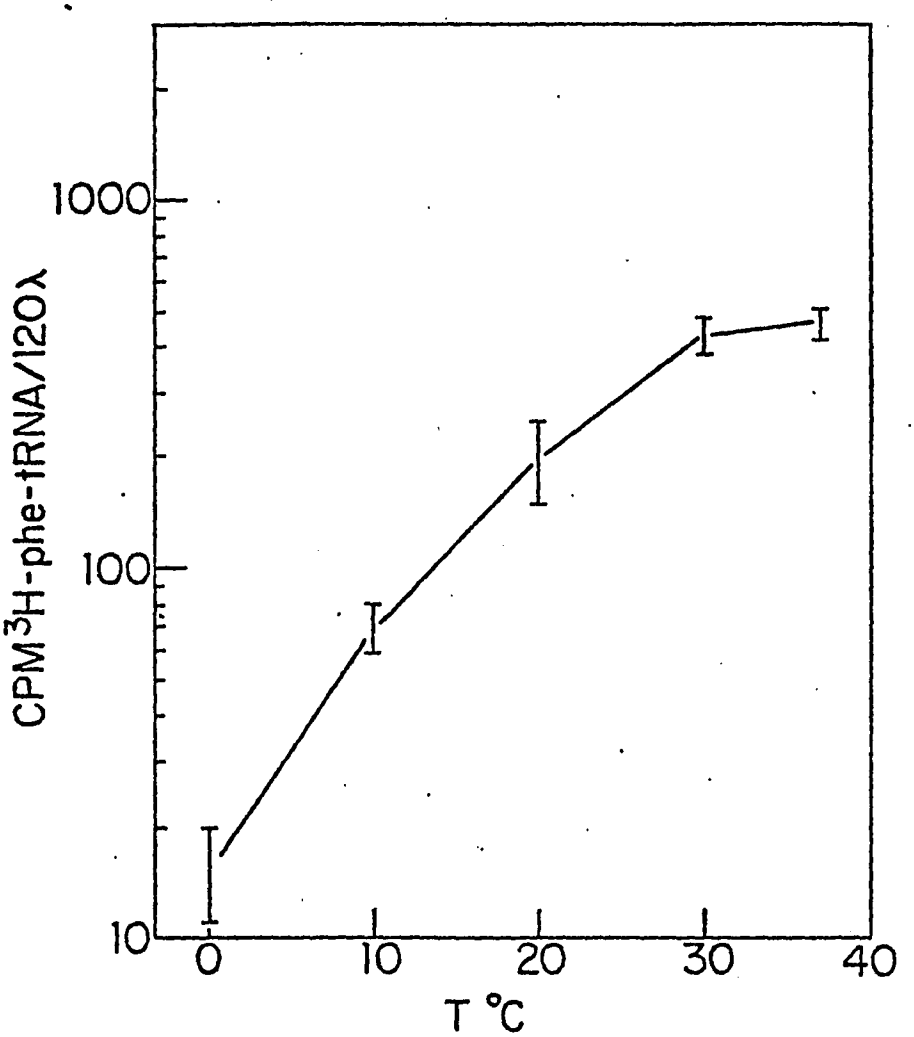


FIGURE 3

Sucrose density gradient analysis of the activities of toadfish liver elongation factors. (a) Distribution of EF-1 binding activity (closed circles) and EF-2 polymerization activity (open circles) in toadfish liver S-100 (20^{o}-acclimated fish). (b) EF-1 binding activity (large form) obtained from Sepharose 6B chromatography. (c) EF-1 (small form) obtained by gradient elution from CM-Sephadex followed by Sepharose 6B; binding activity (closed circles) and EF-2 activity (open circles). 0.2 ml of enzyme preparation or of S-100 diluted 1:2 with 50 mM Tris-HCl (pH 7.5), 2 mM 2-mercaptoethanol was layered on a 5.0 ml 5-20% sucrose gradient in the same buffer and centrifuged 3 hr at 62,500 rpm in a Spinco SW65 rotor. Fractions (0.22 ml) were collected dropwise from the bottom of the tube and were analyzed for binding activity (0.02 ml/assay) and protein (Schaffner and Weissmann, 1973). Bovine serum albumin, human γ-globulin and catalase were run as markers; the position of catalase (11S) is indicated in (c). Calculations of $s_{20,w}$ relative to the markers were made using the tables of McEwen (1967). Recoveries of activity applied to the gradient were 80% for the S-100; 80% for the purified large form, and 30% for the purified small form.

FIGURE 3

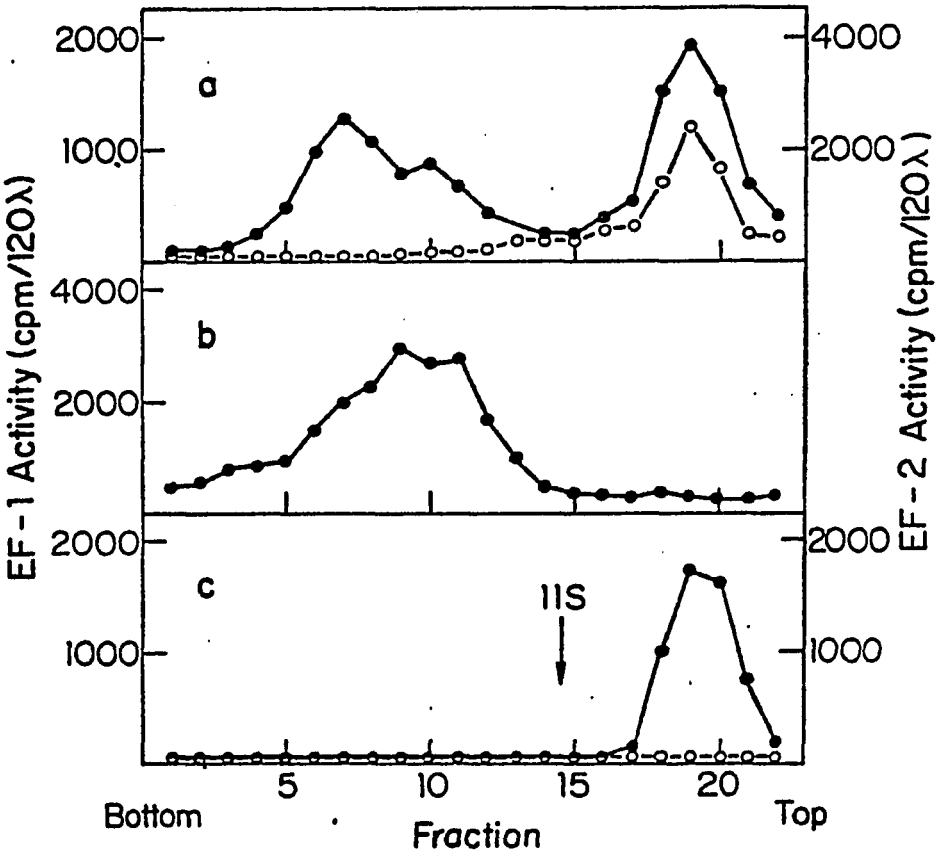

2000
1000
a
4000
2000
b
2000
1000
c
11S
4000
2000
2000
1000
EF-1 Activity (cpm/120λ)
EF-2 Activity (cpm/120λ)
5
10
15
20
Bottom
Top
Fraction

FIGURE 4

Sucrose density gradient analysis of EF-1 binding activity in toadfish liver supernatants. (a) Activity (closed circles) and total protein (open circles) in S-100 of winter 7°-acclimated toadfish. (b) Activity in S-100 of 25°-acclimated winter fish. 0.2 ml of S-100 diluted 1:2 with 50 mM Tris-HCl (pH 7.5), 2 mM 2-mercaptoethanol was layered on a 5.0 ml 5-20% sucrose gradient in the same buffer and centrifuged 3 hr at 62,500 rpm in a Spinco SW 65 rotor. Fractions (0.22 ml) were collected from the bottom of the tube and were analyzed for binding activity (0.02 ml/assay) and protein (Schaffner and Weissmann, 1973). Recovery of activity applied to the gradients was 80%.

FIGURE 4

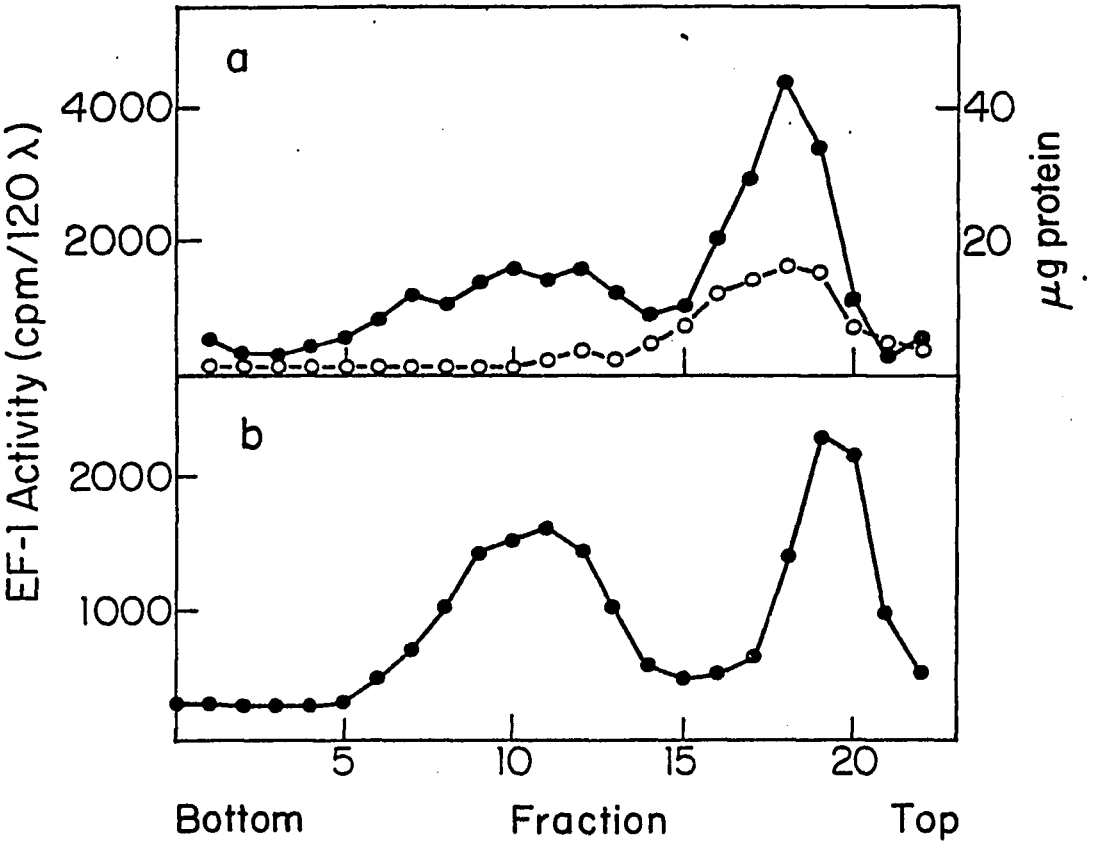

a
b
4000
2000
1000
40
20
EF-1 Activity (cpm/120 λ)
μg protein
5
10
15
20
Bottom
Fraction
Top

FIGURE 5

Characteristics of standard binding assay at 30° with poly(U)-saturated rat liver ribosomes. S-100 from control rats was preincubated with 10 mM N-ethylmaleimide. Non-enzymatic binding was subtracted from plotted results.

(a) Time-course of ^{3}H-phe-tRNA binding at 40 µg/ml S-100 protein.

(b) Dependency on S-100 protein concentration (5 min incubation time).

FIGURE 5

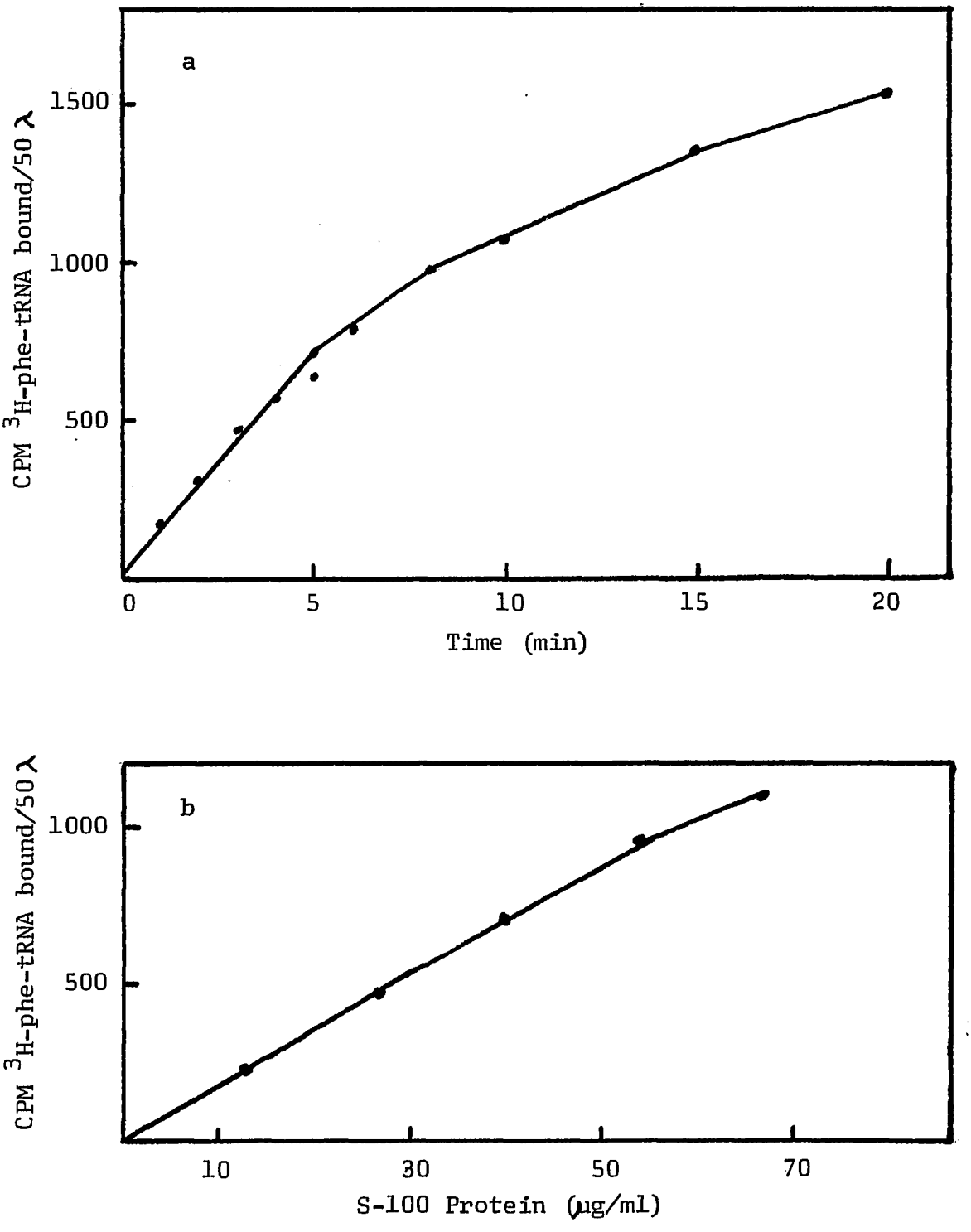

a
1500
1000
500
CPM ^{3}H-phe-tRNA bound/50 λ
0
5
10
15
20
Time (min)
b
1000
500
CPM ^{3}H-phe-tRNA bound/50 λ
10
30
50
70
S-100 Protein (μg/ml)

FIGURE 6

Temperature dependency of binding of ^{3}H-phe-tRNA in the standard poly(U)-saturated rat liver ribosome system (5 min incubation rime) with rat liver S-100 protein at 30 µg/ml. CPM bound ^{3}H-phe-tRNA is given on a logarithmic scale. Standard error bars are based on 5 determinations.

FIGURE 6

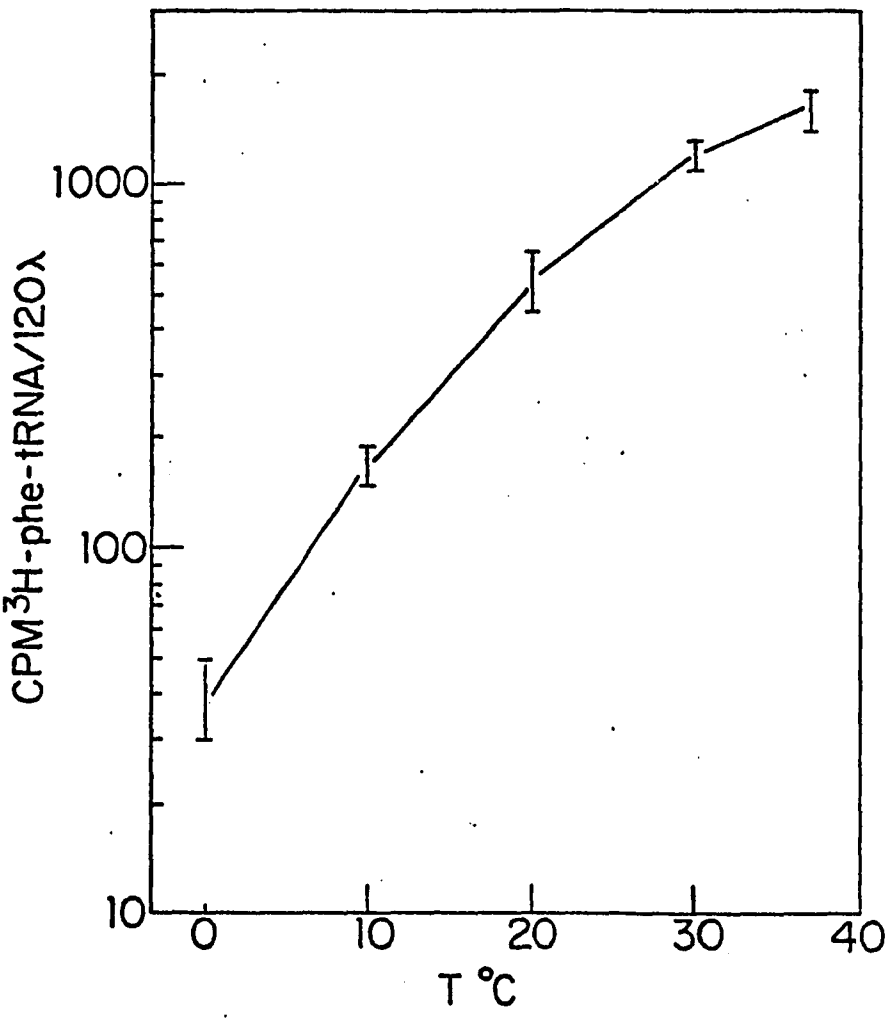


FIGURE 7

Sucrose density gradient of rat liver S-100. 0.2 ml of S-100 in Medium A diluted 1:4 with 0.05 M Tris-HCl (pH 7.5), 0.002 M 2-mercaptoethanol was layered on a 5.0 ml 5-20% sucrose gradient in the same buffer and centrifuged 3 hours at 62,500 rpm in a Spinco SW65 rotor. Fractions (0.22 ml) collected from the bottom of the tube were analyzed for binding activity (0.01 ml/assay) in standard binding assay at 30°C for 5 minutes. 70% of activity applied to the gradient was recovered.

(a) S-100 from non-growing thyroidectomized rat.

(b) S-100 from control rat growing at normal rate of 6 g/day.

FIGURE 7

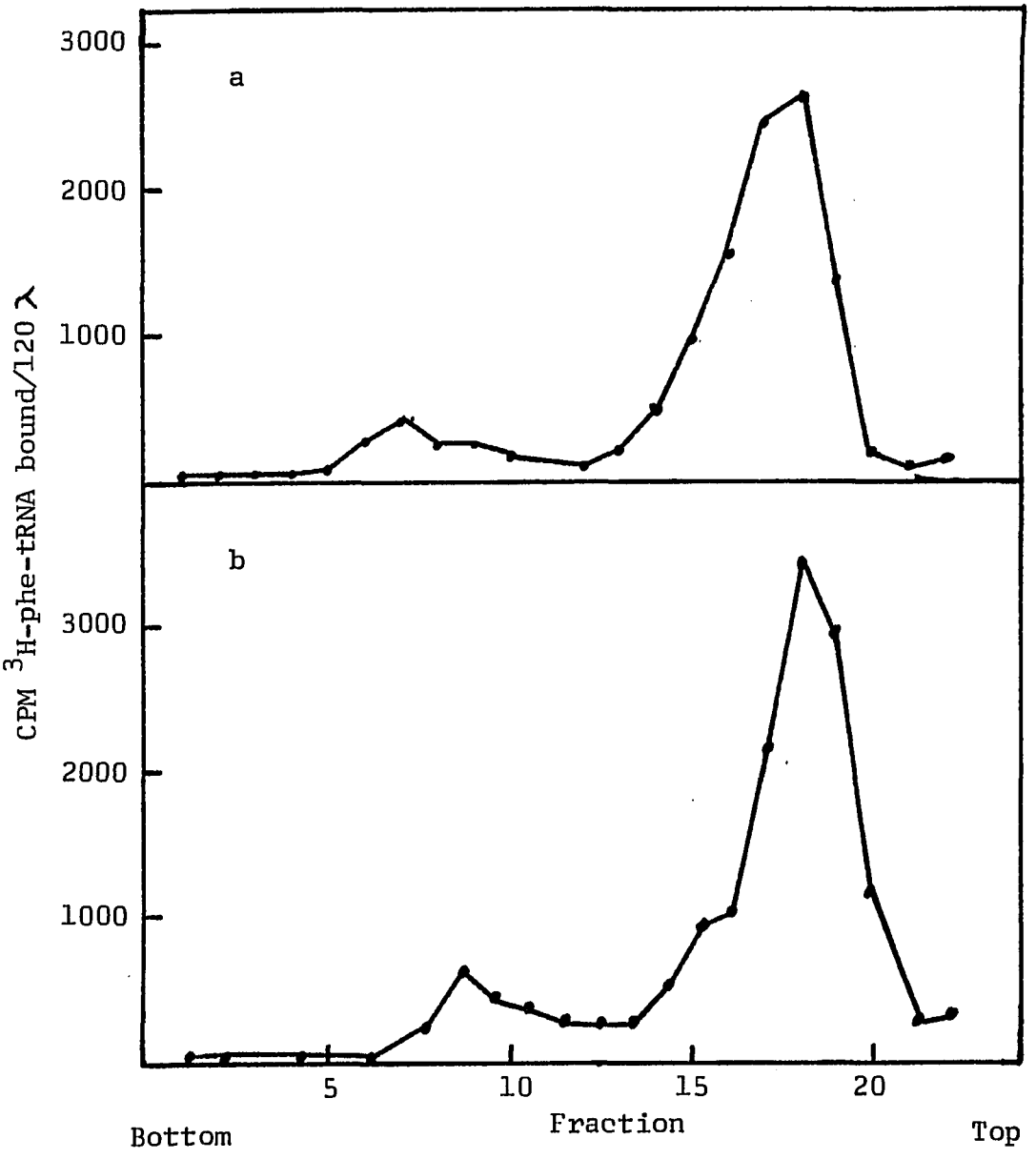
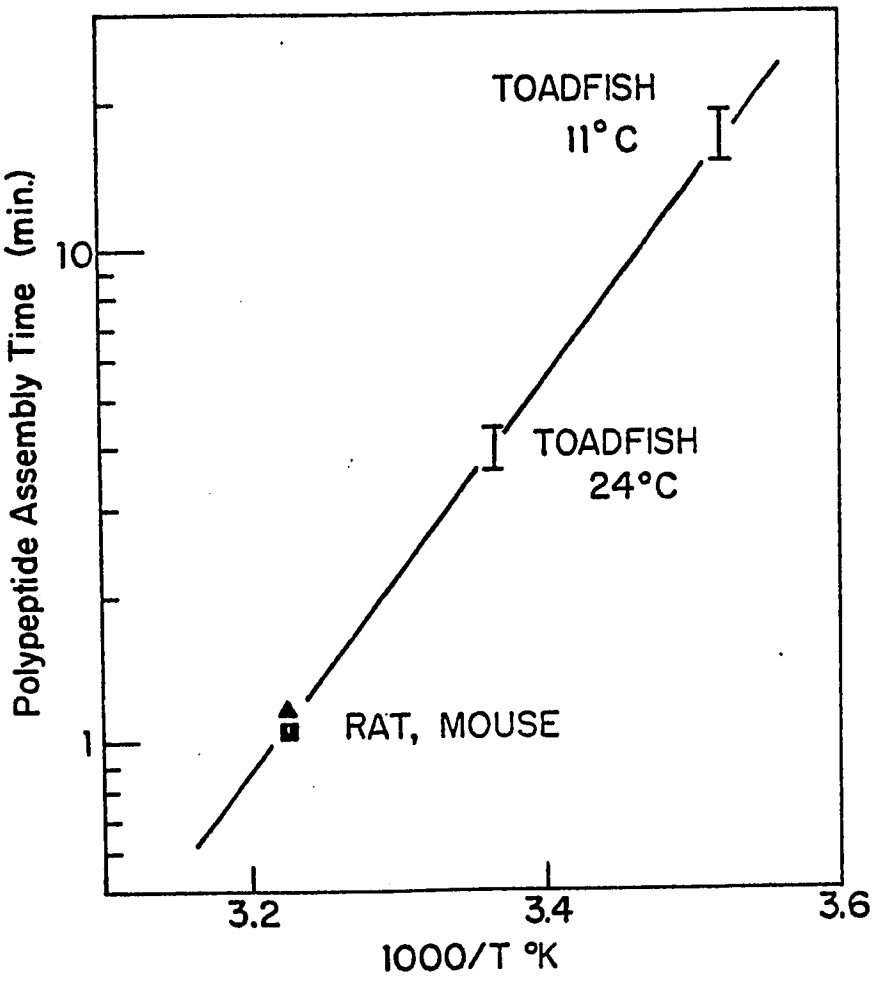

3000
2000
1000
a
b
3000
2000
1000
CPM ^{3}H-phe-tRNA bound/120 λ
5
10
15
20
Bottom
Fraction
Top

FIGURE 8

Arrhenius plot of average polypeptide chain assembly time determined in liver of toadfish (bars), rat (▲), and mouse (■) *in vivo*. Toadfish results presented with standard deviations; references given in text.

FIGURE 8


TOADFISH
11°C
TOADFISH
24°C
RAT, MOUSE
Polypeptide Assembly Time (min.)
10
1
3.2
3.4
3.6
1000/T °K

VIII. APPENDIX

A. EF-1 Activity in Liver from T3-treated and Control Rats

1. Characteristics of Polymerization Assay Using EF-2 Saturated Rat Liver Ribosomes

Initially elongation factor 1 activity from T3-treated and control rats was measured in a polymerization assay using EF-2 saturated rat liver ribosomes. Polyphenylalanine synthesis from ^{3}H-phe-tRNA was carried out as described in Methods. The dependency of the assay on pH 5 supernatant protein concentration is seen in Appendix Figure 1. Very low levels of incorporation were obtained below a protein concentration of 0.10 mg/ml. The dependency on protein concentration was sigmoidal. The approximately 2-fold increase in incorporation between 5 and 10 minutes indicated that the ribosomes were saturated with EF-2. The pH 5 supernatant from T3-treated rats was somewhat more active than that from control rats in this experiment and showed a similar sigmoid concentration dependency.

The temperature dependency of the polymerization assay with EF-2 saturated rat ribosomes is shown in Appendix Figure 2. A marked temperature dependency was noted: the Q_{10} was 7 when calculated for 25°-30°C and only 1.7 for 30°-35°C. Q_{10} values were calculated from the equation $Q_{10} = \left(\frac{K_1}{K_2}\right)^{10/(T_1-T_2)}$. In a similar poly(U) assay system using toadfish liver ribosomes saturated with EF-2,

Haschemeyer (1969c) reported a Q_{10} value of 7 for 20°-30°C. It can also be seen in Appendix Figure 2 that the reaction was linear up to a time of 10 min at the protein concentration used. No difference in temperature dependency was noted with pH 5 supernatant from T3-treated and control rats.

2. Comparison of EF-1 Activity of S-100, pH 5 Supernatant, and 0.5 M KCl Microsomal Wash Fractions

A comparison of EF-1 activity of S-100, pH 5 supernatant, and 0.5 M KCl microsomal wash fractions from T3-treated and control rats is presented in Appendix Table 1. The 0.5 M KCl wash was prepared from the microsomal pellets according to Shafritz and Anderson (1970). The polymerization assay was done with EF-2 saturated ribosomes and the binding assay (performed only on S-100 fractions) was done with the standard puromycin-treated ribosomes. The EF-1 activity of the microsomal washes represented 5% and 8% of that in the S-100 from T3-treated and control rats respectively. The pH 5 supernatants in all cases had a lower specific activity than the comparable S-100 and a total recovery of activity in the range of 40-50%. EF-1 activity was somewhat higher in S-100 from T3-treated as compared to control rats in these initial experiments. S-100 fractions were used for subsequent comparative studies of EF-1 activity in different thyroid states because the recoveries from pH 5 supernatants were too variable to yield reproducible results. The binding assay gave an apparent specific activity for EF-1 approximately 3 times greater than the polymerization assay. This

observation coupled with the problem of non-linearity with protein concentration in the polymerization assay led to the use of the binding assay for subsequent comparative work as discussed under Rat Results.

B. Purification of EF-1 from Rat Liver

Early efforts at EF-1 purification from rat liver pH 5 supernatants gave only marginal increases in specific activity and a very low recovery of total activity. The pH 5 supernatants were prepared from post-ribosomal supernatants in the following manner: titration to pH 5.0 with 1N acetic acid; centrifugation at 12,000 x g for 10 min; and neutralization of the resulting supernatant with 1N NaOH. Initial purification procedures involved adsorption of the neutralized pH 5 supernatant on Hypatite C followed by batch elution with increasing concentrations of potassium phosphate buffer, pH 6.8 (Schneir and Moldave, 1968). The fractions were concentrated by dialysis against solid polyethylene glycol 6000 (Union Carbide) and dialyzed overnight against 0.05M Tris-HCl, pH 8.0 before assay in the polymerization assay using EF-2 saturated ribosomes. The total recovery of activity in the Hypatite C fractions was only 10% of that in the pH 5 supernatant. The specific activity of the 0.25M phosphate eluate was slightly increased (1.5), but this fraction represented only 6% of the loaded activity. Repeated efforts were made with the Hypatite C procedure

under conditions of varying pH and phosphate buffer concentration but activity recoveries were not improved to an acceptable level.

Column chromatography was attempted with hydroxylapatite prepared according to Miyazawa and Thomas (1965). This procedure consisted of the following steps: S-100 was acidified to pH 5.0; pH 5 supernatant was titrated to pH 6.8 and adsorbed on a hydroxylapatite column; column was eluted with 0.05M, 0.125M and 0.25M potassium phosphate, pH 6.8, containing 0.001M 2-mercaptoethanol and 0.0001M EDTA. The active 0.25M phosphate eluate was concentrated by Amicon ultrafiltration using a PM-30 membrane. Phosphate was removed by passage through G-25 Sephadex equilibrated with 0.05M Tris-HCl, pH 8.0. The concentrated hydroxylapatite fraction was only 1.5 to 2-fold higher in specific activity than the pH 5 load and the total recovery of activity from the column was 10-20%.

Since chromatography of the pH 5 supernatant directly on hydroxylapatite gave such poor results, G-200 Sephadex was tried as an intermediate step. The neutralized pH 5 supernatant was precipitated with solid ammonium sulfate and the fraction precipitating at 30-70% saturation was loaded on G-200 Sephadex. The EF-1 activity was spread throughout the G-200 protein profile as seen in Appendix Figure 3. The largest peak, eluting at a total volume of 160 ml, would represent a size of about 200,000 daltons. The smallest

form, which eluted with the hemoglobin peak at a volume of 330 ml, is estimated at 60,000 to 70,000 daltons. At least one additional peak of EF-1 activity of intermediate molecular weight eluted at a volume of 250 ml from the G-200 Sephadex. Schneir and Moldave (1968) similarly observed three active peaks with G-200 Sephadex chromatography of rat liver EF-1. The active fractions from the major peak were pooled and assayed for polyphenylalanine synthesis using EF-2 saturated ribosomes. The specific activity of this fraction was 3-fold higher than the ammonium sulfate load.

This active G-200 fraction was subjected to column chromatography on hydroxylapatite with step-wise elution as described previously. The concentrated hydroxylapatite 0.25M phosphate eluate had a specific activity only half that of the G-200 load with a recovery of total activity of less than 50%.

Since recoveries were so low from both hydroxylapatite and G-200 Sephadex chromatography when pH 5 supernatants were used as the starting material, the pH 5 precipitation step was eliminated. S-100 from control rats was fractionated with solid ammonium sulfate, and the fraction precipitating at 30-70% was chromatographed on 4B Sepharose following McKeehan and Hardesty (1969). A representative profile is presented in Appendix Figure 4. Two peaks of EF-1 activity were observed. The higher molecular weight peak eluted

before EF-2 and the bulk of the S-100 proteins; a smaller EF-1 peak of lower molecular weight eluted in the same region as EF-2. While the larger form of EF-1 appeared to be separated from EF-2 and most of the other S-100 proteins, the specific activity was only slightly greater than the original load. In the polymerization assay, the following specific activity values expressed as (pmoles ^{3}H-polyphe/min/mg protein) were obtained: 37 for S-100; 75 for 30-70% ammonium sulfate; and 93 for the EF-1 large peak. In the binding assay, the specific activity values expressed as (pmoles ^{3}H-phe-tRNA bound/min/mg protein) were 94 for the S-100 and 139 for the EF-1 large peak. Thus an increase in specific activity of only 1.5-2.5 over the S-100 value was obtained with 4B Sepharose. The recovery of total loaded activity in the large EF-1 peak was approximately 2%. Collins et al. (1972) similarly found two peaks of rat liver EF-1 activity of molecular weights 200,000 and 80,000 daltons from 6B Sepharose, but they achieved a 6 to 7-fold purification of the large form activity. Various modifications of this procedure were attempted, such as the addition of bovine serum albumin to all buffers, but recovery of activity was not improved to an acceptable level.

EF-1 purification from rat liver was attempted following the procedure developed for low molecular weight EF-1 λ from pig liver (Iwasaki et al., 1974). This procedure consisted of: fractionation of S-15 in a polyethylene glycol-dextran

phase system, precipitation of the enzyme from the active phase with ammonium sulfate at 0-53% and 53-80% saturation, and chromatography of the ammonium sulfate fractions on carboxymethyl Sephadex by step-wise elution with increasing concentrations of NH_4Cl. With rat liver S-15 the phase separation system followed by ammonium sulfate precipitation produced activity recoveries of only 21% and 13% for the 0-53% and 53-80% ammonium sulfate cuts respectively. However, the fraction eluting from CM-Sephadex at 0.35M NH_4Cl was very active, showing a specific activity of 20-fold over the ammonium sulfate load and 40-fold over the S-15. Approximately 30% of the loaded activity was recovered in the 0.35M fraction, and the active fraction was stable on storage at -20°C or in liquid nitrogen.

The procedure was modified to substitute a pH 5.2 precipitation of the S-100 followed by ammonium sulfate fractionation at 0-30, 30-50, and 50-80% saturation instead of the phase system. The 50-80% ammonium sulfate fraction was passed through G-25 Sephadex and loaded on a CM-Sephadex column. The latter column was eluted step-wise with 0.05M, 0.165M, and 0.35M NH_4Cl containing 0.02M Tris-HCl (pH 7.5), 0.0001M EDTA, 0.005M 2-mercaptoethanol, and 25% glycerol. The results of this purification procedure are seen in Appendix Table 2. While activity was lost in the pH 5 precipitation, the remaining activity was completely recovered in the ammonium sulfate fractions; 70% of the EF-1 activity

precipitated between 50-80% saturation. The peak eluting from CM-Sephadex at 0.35M NH_4Cl had a specific activity increase of 28-fold compared to the ammonium sulfate load and 57-fold compared to the S-100. The recovery of the activity off the CM-Sephadex was 20% of the load, but since only the peak fractions were taken, the maximal recovery could be about 40%.

APPENDIX TABLE 1

Elongation factor 1 activity of liver supernatants (S-100), pH 5.0 fractions, and 0.5M KCl microsomal washes from T3-treated and control rats.

A. 2 animals each group, livers pooled, killed at 100 days of age, not starved.

B. 2 animals each group, livers pooled, killed at 86 days of age, not starved.

Group	No. of animals (pooled)	Average body wt. (grams)	% liver/ body wt.	Protein mg/ml Biuret	Polymerization activity	Binding activity
A. T3 S-100	2	240	3.3	26.5	48.3 ± 6.6 (5)	-
T3 pH 5				13.3	44.8 ± 2.1 (5)	-
T3 microsomal wash				3.5	37.3 ± 6.4 (5)	-
Control S-100	2	239	3.5	27.0	33.1 ± 5.3 (4)	-
Control pH 5				14.6	25.1 ± 2.8 (6)	-
Control microsomal wash				4.6	29.6 ± 7.9 (5)	-
B. T3 S-100	2	264	3.3	25.4	40.9 ± 5.3 (6)	129.1 ± 12.4 (10)
T3 pH 5				15.3	26.4 ± 5.6 (7)	-
Control S-100	2	293	4.0	24.1	31.4 ± 1.8 (2)	107.1 ± 6.9 (10)
Control pH 5				15.8	26.3 ± 4.0 (7)	-

Enzyme activity is expressed as pmoles of ^{3}H polyphe/min/mg protein in polymerization assay and as pmoles ^{3}H-phe-tRNA bound/min/mg protein in the binding assay. Results are given as mean ± standard deviation (number of assays). pH 5 supernatants were treated as described in legend to Appendix Figure 1.

APPENDIX TABLE 2

Purification of EF-1 binding activity from rat liver

Fraction	Volume ml	Protein mg/ml	Specific Activity units/mg	Total Units x 10^{-3}	Yield
S-100	188	27	93	472	100%
pH 5.2 supernatant	188	16	104	313	66%
30-50% AS	11.9	73	107	93	20%
50-80% AS	22.6	53	187	224	47%
0.35M NH_4Cl eluate from CM-Sephadex (50-80% AS load)	8.2	1	5320	44	9%

1 unit = 1 pmole ^{3}H-phe-tRNA bound/min in standard assay at 30°C for 5 min with puromycin-treated ribosomes.

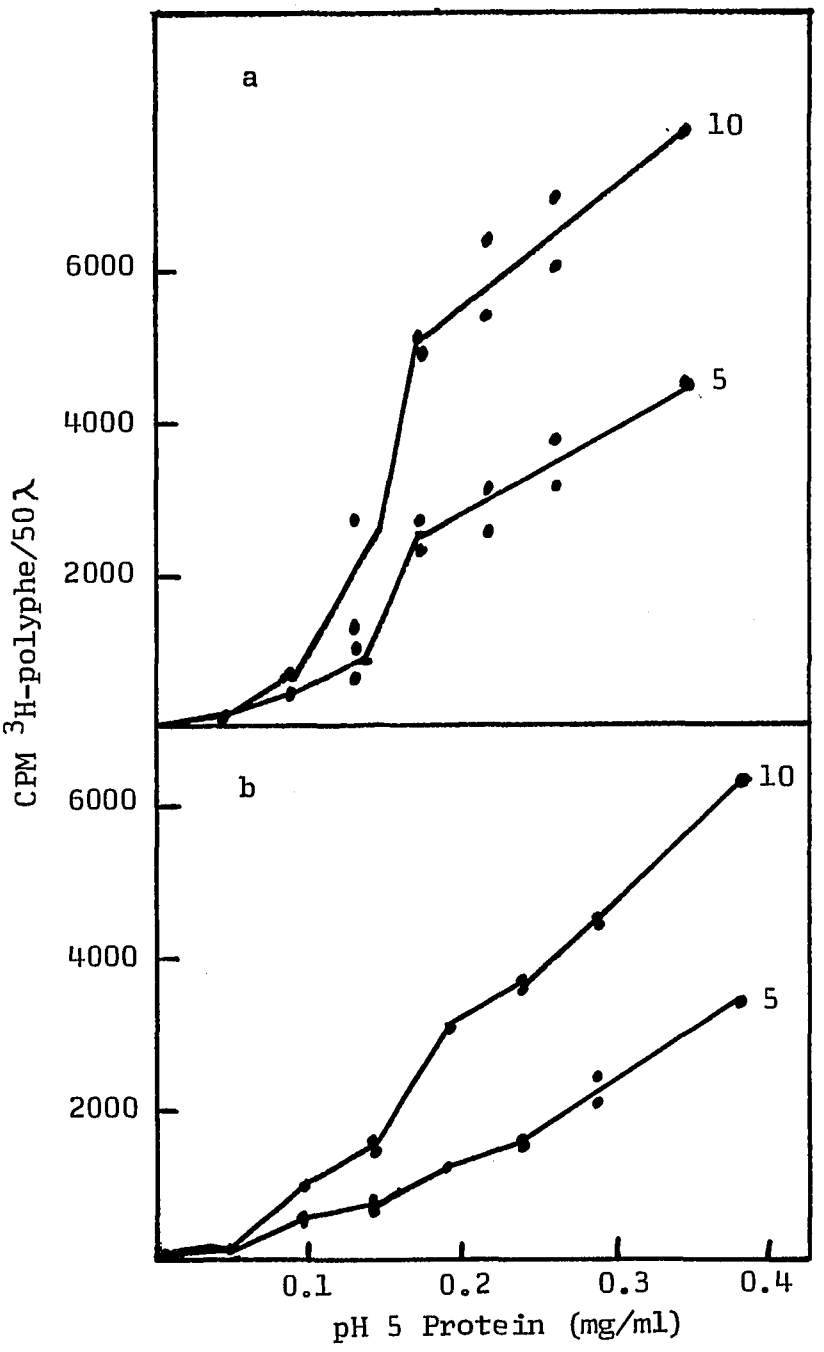
APPENDIX FIGURE 1

Protein concentration dependency of polymerization assay with EF-2 saturated rat liver ribosomes. Non-enzymatic polymerization values (82 cpm at 5 min and 165 cpm at 10 min) were subtracted from plotted results. Rat liver pH 5.0 supernatants were neutralized and passed through G-25 Sephadex equilibrated with 0.05M Tris-HCl, pH 8.0. Aliquots were taken after 5 and 10 min incubations at 30°C.

(a) T3-treated pH 5 supernatant

(b) Control pH 5 supernatant

APPENDIX FIGURE 1



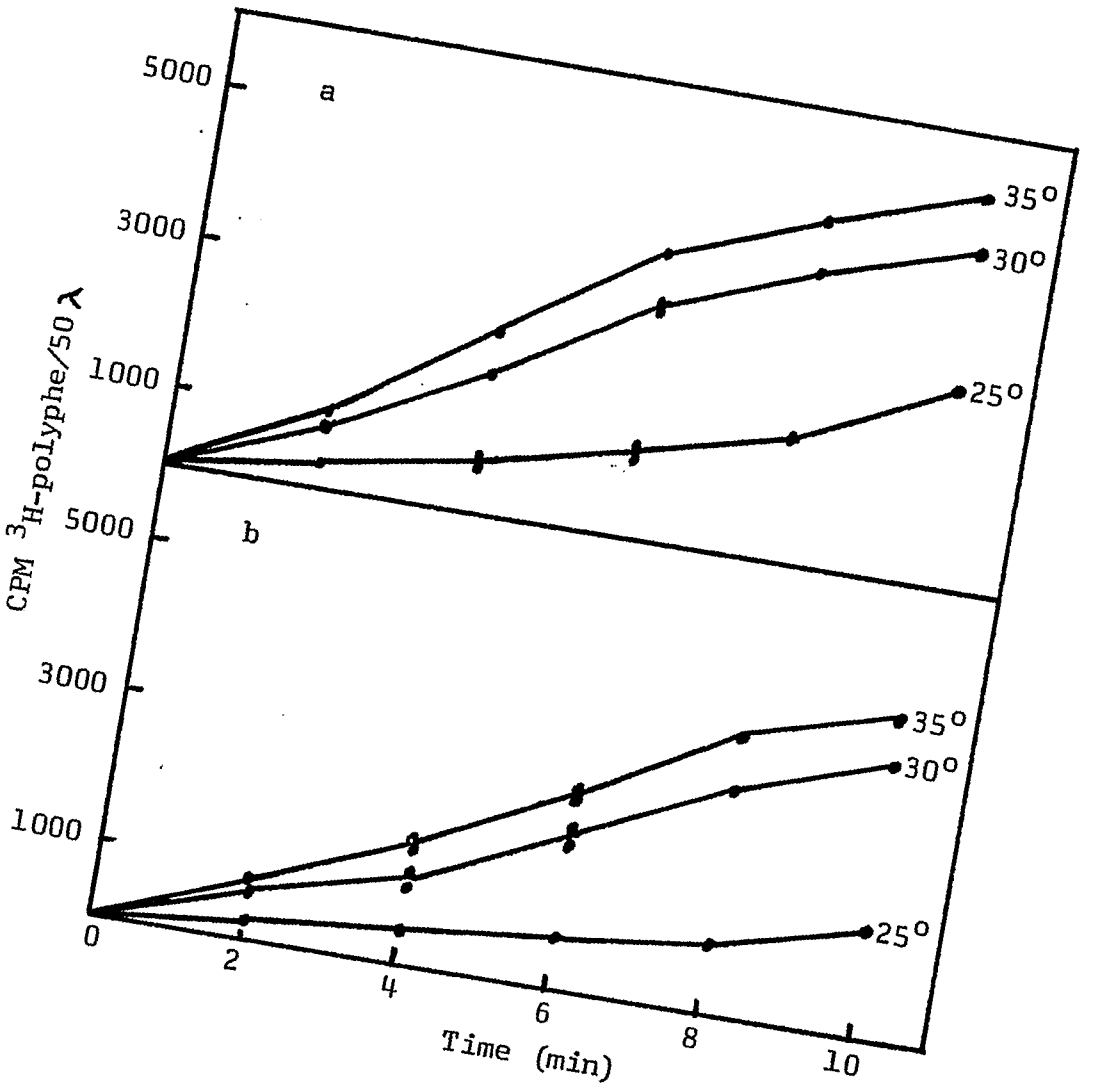
APPENDIX FIGURE 2

Temperature dependency of polymerization assay with EF-2 saturated rat liver ribosomes. Rat liver pH 5.0 supernatants were neutralized and passed through G-25 Sephadex equilibrated with 0.05M Tris-HCl, pH 8.0.

(a) T3-treated pH 5 supernatant at 0.22 mg/ml

(b) Control pH 5 supernatant at 0.24 mg/ml

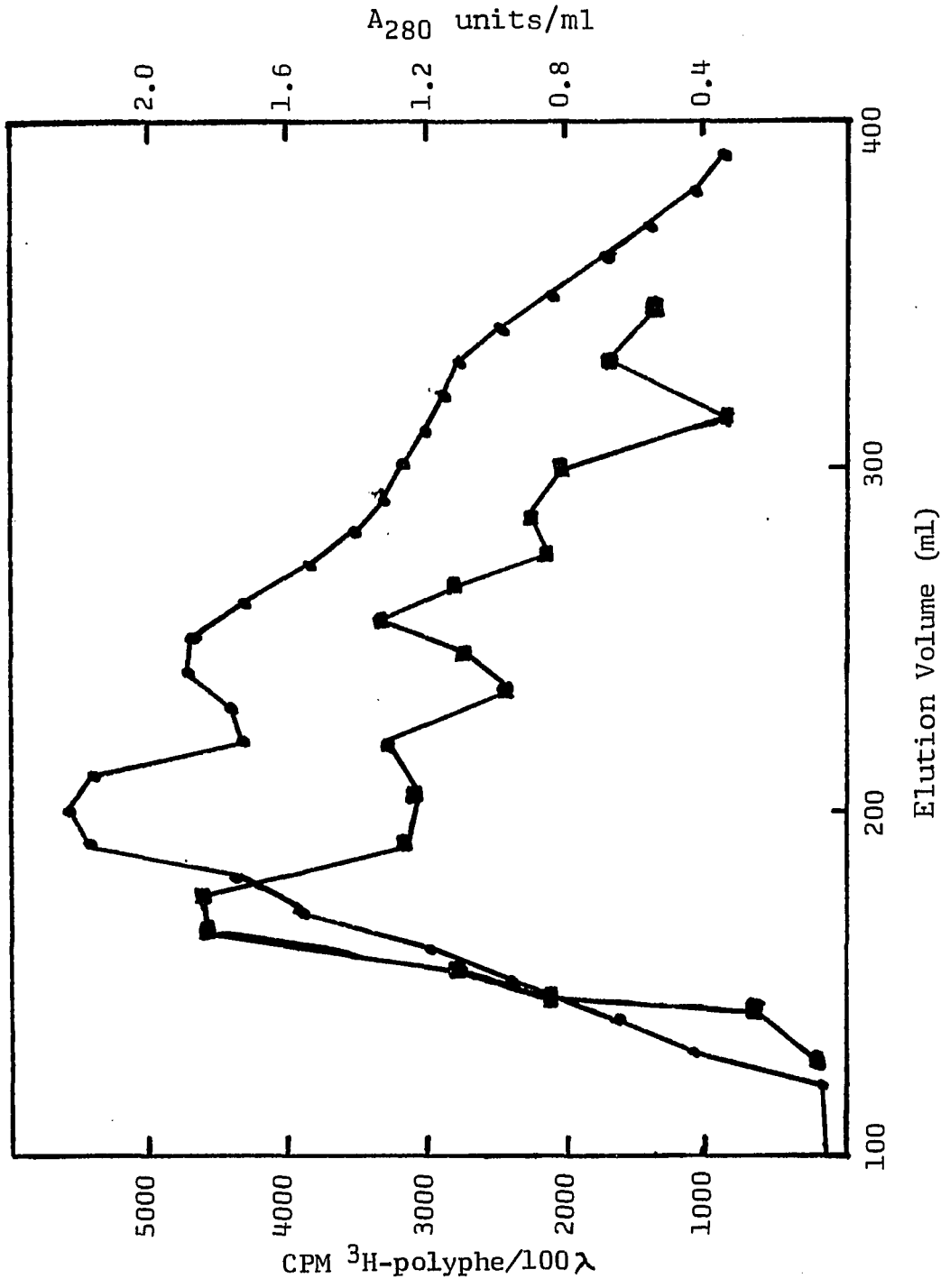
APPENDIX FIGURE 2



APPENDIX FIGURE 3

Chromatography of the 30-70% ammonium sulfate fraction on Sephadex G-200. 7 ml of a 30-70% ammonium sulfate fraction at a protein concentration of 43 mg/ml was passed through G-25 Sephadex and loaded on a 2.5x 83 cm G-200 Sephadex column. The column was equilibrated and eluted with 0.05M Tris-HCl (pH 8.0), 0.001M 2-mercaptoethanol, 0.0001M EDTA, and 0.25M KCl. The void volume of the column as determined by blue dextran was 120 ml. 0.02 ml column fraction was added to a 0.11 ml assay mixture, and 0.10 ml aliquots were taken after 10 min incubation at 30°C. EF-1 activity was assayed in the polymerization assay using EF-2 saturated ribosomes. Absorbance of each fraction was read at 280 nm. Volume of column fractions was 5 ml. (● absorbance at 280 nm, ■ EF-1 activity.)

APPENDIX FIGURE 3

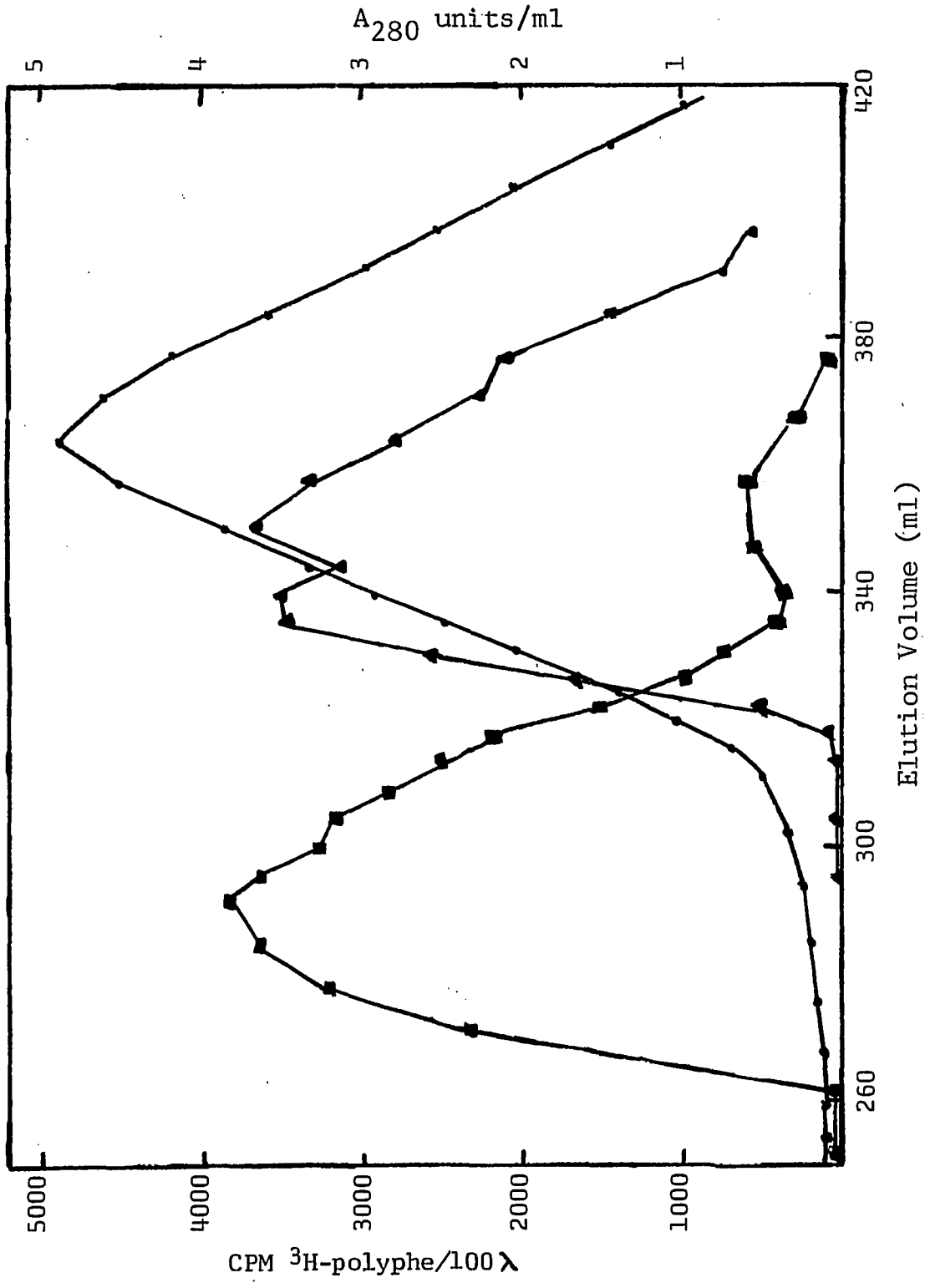


APPENDIX FIGURE 4

Chromatography of the 30-70% ammonium sulfate fraction on 4B Sepharose. 7 ml of a 30-70% saturation ammonium sulfate fraction at a protein concentration of 44 mg/ml was passed through G-50 Sephadex and loaded on a 2.5 x 83 cm 4B Sepharose column. The column was equilibrated and eluted with 0.05M Tris-HCl (pH 7.5), 0.25M KCl, 0.002M 2-mercaptoethanol, 0.0001M EDTA, and 5% glycerol. 0.01 ml of column fraction was added to a 0.11 ml assay mixture, and 0.10 ml aliquots were taken after 15 min incubation at 30°C. Both EF-1 and EF-2 polymerization activities were assayed from the column using puromycin-treated ribosomes and a saturating amount of the complementary factor. Absorbance of each fraction was read at 280 nm. Volume of column fractions was 3 ml. Active fractions were concentrated by Amicon ultrafiltration using a PM-10 membrane, passed through G-50 Sephadex to remove the KCl, and stored in liquid nitrogen.

(● absorbance at 280 nm, ■ EF-1 activity, and ▲ EF-2 activity.)

APPENDIX FIGURE 4



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