Prestayko, A. W., and Busch, H. (1968), *Biochim. Biophys. Acta 169*, 317.
Quincey, R. V., and Wilson, S. H. (1969), *Proc. Nat. Acad. Sci. U. S. 64*, 981.
Shaw, L., and Huang, R. C. (1970), *Biochemistry 9*, 4530.
Shih, T., and Bonner, J. (1969), *Biochim. Biophys. Acta 182*, 30.
Sivolap, Y., and Bonner, J. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 387.
Smith, L. D., Armstrong, J., and McCarthy, B. J. (1968), *Biochim. Biophys. Acta 142*, 323.
Sober, H. A. (1968), Handbook of Biochemistry, Cleveland, Ohio, Chemical Rubber Co.
Szeszák, F., and Phil, A. (1972), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 20*, 177.
Tissières, A. (1959), *J. Mol. Biol. 1*, 365.
Tolstoshev, P., and Wells, J. R. E. (1973), *Biochem. Biophys. Res. Commun. 51*, 223.
Wang, T. Y. (1967), *J. Biol. Chem. 247*, 1220.
Wetmur, J. B., and Davidson, N. (1968), *J. Mol. Biol. 31*, 349.
Wilkes, M. M., Holmes, D. S., and Bonner, J. (1974), in preparation.

Cell-Free Synthesis of Amino-Terminal L-Pyroglutamic Acid†

George H. Jones

ABSTRACT: The cell-free synthesis of amino-terminal L-pyroglutamic acid has been demonstrated using a microsomal protein-synthesizing system prepared from the plasma cell tumor, RPC-20. Glutamic acid is the precursor of the pGlu and the conversion occurs in the RPC-20 microsomal system using RPC-20 or rat liver glutamyl-tRNA. pGlu-containing peptides were isolated by subtilisin digestion of the products of cell-free protein synthesis, and pGlu was released by treatment of these peptides with the bacterial enzyme, pyrrolidonyl peptidase. Two isoaccepting glutamyl-tRNA species were obtained on reverse phase chromatography of RPC-20 tRNA. Both these tRNAs function in cell-free polypeptide synthesis on RPC-20 microsomes and both Glu-tRNAs can participate in the synthesis of pGlu. These results are discussed in terms of previously suggested functions for pGlu in protein biosynthesis.

L-Pyroglutamic acid (pyrrolidonecarboxylic acid) is the amino-terminal amino acid of a number of naturally occurring peptides and proteins. These include the algal peptides eisenine and fastigiatine (Ohira, 1939; Dekker *et al.*, 1949), the hypotensive undecapeptide, eledoisin (Erspamer and Anastasi, 1962), fibrinogen (Blomback *et al.*, 1963), and the heavy and light chains of immunoglobulins (Wilkinson *et al.*, 1966; Apella and Perham, 1968). Although the chemistry of pGlu[1] and pGlu-containing peptides has been studied in some detail, little is known about the biosynthesis of this unusual amino acid. Recently, Twardzik and Peterkofsky (1972) have demonstrated that mouse plasmacytoma cells can incorporate exogenous glutamic acid into N-terminal pGlu. This study marked the first conclusive demonstration of the synthesis of pGlu from glutamic acid. Early studies of pGlu biosynthesis using glutamine (Bernfield and Nestor, 1968) were complicated by the fact that glutamine cyclizes easily to pGlu under midly acidic or basic conditions (Blomback, 1967). With glutamic acid this artifactual cyclization is not a serious problem.

The results of Twardzik and Peterkofsky (1972) identified glutamic acid rather than glutamine as the direct precursor of pGlu, and suggested that pGlu was formed by an enzymatic reaction, but these studies gave no indication of the actual mechanism of cyclization of glutamic acid to pGlu. It has been suggested (Rush and Starr, 1970; Twardzik and Peterkofsky, 1972) that the cyclization occurs after attachment of glutamic acid to a specific glutamyl-tRNA. The pGlu-tRNA thus formed could then participate in the initiation of protein synthesis in certain mammalian systems. To examine this possibility, the synthesis of pGlu has been studied using a cell-free amino acid incorporating system from the mouse plasmacytoma, RPC-20. The specificity of the tumor glutamyl-tRNAs and rat liver tRNA for this synthesis has been studied and the results form the substance of this report.

Material and Methods

Materials. The RPC-20 plasma cell tumor was maintained as previously described (Bridges and Jones, 1973). [^{3}H]Glutamic acid (13.5 Ci/mmol) was supplied by Schwarz/Mann. L-Pyroglutamic acid was purchased from Sigma Chemical Co. and L-pyroglutamyl-L-alanine was supplied by Cyclo Chemical Co. Subtilisin was from Nutritional Biochemicals and ethylenimine was obtained from K & K Chemicals. A sample of dried *Aerobacter cloacea* was generously donated by Dr. Alan Peterkofsky. Pyrrolidonyl peptidase was prepared from *A. cloacea* cells essentially as described by Doolittle and Armentrout (1968) except that the 30,000*g* supernatant was brought to 50% saturation with ammonium sulfate. After Sephadex G-200 chromatography, the enzyme was stored as a slurry in 60% ammonium sulfate and was stable for several months.

Preparation of Components for Cell-Free Protein Synthesis. tRNA, microsomes, and soluble enzymes were prepared from the RPC-20 tumor as previously described (Bridges and Jones, 1973). Rat liver tRNA was prepared by the same procedure as that used for RPC-20 tRNA.

† From the Department of Zoology, University of Michigan, Ann Arbor, Michigan 48104. *Received August 6, 1973.* This work was supported by Research Grant 5R01-CA-12752-02 from the National Cancer Institute, U. S. Public Health Service.

[1] Abbreviations used are: pGlu, L-pyroglutamic acid; pGlu-Ala, L-pyroglutamyl-L-alanine.

Synthesis and Isolation of pGlu. Conditions for protein synthesis on RPC-20 microsomes were essentially as described previously (Bridges and Jones, 1973). Incubation mixtures contained per ml: Tris-HCl, pH 7.6, 50 μmol; $MgCl_2$, 3.75 μmol; KCl, 65 μmol; 2-mercaptoethanol, 5 μmol; ATP, 5 μmol; GTP, 0.5 μmol; creatine phosphate, 10 μmol; creatine kinase, 167 μg; 10 nonradioactive amino acids minus glutamic acid, 0.1 μmol; RPC-20 synthetase preparation, 1 mg; and, RPC-20 microsomes, 1.6 mg of protein. In some experiments, reaction mixtures also contained RPC-20 tRNA, 1.86 A_{260}/ml or rat liver tRNA, 3.06 A_{260}/ml and [^{3}H]glutamic acid, 120 μCi/ml. In other experiments, the RPC-20 isoaccepting glutamyl-tRNA species were present at a level of 10^6 cpm/ml, and [^{12}C]glutamic acid was added to 0.125 μmol/ml.

In preliminary experiments, 0.1-ml incubations were conducted and processed as described previously (Bridges and Jones, 1973). For the preparation of pGlu, incubation mixtures were scaled up to 2–3 ml. After incubation at 37° for 30 min, trichloroacetic acid was added to a final concentration of 10% and the samples were heated for 10 min at 90°. No conversion of glutamic acid to pGlu was caused by this treatment. The protein precipitates were washed and dried as described (Bridges and Jones, 1973) and dissolved in 2.5–5 ml of 8 M urea titrated to pH 8.6. The proteins were then reduced and aminoethylated with ethylenimine by the procedure of Raftery and Cole (1963). For this part of the experiment, the assumption was made that all the proteins present had a molecular weight of 25,000, the molecular weight of immunoglobulin light chains. Derivatized proteins were collected by dialysis and lyophilization.

The dried protein was suspended in 0.8% ammonium bicarbonate and digested with subtilisin at an enzyme–substrate ratio of 1:20. Digestion was continued for 12 hr at 37° after which the digests were lyophilized. Dried subtilopeptides were dissolved in distilled water and applied to 1 × 10 cm columns of Dowex 50 H^+ (200–400 mesh). Nonabsorbed peptides were eluted with distilled water and lyophilized. Fractionation of these peptides was accomplished by paper electrophoresis on Whatman 3MM filter paper in pyridine–acetate (pH 6.5 or 3.5) at 3000 V for 1–1.5 hr, or by ascending paper chromatography on Whatman 3MM filter paper in water-saturated phenol. Papers were cut into 0.5-in. strips and counted in a liquid scintillation counter. In some experiments, peptide peaks were eluted from the papers with distilled water and lyophilized.

Subtilopeptides, before or after separation by paper electrophoresis, were digested with pyrrolidonyl peptidase in a volume of 1 ml containing 50 mM phosphate buffer (pH 7.3), 30 mM 2-mercaptoethanol, and 1 mM EDTA. Digestion was carried out at 32° for 12 hr with the addition of 0.6 mg of enzyme at time zero and an additional 0.6 mg after 6 hr. Digested samples were lyophilized and suspended in water, and insoluble material was removed by centrifugation. pGlu was identified by paper chromatography and electrophoresis as described above.

Reverse-Phase Chromatography. Acylation of RPC-20 tRNA was performed as described previously (Bridges and Jones, 1973). Sixty A_{260} units of RPC-20 [^{3}H]glutamyl-tRNA (26 × 10^6 cpm) were applied to an RPC-5 column (Pearson *et al.*, 1971) and eluted with a linear gradient of 0.5–0.8 M NaCl in 0.01 M sodium acetate–0.01 M $MgCl_2$–0.001 M 2-mercaptoethanol. Aliquots (100 μl) from alternate column fractions were assayed and the isoaccepting peaks of Glu-tRNA were precipitated with 2.5 volumes of cold absolute ethanol. [^{3}H]Glu-tRNAs were collected by centrifugation at 13,000*g* for 10 min, and dissolved in water. Analytical RPC-5 chromatography of rat liver tRNA was performed as described above with 1.5 A_{260} units of rat liver [^{3}H]glutamyl-tRNA and 60 A_{260} units of *Escherichia coli* tRNA (added as carrier).

Other Methods. Liquid scintillation counting was performed in a Beckman LS-250 liquid scintillation spectrometer using toluene–Omnifluor scintillation cocktails. Standard pGlu was identified after paper chromatography or electrophoresis using the starch–iodide reagent of Rydon and Smith (1952).

Results

Synthesis and Identification of N-Terminal pGlu. The RPC-20 tumor produces a λ-immunoglobulin chain of known amino acid sequence (Apella, 1971). This light chain was previously shown to possess amino-terminal pGlu (Apella and Perham, 1968). Since active, tRNA-dependent cell-free protein-synthesizing systems have been prepared from plasma cell tumors (Mach *et al.*, 1967; Bridges and Jones, 1973), the RPC-20 system seemed an excellent one in which to study the synthesis of pGlu. The steps involved in the isolation and analysis of pGlu may be summarized as follows: (1) incorporation of [^{3}H]glutamic acid into protein by RPC-20 microsomes; (2) aminoethylation and subtilisin digestion of the proteins synthesized "*in vitro*;" (3) isolation of acidic (pGlu-containing) subtilopeptides; (4) release of pGlu from the acidic peptides.

As the initial approach to the problem, the incorporation of [^{3}H]glutamic acid by RPC-20 microsomes was optimized. It was reasoned that the incorporation of glutamic acid at or near the amino terminus of the growing polypeptide chain would require conditions which were optimal for the initiation of protein biosynthesis. Thus, incubations were performed at an Mg^{2+} concentration of 3.75 mM as this was found to be the optimum for incorporation of methionine from Met-$tRNA_F$ (unpublished results). The dependency of [^{3}H]-glutamic acid incorporation on added microsomes and on RPC-20 and rat liver tRNAs is shown in Figure 1a,b. Some incorporation was generally observed in the absence of added tRNA, and both RPC-20 and rat liver tRNAs stimulated this incorporation by a factor at at least five (Figure 1b). In addition, some microsomal preparations were totally dependent on added tRNA for activity. The maximum levels of incorporation with RPC-20 and rat liver tRNAs were similar but about 3.5 times as much rat liver as RPC-20 tRNA was required to achieve this level of incorporation (Figure 1b).

Once the optimum conditions for glutamic acid incorporation were established, attempts were made to identify pGlu as one of the products of this incorporation. Subtilisin digestion of the aminoethylated RPC-20 light chain has been shown to produce short pGlu-containing peptides (Apella and Perham, 1968). These pGlu peptides can be separated from the remaining subtilopeptides by passage of the digests through short columns of Dowex 50. The N-blocked peptides are acidic and can be recovered in the effluent after a water wash, while the remaining peptides are retained by the column. This procedure was used to isolate acidic peptides after a large-scale incubation using RPC-20 or rat liver tRNAs. The percentage of the total radioactivity recovered in the effluent after Dowex 50 chromatography is given in Table I, and the electrophoretic pattern of the recovered peptides is shown in Figure 2a for the incubation with RPC-20 tRNA. It can be seen from Table I that 3.5 and 2.7% of the total radioactivity was recovered in the Dowex 50 effluents from the incubations

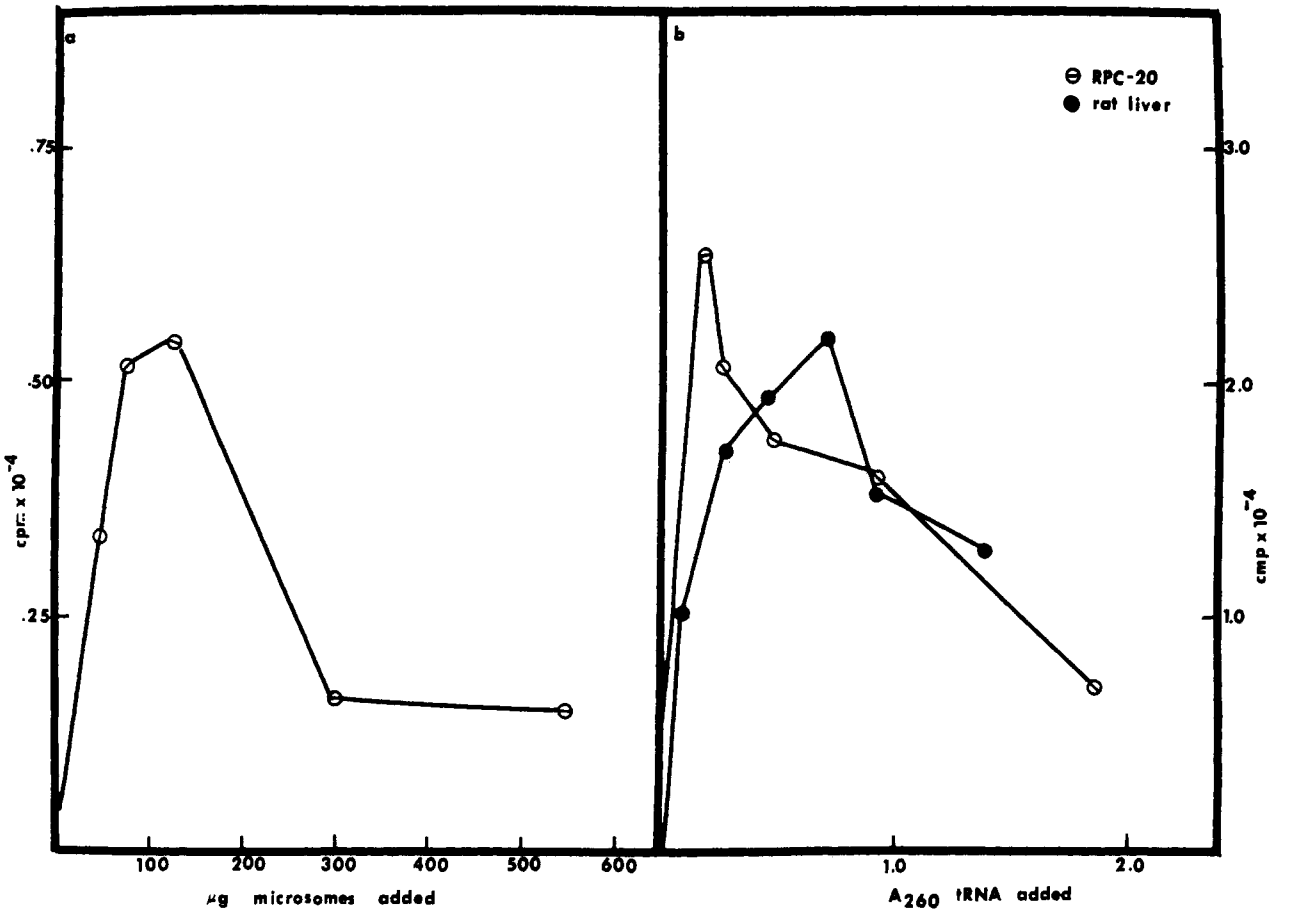

FIGURE 1: Incorporation of [^{3}H]glutamic acid by RPC-20 microsomes. Incubations were conducted in a volume of 0.1 ml and processed as described in Materials and Methods and in Bridges and Jones (1973). In part a the dependency on added microsomes is shown, and in part b the stimulation of incorporation by added RPC-20 and rat liver tRNA is depicted.

with RPC-20 and rat liver tRNA, respectively. The RPC-20 light chain contains about ten glutamic acid residues and it is known that conversion of glutamic acid to glutamine does not occur in this system (Twardzik and Peterkofsky, 1972). Thus, one would predict that 10% of the total radioactivity incorporated into L chain would be converted to pGlu and would be recoverable in the acidic peptide fraction. The recovery of only 2.5–3.5% of the total may reflect the inefficiency of initiation of protein synthesis "*in vitro*" and/or the fact that the RPC-20 system synthesizes proteins other than L chain which contain no pGlu.

The electrophoretic pattern of Figure 2a reveals the existence of two migrating peaks (A and B) in addition to a small amount of radioactivity which remains at the origin. Peaks A and B may contain the two acidic peptides obtained from the purified RPC-20 light chain, whose amino acid sequences are pGlu-Ala-Val-Val and pGlu-Ala-Val-Val-Thr (Apella and Perham, 1968). A similar pattern was observed for the peptides obtained from digests of the incubation with rat liver tRNA. The relative amounts of these two peptide fractions in the Dowex 50 effluents are given in Table II. The products derived from incubations with both RPC-20 and rat liver tRNAs contain peptides A and B in a ratio of about 1:2.

Further analysis of the [^{3}H]glutamic acid labeled acidic peptides was performed using the microbial enzyme, pyrrolidonyl peptidase. This enzyme specifically removes pGlu from the amino terminus of peptides and has a high affinity for the

TABLE I: Recovery of Acidic Subtilopeptides from Incubations of RPC-20 Microsomes with Various tRNA Preparations.[a]

tRNA Source	Incubn Vol (ml)	Total cpm Incorpd	cpm in Acidic Peptides	% of Total cpm in Acidic Peptides
RPC-20	3	574,880	19,854	3.5
Rat liver	2	572,800	15,498	2.7
Glu-tRNA$_I$	2	106,720	6,810	6.3
Glu-tRNA$_{II}$	2	129,440	10,080	7.8

[a] Incubations were as described in Materials and Methods. The acidic peptide fraction represents the radioactive subtilopeptides which pass through Dowex 50 at neutral pH.

TABLE II: Relative Recovery of Peptide Peaks A and B after Paper Electrophoresis of Acidic Subtilopeptides.[a]

tRNA Source	Total cpm (A + B)	%A	%B
RPC-20	2142	30.5	69.5
Rat liver	2170	31.8	68.2
Glu-tRNA$_I$	596	65.3	34.7
Glu-tRNA$_{II}$	937	60.2	39.8

[a] The values in this table were obtained by summing the counts per minute in the A and B regions of the pherograms of Figure 3 and determining the percentage of the total represented by A and B.

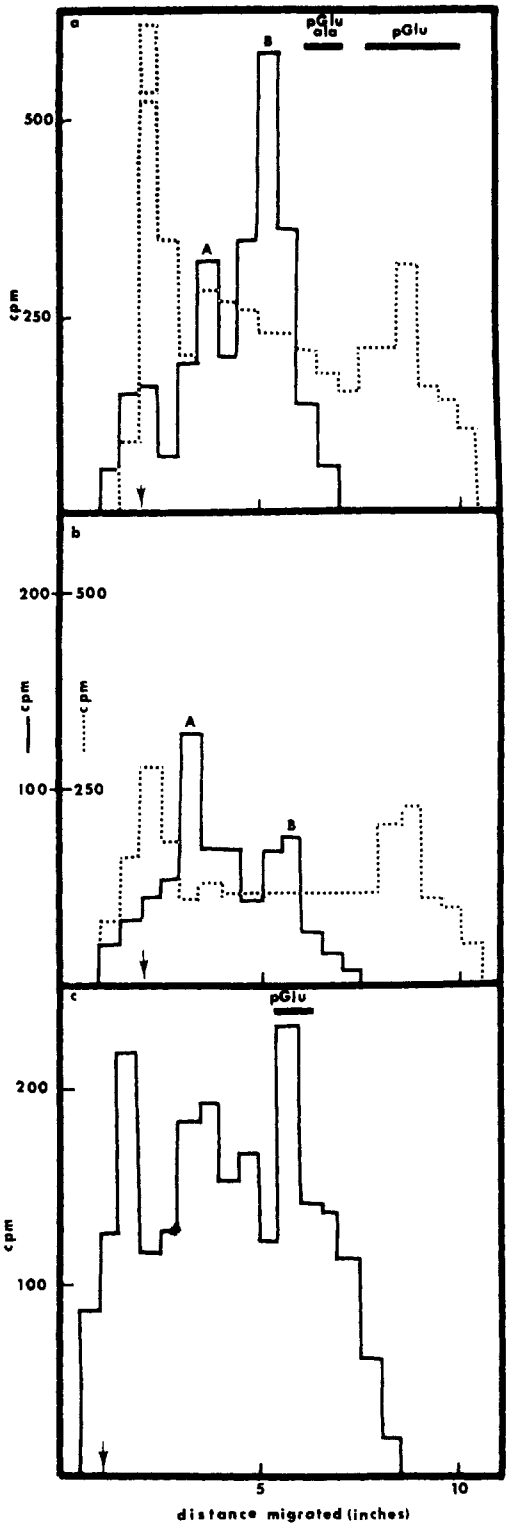

FIGURE 2: Electrophoretic analysis of acidic subtilopeptides and of the pyrrolidonyl peptidase digests of these acidic peptides. (a) Solid line, electrophoresis (3000 V, 40 min, pH 6.5) of the acidic subtilopeptides obtained from a 3-ml incubation of RPC-20 microsomes with RPC-20 tRNA and [^{3}H]glutamic acid (see Materials and Methods); dashed line, electrophoretic analysis of the products of the pyrrolidonyl peptidase digestion of the acidic subtilopeptides described above. (b) Solid line, electrophoretic analysis of the acidic subtilopeptides from the incubation of RPC-20 microsomes with [^{3}H]Glu-tRNA$_I$; dashed line, electrophoretic analysis of the pyrrolidonyl peptidase digest of those acidic peptides. (c) Paper chromatography (ascending, water-saturated phenol) of the pyrrolidonyl peptidase digest of the acidic peptides from the incubation described in part a. The migratory positions of pGlu and pGlu-Ala are shown for comparison. The arrow denotes the origin in each case.

TABLE III: Relative Recovery of pGlu after Digestion of Acidic Subtilopeptides with Pyrrolidonyl Peptidase.[a]

tRNA Source	% pGlu Recovd
RPC-20	27.7
Rat liver	26.8
Glu-I	43.1
Glu-II	40.3

[a] The values in this table were obtained by summing the counts per minute in the pherograms of the pyrrolidonyl peptidase digested acidic peptides (Figure 3a,b). The percentage of the total counts per minute represented by pGlu was then determined.

amino acid sequence pGlu-Ala (Doolittle and Armentrout, 1968). The combined acidic peptides described in Figure 2a were digested with pyrrolidonyl peptidase at 32° for 12 hr. (In a preliminary study, it was shown that both fractions A and B contained pGlu.) The products of this digestion were analyzed by paper electrophoresis at 6.5 and pH 3.5 and by paper chromatography in water-saturated phenol. The results of the pH 6.5 electrophoresis are shown in Figure 2a for the RPC-20 peptides. There remains some radioactivity at the origin and a band of radioactivity is observed with a mobility similar to that of the Dowex 50 acidic peptides. In addition, there is a distinct peak of radioactivity which moves ahead of fraction B and which has a mobility identical with authentic pGlu. This peak is absent from the pherogram of the untreated peptides. Similar results were obtained for the peptides derived from the incubation with rat liver tRNA and when the electrophoretic analysis was performed at pH 3.5. Paper chromatography also revealed the existence of pGlu among the products of pyrrolidonyl peptidase digestion (Figure 2c). The total counts per minute recovered as pGlu for each preparation is given in Table III. Both the RPC-20 and rat liver acidic peptides yielded about 28% pGlu.

Thus, the RPC-20 microsomal system is capable of synthesizing pGlu from exogenous glutamic acid, and both RPC-20 and rat liver tRNAs can function effectively in this synthesis.

Specificity of RPC-20 tRNAs for pGlu Synthesis. Reports from a number of laboratories have suggested the possibility that pGlu formation occurs after the esterification of glutamic acid or glutamine to a specific tRNA (Bernfield and Nestor, 1968; Rush and Starr, 1970; Twardzik and Peterkofsky, 1972). To test this hypothesis, the isoaccepting glutamyl-tRNA species from the RPC-20 tumor were fractionated by reverse-phase chromatography. The pattern obtained is shown in Figure 3 and reveals two peaks of acceptor activity for glutamic acid. A distinctly similar pattern was obtained for rat liver tRNA except that the ratio of the two isoaccepting species was slightly different (Figure 3, inset). These two tRNAs will be referred to as Glu-tRNA$_I$ and Glu-tRNA$_{II}$, corresponding to their order of elution from the RPC-20 column. The relevant fractions from the RPC-20 column run were pooled as indicated in Figure 3 and precipitated with ethanol. The recovery of A_{260} in Glu-tRNA peaks I and II was 4.9 and 3.5 units, respectively.

The [^{3}H]Glu-tRNAs were used for cell-free protein synthesis under the conditions described in Materials and Methods. The dependency of [^{3}H]Glu incorporation on added microsomes is shown in Figure 4. It can be seen that when equivalent amounts of [^{3}H]glutamic acid (as Glu-tRNA) were

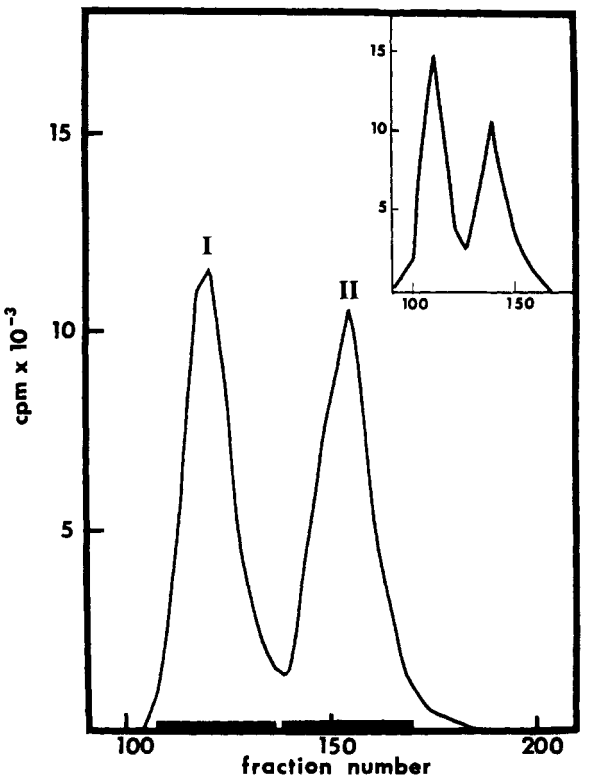

FIGURE 3: Reverse-phase chromatography (RPC-5) of RPC-20 and rat liver [^{3}H]glutamyl-tRNAs. Conditions were as described in Materials and Methods and in Bridges and Jones (1973), and 60 A_{260} units were applied. tRNAs were eluted with a linear gradient of 0.5–0.8 M NaCl in 900 ml, and 2.5-ml fractions were collected. Fractions containing the two RPC-20 Glu-tRNAs were pooled and concentrated. The inset shows the pattern obtained on analysis of rat liver Glu-tRNA by RPC-5 chromatography.

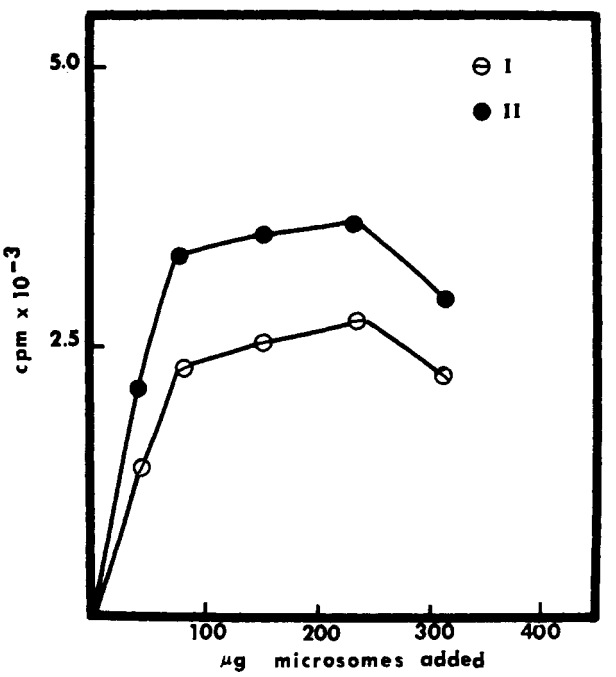

FIGURE 4: Incorporation of [^{3}H]glutamic acid into protein using RPC-20 Glu-tRNAs I and II. Incubation conditions were as described in Materials and Methods. Each 0.1-ml reaction mixture contained 100,000 cpm (about 10 pmol) of [^{3}H]glutamyl-tRNA and 12.5 nmol of [^{12}C]glutamic acid. The quantity of microsomes added was varied in the experiments shown here.

present in the incubation mixtures, the efficiency of incorporation from Glu-tRNA$_{II}$ was some 30% higher than that from Glu-tRNA$_I$.

To examine the specificity of these two tRNAs for pGlu formation, large-scale incubations were performed and analyzed as described above. An acidic peptide fraction was obtained from the incubations with both Glu-tRNAs. The recovery of these peptides after Dowex 50 chromatography is given in Table I and the electrophoretic pattern at pH 6.5 is shown in Figure 2b for Glu-tRNA$_I$. A similar electrophoresis pattern was obtained for Glu-tRNA$_{II}$, and these patterns are essentially identical with those obtained from incubations with total RPC-20 or rat liver tRNAs (Figure 2a). The data of Table I indicate that the percentage of the total radioactivity recovered as acidic peptides is somewhat higher when the isoaccepting Glu-tRNAs are used for protein synthesis as compared with experiments in which total tRNA was used. Further, the ratios of peaks A and B (Figure 2b) from the incubations with the fractionated Glu-tRNAs are different from those obtained with total tRNA. Thus, the ratio of A to B was about 2:1 for Glu-tRNA I and II as compared with a ratio of 1:2 for the peptides obtained from incubations with total tRNA (Table II). These differences may reflect the absence of certain tRNA species from the individual preparations of Glu-tRNAs I and II which are required for efficient elongation in the RPC-20 system.

Pyrrolidonyl peptidase digestion of the acidic peptides from incubations with both Glu-tRNAs I and II released pGlu. A typical electrophoretic analysis is shown in Figure 2b for a Glu-tRNA$_I$ digest. A similar pattern was obtained for the Glu-tRNA$_{II}$ digest. The total radioactivity recovered as pGlu from the Glu-tRNA incubations is given in Table III. Both tRNAs yielded about 40% pGlu, about 15% more than the amount observed after analysis of the products from incubations with total tRNA.

Discussion

The synthesis of pGlu in biological systems has previously been studied by a number of investigators. Moav and Harris (1967) presented some evidence against the direct incorporation of pGlu into protein and favoring the formation of pGlu on tRNA. Similarly, Rush and Starr (1970) reported that free pGlu can be converted to glutamic acid which can subsequently be esterified to tRNA and recyclized. Bernfield and Nestor (1968) have reported an extremely interesting and unusual reaction in which glutaminyl-tRNA can be converted to pGlu-tRNA by an enzyme from papaya latex, glutamine cyclotransferase. This type of enzymatic activity has also been reported in rat liver (Niwaguchi *et al.*, 1964). The data of Twardzik and Peterkofsky (1972), however, would seem to indicate that, in the plasmacytoma system at least, it is glutamic acid rather than glutamine which is the precursor of pGlu.

The present study was undertaken with two objectives in mind, first, to demonstrate the cell-free synthesis of pGlu, and, second, to examine the role of tRNA in this synthesis. Because pGlu is an N-blocked amino acid, and because it possesses some structural similarity to *N*-formylmethionine, it has been suggested that pGlu serves as an initiator amino acid in certain mammalian systems (Rush and Starr, 1970; Baglioni, 1970). It was of particular interest to determine whether the biosynthesis of peptide-bound pGlu involved a specific tRNA. The results presented above show that pGlu can indeed be formed from glutamic acid during protein synthesis on plasmacytoma microsomes. Both plasmacytoma and rat liver

tRNAs were efficient in catalyzing this synthesis and equivalent amounts of pGlu were obtained with each preparation. The amount of pGlu recovered was about 30% of the total counts per minute applied to the paper for electrophoretic analysis. pGlu should be the only amino acid labeled in the two acidic peptides obtained from the RPC-20 light chain, so one would expect that all the radioactivity in the acidic peptide fraction would be converted to pGlu. That only 30% conversion was obtained may indicate that the digestion of the peptide fraction by pyrrolidonyl peptidase was incomplete. Alternatively, there may be pGlu-containing peptides in the acidic fraction, other than those derived from the RPC-20 light chain, which also contain internal glutamic acid residues.

Incorporation studies performed using the fractionated isoaccepting species of glutamyl-tRNA revealed no specificity as regards pGlu formation. Similar percentages of acidic peptides were obtained from subtilisin digests of the polypeptides synthesized in response to both Glu-tRNAs, and pGlu was released from both preparations of subtilopeptides after pyrrolidonyl peptidase treatment. It was noted that higher percentages of acidic peptides were obtained from digests of the polypeptides synthesized in response to the individual Glu-tRNAs than were observed when total RPC-20 tRNA was used, and that the relative recoveries of the acidic peptide peaks A and B were different when the Glu-tRNAs were used. This result might reflect the fact that the isolated Glu-tRNA preparations lack some other tRNAs which are required for efficient elongation. It is known, for example, that the RPC-20 phenylalanine-tRNAs elute much later than Glu-tRNA$_{II}$ (unpublished results). Thus, the microsomal system may be limited to the synthesis of incomplete polypeptide chains when supplied with the individual isoaccepting tRNAs. Since the amount of internal glutamic will decrease as a result of this limitation, the percentage of the total glutamic acid incorporated which is present as pGlu will increase. Similarly, the amount of a particular polypeptide obtained might be expected to be inversely proportional to its chain length.

The results presented here argue against the participation of pGlu in the initiation of plasmacytoma protein synthesis. Recent studies on the initiation mechanism in eukaryotes have implicated methionine as the initiator amino acid in a number of systems (Jackson and Hunter, 1970; Rho and DeBusk, 1971). Milstein *et al.* (1972) have also reported methionine initiation of immunoglobulin light chain synthesis in a reticulocyte cell-free protein synthesizing system primed with plasmacytoma mRNA, and work from several laboratories suggests that the primary translation product of this mRNA is not the light chain itself, but a precursor molecule of slightly higher molecular weights (Milstein *et al.*, 1972; Mach *et al.*, 1973). Recent evidence indicates that this precursor possesses additional amino acids at the amino-terminal end of the light chain (Schechter, 1973). Since pGlu has no free α-amino group, it cannot be involved in an internal peptide bond in the precursor molecule. One can thus envision a mechanism whereby the light-chain precursor is initiated with methionine, with subsequent processing to remove the initiating methionine and a number of additional N-terminal amino acids. N-Terminal glutamic acid would be exposed by this treatment, and the cyclization to pGlu could occur at some later stage of translation. In this regard, attempts have recently been made to determine the site of formation of pGlu during protein biosynthesis in the plasmacytoma. Preliminary results suggest that there is no direct donation of pGlu from tRNA to the growing polypeptide chain. These results, which are consistent with the hypothesis outlined above, will be detailed in a subsequent publication.

Two trinucleotide codons (GAA and GAG) for glutamic acid have been observed in bacterial systems (Nirenberg *et al.*, 1966). Assuming the genetic code is universal, the two isoaccepting tRNAs prepared in this study must represent a pair of degenerate tRNAs for glutamic acid. It seems reasonable that at least one of the two tRNAs responds to both glutamic acid codons, whereas the other may respond to only one of the two, and that the common codon is the one which specifies pGlu. With the recent isolation of the mRNAs for immunoglobulin light chains (Swan *et al.*, 1972; Stavnezer and Huang, 1971), it should become possible to examine this possibility by direct sequence analysis of the message for RPC-20 λ chain.

References

Apella, E. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 590.

Apella, E., and Perham, R. N. (1968), *J. Mol. Biol. 33*, 963.

Baglioni, C. (1970), *Biochem. Biophys. Res. Commun. 38*, 212.

Bernfield, M., and Nestor, L. (1968), *Biochem. Biophys. Res. Commun. 33*, 843.

Blomback, B. (1967), *Methods Enzymol. 11*, 398–411.

Blomback, B., Blomback, M., and Edman, P. (1963), *Acta Chem. Scand. 17*, 1184.

Bridges, K. R., and Jones, G. H. (1973), *Biochemistry 12*, 1208.

Dekker, C. A., Stone, D., and Fruton, J. S. (1949), *J. Biol. Chem. 181*, 719.

Doolittle, R. F., and Armentrout, R.-W. (1968), *Biochemistry 7*, 516.

Erspamer, V., and Anastasi, A. (1962), *Experientia 18*, 58.

Jackson, R., and Hunter, T. (1970), *Nature (London) 227*, 672.

Mach, B., Faust, C., and Vassalli, P. (1973), *Proc. Nat. Acad. Sci. U. S. 70*, 451.

Mach, B., Koblet, H. and Gros, D. (1967), *Cold Spring Harbor Symp. Quant. Biol. 32*, 269.

Milstein, C., Brownlee, G. G., Harrison, R. M., and Mathews, M. B. (1972), *Nature (London), New Biol. 239*, 117.

Moav, B., and Harris, T. N. (1967), *Biochem. Biophys. Res. Commun. 29*, 773.

Nirenberg, M., Caskey, T., Marshall, R., Brimacombe, R., Kellog, D., Doctor, B., Hatfield, D., Levin, J., Rottman, F., Pestka, S., Wildox, M., and Anderson, F. (1966), *Cold Spring Harbor Symp. Quant. Biol. 31*, 11.

Niwaguchi, T., Motohashi, N., and Strecker, H. J. (1964), *Biochim. Biophys. Acta 82*, 635.

Ohira, T. (1939), *Nippon Nogeikagaku Kaishi 15*, 370.

Pearson, P. L., Weiss, J. F., and Kelmers, A. D. (1971), *Biochim. Biophys. Acta 228*, 770.

Raftery, M. A., and Cole, R. D. (1963), *Biochem. Biophys. Res. Commun. 10*, 467.

Rho, H. M., and DeBusk, A. G. (1971), *J. Biol. Chem. 246*, 6566.

Rush, E., and Starr, J. L. (1970), *Biochim. Biophys. Acta 199*, 41.

Rydon, H. N., and Smith, R. W. G. (1952), *Nature (London) 169*, 922.

Schechter, I. (1973), *Proc. Nat. Acad. Sci. U. S. 70*, 2256.

Stavnezer, J., and Huang, R. C. C. (1971), *Nature (London) 230*, 172.

Swan, D., Aviv, H., and Leder, P. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 1967.

Twardzik, D., and Peterkofsky, A. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 274.

Wilkinson, J. M., Press, E. N., and Porter, R. R. (1966), *Biochem. J. 100*, 303.