Translation of Collagen Messenger RNA in a Cell-Free System Derived from Wheat Germ†

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ABSTRACT: A cell-free system for synthesizing protein from wheat germ was used to translate the messenger RNA extracted from 16-day embryonic chick calvaria. A part of the product had properties similar to collagenous peptides and served as a substrate for prolyl hydroxylase, an enzyme specific for collagen. The level of potassium was critical for the synthesis of high molecular weight products with properties similar to pro-α chains. The potassium concentration for optimal protein synthesis, as judged by maximum incorporation of [^{3}H]proline into acid precipitable material, was considerably lower than the concentration required for the synthesis of high molecular weight collagenous peptides.

The polypeptide chains of collagen are synthesized in precursor forms, the pro-α chains. These pro-α chains are estimated to be 140 000–150 000 daltons (Martin et al., 1975), although there are reports of still larger proteins (Tanzer et al., 1974). In the formation of the macromolecule, three pro-α chains are assembled into a procollagen molecule which is then converted by limited proteolytic cleavage to collagen (Lapière et al., 1971). Several additional postribosomal modifications occur including the hydroxylation of certain prolyl and lysyl residues in the molecule.

Messenger RNA for collagenous proteins has been translated previously in cell-free protein synthesizing systems derived from Krebs II ascites cells (Benveniste et al., 1973) and rabbit reticulocytes (Boedtker et al., 1974). The cell-free system derived from wheat germ has potential advantages over these systems for isolating primary gene products since it has low levels of proteases and nucleases (Zehavi-Willner, 1975). Other problems associated with the translation of mRNA[1] for collagenous proteins might be mitigated by using wheat germ extracts. The endogenous level of protein synthesis is low, and collagen does not occur in plants. However, with two exceptions, wheat germ extracts have been unsuccessful in synthesizing proteins larger than 40 000 molecular weight. The hexon protein of adenovirus which has a molecular weight of 120 000 is synthesized in wheat germ extracts, but in smaller quantities than in a mammalian protein synthesizing system (Anderson et al., 1974). Also, small quantities of unidentified proteins up to 100 000 molecular weight are observed in sodium dodecyl sulfate–polyacrylamide gels used to verify synthesis of actin (Paterson et al., 1974).

In the present article we report our studies using the wheat germ system to translate collagen mRNA. We have found that concentrations of K^+ higher than that for optimal protein synthesis were required to support the synthesis of large peptides. A portion of this product had properties similar to collagen proteins.

Materials and Methods

Fresh commercial wheat germ (untoasted) was supplied by General Mills, Inc., Vallejo, Calif., and stored at 2 °C. Sodium *N*-lauryl sarcosinate (sarkosyl) was from K&K Laboratories. Nucleotide triphosphates and tannic acid were obtained from Sigma Chemical Corporation. Urea (Ultra Pure) and radiolabeled amino acids were the products of Schwarz/Mann and New England Nuclear, respectively. All other chemicals were purchased from Calbiochem.

All procedures were performed using sterile techniques. Buffers were treated with 0.05% diethyl pyrocarbonate and heated to 70 °C overnight, and glassware was autoclaved.

Preparation of Chick Embryo Calvaria RNA. RNA was extracted from the calvaria of 16-day chick embryos (Truslow Farms) with phenol–chloroform–isoamyl alcohol as previously described (Benveniste et al., 1973).

Preparation of Wheat Germ Extract. Wheat germ cell-free extracts were prepared as previously described (Shih and Kaesberg, 1973). No attempt was made to remove chaff and broken endosperm and DNase treatment was omitted. The extracts were stored in 0.3-ml aliquots in liquid N_2.

Carrier Collagen. Purified lathyritic chick skin collagen was used as carrier throughout these experiments. This was prepared as previously described (Piez et al., 1963; Kang et al., 1969).

In Vitro Protein Synthesis. A standard 50-μl reaction mixture contained 0.3 A_{280} unit of S_{23} extract, 0.037 A_{260} unit of calvaria RNA, 20 mM Hepes buffer (pH 7.4), 3.4 mM $Mg(OAc)_2$, 70 or 180 mM potassium acetate, as indicated in the individual experiments, 160 μg of creatine phosphate, 8 μg of creatine phosphokinase, 3.3 mM ATP, 0.25 mM GTP, 1 mM CTP, 0.13 mM EDTA, 0.13 mM EGTA, 4.6 mM 2-mercaptoethanol, 40 μM of 19 unlabeled amino acids, and 2 μM 3,4-[^{3}H]proline neutralized with NH_4OH (1.37×10^4 cpm/pmol). The reactions were incubated at 25 °C for 60 min. A 20 000-fold molar excess of cold proline was added to the reaction mixture and incubation was continued for an additional 20 min at 25 °C to chase [^{3}H]proline from prolyl-tRNA. The reaction mixtures were chilled and cold 10% Cl_3CCOOH containing 0.5% tannic acid (0.2 ml) was added. Samples were filtered through glass fiber filters which had been presoaked for 30 min in 5% Cl_3CCOOH containing 1 mg/ml of gelatin, and

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[1] Abbreviations used are: mRNA, messenger ribonucleic acid; CM-cellulose, carboxymethyl-cellulose; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; and MalNEt, *N*-ethylmaleimide.

1 mg/ml of proline. The filters were washed with cold 5% Cl_3CCOOH, dried at 80 °C for 30 min, and counted in a toluene–Liquifluor cocktail in a Beckman liquid scintillation counter.

Molecular Sieve Chromatography. The standard protein synthetic mixture was increased 30-fold and the reaction incubated at the indicated potassium ion concentration for 60 min, followed by a 20-min chase with unlabeled proline, and finally the EDTA concentration was raised to 50 mM. Five milligrams of purified lathyritic chick skin collagen was added to the reaction mixture. The sample was dialyzed for 8 h against 50 mM Tris-HCl buffer (pH 7.4) containing 0.2 M NaCl and then chromatographed at 4 °C on a column of DEAE-cellulose (3 cm × 8 cm) equilibrated with the same buffer. This procedure removed all RNA from the sample. The void volume was pooled, dialyzed extensively against 10 mM Tris-HCl buffer (pH 7.4), and lyophilized. The sample was suspended in 5 ml of 1 M $CaCl_2$, heated for 15 min at 60 °C, and applied to an 8% agarose column (1.5 cm × 190 cm). The column was eluted at room temperature at the rate of 9 ml/h with 50 mM Tris-HCl buffer (pH 7.4) containing 1 M $CaCl_2$. Fractions (3 ml) were collected and the A_{226} of each fraction was determined. Aliquots of 1 ml were counted in Aquasol (New England Nuclear).

CM-Cellulose Chromatography. Ion exchange chromatography was performed using methods previously described (Pontz et al., 1973). Reaction mixtures were prepared and the sample was dialyzed and chromatographed on DEAE-cellulose as described for molecular sieve chromatography, except that 10 mg of lathyritic collagen was added as carrier. The void volume fractions were pooled and dialyzed extensively against 20 mM potassium acetate buffer (pH 4.8) at 4 °C followed by dialysis against the same buffer containing 1 M urea. The sample was heated at 60 °C for 15 min and applied to a column of CM-cellulose (1 cm × 7 cm) equilibrated with the acetate buffer in 1 M urea. The column was maintained at 45 °C and eluted at a flow rate of 80 ml/h with a 200-ml linear gradient from 0 to 0.13 M LiCl and washed finally with acetate buffer containing 1 M LiCl. Two-milliliter fractions were collected and processed as described for the molecular sieve column.

Collagenase Digestion. Purified bacterial collagenase (Advance Biofactors, Lynbrook, N.Y.) was used to determine the amount of collagen present as described previously (Peterkofsky and Diegelmann, 1971).

Sodium Dodecyl Sulfate–Polyacrylamide Electrophoresis. Reaction mixtures were prepared, chromatographed on DEAE-cellulose, dialyzed, and lyophilized as described for molecular sieve chromatography. However, for these experiments no carrier collagen was added. The product was dissolved in distilled water and divided into two aliquots which were incubated for 4 h at 37 °C with or without bacterial collagenase. Following dialysis against 10 mM Tris (pH 7.4) and lyophilization, these samples were dissolved in 0.1 M potassium phosphate buffer (pH 6.8) containing 0.1% sodium dodecyl sulfate and 2 M urea and then electrophoresed according to the procedure of Furthmayr and Timpl (1971). The gels were crushed in 2-mm sections and counted following the addition of 10 ml of Hydromix.

Collagen Prolyl Hydroxylase. The preparation of the enzyme collagen prolyl hydroxylase followed the method of Rhoads and Udenfriend (1970) through the ammonium sulfate step. The 29–60% ammonium sulfate precipitate was dissolved in 50 mM Tris-HCl buffer (pH 7.0) containing 0.2 M NaCl and dialyzed against the same buffer. The enzyme was stored in small aliquots at −20 °C for 2 months without loss of activity.

Protocollagen Substrate for in Vitro Prolyl Hydroxylation. The preparation of a physiological substrate protocollagen for prolyl hydroxylation was obtained following the procedure of Kivirikko and Prockop (1967) with some modification. The 16-day chick embryo calvaria were used as the substrate source, and following incubation with α,α-dipyridyl- and [^{3}H]proline, they were disrupted with a Polytron. This substrate preparation typically contained 10^6 cpm/ml.

The prolyl hydroxylation was carried out in a 1-ml reaction mixture as previously described (Rhoads and Udenfriend, 1970). Cell-free product reactions were prepared for hydroxylation by passing the 6-ml protein synthesizing reaction mixture over a P-2 column (Bio-Rad) using 0.2 M ammonium bicarbonate buffer with carrier bovine serum albumin. Peak fractions were lyophilized and suspended in 1 ml of 0.2 M NaCl and 50 mM Tris (pH 7.4), and used in the prolyl hydroxylation reaction the same day.

Amino Acid Chromatography. The separation of labeled proline and hydroxyproline was achieved by Dowex 50W-X8 column chromatography (Cutroneo et al., 1972).

Results

The frontal and parietal bones (calvaria) were used as a source of RNA. In preliminary assays the ability of the RNA to support protein synthesis was measured by the incorporation of [^{3}H]proline into acid-precipitable material collected by filtration through glass fiber filters. Since collagen is soluble in acid solutions a mixture of Cl_3CCOOH and tannic acid was used for precipitation. In addition, it was found that collagen was bound nonspecifically to glass fiber filters, unless the filters were exposed previously to 1% gelatin solutions.

In the wheat germ system, addition of RNA from chick calvaria stimulated the incorporation of labeled amino acid into acid precipitable material. After an initial lag, the amount of precipitable material was proportional to the amount of added mRNA. Under the conditions used it was not possible to add saturating amounts of RNA. We have observed similar results with the ascites cell-free system (Benveniste et al., 1973). Various other parameters were investigated. Decreased incorporation occurred at temperatures above 20 °C. Initiation in the wheat germ system is highly temperature dependent, and is inhibited entirely at 37 °C, as has been observed previously (Zehavi-Willner, 1975). Although 3.4 mM Mg^{2+} produced a maximum of incorporation there was an increase in protein synthesis above 4.0 mM which was probably due to nonphysiological initiation and miscoding (Nirenberg et al., 1966). Incorporation was 30% greater at 70 mM than at 180 mM K^+. Kinetic experiments indicated that the initial rate of synthesis was more rapid at the higher concentration of K^+. The response to Mg^{2+} concentration was the same at the two levels of K^+.

The amount of collagenous protein synthesized in the wheat germ system was assayed by its susceptibility to digestion with bacterial collagenase (Table I). The collagenase used in these experiments was found to digest authentic collagen purified from the skin of rats injected with [^{14}C]proline. The specificity of the collagenase was assayed also against tryptophan-labeled material synthesized by the cell-free system. While tryptophan may occur in the precursor specific portion of pro-α chains, it is not found in the

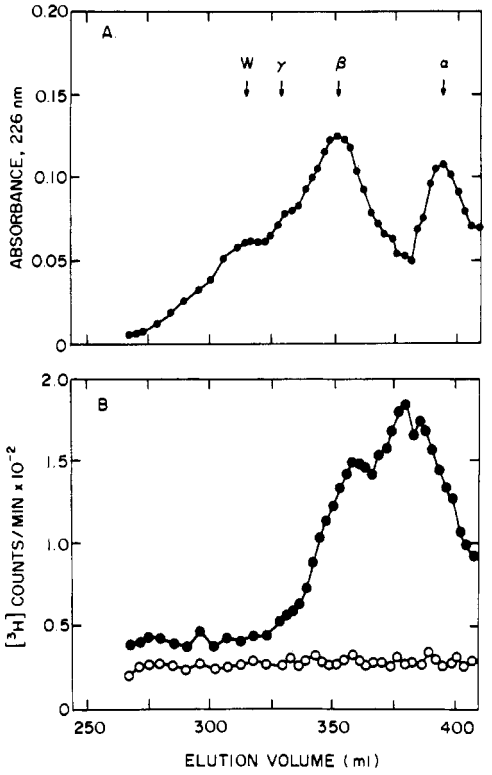

FIGURE 1: Molecular sieve chromatography of the collagenous product synthesized in vitro in the presence of 180 and 70 mM K^+ ion concentration. Chromatography was carried out as described, using 5 mg of purified lathyritic chick skin collagen as carrier: (A) the spectrophotometric profile at A_{226}; (B) radioactivity profile of cell-free product synthesized in the presence of 180 mM (●) and 70 mM (○) potassium concentration. Two samples were chromatographed separately but are presented as a single figure. The absorption profiles which represent collagen carrier primarily were identical in the two chromatographs. Single collagen chains or α chains may be covalently linked as dimers or trimers, termed β and γ chains, respectively. Their elution positions are indicated over the corresponding peaks of chick skin collagen carrier in A.

Table I: Susceptibility of the Cell-Free Reaction Product to Digestion by Purified Bacterial Collagenase.[a]

Preparation	Cl_3CCOOH Precipitable cpm for Digestion Conditions: $CaCl_2$, MalNEt, Control	$CaCl_2$, MalNEt, Collagenase	% Solubilized
[^{3}H]Pro labeled product synthesized in vitro in wheat germ extract at 70 mM potassium	6 330	6 980	0
[^{3}H]Pro labeled product synthesized in vitro in wheat germ extract at 180 mM potassium	3 090	2 080	33
[^{3}H]Pro in vivo labeled collagen from lathyritic rat skin	9 870	920	96
[^{3}H]Trp in vitro labeled product synthesized in wheat germ extract at 180 mM potassium	10 560	10 740	0

[a] The cell-free reaction described under Materials and Methods was terminated by the addition of unlabeled proline and chilled. The subsequent collagenase digestion was carried out as described by Peterkofsky and Diegelmann (1971).

Table II: Ability of the Cell-Free Reaction Product to Serve as a Substrate for Collagen Specific Prolyl Hydroxylase.[a]

Reaction Mixture	cpm Incorpd into ^3H_2O: Cell-Free Product	Physiol. Substrate
Complete no. 1	7890	10 580
no. 2	5890	9 310
Minus α-ketoglutarate	400	20

[a] The cell-free reaction mixture is described under Materials and Methods. A potassium concentration of 180 mM was used. The reaction mixture volume was increased to 3 ml and the product was processed as described. Following P-2 column chromatography and lyophilization, the product was placed in the hydroxylation reaction mixture. Extent of reaction was measured by the ^3H_2O released. The physiological collagen substrate was derived from 16-day chick embryo calvaria with [^{3}H]proline and α,α-dipyridyl. The collagen soluble in 1 M NaCl was used in this assay. The cofactor in the hydroxylation reaction, α-ketoglutarate, was omitted in control reaction mixtures.

collagenous portions (Von der Mark and Bornstein, 1973). None of the tryptophan-labeled substrate was solubilized after incubation with collagenase, indicating that little or no nonspecific enzymes were present in the collagenase preparation.

A portion (33%) of the protein synthesized in vitro at 180 mM K^+ was digested by collagenase while none of the product synthesized at the lower K^+ concentration served as a substrate for the enzyme. This result indicated that the higher K^+ level favored the synthesis of the collagenous portion of pro-α chains.

An entirely different method was used to confirm that collagenous protein was synthesized at the higher K^+ level. Here the products of cell-free synthesis were incubated with partially purified prolyl hydroxylase prepared from embryonic chick wings and legs. The hydroxylation of prolyl residues in the substrate was followed by the formation of tritiated water. One molecule of tritiated water is released for each hydroxyl group incorporated into proline residues. As shown in Table II, the hydroxylation of protocollagen had a specific requirement for α-ketoglutarate which allows one to distinguish between this and unrelated reactions. RNA from calvaria directed the synthesis of a product which was a substrate for prolyl hydroxylase. This reaction had the same requirement for α-ketoglutarate (Table II). In a control reaction, no ^3H_2O was released during enzymatic hydroxylation of the product obtained from a cell-free protein synthesizing system from which the RNA was omitted.

Amino acid analysis of the cell-free product following exposure to prolyl hydroxylase provided more direct evidence for hydroxyproline formation. Proline and hydroxyproline were recovered by Dowex column chromatography from an acid hydrolysate of the substrates following incubation with the prolyl hydroxylase. Only when α-ketoglutarate was present in the hydroxylation reaction were both [^{3}H]hydroxyproline and [^{3}H]proline obtained from hydrolysates using the product derived from wheat germ. The hydroxyproline to proline ratio was 0.16.

The protein synthesized in the in vitro system was characterized further by chromatography on molecular sieve and ion exchange columns. The elution profiles from molecular sieve chromatography of both carrier collagen and the products synthesized in the cell-free system are illustrated in Figures 1A and 1B, respectively. At 180 mM K^+ three major peaks of radiolabeled protein eluted between the α and β peaks of the chick carrier collagen used as markers.

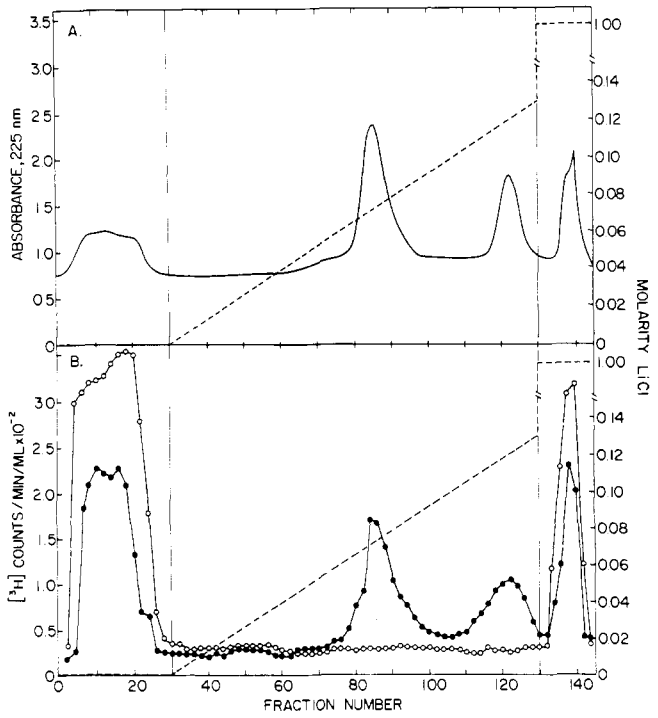

FIGURE 2: CM-cellulose chromatography of the collagenous product synthesized in vitro in the presence of 180 and 70 mM K^+ ion concentration. Chromatography was carried out as described under Materials and Methods using 10 mg of purified lathyritic chick skin collagen as carrier: (A) the spectrophotometric profile at A_{226}; (B) radioactivity profile of cell-free product synthesized in the presence of 180 mM (●) and 70 mM (○) K^+ concentration. Profiles from two separate chromatograms are presented in the single figure. The absorption profiles representing the collagen carrier were identical in the two chromatographs. The recovery of radioactive label was approximately 50%. The skin carrier collagen, as well as collagen of tendon and bone, are composed of three α chains, two of which are the same (α1) and one which differs (α2). The formula for this mature triple helical molecule may be written $(\alpha 1)_2\alpha 2$. The elution positions of α1 and α2 chains are indicated over the corresponding peak in A.

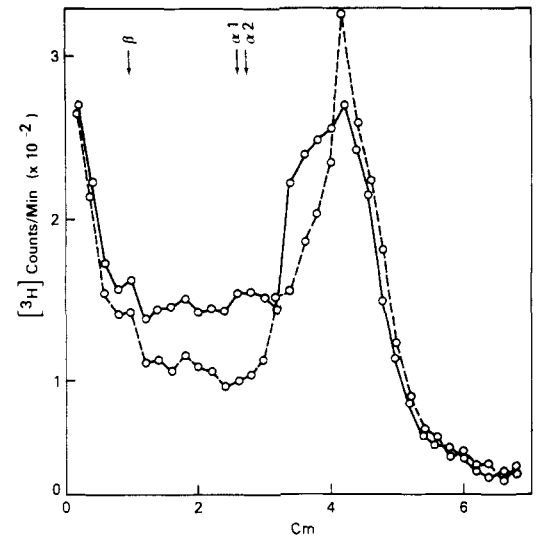

FIGURE 3: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of in vitro synthesized protein. Protein synthesized at 180 mM K^+ was processed as described for molecular sieve chromatography except that no carrier collagen was added. The sample was divided into two aliquots which were incubated for 4 h at 37 °C with or without bacterial collagenase. After dialysis and lyophilization the samples were electrophoresed on 5% sodium dodecyl sulfate gels (Furthmayr and Timpl, 1971). The gels were crushed in 2-mm sections and counted following the addition of 10 ml of Hydromix. The radioactivity in the gels is illustrated: collagenase treated (○ - - ○); control (○—○). The positions of α and β chains are indicated.

These peaks were compatible with proteins of molecular weight of approximately 155 000, 120 000, and 110 000. The product synthesized at 70 mM K^+ contained no protein of comparable molecular weight. Proteins synthesized under these conditions were smaller than 30 000 daltons. Figure 2 illustrates the profiles from CM-cellulose chromatography of carrier collagen and the products synthesized at both high and low K^+ concentrations. The products synthesized at 180 mM K^+ contained two peaks of protein which eluted at the positions of the carrier collagen α1 and α2 peaks. These peaks of radiolabeled protein were present in a ratio of 2:1, respectively. No such material was detected in the reaction products synthesized at 70 mM K^+.

Finally, the product synthesized at 180 mM K^+ was also examined on sodium dodecyl sulfate-polyacrylamide gels. One-half of the sample served as the control and the other half was digested with collagenase prior to electrophoresis. Although no sharp peaks of radioactivity were observed in the region where authentic pro-α chains migrate, a considerable portion of the protein in this section of the gel was destroyed by collagenase (Figure 3). In addition, there were collagenase digestible peptides smaller than complete α chains.

Discussion

The mechanism by which collagen synthesis is regulated is not known but various tissues show marked differences in collagen content, in normal as well as pathological conditions. Furthermore, there are several genetically and chemically distinct collagens and their synthesis varies with age, tissue source, and disease state (Grant and Prockop, 1972; Gross, 1972; McKusick and Martin, 1975). We have used embryonic bones from chicks as a source of RNA since 60% of the proteins synthesized by this tissue is type I collagen (Diegelmann and Peterkofsky, 1972; Miller and Matukas, 1974). Presumably the levels of mRNA for the pro-α1 (I) and pro-α2 chains will be present as a proportionate percentage of the total mRNA. Collagen synthesis in embryonic calvaria parallels the synthesis of hemoglobin (Anderson, 1974; Nienhuis et al., 1974), silk fibroin (Suzuki and Brown, 1972), ovalbumin (Harris et al., 1973), and δ-crystallin (Zelenka and Piatigorsky, 1974). In each case, the protein is the predominant species synthesized in the tissue of origin.

Previously, mRNA for collagenous protein has been extracted from chick calvaria and translated in heterologous cell-free systems derived from Krebs II ascites cells (Benveniste et al., 1973) and in rabbit reticulocytes (Boedtker et al., 1974). In the former system a portion of the protein formed was found to be susceptible to collagenase but only 2–3% of the protein synthesized was as large as α chains and none of the synthesized product was as large as pro-α chains. In addition, we have found that collagenous protein is synthesized by Krebs II ascites cells (unpublished observations). These cells may contain the specific proteases that normally degrade the initial translation products and difficulties might occur in distinguishing endogenous from exogenous mRNA translation.

The reticulocyte lysate is more efficient in translating mRNA than the ascites system, but since it cannot be preincubated, there is a high level of endogenous activity. Translation of chick calvaria mRNA in the reticulocyte system generates a product of which a small portion is similar

in size to pro-α chains. The amount of product is comparable to the level of ovalbumin synthesis generated by ovalbumin mRNA in the rabbit reticulocyte system (Palmiter, 1973).

We became interested in the cell-free preparation from wheat germ as a possible system for translating pro-α chain mRNA since it contains low levels of proteases and nucleases (Davies and Kaesberg, 1973; Zehavi-Willner, 1975) and collagen synthesis does not occur in the intact tissue. In preliminary experiments, we found that while total protein synthesis was lower at 180 mM K^+ than at 70 mM, only at the higher K^+ concentration were both large molecular weight protein and collagenous protein produced. Susceptibility to collagenase represents a convenient method for monitoring the collagenous portion of a pro-α chain but the amino-terminal portion of the pro-α1 (I) chain contains some 150 additional amino acids that are not cleaved by the enzyme. Tryptophan is present only in the noncollagenous portion of the pro-α chain. Since none of the tryptophan-labeled protein synthesized in vitro was solubilized by collagenase, the enzyme preparation used in these experiments could be assumed to be free of nonspecific proteases. Therefore, the collagenase digestion data indicated that at low K^+ concentrations the pro-α chain mRNA either was not translated or that translation did not extend into the collagenous portion of the pro-α chain. The data from the ion exchange chromatography corroborated that a high K^+ concentration was needed for the synthesis of collagenous proteins. Preliminary data suggest that pro-α1 coelutes with mature α1 chains and pro-α2 coelutes with α2 chains (P. Byers and K. McKenney, personal communications). Further characterization of the products by molecular sieve chromatography also indicated that large size peptides were synthesized only at the high K^+ level. The sodium dodecyl sulfate gels showed that high molecular weight proteins synthesized under these conditions were indeed the peptides that were also collagenase digestible.

The highest molecular weight form (155 000) observed by molecular sieve chromatography was consistent with pro-α chains. These molecules contain both amino- and carboxy-terminal sequences which normally are enzymatically removed during the maturation of the polypeptide (Fessler et al., 1975; Davidson and Bornstein, 1975). The amino- and carboxy-terminal peptides have approximate molecular weights of 13 000 and 35 000, respectively. Partial degradation of pro-α chains seems to be inevitable during purification. Molecules with *either* the amino *or* carboxy terminus are present in final preparations as well as totally intact chains. It has proven difficult to prevent these cleavages despite the presence of proteolytic inhibitors. The increased handling required for collagenase digestion and the parallel controls may have resulted in increased degradation of the pro-α chains. This would account for the failure to find discrete peaks of collagenase sensitive protein in the sodium dodecyl sulfate gels (Figure 3). The peaks of radioactive protein observed by molecular sieve chromatography, which have molecular weights of 120 000 and 110 000, may represent pro-α chains without their amino- or carboxy-terminal peptides, respectively. At present we cannot exclude the possibility that both these molecules have intact amino termini but were not translated in their entirety.

The presence of a collagenous protein was also verified by testing whether the in vitro product synthesized at 180 mM K^+ could serve as a substrate for the highly specific enzyme, prolyl hydroxylase. This enzyme catalyzes the hydroxylation of selected proline residues in nascent collagen chains. The reaction has an absolute requirement for α-ketoglutarate as well as a reducing agent, O_2, and iron (Hutton et al., 1967). As shown in Table II proline in the product synthesized in the wheat germ system was hydroxylated by this enzyme. This is the first report of a physiological posttranslational modification of a collagenous protein synthesized in vitro. Since many plant cell wall proteins (Lamport and Northcote, 1960; Heath and Northcote, 1971; Allen and Neuberger, 1973) are rich in hydroxyproline, the wheat germ extract might contain a prolyl hydroxylase activity. However, we determined in preliminary experiments that no prolyl hydroxylase was present in the S_{23} preparation from wheat germ. In addition, we established that the cell-free reaction mixture containing chick calvaria mRNA did not generate a prolyl hydroxylase activity during the course of the protein synthesizing incubation (unpublished observations).

The data presented here indicate that the synthesis of large peptides and collagenous proteins depends on high K^+ concentrations in the wheat germ cell-free system. On the basis of a preliminary report of these results (Benveniste et al., 1974) other investigators have confirmed our findings (Zeichner and Rojkind, 1976). The failure to synthesize large collagenous proteins at low K^+ concentrations suggests that K^+ ions can affect the functionality of mRNA and determine whether it can be translated. Support for this hypothesis is obtained from previous studies in which the relative synthesis of the α and β chains of hemoglobin is affected by the ionic environment (Anderson, 1974; McKeehan, 1974). An alternative explanation for our results is obtained from the experiments of Mathews and Osborn (1974). They have shown in the Krebs II ascites cell-free protein synthesizing system that elevated K^+ concentrations increase the rate of protein synthesis and the appearance of high molecular weight proteins. Perhaps the high K^+ levels stabilize the integrity of the mRNA–ribosome structure so that the rate of translation is increased and premature termination is decreased. At the present time it is impossible to determine which mechanism is operative.

Observations similar to those presented in this article, describing the requirement for elevated K^+ concentrations for the cell-free synthesis of large collagenous proteins, have recently appeared (Harwood et al., 1975). These authors do not cite the reference to our preliminary communication (Benveniste et al., 1974), which summarizes these same data.

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