# Differential Effect of Hypertonic Initiation Block on the Synthesis of Collagen Chains by Cultured Chick Embryo Cells†

Philip J. Pawlowski

ABSTRACT: The major type of collagen synthesized by fibroblasts or bone cells, type I collagen, consists of two chains normally found in a 2:1 ratio designated $\alpha_1(I)_2\alpha_2(I)$ or more simply $\alpha_1(I)_2\alpha_2$. I have analyzed the relative synthesis of type I chains in these cells under conditions which reduce the initiation of protein synthesis. It was found that in bone cells, which make a large amount of collagen, the $\alpha_1(I)$:$\alpha_2$ ratio is unaltered whereas in fibroblasts, which make smaller amounts of collagen, the $\alpha_2$ chain is particularly sensitive to these same conditions. Examination of the collagen secreted into the medium, under these same conditions, also revealed an altered chain ratio from cells making low amounts of collagen.

There is much evidence showing that different messenger ribonucleic acids (mRNAs) vary in their efficiency to initiate protein synthesis [for a review, see Lodish (1976)], with some proteins being resistant and some more susceptible to factors reducing initiation. These studies, using both intact cells and cell-free protein-synthesizing systems, have led to models pertaining to the regulation of protein synthesis at the translational level (Lodish, 1974). Previous work in this laboratory has shown that type I collagen synthesis, in cultured chick cells, is more sensitive to hypertonic initiation block (HIB)[1] than the majority of cellular proteins (Koch et al., 1977). As there are several reports of the synthesis of a collagen molecule containing three $\alpha_1(I)$ chains rather than the commonly found $\alpha_1(I)_2\alpha_2$ (Mayne et al., 1975, 1976; Benya et al., 1977; Crouch & Bornstein, 1978), I closely examined the chain ratio under conditions of reduced initiation to see if there was a difference in initiation efficiency which might explain the occurrence of $[\alpha_1(I)]_3$ molecules. It was found that HIB could alter the ratio of the collagen chains synthesized by cultured chick cells but that this effect was dependent on the cell type. Cells making large amounts of collagen, upward of 40%, did not alter the chain ratio while cells making lesser amounts of collagen, near 15%, were found to respond with a decrease in $\alpha_2$ synthesis, relative to $\alpha_1(I)$.

In additional studies, employing cultures with a mixture of bone, type I, and chondrocyte, type II, cells, it was found that type II synthesis was more resistant to HIB than type I.

## Experimental Procedures

*Cell Culture.* Calvaria were dissected from 14-day-old chick embryos (Spafas) and incubated at 37 °C in 0.2% trypsin–0.2% collagenase (Worthington) in phosphate-buffered saline for 5 min. The solution was removed and discarded, and the calvaria were again digested with fresh trypsin–collagenase for 40 min at 37 °C. The released cells were collected by centrifugation and plated onto 35-mm petri dishes at $5 \times 10^5$ cells/plate in 1.5 mL of Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Cells were grown at 37 °C in 10% $CO_2$ until they were about 75% confluent (5–6 days), at which stage they have a rounded or somewhat squarish shape.

Leg cells were derived from 14-day-old chick embryos as described for calvaria cells. They were plated at the same density as calvaria cells and reached 75% confluency after 3–4 days. These cells have the characteristic spindle shape appearance of fibroblasts probably as a result of adhering tissue left on the bone after dissection.

If leg bones were meticulously cleaned and then minced before trypsin–collagenase digestion, cultures contained a mixture of cells resembling those of calvaria and chondrocytes. These polygonal chrondrocyte-like cells resemble those described by Chacko et al. (1969) and, similar to their report, would liberate "floaters" into the culture medium.

*Cell Labeling.* For cell labeling, the medium was removed and replaced with 1 mL of fresh medium containing the desired amount of excess NaCl. After 15 min, [2,3-$^3H_2$]proline (New England Nuclear) was added, and the cultures were incubated for 30 min. The medium was then removed, and the cultures were rinsed twice with ice-cold buffered salt solution. The cells were scraped from the plates with a policeman in 1 mL of buffered saline, and 1.5 mL of 10% trichloroacetic acid was added. After 15 min on ice, the precipitate was pelleted at 10000*g* for 5 min, and the pellets were washed once in 5% $Cl_3CCOOH$ and once in ether and vacuum dried. The pellets were then dissolved in gel sample buffer (0.01 M Tris, pH 8.1, and 1% $NaDodSO_4$) for electrophoresis or 0.1 N NaOH for collagenase digestion.

For examination of the secreted products, cultures were washed once with serum-free medium, and serum-free medium containing the appropriate NaCl concentration was added. After 15 min, radioactive proline was added, and the cultures were incubated for 2 h. The medium was then removed and centrifuged for 5 min at 10000*g*, and the supernatant was made 7.5% in $Cl_3CCOOH$ after addition of 100 μg of bovine serum albumin to serve as carrier protein. After 20 min on ice, the precipitate was centrifuged and prepared for electrophoresis as described for the cell layer.

*Gel Electrophoresis.* For display of the labeled proteins, samples were heated for 5 min at 95 °C and electrophoresed on 5–12.5% acrylamide–$NaDodSO_4$ slab gels (Laemmli, 1970). The gels were prepared for autoradiography according to Laskey & Mills (1975) and exposed to X-ray film for various lengths of time at −70 °C. The autoradiographs were scanned on an ORTEC 4310 integrating densitometer, and several different exposures of each gel were used to ensure

† From the Biology Department, Wesleyan University, Middletown, Connecticut 06457. *Received June 22, 1981.* Supported by Grants HL-21662 and AM 16162 from the National Institutes of Health.

[1] Abbreviations: HIB, hypertonic initiation block; $Cl_3CCOOH$, trichloroacetic acid; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate; $p\alpha_1$ and $p\alpha_2$, procollagen containing the amino- and carboxy-terminal extensions; BrdU, 5-bromo-2-deoxyuridine.

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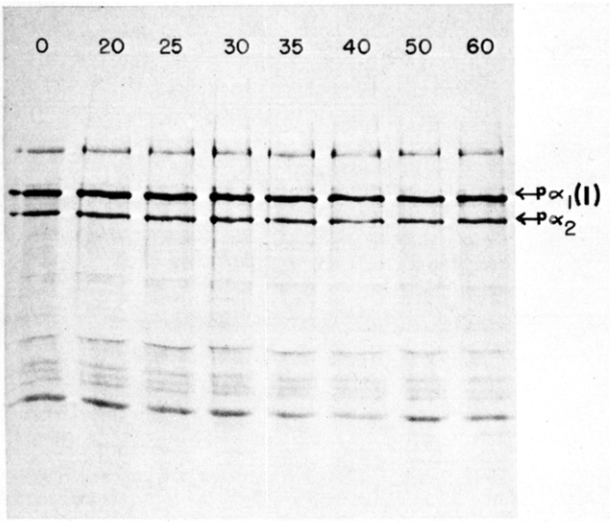


FIGURE 1: Effect of HIB on the synthesis of $p\alpha_1(I)$ and $p\alpha_2$ subunits in cultured calvaria cells. Primary cultures of calvaria cells were treated with excess NaCl at the concentrations (mM) given above each lane. After 15 min, cells were labeled for 30 min with [2,3-$^3H_2$]proline (25 $\mu$Ci/mL), harvested, and electrophoresed as described under Experimental Procedures.

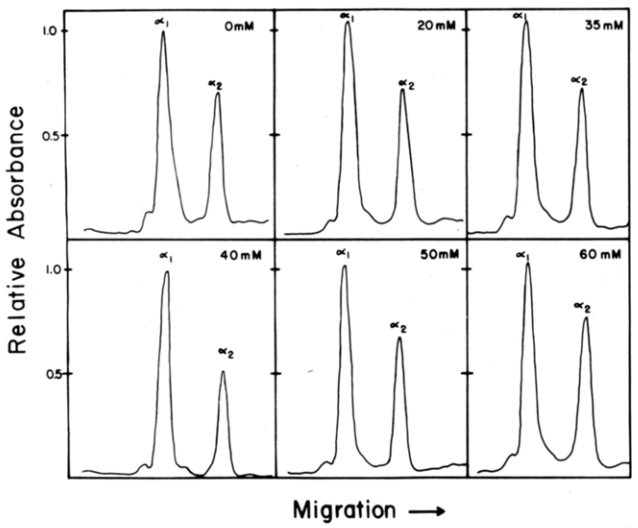


FIGURE 2: Densitometer scan of the $p\alpha_1(I)$ and $p\alpha_2$ regions of a gel autoradiogram produced by calvaria cells labeled at various excess NaCl concentrations. The $p\alpha_1(I)$ and $p\alpha_2$ regions of a gel autoradiogram of calvaria cells, such as those in Figure 1, were scanned by using on ORTEC 4310 recording, integrating densitometer.

linearity between radioactivity and film density.

*Collagenase Digestion.* The samples were digested with collagenase, which had been repurified, according to Peterkofsky & Diegelmann (1971).

## Results

*Effect of HIB on Calvaria Cell Collagen.* When calvaria cells were treated with excess NaCl, the relative amounts of proline incorporation at 20, 30, 40, and 50 mM excess NaCl were found to be 85, 80, 62, and 52% respectively of control values. Collagenase digestion of untreated cells revealed that 40–45% of the proline-labeled protein was susceptible to digestion. A typical labeling pattern seen after gel electrophoresis is shown in Figure 1, and a densitometer scan of the collagen region is presented in Figure 2. The $\alpha_1(I)$:$\alpha_2$ ratios obtained from four experiments were averaged and are presented in Figure 5. The results indicate that little change in the ratio was found except for a slight decrease at higher salt concentrations.

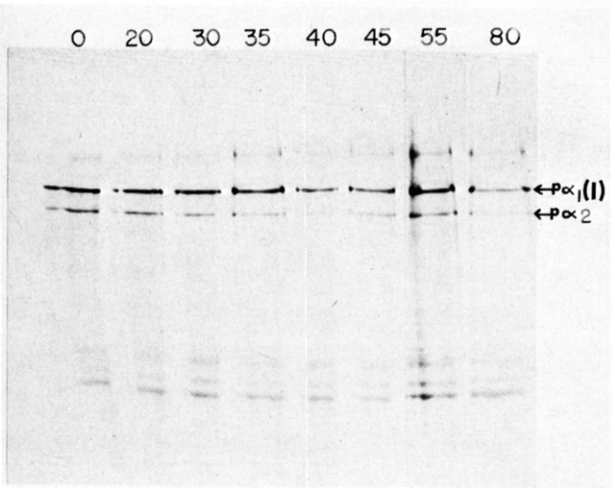


FIGURE 3: Effect of HIB on the synthesis of $p\alpha_1(I)$ and $p\alpha_2$ subunits in cultured leg cells. Primary cultures of leg cells were treated with excess NaCl, labeled, and electrophoresed as described in Figure 1 and under Experimental Procedures.

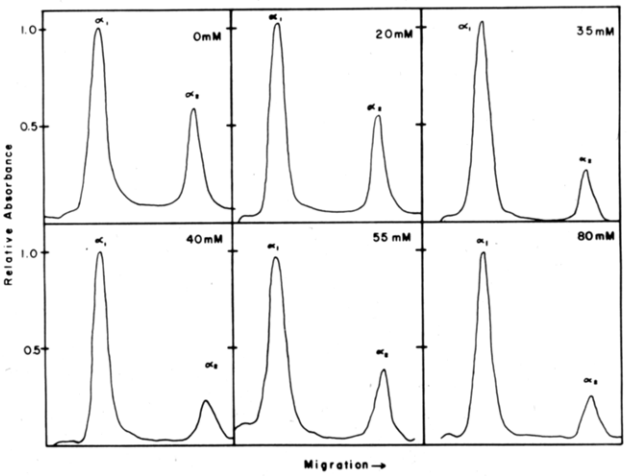


FIGURE 4: Densitometer scan of the $p\alpha_1(I)$ and $p\alpha_2$ regions of a gel autoradiogram produced by leg cells labeled at various excess NaCl concentrations. The $p\alpha_1(I)$ and $p\alpha_2$ regions of a gel autoradiogram, such as those in Figure 3, were scanned with on ORTEC 4310 densitometer.

*Effect of HIB on Leg Cell Collagen.* When primary cultures of leg cells were treated with NaCl, the relative amounts of proline incorporation at 30, 40, 50, and 60 mM excess NaCl were found to be 88, 80, 71, and 62%, respectively, of control values. Collagenase digestions of control cells revealed that 15–17.5% of the incorporated label was susceptible to collagenase digestion. The somewhat higher values of proline incorporation for leg cells compared to calvaria, at the same NaCl concentration, are most likely a result of collagen's increased susceptibility to HIB (Koch et al., 1977) and a larger amount of collagen synthesized by calvaria cells.

A labeling pattern obtained after $NaDodSO_4$ gel electrophoresis of labeled leg cells is shown in Figure 3 with a densitometer scan of the collagen region presented in Figure 4. It can be seen that, as the NaCl level is raised, incorporation of radioactivity into the $\alpha_2$ chain is decreased in relation to that into the $\alpha_1(I)$ chain. The average ratio obtained from four separate experiments is presented in Figure 5 and indicates that NaCl concentrations over 20 mM can begin to effect a decrease in $\alpha_2$ synthesis. At salt concentrations over 50 mM, the ratios were found to vary between 3 to 1 and 4.5 to 1 in these cultures. As presented below in populations of cells

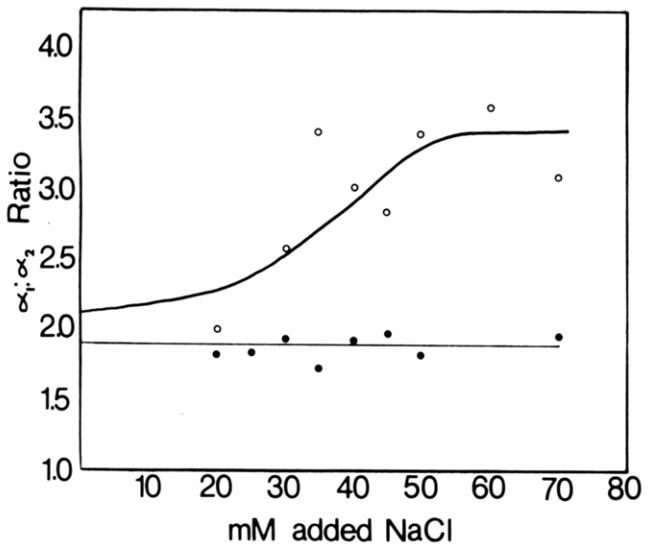


FIGURE 5: $p\alpha_1(I):p\alpha_2$ ratios of calvaria and leg cells during treatment with increasing HIB. Areas under the peaks, as shown for Figures 2 and 4, were integrated from several separate experiments and the $p\alpha_1(I):p\alpha_2$ ratios averaged: (●) calvaria; (○) leg cells.

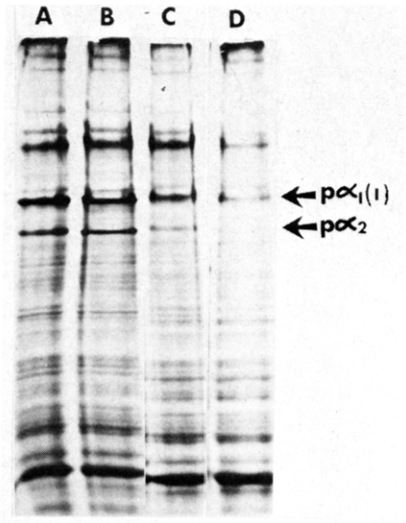


FIGURE 6: Gel electrophoretogram of cultured cells labeled with and without $\alpha,\alpha'$-dipyridyl and excess NaCl. Primary cultures of cells, derived from 14-day-old chick embryo muscle tissue as described for leg cells, were labeled 30 min with [2,3-$^3H_2$]proline (50 $\mu$Ci/mL) and electrophoresed as described in Figure 1. (A) No treatment; (B) $\alpha,\alpha'$-dipyridyl (4.8 mM) added 45 min before labeling; (C) cells treated with 60 mM excess NaCl; (D) cells treated with 90 mM excess NaCl.

derived from embryonic muscle or skin, it is often found that at high NaCl concentration (above 80 mM) the $\alpha_2$ chain can be almost undetectable.

*Effect of HIB on Collagen Secretion.* As the above studies involved examination of the intracellular chain ratio after relatively short labeling times, the effects of HIB on the collagen secreted to the extracellular space after longer periods of treatment were also examined. Such studies were performed by using a population of cells derived from embryonic skin or muscle since it was found that such cells would give a particularly acute response to HIB. A gel electrophoretogram of the [$^3$H]proline-labeled proteins synthesized by cultured muscle-derived cells, in response to HIB, is shown in Figure 6. It is seen that as the NaCl concentration is raised from isotonic (lane A) to 60 and 90 mM above isotonicity (lanes C and D, respectively) synthesis of the $\alpha_2$ chain is greatly reduced.

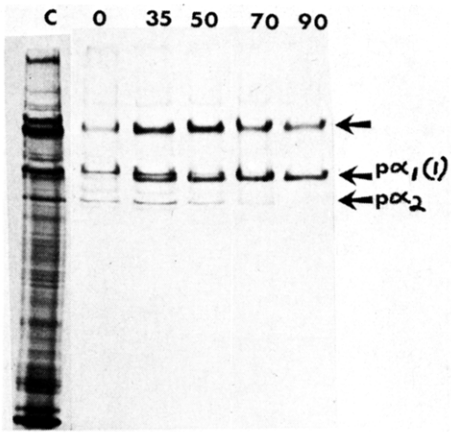


FIGURE 7: Labeling pattern of proteins secreted by cultured cells treated with HIB. Primary cultures as described in Figure 6 were labeled for 2 h with [2,3-$^3H_2$]proline (50 Ci/mL) at different NaCl concentrations and proteins secreted into the culture medium examined as described under Experimental Procedures. Arrows marked $p\alpha_1(I)$ and $p\alpha_2$ indicate the collagen chains which first appear in the medium whereas at longer labeling times processed collagen intermediates began to accumulate. The lane marked C represents the labeling pattern in the cell layer of control cells after this same labeling time. The unmarked arrow represents a protein, tentatively identified as fibronectin, which is also relatively resistant to HIB.

If similar cells are labeled for 2 h and the secreted products examined by gel electrophoresis, as described under Experimental Procedures, results as shown in Figure 7 are obtained. It is found that the chain ratio of secreted collagen accurately reflects that found within the cell. These results indicate that the decrease in $\alpha_2$ synthesis resulting in an excess of $\alpha_1(I)$ is not merely an intracellular event but that the excess $\alpha_1(I)$ chains may be normally secreted into the extracellular space, presumably as $\alpha_1(I)$ trimer. It is unknown whether all excess $\alpha_1(I)$ is secreted or if some intracellular degradation takes place. Also after prolonged exposure of the gel autoradiograms or longer periods of incubation, bands representing the type I processing intermediates began to appear. Traces of such intermediates can be seen in Figure 7 at 0, 35, and 50 mM as faint bands between the $p\alpha_1$ and $p\alpha_2$ bands and just ahead of the $p\alpha_2$ band. It seems likely, therefore, that normal processing of secreted collagen can take place under HIB conditions.

It should be noted that the secretion of a high molecular weight protein (Figure 7, unmarked arrow) which migrates with the mobility expected of fibronectin is not inhibited by HIB, another indication of the differential effects of HIB on cellular protein synthesis.

*Effect of HIB on Type II Collagen.* A number of studies have shown that the type II collagen chain can migrate, in gel electrophoresis, to a position between the type I collagen chains (Cheah et al., 1979; Paglia et al., 1981; Pawlowski et al., 1981). This behavior of the type II chain is due to underhydroxylation of the chain and makes it possible to directly compare type I and II collagen syntheses by gel electrophoresis. For comparison of the effect of HIB on type II collagen synthesis with type I synthesis, cultures derived from leg bone, as described under Experimental Procedures, were used. When such cultures are labeled, under HIB conditions, a pattern as shown in Figure 8 is observed.

A densitometer scan of the collagen region of Figure 8 is shown in Figure 9. It can be seen that the synthesis of $\alpha_1(II)$ is more resistant to HIB than $\alpha_1(I)$ or $\alpha_2$ and becomes a greater proportion of the three at higher NaCl levels. It remains to be determined whether this resistance is a consequence of type II collagen or of the fact that those cells are

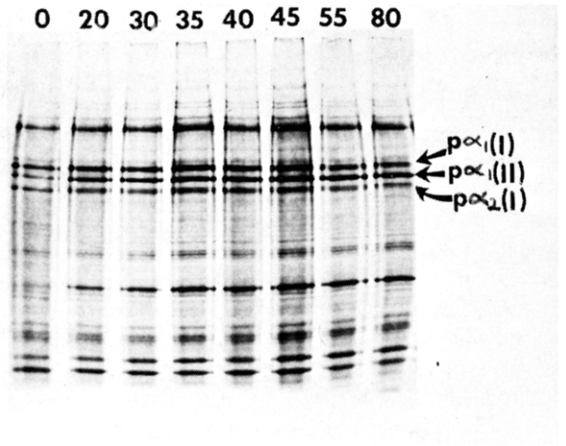


FIGURE 8: Labeling pattern of a cell population synthesizing type I and type II collagen at different NaCl concentrations. Mixed cells derived from leg bones, as described under Experimental Procedures, were treated with excess NaCl, labeled, and electrophoresed as described under Experimental Procedures.

making a higher amount of collagen than the type I producing cells. In populations of purified chondrocytes, it has been found that 48–60% of incorporated proline is collagenase susceptible.

In work to be presented elsewhere, it is shown that the $\alpha_1$(II) chain will migrate in this gel system to the position of the $\alpha_1$(I) subunit if fresh ascorbate is added to the culture medium. Addition of $\alpha,\alpha'$-dipyridyl, an inhibitor of proline hydroxylation (Juva et al., 1966), after addition of ascorbate, will again result in migration of $\alpha_1$(II) to the position shown in Figure 8. Since the question arises whether the $\alpha_1$(I) bands contain $\alpha_1$(II) chains, cells were labeled in the presence and absence of $\alpha,\alpha'$-dipyridyl to see if an $\alpha_1$(II) band appeared. The results of such an experiment are presented in Figure 6A,B and indicate that a band between $\alpha_1$(I) and $\alpha_2$ does not appear after treatment with $\alpha,\alpha'$-dipyridyl. These results indicate that the relative resistance of the $\alpha_1$(I) chain is not due to the comigration of a more resistant $\alpha_1$(II) present in these cultures.

## Discussion

The maintenance of the proper amount and chain ratio of collagen provides a useful system in which to study the coordination of gene activity. The results reported here indicate that the synthesis of the $\alpha_2$ collagen chain is more susceptible to inhibition of initiation than its complementary $\alpha_1$(I) subunit. This sensitivity, however, seems to depend on the amount of collagen synthesized by the cell, with cells making relatively large amounts of collagen being resistant to alterations in the chain ratio.

The mechanism by which HIB inhibits the initiation of protein synthesis is unknown, but its use in many different systems has shown a wide response in the synthesis of individual proteins (Saborio et al., 1974; Nuss & Koch, 1976a,b). It has also been shown that HIB is readily reversible even at NaCl concentrations much higher than those used in this study (Saborio et al., 1974; Koch et al., 1977), indicating that it does not affect cell viability.

The findings that the excess $\alpha_1$(I) chain may be secreted into the extracellular space would seem to indicate that this collagen is not completely degraded within the cell as already indicated by the findings of $\alpha_1$(I) trimer in a number of instances (Mayne et al., 1975, 1976; Benya et al., 1977; Crouch & Bornstein, 1978). The finding of normal secretion under HIB conditions is similar to that of Nuss & Koch (1976a) in their examination of the effects of HIB on the synthesis and secretion of the heavy and light immunoglobulin chains in mouse plasmacytoma cells.

Whether or not translational effects, as produced by HIB, could be responsible for regulation of collagen synthesis or production of $[\alpha_1(I)]_3$ trimer in the intact animal is unknown. Studies by Paglia et al. (1979, 1981), however, have shown that procollagen peptides can effect a specific reduction in collagen synthesis at translation both in vitro and in vivo, further implicating translation as a possible site of control of collagen synthesis.

Type I trimer has been found to be synthesized by cultured chondrocytes which have been grown in 5-bromo-2-deoxyuridine (BrdU), which shifts the type of collagen synthesized

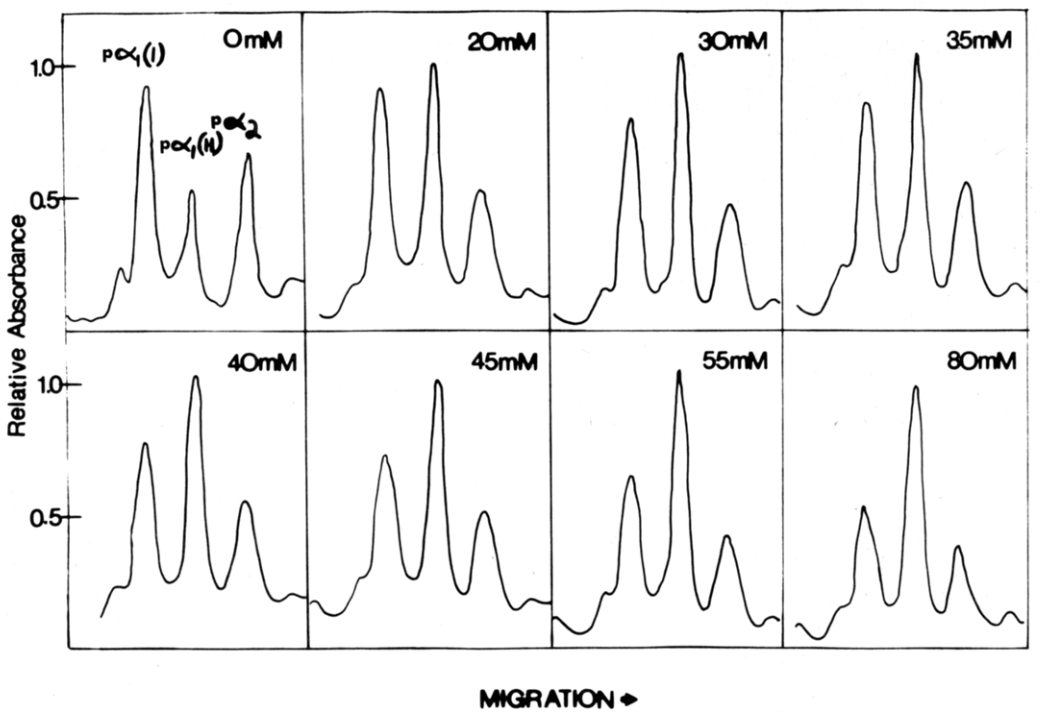


FIGURE 9: Densitometer scan of the collagen region of a gel autoradiogram given by a mixed cell population as described in Figure 6.

from type II to type I (Mayne et al., 1975). In studies presented elsewhere (Pawlowski et al., 1981), the collagen mRNA population of BrdU-grown chondrocytes was examined by using a cell-free protein-synthesizing system. It was found that the BrdU-treated chondrocyte message would give an excess of $\alpha_1$(I), indicating that in this system an altered subunit ratio is not due to translational effects. These seemingly contradicting findings may be the result of different types of controls being operable between cells starting to synthesize a new protein (BrdU system) and cells regulating the amounts of a protein they are already synthesizing. Direct examination of the mRNA levels is necessary, however, to precisely examine these findings.

The difference in the response to HIB among cells making different amounts of collagen presents several possibilities. Candidates for further study would include factors such as the presence of "cap" structures and translational control RNAs and the concentrations of protein initiation factors (Bergmann & Lodish, 1979; Heywood & Kennedy, 1976). Analysis, using cell-free protein-synthesizing systems, as well as direct examination of the mRNA concentration and structure should be able to distinguish among these possibilities.

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# Identification and Characterization of the Direct Folding Process of Hen Egg-White Lysozyme†

Shingo Kato, Nobuo Shimamoto, and Hiroyasu Utiyama*

ABSTRACT: Refolding kinetics of hen egg-white lysozyme (HEWL) have been studied by means of the stopped-flow method with guanidinium chloride as the denaturant. We show here that the three-species model $U_1 \rightleftarrows U_2 \rightleftarrows N$ ($U_1$ and $U_2$ = unfolded; N = native) now established for pancreatic ribonuclease A is also valid for HEWL on the basis of the following lines of evidence: (1) refolding kinetics outside the transition region are biphasic; (2) dependence of the fractional amplitude for the fast phase on the ratio of the time constants of the two phases agrees with theory; (3) unfolding kinetics outside the transition region are of single phase; (4) direct evidence for the $U_2 \rightarrow U_1$ transformation is obtained by double-jump experiments; (5) the time constant of the binding reaction of a substrate analogue, 4-methylumbelliferyl *N*,*N'*-diacetyl-$\beta$-chitobioside, to HEWL molecules during refolding reaction agrees with the time constant of the direct refolding phase $U_2 \rightarrow N$. The characteristic properties of the nucleation-controlled reaction of refolding of small globular proteins are discussed in general. The results of the discussion are used to suggest that the direct folding process is nucleation controlled from the experimental results of the temperature dependence of the refolding rate.

The rate of folding reaction of small globular proteins usually ranges from 10 to 100 $s^{-1}$. Therefore, the folding mechanism is often discussed based on the kinetic results obtained by using the stopped-flow technique. The important character of the models put forward heretofore may be represented by reactions A and B.

$$U \underset{k_{21}}{\overset{\text{fast } k_{12}}{\rightleftharpoons}} I \underset{k_{32}}{\overset{\text{slow } k_{23}}{\rightleftharpoons}} N \qquad \text{(A)}$$

(U = unfolded; I = reaction intermediate; N = native.) Reaction A is biphasic: the faster reaction produces an in-

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