*EVIDENCE FOR A LOW-MOLECULAR-WEIGHT COLLAGEN PRECURSOR**

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Collagen is known to contain two amino acids, hydroxyproline and hydroxylysine, which are not incorporated directly by activation.[1, 2] It now appears that the hydroxylation occurs after proline or lysine is incorporated into a polypeptide chain.[3, 4] However, the question as to the length of polypeptide chain required for initiating hydroxylation still remains unanswered.[5]

In this communication evidence is presented that collagen-proline hydroxylation occurs on polypeptides of low molecular weight. Thus, the hydroxylation process is initiated prior to the completion of the synthesis of collagen α-chains.

The present data rule out the participation of any proteolytic enzymes in obtaining low-molecular-weight polypeptides containing hydroxyproline.

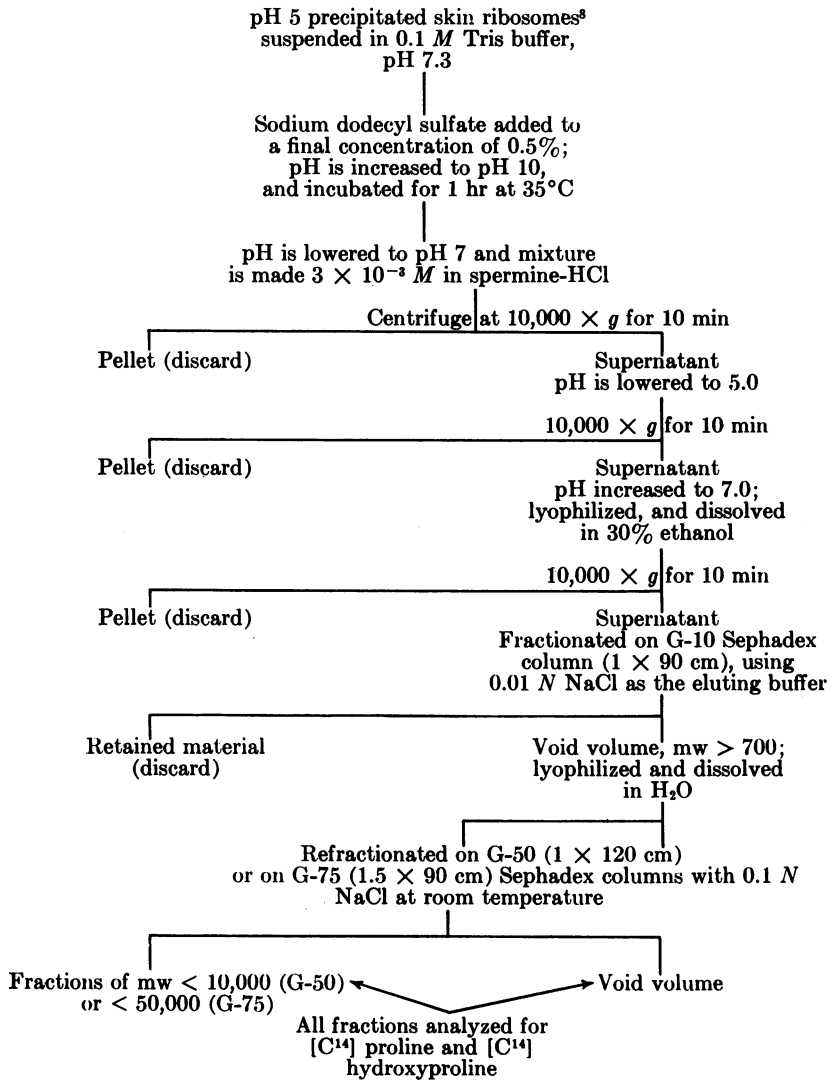
Materials and Methods.—The preparation of the skin, its method of incubation, the preparation of the microsomal fractions, and the isolation of proline-C^{14} and hydroxyproline-C^{14} were carried out as described in a previous communication.[6]

The isolation of 0.5 M NaCl soluble collagen and the measurement of free hydroxyproline: The incubated skin tissue (0.5 gm in weight) was homogenized in cold 0.5 *N* NaCl for 20 min in a VirTis homogenizer at medium speed. The homogenate was maintained at 4°C for 24 hr and then centrifuged at 10,000 rpm to remove insoluble material. The noncollagenous protein was precipitated at pH 3.5 with 2.4% trichloroacetic acid (TCA) in the cold, and the precipitate removed by centrifugation at 20,000 rpm for 1 hr. The TCA from the supernatant was removed by extraction with ether, and the pH restored to neutrality. Collagen was precipitated by the addition of enough cold 95% ethanol to give a final concentration of 14%. It was then kept at 4°C for 24 hr, and the precipitate collected by centrifugation at 20,000 rpm for 1 hr. The supernatant was evaporated to dryness and redissolved in 5 ml of 1% picric acid to recover the free amino acids.[6] The collagen precipitate was further washed with 5% TCA and ethanol:ether, and extracted with hot 5% TCA. The hot TCA supernatant was analyzed for bound [C^{14}] hydroxyproline.

The inhibition of protein synthesis in skin by puromycin: Puromycin (General Biochemicals, Inc.), which is known to inhibit the incorporation of sRNA-bound amino acid into ribosomal polypeptides,[7] was used to study its effect on protein synthesis in embryonic skin. The concentration of puromycin to bring about complete inhibition of protein synthesis was 100 μg/ml as determined in a separate experiment before examining its mode of action on the formation of 0.5 *N* NaCl soluble collagen and free hydroxyproline (Fig. 1). Sections of skin were incubated with labeled proline for 2 hr at which time 100 μg/ml of puromycin was added, and samples were taken for analysis at 30-min intervals thereafter.

Fractionation of ribosomal-bound polypeptides on Sephadex G-50 and G-75: The fractionation of ribosomal-bound polypeptides was preceded by their dissociation either by incubation with puromycin, or by incubation at pH 10 for 1 hr. The labeled ribosomes were detached from microsomes by deoxycholate treatment and precipitated at pH 5.[8] The pH 5 precipitate was suspended in 0.1 *N* Tris buffer, pH 7.3, and reincubated for 15 min at 37°C in a cell-free system with puromycin and rat liver pH 5 enzymes.[6] The pH of the incubation mixture was then lowered to pH 5, and the precipitate was discarded. The pH 5 supernatant was analyzed for peptide-bound [C^{14}] hydroxyproline. It was fractionated at room temperature on a calibrated G-50 Sephadex or G-75 column using 0.1 *N* NaCl solution as the effluent buffer. The calibration of G-50 is shown in Figure 2. There is a linear relationship between molecular weight and effluent volume.

The procedure for the dissociation of ribosomal bound polypeptides at pH 10 is shown in the following diagram. Special care had to be taken for the complete removal of free [C^{14}] proline and hydroxyproline. Following fractionation on Sephadex, three fractions were pooled, dried, hydrolyzed, and analyzed for [C^{14}] proline and [C^{14}] hydroxyproline, as previously described.



Results.—The results in Figure 3 (control) indicated that the specific activity of free hydroxyproline could surpass that of soluble collagen. In this experiment no attempt was made to inhibit the activity of proteolytic enzymes. It involved the measurements of the endogenous free hydroxyproline, and soluble collagen hydroxyproline as products of metabolism in the rabbit embryo skin. Initially, it was observed that the specific activity of free hydroxyproline was lower than that of soluble collagen, signifying a dilution of the new labeled free hydroxyproline by the pre-existing unlabeled free hydroxyproline pool. This was followed by an

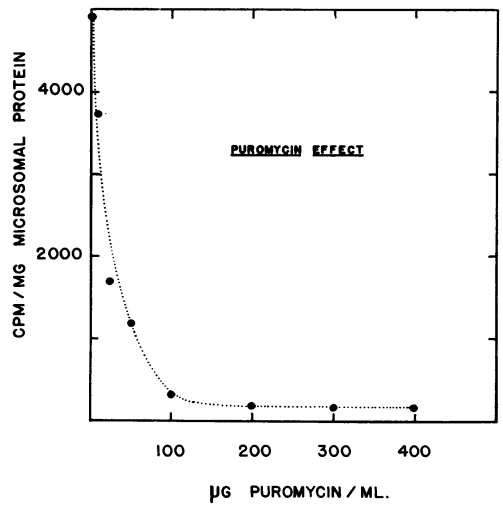


FIG. 1.—The effect of varying the concentration of puromycin on protein synthesis in the rabbit embryo skin *in vitro*. Various concentrations of puromycin up to 400 µg/ml were added together with [C^{14}] proline to the incubation medium following a 2-hr preincubation period of the skin in the absence of these two additives.

increase in the specific activity suggesting that the origin of this imino acid was a collagen precursor. The decrease observed after four hours would be expected if soluble collagen were degraded. Thus the specific activity of free hydroxyproline would ultimately reach that of soluble collagen or lower. Published data had shown that the specific activity of free hydroxyproline could exceed that of soluble collagen.[9] Contradictory evidence was also published showing that the specific activity of free hydroxyproline was lower than that of soluble collagen.[10] However, in these latter studies, no distinction was made between newly formed free hydroxyproline and "old" hydroxyproline.

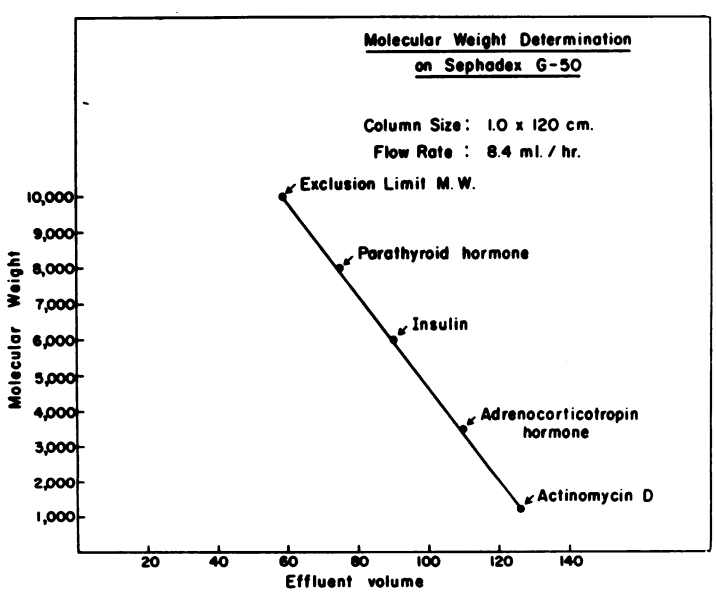


FIG. 2.—The elution pattern of amino acid polymers of varying molecular weights on Sephadex G-50 (1 × 120 cm).

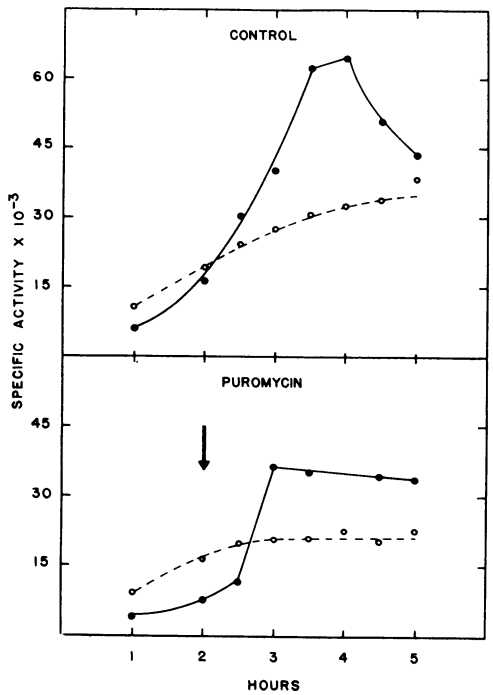


FIG. 3.—●, Free hydroxyproline; ○, 0.5 *N* NaCl soluble collagen hydroxyproline. The effect of puromycin on the change in the specific activity (cpm/μmole) of free hydroxyproline and soluble collagen hydroxyproline, as compared to that of the control. Arrow indicates time of addition of puromycin (100 μg/ml) to the incubation mixture of 0.5 gm of skin.

Additional experiments were performed to investigate the nature of the increase in the specific activity of free hydroxyproline. It was assumed that there were two possible sources of free hydroxyproline: (1) soluble collagen undergoing degradation, and (2) degradation of polypeptides prematurely released from polysomes, owing to rapid decay of collagen messenger RNA.[6] Release of such "incomplete" peptides would be promoted by addition of puromycin. In the studies on collagen using puromycin, the skin was preincubated for two hours, and [C^{14}] proline was then added, and incubation was continued for an additional period of five hours. Puromycin was added to the incubation mixture two hours following the addition of labeled proline. Samples were taken at intervals of half an hour. Puromycin inhibited collagen synthesis 30 minutes following its addition; no increase in the specific activity of soluble collagen hydroxyproline was observed. The inhibition of collagen synthesis by puromycin was accompanied by a lag in the formation of free hydroxyproline, followed by a sudden increase in its specific activity during a time when no synthesis was occurring, between $2^1/_2$ and 3 hours. Since during this time the specific activity of free hydroxyproline became much greater than that of soluble collagen, it was evident that free hydroxyproline must arise from the release and degradation of newly synthesized microsomal bound polypeptides containing hydroxyproline of high specific activity.

Polypeptides containing hydroxyproline were obtained from ribosomes by breaking the bonds between sRNA and the polypeptide chains in the ribosomal fraction either with puromycin or by incubation at pH 10 in a cell-free system. The peptides released by puromycin were fractionated on G-50 Sephadex. The results in Figure 4 suggested that [C^{14}] hydroxyproline was present on peptides of an average molecular weight of 6000 or greater. The specific activity of [C^{14}] proline

was higher in these peptides of low molecular weight, suggesting that these represent newly formed consecutive polypeptides. When these polypeptides were dissociated from the ribosomes at pH 10, and fractionated on G-50 Sephadex as described in *Methods*, the results shown in Figure 5 were obtained. Again, most of the [C^{14}] hydroxyproline was found on polypeptides of molecular weight 6000 and higher. No significant counts of [C^{14}] hydroxyproline were found on the polypeptides of a lower molecular weight. When a chemical detection method for hydroxyproline

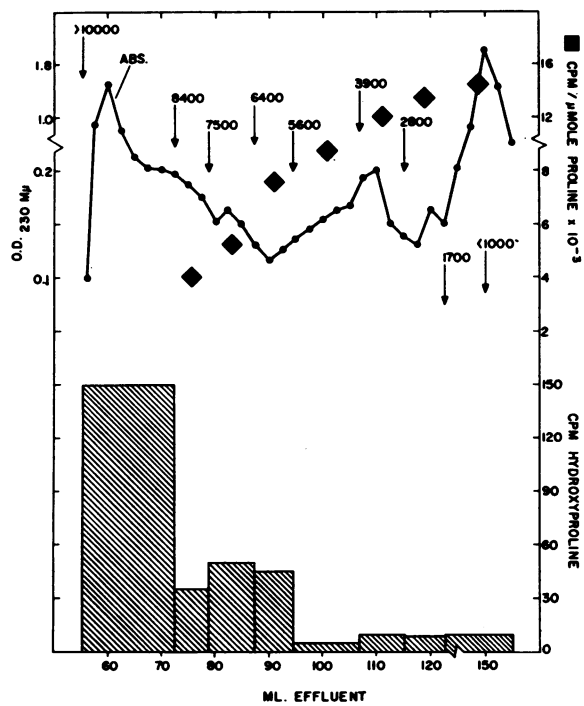


FIG. 4.—The elution of puromycin-dissociated polypeptides from skin ribosomes on Sephadex G-50. Pooled ribosomes from about 40 gm of [C^{14}] proline-labeled embryonic skin were suspended in 10 ml of medium A, and incubated in a cell-free system with: 50 μg/ml of puromycin; 1.0 mg/ml of rat liver pH 5 enzymes; 2 μmoles ATP/ml; 5 μmoles of creatine phosphate/ml; and 0.025 mg of creatine phosphokinase/ml. Incubation was for 15 min at 37°C. Figures above the arrows indicate approximate molecular weights of the fractions.

was used,[11] the results shown in Figure 6 were obtained, again suggesting that hydroxyproline was present on polypeptides of molecular weight of less than 10,000.

Discussion.—These results suggest a mechanism for biosynthesis of collagen which incorporates the ideas of a labile messenger RNA, the conversion of microsomal-bound proline to hydroxyproline, and also some information about the size of peptide required for hydroxylation. It is proposed that (1) the hydroxylation of proline occurs on a peptide of a molecular weight less than 10,000 and (2) that the hydroxylation is sequential, proceeding as the polypeptide chain grows in size.

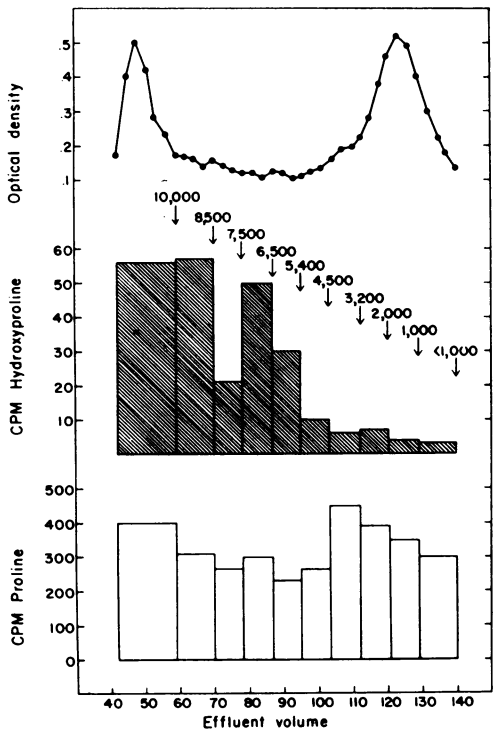


FIG. 5.—The elution of pH 10 dissociated polypeptides from ribosomes on Sephadex G-50 (1 × 120 cm). The polypeptides were obtained from the ribosomes as described in *Methods*. The ribosomes were obtained from 60–80 gm of pooled skin samples of 0.5 gm each, incubated with 1 μc of [C^{14}] proline for 3 hr following a preincubation period of 2 hr. Figures above arrows indicate approximate molecular weights of the fractions. Optical density is read at 230 mμ.

Previous studies[6, 12] have indicated that collagen biosynthesis in the embryonic skin is a process which is sensitive to actinomycin D. The suggested relatively short half life of collagen messenger RNA, taken with the observation that the specific activity of free hydroxyproline is higher than that of soluble collagen (Fig. 3), points to an origin of this high activity in the release and degradation of "incomplete" polypeptides containing [C^{14}] hydroxyproline. "Incomplete" polypeptides simply indicate polypeptides that are stopped short from being a complete and active protein due to inhibition in its synthesis. This inhibition may be produced either *in vitro* by incubation with puromycin, or may be set about by degradation of the messenger RNA with ribonuclease whether *in vivo* or *in vitro*. The process of possible degradation of messenger RNA *in vivo* is being designated as "half life" in this communication. A short half life of messenger RNA indicates that a rapid degradation of an *in vivo* messenger RNA may be occurring by an *in vivo* mechanism utilizing the enzyme ribonuclease or one like it that may be present in the rabbit embryo skin.

Inhibition of collagen formation by puromycin (Fig. 3) leads to an increase in the specific activity of free hydroxyproline. This indicates that free hydroxyproline can arise from the degradation of released ribosome-bound polypeptides, and this hydroxyproline is expected to have a higher specific activity than that of soluble collagen. Therefore, under normal conditions, the rapid decay of collagen messenger RNA can give rise to "incomplete" polypeptides containing hydroxyproline, and in turn to free hydroxyproline. It is known that an increase in urinary hydroxyproline peptides and in free hydroxyproline occurs during growth or following the

administration of an anabolic hormone.[13] These urinary peptides are believed to originate from soluble collagen. However, the results presented here suggest that microsome-bound polypeptides can also constitute precursors both for peptides containing hydroxyproline and for free hydroxyproline. The occurrence of ribosomal-bound low-molecular-weight polypeptides containing hydroxyproline is highly significant, as this observation implies that biosynthesis of collagen does not normally proceed via a "proline-rich precursor" which is hydroxylated after complete formation of a collagen peptide chain. The results reported under conditions which inhibited hydroxylation[14] simply indicate that peptide synthesis can go on

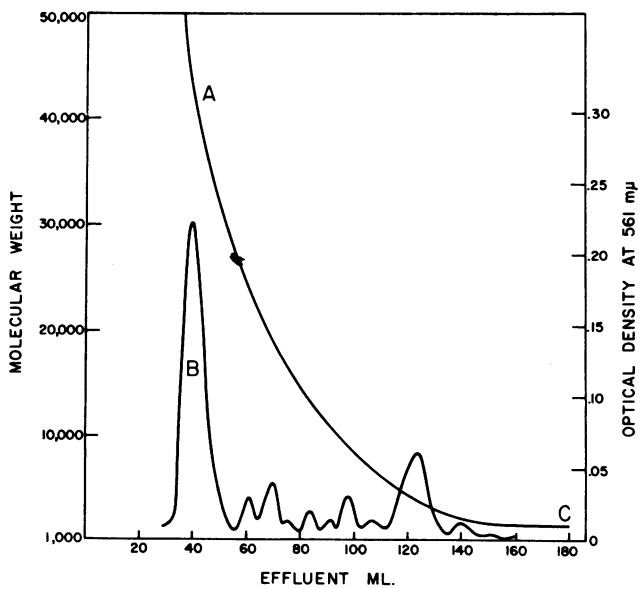


FIG. 6.—The distribution of *in vivo* peptide-bound hydroxyproline on Sephadex G-75, as dissociated from ribosomes of pH 10 treatment. (*A*) Calibration of a Sephadex G-75 column 1.5 × 90 cm, eluted with 0.1 *N* NaCl at room temperature; (*B*) amount of hydroxyproline in the void volume. The region between (*B*) and (*C*) represents that of hydroxyproline found at different positions on the column. Free hydroxyproline is eluted between 180 and 200 ml. An absorption of 0.05 OD at 561 mμ is equivalent to 1.0 μg of hydroxyproline. A total of 180 gm of embryonic skin was used for the isolation of the ribosomal peptides.

in the absence of hydroxylation. In addition, the present data also suggest that protocollagen, a nonhydroxylated collagen precursor similar to the α-chain of collagen,[14] may not exist *in vivo* where collagen synthesis is proceeding under normal conditions. There is no real evidence that a proline-rich polypeptide is found *in vivo*. The two experiments that are believed to somewhat establish the presence of a low-molecular-weight polypeptide containing hydroxyproline are those presented in Figures 3 and 6. Figure 3 shows that free hydroxyproline can have a higher specific activity than that of soluble collagen, and Figure 6 indicates that a polypeptide of a molecular weight of less than 10,000 is found to contain hydroxyproline. This is significant, because the procedure used to obtain such polypeptides

minimizes the action of any proteolytic enzyme. This conclusion is based on our findings that these preparations lack the presence of trypsin, pepsin, and α-chymotrypsin.[15] Although we have found significant amounts of the exopeptidase enzyme leucine amino peptidase in these preparations, the use of 0.5 per cent sodium dodecyl sulfate as shown in the diagram completely inhibited its action.[15] Carboxypeptidase is also inhibited by 0.5 per cent sodium dodecyl sulfate.[15] The possibility that these peptides are still products of the action of an endopeptidase on the ribosomes appears to be unlikely, because not only the ribosomal fractions but also the microsomal fractions obtained from the skin[6] lack endoproteolytic activity.[15] Therefore, the occurrence of such a polypeptide cannot be disregarded.

Our results appear to be in contrast with those reported,[16, 17] suggesting that collagen α-chain is the substrate for the hydroxylase. The approach used in our work has made it possible to detect the more likely substrates of hydroxylase activity, the microsome-bound growing polypeptides; without special techniques they would easily be overlooked for they comprise only a small fraction of the total cellular protein, and they are transitory intermediates whose release from microsomes depends on the half life of collagen messenger RNA.

Summary.—The mechanism of the formation of collagen in the rabbit embryo skin was studied *in vitro*. It was found that free hydroxyproline possessed a higher specific activity than that of soluble collagen hydroxyproline and that puromycin accentuated this finding. Other experiments indicate that hydroxylation of proline to form hydroxyproline is first initiated on a peptide of a molecular weight of less than 10,000 and that this hydroxylation is sequential; that is, the hydroxylation process takes place as the polypeptide grows in size.

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[1] Sinex, F. M., and D. D. van Slyke, *J. Biol. Chem.*, **216**, 245 (1965).

[2] Stetten, N. R., *J. Biol. Chem.*, **181**, 31 (1949).

[3] Peterkofsky, B., and S. Udenfriend, *J. Biol. Chem.*, **238**, 3966 (1963); Peterkofsky, B., and S. Udenfriend, *Biochem. Biophys. Res. Commun.*, **12**, 257 (1963).

[4] Prockop, D. J., E. Weinstein, and T. Mulveny, *Biochem. Biophys. Res. Commun.*, **22**, 124 (1966).

[5] Bhatnagar, R. S., K. I. Kivirikko, I. Rosenbloom, and D. J. Prockop, these PROCEEDINGS, **58**, 248 (1967).

[6] Bekhor, I. J., Z. Mohseni, M. E. Nimni, and L. A. Bavetta, these PROCEEDINGS, **54**, 615 (1965).

[7] Nathans, D., *Federation Proc.*, **23**, 984 (1964).

[8] Warner, J. R., and A. Rich, these PROCEEDINGS, **51**, 1134 (1964).

[9] Daughaday, W. H., and I. K. Mariz, *J. Biol. Chem.*, **237**, 2831 (1962).

[10] Prockop, D. J., B. Peterkofsky, and S. Udenfriend, *J. Biol. Chem.*, **237**, 1581 (1962).

[11] Grant, R. A., *J. Clinic. Pathol.*, **17**, 685 (1964).

[12] Bekhor, I. J., and L. A. Bavetta, these PROCEEDINGS, **53**, 613 (1965).

[13] Smiley, J. D., and M. Ziff, *Physiol. Rev.*, **44**, 30 (1964).

[14] Prockop, D. J., and K. Juva, these PROCEEDINGS, **53**, 661 (1965).

[15] Bavetta, L. A., and Z. Mohseni, to be published.

[16] Juva, K., D. J. Prockop, G. W. Cooper, and J. W. Lash, *Science*, **152**, 92 (1966).

[17] Lukens, L. N., *Federation Proc.*, **25**, 715 (1966).