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BIOSYNTHESIS, *IN VITRO*, OF "HISTIDINE-PROTEIN" — A BIOCHEMICAL MARKER IN EPIDERMAL DIFFERENTIATION

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SUMMARY

Unusual protein containing a high level of histidine and eight other amino acids (*i.e.* glycine, alanine, serine, threonine, arginine, glutamic acid, aspartic acid and tyrosine) is localized in the epidermal granular cell layer of the newborn rat. It appears that the formation of "histidine-protein" is a step in the sequence of epidermal differentiation terminating in keratinization. This paper reports the biosynthesis of "histidine-protein" by granular cells, *in vitro*. The protein, having a molecular weight of about 30 000, is obtained by extraction of homogenized epidermis in 8 M urea, dialysis of the extracted protein, lyophilization of the non-dialyzable material, extraction of the dried residue at 24° with 0.1 M $HClO_4$, precipitation at pH 4.5 and purification on Sephadex G-100. Synthesis of "histidine-protein" appears to occur in the "large" microsomal fraction and to involve at least two steps. A "peptide precursor", formed initially in a reaction sensitive to puromycin, is incorporated into "histidine-protein" by a mechanism not blocked by this inhibitor.

INTRODUCTION

Unusual protein, containing a high level of histidine, is found[1] at a localized stage of differentiation — the granular layer — leading to keratinization in the mammalian epidermis. This "histidine-protein", obtained by homogenizing the epidermis in 8 M urea, is non-dialyzable and, after lyophilization, is soluble in 0.1 M $HClO_4$ at 24°. It precipitates at pH 4.5 and, in addition to histidine, contains glycine, alanine, serine, threonine, arginine, glutamic acid, aspartic acid and tyrosine[2]. No sulfur-containing amino acids are present[2].

Radioautographic studies[3,4] have revealed that intraperitoneally injected amino acids localize in different layers of the epidermis of newborn rats. Phenylalanine, leucine, methionine, and valine, not present in "histidine-protein", are initially incorporated in the basal and lower spinous layers, whereas histidine, glycine and serine, present in the protein, first appear in the granular and upper spinous layers. Tyrosine, a component of this "histidine-protein", is generally distrib-

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uted in all viable cellular layers. Labeling in the cornified layer from these amino acids occurs only after all the other epidermal layers are extensively labeled. The rapid biosynthesis of "histidine-protein" in the upper layers could account for the initial localization of its constituent amino acids seen in the upper epidermal viable cell layers.

Since the location of "histidine-protein", immediately below the stratum corneum suggests that the protein might play a role in keratinization, an investigation of its biosynthesis, *in vitro*, was initiated. This paper reports the results of such initial studies using minced epidermis consisting mainly of granular cells and stratum corneum.

MATERIALS AND METHODS

Materials

L-[^{14}C]histidine (0.1 mC/0.067 mg) and [^{3}H]amino acid mixture were obtained from the New England Nuclear Corporation, Boston, Mass.; puromycin, from the Nutritional Biochemical Corp., Cleveland, Ohio; trypsin (twice crystallized), from the Mann Research Laboratories, New York, N.Y.; trypsin inhibitor (twice crystallized) and ribonuclease from the Sigma Chemical Corp., St. Louis, Mo.

Preparation of minced epidermis

The skin of newborn rats (Fig. 1A) (7–7.5 g) (3–4-days-old) of the CFN strain (Carworth Farms) was exposed for 60–80 min at 0° to 0.01 % trypsin in Earle's

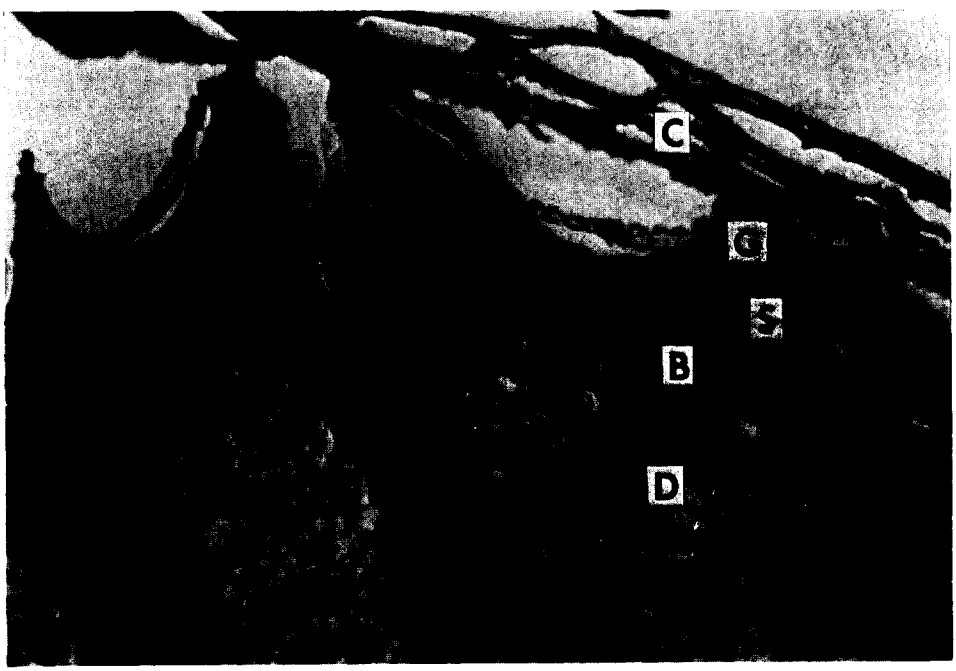


Fig. 1A. For legend see next page.

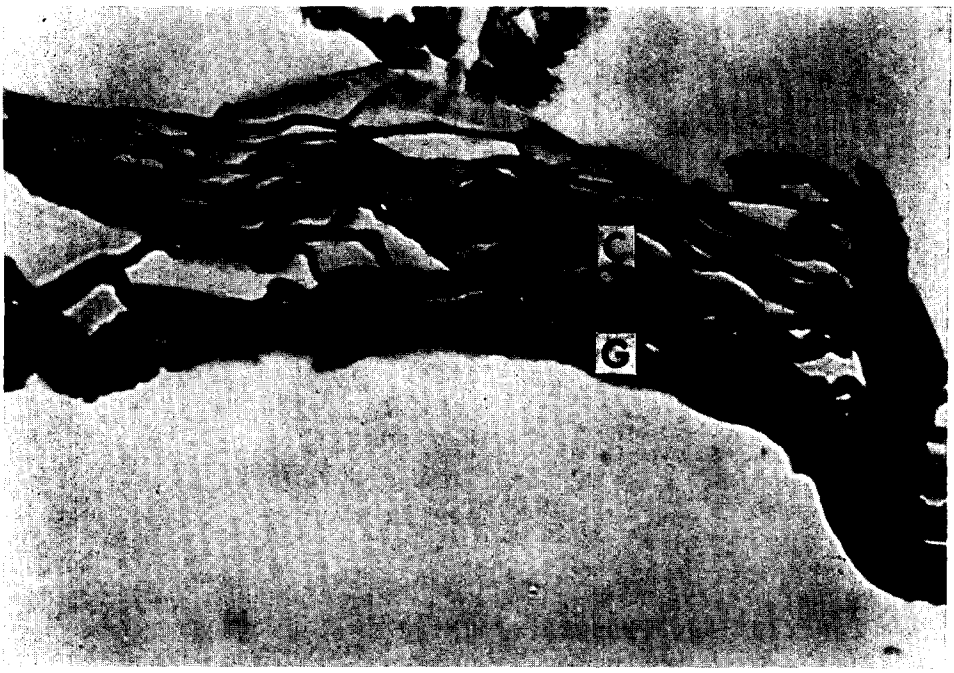


Fig. 1. Histological preparations of whole skin (A) and separated epidermis (B), containing mainly granular and cornified cells, as obtained by the trypsin technique (see text for methodology). Stained with haemotoxylin and eosin. About 400× magnification. D, dermis; B, S, G and C, basal, spinous, granular and cornified layers of the epidermis from the newborn rat, respectively.

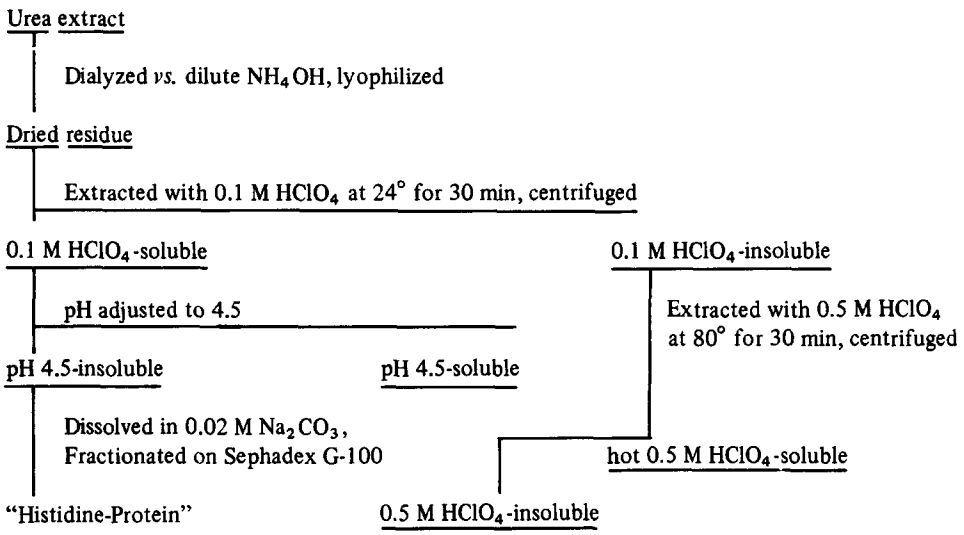
isotonic solution to separate the epidermis from the dermis. The epidermis, lifted from the dermis with forceps, was incubated for 10 min at 0° in 0.01 % soybean trypsin inhibitor[5] and then washed in Earle's solution. The resulting preparation, consisting mainly of stratum corneum and adhering layers of granular cells (Fig. 1B), was minced with a razor blade.

Incubation

The minced tissue was suspended in Earle's isotonic solution (0.2–0.3 g of tissue per 2 ml of solution) containing 0.25 μmole/ml of alanine, glycine, serine, threonine, aspartic acid, glutamic acid, tyrosine and arginine, respectively, and 300 units/ml of penicillin. Incubations were carried out aerobically at 37° in a shaking incubator after the addition of [^{14}C]histidine.

Isolation of "histidine-protein"

The reaction mixture, after incubation, was adjusted to pH 4.5 at 0° and was centrifuged at low speed. The sedimented material was submitted to extraction with 8 M urea–0.2 M Tris (pH 8.5) and the extracted protein was fractionated, according to the following flow diagram, by a modification (ribonuclease was not used) of a previously published[2] procedure:



Preparation of subcellular fractions

The minced epidermis, after incubation with [^{14}C]histidine, was homogenized in 3 vol. of ice cold 0.25 M sucrose in 50 mM Tris (pH 7.6) containing 5 mM magnesium acetate[6], and centrifuged at 700 $\times g$ for 10 min at 0–4°. The sedimented fraction was homogenized again in the same medium and again centrifuged. The supernatant solutions were combined and centrifuged for 10 min at 11 000 $\times g$ at 0–4° to sediment a mitochondrial fraction. The postmitochondrial fraction was treated with 0.5 % sodium deoxycholate (final concentration) and centrifuged 90 min at 40 000 $\times g$ at 0–4° to yield a "large" microsomal fraction (containing 0.2 mg of protein from the epidermis of 8 newborn rats). A "small" microsomal fraction was sedimented by centrifugation at 105 000 $\times g$ for 2 h at 0–4°.

Protein estimation

The LOWRY method[7] with bovine serum albumin as a standard was used for soluble protein. Low concentrations of protein were determined spectrophotometrically using the formula of WARBURG and CHRISTIAN[8] as modified by LAYNE[9], or the formula of WADDELL[10]. Insoluble protein was estimated by dry weight.

Analysis of protein for radioactivity

The protein of each fraction was precipitated with trichloroacetic acid in a final concentration of 10 % (containing 1 g of histidine and 0.5 g of urocanic acid per l) at 0°. The precipitate was washed with cold 5 % trichloroacetic acid, treated with 5 % trichloroacetic acid at 90° for 10 min, and allowed to stand at 0° for 30 min. The precipitated protein was then dissolved in 0.1 M NH_4OH (containing unlabeled histidine and urocanic acid) and reprecipitated with 10 % trichloroacetic acid. The final precipitate was treated with 95 % ethanol or 100 % acetone, followed by 3 : 1 ethanol–ether for 3 min at 60° and washed with ether (SCHNEIDER[11]). For determination of radioactivity, the isolated proteins were dissolved or swollen in 90 % formic acid, spread evenly on a weighed aluminum planchet and counted with a Geiger-Müller gas flow counter to a significance of at least ± 3 %.

RESULTS

Selection of experimental conditions

The results of preliminary experiments indicated that very little [^{14}C]histidine was incorporated into protein in epidermis separated from dermis by incubation in 0.24 M NH_4Cl (pH 9.5)[2]. A much higher incorporation was obtained when the epidermis was separated with trypsin[1]. It was also clear from early experiments that the tissue had to be homogenized in 8 M urea in order to solubilize most of the radioactive protein. Dialysis of the urea extract, lyophilization of the dialyzed residue and extraction of the dried residue with 0.1 M $HClO_4$ yielded a great deal of radioactivity in the higher molecular weight fraction. On the other hand, direct extraction of lyophilized tissue with 0.1 M acetic acid or 0.1 M $HClO_4$ gave radioactive components which were retarded when chromatographed on Sephadex G-25 and were obviously of smaller molecular weight. In all subsequent experiments, therefore, the epidermis was separated from the dermis by treatment with 0.01 % trypsin at 0–4° and homogenized in 8 M urea for isolation of "histidine-protein".

The isolation of labeled "histidine-protein" from the tissue

Table I shows the distribution of protein and radioactivity in the various fractions obtained from epidermis which had been incubated with [^{14}C]histidine, *in*

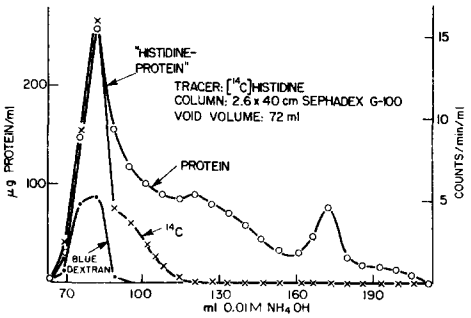
TABLE I

INCORPORATION OF [^{14}C]HISTIDINE INTO VARIOUS FRACTIONS OF EPIDERMAL PROTEIN

Protein fractions	*Total protein (mg)*	*Total counts/min*	*Counts/min per mg protein*
Urea-insoluble	800	4 120	5.2
Urea extract			
0.1 M $HClO_4$-soluble			
pH 4.5,-insoluble*	23	1 860	81.0
pH 4.5,-soluble	226	1 700	7.5
Hot 0.5 M $HClO_4$-soluble	120	1 670	14.0
0.5 M $HClO_4$-insoluble	891	28 800	32.0

* First peak from Sephadex G-100 column. For details of procedures see text.

vitro. A higher specific activity and a lower amount of protein were found in the 0.1 M $HClO_4$-soluble, pH 4.5-insoluble fraction (containing the "histidine-protein") as compared with any other protein fraction. The sharp protein peak, representing the "histidine-protein", obtained on a column of Sephadex G-100 (Fig. 2) was slightly retarded and was highly radioactive. In order to further test the homogeneity of this protein, highly labeled fractions from this peak were applied to a column of Sephadex G-200. A single peak with a molecular weight estimated at 30 000 was obtained on elution. These results coincide with the previous data[12] obtained, *in vivo*, for "histidine-protein". Amino acid analysis was not done since insufficient material was available.



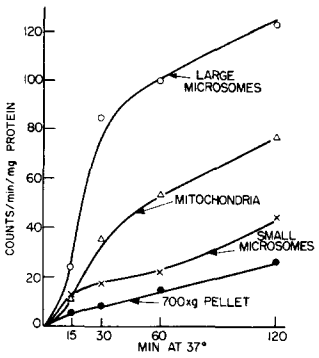


Fig. 2. Gel filtration of the 0.1 M $HClO_4$-soluble fraction. Epidermis, obtained by the trypsin technique, was incubated with [^{14}C]histidine, the pH adjusted to 4.5 and the insoluble fraction homogenized in 8 M urea. The urea extract was dialyzed, and lyophilized and the dried residue extracted with 0.1 M $HClO_4$ at room temperature. This extract was chromatographed on a column of Sephadex G-100 equilibrated and eluted with 0.01 M NH_4OH.

Fig. 3. Incorporation of [^{14}C]histidine into protein fractions in minced epidermis. Minced epidermis was incubated as described in the text with 2.5 μC of [^{14}C]histidine. At appropriate times, the incubation mixtures were adjusted to pH 4.5 at 0°. The insoluble material was then homogenized in 0.2 M sucrose and fractionated into the various subcellular elements as described in the text.

Time course of [^{14}C]histidine incorporation into the subcellular elements

As shown in Fig. 3, the "large" microsomal fraction (containing most of the microsomal protein) was the most active for protein synthesis in the epidermis as measured by the incorporation of [^{14}C]histidine. The mitochondrial fraction also incorporated significant levels of labeled histidine. The "large" microsomal fraction had an absorption spectrum close to that of ribosomes[13] with a 260 nm/235 nm ratio of about 1.63. In the presence of puromycin (50 μg/ml) the ability of these microsomes to incorporate histidine was reduced to 23 % of the control value.

The effect of puromycin and unlabeled histidine on the [^{14}C]histidine incorporation into epidermal protein fractions

Fig. 4 indicates the results obtained when puromycin (50 μg/ml) was added to the incubation mixture after 15 min of preincubation in the presence of [^{14}C]histidine. The specific activity of the 0.1 M $HClO_4$-soluble, pH 4.5-insoluble fraction (assumed to mirror "histidine-protein") continued to increase for 15 min at a rate less than that of the control after which the specific activity remained constant. The specific activity of the 0.1 M $HClO_4$-insoluble fraction was not significantly altered from the control during the initial 30 min after the addition of puromycin. In contrast, the addition of the inhibitor consistently caused an immediate, progressive decrease in specific activity of the 0.1 M $HClO_4$-soluble, pH 4.5-soluble fraction.

A consideration of the technique by which the incubation mixture was fractionated, indicated the possibility that some of the radioactive protein of the 0.1 M $HClO_4$-soluble, pH 4.5-soluble fraction could escape from the minced tissue and could appear in the pH 4.5-supernatant solution of the reaction mixture. Support for this idea derived from data obtained in a "cold chase" experiment (*i.e.* addition of a very large concentration of [^{12}C]histidine after preincubation with [^{14}C]histidine),

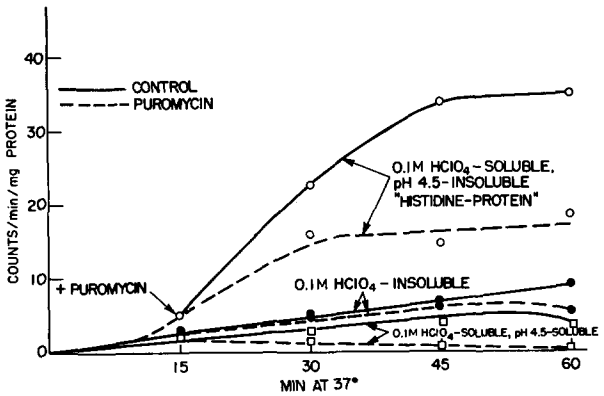


Fig. 4. Effect of puromycin on the incorporation of [^{14}C]histidine. Minced epidermis was preincubated with [^{14}C]histidine for 15 min followed by addition of puromycin (50 μg/ml). For assay of the various fractions, the incubation mixture was adjusted to pH 4.5 at 0°, centrifuged and the sedimented material submitted to extraction with 0.1 M $HClO_4$.

in which the specific activities of the pH 4.5-soluble fraction from the incubation mixture and the 0.1 M $HClO_4$-soluble, pH 4.5-soluble fraction of the urea extractable protein showed similar kinetics (Fig. 5). In both cases, the specific activity began to decrease immediately after addition of the unlabeled histidine. As in the experiment with puromycin, the specific activity of the 0.1 M $HClO_4$-soluble, pH 4.5-insoluble fraction continued to increase at a rate less than control for the first 15 min after the addition of the "cold chase" after which the specific activity did not change

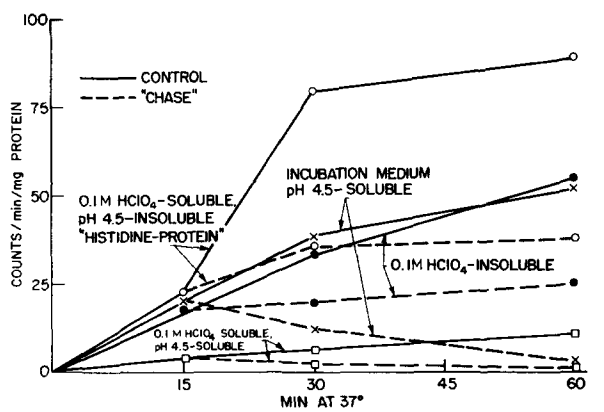


Fig. 5. "Chase" of labeled proteins after a preincubation with [^{14}C]histidine. Minced epidermis was incubated with 1 μC [^{14}C]histidine for 15 min at 37° followed by addition of unlabeled histidine to a concentration of 6 mM and further incubation. At various times samples were taken and treated as in Fig. 4.

DISCUSSION

The following may be tentatively concluded from the data in this report: (a) The biosynthesis of "histidine-protein" has been demonstrated *in vitro*, using a

preparation of minced epidermis consisting mainly of granular cells and stratum corneum; (b) studies of "pulse-chase" kinetics suggest that protein in the pH 4.5-soluble fraction is a precursor of the "histidine-protein"; (c) conversion of the pH 4.5-soluble protein to "histidine-protein" does not appear to involve ribosomal protein synthesis, and (d) synthesis of the pH 4.5-soluble protein takes place by a mechanism basically similar to the usual ribosomal process.

The mammalian epidermis is a continuously differentiating tissue in which, statistically, one of the two daughter cells produced by mitosis in the basal layer, is carried through a series of distinct morphological, and therefore biochemical, states to ultimately cornify and be sloughed from the external surface. As the cell passes through these stages, the tonofibrils present in the spinous layer and the so called keratohyalin granules, which appear in the granular layer, the last stage before loss of the nucleus, combine to produce the "keratin" fibers which fill the fully keratinized cell of the stratum corneum[14]. The complete chemical characterization of epidermal keratin is yet to be accomplished although numerous studies on solubilized protein from mammalian stratum corneum have been reported. The detailed role of "histidine-protein" in the biosynthesis of epidermal keratin is still unclear but the involvement of this unique protein in the biosynthetic process is suggested by kinetic radioautography[15]. The availability of an *in vitro* system should be extremely useful in future study of the relationship of epidermal "histidine-protein", keratohyalin and keratin[16,17].

At the present time, criteria for "histidine-protein" are solubility in 8 M urea, non-dialyzability, solubility in 0.1 M $HClO_4$ after drying *in vacuo*, exclusion on Sephadex G-50, but retardation on Sephadex G-100 (molecular weight estimated at about 30 000), and a composition consisting of glycine, alanine, arginine, serine, threonine, tyrosine, glutamic acid and aspartic acid as well as un unusually high level of histidine. With the exception of amino acid analysis, for which insufficient material was isolated, the characteristics of the protein into which [^{14}C]histidine was incorporated *in vitro* in this investigation coincided with all of these criteria. Since "histidine-protein" is primarily localized in the granular layer[1], could not be isolated from the cornified layer[12] which does not appear to synthesize protein *de novo*[3], and was synthesized by a preparation in which granular cells made up the major viable component, it is reasonable to assume that synthesis of this material is restricted to the granular cell.

At the beginning of this study, efforts were made to extract "histidine-protein" from the tissue without prior homogenization in urea. However, the low yield of labeled protein obtained under these conditions suggests that extraction in urea is a necessary prerequisite for obtaining the protein.

The inhibition of [^{14}C]histidine incorporation by puromycin and the high specific radioactivity of microsomal protein indicate that, at least the first step in the synthesis of "histidine-protein" occurs by the usual ribosomal mechanism for protein synthesis. The disappearance of ^{14}C from the pH 4.5-soluble fractions after the addition of puromycin or [^{12}C]histidine while the specific activity of the protein in the 0.1 M $HClO_4$-soluble, pH 4.5-insoluble fraction continued to increase in radioactivity for a short period, clearly suggest the hypothesis that the "histidine-protein" is formed from a pH 4.5-soluble precursor peptide in a reaction other than the ribosomal process. The metabolic relationships of the protein in these fractions with the

protein in the 0.1 M $HClO_4$-insoluble and the urea-insoluble fractions are presently unknown.

Tentative confirmation of the role of pH 4.5-soluble protein as precursor of the pH 4.5-insoluble protein was obtained in a preliminary experiment, shown in Table II, in which the pH 4.5-soluble protein in the medium, labeled from a mixture of

TABLE II

CONVERSION OF pH 4.5-SOLUBLE PROTEIN INTO "HISTIDINE-PROTEIN"

Minced epidermis (from 18 rats) was shaken with a [^{3}H]amino acid mixture (65 μC, 18 amino acids) at 37° for 2 h. The incubation mixture was adjusted to pH 4.5 at 0°, centrifuged and the protein in the supernatant was applied to a column of Sephadex G-25. The excluded protein fraction (11 mg, 4600 counts/min per mg protein) was used as the ^{3}H-labeled precursor protein. For the conversion reaction, homogenized epidermis (from 3 newborn rats) in 10 ml of Earle's solution containing cycloheximide (50 μg/ml) and unlabeled amino acid mixture (0.25 μmole/ml) was incubated at 37° for 3 h. The reaction mixture was adjusted to pH 4.5 at 0°, centrifuged and the sedimented residue was submitted to the described procedure for obtaining the "histidine-protein" which was assayed for protein[7] and for radioactivity (by liquid scintillation spectrometry)

	Total protein (mg)	*Total counts/min*	*Counts/min per mg protein*
Zero time	4.3	193	45
After 180 min of incubation	1.9	330	177

[^{3}H]amino acids, was separated by exclusion on a column of Sephadex G-25, and was then incubated with homogenized epidermis, cycloheximide (50 μg/ml) and a mixture of unlabeled amino acids. A small amount of the ^{3}H originally in the pH 4.5-soluble fraction was converted to 0.1 M $HClO_4$-soluble, pH 4.5-insoluble protein which was slightly retarded on Sephadex G-100 appearing in the position of "histidine-protein". Unfortunately, the zero time control was relatively high and more convincing experiments on this point are being attempted.

Further studies to elucidate the biosynthetic mechanism for "histidine-protein" require purification and characterization of the protein as well as the use of purified enzymatic components. Before the latter can be accomplished, the reported[18,19] difficulties in obtaining active components for protein synthesis from the epidermis will have to be overcome.

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REFERENCES

1 J. GUMUCIO, C. FELDKAMP AND I. A. BERNSTEIN, *J. Invest. Dermatol.*, 49 (1967) 545.
2 J. K. HOOBER AND I. A. BERNSTEIN, *Proc. Natl. Acad. Sci. U.S.*, 56 (1966) 594.
3 K. FUKUYAMA, T. NAKAMURA AND I. A. BERNSTEIN, *Anat. Record.*, 152 (1965) 525.
4 K. FUKUYAMA AND W. L. EPSTEIN, *J. Invest. Dermatol.*, 47 (1966) 551.

5 C. FELDKAMP, *Relation of "Histidine-Rich Protein" to Epidermal Keratohyalin in the Newborn Rat*, Thesis, The University of Michigan, Ann Arbor, Mich., 1969.
6 D. C. N. EARL AND A. KORNER, *Biochem. J.*, 94 (1965) 721.
7 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol., Chem.*, 193 (1951) 265.
8 O. WARBURG AND W. CHRISTIAN, *Biochem. Z.*, 310 (1941) 384.
9 E. LAYNE, *Methods Enzymol.*, 3 (1957) 447.
10 W. J. WADDELL, *J. Lab. Clin. Med.*, 48 (1956) 311.
11 W. C. SCHNEIDER, *J. Biol. Chem.*, 161 (1945) 293.
12 S. G. CHAKRABARTI AND I. A. BERNSTEIN, *Abstr. 152nd Natl. Meeting, Am. Chem. Soc., New York, N.Y.*, 1966.
13 M. L. PETERMAN, *The Physical and Chemical Properties of Ribosomes*, Elsevier, New York, 1964.
14 I. BRODY, *J. Ultrastruct. Res.*, 2 (1959) 482.
15 K. FUKUYAMA AND W. L. EPSTEIN, *J. Invest. Dermatol.*, 49 (1967) 595.
16 I. A. BERNSTEIN, S. G. CHAKRABARTI, K. K. KUMAROO AND L. A. SIBRACK, *J. Invest. Dermatol.* 55 (1970) 291.
17 I. A. BERNSTEIN, *J. Soc. Cosmetic. Chemists.*, 21 (1970) 583.
18 H. P. BADEN AND C. PEARLMAN, *J. Invest. Dermatol.*, 44 (1965) 145.
19 I. M. FREEDBERG, I. H. FINE AND F. H. CORDELLE, *J. Invest. Dermatol.*, 48 (1967) 55.