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Evidence of a Precursor Form of Stratum Corneum Basic Protein in Rat Epidermis†

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ABSTRACT: The fully differentiated anucleate cells of the stratum corneum of newborn rat epidermis contain a cationic protein called stratum corneum basic protein (SCBP). This protein has a molecular weight (49 000) and an amino acid composition similar to a protein extracted from the less differentiated cell layers of the epidermis. Pulse–chase experiments with radiolabeled histidine were undertaken to test the possibility that SCBP is derived from a preexisting protein. A protein of 52 000 daltons is rapidly but transiently labeled in extracts of the less differentiated cell layers. As the amount of label in the 52 000-dalton protein decreases, an increase in radiolabel is observed in extracts of the fully differentiated cells. This label is found in SCBP, a protein of lower molecular weight (49 000) than that initially labeled. These proteins are immunologically related and both are resistant to cyanogen bromide cleavage. They differ in apparent molecular weight on sodium dodecyl sulfate–polyacrylamide gels and in their net charge. The results are consistent with the conversion of a precursor protein into SCBP.

Keratinization is an intracellular differentiation process which culminates in the formation of a layer of fully differentiated anuclear cells, the epidermal stratum corneum. These cells contain the filamentous protein α-keratin and a recently isolated basic protein called stratum corneum basic protein (SCBP)[1] (Dale, 1977). The derivation of SCBP from a precursor is the subject of this report.

Keratinization of normal mammalian epidermis is associated with discrete morphologic changes. Cells at different stages of keratinization are arranged in distinct layers. The basal cells, which lie on the basement membrane separating the

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[1] Abbreviations used: SCBP, stratum corneum basic protein; PMSF, phenylmethanesulfonyl fluoride; $NaDodSO_4$, sodium dodecyl sulfate; KHG, keratohyalin granule; cpm, counts per minute.

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dermis and epidermis, form the innermost layer. These differentiate sequentially on their way to the epidermal surface, first into spinous cells, characterized by cytoplasmic filaments, then into granular cells, characterized by both keratohyalin granules (KHG's) and filaments, and finally into the fully keratinized cells of the stratum corneum. The change from granular to keratinized cells involves the loss of nucleus, keratohyalin granules, mitochondria, and other cellular organelles, the thickening of the cell membrane, and the rearrangement of the filaments into the "keratin pattern" of densely packed filaments embedded in an amorphous matrix (Brody, 1959a; Rhodin & Reith, 1962; Odland, 1964). It has been suggested, on morphological evidence, that KHGs contribute to the amorphous (interfilamentous) matrix material or possibly to the filamentous component itself (Brody, 1959a,b; Rhodin & Reith, 1962; Odland, 1964; Fukuyama & Epstein, 1967).

The role of KHG's in keratinization is not yet understood at the biochemical level. Autoradiographic studies show preferential incorporation of labeled histidine into KHG's. With increased time of exposure, the label is detectable inside the cells of the stratum corneum (Fukuyama & Epstein, 1967, 1975). Several proteins rich in histidine and arginine have been isolated from the granular cell layer or from extracted KHG's (Hoober & Bernstein, 1966; Ugel & Idler, 1972; Sibrack et al., 1974; Tezuka & Freedberg, 1974; Bhatnagar & Freedberg, 1976; Balmain et al., 1977). A protein has been isolated from the stratum corneum of newborn rat epidermis which is similar to the material extracted from KHG's (Dale & Stern, 1975; Dale, 1977). This protein, SCBP, has a similar mobility to KHG proteins on sodium dodecyl sulfate–polyacrylamide gels and also has a relatively high histidine and arginine content. Evidence has been presented that SCBP is a component of the interfilamentous matrix of the stratum corneum (Dale et al., 1978). In the work presented here, we describe experiments designed to test the hypothesis that the SCBP is derived from a protein synthesized in the nucleated cell layers which may be localized in the keratohyalin granules.

Materials and Methods

Materials. [^{3}H]Histidine, [^{14}C]histidine, [^{14}C]iodoacetic acid, and Aquasol were obtained from New England Nuclear. Urea, 8.8 M, was deionized with Bio-Rad mixed-bed resin, stored in the cold, and used within 1 month.

Pulse–Chase Labeling Experiments. Sprague–Dawley, Berkeley strain, rats less than 24 h old were injected subcutaneously with 5 μCi of [^{14}C]histidine (323 μCi/μmol) or 20 μCi of [^{3}H]histidine (11.3 Ci/mmol) in 0.08 M NaCl and after 2 h with 0.15 μmol of unlabeled histidine (10-fold excess for [^{14}C]histidine and 100-fold excess for [^{3}H]histidine). The animals remained with the mother until they were sacrificed. The animals were killed by cervical dislocation, and the dorsal skin was rapidly removed and chilled. Epidermis was separated by the NH_4Cl technique (Sibrack et al., 1974) and then was extracted with 1 M potassium phosphate, pH 7, containing 10 μg/mL phenylmethanesulfonyl fluoride (PMSF) for 30 min at 37 °C. In later experiments 0.22 M histidine was added during this extraction.[2] The tissue was then treated with 1% sodium dodecyl sulfate ($NaDodSO_4$) in 0.05 M sodium phosphate, pH 7.0, containing 10 μg/mL PMSF for 45 min at 25 °C. $NaDodSO_4$ treatment solubilizes the remaining contents of the nucleated cell layers of the epidermis (basal, spinous, and granular) (Dale et al., 1977; also see Results). Proteins of the residual tissue (stratum corneum) were extracted with 4 M urea in 0.05 M sodium phosphate, pH 7, containing 10 μg/mL PMSF overnight at 25 °C, with stirring. The resulting residue was reextracted with the same mixture for 4 h. The residue was homogenized in 8 M urea containing 0.1 M 2-mercaptoethanol, 1 mM dithiothreitol, and PMSF in the same buffer. A flow sheet of the extractions and treatment of the extracts is shown in Figure 1. Whenever possible, supernatants were dialyzed vs. distilled water, lyophilized, and redissolved in a small volume of phosphate buffer, pH 7, or 4–8 M urea containing phosphate buffer. The incorporated radioisotope was assayed by scintillation counting. Portions (10 μL) of the protein samples were counted in 10 mL of Aquasol. No precipitation of protein occurred in Aquasol. Duplicate aliquots of [^{3}H]histidine or [^{14}C]histidine were counted in 10 mL of Aquasol containing 10 μL of phosphate buffer with 4 M, 8 M, or no urea added. No quenching was observed with urea.

Protein Determination. Protein was determined by the method of either Lowry et al. (1951) or Bramhall et al. (1969) for samples containing mercaptoethanol and 8 M urea. Bovine serum albumin was used as the standard.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. Electrophoresis was performed in 7.5% polyacrylamide slab gels according to the method of Laemmli (1970) at pH 8.8. Samples for electrophoresis were dissolved in 4 M urea and 0.03 M Tris-HCl, pH 6.8, with 1.5% $NaDodSO_4$ and 1.5% 2-mercaptoethanol and boiled for 2–3 min. Protein (30–60 μg) was applied per lane. Electrophoresis was carried out at 20 mA for 1 h and then at 35 mA (or maximum of 200 V) for approximately 2 h. Gels were stained with Coomassie brilliant blue.

Fluorography of Polyacrylamide Slab Gels. Scintillation autography (fluorography) of polyacrylamide slab gels was done according to the method of Bonner & Laskey (1974) with modifications suggested by Laskey & Mills (1975). Gels were stained and destained as usual, then dehydrated in two changes of dimethyl sulfoxide, and impregnated with 2,5-diphenyloxazole by soaking in a 22.2% solution (w/v) in dimethyl sulfoxide for 3 h. The gel was immersed in water to precipitate the diphenyloxazole and vacuum dried. The gel was then placed in contact with RP Royal "X-Omat" X-ray film, preexposed with an electronic flash unit (Laskey & Mills, 1975), and exposed at −70 °C. For the pulse–chase experiments, film was generally exposed for 1–3 weeks. After development, the X-ray film fluorograph was scanned on a densitometer (Helena Instruments, QuikScan or Ortec, Model 4310, with digital integration) for quantitation of radioactivity per protein band. The quantitation and linearity of the method were checked in several ways. The response of the X-ray film to light (electronic flash) was linear up to a film absorbance of approximately 1.0 at 525 nm, determined by comparison with a commercially prepared step tablet of increasing density (Helena Instruments). The response of the X-ray film to light emitted by the scintillant was tested by exposure with a standard step polyacrylamide gel processed for fluorography as described above (Laskey & Mills, 1975). This step gel was formed by mixing a known quantity of [^{14}C]-S-carboxymethylated bovine serum albumin with unpolymerized polyacrylamide, pouring the gel, and repeating with decreasing quantities of labeled protein to form seven steps with known counts per minute per unit area. Linear response of the X-ray film was obtained below 0.5 absorbance units. Thus, quan-

[2] The addition of histidine resulted in better electrophoretic separation of extract B without altering the protein profile of other extracts on the gels. The reason for the improved resolution is not known.

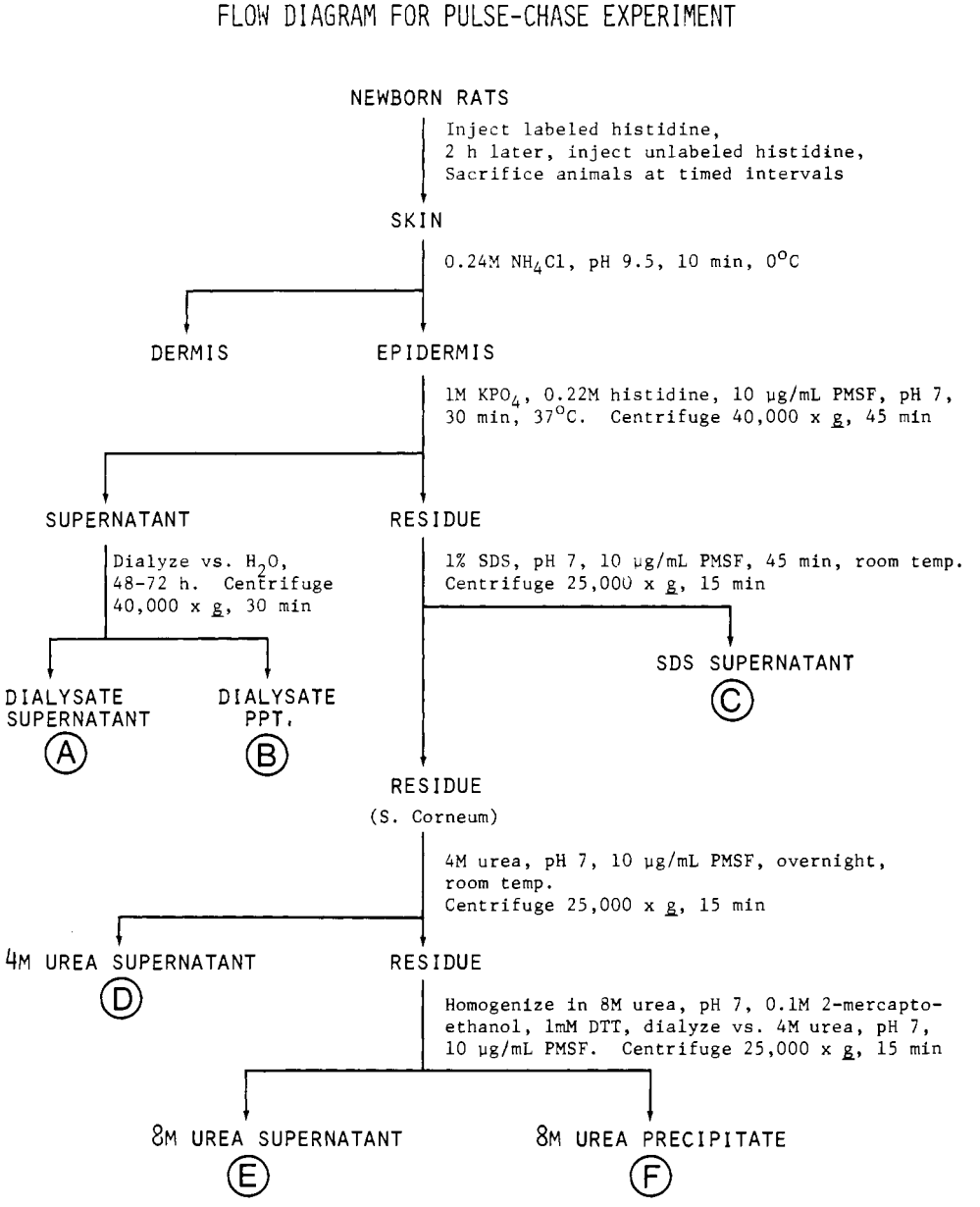

FIGURE 1: Flow diagram for the pulse–chase experiment. SDS, sodium dodecyl sulfate; KPO_4, potassium phosphate; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol.

titation of radioactivity could be obtained for the labeled protein bands in this study by varying exposure time so that the maximum density of the bands was less than 0.5 absorbance units at 525 nm. All quantitation reported was performed using films with densities in the linear range. Quantitation was also checked using increasing amounts of [^{14}C]-S-carboxymethylated bovine serum albumin electrophoretically separated on sodium dodecyl sulfate–polyacrylamide slab gels processed as described above.

Amino Acid Analysis. Samples for amino acid analysis were dialyzed vs. distilled water, lyophilized, and then hydrolyzed in 6 N HCl at 104–108 °C for 24 h. Composition was determined on a Beckman 120 °C amino acid analyzer. The results were not corrected for the loss of residues on hydrolysis.

Cyanogen Bromide Treatment. Protein samples for cyanogen bromide treatment were dialyzed vs. water, divided into three portions, and then lyophilized in test tubes. One portion was treated with a fivefold excess of cyanogen bromide in 70% formic acid (Epstein, 1974). A second portion served as the formic acid control, and a third portion served as the untreated control. After incubation and lyophilization, the three samples were redissolved in 8 M urea–0.063 M Tris-HCl buffer, pH 6.8, containing 1.5% $NaDodSO_4$ and 1.5% 2-mercaptoethanol for electrophoresis on slab gels.

Immunologic Analysis. Antiserum against SCBP was elicited in a goat, by using chromatographically purified SCBP (Dale & Ling, 1979). The antiserum used for immunoelectrophoresis was purified by ammonium sulfate precipitation and chromatography on DEAE-cellulose (Peterson & Sober, 1960). Adsorbed antiserum was prepared by incubation with SCBP linked to cyanogen bromide activated Sepharose 4B (Cuatrecasas, 1970).

Immunoelectrophoresis was performed according to Grabar & Williams (1953) in agarose containing 0.04 M barbital buffer, pH 8.0.

Results

Tissue Origin of Epidermal Extracts. The extraction sequence of the pulse–chase experiments was designed to maximize information on the localization of labeled proteins in the epidermis. The 1 M potassium phosphate treatment has been shown by histologic methods to extract the contents

Table I: Protein Yields and Probable Origin of Epidermal Extracts

extract[a]	protein yield[b] (mg)	protein origin
(A) 1 M KPO_4, dialysis supernatant	1.5 ± 0.3	proteins of nucleated cells soluble at high salt concentration
(B) 1 M KPO_4, dialysis precipitate	3.5 ± 0.4	keratohyalin granules[c]
(C) 1% $NaDodSO_4$	7.2 ± 1.3	remaining contents of nucleated cells[d]
(D) 4 M urea	34.5 ± 3.5	SCBP and fibrous proteins from stratum corneum[e]
(E) 8 M urea	8.7 ± 2.5	remaining contents of stratum corneum
(F) 8 M urea, residue	1.7 ± 0.3	remaining contents of stratum corneum
total A-F	56.8 ± 4.1	

[a] Extracts A-F are identified as shown in Figure 1. [b] The mean and standard deviation for five extractions of four newborn rat epidermis. [c] Sibrack et al. (1974). [d] Dale et al. (1977). [e] Dale (1977).

of KHGs (Ugel, 1969; Sibrack et al., 1974). When dialyzed, the precipitate fraction has the staining and morphologic characteristics of KHGs. Thus, extract B and possibly extract A contain KHG material (Table I and Figure 1). They may also contain other proteins soluble at high salt concentration. Treatment of epidermis with 1% $NaDodSO_4$ was shown by Dale et al. (1977) to yield stratum corneum preparations essentially free of nucleated cells. Thus, extract C contains proteins from the nucleated cell layers while extracts D–F contain stratum corneum proteins. These conclusions as well as the yield of protein in the extracts are summarized in Table I.

Trial extraction and autoradiographic studies were conducted to confirm these conclusions. Autoradiography was performed 2, 6, and 20 h after injection of [^{14}C]histidine (chased with a 10-fold excess of unlabeled histidine at 2 h). At 2 h, grains were seen in the nucleated cell layers, especially the granular layer. After 6 h, some grains were also visible in the lowest layer of the stratum corneum. After 20 h, many grains were seen in both the granular and the cornified layers. The $NaDodSO_4$ extracts prepared at these times had 110 000, 167 000, and 150 000 cpm, respectively (the potassium phosphate step was omitted for simplicity). These values corresponded to 71, 56, and 40% of the total label recovered at 2, 6, and 20 h, respectively. The positive correlation between the proportion of grains in the nucleated cell layers and the percentage of label in $NaDodSO_4$ extracts suggests that nucleated cell proteins are solubilized by this treatment.

In a parallel experiment, potassium phosphate treatment resulted in extraction of 43 400, 85 200, and 59 800 cpm corresponding to 50, 38, and 28% (extracts A + B) of the total label at 2, 6, and 20 h. Thus, there is also a strong correlation between the proportion of grains in the nucleated cell layers and the percentage of label extractable with potassium phosphate, but the absolute value of the counts is less than that for $NaDodSO_4$ treatment alone.

An additional trial experiment was performed to compare the protein yield of the 1 M potassium phosphate treatment of whole epidermis vs. that of isolated stratum corneum. The potassium phosphate extract of whole epidermis (extracts A + B) contained 7.6% of the total epidermal protein, whereas that of the stratum corneum contained only 0.3%. Thus, potassium phosphate is a very ineffective method for extracting stratum corneum proteins. This method yields proteins from the nucleated cell layers, including KHG proteins.

Table II: Labeling of Epidermal Extracts

extract[a]	time after injection (h)	cpm × 10^{-4}	cpm/μg of protein	change in sp act.
A	0.5	1.45	11.2	1
	2	2.06	19.1	1.71
	5	2.90	20.3	1.81
	8	3.43	20.8	1.86
	24	3.09	15.4	1.38
B	0.5	5.91	17.4	1
	2	11.42	26.9	1.55
	5	13.56	42.4	2.44
	8	20.11	58.3	3.35
	24	8.56	26.4	1.52
C	0.5	10.01	18.9	1
	2	18.39	30.7	1.62
	5	22.93	29.4	1.56
	8	33.92	41.9	2.22
	24	17.97	20.9	1.11
D	0.5	6.96	1.93	1
	2	12.04	3.96	2.05
	5	11.52	3.43	1.78
	8	25.79	6.38	3.31
	24	49.75	13.67	7.08
E	0.5	0.44	0.52	1
	2	0.60	0.77	1.48
	5	0.71	1.05	2.02
	8	0.52	0.73	1.40
	24	2.11	1.56	3.00
F	0.5	0.15	0.90	1
	2	0.25	1.54	1.71
	5	0.24	1.85	2.06
	8	1.08	4.85	5.39
	24	2.80	6.44	7.16

[a] Extracts are identified (A-F) as shown in Figure 1.

Pulse–Chase Labeling Experiments. In vivo pulse–chase experiments were performed to determine if the SCBP was derived from a preexisting protein present in the potassium phosphate extract. The procedure for the experiment is shown diagrammatically in Figure 1. The recovered label and the specific activity of labeled extracts for a representative experiment are shown in Table II. Extract C has the greatest number of counts recovered at each time point (except at 24 h). The specific activities of extracts A–C increase to a maximum at 8 h after the initial injection and then decrease. However, those of extracts D–F are still increasing at 24 h. The extracts A–C, which represent proteins of the nucleated cell layers, contained 72 ± 12% (mean and standard deviation for four experiments) of the total label recovered at 2 h after injection, whereas these extracts contained only 35 ± 10% after 24 h. The label in extract D, representing proteins of the stratum corneum, increased from 27 ± 12% at 2 h to 56 ± 2% at 24 h. Extracts E and F contained less than 10% of the total label.

The "specific labeling" of the individual extracts is shown in Figure 2 where "specific labeling" is defined as the counts per minute per milligram of total epidermal protein at that time point (the sum of protein present in extracts A–F). When plotted in this way, an overall view of the extractability of label with respect to all epidermal proteins can be obtained. Extract B has the greatest specific labeling at early times, increasing to a maximum at 8 h. Extract D has the greatest specific labeling at 24 h.

Labeled proteins were identified by electrophoresis on slab gels which were processed for scintillation autography (fluorography). Photographs of the gels, stained with Coomassie brilliant blue, and the corresponding X-ray film

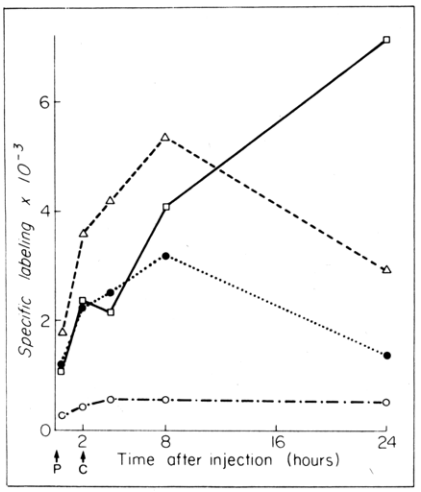

FIGURE 2: Labeling of the epidermal extracts relative to total epidermal protein. [^{3}H]Histidine (20 μCi) was injected subcutaneously at 0 time (arrow, P) and chased with unlabeled histidine at 2 h (arrow, C). At various times the dorsal skin was removed and the epidermis was sequentially treated as shown in Figure 1. (○) Extract A; (●) extract B; (△) extract C; (□) extract D. Extracts E and F had very little label and are not shown.

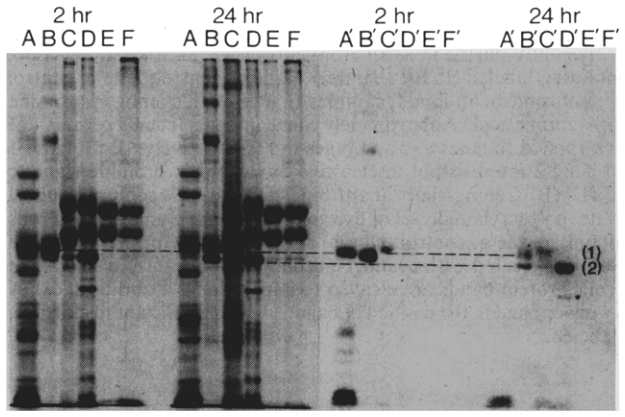

FIGURE 3: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and fluorography of epidermal extracts obtained 2 and 24 h after injection. Left: slab gels stained for protein with Coomassie brilliant blue; lanes are labeled by extract letter A–F, as shown in Figure 1. Right: scintillation autography of the same slab gel; corresponding lanes are labeled A′–F′. The most highly labeled bands are numbered (1) and (2). The band marked with an asterisk is newly labeled at 24 h.

fluorographs are shown for the 2-h and the 24-h extracts (Figure 3). The bands labeled 1 and 2 were the most highly labeled at early and late times, respectively. Scans of X-ray film fluorographs of extracts B–D in the region of bands 1 and 2 are shown in Figure 4. Band 1 is labeled 0.5 h after injection, whereas band 2 is not labeled until 5 h after injection. A third labeled band (*) is seen at 24 h in extract D. All these bands are clearly identified at all times in the Coomassie brilliant blue stained gels.

Figure 5 shows the relative change in radioactivity with time for bands 1 and 2. Each activity is compared with the total amount of radioactivity that was incorporated in the epidermis. Thus, the percentage of label in a particular band in one extract, determined from quantitative scanning of a fluorograph, is multiplied by the amount of label in the extract to obtain counts per minute. These values were added for extracts A–F. The value at each time point is expressed as a percentage of the maximum label incorporated in the whole epidermis (generally the value at 8 h) and plotted against time after injection.

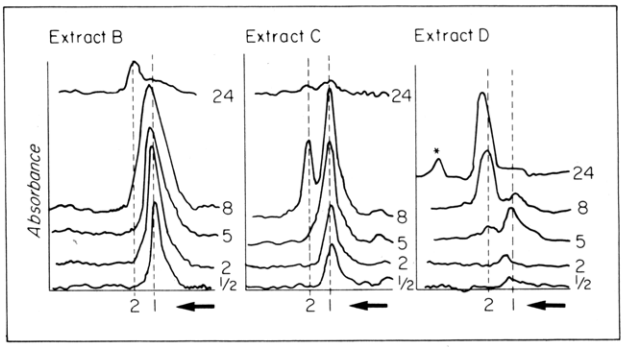

FIGURE 4: Scans of scintillation autographs of slab gels. Extracts B–D were obtained 0.5, 2, 5, 8, and 24 h after initial injection of [^{3}H]histidine. Each extract was run on a 7.5% sodium dodecyl sulfate–polyacrylamide slab gel (the direction of migration is shown by the arrows) and processed for autography. Densitometry was performed on an Ortec Model 4210 at 575 nm. The position of bands 1 and 2 is indicated. Band 1 is rapidly labeled after injection. Band 2 is initially labeled at 5 h after injection. As the labeling of band 2 increases, that of band 1 decreases. The band marked with an asterisk is newly labeled at 24 h.

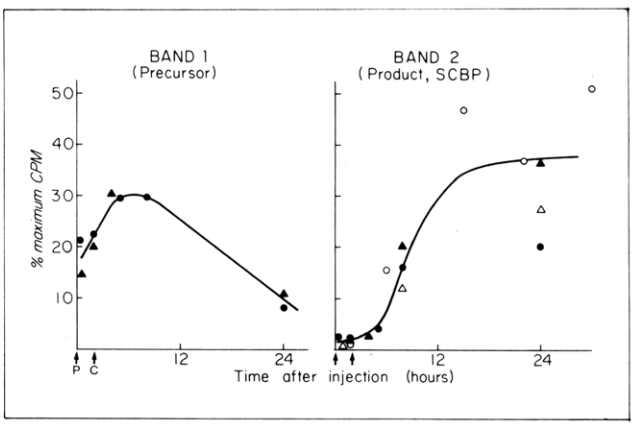

FIGURE 5: Relative labeling of bands 1 and 2. The label incorporated into bands 1 and 2 was calculated from quantitation of scintillation autographs and expressed as a percentage of the maximum label incorporated into the epidermis. Arrows marked P and C indicate the time of pulse and chase, respectively. Experiment 1 (○); 2 (△), 3 (▲); 4 (●). Only data from experiments 3 and 4 are shown for band 1, because of technical problems with electrophoretic separation of labeled protein in extract B prior to use of histidine in the extraction mixture.[2] This did not affect quantitation of band 2.

Initially band 1 is the more highly labeled. Band 2 is essentially unlabeled for the first 5–6 hours, after which its relative labeling begins to increase dramatically. At about the same time (5–6 h) the label in band 1 begins to level off and then decline. These results suggest that band 1 is a direct precursor of band 2.

Purification of Labeled Bands. To verify that labeled band 2 is SCBP, we purified the labeled protein in extract D obtained 24 h after injection of [^{3}H]histidine using the two-column chromatographic procedure previously described for SCBP (Dale, 1977). Sixty-five percent of the label did not adsorb to the DE-52 column in 50 mM phosphate buffer, pH 7, with 4 M urea. When this material was chromatographed on CM-52, one peak of radioactivity was recovered which corresponded in position to both the major protein peak eluted at 0.2 M NaCl and the known elution of SCBP (Dale, 1977). The peak fractions were pooled and analyzed for purity by gel electrophoresis and for amino acid composition. The preparation contained one predominant protein band which had the same electrophoretic mobility as SCBP. It had an amino acid composition essentially identical with that previously

Table III: Amino Acid Composition of SCBP and KHG's

	residues per 100 residues		
	^{3}H band 2	SCBP[a]	KHG prepn[b]
half-cystine	0	0	0.5
lysine	0.06	0	4.3
histidine	6.9	7.9	5.2
arginine	13.6	13.9	10.9
aspartic acid	3.9	3.6	6.8
threonine	6.3	5.7	5.3
serine	16.5	17.5	13.2
glutamic acid	19.9	20.5	18.5
proline	3.6	2.8	3.4
glycine	15.2	14.4	12.1
alanine	11.4	11.8	9.9
valine	0.3	0.6	2.4
methionine	0.1	0	0.7
isoleucine	1.8	1.4	2.4
leucine	0.1	0	3.0
tyrosine	0.3	trace	0.3
phenylalanine	0	0	1.1

[a] From Dale (1977). [b] From Dale & Stern (1975). This preparation is similar to extract B.

obtained for SCBP (Table III). A duplicate sample was chromatographed on an amino acid analyzer, and fractions were collected for sinctillation counting. All of the label was present in fractions corresponding to the elution of histidine from the column.

Chromatographic purification of band 1 from extract B was attempted. The method for purification of SCBP was used except that the initial buffer concentration for the DE-52 column was 10 mM phosphate in 4 M urea instead of 50 mM phosphate in 4 M urea. A minor unadsorbed peak with electrophoretic mobility corresponding to SCBP was recovered in 10 mM phosphate buffer. A labeled peak with mobility corresponding to band 1 was eluted with 250 mM phosphate buffer. Thus, band 1 was separable from band 2 by ion-exchange chromatography. Further purification and characterization of this band will be reported separately.

Cyanogen Bromide Treatment of Epidermal Extracts. One of the unusual properties of SCBP is the absence of the amino acid methionine (Dale, 1977). As a consequence, this protein should be resistant to cleavage by cyanogen bromide in acid. Furthermore, any precursor should yield a protein the same size or larger than SCBP when treated with cyanogen bromide. Extract B, obtained 2 h after injection of [^{3}H]histidine, contained labeled band 1, the possible precursor of SCBP. Labeled band 2, SCBP, was purified from extract D, obtained 24 h after injection of labeled histidine. These samples were tested for susceptibility to cyanogen bromide cleavage. The untreated control, the formic acid control, and the cyanogen bromide treated samples were analyzed by $NaDodSO_4$ gel electrophoresis and fluorography. The results are shown in Figure 6A. Extract E, which contained mainly unlabeled fibrous protein, was similarily treated (Figure 6B). As predicted, the SCBP (molecular weight 49 000) is resistant to cleavage, in contrast to the fibrous proteins. Band 1 (molecular weight 52 000) in extract B is also resistant to cyanogen bromide cleavage.

Immunoelectrophoresis. Extract B and SCBP were tested for reaction with antibody against SCBP. We have shown elsewhere (Dale & Ling, 1979) that these samples yield a reaction of identity in Ouchterlony diffusion and that the immunoreactive protein in extract B is the 52 000 molecular weight protein, band 1. Both samples gave a single arc in immunoelectrophoresis (Figure 7). SCBP migrated toward

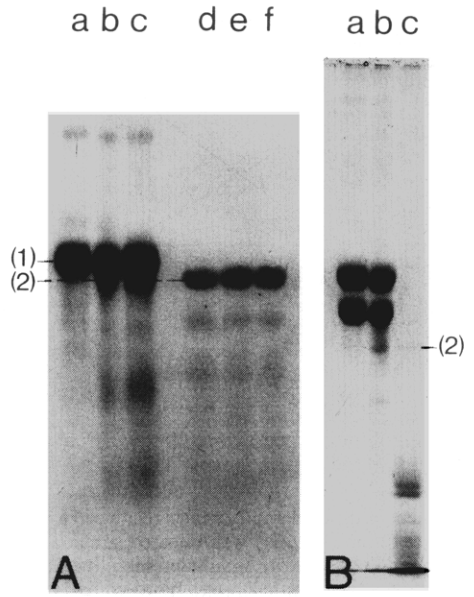

FIGURE 6: Cyanogen bromide cleavage of epidermal proteins. (A) Fluorograph of a 10% sodium dodecyl sulfate–polyacrylamide gel. Lanes a–c, extract B, 2 h after injection, containing labeled band 1: lane a, untreated control; lane b, control in 70% formic acid; lane c, samples treated with cyanogen bromide in 70% formic acid. Lanes d–f, partially purified SCBP from extract D, 24 h after injection, containing labeled SCBP (band 2): lane d, control; lane e, control in 70% formic acid; lane f, samples treated with cyanogen bromide in 70% formic acid. Approximately equal amounts (counts per minute) were applied to lanes a–c and lanes d–f, respectively. Both band 1 and band 2 are resistant to cleavage by cyanogen bromide (lanes c and f). (B) Coomassie brilliant blue stained 7.5% sodium dodecyl sulfate–polyacrylamide gel of extract E, containing unlabeled fibrous proteins. Lane a, control; lane b, control in 70% formic acid; lane c, samples treated with cyanogen bromide in 70% formic acid. The fibrous protein bands are cleaved (compare lanes a and b vs. c). A very minor band in the position of band 2, which is resistant to cleavage, is labeled.

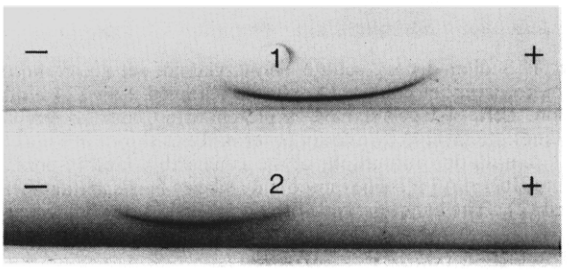

FIGURE 7: Immunoelectrophoresis of extract B, well 1, and partially purified SCBP, well 2, at pH 8.0. After electrophoresis at 5 V/cm for 1.5 h, the troughs were filled with antibody against SCBP and permitted to diffuse for 36 h. The immunoreactive protein in well 1 has a net negative charge, while that in well 2 has a net positive charge.

the cathode, as expected of a basic protein. In contrast, the immunoreactive protein in extract B migrated toward the anode.

Discussion

Newborn rat SCBP has been characterized as a protein relatively rich in histidine and arginine. SCBP interacts with keratin filaments to form large fibrous aggregates indicative of its probable function as an interfilamentous matrix protein of keratin (Dale et al., 1978). The amino acid composition of SCBP from the anucleate stratum corneum is similar to that of material extracted with 1 M potassium phosphate from the nucleated cell layers of epidermis (Sibrack et al., 1974; Dale,

1977). The experiments reported here suggest that the SCBP is derived from a precursor protein extractable with potassium phosphate. Previous studies with sodium dodecyl sulfate–polyacrylamide gel electrophoresis using a continuous buffer system showed that SCBP and the major component of the potassium phosphate extract were not separable and therefore had identical apparent molecular weights (Dale & Stern, 1975; Dale, 1977). In this study, a discontinuous buffer system was used for electrophoresis. This permitted separation of the protein bands, and we conclude that the presumed precursor present in the potassium phosphate extract has a greater apparent molecular weight than does SCBP.

We can draw several conclusions about the biosynthesis of the histidine-rich protein, SCBP, from the present radiolabeled histidine incorporation studies. SCBP (band 2) is the only protein in the stratum corneum which is labeled to any significant extent, even after 24 h. However, this incorporation is only observed after a lag phase of approximately 5 h. In contrast, a protein (band 1) isolated from extracts containing the contents of the nucleated cell layers and keratohyalin granules is rapidly labeled (within 0.5 h). The specific radioactivity of band 1 reaches a maximum at about 6–8 h after initial injection, after which it declines, while that of SCBP increases. This inverse relationship and the lag time in the labeling of SCBP suggest that a precursor–product relationship may exist between band 1 and SCBP (band 2).

Biochemical and chemical data were sought to support the hypothesis that band 1 is the precursor of band 2. The two proteins are clearly not identical. They have different molecular weights by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, different solubilities in dilute salt solutions, and different charges as exemplified both by immunoelectrophoresis and by their different elution patterns on DE-52 cellulose. Nevertheless, the two proteins are similar. Immunodiffusion experiments gave lines of identity with no obvious spurs (Dale & Ling, 1979). The resistance of both SCBP and band 1 to cyanogen bromide suggests that neither protein contains the amino acid methionine. Indeed, the amino acid composition of SCBP and that of the extract containing precursor show many similarities (Table III). The primary amino acid sequence may also be similar. Peptide maps show similar, though not identical, patterns (J. D. Lonsdale-Eccles, personal communication).

The histidine-rich protein(s) have long been known as specific, differentiated products of granular cells (Hoober & Bernstein, 1966; Ugel & Idler, 1972; Sibrack et al., 1974; Tezuka & Freedberg, 1974; Bhatnagar & Freedberg, 1976; Balmain et al., 1977). They are altered in some disease states (Voorhees et al., 1968; Baden et al., 1974). The precursor identified here (band 1) is probably equivalent to the histidine-rich protein (HRP_1) of the granular layer (Gumucio et al., 1967; Sibrack et al., 1974; Ball et al., 1978). The main difference between band 1 and HRP_1 is in their molecular weights: band 1, M_r 52 000; HRP_1, M_r range 80 000 to 10^6 (Ball et al., 1978) or M_r 390 000 (Bernstein & Sibrack, 1972). Several bands in the gels and fluorographs of extract B appear to be multimers of band 1. This protein may form multimers by noncovalent association similar to those observed by Ugel (1971) in extracts of bovine keratohyalin. The native state of this material may be as a large aggregate. However, under the conditions used here, band 1 is primarily seen as a monomer. The SCBP is similar in molecular weight to HRP_2 [60 000 (Ball et al., 1978)], and the amino acid compositions of SCBP and HRP_2 are also very similar, suggesting that these proteins may be identical.

Conversion of a precursor to SCBP may effect a change in the conformation and functional state of the protein, as well as a change in solubility. As the insoluble precursor present in KHG's is converted to the more soluble product, SCBP, the KHG's disappear, and SCBP is available to diffuse throughout the cell and to associate with the network of filamentous protein. The conversion of the precursor to SCBP, possibly by limited proteolysis, may be a critical step in the conversion of the granular cell to a cornified cell.

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A Structural Model for Heme in High-Spin Ferric Hemoproteins. Iron Atom Centering, Porphinato Core Expansion, and Molecular Stereochemistry of High-Spin Diaquo(*meso*-tetraphenylporphinato)iron(III) Perchlorate†

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ABSTRACT: The synthesis and characterization of a six-coordinate high-spin ferric porphyrin, diaquo(*meso*-tetraphenylporphinato)iron(III) perchlorate ($[Fe(TPP)(OH_2)_2]ClO_4$) is described. Magnetic, Mössbauer, ESR, and structural characterization support a high-spin state ($S = {}^5/_2$) assignment. Most importantly, the structural characterization of the title compound demonstrates that the large high-spin iron(III) atom is *centered* in the heme plane, in contrast to the commonly expected out-of-plane displacement of the iron(III) atom based on the structures of a number of other high-spin ferric porphyrins. In $[Fe(TPP)(OH_2)_2]^+$, the large size of the high-spin iron(III) atom is accommodated by a radial expansion of the porphinato core with no displacement of the iron atom. The average Fe–N distance in the centrosymmetric molecule is 2.045 (8) Å, corresponding to an increase in the radius of the central hole of the porphinato ligand of ~0.055 Å relative to low-spin ferric derivatives. The axial Fe–water bond distance is 2.095 (2) Å. Crystal data for $[Fe(TPP)(OH_2)_2]ClO_4 \cdot 2THF$: $a = 16.812$ (2) Å, $b = 12.850$ (2) Å, $c = 21.346$ (2) Å, orthorhombic, space group *Pbcn*, $Z = 4$, $\rho_{calcd} = 1.38$ g/cm^3, $\rho_{obsd} = 1.39$ g/cm^3. Porphinato core expansion and concomitant centering or near centering of the iron(III) atom in the heme plane are likely to occur in a number of high-spin ferric hemoproteins, including derivatives with thermal spin equilibria. The stereochemical features of $[Fe(TPP)(OH_2)_2]^+$ provide a model for the geometry of heme in aquomethemoglobin. The data also suggest that considerable caution must be used when heme stereochemistry (coordination number) is assigned solely on magnetic properties.

The stereochemistry of porphinato complexes is of continuing interest. Especially important are studies of the stereochemistry of porphinato complexes as the metal center and axial ligands are varied systematically (Hoard, 1975; Scheidt, 1977, 1978). The structures of the iron derivatives are of immediate interest with respect to understanding the structure and function of the hemoproteins (Hoard, 1971).

For ferric porphyrins, the spin state and stereochemistry of the iron(III) center are controlled by the nature of the axial ligands. Some aspects of these phenomena are well-known. The coordination of two strong field ligands leads to low-spin six-coordinate hemes, e.g., bis(imidazole)iron(III) derivatives. Weaker field ligands, typically anionic ones such as chloride, azide, etc., lead to five-coodinate high-spin derivatives. The stereochemical features of these two classes of ferric porphyrins are also well-known, and a number of examples of each class have been structurally characterized (Hoard, 1975; Scheidt, 1978). The high-spin five-coordinate species have the iron(III) atom displaced out of the porphinato plane by ~0.5 Å with concomitant long Fe–N bonds. The low-spin six-coordinate complexes have the iron(III) atom essentially centered in the porphinato plane and relatively short Fe–N bonds. The stereochemical differences are associated with the population of the $3d_{x^2-y^2}$ orbital in the high-spin complexes and its depopulation in the low-spin complexes.

Recently we have been systematically studying ferric porphyrins coordinated to a variety of axial ligands, all of which can be described as weak field ligands. When the axial ligands are extremely weak field anionic ligands such as perchlorate (Reed et al., 1979; Dolphin et al., 1977) or tricyanomethanide $[C(CN)_3^-]$ (Summerville et al., 1978), the resulting ferric porphyrin derivatives are not high spin. Rather these complexes have magnetic properties, Mössbauer spectra, and stereochemical features consistent with a ground-state assignment of intermediate spin ($S = {}^3/_2$) or a quantum mechanical admixture of $S = {}^3/_2$ and $S = {}^5/_2$ spin states (Maltempo, 1974). Both five-coordinate and six-coordinate complexes with this ground state have been structurally characterized and represent new structural types for ferric porphyrins.

The coordination of two weak field ligands to ferric porphyrins leads to still another structural type for iron(III) porphyrins, high-spin six-coordination. Ligands which lead to this type are typically neutral oxygen donor ligands (Mashiko et al., 1978): sulfoxides, dimethylformamide, pyridine *N*-oxide, triphenylphosphine oxide, and water. We report herein the preparation, characterization, and structure of one member of this class, diaquo(*meso*-tetraphenyl-

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