Biochemical and Immunological Analysis of a Basic Protein from Newborn Rat Epidermis†

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ABSTRACT: A basic protein, solubilized in buffered salt solutions from keratohyalin granules of newborn rat epidermis, has been purified by ion-exchange chromatography. The relative molecular weight of the protein was determined as 12 800 ± 200 from its mobility on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The protein is relatively rich in lysine, glycine, alanine, and valine which together comprise about 60% of the total amino acid residues. Using an antibody to this protein, which we have designated fraction 4, we have found that it is specific to rat epidermis and is not present in any other rat tissues or in epidermal extracts from other species. The cells of the four epidermal layers were separated and the amount of fraction 4 in each cell layer was measured by radioimmunoassay. The protein is localized mainly in the upper layers of epidermis. The protein, which binds to DNA, appears in the epidermis just prior to birth, increases during the first week of post-natal life and declines sharply thereafter. Fraction 4 represents about 7% of the total solubilized protein in 7-day-old rat epidermis.

The epidermis, the outermost component of skin, consists of four cell layers: the basal layer next to the dermis, the spinous layer, the granular layer, and the fully keratinized stratum corneum. The epidermis is a constantly replicating tissue in which cell division occurs in the basal layer and the cells subsequently migrate through the tissue to the surface. During this migration process, maturation and cell death occur and several characteristic components are produced among which are the filamentous components of keratin, deposited intracellularly in the form of tonofilaments, and the electron dense amorphous bodies known as keratohyalin granules.

The tonofilament proteins, which are helical in configuration, have been isolated and characterized by a number of investigators (Huang et al., 1975; Lee et al., 1975; Matoltsy, 1965; Steinert and Idler, 1975) and the polypeptides isolated from a variety of species have been found to be similar. In contrast, studies of keratohyalin granules have shown them to be heterogeneous (Fukuyama and Epstein, 1975; Matoltsy, 1975; Ugel, 1975). Histidine-rich proteins (Bhatnagar and Freedberg, 1976; Sibrack et al., 1974) have been identified as well as cysteine- and proline-rich species (Matoltsy and Matoltsy, 1970). In our previous studies of keratohyalin granules from newborn rat epidermis, we have solubilized six relatively low molecular weight fractions from these organelles (Bhatnagar and Freedberg, 1976). Four of the fractions have been isolated and at least partially characterized. Two have amino acid compositions similar to the histidine-rich protein studied by others (Sibrack et al., 1974) and one of these has ribonuclease activity. The other two fractions are basic proteins and, of them, fraction 4 is the most predominant of the low molecular weight species (Bhatnagar and Freedberg, 1976). In this paper we shall present biochemical and immunological data which indicate that fraction 4 may be involved in control of epidermal development.

Materials and Methods

Newborn, pregnant, and adult rats were purchased from Charles River Breeding Laboratories in Wilmington, Mass. Guinea pigs (2 days old) were purchased from Elm Farm, Chelmsford, Mass. Human skin was obtained from surgical specimens (leg amputations). Callus tissue was obtained by scraping the soles of the feet of normal volunteers. Psoriatic scales were collected from patients by scraping.

DEAE[1]-cellulose, bovine serum albumin, ovalbumin α-chymotrypsinogen A, cytochrome *c*, calf thymus histones (type II-A), and calf thymus DNA (type I) were purchased from Sigma Chemical Co. Ribonuclease A was obtained from Worthington Biochemicals. Acrylamide and iodoacetamide were from Eastman Kodak Co.; Coomassie blue was purchased from Schwarz/Mann. [^{14}C]HeLa cell DNA was a gift from Dr. Lowell Schnipper.

Extraction of Solubilized Proteins from Newborn Rat Epidermis. Newborn rats (2 to 7 days) were sacrificed by cervical dislocation and decapitation. The skin was rapidly removed, washed with cold physiological saline, placed on ice-cold aluminum foil, and stored for 2 h at −20 °C.

The epidermis was removed from the skin by soaking the skin for 20 min in a solution of 0.24 M ammonium chloride, pH 9.4 (Hoober and Bernstein, 1966), or by a modification of the tetraphenylboron method used previously in our laboratory (Tezuka and Freedberg, 1972). The skin was placed on an iced-glass plate and the epidermis removed from the dermis by blunt dissection. The epidermal sheets were collected by filtration on nylon nets and were minced manually. The tissue was subsequently homogenized (Kontes glass–glass) at 4 °C in 3 volumes of 50 mM Tris-HCl, pH 8.8, containing 1 M NaCl and 10 mM 2-mercaptoethanol. The homogenates were centrifuged at 13 000*g* for 20 min in a Sorvall refrigerated

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[1] Abbreviations used: DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

centrifuge. The pellet was extracted once with the same solution and both supernatants were combined and centrifuged at 105 000*g* in a Beckman L2-65 ultracentrifuge for 1 h.

The resulting high speed supernatant fractions were S-alkylated with iodoacetamide at pH 8.5 (Crestfield et al., 1963) and subsequently dialyzed for 36 h at 4 °C. Any precipitate which formed during dialysis was removed by centrifugation at 13 000*g*.

Isolation of Solubilized Proteins from Adult Rats and Fetuses. Pregnant rats were sacrificed at 17 to 21 days of gestation, the fetuses removed and decapitated, and their skin removed. The homogenate of whole skin was prepared from fetuses less than 19 days. The epidermis from fetuses of 20–21 days was separated from dermis by blunt dissection after soaking the skin for 20 min in a solution of 0.24 M ammonium chloride, pH 9.4. The tissue was washed with 50 mM Tris, pH 8.8, containing 1 M NaCl and collected by filtration on nylon nets and subsequently homogenized at 4 °C in 3 volumes of 50 mM Tris-HCl, pH 8.8, containing 1 M NaCl and 10 mM 2-mercaptoethanol. The solubilized proteins were obtained from the homogenates as described above.

Adult rats were shaven and epilated with a rosin–wax mixture as previously described (Freedberg, 1970). The epidermis was separated from dermis by the stretch technique (Freedberg and Baden, 1962). The tissue was placed immediately into ice-cold 50 mM Tris-HCl, pH 8.8, containing 1 M NaCl and was collected on nylon nets. Homogenization was done in 50 mM Tris-HCl, pH 8.8, containing 1 M NaCl and 10 mM 2-mercaptoethanol and the solubilized proteins were obtained as described above.

Other Protein Preparations. Solubilized proteins from newborn and adult mouse, guinea pig, and human epidermis including psoriatic scales and callus were prepared by a method similar to that described for rat epidermis. Tonofilament proteins of newborn rat epidermis were prepared in our laboratory (Freedberg and Tezuka, 1971). Rat hair roots and epidermal ribosomes were isolated according to techniques previously described (Gilmartin and Freedberg, 1975) and hair root and ribosomal proteins were extracted with 1% sodium dodecyl sulfate containing 6 M urea, 0.1% 2-mercaptoethanol, and 50 mM Tris-HCl, pH 7.9.

Separation of Cells of Epidermis. Epidermal cell fractions were obtained from 2-day-old newborn rat skin by trypsinization (0.1%) in Earle's balanced salt solution, pH 7.4, of whole skin followed by sequential elution of basal, spinous, and granular cells by incubation in buffered 0.01% or 0.1% EDTA (Stern and Sekeri-Pataryas, 1972). The residual cornified cell fraction was treated with 0.05% soybean trypsin inhibitor in Earle's balanced salt solution, pH 7.4. The cells were collected by centrifugation at 6000*g* for 10 min and were homogenized at 4 °C with 1 M sodium chloride or extracted at 37 °C with 1% sodium dodecyl sulfate in 10 mM Tris-HCl, pH 7.9, and 0.1% 2-mercaptoethanol. Solubilized proteins were obtained by centrifugation at 6000*g*.

Amino Acid Analyses. Amino acid analyses were performed with a Beckman Model 120C amino acid analyzer. Samples were hydrolyzed in 6 M HCl for 24, 48, and 72 h at 110 °C in sealed evacuated tubes. Methionine and cysteine were determined by performic acid oxidation (Moore, 1963). Tryptophan was analyzed spectrophotometrically (Edelhoch, 1967).

Protein Concentration. Protein concentrations were determined by the procedure of Lowry et al. (1951) using bovine serum albumin as standard.

Electrophoresis. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed as described by Weber and Osborn (1969) using Tris-glycine buffer. Electrophoresis was carried out either in 10 × 0.5 cm tubes or on 10 × 8.0 × 0.2 cm slab gels containing 12.5% polyacrylamide, 0.1% sodium dodecyl sulfate, and 5 mM Tris-glycine buffer, pH 8.2. For separation of subunits or molecular weight estimates, the proteins were reduced with 2-mercaptoethanol prior to electrophoresis. Gels were stained overnight with 0.1% Coomassie blue and destained with 25% 2-propanol and 7% acetic acid (Fairbanks et al., 1971).

Electrophoresis at low pH was done using the procedure of Panyim and Chalkley (1969a) on 10 × 0.5 cm tubes containing 15% acrylamide, 2.5 M urea, and 0.9 M acetic acid. The gels were prerun with 0.9 M acetic acid prior to loading of the samples. Protein samples were dissolved in 6 M urea, 0.9 M acetic acid, and 10 mM 2-mercaptoethanol. Gels were stained with 0.05% amido black for 12 h and destained with 7% acetic acid.

Preparation of Antibody. Antibody against purified fraction 4 was raised in rabbits. Approximately 800 μg of purified protein was emulsified with an equal volume of Freund's complete adjuvant. The animals were immunized by subcutaneous injections in the foot pads (0.3 mL/pad) and were boosted with antigen in complete adjuvant and bled weekly for 5 weeks. The blood was stored at 4 °C overnight before the antiserum was collected. Immunoglobulin fractions were obtained from the serum by precipitation with 2 M ammonium sulfate. The precipitate obtained after centrifugation at 6000*g* was dialyzed at 4 °C against 0.1 M sodium bicarbonate and was stored at −20 °C.

Double Diffusion Analysis. The immunodiffusion medium was prepared by mixing a hot sterilized 2% solution of Ionagar (Consolidated Laboratories, Chicago, Ill.) in water (w/v) with an equal amount of 0.05 M Tris-HCl, pH 8.0, and 1% sodium azide. The solution was poured into disposable polystyrene Petri dishes to a depth of 4 mm and allowed to solidify. The dishes were stored at 4 °C. Two-millimeter diameter wells were punched with a template resulting in a pattern of wells with center to center distances of 10 mm. After the wells were filled with the appropriate proteins, the plates were incubated in a humid atmosphere at 21 °C for 1 to 3 days.

Radioimmunoassay. Purified fraction 4 was radiolabeled by ^{125}I using the Chloramine-T method of Hunter and Greenwood (1962). In a typical preparation, 5 μL of a solution of fraction 4 (1 mg/mL) was added to 20 μL of 0.3 M sodium phosphate buffer, pH 7.5, in a radiolabeling vial. Approximately 0.5 mCi of $Na^{125}I$ (New England Nuclear Corp.) was added. The incorporation of radioactive iodine was accomplished by addition of a 10-μL aliquot of fresh solution of Chloramine-T (0.25 mg/mL in sodium phosphate buffer, pH 7.5) and 10 s later 10 μL of sodium metabisulfite (0.25 mg/mL in sodium phosphate buffer, pH 7.5) was added to stop the reaction. To this mixture 50 μL of normal serum, 10 μL of 0.1% bromophenol blue, and 50 μL of sucrose (10%) were added. The reaction mixture was desalted on a column (50 × 1.5 cm) of Sephadex G-75 equilibrated in 0.05 M sodium phosphate buffer, pH 7.5, containing 0.15 M sodium chloride, 0.01 M EDTA, and 0.1% bovine serum albumin (phosphate–BSA buffer). Fractions of 0.5 mL were collected. ^{125}I-labeled fraction 4 was recovered in the peak after the void volume. When analyzed on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, the ^{125}I-labeled fraction 4 migrated as a single radioactive peak having a mobility identical with that of the unlabeled protein.

For radioimmunoassays the reaction mixture (1.0 mL) contained phosphate–BSA buffer, pH 7.5, 0.1 mL of 1/5000 dilution of anti-fraction 4 (final dilution 1/50 000) and 1 to 200 ng of purified fraction 4. Assay tubes were incubated at

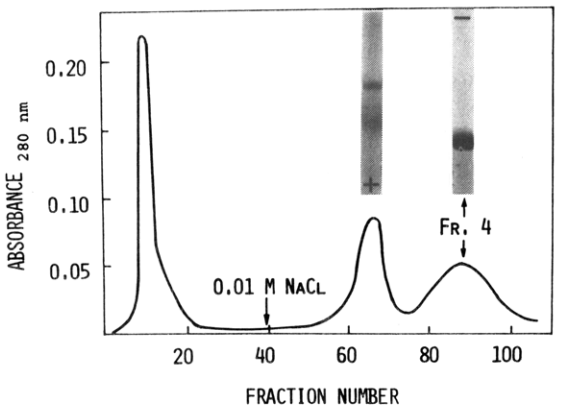

FIGURE 1: Separation of fraction 4 on a DEAE-cellulose column. The column (2.0 × 15 cm) was equilibrated with 10 mM Tris-HCl, pH 7.9, at 4 °C and the solubilized proteins of newborn rat epidermis were eluted with 10 mM NaCl. Peak 1 containing the unadsorbed protein was discarded. Peak 2 (tubes 60–75) and peak 3 (80–96) were separated, pooled, and lyophilized. Samples for electrophoresis were prepared by dialyzing proteins against 5 mM Tris–maleate, pH 8.2, 0.1% sodium dodecyl sulfate, and 5 mM 2-mercaptoethanol. Fraction 4 was identified by polyacrylamide gel electrophoresis (insert) in the presence of sodium dodecyl sulfate.

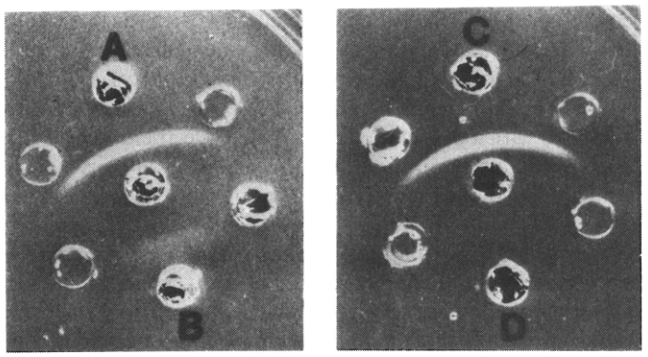

FIGURE 2: Immunodiffusion analysis of newborn (A), adult rat epidermis extracts (B), fraction 4 (C), and guinea pig epidermis (D) extract. The center well contained 20 μL of anti-fraction 4. The peripheral wells contained 20 μL (1.0 mg/mL) of different test reactants.

TABLE I: Amino Acid Composition of Purified Fraction 4.

Amino acid	Residues/100 at h of hydrolysis 24	48	72	Extrapolated[c]
Lys	13.6	15.4	13.7	15.84 (16)
His	3.1	3.1	3.2	3.46 (3)
Arg	3.3	3.0	3.1	3.44 (3)
Cysteic acid[a]	0.5	0.5	0.5	0.56 (1)
Asp	5.3	5.6	5.3	5.99 (6)
Thr	0.6	0.5	0.5	0.61 (1)
Ser	TR	TR	TR	0 (~0)
Glu	8.2	8.3	7.9	9.02 (9)
Pro	6.0	5.4	5.4	6.16 (6)
Gly	17.4	16.6	18.6	19.46 (19)
Ala	11.5	11.1	11.5	12.61 (13)
Val	13.4	13.0	13.2	14.65 (15)
Met[a]	1.4	1.4	1.4	1.55 (2)
Ile	2.7	3.0	2.6	3.07 (3)
Leu	9.2	9.2	9.3	10.25 (10)
Tyr	1.5	1.7	1.6	1.78 (2)
Phe	2.3	2.2	2.2	2.55 (3)
Trp[b]				0

[a] Determined by performic acid oxidation. [b] Determined spectrophotometrically. [c] Amino acid calculated for 111 residues and rounded to the nearest integer.

4 °C for 24 h and then 0.1 mL of ^{125}I-labeled fraction 4 (5000 cpm) containing normal rabbit serum (final dilution 1/2000) was added and the incubation was continued for another 24 h. Sheep anti-rabbit antiserum (Arnel Products, New York, N.Y., final dilution 1/100) was added and incubation at 4 °C was continued for another 16 h. The assay tubes were centrifuged at 3000 rpm in a Sorvall GLC centrifuge. The supernatant was decanted. Both the supernatant and the pellet were counted in a Packard Auto-Gamma spectrometer and free and bound radioactivity were determined. Assays were performed in duplicate.

In experiments designed to study the displacement characteristic produced by different epidermal extracts, varying concentrations of the extract were incubated at 4 °C in phosphate–BSA buffer with antibody to fraction 4. ^{125}I-labeled fraction 4 was added after 24 h of incubation and the assays were performed as described above.

Isolation of Chromatin and Extraction of Histones. Chromatin was prepared from newborn rat epidermal nuclei by a combination of the methods of Bekhor et al. (1969) and Shaw and Huang (1970). Epidermal sheets were homogenized (Kontes glass homogenizer) for 2 min at 4 °C in 10 mL/g of TSKM buffer (10 mM Tris, 25 mM KCl, 5 mM $MgCl_2$, 50 mM $NaHSO_3$, and 0.32 M sucrose), pH 7.8, which contained 1% Triton X-100. The homogenate was filtered through Nitex filter cloth and centrifuged at 5000 rpm for 10 min. The pellet which contained nuclei was washed three times by alternate suspension in TSKM buffer and centrifugation at 1500 rpm. This preparation of crude nuclei was sedimented through 2.3 M sucrose in TKM buffer (TSKM buffer, without 0.32 M sucrose) at 25 000 rpm for 60 min using an SW 65 rotor in a Beckman L-2 65B centrifuge. The pellet was washed once with a buffer containing 10 mM Tris-HCl and 0.14 M NaCl, pH 7.8, and two times with 10 mM Tris-HCl, pH 7.8, containing 1 mM EDTA by alternate suspension and centrifugation at 5000 rpm for 10 min.

Histones were extracted from the chromatin pellet by the addition of 0.25 N HCl. The samples were homogenized and left standing for 2 h at 4 °C. The homogenate was centrifuged for 30 min at 5000 rpm. The supernatant fraction was decanted and saved while the insoluble pellet was again extracted with acid and centrifuged. The supernatants were combined. The extracted histones were dialyzed against two changes of 1 mM HCl for 24 h at 4 °C and lyophilized.

Fraction 4–DNA Complexes. Complexes of DNA (calf thymus DNA or ^{14}C-labeled HeLa-cell DNA) with fraction 4 were reconstituted with and without histones at 4 °C by mixing the components at the desired concentrations and ratios under dissociating conditions (in 3 M NaCl, 5 M urea, 50 mM $NaHSO_3$ and 10 mM Tris-HCl, pH 7.8) and gradually lowering the urea concentration by gradient dialysis (Rubin and Moudrianakis, 1972). The mixture was dialyzed against 0.14 M NaCl, 10 mM Tris-HCl, pH 7.8, and 50 mM $NaHSO_3$ and the complexes were recovered by centrifugation at 105 000*g* for 1 h and washed two times with 10 mM Tris-HCl, pH 7.8, containing 50 mM $NaHSO_3$ by alternate suspension and centrifugation. When ^{14}C-labeled HeLa-cell DNA was used in complex formation, the amount of radioactivity bound in the precipitate was measured by dissolving the pellet in Aquasol and counting the radioactivity in a Packard TriCarb liquid scintillation spectrophotometer. In experiments in which calf thymus DNA was used, the reconstituted pellet (105 000*g*) containing protein–DNA complexes was extracted with 0.5 N HCl. The proteins recovered in the supernatant

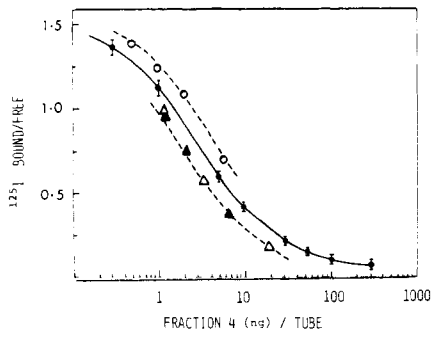

FIGURE 3: Standard radioimmunoassay curve of fraction 4 (●) and its comparison to the inhibition curves produced by extracts of rat epidermis of 3 day (▲), 7 day (△), and adult animal (○). On the ordinate are indicated the ratios of bound to free ^{125}I-labeled fraction 4 and on the abscissa varying concentrations of fraction 4 on a log scale for the standard curve. Assays of epidermal extract for 3 or 7 day rats and from adults contained 20–500 ng and 150–1000 ng of protein, respectively. The assays were performed as described in Materials and Methods.

were subjected to electrophoresis as described by Panyim and Chalkley (1969a).

Results

We have previously reported on the isolation of four of the low molecular weight proteins of newborn rat keratohyalin granules (Bhatnagar and Freedberg, 1976). In that study fraction 4 and the three other proteins were isolated and purified by a series of molecular sieve and ion exchange columns. The apparent molecular weights of the proteins resolved were between 12 000 and 18 000 and the fractions showed variable heterogenity (Bhatnagar and Freedberg, 1976). Using the biochemical characteristics of fraction 4 at low ionic strength, we have now developed an isolation procedure for this protein which results in its selective separation from the other components. The purity of this preparation has permitted us to reexamine its molecular weight and amino acid composition.

Isolation of Fraction 4. The soluble proteins from newborn rat epidermis obtained, as described in Materials and Methods, were applied to a DEAE-cellulose column. Figure 1 demonstrates a typical elution diagram. The protein peak not bound to DEAE was discarded as it contained a mixture of proteins. The column was washed with 0.1 M NaCl and the two peaks obtained were pooled separately and lyophilized. The sodium dodecyl sulfate–polyacrylamide gel electrophoretograms of these two peaks are shown also in Figure 1. Fraction 4 was identified as a single protein band in the second of the two peaks. This electrophoretic pattern indicated selective isolation of fraction 4.

Molecular Weight. In sodium dodecyl sulfate gel electrophoresis, purified fraction 4 had a mobility which corresponded to a molecular weight of 12 800 ± 200. This was calculated from a semilogarithmic plot of the molecular weight vs. the mobilities of four standard protein markers: ovalbumin, α-chymotrypsinogen A, ribonuclease A, and cytochrome *c*.

Amino Acid Analysis. Results are listed in Table I. Lysine, glycine, alanine, and valine comprise about 60% of the total residues of fraction 4. The protein is characterized by traces of serine and threonine and absence of tryptophan.

Ouchterlony Double Diffusion Analysis. Figure 2 shows the immunological relationships among extracts of newborn and adult rat epidermis, guinea pig epidermis, purified fraction 4, and the fraction 4 antibody. A single distinct precipitin line was observed with extracts of newborn rat epidermis or with purified fraction 4. Adult rat epidermis preparations produced a faint precipitin line. The antibody did not react with guinea pig epidermal extracts.

TABLE II: Localization of Fraction 4 in Newborn Rat Epidermis.

Cell fraction[a]	ng of Fr-4/μg of protein	
	$NaDodSO_4$ extract	NaCl extract
1. Basal	1.6	3.1
2. Spinous	1.5	1.3
3. Granular	1.4	3.9
4. Corneum	12.6	31.2

[a] Histologically the basal cells and stratum corneum were isolated as pure fractions, but cells of layers 2 and 3 contained a mixture of spinous and granular cells. The amount of fraction 4 was determined using radioimmunoassay for fraction 4 (Figure 3) on an equal amount (5 μg/mL) of sodium dodecyl sulfate and sodium chloride solubilized protein extracts. The values shown are the averages of closely duplicate determinations.

Ouchterlony double diffusion analysis was done with various tissue extracts using fraction 4 antibody. Except for rat epidermal extracts which showed a positive precipitin reaction, the epidermal extracts of mouse, guinea pig, or human skin including psoriatic scales and callus were negative. The anti-fraction 4 did not react with any other tissues of newborn or adult rat. Extracts of rat epidermal ribosomes, nuclei, tonofilaments, and rat hair-root cells failed to react with the antibody.

Radioimmunoassay of Fraction 4. The interaction of fraction 4 with the antibody was studied by radioimmunoassay. The antibody was diluted 1/50 000 into the assay solution and incubated with varying concentrations of fraction 4 and a constant amount of ^{125}I-labeled fraction 4 (5000 cpm). The curve showing the competition of ^{125}I-labeled fraction 4 for fraction 4 is shown in Figure 3. With antibody and ^{125}I-labeled fraction 4 a bound:free ratio of approximately 1.5 was measured. A bound:free ratio of 0.15 to 0.2 was measured for blank controls which contained no antibody. The data were corrected for these blank controls. Also shown in Figure 3 are the inhibition curves produced by extracts of 3 day, 7 day, and adult epidermis. The relationship observed for each of the extracts was parallel to that of purified fraction 4.

The distribution of fraction 4 in various layers of epidermis as determined by radioimmunoassay is shown in Table II. More fraction 4 was solubilized when the cells were homogenized with sodium chloride than when they were extracted with sodium dodecyl sulfate. Although fraction 4 was distributed in all layers, it was present in small amounts in the first three layers and was found in highest concentration in the outermost layer, the stratum corneum. Approximately 3% of the total solubilized protein of the stratum corneum of 2-day old rats is fraction 4.

The amount of fraction 4 in rat epidermis of different ages was also quantitated by radioimmunoassay. The data are shown in Figure 4. Fetal skin from rats of less than 19 days gestation does not contain fraction 4. The protein is present in epidermis at the time of birth and it increases during the post-natal period for 1 week at which time it declines sharply to adult levels. At 1 week the protein is about 7% of the total solubilized epidermal protein.

The Appearance of Fraction 4 during Development. Our observations that fraction 4 appears at the time of birth in rat epidermis, increases during the first week of post-natal life and declines thereafter are illustrated on the sodium dodecyl sulfate–polyacrylamide gel electrophoretograms reproduced in Figure 5a. The band corresponding to fraction 4 appears in the

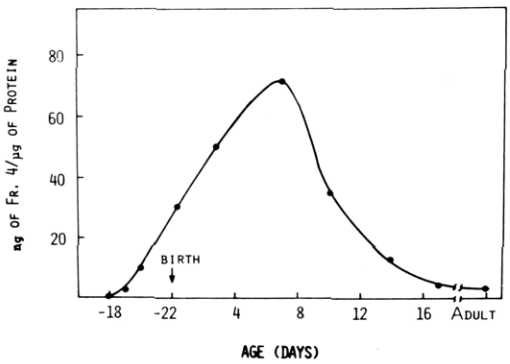

FIGURE 4: Quantitative estimation of fraction 4 during development of rat epidermis. The solubilized proteins were obtained as described in Materials and Methods. Extracts of 3 day, 7 day, and adult rat epidermis showed inhibition curves closely parallel to that of fraction 4. It was assumed that the curves for other extracts also paralleled that of purified fraction 4. The concentration of fraction 4 was determined from the standard curve (Figure 3). The values reported represent an average of the concentration determined from a minimum of three dilutions. The results are expressed as ng/μg of tissue protein extract.

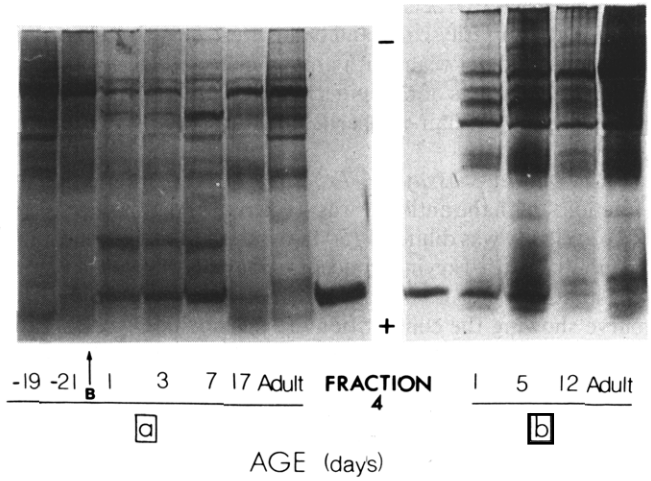

FIGURE 5: Electrophoretic pattern on sodium dodecyl sulfate–polyacrylamide gels of extracts of different ages of rat (a) and mouse (b) epidermis. The solubilized proteins were prepared as described in Materials and Methods. Samples for electrophoresis were prepared as described in Figure 1. Equal amounts (50 μg) of proteins were introduced on each gel. Purified fraction 4 (10 μg) was included to show the relative migration of this fraction in epidermal extracts.

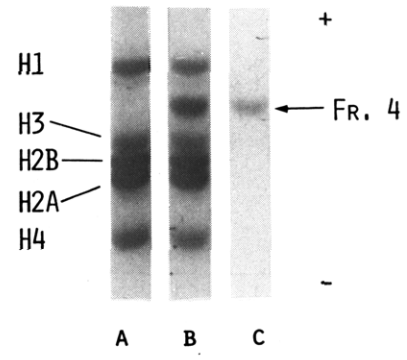

FIGURE 6: Polyacrylamide gel electrophoretograms of proteins recovered from protein–DNA complexes. Electrophoresis was carried out according to *Panyim and Chalkley* (1969a). Fraction 4 and calf thymus DNA complexes were prepared with and without histones. Protein and DNA were at a concentration of 500 mg each in Tris–NaCl–urea buffer. Histone solution and fraction 4 or fraction 4 alone were added to DNA solution dropwise until the DNA/protein ratio was 1 and the mixture was stirred for 15 min at 4 °C. Reconstitution was performed by dialysis and proteins were released from DNA–protein complexes as described in Materials and Methods. (A) Rat epidermal histones; (B) histones with fraction 4; (C) fraction 4.

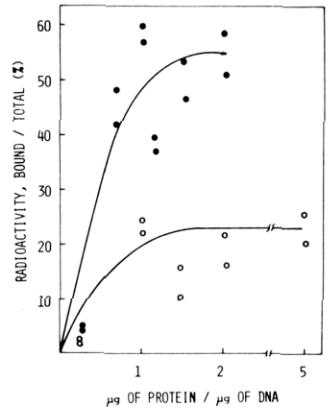

FIGURE 7: Measurement of binding of ^{14}C-labeled HeLa-cell DNA to calf thymus histones (●—●) and fraction 4 (○—○). Reconstitution experiments were similar to those described in Figure 6 and under Materials and Methods. Protein–DNA complexes were obtained as in the 105 000*g* pellet. Radioactivity was measured in pellet and supernatant.

epidermal extracts prior to birth; it reaches a maximum at 1 week of post-natal life and then begins to disappear.

Although immunodiffusion analysis using the antibody to fraction 4 did not show any reactivity with other epidermal extracts, we examined the solubilized epidermal proteins of mice of different ages. The polyacrylamide gel electrophoretograms shown in Figure 5b indicate a band similar in mobility to fraction 4 which is present in newborn mouse epidermis and which decreases in concentration with age.

Studies of the Interaction between Fraction 4 and DNA. Polyacrylamide gel electrophoresis of rat epidermal nuclear histones show five major species (Figure 6) identical with the five major histones of calf thymus. Reconstitution experiments have been performed in which histones from rat epidermis or calf thymus or fraction 4 were added to a constant amount of DNA to form a protein–DNA complex. The proteins released from these complexes are displayed in the electrophoretograms shown in Figure 6. The results show that fraction 4 does bind to DNA and that when it is released it migrates as a single band between histones H1 and H3. Both rat epidermal and calf thymus histones produced the same result. As shown in Figure 7, when ^{14}C-labeled HeLa cell DNA was used for such studies about 20% of the DNA was bound to fraction 4, in contrast to 60% binding obtained with calf thymus histones. The maximum binding occurred at a protein–DNA ratio of approximately 1:1.

Discussion

Differentiation of epidermis is a highly organized process which results in the coordinated synthesis of several phenotypic organelles including tonofilaments and keratohyalin granules. A number of studies of keratohyalin granules and their proteins have appeared within recent years (Bhatnagar and Freedberg, 1976; Dale and Stern, 1975; Fukuyama and Epstein, 1975; Matoltsy and Matoltsy, 1970; Matoltsy, 1975; Sibrack et al., 1974; Ugel, 1975) but there is as yet no unanimity of opinion concerning the composition or function of these organelles.

We have previously identified, among other protein species, six proteins from keratohyalin granules with apparent molecular weights between 10 000 and 18 000 (Bhatnagar and Freedberg, 1976). In this paper we have focused upon one of the proteins (fraction 4) and have clarified its biochemical and

immunological characteristics. We have modified the method by which the fraction is prepared and have been able to isolate it selectively (Figure 1). The purified fraction has been found to have an amino acid analysis (Table I) somewhat different (Bhatnagar and Freedberg, 1976) from that of the impure material although its molecular weight is not changed. Fraction 4 is a lysine-glycine-rich protein with a molecular weight of 12 800 ± 200.

The antibody we have produced to fraction 4 has enabled us to show that this fraction is found only in rat epidermis and our immunodiffusion analyses indicate that the antibody does not react with any other rat tissues or epidermal extracts from other species. A band corresponding in migration to fraction 4 of newborn rat epidermis can be visualized on sodium dodecyl sulfate-polyacrylamide gel electrophoretograms of newborn mouse epidermal extracts (Figure 5b) and it is possible that corresponding proteins will be found in other species as well.

The quantitative studies noted in Table II indicate that, although we originally isolated fraction 4 from keratohyalin granules (Bhatnagar and Freedberg, 1976), the bulk of the protein is localized in the stratum corneum. This raises the possibility which has been raised by others that components of keratohyalin granules are involved in the later stages of maturation of stratum corneum. Dale and Stern (1975) in their comparative studies on the proteins of keratohyalin granules extracted by potassium phosphate (Ugel, 1969) and citric acid-detergent procedures (Matoltsy and Matoltsy, 1970) have raised similar possibilities.

Newborn rat epidermis has been used as a model of epidermal development by several investigators who have focused upon both morphological (Menefee, 1957; Bauer, 1972), and biochemical changes (Dale et al., 1976). Tonofilaments have been first seen at 17 days gestation, the same time that protein bands corresponding to stratum corneum fibrous proteins have been identified. Keratohyalin granules are seen on the 18th day, and by the 19th and 20th days a true stratum corneum is evident. Our studies indicate that the concentration of fraction 4 protein in newborn rat epidermis undergoes major changes at this same time. The material appears on the 19th day, increases in concentration until the 7th day post partum, and subsequently falls to low adult levels (Figure 4). Bhargava and Fiegelson (1976) have shown that epidermal histidase follows a similar developmental course but fraction 4 has none of the biochemical or physicochemical characteristics of histidase.

The studies summarized in Figure 6 indicate that the electrophoretic migration of epidermal histones is similar to that of the five major histones (Bradbury, 1975) of other tissues. We undertook these studies because we found that fraction 4 was a basic protein rich in lysine, void of tryptophan, and of low molecular weight. These characteristics are similar to those of histones and we were concerned that fraction 4 might represent only nuclear contamination of our keratohyalin preparations. Species and tissue variability has been found in lysine-rich histones. A unique histone similar to fraction 4 in electrophoretic mobility but not in amino acid composition has been reported in several tissues with a low rate of cell division (Panyim and Chalkley, 1969b). The studies shown in Figure 6 indicate, however, that this is not the case since fraction 4 has electrophoretic mobility distinct from that of epidermal histones. The binding studies shown in Figure 7 indicate that, although fraction 4 is not a histone, it does bind to DNA. It is exciting to consider the possibility that fraction 4 may be involved in controlling DNA function in the upper layers of epidermis although firm data concerning this possibility are not yet available.

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Physarum Actin. Observations on Its Presence, Stability, and Assembly in Plasmodial Extracts and Development of an Improved Purification Procedure†

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ABSTRACT: Actin is readily extracted from plasmodia of *Physarum polycephalum* by low ionic strength solutions which do not solubilize the plasmodial myosin. The actin in such extracts exists predominantly as a monomer which slowly denatures, apparently via removal of bound nucleotide, and is subsequently proteolyzed. However, the native monomeric actin can be induced to assemble into polymeric arrays under appropriate solvent conditions. Actin assembly is dependent on the addition of ATP and is a function of KCl and $CaCl_2$ concentrations. These observations have allowed the development of an improved actin purification scheme which is simple, rapid, and efficient, yielding ~60 mg of protein from 100 g of plasmodium. The actin thus obtained is pure, stable, and comparable to that obtained by previously described procedures. Furthermore, the observations suggest that actin polymers may be metastably assembled in vivo and raise the possibility that actin assembly, and plasmodial movements, could be regulated via alterations in intracellular concentrations of nucleotide and/or divalent cation.

Since the demonstration, in the late 1960's, that plasmodia of the myxomycete slime mold *Physarum polycephalum* contain actin and myosin (Hatano and Oosawa, 1966; Adelman and Taylor, 1969a,b; Hatano and Ohnuma, 1970) such proteins have been isolated from a wide variety of other nonmuscle sources (Pollard and Weihing, 1974; Goldman et al., 1976). These nonmuscle actins and myosins closely resemble their muscle counterparts, the degree of evolutionary conservation being particularly striking in the case of the actins. Thus, for example, *Physarum* plasmodial actin is like its muscle homologue in terms of sedimentation and diffusion coefficient, molecular weight, nucleotide binding, filament formation, and interaction with heterologous as well as homologous myosin (Hatano and Oosawa, 1966; Adelman and Taylor, 1969a,b; Hatano, 1973). Moreover, the two proteins show substantial similarity in amino acid composition (Hatano and Oosawa, 1966) and at least partial sequence homology (Jockusch et al., 1974).

In contrast to the similarity of their constituent actins and myosins, nonmuscle systems show considerable diversity, both among themselves and in comparison with muscle, in the details of their movements. It is of obvious interest to determine the extent to which this phenomenological diversity reflects subtle differences between the constituent macromolecules and to this end it is necessary to characterize at least a few nonmuscle actins and myosins in detail. For such studies *Physarum* plasmodia remain a particularly suitable system, for reasons which dictated their earlier use; they manifest extremely vigorous protoplasmic streaming (Kamiya, 1959) and can be easily cultured in large quantities. Furthermore, the various stages of the *P. polycephalum* life cycle display a broad range of interesting motility phenomena and the availability of genetic approaches to this system makes it a logical choice for systematic studies of motility-related macromolecules (Jacobson et al., 1976).

This paper, the first of a new series on motility-related proteins from *Physarum,* describes observations on the actin present in crude plasmodial extracts which may have bearing on our understanding of the in vivo organization of plasmodial actin and which have allowed the development of an improved protocol for its purification. These results also form the basis for a refined plasmodial myosin isolation procedure and for studies of factors which interact with, and modulate the assembly and/or interaction of, the actin and myosin (manuscripts in preparation). Some aspects of these studies have appeared in preliminary form (Adelman, 1974a,b; Jacobson et al., 1976).

Materials and Methods

Culturing and Harvesting of Plasmodia. The *P. polycephalum* cultures used for these experiments were all derived from a single sample of M_3c VIII spherules supplied by Dr. Joyce Mohberg (McArdle Laboratory, University of Wisconsin). Spherule and microplasmodial stocks (used primarily for restarting plasmodial cultures) were maintained according to Daniel and Baldwin (1964). Plasmodia were cultured on oatmeal flakes and harvested after migration over agar surfaces (Adelman and Taylor, 1969a). As in the earlier work, freshly harvested plasmodia were used for all experiments; however, plasmodia were not washed with water prior to weighing and homogenization.

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