Biochimica et Biophysica Acta, 668 (1981) 98—106
© Elsevier/North-Holland Biomedical Press

BBA 38648

SIMILARITIES BETWEEN STRATUM CORNEUM BASIC PROTEIN AND HISTIDINE-RICH PROTEIN II FROM NEWBORN RAT EPIDERMIS

BEVERLY A. DALE [a], BABU VADLAMUDI [b], LINDA W. DeLAP [b] and I.A. BERNSTEIN [b]

[a] *Departments of Periodontics and Medicine, University of Washington, Seattle, WA 98195 and* [b] *Departments of Environmental and Industrial Health and of Biological Chemistry, University of Michigan, Ann Arbor, MI 48109 (U.S.A.)*

(Received August 7th, 1980)
(Revised manuscript received November 17th, 1980)

Key words: Stratum corneum basic protein; Basic protein; Histidine-rich protein II; Keratinization; (Rat epidermis)

Summary

The stratum corneum basic protein and histidine-rich protein II were each isolated from newborn rat epidermis and compared by biochemical and immunologic methods. The proteins were indistinguishable by immunodiffusion using antiserum elicited to either protein. The migration of the proteins on SDS-polyacrylamide gel electrophoresis was identical giving a molecular weight of 49 000. These proteins, which have similar but unusual amino acid compositions, give very similar tryptic peptide maps. Both proteins aggregate with keratin filaments to form macrofibrils. These results suggest that histidine-rich protein II and stratum corneum basic protein are the same protein. We suggest that this protein be called histidine-rich basic protein.

Introduction

Histidine-rich proteins have been isolated from the epidermis by several laboratory groups [1—4] and are considered to be specific differentiated products of the epidermis [5]. One of them has been suggested to function as the interfilamentous matrix material [6]. Two of them, the stratum corneum basic protein [3] and histidine-rich protein II [5], were extracted from the stratum corneum of the newborn rat. In this report the characteristics of these two proteins are compared to see whether they may be the same protein.

The search for a histidine-rich protein in the epidermis began after autoradiographic studies showed that histidine was one of several amino acids

rapidly and preferentially incorporated into the granular layer and only later into the cornified layer of epidermis [7—9]. Morphologically, the granular layer is distinguished by its characteristic keratohyalin granules, which disappear when its cells are converted to those of the stratum corneum.

The first histidine-rich protein was isolated from newborn rat skin by Hoober and Bernstein [1] and was shown to be derived from the upper layers of epidermis [10]. Histidine-rich proteins have been readily extracted by 1 M potassium phosphate from bovine and rat epidermis [11—13] and by various other techniques from mouse, rat, and human epidermis [2,4,14,15]. The association between histidine-rich proteins and keratohyalin granules has been shown by their coincident appearance during development [16,17], by antibody localization [2,18,19], and by preferential extraction techniques [12,20].

A protein which is similar in its amino acid composition to the histidine-rich proteins previously studied was isolated from the rat stratum corneum [3]. This protein, called stratum corneum basic protein because of its source and its cationic nature, has an apparent molecular weight of 49 000 by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. A similar protein, called histidine-rich protein II, which has a molecular weight of approx. 60 000 by gel filtration, was isolated from rat stratum corneum by Ball, Walker, and Bernstein [5]. Histidine-rich protein II and stratum corneum basic protein were both shown by radiolabeling experiments to be derived from a precursor protein present in the living cell layers of the epidermis [5,21]. In each case, the precursor had a higher molecular weight than did the product, although the precursors isolated in the two studies differed substantially in size. Stratum corneum basic protein and its precursor are immunologically related [22]. The precursor has been obtained by techniques known to extract the contents of keratohyalin granules [21]. Murozuka et al. [19] confirmed that the histidine-rich proteins from the living cell layers and the stratum corneum are immunologically related.

Thus, the source, the amino acid compositions, and the apparent derivation of histidine-rich protein II and stratum corneum basic protein from precursors suggest that the two proteins are similar. However, their reported molecular weights differ. The biochemical, immunologic, and functional properties of these two proteins are compared in parallel here, in order to see whether they are the same cellular product.

Methods

Isolation of proteins. The procedure used for isolating histidine-rich protein II from the stratum corneum was essentially that of Bernstein and co-workers (cf. Ref. 5). Epidermis was scraped to remove nonkeratized cells as previously described. Gel filtration was carried out on a column of Sepharose 4B rather than Sepharose 6B. The resulting material was further purified by preparative SDS-polyacrylamide gel electrophoresis [23]. The major protein band (M_r 50 000), located by staining of a small longitudinal slice of the gel with Coomassie blue, was cut out and extracted with 0.1% SDS, 0.01 M sodium phosphate, pH 7.5, and then dialyzed extensively against 0.01 M NH_4OH and lyophilized.

Stratum corneum basic protein was purified by ion-exchange and gel filtration chromatography as previously described [3], with modifications to avoid the use of urea [24]. Briefly, newborn rat epidermis was extracted with 9% formic acid. This material was lyophilized, treated with cyanogen bromide in 70% formic acid, then diluted and applied to a Sephacryl S200 column. Stratum corneum basic protein was eluted in the first large peak which was lyophilized and then was further purified on DE-52 cellulose and CM-52 cellulose as previously described, except that the buffers contained no urea. A final gel filtration step on Sephacryl S300 yielded a preparation that contained mainly the M_r 49 000 stratum corneum basic protein.

Antiserum preparation. Purified stratum corneum basic protein in phosphate-buffered saline (4—10 mg/ml) was mixed with an equal volume of complete Freund's adjuvant and 0.1 ml was injected into the foot pads of New Zealand white rabbits. Animals received booster injections of 0.1 mg in incomplete Freund's adjuvant at 2-week intervals for 8 weeks and occasionally for up to 6 months. Antiserum was collected at 2-week intervals, starting at week 5 after primary inoculation.

Antiserum to histidine-rich protein II was prepared in New Zealand white rabbits by intradermal injection of 300 μg of the purified protein dissolved in 0.5 ml of 0.05 M sodium phosphate, pH 7.5, mixed with an equal volume of complete Freund's adjuvant. Antiserum was obtained after six injections over the course of 2 months.

Double immunodiffusion. Ouchterlony plates contained 1% agarose, 0.1% SDS, 0.5% Triton X-100, 0.9% NaCl and 0.05% sodium azide [25]. The histidine-rich protein II-antiserum was concentrated 3-fold to intensify the precipitin bands. The plates were allowed to develop for 2 days at room temperature and then washed extensively in 0.14 M NaCl, 0.5% Triton X-100, 0.01 M sodium phosphate, pH 7.5, stained with amido black, and destained in 10% acetic acid.

SDS extraction and assay. The SDS concentration in protein samples was measured by the method of Waite and Wang [26]. Twice recrystallized SDS was used as a standard. The range of the assay was approx. 2—50 nmol SDS.

SDS was extracted by ion-pair formation [27]. Lyophilized protein samples (approx. 0.5 mg each) were extracted with a mixture of cold acetone/triethylamine/acetic acid/water (85:5:5:2, by vol.) in 1 ml total volume. After the sample had stood in ice for 4 h, the protein precipitate was removed by centrifugation and reextracted. After 15 min in ice, the samples were again centrifuged and the protein was redissolved in 5 mM Tris-HCl (pH 7.6) to approx. 1 mg/ml, then dialyzed overnight against the same buffer in the cold. To test the effects of the extraction method on the protein, stratum corneum basic protein was used as a control. 1 mg samples were incubated with and without 1.5% SDS for 5 min at 100°C in 8 M urea, then dialyzed overnight against distilled water with several changes. Small samples were removed and the remaining material was lyophilized for SDS extraction. Samples were assayed for SDS, for protein and for fiber formation before and after SDS extraction.

Protein concentration was measured by the method of Lowry et al. [28], using bovine serum albumin as a standard.

Peptide analysis. Residual SDS was removed from histidine-rich protein II by

ion-pair extraction [27]. Tryptic digestion [5] of the histidine-rich protein II and stratum corneum basic protein was separately carried out by incubation of 200 μg of each protein in 0.4 ml of 0.2 M ammonium bicarbonate with 2 μg of diphenylcarbamyl chloride-treated trypsin (Sigma Chemical Company) at 37°C. After 6 h, half the solution was removed, added to 50 μl of glacial acetic acid, and frozen. The remaining solution was incubated for another 6 h after addition of 1 μg of trypsin and the reaction was terminated by addition of 50 μl of glacial acetic acid. The 6-h and 12-h samples were lyophilized and subjected to one-dimensional peptide mapping on a Dionex amino acid and peptide analyzer with detection of the fluorescent products of reaction with *o*-phthalaldehyde [29,30].

Aggregation with keratin filaments. Keratin filaments were prepared by dialysis from 8 M urea extracts of newborn rat epidermis [6,31]. Any precipitated material was removed by centrifugation at 10 000 × *g* and then the filaments were collected by centrifugation at 105 000 × *g* for 90 min. These were redissolved in 8 M urea, 0.1 M 2-mercaptoethanol, 0.1 M Tris-HCl (pH 7.6) and reformed by dialysis against 5 mM Tris (pH 7.5) with 10 mM 2-mercaptoethanol. Reformed filaments were used in all the experiments described here.

Samples (5 μl) of keratin filaments in suspension (approx. 1 mg/ml) were dispensed on glass cover slips. Samples of test proteins (5 μl) at varying concentrations were added. Fiber formation was observed with slide lighting against a dark background and by dark-field microscopy. Optimal protein concentrations are 0.5—1.5 mg/ml filament protein and 0.3—1.0 mg/ml stratum corneum basic protein or histidine-rich protein II.

For observation of macrofibrils by electron microscopy, the mixtures of keratin filaments and stratum corneum basic protein or histidine-rich protein II were prepared in test tubes, diluted to 0.1 mg/ml filament protein, placed dropwise on grids, and negatively stained as described previously [6].

Results

Polyacrylamide gel electrophoresis

Stratum corneum basic protein and histidine-rich protein II had identical electrophoretic mobility on SDS-polyacrylamide gels. Only one band was present. Mixtures of the proteins showed no separation. A molecular weight of 49 000 was estimated from a standard curve using bovine serum albumin (68 000), pyruvate kinase (57 000), ovalbumin (43 000) and cytochrome *c* (12 500) on 7.5% and 10% polyacrylamide gels. The two proteins also had identical electrophoretic mobility on polyacrylamide gels in 4 M urea at pH 4.5 [32].

Immunodiffusion

Stratum corneum basic protein and histidine-rich protein II were compared by Ouchterlony double immunodiffusion using antiserum to each of these two proteins. When these two antigens were placed in adjacent wells a confluent precipitin line formed (Fig. 1). This reaction of identity was also seen with histidine-rich protein I, the precursor of histidine-rich protein II [5]. There was evidence of an additional precipitin line for stratum corneum basic protein

Fig. 1. Double immunodiffusion plate showing the reaction between rabbit antiserum prepared against stratum corneum basic protein (A) and histidine-rich protein II (B) and the antigens stratum corneum basic protein (0.25 mg/ml, well 1), histidine-rich protein II (0.2 mg/ml, well 2), histidine-rich protein I (0.2 mg/ml, well 3; 1 mg/ml, well 4), a urea extract of newborn rat epidermis (well 5), and the solution in which the antigens were dissolved (0.1% SDS, 0.01 M sodium phosphate, pH 7.5, well 6).

Fig. 2. Double immunodiffusion plate showing the reactions between rabbit antisera prepared against stratum corneum basic protein (well 2) and against histidine-rich protein II (well 4) and the antigens stratum corneum basic protein (0.1 mg/ml, well 1) and histidine-rich protein II (0.1 mg/ml, well 3).

(Fig. 1, well 1), for histidine-rich protein II (well 2) and the urea extract (well 5) using either antiserum, and for histidine-rich protein I (well 3) using the antiserum against stratum corneum basic protein. To check the possibility that the two antisera were directed against different but shared components of the preparations of stratum corneum basic protein and histidine-rich protein II, these antisera were put into adjacent wells of an immunodiffusion plate (Fig. 2). There was no evidence of spur formation or cross-over of the precipitin lines, indicating that the antisera react with the same protein.

Peptide mapping

Stratum corneum basic protein and histidine-rich protein II were each digested with trypsin and the resulting peptides were separated by ion-exchange chromatography. The elution patterns of samples digested with trypsin for 12 h are shown in Fig. 3. Samples digested for 6 h gave essentially the same results. In duplicate analyses of the same sample, the elution times of some of the peptides varied by as much as two minutes. Despite this variability, the peaks can be identified from their shape and relationship to other peaks. The profiles of peptides from stratum corneum basic protein and histidine-rich protein II were similar, except for the presence of peaks a and j from histidine-rich protein II. However, peak a was not found in all histidine-rich protein II peptide samples, and peak j was seen in some of the stratum corneum basic protein peptide samples. Although there were differences in relative peak heights for the stratum corneum basic protein and histidine-rich protein II peptides, the similarity in the elution profiles suggests that stratum corneum basic protein and histidine-rich protein II are similar in structure.

Keratin filament aggregation

Stratum corneum basic protein aggregates with keratin filaments to form

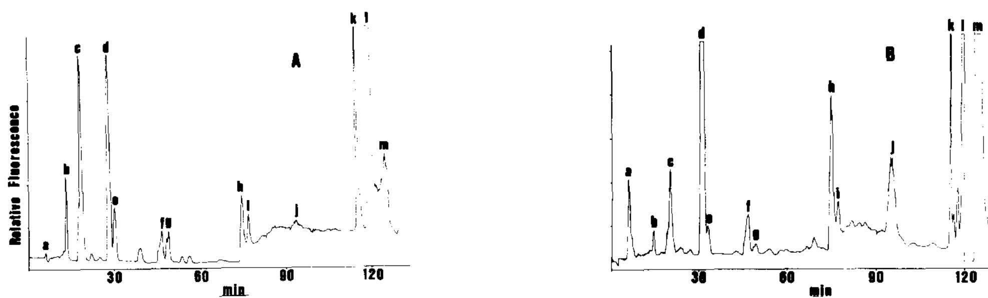

Fig. 3. Elution patterns from ion-exchange chromatography of tryptic peptides of stratum corneum basic protein (A) and histidine-rich protein II (B).

visible fibers. These fibers can be seen by eye and by dark-field microscopy (Fig. 4a). When histidine-rich protein II was tested in place of stratum corneum basic protein at equivalent protein concentration, no fiber formation occurred. Since the purification of histidine-rich protein II included a preparative SDS gel electrophoresis step, SDS could be bound to the protein and interfere with its ability to aggregate with keratin filaments. Therefore, SDS was assayed colorimetrically in histidine-rich protein II and stratum corneum basic protein (previously dialyzed against 5 mM Tris-HCl, pH 7.6) at several protein concentrations [26]. Stratum corneum basic protein had the same absorbance as did buffer alone. Histidine-rich protein II contained 0.27 mg SDS/mg protein.

SDS was extracted from histidine-rich protein II by ion-pair method [27]. Stratum corneum basic protein was used as a control to test for any effects of

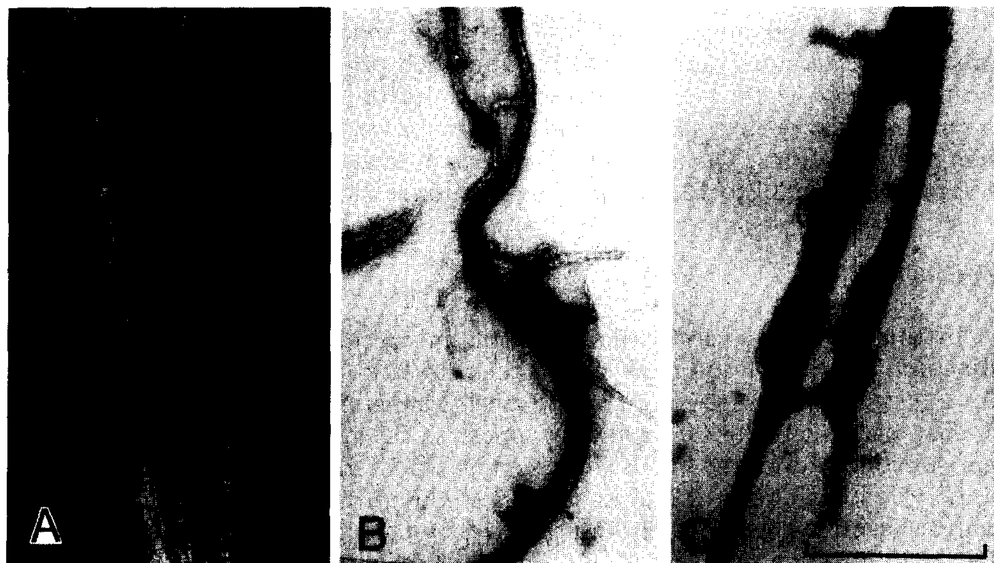

Fig. 4. Association of stratum corneum basic protein and histidine-rich protein II with keratin filaments. A. Dark field microscopy of a fiber (also visible by eye) formed by filaments and stratum corneum basic protein (X 70). B. Macrofibril of filaments and histidine-rich protein II (X 47 500). C. Macrofibril of filaments and stratum corneum basic protein (X 47 500). Bar, 0.5 μm.

TABLE I

EFFECT OF SDS EXTRACTION ON SDS CONCENTRATION AND FIBER FORMATION

SDS was extracted by the ion-pair method [27]. The SDS concentration was measured colorimetrically [26], protein concentration was measured by the method of Lowry et al. [28]. Fiber formation with keratin filaments was assessed microscopically in 5 mM Tris-HCl, pH 7.6.

Sample	Treatment	Before extraction		After extraction	
		SDS⁻ (mg/mg protein)	Fiber formation	SDS (mg/mg protein)	Fiber formation
Histidine-rich protein II	None	0.27	—	0.02	++
Stratum corneum basic protein	None	0	++	0	++
Stratum corneum basic protein	5 min, 100°C, + 8 M urea, dialyzed vs. H_2O, lyophilized	0	++	0	++
Stratum corneum basic protein	5 min, 100°C, + 1.5% SDS and 8 M urea, dialyzed vs. H_2O, lyophilized	4.32	—	0.01	++

the extraction procedure on the function of the protein. A summary of the results is shown in Table I. The extraction method reduced the SDS concentration to 0.02 mg/mg protein or less. This was greater than a 10-fold reduction of SDS in histidine-rich protein II and approx. 500-fold reduction of SDS in the samples of stratum corneum basic protein heated with SDS. After SDS extraction, all protein samples gave a clear positive test for fiber formation with keratin filaments at the light microscopic and visible level.

At the electron microscopic level, however, macrofibrils were seen even in mixtures of keratin filaments and histidine-rich protein II prior to SDS extraction (Fig. 4b). These macrofibrils were similar to those formed with stratum corneum basic protein (Fig. 4c) but formed in lower yields at equivalent protein concentrations. The macrofibrils had an average diameter of approx. 50 nm but ranged from 35 to 150 nm in diameter under the conditions used here.

Discussion

In this report we have compared stratum corneum basic protein and histidine-rich protein II and have shown them to have the same molecular weight by SDS gel analysis, to be immunologically indistinguishable and functionally similar. Although the two proteins appear to be pure by SDS-polyacrylamide gel electrophoresis, two bands were seen by Ouchterlony double diffusion. This may have been due to the presence of a second protein, not separated by SDS-polyacrylamide gel electrophoresis, shared by both stratum corneum basic protein and histidine-rich protein II. Another explanation is that both of these proteins, which are extremely sensitive to proteolysis, are partially degraded by proteases in the immune sera. Any resulting lower molecular weight peptides could diffuse differently and cause the formation of a second immunoprecipitin band.

The two proteins are effective in aggregating keratin filaments to form macrofibrils visible at the electron microscopic level. At optimal protein concentrations macrofibrils form larger thread-like fibers which are visible by eye. However, in the case of histidine-rich protein II, it was first necessary to remove residual SDS in order to achieve visible fibers. The inhibition of visible fiber formation by SDS was confirmed by treating stratum corneum basic protein with SDS. When the SDS-treated stratum corneum basic protein was added to keratin filaments, no fibers were formed. However, subsequent removal of the SDS regenerated the fiber-forming potential of the stratum corneum basic protein. Incubation at 100°C or extraction of stratum corneum basic protein with acetone also had no irreversible effect on fiber formation.

Additional evidence of the similarity between stratum corneum basic protein and histidine-rich protein II is apparent from their amino acid compositions. Both proteins have an unusually low content of nonpolar amino acids and an unusually high content of glutamic acid, glycine, serine, arginine and histidine [1,3,5]. Arginine contents of 13.1% for stratum corneum basic protein and 13.9% for histidine-rich protein II were reported; the histidine contents were 7.9% and 7.3%, respectively. No methionine, cysteine, or phenylalanine was detected in either protein. The quantitative differences found in the tryptic peptide maps of stratum corneum basic protein and histidine-rich protein II may be due to the presence of minor contaminants, rather than differences in primary structure.

Both stratum corneum basic protein and histidine-rich protein II have been isolated from newborn rat epidermis and previous results have suggested that both were found in highest concentration in the stratum corneum. Furthermore, the proteins are both derived from precursor proteins found in the keratohyalin granules of the less differentiated granular cell layer [5,12,21,22]. These precursors, however, do not appear to be identical, since the molecular weight of the stratum corneum basic protein-precursor is 53 000, while that of histidine-rich protein I is so large that it only just enters a 7.5% SDS-polyacrylamide gel [5]. The predisposition of stratum corneum basic protein-precursor to polymerize [22,23] may be related. The nature of this polymerization is as yet unknown, as the yield of polymeric stratum corneum basic protein-precursor is variable and in vitro depolymerization has not yet been accomplished. A partial characterization of the stratum corneum basic protein-precursor has shown that it is a phosphoprotein in which serine residues are phosphorylated [33]. Covalent phosphate has also been found in an acidic acid peptide of histidine-rich protein I (Vadlamudi, B., unpublished results). The stratum corneum basic protein-precursor and histidine-rich protein I may be different forms of the same protein. Unlike their respective precursors, neither stratum corneum basic protein nor histidine-rich protein II contains phosphate.

Thus, the available evidence suggests that stratum corneum basic protein and histidine-rich protein II are derived from a phosphorylated precursor protein localized in keratohyalin granules and are identical. As a result of this work we suggest that both stratum corneum basic protein and histidine-rich protein II be called histidine-rich basic protein.

Acknowledgements

We wish to thank at the University of Washington Dr. Karen Holbrook, Departments of Biological Structure and Medicine, for electron microscopic analysis, Ms. Julie Haugen for excellent technical assistance, and Dr. John D. Lonsdale-Eccles for critical reading of the manuscript and, at the University of Michigan, Ms. Phyllis Foster for preparing some of the histidine-rich protein II used in this study, and Ms. Jane Fedor for help with immunological studies.

This investigation was supported by grants DE-04660 and DE-02600 (Center for Research in Oral Biology) from the National Institute for Dental Research and by AM-21557 (University of Washington) and AM 07236 and AM 15206 (University of Michigan) from the National Institute for Arthritis, Metabolic and Digestive Diseases, U.S.P.H.S.

References

1 Hoober, J.K. and Bernstein, I.A. (1966) Proc. Natl. Acad. Sci. U.S.A. 56, 594—601
2 Tezuka, T. and Freedberg, I.M. (1974) J. Invest. Dermatol. 63, 402—406
3 Dale, B.A. (1977) Biochim. Biophys. Acta 491, 193—204
4 Balmain, A. (1976) J. Invest. Dermatol. 67, 246—253
5 Ball, R.D., Walker, G.K. and Bernstein, I.A. (1978) J. Biol. Chem. 253, 5861—5868
6 Dale, B.A., Holbrook, K.A. and Steinert, P.M. (1978) Nature 276, 729—731
7 Fukuyama, K., Nakamura, T. and Bernstein, I.A. (1965) Anat. Rec. 152, 525—529
8 Fukuyama, K. and Epstein, W.L. (1966) J. Invest. Dermatol. 47, 551—560
9 Fukuyama, K. and Epstein, W.L. (1967) J. Invest. Dermatol. 49, 595—604
10 Gumucio, J., Feldkamp, C. and Bernstein, I.A. (1967) J. Invest. Dermatol. 49, 545—551
11 Ugel, A.R. and Idler, W. (1972) J. Cell Biol. 52, 453—464
12 Sibrack, L.A., Grey, R.H. and Bernstein, I.A. (1974) J. Invest. Dermatol. 62, 394—405
13 Dale, B.A. and Stern, I.B. (1975) J. Invest. Dermatol. 65, 223—227
14 Tezuka, T. and Freedberg, I.M. (1972) Biochim. Biophys. Acta 261, 402—417
15 Tezuka, T. (1977) in Biochemistry of Cutaneous Epidermal Differentiation (Seiji, M. and Bernstein, I.A., eds.), pp. 467—477, University Park Press, Baltimore
16 Freinkel, R.K. and Weir, K.A. (1975) J. Invest. Dermatol. 65, 482—487
17 Balmain, A., Loehren, D., Fischer, J. and Alonso, A. (1977) Devel. Biol. 60, 442—452
18 Guss, S.B. and Ugel, A.R. (1972) J. Histochem. Cytochem. 20, 97—106
19 Murozuka, T., Fukuyama, K. and Epstein, W.L. (1979) Biochim. Biophys. Acta 579, 334—345
20 Ugel, A.R. (1969) Science 166, 250—251
21 Dale, B.A. and Ling, S.Y. (1979) Biochemistry 18, 3539—3546
22 Dale, B.A. and Ling, S.Y. (1979) J. Invest. Dermatol. 72, 257—261
23 Laemmli, U.K. (1970) Nature 227, 680—685
24 Dale, B.A., Lonsdale-Eccles, J.D. and Holbrook, K.A. (1980) in Biochemistry of Normal and Abnormal Epidermal Differentiation (Bernstein, I.A. and Seiji, M., eds.), University of Tokyo Press, Tokyo, pp. 311—325
25 Yen, S.-H., Dahl, D., Schachner, M. and Shelanski, M.L. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 529—533
26 Waite, J.H. and Wang, C.-Y. (1976) Anal. Biochem. 70, 279—280
27 Henderson, L.E., Oroszlan, S. and Konigsberg, W. (1979) Anal. Biochem. 93, 153—157
28 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
29 Hare, P.E. (1977) Methods Enzymol. 47, 3—18
30 Benson, J.R. (1977) Methods Enzymol. 47, 19—31
31 Steinert, P.M., Idler, W.W. and Zimmerman, S.B. (1976) J. Mol. Biol. 108, 547—567
32 Reisfeld, R.A., Lewis, U.L. and Williams, D.E. (1962) Nature 195, 281—283
33 Lonsdale-Eccles, J.D., Haugen, J.A. and Dale, B.A. (1980) J. Biol. Chem. 255, 2235—2238