KERATINIZATION

A Biochemical, Immunological and Morphological Investigation

FRANZ WIENAND BAUER

GEBOREN TE UBACH OVER WORMS

19 72

Keratinization

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PROMOTORES:

PROF. DR. CH. M. A. KUYPER

PROF. DR. J. W. H. MALI

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PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
IN DE WISKUNDE EN NATUURWETENSCHAPPEN AAN DE
KATHOLIEKE UNIVERSITEIT TE NIJMEGEN,
OP GEZAG VAN DE RECTOR MAGNIFICUS
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HOOGLERAAR IN DE FACULTEIT DER
SOCIALE WETENSCHAPPEN,
VOLGENS BESLUIT VAN DE SENAAT
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DES NAMIDDAGS TE 2 UUR PRECIES

DOOR

FRANZ WIENAND BAUER

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To Bertie

and My Parents

Contents

De hoornlaag, de buitenste laag van onze huid, bestaat uit dode afge-
platte cellen en vormt een beschermende barriere zonder welke we niet
zouden kunnen leven. Deze hoornlaag is via een gecompliceerd proces ont-
staan uit de cellen van de basale laag van de epidermis. Dit proces wordt het
verhoorningsproces genoemd (keratinisatieproces). De cellen van de hoorn-
laag zijn voor ± 65% gevuld met fibrilaire eiwitten die men alleen door vrij
drastische methoden in oplossing kan brengen. Men noemt deze eiwitten
de zachte keratines.

De effectiviteit van de barriere wordt voor een groot gedeelte bepaald
door de eigenschappen van deze eiwitten.

In dit proefschrift zijn de resultaten beschreven van een onderzoek
enerzijds naar de structuur van deze zachte keratines in normale en patho-
logisch verhoornde huid (biochemische en immunologische methodes) en
anderzijds zijn de resultaten vermeld van een vergelijkend morphologisch
onderzoek van het verhoorningsproces in vivo en in vitro, een studie dus
van de processen die uiteindelijk leiden tot de vorming van de zachte keratines.

In hoofdstuk I zijn de resultaten beschreven van het onderzoek van kera-
tinefracties uit normale humane huid, pathologisch verhoornde huid en huid
verkregen van enkele andere zoogdieren.

Hoewel we na extractie van humane huid met loog een viertal keratine-
fracties konden isoleren die verschilden in Isoelectrisch punt (pH 6,3, 5,5,
5,0, 4,5) en hoewel deze fracties verre van homogeen waren, zoals bleek uit
de resultaten met behulp van Sephadexgel chromatografie, bleek uit de
resultaten van de polyacrylamidegel electrophorese, dat deze fracties allen
waren opgebouwd uit een of twee subunits en dat de verschillen tussen de
fracties waarschijnlijk berustten op verschillen in aggregatiegraad en/of con-

formatietoestand. Het was niet mogelijk om met zekerheid vast te stellen of de zachte keratines uit een of twee subunits zijn opgebouwd. We konden weliswaar na polyacrylamidegel electrophorese van de keratinefracties twee eiwitbandjes identificeren, maar beide bandjes hadden dezelfde aminozuursamenstelling en na hernieuwde electrophorese van elk van de bandjes afzonderlijk splitsten deze opnieuw in twee bandjes.

Met behulp van de door ons gebruikte methoden waren er geen verschillen aan te tonen tussen de keratinefracties van normale verhoornde humane huid en pathologisch verhoornde humane huid. Ook vonden we geen verschillen tussen humane huid en andere zoogdierhuid voor zover we die hebben onderzocht.

Uit de resultaten van een polyacrylamidegel electrophorese van een vers gemalen hoornlaag kregen we bovendien aanwijzingen dat de keratinefracties niet gedenatureerd waren.

Tenslotte maakten de gegevens verkregen uit een vergelijkend onderzoek van een extractie van de huid met loog enerzijds en met zuur anderzijds het onwaarschijnlijk dat de keratohyalinekorrels uiteindelijk een component zouden leveren voor de hoornlaag zoals in de literatuur wordt aangenomen. Beide extracties bevatten namelijk dezelfde eiwitten, terwijl bij de zure extractie de keratohyalinekorrels achterblijven.

In hoofdstuk II zijn de resultaten vermeld van een vergelijkend onderzoek tussen allerlei keratinefracties met behulp van immunologische methodieken. Daartoe werden er tegen de humane pH 5,5 fractie antilichamen gevormd in konijnen.

Uit de resultaten van dit onderzoek bleek dat alle tot nu toe door ons onderzochte keratinefracties immunologisch identiek waren met de humane pH 5,5 fractie.

Hoewel dit niet inhoudt dat deze stoffen dan ook chemisch identiek zijn, geven deze bevindingen samen met de resultaten van de biochemische methoden sterke aanwijzingen dat er in de subunit(s) van het keratinemolecuul weinig verschil zal zijn in het zoogdierrijk. De pathologische verhoorning zal daarom waarschijnlijk niet het gevolg zijn van een fout in de synthese van de subunit (eventueel wel in de snelheid) maar veeleer het gevolg zijn van een onjuiste interactie van de stoffen die bij deze formatie een rol spelen (eiwitten, vetten, water, afbraakprocessen). Uit een vergelijkend immunoelectrophoretisch onderzoek van de met loog verkregen keratinefracties met eiwitfracties verkregen na een electrophorese van versgemalen hoornlaag gesuspendeerd in electrophoresebuffer bleek, dat de met loog verkregen fracties niet gedenatureerd waren.

In hoofdstuk III zijn de resultaten van een lichtmicroscopisch onderzoek beschreven van foetale rattenhuid in vivo en in vitro. Omdat in de rat het hele keratinisatieproces in enkele dagen verloopt (17de–21ste dag van het foetale leven) vormt deze soort een geschikt studieobject voor de processen die gepaard gaan met de formatie van de hoornlaag. De snelheid van het verhoorningsproces was in vivo en in vitro niet helemaal gelijk maar het eindproduct (de hoornlaag) was in beide gevallen identiek. Met behulp van DNA, RNA en eiwit precursors is aangetoond dat de huid in vitro tijdens de kweekduur metabolisch intact bleef. Actinomycine D en puromycine waren in concentraties van 0,1 μg/ml medium en 1,0 μg/ml medium respectievelijk, toxisch voor de foetale huid gekweekt in vitro.

Uit experimenten met ^{3}H thymidine bleek, dat de bouwstenen van het DNA dat afgebroken wordt in het stratum granulosum (de levende cellaag direct onder de hoornlaag, gekarakteriseerd door zijn afgeplatte kernen en zijn keratohyalinekorrels), opnieuw werden gebruikt voor de synthese van het DNA van de basale cellaag. In hoeverre deze reutilisatie berustte op een systemische recirculatie of een directe diffusie naar de basale cellaag, is verder niet nagegaan.

In hoofdstuk IV zijn de resultaten beschreven van het electronenmicroscopisch onderzoek van het verhoorningsproces in vivo en in vitro van foetale rattenhuid. Onze speciale aandacht werd daarbij gevestigd op de diverse soorten van granula die er tijdens de ontwikkeling tot adulte huid optraden. We meenden daarbij onderscheid te kunnen maken tussen een drietal typen granula.

1^e Een samengesteld granulum, bestaande uit een amorphe, electronendichte matrix en lipidachtige electronentransparante insluitsels. Dit granulum komt slechts gedurende een bepaalde periode van het foetale leven voor. De betekenis ervan is niet erg duidelijk.

2^e De keratohyalinekorrels. Deze korrels ontstaan rond de 18de dag van het foetale leven in de granulaire laag. Onze interpretatie is dat deze korrels zijn ontstaan uit een samenklontering van ribosomen. Men zou zich voor kunnen stellen dat in de granulaire laag waar een afbraak plaats vindt van allerlei celorganellen (o.a. nucleus, mitochondieën), de ribosomen die niet langer een synthese functie hebben eveneens worden afgebroken. Dit zou betekenen dat de keratohyaline korrels geen component leveren die uiteindelijk in de hoornlaag terecht komt. Deze interpretatie is in overeenstemming met de resultaten verkregen uit de biochemische experimenten (zie boven).

3e Een klein bolvormig lipidachtig electronentransparant granulum. Dit
komt voor vanaf de 21ste dag van het foetale leven. Het onderzoek sug-
gereerde dat deze granula afkomstig zijn uit de keratohyaline korrels. In
dit geval zouden ze afbraak produkten van RNA en eiwit kunnen be-
vatten.

In vitro waren de keratohyaline korrels niet zij talrijk als in vivo en soms
bijna geheel afwezig terwijl er toch een morphologische normale hoornlaag
aanwezig was. Dit is een derde argument dat de keratohyaline korrels voor
de formatie van de hoornlaag niet noodzakelijk zijn.

Studies on Isolated Keratin Fractions from Mammalian Epidermis

I. Physicochemical Properties of the Fractions

F. W. BAUER

Department of Chemical Cytology (Head: Prof. CH. M. A. KUYPER) and
Department of Dermatology (Head: Prof. J. W. H. MALI), Catholic University,
Nijmegen

Abstract. A protein fraction precipitated at pH 5.5 from an alkaline extract of human epidermis or stratum corneum was not homogeneous on Sephadex gel filtration. The inhomogeneity was due to aggregation phenomena since polyacrylamide gel electrophoresis of all Sephadex fractions gave the same results: a highly-aggregated immobile protein plus two mobile protein bands that represented lower aggregates or subunits. This electrophoretic pattern was found for all the isolated fractions even for fractions with different iso-electrical points (IEP) and from different species. Freshly-ground callus gave essentially the same pattern, indicating that the bands are not artefacts caused by denaturation. The significance for the process of keratinization is discussed.

Key Words
Sephadex chromatography
Polyacrylamide gel electrophorese
Amino acid analyses
Keratin subunit
S-value
Aggregation
Protein denaturation

Introduction

Epidermal keratin, or soft keratin, forms the major component of the stratum corneum, a protective barrier without which we could not survive.

There are various definitions of the term keratin which are reviewed by MERCER [1961]. In agreement with CROUNSE [1963] we like to define keratin 'as an insoluble fibrous macromolecule synthesized as the final protein product of epidermal differentiation and growth'. This definition leaves it an open question whether keratin is the result of aggregation from only one kind

of molecules or that several classes of molecules, synthesized at different sites in the epidermis contribute to its formation.

In order to understand more about the chemical nature of soft keratin, several investigators have isolated protein fractions of the stratum corneum or the entire epidermis. CARRUTHERS *et al.* [1955], for instance, found after urea extraction of cow nose epidermis, fractions with iso-electrical points (IEP) at pH *6.3, 5.5, 5.1* and *4.5.* ROTHBERG [1960] found by alkaline extraction of human skin IEP at *5.5, 5.0,* and *4.5.* CROUNSE [1963] operating with different alkali concentrations found IEP at *6.3, 5.5, 5.0,* and *4.5.* The same values have been reported for swine epidermis [WEINSTEIN, 1966]. MATOLTSY [1964, 1965] obtained by citric acid extraction at pH 4.5, a protein fraction from cow nose epithelium which he called prekeratin.

So, in spite of the remarkable differences in sample source, sample collection and extraction procedure, the isolated protein fractions have approximately similar IEP. Studies by means of electrophoresis and with the analytical ultracentrifuge showed that the 'pH 6.3 fraction' from alkaline extracts and the 'prekeratin fraction' from acidic extracts behaved as a single band in moving boundary electrophoresis and that they had a high molecular weight [CROUNSE, 1963; MATOLTSY, 1964; 1965]. The 'pH 5.5 fraction' is thought to be the subunit of both with a molecular weight of 50,000 [CROUNSE, 1966]. The relation between the fractions with different IEP and the question whether these isolated fractions represent proteins which are identical with the protein *in vivo*, is not yet answered. We have to consider the possibility of denaturation and alternations by the extraction methods employed, although CROUNSE [1963, 1965a] argued that the 'pH 6.3 fraction' shares several properties with the whole epidermis: It required a time of hydrolysis comparable to that of the whole epidermis, and 'buttons' prepared from the 'pH 6.3 fraction' showed water vapour transport rates comparable to those of human epidermis *in vitro*. However, these were only preliminary results.

In the work reported in this article we have further characterized the isolated fractions by means of Sephadex gel filtration, polyacrylamide gel electrophoresis and amino acid analysis in order to understand more about the keratinization process, and the proteins involved in it. Besides this, we give further evidence that the isolated fractions share properties with freshly-ground callus which has not undergone any chemical treatment.

This article will be followed by an immunological and immuno-electrophoretical study of isolated keratin fractions from normal and pathological skin with further evidence of the native structure of the isolated fractions. A review article is published elsewhere [BAUER, 1971].

Materials and Methods

Isolation Procedures

The chemical used were Pro Analysi Grade from Merck Normal human epidermis from the back was collected from cadaver skin by the stretch technique of VAN SCOTT [1952], modified by SPRUIT [1964] In some of the experiments we prepared sheets of human stratum corneum by the method of KLIGMAN and CHRISTOPHERS [1964] Stratum corneum flakes of normal skin, psoriatic and ichthyotic skin and callus were scraped from the skin surface of the subjects The steps for extracting the tissues were in general those described by ROTHBERG [1960] The ground freeze-dried material was thoroughly defatted with diethyl-ether and then suspended and dialysed against phosphate buffer [ROTHBERG, 1960] The remaining 'insoluble material' was then extracted with 0 02 N NaOH for 24 h at room temperature The sediment after centrifuging was suspended in 0 02 N NaOH for another 24 h This procedure was followed each day for a week The supernatants were acidified with 0 1 N or 1 N acetic acid In other experiments we extracted the insoluble material with 0 02 N NaOH by powerful homogenizing in a Waring blendor for 10–30 min The fractions are named after the pH at which they precipitate

Prekeratin was prepared according to MATOLTSY [1964, 1965] from cow nose epidermis by extracting with 0 1 N citrate buffer pH 2 6

Electrophoretic Separation

Some of the epidermal proteins extracted in alkali were subjected to disc electrophoreses on polyacrylamide gels according to the methods of ORNSTEIN [1964] and DAVIS [1964], with tris-HCl buffer at pH 8 3 using a 5-percent gel In some experiments the protein sample was applied to the gel in concentrated sucrose, in other experiments the protein was mixed with 5% acrylamide solution and applied to the already-polymerized gel After polymerization of this upper gel the electrophoresis could start

The polyacrylamide electrophoresis was carried out at room temperature for 30 min using 3 mA per tube (6 mm diameter and 70 mm length), or for preparative procedure, on an Uniphor column (LKB) at 4 °C, height 20 cm, diameter 2 5 cm In this case the elution rate was approximately 3 ml/h The separation took 36 h using 600 V at 10 mA

The gels were stained for protein with 0 1% amido black in 7% acetic acid and the excess dye was removed with 1% aquous acetic acid For lipids the gels were stained in 0 1% Nile blue in 1% sulphuric acid and the excess dye removed with 1% sulphuric acid

Sephadex Gel Filtration

20 mg of protein were dissolved in 2 ml buffer and placed on a Sephadex G-200 column of 30 cm height, diameter 2 5 cm The column was rinsed with the desired buffer before use and eluted with the same buffer The exact data are given in the text Absorption was measured at 280 nm

Amino Acid Analysis

Amino acid analysis was performed, after 24 h hydrolysis of 1 mg protein with 1 ml 5 7 N HCl at 105 °C, with a Technicon Auto-Analyzer

Sedimentation Velocity

For the determination of S-value 6 mg protein was dissolved in 1 ml 0 01 N NaOH to give a clear solution

Results

Precipitation

Since the precipitation behaviour after acidifying the alkaline protein solution was different from that reported in the literature [ROTHBERG, 1960; CROUNSE, 1963] we briefly describe our findings.

a) Alkali Extraction

The supernatant, after extracting the 'insoluble material' with 0.02 N NaOH, was acidified with 0.1 N acetic acid; the clear solution became more and more turbid from ± pH 10.0 down to pH 6.5. At this pH we centrifuged the suspension. The sediment had a gelatinous appearance. The supernatant was acidified further and at pH 5.5 about 90% of the protein precipitated. Minor precipitates were obtained at pH 5.0 and 4.5.

The gelatinous sediment obtained at pH 6.5 was redissolved in 2–3 times the amount 0.02 N NaOH that was used for extraction, and the diluted solution was acidified. Now the solution remained clear and sharply-defined precipitates occurred at pH 6.3 und 5.5. In more diluted protein solutions only a precipitate at pH 5.5 occurred. This is in agreement with the findings of CROUNSE [1963]. The gelatinous material in the concentrated protein solution probably represent such large aggregates of molecules that they sediment already by centrifugation, although the solution has not yet reached the IEP.

More than 90% of the 'insoluble' material was ultimately soluble in 0.02 N NaOH without change in the amino acid composition. Shaking in a Waring blendor for 10 min was just as effective for extraction as 24 h stirring at room temperature.

We found no differences between fractions isolated from whole epidermis and fractions derived from stratum corneum alone.

b) Acid Extraction

According to MATOLTSY [1965] the precipitate obtained by adding 1 N NaOH to citrate buffer extracts at pH 2.6 is insoluble in the pH range 3.4–11.5. We found, however, that this only holds true for concentrated solutions.

By lowering the protein concentration the precipitate redissolves above pH 6. On addition of 1 N HCl to dilute protein solutions of pH 12 all protein precipitated at pH 5.5.

Sephadex Experiments

The results of one typical experiment are shown in figure 1. 20 mg of the human pH 5.5 fraction were dissolved in 0.1 M carbonate-bicarbonate buffer
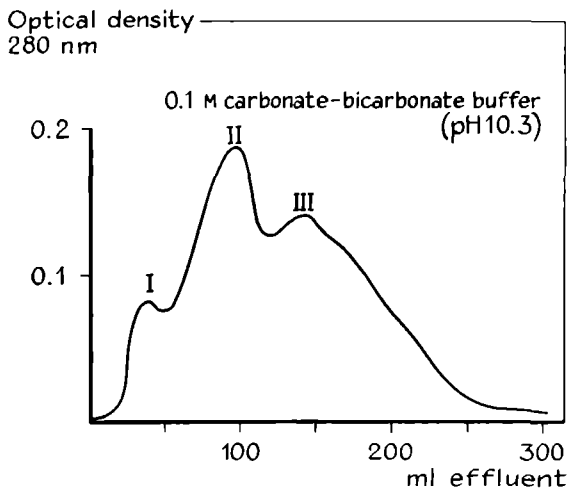
Fig. 1. Fractionation of human pH 5.5 protein on Sephadex G-200.

at pH 10.3, placed on a Sephadex G-200 column, equilibrated and eluted with the same buffer. The overall elution pattern was not exactly reproducible, but the same characteristics were always seen. Immediately after the void volume (determined with dextran blue in a separate experiment) the high molecular weight protein was eluted from the column, but at least one fraction of lower molecular weight could always be seen.

Electrophoresis

After applying the pH 5.5 fraction to the gel in the electrophoresis buffer saturated with sucrose, on staining for protein it became clear that some protein did not migrate into the gel. Apart from this protein, 2 well-defined bands (A and B) became visible; staining for lipids was negative. After applying the protein in a 5-percent upper gel, it was indeed clear that a considerable amount could not enter the gel (fig. 2).

When the upper gel was separated from the lower gel after the electrophoretic run, placed upon a new 5-percent lower gel and subjected to a second run, the same results were obtained (fig. 2). This process could be repeated several times. Some protein again remained in the upper gel, but two bands with the mobility of the bands A and B were also visible; apparently the proteins in these bands are derived from the protein remaining in the upper gel in the first electrophoretic run. After electrophoresis of the pH 5.5
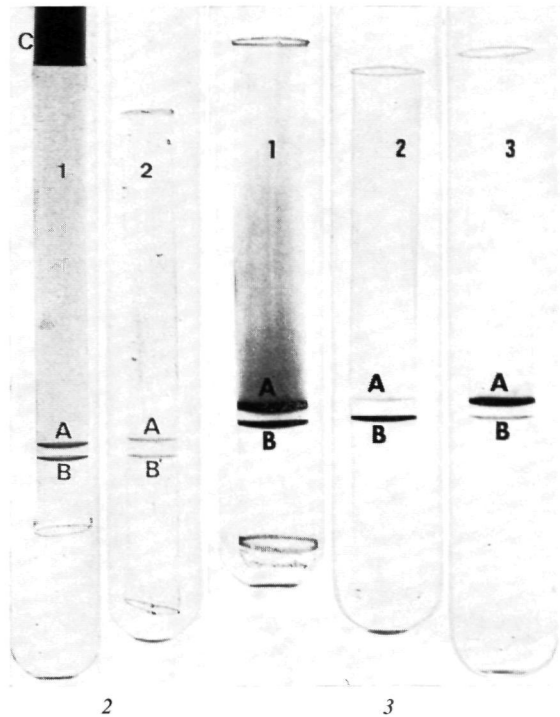
Fig. 2. Electrophoretic patterns of keratin fractions: (1) pH 5.5 protein. (2) Re-electrophoresis of the upper gel C.

Fig. 3. Electrophoretic patterns of: (1) Bands A and B together with pH 5.5 protein. (2) Band B only. (3) Band A only.

protein, redissolved in 0.02 N NaOH containing 6 M urea, in a 5-percent gel also containing 6 M urea, the amount of protein in the upper gel was clearly decreased whereas the bands A and B contained more protein. This is an indication that aggregation phenomena occur.

The freeze-dried Sephadex fractions I, II and III (fig. 1) were also investigated by polyacrylamide gel electrophoresis. Each fraction gave the same pattern, i. e. the same two bands, except the high molecular weight fraction I, which gave, besides the two bands, an immobile protein fraction in the 5-percent upper gel. The significance of this will be discussed later on.

The pH 5.5 fraction from different sources such as rabbit, rat, cow nose epithelium, psoriatic lesions, ichthyotic skin and from normal human skin

and those from pooled callus prepared according to THIELE and BAUER [in preparation], all gave identical results. Moreover, the pH 6.3 fraction mentioned above and the pH 5.0 and 4.5 fraction gave the same results. The prekeratin gave the same electrophoretic pattern as well.

Even in the same electrophoretic run the ratio of protein A: protein B was not always constant. Obviously A could form B and vice versa. This was investigated further by the following procedure: The two bands were cut off and new electrophoretic runs were performed with each band. Band A and B *each* gave, now, two bands with the mobility of the forementioned bands A and B. This result was confirmed by mixing A, B and the pH 5.5 protein and placing it on a 5-percent gel; only two bands were visible (fig. 3).

After oxidizing the pH 5.5 protein according to the method of HARRIS [1960] or HIRS [1956] the two bands A and B were still found.

From freshly-ground callus suspended in electrophoretic buffer we obtained beside other bands, two bands with the same electrophoretic mobility as the bands A and B as was confirmed by co-electrophoresis of the pH 5.5 fraction and the freshly-ground callus.

Amino Acid Analysis

For amino acid analysis of the proteins from bands A and B we homogenized the gel containing either band A or B. After centrifugation amino acid analyses were performed on the supernatants (table I). The chromatograms of the hydrolyzed protein from A and B showed some unidentified peaks, due to interference from the polycrylamide or the tris buffer. Beside this the glycine, forming part of the electrophoresis buffer, moves in the electrical field and has about the same position in the gel as the bands A and B. In the table, therefore, the molar percentages of the amino acids minus glycine are given. In the table the amino acid analysis of the freeze-dried fractions A and B separated by preparative polyacrylamide electrophoresis and an analysis of the pH 5.5 protein are also given.

With respect to the discussion of whether the acid and the alkaline extracted proteins are similar [CROUNSE, 1966], amino acid analyses were done on the prekeratin (acid extraction) and the pH 5.5 protein (alkaline extraction), both prepared from calf nose epithelium. The amino acid analyses showed no significant differences.

S-Values

The S_{20} value of human pH 5.5 protein (and prekeratin) solubilized in 0.01 N NaOH was 2.5 S. This could indicate a molecular weight of about

Table I. Amino acid composition[1] (molar %) of some keratin fractions

	pH 5.5 fraction	upper gel (homogenized)	A		B	
Asp	12.6	14.5	15.7[2]	(14.9)[3]	16.3[2]	(16.2)[3]
Thr	4.8	5.9	6.3	(5.0)	5.6	(5.7)
Ser	10.9	11.7	11.6	(10.2)	9.8	(9.0)
Glu	17.8	14.7	18.3	(18.4)	18.4	(21.2)
Pro	2.0	2.8	3.2	(2.2)	2.5	(2.3)
Ala	7.3	7.9	10.9	(8.8)	8.2	(8.6)
Val	4.9	5.8	5.3	(5.4)	5.0	(5.5)
Cys	+	+	+	(+)	+	(+)
Met	1.5	+	1.9	(+)	2.1	(+)
Ileu	5.3	5.9	4.4	(5.6)	5.0	(7.3)
Leu	11.9	12.6	8.0	(12.2)	10.9	(11.4)
Tyr	2.8	+	1.6	(+)	2.3	(+)
Phe	4.1	5.0	2.0	(4.8)	2.8	(3.7)
Orn	0.2	+	+	(+)	+	(+)
Lys	6.0	6.5	4.6	(6.4)	5.9	(4.3)
His	1.2	0.8	1.3	(1.3)	1.0	(1.6)
Arg	6.5	6.0	4.0	(4.8)	4.2	(3.1)

[1] Amino acid composition (molar %) minus glycine.

[2] Amino acid composition from A and B after preparative electrophoresis.

[3] In parentheses the amino acid composition after homogenizing the gel containing A and B.

50.000, the value found by CROUNSE [1966]. There was only one sharp peak. No high molecular weight fraction, as was seen in the Sephadex experiments, was found.

Discussion

All of the isolated fractions from different species, even those with different IEP gave two bands A and B after polyacrylamide gel electrophoresis, whereas a considerable amount of protein did not enter the gel.

Freshly-ground callus material suspended in electrophoresis buffer, gave, on electrophoresis, beside other bands, two bands with exactly the same mobility as band A and B.

Since the pH of the electrophoresis buffer is much higher than the pH at which the proteins precipitate (pH 6.3, 5.5, 5.0, 4.5) and, therefore, the

protein has a negative charge, the reason for the immobility must be the higher molecular weight of the protein in the upper gel. This immobile protein must consist of some complex of the proteins A and B, since a second electrophoresis of the upper-gel protein gave again two bands that had exactly the same electrophoretic mobility as the bands A and B. This procedure could be repeated 3 or 4 times with the same result. There must exist an equilibrium between an aggregated form and the subunits which is affected by the electrophoretic conditions; e.g. in such a way that the equilibrium cannot be established during the run. After the run some subunits are again formed which in turn can move in the electrical field.

Very striking is the finding that protein A and B on repeated electrophoresis each gave two bands with the electrophoretic mobility of the proteins A and B. This has only been investigated for the human pH 5.5 fraction but might be true, too, for all of the fractions. The proteins A and B have a very similar amino acid composition. Therefore, A and B can either be the monomer and dimer (or polymer) or A and B may both be monomers that can be transformed into each other, by some change in tertiary structure. Reduction or oxidation of an S-S-bridge cannot play a role; this possibility is excluded by our oxidation experiment.

The following conclusions can be drawn from the electrophoretical data:

(a) The differences found in the IEP of the alkali-extracted fraction (pH 6.3, 5.5, 5.0, 4.5) depend on the degree of aggregation and/or on the conformation of the proteins and ionic concentration of the solutions, whereas the subunit(s) in all the preparations is (are) the same [see also THIELE and BAUER, 1970]. This is confirmed since prekeratin (acidic extraction with IEP 4.5) and an alkali-extracted pH 5.5 fraction from the same material (cow nose epithelium) have the same amino acid composition and since, furthermore, the prekeratin could be precipitated at pH 5.5 after resolubilization in 0.02 N NaOH.

These findings are not in accordance with those of CROUNSE [1963] who states that the 5.0 and 4.5 fractions are degradation products which can be derived from the 5.5 protein by splitting off some of the N-terminal amino acids. Nor are they concordant with those of ROTHBERG [1964] who claims that the fractions pH 5.0 and 4.5 are synthesized in the lower epidermis and that the pH 5.5 fraction is the result of splitting off some peptides from the original proteins.

(b) The isolated fraction share properties with the *in vivo* protein because freshly-ground callus gave, besides other bands, two bands with the same

electrophoretic mobility as the bands A and B of the isolated fractions, and since immunological experiments [BAUER, in press] showed that the bands from the isolated fractions and those from the fresh material represent the same proteins.

(c) The subunit(s) of the keratin molecule are very uniform in mammalian skin. Of interest in this connection are the findings of MATOLTSY and MATOLTSY [1963] who showed that powder of stratum corneum, nails, hair and callus, suspended in electrophoresis buffer gave two fast-moving bands dominant in all horny tissues after electrophoresis on 5% polyacrylamide gel. So identical subunits are used not only for the formation of soft keratin, but also for hard keratin.

(d) The subunits have a capacity to aggregate as is shown by the electrophoresis experiment and by the formation of a gelatinous material during the titration of the protein to its IEP. This is further supported by our findings in the Sephadex experiment. The observed inhomogeneity of the pH 5.5 fraction is also due to aggregation phenomena. The different peaks must represent different degrees of aggregation since, after concentration by freeze drying, they again gave the same electrophoretic pattern of bands A and B. A third argument for the aggregation is given by the sedimentation experiment. The pH 5.5 protein and the prekeratin solubilized in 0.01 N NaOH gave an S-value of 2.5. Prekeratin, dissolved in citrate buffer pH 2.6 gave an S-value of 5.4 [MATOLTSY, 1964; 1965]. So obviously the sedimentation value (or molecular weight) is influenced by the pH of the solution.

From this work it is reasonable to assume that the subunit proposed by CROUNSE [1965b, 1966] really exists. From our work it is not clear whether there are one or two distinct subunits, for we obtain two bands after electrophoresis which can, however, transform into each other and which probably have the same amino acid compositions. The subunit is probably synthesized in the epidermal cell on the polysomes and aggregates to form tonofilaments and tonofibrils and seems to be quite uniform in the investigated species. In a manner as yet unknown it then takes up its definitive position in the stratum corneum.

CROUNSE [1963] states that this work 'in no way detracts from (or adds to) the concept of the two component systems of keratinization, since either both components might act as a single whole under properly mild conditions or the so-called high sulphur matrix may not be extracted at all'. His own observations, however, leave little place for the second suggestion since the amino acid analysis of the pH 5.5 protein and whole callus are essentially similar and since we can extract up to 90% of the 'insoluble material'. The

small differences still present might be explained for instance by assuming that the proteins from the cell membranes do not all pass into solution. If this were so, only his first supposition holds true. That, however, is more or less excluded since the prekeratin, which is extracted from the Malpighian cells in which the second component (the keratohyalin granules) represents only a minor quantity, shows the same properties as the pH 5.5 protein. So, in conclusion, our work does not appear to support the concept of a two-component system.

Acknowledgements

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Author's address: Dr. F. W. BAUER, Department of Dermatology, Catholic University, Philip van Leijdenlaan 25, *Nijmegen* (The Netherlands)

Studies on Isolated Keratin Fractions
from Mammalian Epidermis

II. Immunological and Immuno-Electrophoretic Comparison of the Fractions

F. W. Bauer

Department of Dermatology (Head: Prof. J. W. H. Mali) and
Department of Chemical Cytology (Head: Prof. Ch. M. A. Kuyper),
Catholic University, Nijmegen

Abstract. Immunological and immuno-electrophoretic studies of various keratin fractions from different mammalian sources showed that these fractions were all immunologically identical with the human pH 5.5 protein. That means that the differences in iso-electric points (IEP) have to be explained by differences in degree of aggregation or by differences in conformation.

Key Words
Keratin subunit
Native structure
Antibody formation
Psoriasis
Ichtyosis

Introduction

In our study of the chemical nature of the soft keratins, i.e. the keratins which form an important part of the stratum corneum, we have shown that human keratin fractions with different iso-electric points (IEP) and analogous fractions from different mammalian species behave similarly on polyacrylamide gel electrophoresis. Even untreated callus powder suspended in electrophoresis buffer gave the same electrophoretic pattern [Bauer, in press]. These findings indicate that the keratin subunit is very uniform in mammals and has not undergone severe changes during evolution.

In this communication we give further support to this proposition by an immunological and immuno-electrophoretic study of the fractions. Furthermore, by comparing the isolated proteins with chemically-untreated callus we give new evidence that the isolated proteins are not denatured by the extraction procedures.

Materials and Methods

Normal human epidermis and rabbit epidermis were obtained from cadaver skin via the stretch technique of VAN SCOTT [1952], as modified by SPRUIT [1964]. Stratum corneum flakes of normal psoriatic and ichtyotic skin were scraped from the skin surface of the subjects.

Cow nose epithelium was cut from the nose of a fresh cadaver with a keratotome. Rat epidermis was prepared from pieces of newborn rat skin after 45 min incubation at 37 °C in a solution of 1.25% trypsin (Difco 1:250) in Hank's solution without magnesium and potassium ions, after adjustment of the pH to 7.8.

Keratin fractions were prepared from the epidermis with IEP at pH 6.3, 5.5, 5.0, and 4.5 [ROTHBERG, 1960; BAUER, in press]. Prekeratin (IEP 4.5) from cow nose epithelium was prepared according to MATOLTSY [1965].

Precipitating antibodies were prepared by footpad immunization in New Zealand rabbits in the following way: Human pH 5.5 fraction was dissolved in 0.9% NaCl pH 8.0 (after adding a small amount of 0.02 N NaOH) at a concentration of 2 mg/ml. Of this solution, 0.6 ml were mixed with 0.6 ml complete Freund's adjuvant and 0.5 ml was injected intracutaneously in each footpad, in each of the axillae and in the groins. At intervals of 3 weeks booster injections were given (5 mg of pH 5.5 protein in 1 ml 0.9% NaCl). Three or 4 days after the booster injections, the antisera were tested with the double diffusion technique of OUCHTERLONY [1953] on microscope slides in veronal buffer pH 8.4 or in 0.9% NaCl. For some experiments, immunoglobulins were prepared from the serum: to the serum was added ammoniumsulphate until there was reached a saturation of 40% [STEFFEN, 1968].

Absorption of the immunoglobulins was accomplished by first incubating the antigens and the immunoglobulins in a test tube for 1 h at 37°C. The supernatant remaining after centrifugation was used as pretreated antiserum. For absorption of 5 mg immunoglobulins, 1, 5 and 10 mg of each of the pH 5.5, 5.0 and 4.5 fractions were used, respectively.

For immuno-electrophoresis, the antigens were separated on 5% polyacrylamide gel according to ORNSTEIN [1964] and DAVIS [1964], with a minor modification [BAUER, in press].

Discs of 2 mm were then cut transversely from the gel and placed, in the form of hexagon, on a microscopic slide. These discs were then embedded in agar; after 24 h a well was cut in the center for the antiserum. After application of the antisera, the slides were incubated at room temperature for 24 or 48 h in a moist atmosphere. The slides were then washed in saline, to remove the soluble proteins, covered with filter paper strips and dried. Amido Black was used to stain the bands of precipitated protein.

Results

Immunodiffusion

If the serum was tested 14 days after the footpad immunization, in general a weak precipitation line was visible. A higher titer was obtained after one booster injection. Only after repeated booster injections more than one pre-

cipitation line was visible. This indicates that our antigen preparation was rather pure. The immunological comparison was made at the time that only one precipitation line was obtained.

All fractions investigated hitherto by the double-diffusion test showed one precipitation line with the antiserum against the pH 5.5 fractions. These fractions are summarized in table I. Furthermore, cross reactions revealed that these fractions were immunologically identical and some of these reactions are shown in figure 1.

Pretreatment of the antibody-containing serum or the immunoglobulins obtained from that serum with different concentration of some of the antigens showed in all cases a complete inhibition of the precipitation lines in the double-diffusion test (table II).

Table I. Survey of the epidermal fractions tested against human pH 5.5 antiserum

	Healthy epidermis	Psoriasis scales	Ichtyotic scales	Rat epidermis	Rabbit epidermis	Cow nose epidermis
Fraction 6.3	+	●	●	●	●	●
Fraction 5.5	+	+	+	●	+	+
Fraction 5.0	+	+	+	+	●	●
Fraction 4.5	+	+	+	●	●	●
Prekeratin	●	●	●	●	●	+

+ Means a positive reaction (one precipitation line) in the double-diffusion test; ● means that this fraction was not obtained or not tested.

Table II. Immunodiffusion pattern of some human epidermal proteins with pH 5.5 antiserum before and after pretreatment with varying concentrations of these proteins

Antigen fractions	Untreated serum	Serum pretreated with fractions		
		pH 5.5	pH 5.0	pH 4.5
pH 5.5	+	–	–	–
pH 5.0	+	–	–	–
pH 4.5	+	–	–	–

+ Means a positive reaction (one precipitation line) in the double-diffusion test; – means no visible precipitation reaction.

Fig. 1. Electrophoretic pattern (polyacrylamide) of the human pH 5.5 proteins. C = immobile protein remaining in the 'upper gel'.

Immuno-Electrophoresis

In order to check the purity of the antigen preparation and the minimum number of antibodies present, electrophoresis was performed with the human pH 5.5 protein. Polyacrylamide gel electrophoresis was chosen since we obtained no satisfactory separation on agar gel electrophoresis according to WIEME. As already described in a previous paper [BAUER, in press] two bands were found after staining for protein while a considerable amount of protein failed to enter the gel (fig. 2). The normal procedure, i.e. adding of
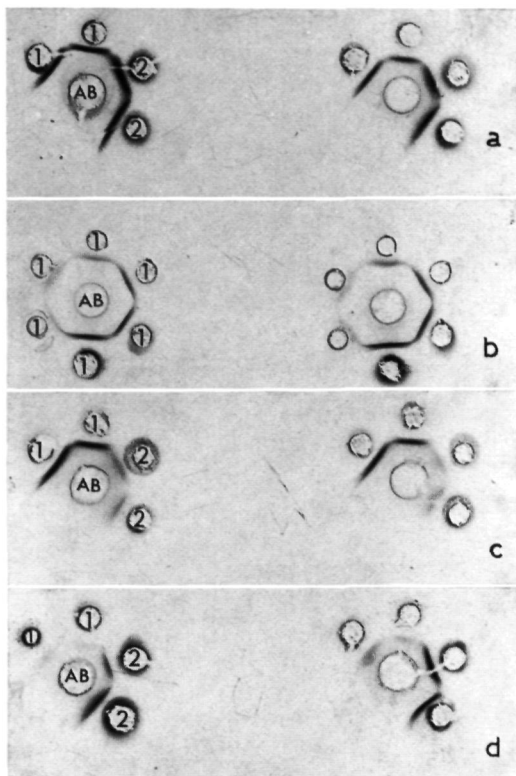
Fig. 2. Immunodiffusion patterns of the antiserum (AB) prepared against the human pH 5.5 protein *versus* varying human keratin fractions. The peripheral wells contain the antigens. *a* 1 = Human pH 5.5 fraction; 2 = human pH 5.0 fraction. *b* 1 = Human pH 5.5 fraction in different concentrations. *c* 1 = Human pH 5.5 fraction; 2 = human pH 4.5 fraction. *d* 1 = Human pH 4.5 fraction; 2 = human pH 5.0 fraction. All fractions show a reaction of identity.

the antibody in a well parallel to the direction of migration of antigens, could not be performed without deformation of the gel since the 5-percent gel had not enough tensile strength to be cut lengthwise. Only occasionally a satisfactory result could be obtained. A better procedure, therefore, was to cut the gel transversely in slices of 2 mm and embed these discs in agar round a well containing antiserum. The following results were obtained.

Precipitation lines were obtained only against the protein in the upper gel and against the proteins in bands A and B. The same results were ob-

tained with the eluted proteins by homogenizing the gel with small amounts
(0.1 ml) of aqua dest. Again cross reactions revealed that the protein in the
upper gel and the proteins in A and B were immunologically identical.

Comparison of Isolated Fractions to 'Native Proteins'

Freshly-ground callus suspended in electrophoresis buffer gave an electro-
phoretic pattern which, besides other bands, gave two bands with the same
mobility as the bands A and B of the isolated pH 5.5 protein [see also BAUER,
in press]. Also a precipitation line occured against these bands and, further-
more, cross reactions of the proteins in these two bands with the bands
A and B of the pH 5.5 protein showed a reaction of identity. Figure 3 shows
the reaction of band A and B of the isolated fraction, band A and B of
freshly-ground callus, and the protein remaining in the upper gel after electro-
phoresis of the pH 5.5 protein.
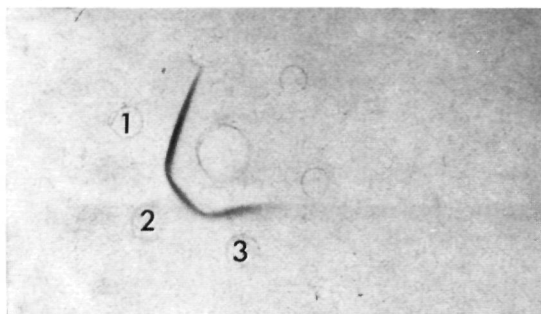
Fig. 3. Immunodiffusion patterns of the antiserum (AB) prepared against the human
pH 5.5 protein *versus* the eluted proteins A, B and C after electrophoresis of human pH
5.5 protein and fresh callus. The peripheral wells contain the antigens. 1 = Human pH 5.5
protein. 2 = Eluted proteins from the bands A and B after polyacrylamide gel electro-
phoresis of the human pH 5.5 fraction. 3 = Eluted proteins of the bands A and B after
polyacrylamide gel electrophoresis of the fresh-ground callus. The fractions show a reaction
of identity.

Discussion

In a previous paper [BAUER, in press] we stated that the various keratin
fractions with different IEP and from different sources consist of the same
subunit and that this molecule is very similar in the skin of various mammals.

Furthermore, based on comparison of the electrophoretic pattern of the various fractions, we postulated that these fractions were not denatured by the alkali treatment.

The data from this study support this statement for the following reasons:

(a) All of the fractions investigated hitherto showed in the double-diffusion test a reaction of identity with the human pH 5.5 protein. That means that at least an (unknown) portion of the molecule has the same tertiary structure (the antigenic site).

(b) After pretreatment of the antiserum or immunoglobulins against the pH 5.5 protein, with varying concentrations of all of the protein fractions tested (human pH 5.5, 5.0 and 4.5) the precipitation was completely inhibited in the double-diffusion test, indicating that the serum contains only one antibody that reacts with antigenic sites present in all of the protein fractions. The reverse situation, i.e. the formation of antibodies against fractions other than the pH 5.5 protein and a comparison of the immunological reactions of the fractions against these antibodies, has not yet been performed but it is unlikely that it would give rise to different conclusions. This could only be expected if the other fractions had extra antigenic sites which are not present in the pH 5.5 protein.

(c) The three precipitation lines obtained after immuno-electrophoresis are in fact caused by a precipitation reaction of the same antibody with the same antigenic site. Whether the antibody reacts with the subunit or with the aggregate is not certain, for, either in the immobile protein in the upper gel are subunits still present or alternatively, the subunits aggregate during diffusion in the agar.

(d) The demonstration that after electrophoresis of freshly-ground callus the two bands, with the same mobility as the bands obtained from the isolated pH 5.5 fractions of normal human skin, gave a precipitation line with antibody against the isolated pH 5.5 fraction, shows beyond doubt that the proteins of the fresh material represent keratin fractions. Since the fresh material has not undergone any severe chemical treatment this can only mean that the antigenic site of the isolated fractions is not denatured during the alkali treatment; as the antigenic site is considered to be very labile, we may conclude that there is no denaturation at all.

Recently CARRUTHERS [1970] has compared the human protein fractions obtained by different extraction procedures [RUDALL, 1952; ROE, 1956a and b; CARRUTHERS *et al.*, 1955; WOERNLY *et al.*, 1957]. He concluded that either the fractions are strongly related, or similar proteins were extracted in different amounts. He also found a reaction of identity. A possible explana-

tion of these phenomena could simply be that these fractions also are built up from the same subunit and that the differences in IEP and the differences found in physical properties may be due to differences in degrees of aggregation or in conformation of the proteins (without affecting the antigenic site).

From the medical point of view our findings may be somewhat disappointing, since it is not likely that immunological studies can serve as a diagnostic tool for differentiation between normal and pathological human skin. It is unlikely that great differences exist in the keratin subunit in the pathological disorders we studied thus far. It is more likely that there are differences in aggregation behaviour and conformation of these units which lead to abnormal keratinization and stratum corneum formation. For instance the incomplete removal of subcellular components in psoriasis might create a situation in which normal aggregation and the further processes leading to a healthy stratum corneum are inhibited. On the other hand, however, the similarity between the various mammalian fractions enables us to perform physicochemical studies on isolated fractions from laboratory animals and to extrapolate the results to normal human skin.

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Author's address: Dr. F. W. Bauer, Department of Dermatology, Catholic University, Philip van Leijdenlaan 25, *Nijmegen* (The Netherlands)

Differentiation and Keratinization of Fetal Rat Skin

I. Light Microscopic Study on Skin cultured *in vitro*

F. W. BAUER and CH. M. A. KUYPER

Department of Dermatology (Head: Prof. Dr. J. W. H. MALI) and
Department of Chemical Cytology (Head: Prof. Dr. CH. M. A. KUYPER),
Catholic University, Nijmegen

Abstract. (1) Fetal rat skin of 17-day-old embryos was cultured up to 8 days *in vitro*.

(2) Within 3–4 days a complete adult epidermis was formed *in vitro*. The stages that lead to this formation and the time necessary for it were slightly different *in vitro* and *in vivo*. No skin adnexa were formed.

(3) Actinomycin D (at 0.02 μg/ml) and puromycin (at 0.1 μg/ml) failed to inhibit the differentiation and the morphological effect of it: i.e. stratum corneum formation. At higher doses a total degeneration of the stratum corneum occurred.

(4) It was clear from the incorporation experiments with ³H-thymidine that a labelled component released from DNA breakdown in the stratum granulosum was re-used for DNA synthesis in the nuclei of the basal cell layer.

Key Words
Organ culture
DNA synthesis
Protein synthesis
Autoradiography
Actinomycin D
Puromycin

Introduction

Keratinization in fetal rat skin *in vivo* is an event which happens between the 17th and 21st day of fetal life. On the 17th day the epidermis consists of 3 strata: stratum germinativum, stratum intermedium and the periderm, so differentiation and maturation occur in a relatively short time. Since the possibility of culturing skin *in vitro* is still a matter of days rather than of weeks, and since we may expect maturation of the skin *in vitro* to be completed in a rather short time in the rat, we chose this skin as a model for the study of differentiation and keratinization.

In this paper we describe our experience with fetal rat skin in culture;

we tested the viability of the skin by means of labelling experiments with radioactive thymidine, uridine and amino acids, and we tested the effect of some inhibitors of protein biosynthesis This paper will be followed by a comparative electron microscopic study on the keratinization of fetal rat skin *in vitro* and *in vivo*

Materials and Methods

Fetal Rat Skin

Pregnant Wistar rats (17 days post-mating) were obtained from the Animal Laboratory, Nijmegen, The Netherlands Flank skin of 17-day-old rat fetuses was dissected with aseptic precautions

Culture Method

After washing the piece of skin in Hanks' solution pH 7 6 (Hanks' BSS) it was placed, epidermal surface up, on a millipore filter (Birn N V , Belgium, pore diameter 3 μm) and laid on a cube of coarse sintered glass (base 10×10 mm, height 7 mm) resting in a flat bottomed watch glass Medium (about 5 ml) was added to just beneath the millipore filter so that it reached the skin by capillary flow The cultures were placed in an air tight perspex container which depending on the pH of the medium, was flushed from time to time with 5% CO_2 in air or in oxygen The gas mixture was first passed through sterile water for humidification Besides this a large Petri dish with sterile water or Hanks' solution was placed in the perspex box The cultures were incubated at 37°C Medium was changed every second day The box was made of a type of perspex which did not absorb UV Therefore, for sterilization we simply placed an UV lamp over the box before use

Medium

Medium M 199 (Morgan, Morton and Parker) was used without any addition of protein 200 units penicillin G (sodium penicillin G, Mycofarm, The Netherlands) and 100 μg streptomycin (streptomycin sulphate, Mycofarm, The Netherlands) per ml were added to the medium

Viability of the Culture

To study protein synthesis in the skin *in vitro* we added at different times after the beginning of the culture, a mixture of 5 tritium-labelled amino acids (The Radiochemical Centre, Great Britain) The amino acids used were 0 1 mCi glycine (spec act ± 1,000 mCi/mmol), 0 08 mCi L-leucine (spec act ± 750 mCi/mmol), 0 1 mCi D-L-isoleucine (spec act ± 200 mCi/mmol), 0 04 mCi D-L-arginine-HCl (spec act 1,000 mCi/mmol), 0 1 mCi D-L-lysine (100 mCi/mmol) The final activity was 3 μCi ³H-amino acids/ml medium After 1 5 h exposure the cultures were either harvested or transferred to medium M 199 without radioactive amino acids

RNA Synthesis

To study RNA synthesis in the organ culture we added ³H-uridine (NEN, USA, spec act 10 7 Ci/mmol) at different times after the start of the culture (2–5 μCi/ml medium) After 1 5 or 6 h the samples were harvested
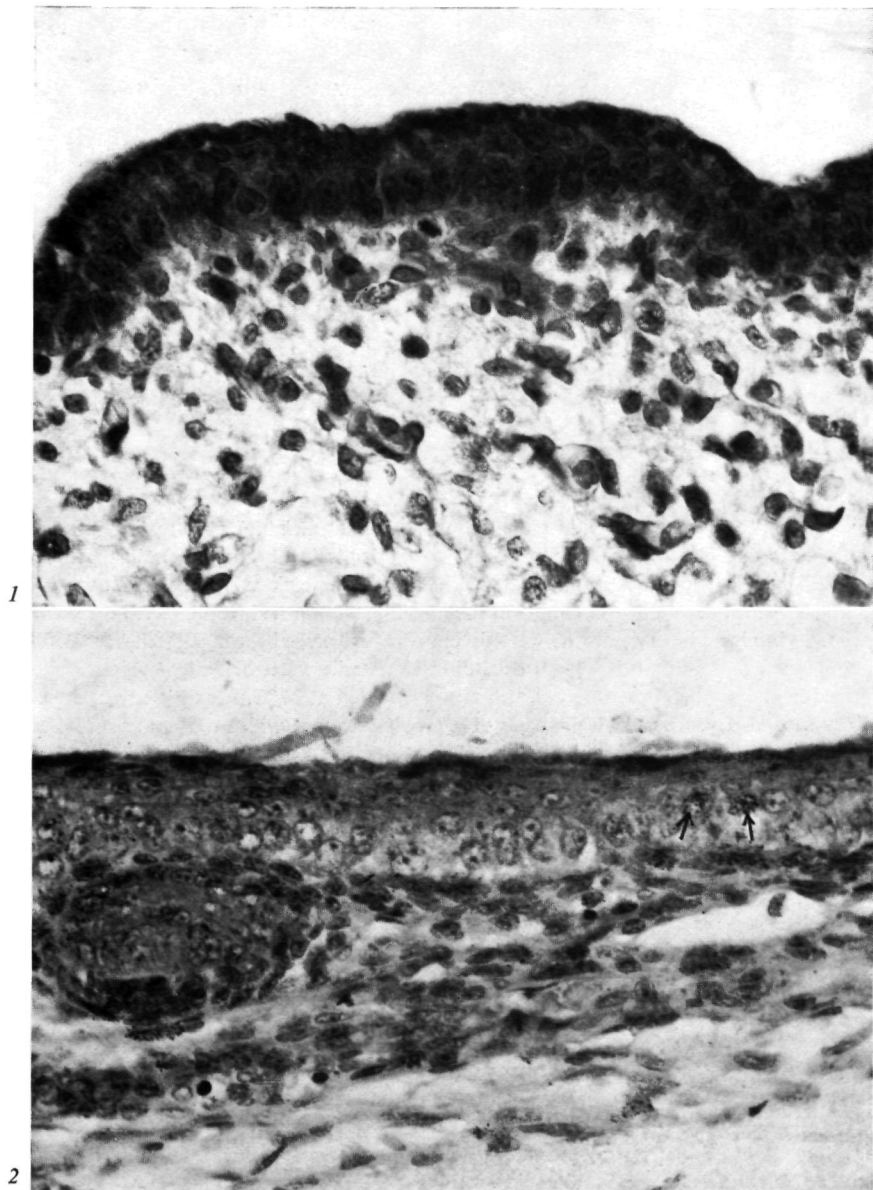
Fig. 1. 17-day-old fetal rat skin. HE. ×410.

Fig. 2. 17-day-old fetal rat skin after 1 day in culture. Autoradiography. Arrows show nuclei labelled with ³H-thymidine. HE. ×410.

DNA Synthesis and Epidermal Turnover

[3]H-thymidine (NEN, USA; spec. act. 18.7 Ci/mmol) was added (4 μCi/ml medium) at different times after the start of the culture to detect the site of DNA synthesis in the developing skin in culture and the turnover of the epidermis *in vitro*. After a 1-hour exposure the cultures were either harvested or transferred to medium M 199 without ^{3}H-thymidine.

Histology and Autoradiography

Sample explants were harvested after 1, 2, 3, 4, 5, 6, and 8 days of culture and fixed in Bouin-Hollande fluid. After fixation, the specimens were dehydrated and oriented in paraffin blocks in the usual manner and transverse sections of 5 μm thickness were cut. For light microscopy sections were stained with haematoxylin-eosin (HE). For autoradiography the mounted slides were coated with Kodak AR 10 film or dipped in Kodak NTB-2 emulsion, developed after 14 or 21 days, stained with Mayer's haematoxylin, counterstained with eosin and embedded in Rhenohistol (Merck, Western Germany).

Inhibitors of DNA and RNA Synthesis

Actinomycin D (gift from Merck, Sharp and Dohme Research Laboratories, USA) or puromycin dihydrochloride (Sigma, USA) were added to the medium a different concentrations at the beginning of the culture. In some experiments the cultures were transferred after 24 h of exposure to medium M 199 without the inhibitors. The exact data are given in the text.

Results

I. General Morphology *in vitro*

At 17 days of fetal life, the onset of our culture experiments, the (epidermis consists of 3 strata: stratum germinativum, stratum intermedium and periderm according to the terminology of HANSON [1947]. In our opinion there is no difference in morphology between the cells of the stratum germinativum and stratum intermedium (fig. 1). ^{3}H-thymidine pulse label at this moment shows that all layers contain cells capable of DNA synthesis, although very few cells in the periderm are labelled. As soon as the capacity for division is restricted to the basal cell layer and a stratum spinosum is also present, we employ the more common term 'stratum basale' in the text instead of 'stratum germinativum'.

One-Day-Old Culture

Stratum intermedium and stratum germinativum together consist of 4–5 cell layers. There are still many mitoses present, and DNA synthesis takes place outward from the basal cell layer, so that at this moment one cannot yet speak of a stratum spinosum (fig. 2).
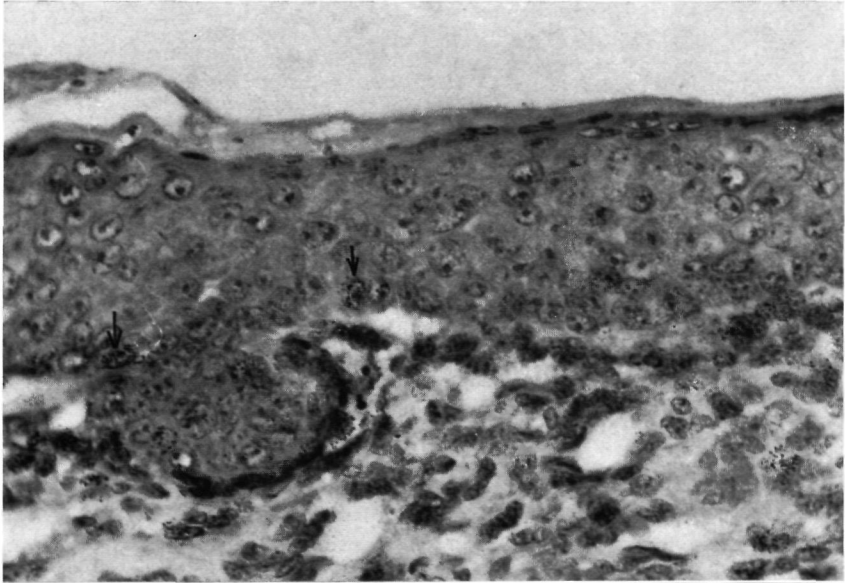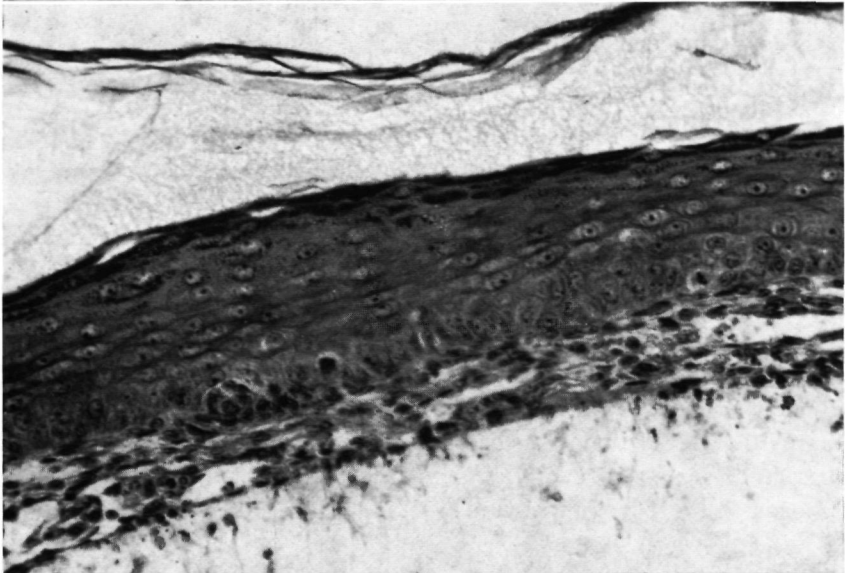
Fig. 3. 17-day-old fetal rat skin after 2 days in culture. Autoradiography. Arrows show cells labelled with ^{3}H-thymidine. Label is restricted to stratum basale (arrow) and dermal cells. Note the beginning formation of stratum granulosum. HE. ×410.

Fig. 4. 17-day-old fetal rat skin after 3 days in culture. Adult appearance of the skin. HE. ×256.

In vivo after 18 days of fetal life, the formation of the stratum spinosum outward from the stratum intermedium is beginning, probably caused by the differentiation of this stratum intermedium, but *in vivo*, too, there are still a few mitoses above the basal cell layer.

Two-Day-Old Culture

The epidermis is now 7–9 cell layers thick (fig. 3). A clear stratum basale is present. From this moment DNA synthesis is almost restricted to this stratum (1 cell layer in thickness). This layer is more basophilic than the now existing stratum spinosum. A stratum granulosum is sometimes present and even a thin layer of stratum corneum. *In vivo* at 19 days of embryonic life no stratum corneum is present [HANSON, 1947]. The granulation in the stratum granulosum is not as coarse as that seen *in vivo* at 19 days. Because the total number of cells has almost doubled during the last 24 h this means that the duration of the germinative cell cycle is approximately 30 h. This is very rapid when we compare it to the value of 163 h in normal human skin, but in agreement with the value of 37 h in psoriatic skin [WEINSTEIN, 1968].

Three-Day-Old Culture

The skin has an adult appearance. A stratum corneum can be seen and the total epidermis minus stratum corneum is 7–10 cell layers thick (fig. 4). Individual differences in thickness are sometimes seen in skin specimens of the same age. On the stratum corneum one can sometimes see the remainders of the periderm which are clearly not keratinized. The condition of the dermis is quite variable; however, this is without effect on the morphology of the epidermis. *In vivo* the stratum corneum is not yet present.

Four-Day-Old Culture and Longer

Mitoses are still frequently seen in the basal cell layer (fig. 5). A prominent stratum granulosum is present. Number of cell layers of stratum basale: 1; of stratum spinosum: 3–5; of stratum granulosum: approx. 3 layers. In the last two days in culture the thickness of the viable epidermis has decreased. A further decrease is seen after 8 days of culture. At this time a thick stratum corneum has formed (fig. 6).
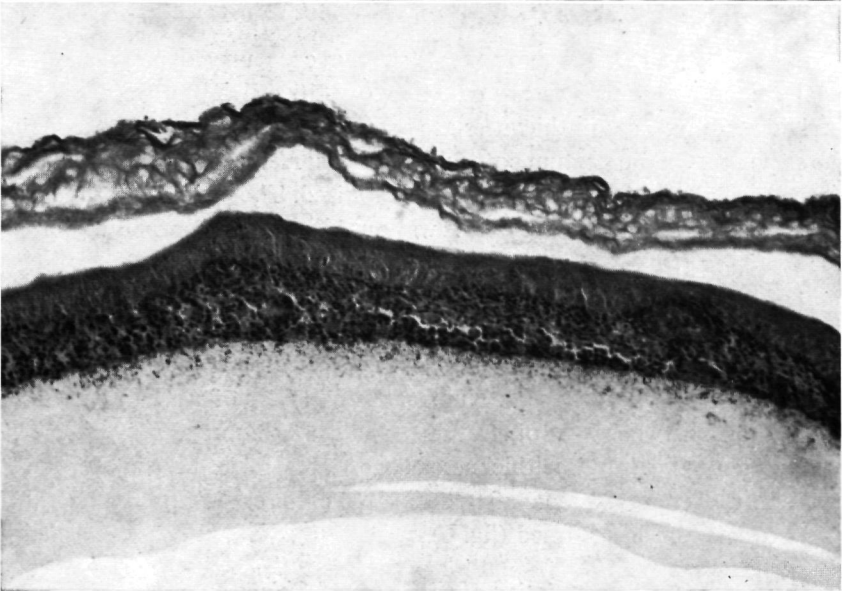
Differences in Differentiation of the Cultured Skin

Three main differences are seen when comparing the skin *in vivo* and *in vitro*. (1) There is little development of skin appendages *in vitro*. (2) The dermis is not as well preserved as the epidermis but there is a big variation
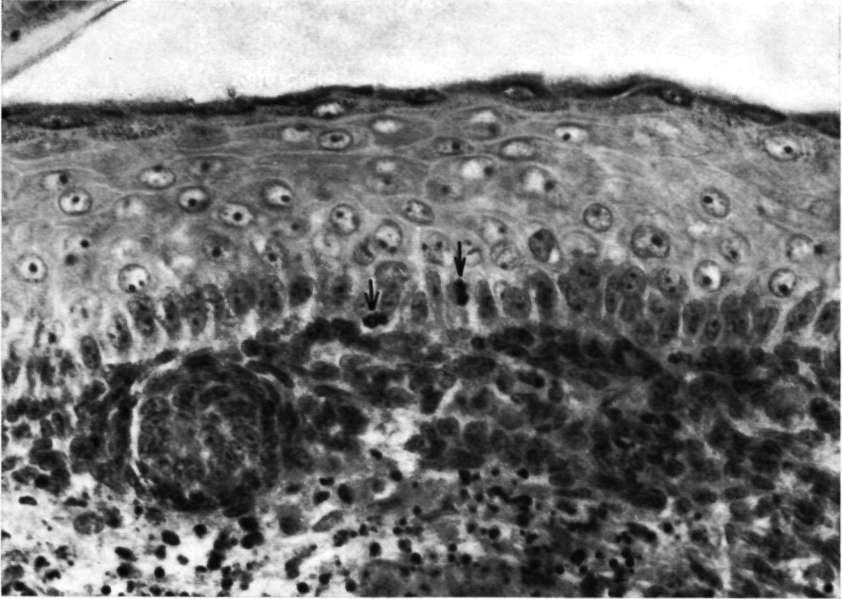
Fig. 5. 17-day-old fetal rat skin after 4 days in culture. Note pycnosis of the dermis. Arrows show mitosis in the basal cell layer. HE. ×410.

Fig. 6. 17-day-old fetal rat skin after 8 days in culture. A thick stratum corneum has formed. HE. ×128.

between different specimens. (3) The differentiation proceeds *in vitro* slightly differently and faster than *in vivo*, although the end product (adult skin) is indistinguishable.

II. RNA and Protein Synthesis

Labelling for 1.5 h with ³H-uridine (RNA precursor) and ³H-amino acids also confirm the viability of the skin in culture.

When we add ³H-uridine (5 μCi/ml medium) for 1.5 h 2 days after beginning the culture, a heavy label is seen in the nucleolus, but some label also appears over the chromatin and the cytoplasm of the epidermis (fig. 7). This may reflect a transport through the nucleus but it may also be partly an incorporation of uridine breakdown products into protein [SAKAI and KIHARA, 1968]. The dermal cells are heavily labelled as well. Skin for 5 days in culture also incorporates ³H-uridine, (2 μCi/ml medium during 6 h) but almost no label is seen in the stratum granulosum (fig. 8). Much more label is now seen in the cytoplasm as might be expected.
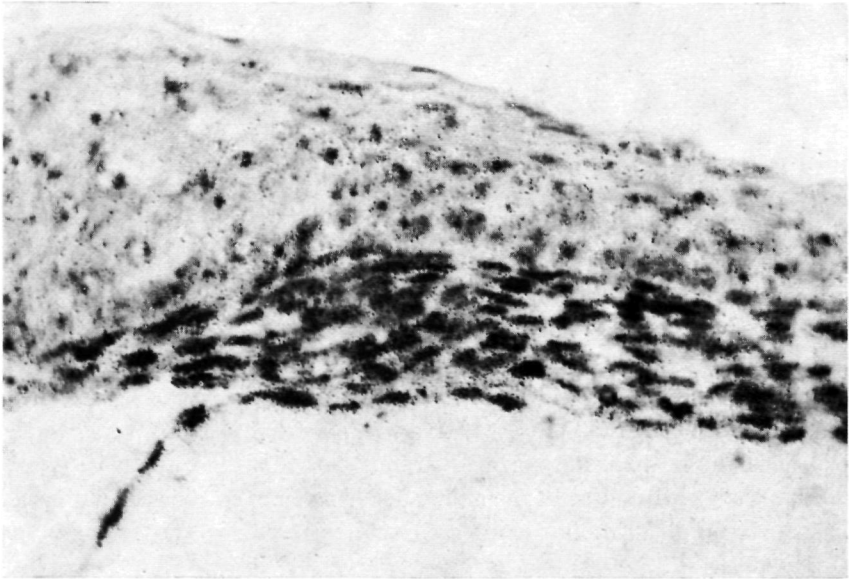
Fig. 7. 17-day-old fetal rat skin, labelled with ³H-uridine for 1.5 h after 2 days in culture. Heavy label in dermal cells. In the epidermis the nucleolus is especially labelled. Autoradiography. HE. ×410.

Fig. 8. 17-day-old fetal rat skin labelled with ³H-uridine for 6 h. Most of the label is now in the cytoplasm of the epidermal cells. Autoradiography. HE. ×716.

In one particular experiment ³H-amino acids were added to the skin after 2 days in culture. After 27 h a very heavy labelling was seen over the whole epidermis and in the freshly-formed stratum corneum (fig. 9). Nuclei (were also labelled, because one of the amino acids (glycine) can be used in purine biosynthesis and all amino acids are probably used for the synthesis of nuclear proteins.

Figure 10 shows the labelling of the nucleus with ³H-thymidine. In this case the skin was labelled with ³H-thymidine for 1.5 h after 1 day in culture and, thereafter, the explant was placed on fresh medium without ³H-thymi-
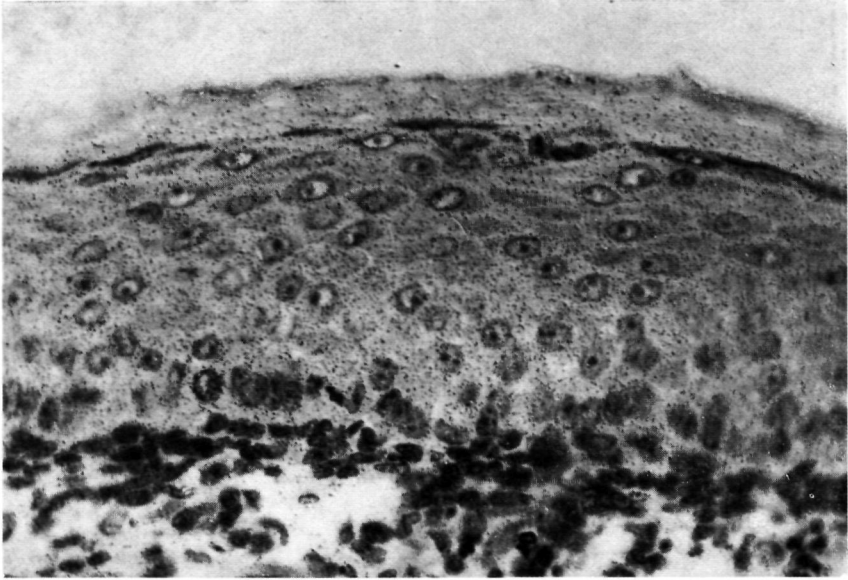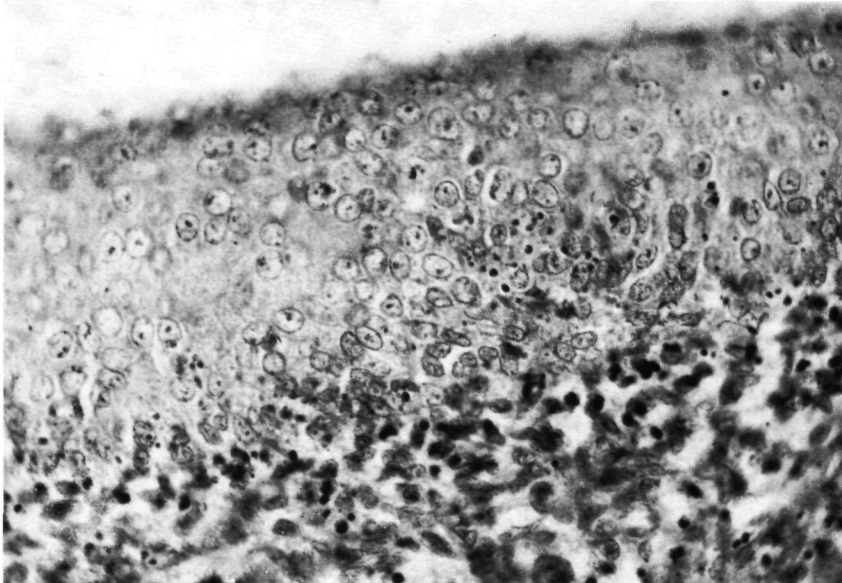
Fig. 9. 17-day-old fetal rat skin after 3 days in culture labelled for 27 h with an ³H-amino acid mixture, 2 days after the start of the culture. Note also label in the freshly-formed stratum corneum. Autoradiography. HE. × 716.

dine; 48 h later the skin was fixed and processed. Unevenly throughout the whole viable epidermis labelled nuclei were seen, as might be expected since after 1 day in culture cells in all layers of the epidermis show DNA synthesis and the capacity for division. Almost no label, however, was seen in the newly-formed stratum corneum. On the other hand, in many nuclei of the cells from the stratum basale a slight scattered labelling was seen. This could be caused by dilution of the label by subsequent cell division or, partially, by recycling of the DNA breakdown products in the epidermis. This was even more evident in skin pulse-labelled with ³H-thymidine at the beginning of the culture. After 4 days, only a few cells of the stratum granulosum had a heavy label while many nuclei of the cells of the stratum basale had a scattered label. Almost no label was present in the stratum spinosum.

According to BADEN [1967] thymidine is not broken down in the epidermis and only a minor quantity of the radioactivity is shed with the stratum corneum. An explanation of the fact that this label is not detected in the stratum corneum by autoradiographic techniques after some fixation methods is offered by SUSI [1968].
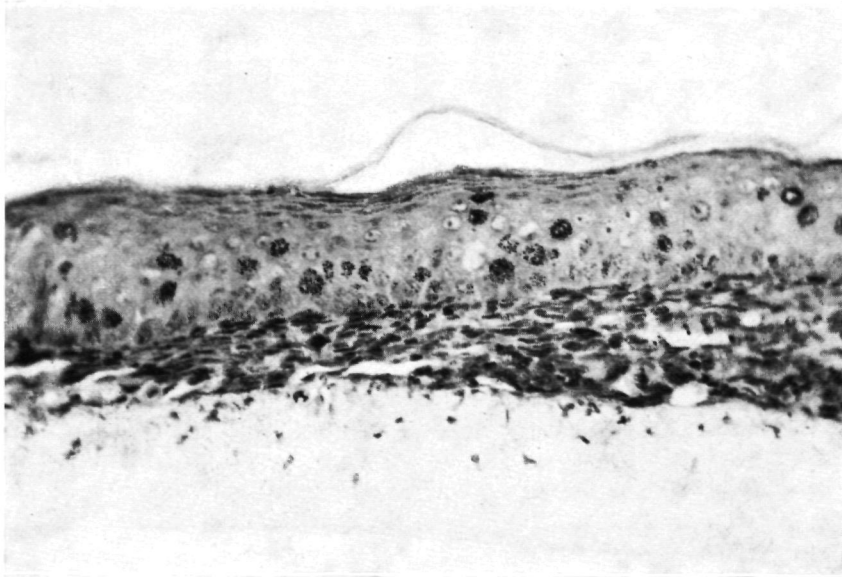
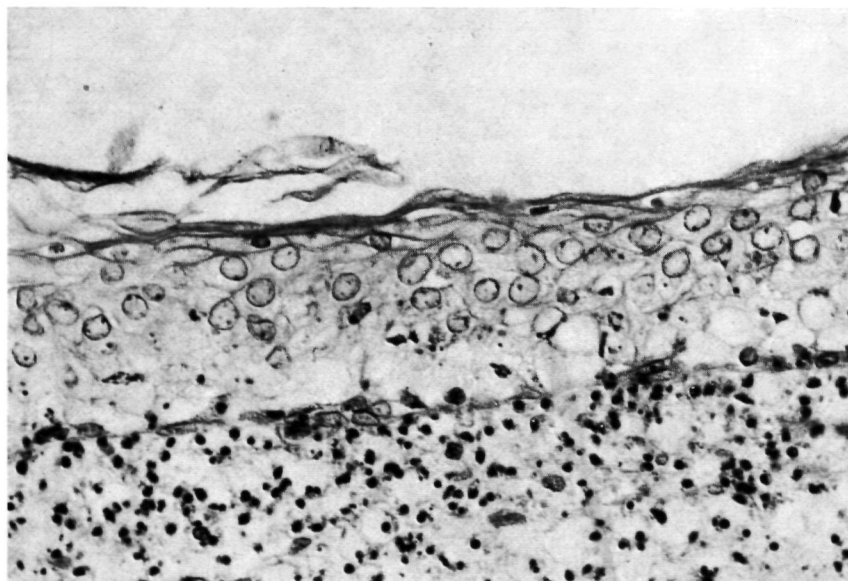Fig. 10. 17-day-old fetal rat skin after 3 days in culture. Labelling with ³H-thymidine for 1 h after 1 day in culture. Note scattered label of the cells of the stratum basale. Autoradiography. HE. ×256.

Fig. 11. 17-day-old fetal rat skin after 2 days in culture in the presence of 0.2 µg actinomycin D/ml medium. Decreasing basophilia. Note the appearance of dark spots in the epidermal cells. HE. ×410.

III. Inhibitors of Protein Biosynthesis

Actinomycin D and puromycin are known inhibitors of protein bio-synthesis. Actinomycin D inhibits the DNA dependant RNA synthesis whereas puromycin acts at the translational level.

Actinomycin D at a concentration of 0.02 µg/ml medium during 24 h, 48 h, or during the whole culture period (4 days) had no effect on differentiation. No difference was seen in the controls without actinomycin D. An increase in the concentration up to 0.1 or 0.2 µg/ml of actinomycin D had already a profound effect on the skin after 24 h in culture (table I). After 2 days dark spots were seen throughout the whole epidermis, while a slight karyo-lysis was also seen (fig. 11). At this time no RNA synthesis (^{3}H-uridine incorporation) could be detected, whereas a small amount of protein synthesis (amino acid incorporation) was still present. Karyolysis was quite obvious on the 3rd day in culture (fig. 12). The nuclei of the epidermal cells were almost empty, while on the other hand the dermal cells showed pykno-sis. The epidermis showed a strong eosinophilia and the skin specimen was essentially dead. At this time no RNA synthesis or amino acid incorporation

Fig. 12. 17-day-old fetal rat skin after 3 days in culture in the presence of 0.2 µg actinomycin D/ml medium. No viable skin exists. HE. × 410.

Table I. Influence of actinomycin D on the differentiation and keratinization of fetal rat skin

Experiment	Keratinization	Concentration actinomycin D, µg/ml	Remarks
0 1 2 3 4	+ +	—	—
0 1 2 3 4	+ +	0 02	—
0 1 2 3 4	+ +	0 02	—
0 1 2 3 4	+ +	0 02	—
0 1 2 3 4	— —	0 2	necrosis after 1 day
0 1 2 3 4 Days	— +	0 1	decreasing basophilia, epidermis, although thinner, is differentiated

Duration of actinomycin D, application in days

could be detected. The stratum corneum-like material must have been formed during the preceding day in culture.

Puromycin in a concentration of 0.1 µg/ml medium or less has no effect on differentiation and keratinization, whereas 1 µg/ml was fatal to the culture; about the same morphology was seen as with actinomycin D.

Discussion

With some exceptions [TROWELL, 1958; PULLAR, 1964] almost every study on organ culture on adult skin or fetal skin has been performed either with plasma clots (natural media) or synthetic media with serum addition. The disadvantage of this kind of experiments is the unknown composition of the media and the common feature of epiboli, the encapsulation of the dermis by the epidermis by migration or outgrowth of the epidermis from the edges of the explant. In our hands, contrary to the findings in fetal pig skin [CHANG *et al.*, 1967], these difficulties leading to central necrosis have been overcome by the use of purely synthetic medium. An additional advantage is the more

homogeneous distribution of added substances (for instance radioactive amino acids and nucleosides). With this type of experiments we have proved the viability of the epidermis on other grounds than purely histological parameters [TROWELL, 1958; PULLAR, 1964].

Undifferentiated skin of 17-day-old rat fetuses differentiates under organ culture conditions within 3–4 days to apparently normal adult skin. The viability of the epidermis is beyond doubt. Mitoses are still frequently seen after 4 days, as well as DNA, RNA and protein synthesis. The condition of the dermis, however, is quite variable under our culturing conditions.

Although the cultured skin differentiates to an adult skin indistinguishable from adult skin *in vivo*, the stages that lead to this formation are slightly different. Also the time which is necessary for the differentiation to adult skin is shorter *in vitro* than *in vivo*. After 48 h in culture a hypertrophy of the epidermis, originating in the cells of the stratum germinativum and the stratum intermedium is seen, compared to the situation *in vivo*. By definition a stratum spinosum is now present because at this time only the cells of the basal cell layer (stratum basale, 1 cell thick) show DNA synthesis. *In vivo* the formation of the stratum spinosum starts in the 18-day-old fetal rat skin, thus earlier than *in vitro*, which explains the difference in thickness of the epidermis. After 48 h in culture, however, the process leading to a complete differentiation to adult skin, proceeds much faster than *in vivo*. 72 h after the beginning of the culture there exists an apparently adult skin. This situation is reached *in vivo* not earlier than about parturition (approx. 21st–22nd day of pregnancy).

In spite of the high mitotic rate and the short duration of the germinative cell cycle (approx. 30 h) comparable with the duration of the cycle in psoriasis, generally a normal stratum corneum is observed without parakeratosis.

Although in culture one can see the formation of hair premordia, there is no development to a complete hair or other adnexa, a feature also noted in fetal mouse skin [PULLAR, 1964]. This might be due to an insufficiency in the medium or, especially for hair, it might be caused by the degeneration of the dermis, which normally contributes to the formation of hair.

The condition of the dermis is quite variable in our experiments. Sometimes after 4 days of culture it is just as cellular as at the beginning of the culture or even more so, but on occasion it shows degenerative changes as indicated by the many pycnotic nuclei. However, this might reflect the situation *in vivo* where the 17-day-old fetal dermis is much more cellular than in later fetal life. A decrease in the thickness of the dermis is always seen,

probably caused by the weight of the epidermis or the structural weakness of the dermis. The condition of the dermis, however, has no effect on differentiation to an adult epidermis.

In the labelling experiments with ^{3}H-thymidine added for 1 h at the start of the culture it was seen that after 4 days the label had disappeared almost completely with the exception of the stratum basale; a scattered label over nearly all the cells of this layer was seen. This means that either a recycling of the DNA breakdown products occurs within the epidermis, or a sort of 'systemic circulation' is operative [BADEN, 1967], i.e. the thymine or thymidine diffuses into the medium and in turn is incorporated into the cutis and stratum basale. With suitable techniques this sort of problems can easily be attacked with *in vitro* experiments.

Actinomycin D which inhibits DNA dependant RNA synthesis was added in order to see whether the differentiation (i.e. stratum corneum formation) was inhibited. SORIANO [1967] found such inhibition in embryonic mouse oesophagus epithelium *in vitro* without necrosis of the epithelium or mesenchyme. Working at the same concentration, we were not able to demonstrate these phenomena in rat skin. This could mean that:

(a) The messenger RNA for keratin synthesis or the keratin subunit [BAUER, in press] is already present in 17-day-old rat skin; this messenger RNA is quite stable and the addition of actinomycin D has no effect, since the formation of new messenger RNA is not essential for keratin synthesis.

(b) The messenger RNA for keratin synthesis is synthesized at a later stage in fetal life and the actinomycin D is inactivated before this synthesis starts. This is unlikely for in some experiments we refreshed the medium after 48 h and again added actinomycin D. Normal differentiation and stratum corneum formation were seen. Immunofluorescence skin studies on fetal rat skin with antibody against isolated human keratin fractions also show that keratin synthesis starts in the epidermis at 16 and 17 days of fetal life. So at this time the messenger RNA for keratin synthesis must be present [BAUER, in prep.].

(c) The concentrations we used are too low for skin. To test this possibility we increased the concentration. But with 0.1 and 0.2 µg actinomycin D/ml medium, great alterations were seen in the skin. The keratinization process was not selectively inhibited but apparently the synthesis of all kinds of RNA for all important processes are inhibited as well, so that the epidermis did not survive.

Puromycin, which was expected to inhibit protein synthesis, showed at a concentration of 0.1 µg/ml no inhibition of differentiation at all, whereas

concentrations 10 times higher completely destroyed the embryonic skin in culture. At these concentration, the synthesis of enzymes for other vital processes must be inhibited.

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Authors' address: F. W. BAUER, Department of Dermatology, Catholic University, *Nijmegen* (The Netherlands)

Differentiation and Keratinization of Fetal Rat Skin

II. Ultrastructural Study of the Epidermis *in vivo* and *in vitro*

F. W. BAUER

Department of Dermatology (Head: Prof. J. W. H. MALI) and
Department of Chemical Cytology (Head: Prof. CH. M. A. KUYPER),
Catholic University, Nijmegen

Abstract. Study of epidermis from fetal rats from 17 days until birth includes the whole keratinization process. In 18-day fetal skin, so-called 'compound' granules appear. These are different from keratohyalin granules. The layer containing these granules undergoes a flattening in 20-day fetal skin but the individual granules remain visible.

Key Words
Keratohyalin granule
Compound granule
'Clumping' of ribosomes
Stratum corneum formation

On the 18th day of fetal life, the synthesis of keratohyalin granules starts. They seem to originate by the clumping of ribosomes. While growing larger (19-day fetal skin) they become patchy. Many of the granules are in contact with fibrillar material, in particular when the cells become filled with this fibrillar material (21-day skin). Whether this contact happens by accident or whether this material becomes incorporated in these granules is not yet resolved.

In 20-day fetal skin a stratum corneum is present.

In 17-day fetal skin cultured *in vitro,* differentiation to morphologically adult skin proceeds faster than *in vivo.* Already after 2 days in culture a stratum corneum is present which shows most of the ultrastructural characteristics present *in vivo.* However, at that time the scantiness of keratohyalin granules and of fibrillar material is striking. During the 3rd and 4th day in culture, the amount of fibrillar material increases, as do the number of keratohyalin granules.

Our findings on keratohyalin granules are discussed in relation to the current ideas about the role of these keratohyalin granules.

Introduction

Since in the rat the steps leading to keratinization take place in a rather short time (days 17–20 inclusive, fetal life) [HANSON, 1947; STERN *et al.,* 1971;

BAUER and KUYPER, in press], this species is particularly suitable for the study of this process.

To our knowledge, with one exception [BONNEVILLE, 1968] no ultra-structural studies dealing with the differentiation of fetal rat skin *in vivo* have been reported. No ultrastructural study at all is available about the differentiation of fetal rat skin under organ culture conditions.

As organ culture is regarded as a model for the *in vivo* situation, it would be worth while to compare the differentiation both *in vivo* and *in vitro*. Such a study is presented here.

Materials and Methods

Fetal Rat Skin

The morphological observations on skin development *in vivo* reported here were made on flank skin of 17- to 21-day-old fetuses.

For the *in vitro* study, flank skin of 17-day fetal rat skin was cultured for a further 1, 2, 3 or 4 days.

Age Determination

The data on oestrus and conception were determined by vaginal smears. The first day *post coitum* is counted as day 1 of fetal life.

Culturing Conditions

The culturing conditions have been described in detail elsewhere [BAUER and KUYPER, in press].

Fixation Procedure

Small pieces of skin were fixed in 2% glutaraldehyde in cacodylate buffer for 2 h. After thorough rinsing in cacodylate buffer, the tissue was postfixed in 2% OsO_4 [PALADE, 1952]. The pieces were then dehydrated in ethanol and embedded in Epon 812. Sections were made on a Porter-Blum ultramicrotome and stained with a saturated aqueous solution of uranyl acetate, followed by lead citrate [VENABLE and COGGESHALL, 1965; or REYNOLDS, 1963]. The specimen were studied in a Philips EM 200 or a Zeiss EM 9 electron microscope.

Observations

For the sake of clarity we will first present our data on the development of fetal rat skin *in vivo*. Then our observations on cultured skin will be presented, but only insofar as they are different from the *in vivo* differentiation.

The youngest fetuses examined were 17 days old. This was chosen since in fetuses of this age we saw no sign of keratinization with light microscopy

[BAUER and KUYPER, in press]. This is in agreement with the findings of HANSON [1947] but not with the observations of BONNEVILLE [1968] who found that keratinization starts at about the 11th day of fetal life. The reason for this difference is discussed later.

On the 17th day of fetal life, the epidermis is covered by a one-cell thick flattened periderm with villus-like protrusions on its free surface. The other cells of the epidermis give a rather uniform appearance. Large glycogen deposits are distributed throughout the epidermis (fig. 1). Ribosomes and
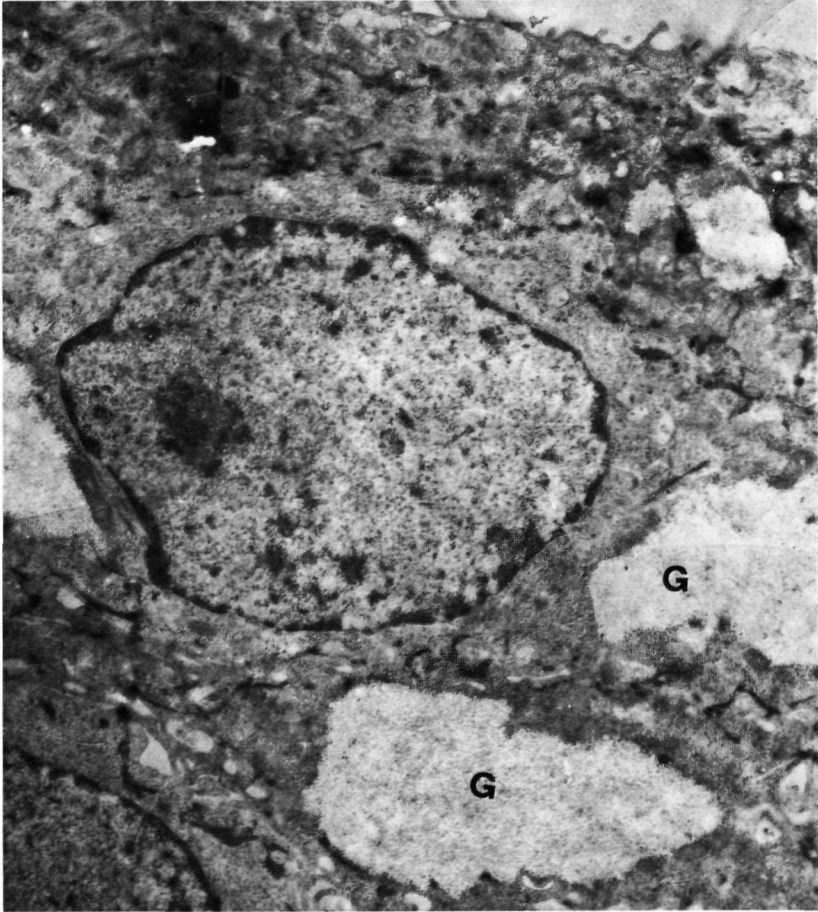
Fig. 1. 17-day fetal rat skin. Large glycogen depots (G) are distributed throughout the epidermis. The periderm covers the epidermis. × 10,080.

polysomes are abundant, only small traces of rough endoplasmatic reticulum are present as might be expected in a retaining cell type. Tonofilaments are present albeit not in great amounts. They have not yet aggregated to tonofibrils.

The plasma membrane of the epidermal cells possesses many microvilli. The intercellular space is sometimes wide and appears to be empty. Many desmosomes join the cells.
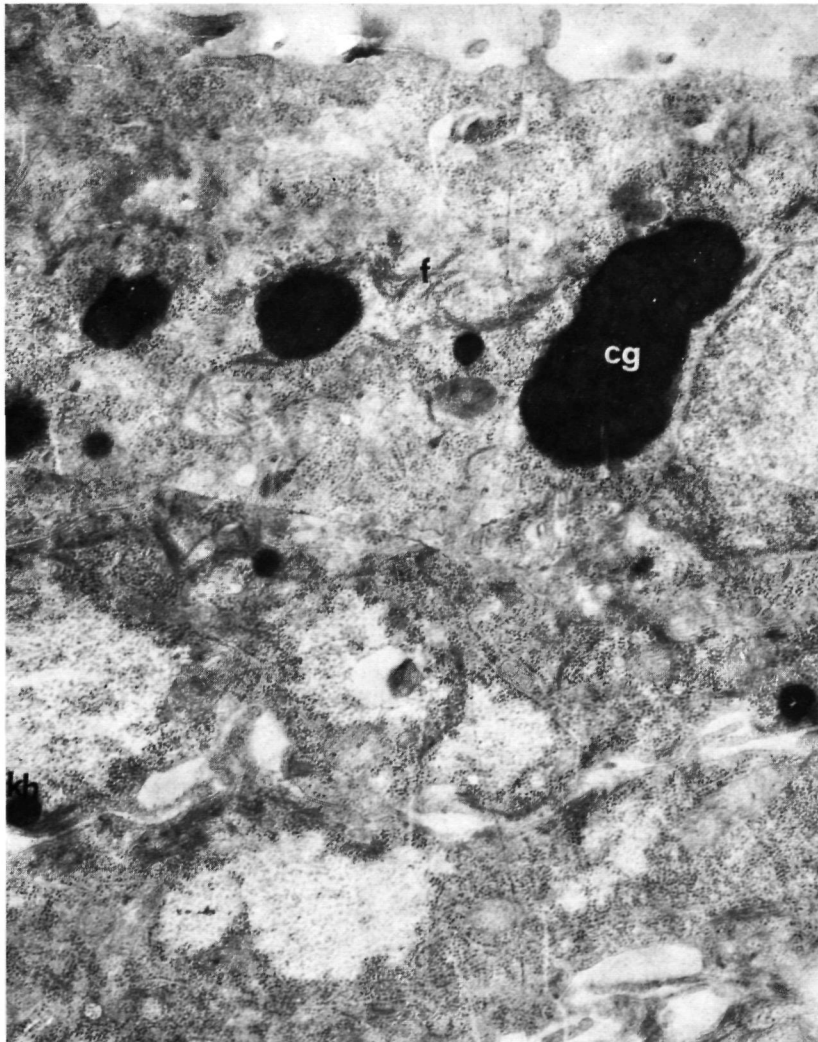
18-Day Fetal Rat Skin

A remarkable phenomenon at this stage is the abrupt occurrence of typical granules (fig. 2 and 3). They are confined to the cell layer immediately beneath the periderm. The layer containing these granules is generally one-cell thick and most likely arises as the result of the differentiation of cells from the stratum intermedium. These granules have been described by BONNEVILLE [1968] and would, in her opinion, already be present in 13- to 14-day fetal rat skin. The granules in question, which are composed of at least two different components (fig. 2 and 3), have a round or ovoid shape; sometimes, however, they are irregular. In this study we will call them 'compound granules'. Their longest diameter varies from 0.2 to approximately 1.6 µm. As a rule they consist of homogeneous, rather electron lucent, lipid-like globules embedded in a strongly electron dense matrix. We do not consider them to be keratohyalin granules since their formation and occurrence are limited to a rather short time interval. Generally, they are not in close association with tonofilaments although some are, perhaps accidentally. No precursor stage of this granule was found in 17-day skin.

Tonofilaments are scarce and located on the periphery of the cell. As in the 17-day skin, glycogen deposits are present throughout the entire epidermis with the exception of the most superficial layers.

In the deeper layers another granule is formed (fig. 2). The diameter of this granule varies between several hundred Ångström units to about 0.3 µm. These seem to originate through the clumping of ribosomes (fig. 4, 10, 12 and 13). As soon as these granules increase in size the ribosomes seem to fuse and an homogeneous electron dense mass remains. Only at the border are the ribosomes discernible. In our opinion these granules are 'true' keratohyalin granules. Granules of this type are sometimes associated with tonofilaments.

All cell membranes, especially those of the lower part of the epidermis, have many microvilli. In these layers tonofilaments or tonofibrils are extremely scarce.

In the nuclei of the granular layer electron dense bodies of varying size are regularly seen. These granules resemble the keratohyalin granules.

Fig. 2. 18-day fetal rat skin. Survey of the uppermost layers of the epidermis. Compound granules (cg), consisting of lipid-like globules embedded in electron dense homogeneous material. Tonofilaments (f) are scanty. Small keratohyalin granules (kh) are present deeper in the granular layer. $\times$ 24,000.

19-Day Fetal Rat Skin

The compound granules are still present and have somewhat larger dimensions than on the day before. The largest granules have a long axis of approximately 2.5 μm. The biggest globules are approximately 0.6 μm in
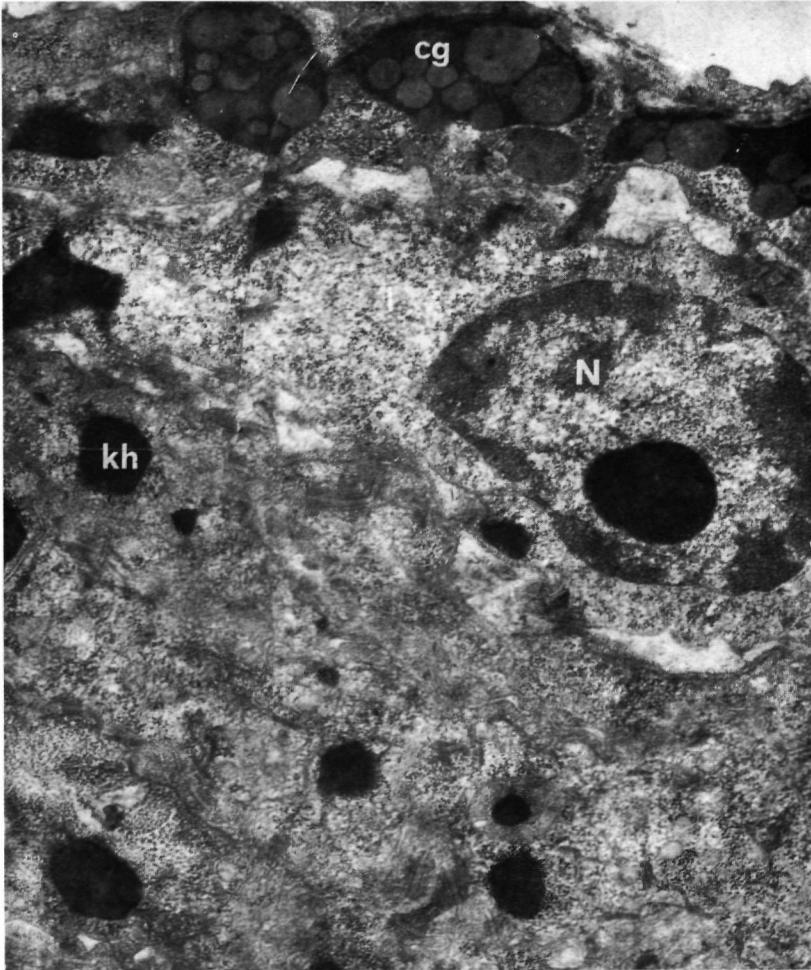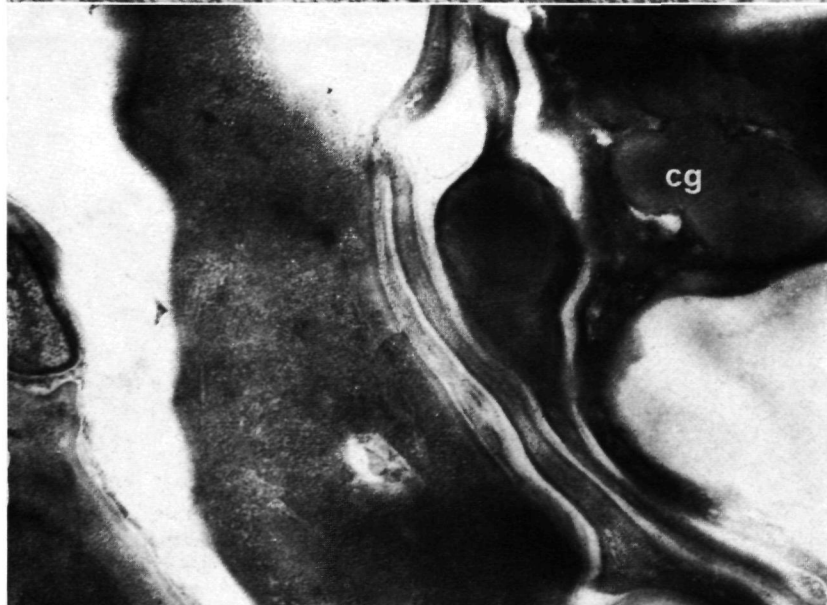

Fig. 3. 19-day fetal rat skin. The compound granules (cg) have increased in size. The lipid-like globules are larger and some of them lay free in the cytoplasm. Keratohyalin (kh) granules are larger than the day before and become patchy with increasing size. Note the keratohyalin-like granule in the nucleus (N). × 24,000.
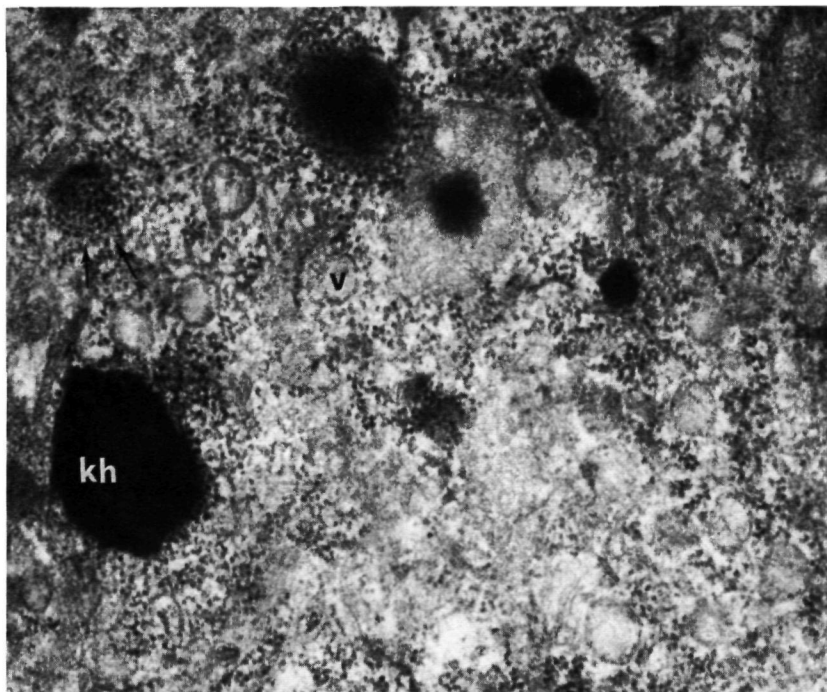
4

5

diameter, and some of them appear not to be incorporated in electron dense material. These compound granules are still confined to a one-cell thick layer.

Beneath this layer the 'true' keratohyalin granules of varying size are seen. Especially when they are small, they appear homogeneous, but when they get larger they become patchy. They are not uniformly electron dense (fig. 3).

As in 17- and 18-day skin tonofilaments are scarce, especially in the deeper layers of the epidermis. In the granular cells they are not abundant either.

Sometimes large dense structures resembling the patchy keratohyalin granules are present in the nuclei (fig. 3).

20- to 21-Day-Old Fetal Rat Skin

In 20-day fetal skin the cell layer containing the compound granules has been shed from the viable epidermis but can still be identified just beneath the periderm which is not keratinized (fig. 6). These findings are in accordance with those of BONNEVILLE [1968]. Beneath these two layers a stratum corneum is present. In this layer an enormous diversity in cell height and in electron opacity is striking. Especially in 21-day fetal skin the keratin pattern substructure can be recognized in some cells of the stratum corneum (fig. 6–8). In the viable epidermis a dramatic increase in the amount of fibrous material has occurred. Tonofilaments as well as tonofibrils are now present. In 21-day fetal skin in both the cytoplasm and in the nucleus of the granular layer a round globule that is less electron dense than the keratohyalin granules can be seen (fig. 8 and 11). These globules resemble in density the globules of the compound granules. However, they are never observed in the stratum corneum.

The different granules found in the epidermis are illustrated in figures 9–13, with the exception of compound granules. Figure 9 represents a transversal section through the granular layer of the epidermis. In the inset of figure 10, and in figures 12 and 13, it can be seen how the keratohyalin granules most likely originate through the clumping of ribosomes. While

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Fig. 4. 19-day fetal rat skin. Note the dense body inside the mitochondrion. Note the aggregation of ribosomes, which may give rise to the keratohyalin granules (kh). × 46,500.

Fig. 5. 20-day fetal skin. Survey of the stratum corneum. The cell layers containing compound granules (cg) are shed. × 40,500.
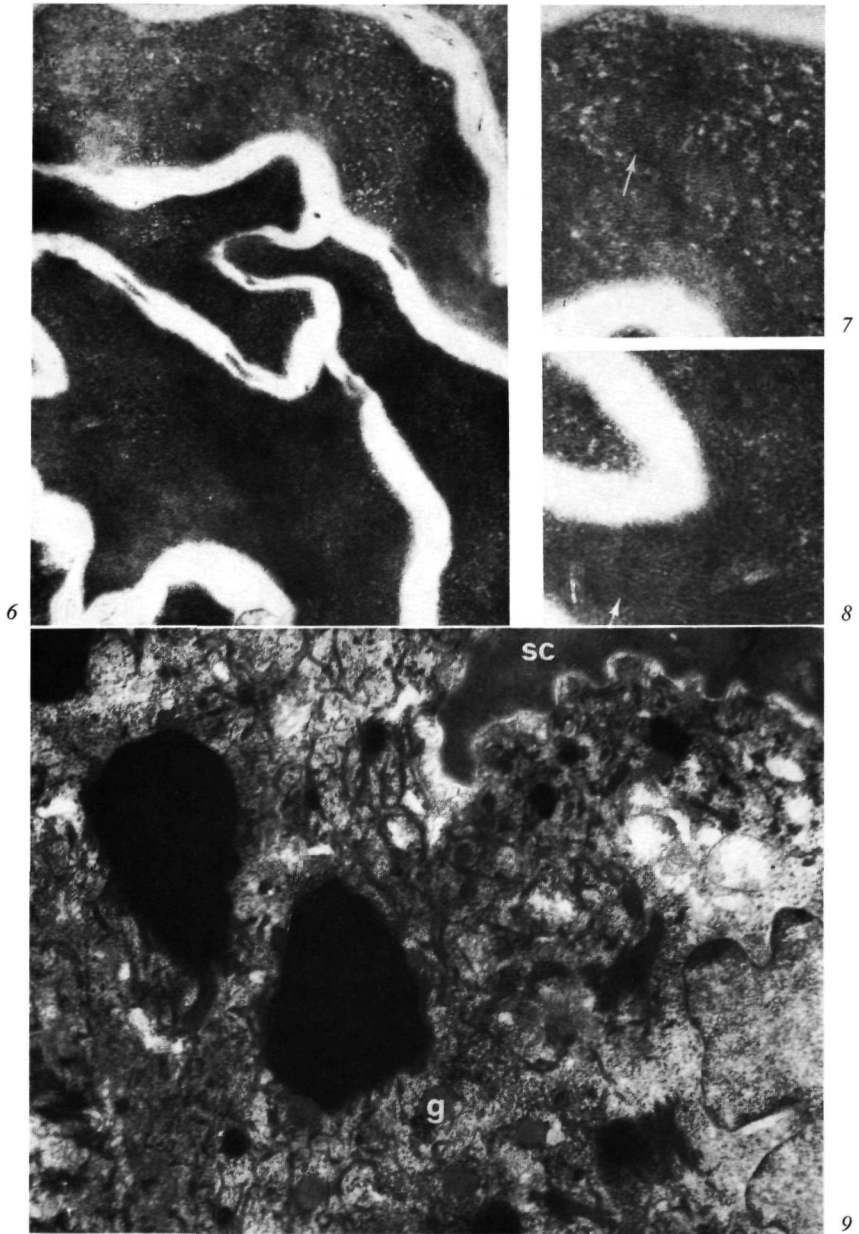
Fig. 6. 21-day fetal rat skin. Survey of the stratum corneum. ×24,000.

getting larger they become homogeneous, are sometimes in contact with fibrillar material and ultimately they become somewhat patchy. At that time occasionally globules are seen which might have been released from the granules.

17-Day Fetal Skin in Culture for 1 Day

Skin cultured for 1 day shows almost the same ultrastructural characteristics as 18-day fetal skin *in vivo*. The slight differences described in an earlier paper might reflect variations in fetal age [BAUER, in press].

17-Day Fetal Skin Cultured for 2 Days

The epidermis has increased in thickness as has been described in a previous paper [BAUER, in press]. The cytoplasm of the stratum spinosum cells contains numerous vesicles and the endoplasmic reticulum is vacuolated (fig. 14). A prominent Golgi apparatus is present. Mitochondria are sometimes swollen and some of them appear empty. The nuclear envelope is widened. In the granular layer tonofilaments are scarce, as are keratohyalin granules. Keratinosomes are present in large amounts (fig. 15) Some mitochondria show a dense structure inside. A stratum corneum is present, so at least 24 h before this situation is reached *in vivo*. In this stratum not all of the subcellular components have disappeared. Remnants of mitochondria, lipid-like droplets, lysosome-like structures and of nuclei are regularly visible. As *in vivo* there is a great divergency in electron opacity between individual stratum corneum cells. The cell membranes show the characteristic thickening. Locally, in the intercellular spaces, electron dense material is present (fig. 16).

Fig 7 21-day fetal rat skin An enlargement of figure 6 The typical keratin structure of 'fibrils embedded in a dense matrix' is visible (→) × 48,000

Fig 8 21-day fetal rat skin Enlargement of figure 6 × 48,000 Some of the fibrils are cut lengthwise

Fig 9 21-day fetal rat skin. Portion of the uppermost granular layer. The stratum corneum (sc) is just visible Note the increase in fibrillar material. The keratohyalin granules are in contact with tonofibrils. Small globules (g) surrounded by ribosomes appear in the cytoplasm. × 24,000.
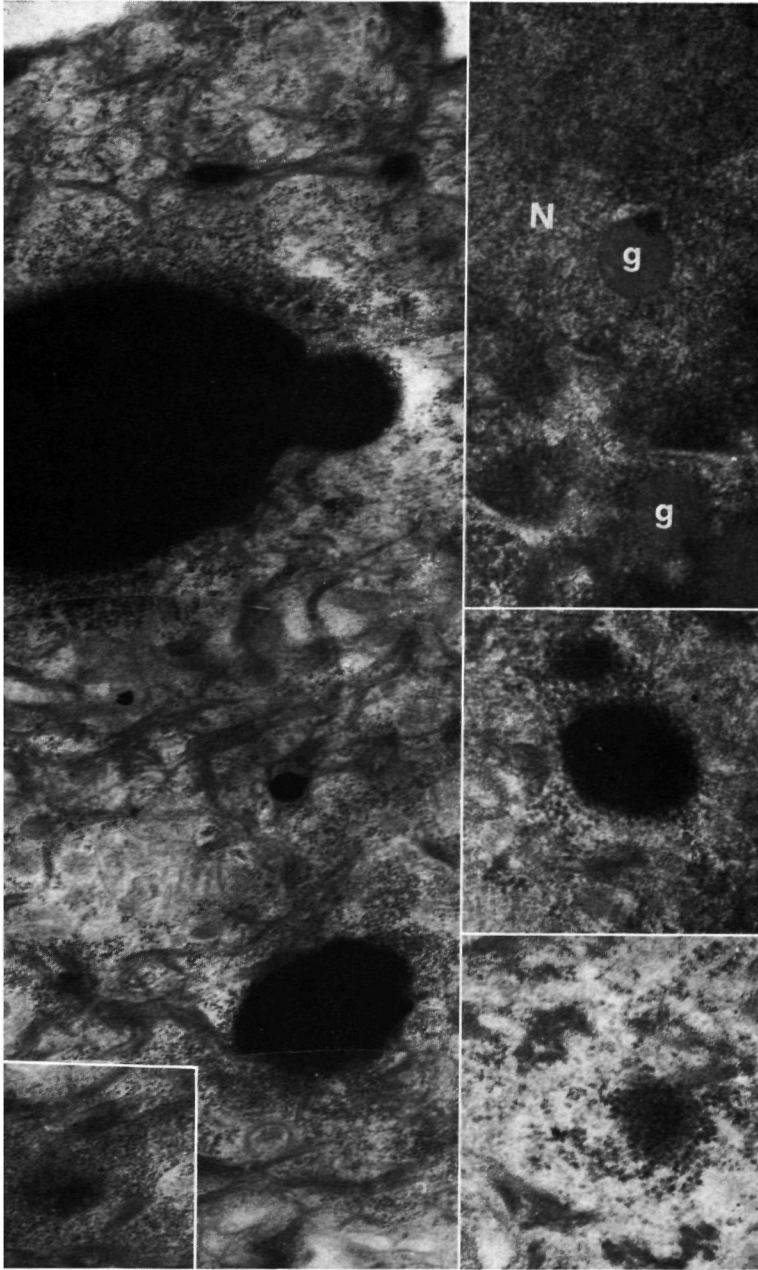
17-Day-Old Skin Cultured for 3 and 4 Days

The basal cell layer contains some fibrillar material. More fibrillar material is present especially in the uppermost layers of the stratum spinosum and the stratum granulosum.

In the 17-day fetal skin cultured for 3 days, this material is present as tonofilaments. In skin cultured for 4 days these have partly aggregated into tonofibrils.

Keratohyalin granules are present, but in general they are smaller than *in vivo* and less numerous. As *in vivo* the larger granules have a patchy appearance. The most superficial layers of the stratum granulosum are less electron dense. In this layers the number of single ribosomes decreases while many polysomes are seen, some of them in an apparently helical configuration (fig. 17). Desmosomes are seen regularly and they are in contact with fine tonofilaments (fig. 18).

As is the case in skin cultured for 2 days great variations in structure and overall electron density exist in the stratum corneum. Some of the cells contain fibrillar material while in others this material seems to be absent (fig. 19).

Figure 20 shows the border between dermis and epidermis in skin cultured for 4 days. Collagen is present in the cutis. The basal cell layer and the basement membrane are intact and mitochondria appear healthy.

Discussion

The differentiation of a relatively-undifferentiated skin of 17-day fetal rat skin, consisting of a basal cell layer, 2 or 3 layers of the stratum intermedium, and a one-cell thick periderm to an adult appearing skin, takes

Fig. 10. 21-day fetal rat skin. Survey of the diverse types of keratohyalin granules. The inset is located just beneath the right hand corner. Inset shows the possible origin of the keratohyalin granules. × 24,000.

Fig. 11. 21-day fetal rat skin. Portion of nucleus (N) and surrounding cytoplasm. Both in the nucleus and in the cytoplasm identical globules (g) are present. In the cytoplasm they are surrounded by ribosomes. × 29,000.

Fig. 12. 21-day fetal rat skin. This picture shows again the clumping of ribosomes, showing that this phenomenon is not accidental. × 44,000.

Fig. 13. 21-day fetal rat skin. See figure 12. × 44,000.
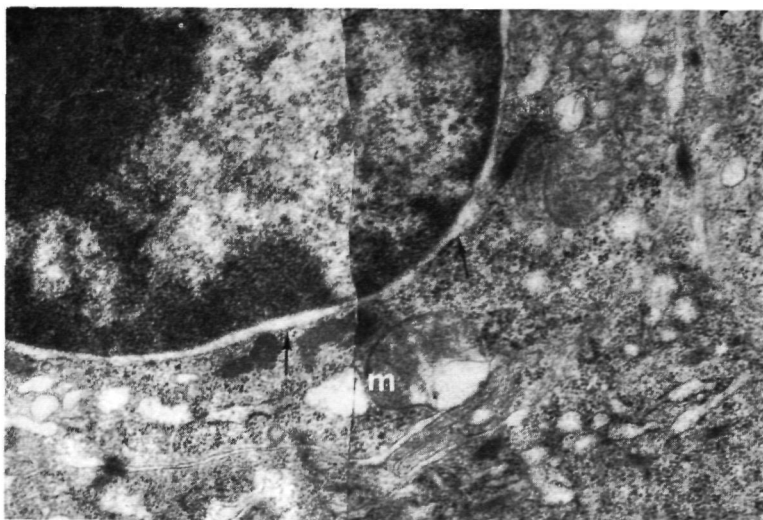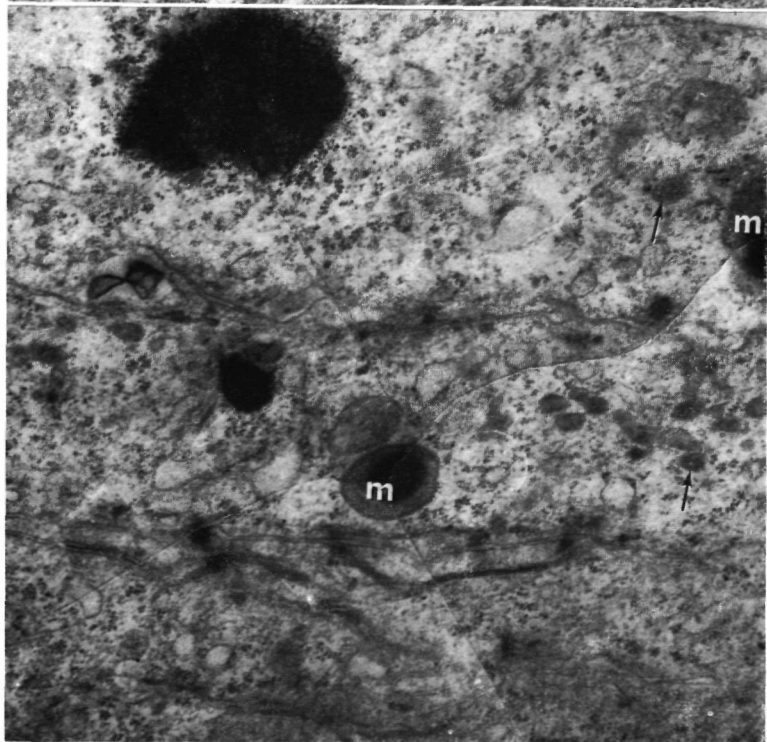
place between 17 and 20–21 days of fetal life. These data fit well with the light microscopic observations made by HANSON [1947] and by STERN et al. [1971]. On the other hand, our results more or less agree with the observations of BONNEVILLE [1968] but only insofar as the sequence of events is concerned. When we compare the findings in terms of time, our results differ greatly. Whether these great differences reflect strain differences in the experimental animals is uncertain. In our experiments the first day *post coitum* is counted as day 1. The exact time of coitus or of fertilization is not precisely known. Hence a small variation in the estimation of fetal age is possible, but it will not exceed 1 day. Furthermore, this study is based upon several series of fetal rat skin, thus excluding an uncorrect interpretation of the sequence of events. For a better understanding of the events leading to keratinization *in vivo*, we have made a simplified illustration (fig. 21). This may serve possibly as a guide to the photographs.

In vivo the stratum granulosum and stratum spinosum are present at 18 days; the stratum corneum at 20–21 days of fetal life.

A remarkable phenomenon in 18- and in 17-day fetal skin cultured for 1 day is the sudden appearance of a very typical granule consisting of more or less homogeneous globules embedded in a dense matrix (fig. 2, 3 and 21). These 'compound' granules are, according to BONNEVILLE [1968], already present in 13- to 14-day fetal skin. The layer containing this granule is shed in 20- to 21-day fetal rat skin and is visible just above the uppermost layer of the then existing stratum corneum (fig. 5 and 21d). The dimensions in 18-day skin are about 0.2–1.5 μm and up to 2.5 μm in 19-day skin, and no precursors of this type of granule are seen in the epidermis of 17-day skin. BONNEVILLE [1968] considers the compound granule as a keratohyalin granule although it may be that the processes leading to its formation are not repeated later in fetal life. In our opinion these granules have nothing to do with the true keratohyalin granule. The significance of the compound granules is not known. Perhaps they are a relic of evolutionary changes. Small keratohyalin granules are first observed in 18-day fetal skin.

Fig. 14. 17-day fetal skin in cultured for 2 days. Part of a cell of the stratum spinosum is shown. Many small ER-elements are present; some of the mitochondria are swollen (m). The nuclear envelope is widened. × 24,000.

Fig. 15. 17-day fetal skin cultured for 2 days. Detail of the uppermost layers of the stratum granulosum. Note the dark granules in the mitochondria (m) and the scantiness of fibrillar material. Keratinosomes are present in fairly large numbers (→). × 30,000.
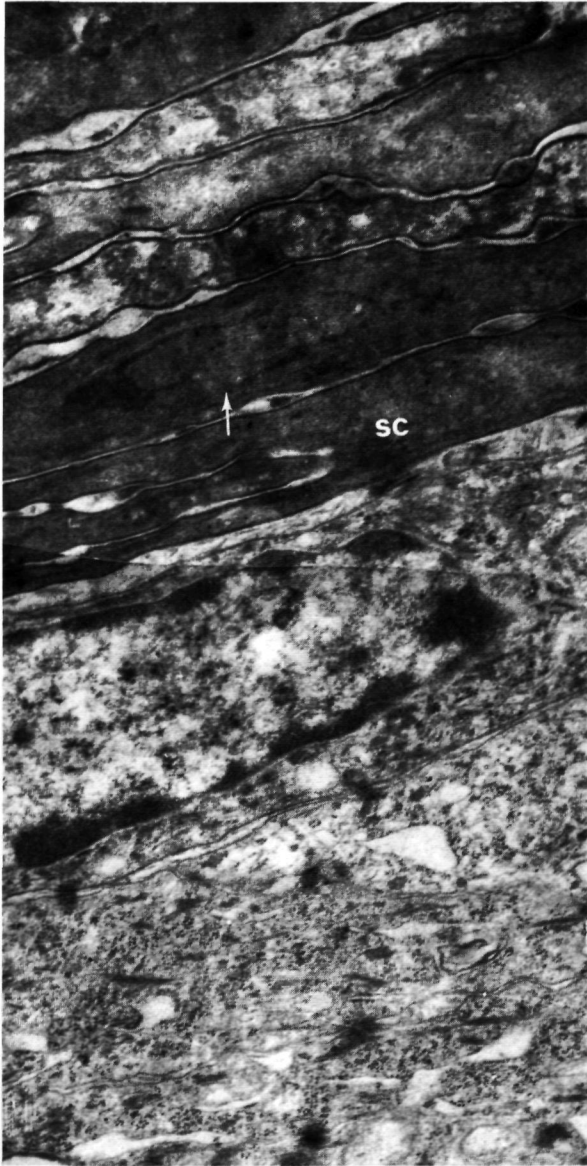
Fig. 16. 17-day fetal rat skin cultured for 2 days. A prominent stratum corneum (sc) is present. Note the remnants of a nucleus in one of the stratum corneum cells (→). In the granular layer cells the scantiness of fibrillar material and of keratohyalin granules is striking. × 24,000.

In 19-day fetal skin these small granules are located in the deeper parts of the granular layer. In the higher levels larger granules of varying size are visible. A part of them is in contact with fibrillar material, especially later in fetal life and in general the larger the granules, the more patchy they are (fig. 3). The small granules are considered to be the precursor of all these granules and are the classic keratohyalin granules. Morphologically identical granules are sometimes present in the nuclei. In 20-day fetal skin a stratum corneum is present and a gradual increase in fibrillar material is observed.

In 21-day fetal skin round bodies are detectable, both in the cytoplasm and nucleus (fig. 11, 21e). They might be released from the large keratohyalin granules (fig. 21b, 9).

In vitro the processes leading to keratinization proceed faster. Already after 2 days a stratum corneum is present *in vitro* at least 24 h before this situation is reached *in vivo*. Occasionally cell remnants are visible in this stratum. However, this stratum corneum shows most of the ultrastructural characteristics of normal adult rat skin. A number of the stratum corneum cells are rather electron dense and the problem arises as to where this dense material comes from. Tonofilaments and tonofibrils are extremely scarce and not nearly enough to account for the electron density after their passage into the stratum corneum. The keratohyalin granules are scarce as well. One explanation might be that the precursors of the tonofilaments and tonofibrils (the keratin subunits [BAUER, in press]) are already present but have not yet aggregated.

Also in skin cultured for 3 and 4 days, keratohyalin granules are as a rule not abundant and generally they are not very large. This might be explained by the conditions of the culture; slight variations in the pH of the culture medium have an effect on the keratohyalin content [REAVEN *et al.*, 1965].

In skin cultured for 4 days the tonofilaments aggregate to tonofibrils. In general, we may conclude that *in vitro* the skin shows no severe degenerative changes. All of the known epidermal cytoplasmatic structures are present and rather well preserved. The events leading to maturation of the epidermis are the same in outline although the time course is somewhat different.

In the light of our findings on keratohyalin granules we shall briefly discuss the possible rule of these granules. There are two main points of view current. The first one is that keratohyalin is ultimately an essential component of the stratum corneum. The supporters of this view do not agree as to the manner in which this could occur; according to BRODY [1959] the amorphous electron dense keratohyalin spreads over the fibrillar material to give the typical keratin pattern. On the other hand MERCER [1961] states that the
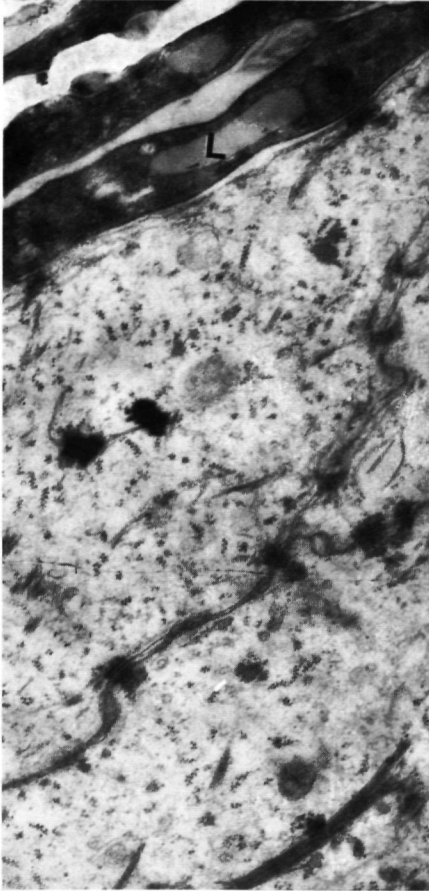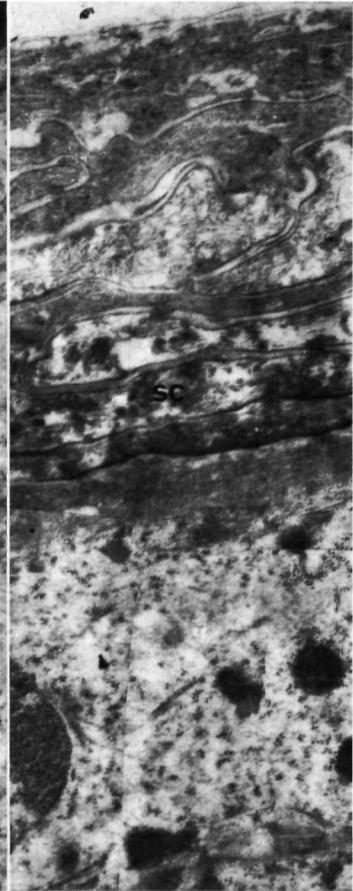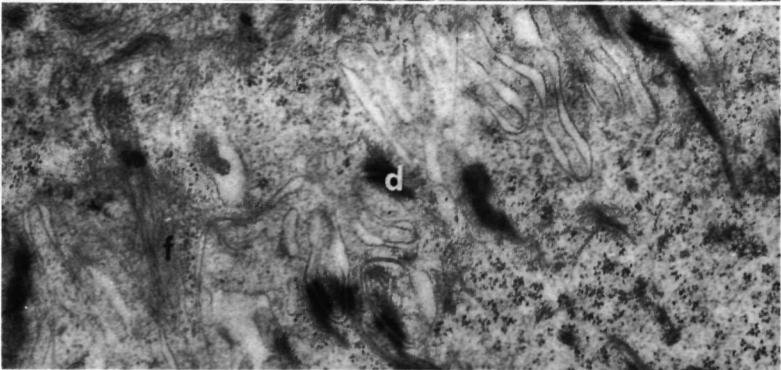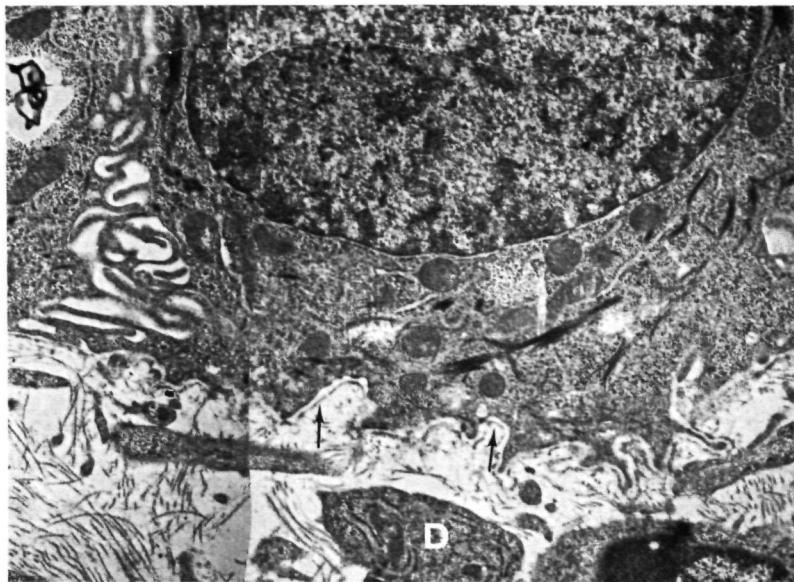
Fig. 20. 17-day fetal rat skin cultured for 4 days. Detail of the border between dermis (D) and epidermis. Note the large number of well-preserved mitochondria. × 11,200.

keratohyalin granules undergo a fibrillar transformation and are a direct precursor of the fibrous keratin.

The second view current, partly based on the findings that keratinization can occur without keratohyalin is that keratohyalin granules are degradation products of superfluous intracellular substances which will be eliminated from the granular layer.

In this study we frequently found keratohyalin granules surrounded by

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Fig. 17. 17-day fetal rat skin cultured for 3 days. Note the polysomes in the stratum granulosum (→). The dense stratum corneum cells have some lipid-like inclusion bodies (L). × 24,000.

Fig. 18. 17-day fetal rat skin cultured for 3 days. Detail of the border of two cells of the stratum spinosum. Tonofilaments (f) are present here and some of them appear to be in contact with the desmosomes (d). × 24,000.

Fig. 19. 17-day fetal rat skin cultured for 3 days. Portion of the stratum corneum and stratum granulosum. Note the divergency in structure and in electron density of the stratum corneum cells. × 24,000.
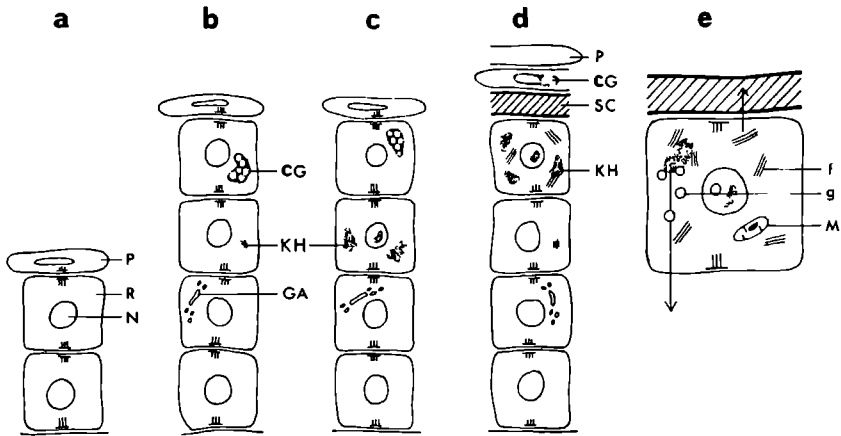
Fig 21. Schematic illustration of the main changes during fetal development. N = nucleus; R = ribosomes; P = periderm; GA = Golgi apparatus; CG = compound granules; KH = keratohyalin granule, SC = stratum corneum, f = fibrillar material; M = mitochondria, g = smooth globule. *a* Undifferentiated 17-day fetal skin *b* 18-day fetal skin. The sudden appearance of the compound granules; beginning of keratohyalin formation by clumping of ribosomes *c* 19-day fetal skin Growing of keratohyalin granules and their transformation into patchy granules perhaps indicating a breakdown of the granules Morphologically identical granules are present in the nucleus *d* 20-day fetal skin. Shedding of the compound granules. These granules are no longer present in the viable epidermis Increasing formation of fibrillar material *e* 21-day fetal skin. Appearance of smooth globules which may contain breakdown products of nucleic acids and protein. These products may come into recirculation (↓) The fibrillar material is used for stratum corneum formation (↑)

ribosomes, a feature also observed earlier by RHODIN and REITH [1962] and ROGERS [1964]. This could indicate that the ribosomes synthesize these granules which would agree with the first view in that this material finally becomes a part of the stratum corneum. However, it is difficult to imagine how apparently unordered ribosomes could have a co-ordinated synthetic function. Moreover we found in this study considerable evidence that the keratohyalin granules originate by a mechanism of clumping of the ribosomes (fig. 4, 10, 12, 13); even larger granules are sometimes seen to be composed of ribosomes; thus the second view cannot be ruled out at this moment. This interpretation is not necessarily in conflict with the ultrastructural findings on keratohyalin granules reported by LAVKER and MATOLTSY [1970], demonstrating a fine granular substructure of the kerato-

hyalin granules with a smaller diameter than the ribosomes. Shrinking of the ribosomes in the center of the granule caused by water loss of stratum granulosum cells or, alternatively, a breakdown of the RNA or protein component of the ribosomes could account for a decrease in size and eventually for the patchy appearance of the granules, the less electron dense areas being those areas where RNA and protein are partially broken down. The apparent contact of fibrillar material with these granules could then be per accident or be caused by preferential adherence to the sticky surface of the granules. This could also explain the occurrence of morphologically identical granules in the nuclei and even in the mitochondria. A similar breakdown of nucleic acid and protein as in the cytoplasm could occur.

The round globule in 21-day skin, less electron dense than the keratohyalin granule, could be released from it and contain breakdown products from proteolytic enzymes (fig. 9). In this concept, the keratohyalin granules are not prospective components of keratin, but they are more or less necessary for the normal progress and outcome of the stratum corneum formation. The protein material in the stratum corneum should then be derived from the fibrillar material.

This concept fits entirely with the histochemical findings on the keratohyalin granules [LEUCHTENBERGER and LUND, 1950]. A survey of this literature [HARMS, 1965] shows that these granules contain RNA and protein (as do the ribosomes). The RNA is not confined to the borders of the granules, which would be expected if one assumed that the granules are surrounded by ribosomes synthesizing an amorphous matrix protein.

Also our *in vitro* data suggest that keratohyalin granules are not essential for the formation of the stratum corneum. Especially in skin which has been 2 days in culture, keratohyalin granules are scarce; a well-developed stratum corneum is present. Probably *in vitro* a 'clumping mechanism' is not as active as *in vivo*. The ribosomal breakdown by RNase and proteolytic enzymes could, however, still occur. If one assumes that the keratohyalin granules represent a material in which the fibrillar material has to be embedded, an explanation would be much more difficult.

Acknowledgements

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Author's address Dr F W BAUER, Department of Dermatology, Catholic University, Philip van Leijdenlaan 25, *Nijmegen* (The Netherlands)

STELLINGEN

I

De conclusie van Baden, dat het keratine macromolecuul opgebouwd is uit verschillende eiwitten is niet in overeenstemming met zijn eigen resultaten.

Baden, H.P., (1971) Acta Dermatovener. 51, 327.

II

De conclusie van Crounse, dat het keratine macromolecuul is opgebouwd uit één type subunits, is waarschijnlijk juist, maar de gegevens waarop hij deze conclusie baseert zijn gedeeltelijk onjuist.

Crounse, R.G., (1966) Nature 211, 1301.

Matoltsy, A.G., in Biology of Skin and Hair Growth, Edit. Angus and Robertson, Sydney, 1965.

III

De opvatting, dat de dermis noodzakelijk is voor de keratinisatie van de epidermis, is onjuist.

Briggaman, R.A., Wheeler, C.E., (1968) J. Invest. Derm. 51, 454.

IV

Het is mogelijk om keratinocyten in monolayer te laten groeien, maar, wat belangrijker is, men kan uit de cellen die in monolayer groeien opnieuw een verhoornend epitheel reconstrueren.

V

De verwachting, dat gekweekte huidcellen in de nabije toekomst kunnen worden benut voor huidtransplantatie, lijkt gewettigd.

VI

De biochemische kennis van antimetabolieten wordt in de dermatologische klinieken niet optimaal benut.

Weinstein, G.D., (1972) in Advances in Biology of Skin, Vol. 12, 287. Edit. W. Montagna, E.J. van Scott and R.B. Stoughton, New York 1969.

VII

Celfusie geïnduceerd door eenvoudige chemicaliën zoals lysolecithine, is in principe te verkiezen boven celfusie door middel van virussen.

VIII

Nu de 'cellmarker' technieken verder zijn verfijnd verdient het aanbeveling in geval van genetisch bepaalde dermatopathieën een karyo-typering te doen met deze nieuwe technieken.

> Sumner, A.T., Evans, H.J. and Buckhand, R.A., (1971) Nature New Biol. 232, 31. Seabright, M., (1971) Lancet II, 971.

IX

De conclusie van Kelly, dat in erythrocyten van heterozygoten voor het Lesh-Nyhan syndroom (de draagsters) normale HG-PRT activiteiten aanwezig zijn, is niet in overeenstemming met de resultaten van Emmerson et al. De resultaten van deze laatste auteurs zijn ook beter in overeenstemming met de resultaten van onderzoek aan fibroblasten en haarwortels.

> Kelley, W.N., (1968) Fed. Proc. 27, 1047.
>
> Emmerson, B.T., Wijngaarden, V.N., Nyhan, W.L., Young, W.J. and Childa, B., (1968) Science 160, 425.

X

De selectie op grond van eindexamenresultaten voor de toelating tot wetenschappelijk onderwijs is op dit moment niet geoorloofd.

XI

Wanneer de stellingen bij een proefschrift niet ingebonden zijn, is het wenselijk dat de naam van de promovendus op het losse inlegvel aanwezig is.

F.W.Bauer
Nijmegen, 29 september 1972