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PRODUCTS OF EPIDERMAL PROTEIN SYNTHESIS

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Abstract. The products of epidermal protein synthesis in tissue slice and cell-free systems have been characterized by chromatographic, electrophoteric, and immunologic methods. Radiolabeled proteins of heterogeneous size are found in the supernatant and particulate fractions from both systems. The major labeled proteins in the supernatant fraction have molecular weights of approximately 43 000 and 65 000. The natively-insoluble proteins of the particulate fraction were solubilized either with desoxycholate or alkaline urea. Subsequent analysis of these fractions yielded a labeled, high molecular weight, urea-soluble protein whose electrophoretic mobility was slower than that of the heavy chain of myosin. The immunologic reaction between the radiolabeled fraction and antibody raised against filamentous protein suggests that synthesis of proteins identical to epidermal filamentous protein occurs in the in vitro systems.

Key words: Protein synthesis; Epidermis; Skin; Proteins; Keratin

The mechanism of protein synthesis in epidermis and hair root cells has been studied extensively in many laboratories during the past several years and it has been shown that epithelial proteins are synthesized by a process which is basically similar to that in other tissues (8, 12, 15). The unique findings in systems of epithelial origin are undoubtedly related to the specific differentiation of epidermis and hair root cells: tissues whose major function is the production of large, insoluble macromolecules.

One of the major remaining questions involves the relationship between the studies of in vitro epithelial protein synthesis and the actual in vivo events which result in the production of the markers of epithelial differentiation: keratin and keratohyalin. One of several possible approaches to the answer depends upon the isolation and characterization of the earliest reaction products of the protein biosynthesis in epidermis and hair root cells and the elucidation of the relationship between these early synthesized proteins and the final, biologically-active products. The validity of this approach has been demonstrated in studies of other protein synthesizing systems, such as reticulocytes (11) and muscle (14) as well as in epidermal derivatives (16). In this communication, we are reporting our data on the products of the in vitro protein synthesis reaction in epidermis and on the relationship between the in vitro and in vivo synthesis reactions.

MATERIALS AND METHODS

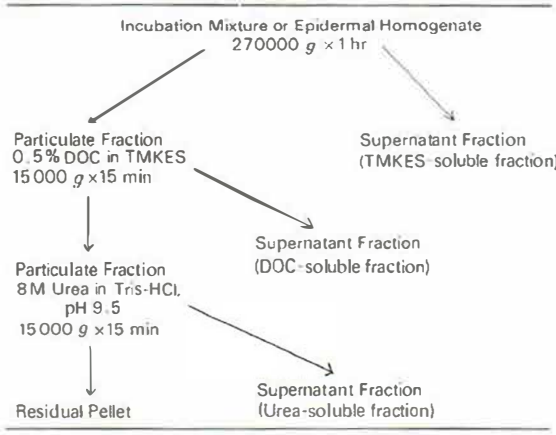
Details of the in vitro incubation of epidermal tissue slices and the procedure for subsequent separation of epidermis from dermis using the heat method have been described previously (7). The slices used in the in vitro system were obtained from either 3-day-old CD rats (Charles River Laboratories, Wilmington, MA) or from latex-epilated, male, albino guinea pigs (Elm Farm, Chelmsford, MA). Fifty micron slices of guinea pig epidermis were obtained with a Staddie–Riggs microtome. The rat epidermis was cleaned of subcutaneous fat and membranes and used without microtome slicing. Tissue slices were incubated for varying periods of time in the presence of either ^{3}H or ^{14}C labeled L-amino acid mixtures (NET-250 or NEC-445, New England Nuclear Corporation, Boston, MA) in Krebs–Ringer phosphate buffer, pH 7.4.

The cell-free incorporation methods used in this paper are those developed in our laboratory during the past several years (9, 10). Stretch-separated guinea pig epidermis was homogenized and fractionated by differential centrifugation into ribosomal and high-speed supernatant fractions. The particulate and soluble components were incubated for 30 min in the presence of ATP, an ATP generating system, and labeled amino acids.

Fractionation of tissues following incubation

The epidermis from tissue slice experiments was homogenized in TMKES buffer (10 mM Tris–HCl, pH 7.5; 3 mM magnesium acetate; 10 mM potassium chloride; 5 mM 2-mercaptoethanol; and 250 mM sucrose) and this homogenate as well as the incubation mixture from the cell-free incorporation experiments was fractionated according to the scheme outlined in Table 1. Subsequent to incubation the labeled mixtures were centrifuged at 270 000 *g* for 1 hour to obtain a TMKES-soluble supernatant fraction as well as a high-speed pellet. The latter was extracted with 0.5% sodium desoxycholate (DOC; Difco Laboratories,

Table I. *Fractionation scheme*



Detroit, Michigan) and centrifuged at 15 000 *g* for 15 min. A DOC-soluble fraction and a residual pellet were obtained. The pellet was washed to remove residual DOC and was treated with puromycin or urea in order to free the nascent proteins from the ribosomes.

Puromycin was used at a concentration of 10^{-3} M in TMKES. The samples were incubated for 30 min at 37°C and centrifuged at 270 000 *g* to obtain the released polypeptides. Urea was used at 8 M over a range of pH values. The protein content of the several fractions was determined by the method of Lowry et al. (13) and RNA content was measured by the orcinol reaction (1). Radioactivity in the tissue fractions was determined in a Packard Tri-Carb Liquid Scintillation Spectrometer following precipitation of the samples in 10% trichloroacetic acid (TCA) and collection on 0.45 μm Millipore filters.

Molecular sieve chromatography of nascent polypeptides

The several soluble fractions were placed on 2 × 35 cm columns packed in the usual manner with dextran beads of various exclusion limits (Sephadex G-25, G-50, G-100, and G-200; Pharmacia Fine Chemicals, Piscataway, N.J.). The columns were eluted with TMKES containing 6 M urea in the indicated experiments and absorption of the eluates was monitored at 280 nm. Radioactivity in the several fractions was determined according to the procedures described above.

Acrylamide gel electrophoresis

The technique of Davis was used for routine disc gel electrophoresis (5). Acrylamide concentration was varied from 4.0% to 7.5% with corresponding changes in the concentration of the *bis*-acrylamide cross linker. Samples were electrophoresed in a Buchler Polyanalyst electrophoresis apparatus (Buchler Instruments, Fort Lee, N.J.). In addition, samples were analyzed by gel electrophoresis in the dodecyl sulfate-polyacrylamide system described by Weber & Osborn (18).

The following commercially available proteins were used to standardize the gels for molecular weight determinations; rabbit muscle phosphorylase A (Worthington Biochemical Corporation; mol. wt 94 000), egg white ovalbumin (Worthington Biochemical Corporation; mol. wt 43 000), bovine pancreatic α-chymotrypsinogen A (Sigma Chemical Company; mol. wt 36 000), and bovine serum albumin (Schwarz/Mann; mol. wt 68 000). Cardiac myosin of molecular weight 200 000 was supplied by Dr Eugene Morkin.

The acrylamide concentration was lowered to 4.0% to permit entry into the gel of high molecular weight proteins labeled in the in vitro system. Isoelectric focusing in acrylamide gels was performed according to the procedure of Drysdale (6) in a Metalloglass isofocusing apparatus (Metaloglass, Inc., Boston, MA). Ampholytes were obtained from LKB (LKB-Produkter AB, Stockholm, Sweden) and were used at a concentration of 4.0% in order to insure stability and reproducibility of the pH gradients. Immunoelectrophoresis was performed following polyacrylamide electrophoretic separation of the samples by placing the gels on a 9.5 cm Petri dish to which 1% agar in 0.025 M Tris-HCl, pH 8.0, was added and allowed to solidify. Longitudinal wells for antibody were cut parallel to the gels and immunodifusion proceeded in a manner essentially similar to the Ouchterlony technique (3). The contents of the Petri dish were subsequently washed to remove non-precipitated protein and were stained for 30 min in 0.5% Amido Black in 5% acetic acid and destained by repeated washing with 7% acetic acid (4). Antibodies to the fibrous epidermal proteins were prepared as previously described (17).

In order to localize radioactivity following electrophoresis the gels were removed from the glass tubes, frozen, and sliced on a Mickel gel slicer (Buchler Instruments, Fort Lee, N.J.). The gel slices were either dried on Whatman no. 1 filter paper, pelleted in a Parr press, and combusted in a Packard Tri-Carb Sample Oxidizer—or solubilized in a toluene-based scintillation fluid containing 3% Protosol (New England Nuclear Corp.).

RESULTS AND DISCUSSIONS

Release of nascent polypeptides

In the cell-free system currently in use in our laboratory, ribosomes isolated from guinea pig epidermis or hair root cells are incubated in the presence of a supernatant fraction and appropriate co-factors. In this system, labeled amino acids are incorporated into TCA-precipitable peptides. To characterize the peptides which were labeled in this system we initially localized them in the several fractions of the cell-free incubation mixture.

The post-incubation mixture was separated by centrifugation (Table I) into particulate and supernatant fractions. Analysis of the fractions indicated that 88% of the incorporated activity in the epidermal system was present in the particulate fraction while 12% was found in the TMKES-supernatant fraction. Data from the hair root system were similar. There are two explanations for these results. The cell-free system as used in our laboratory may be defi-

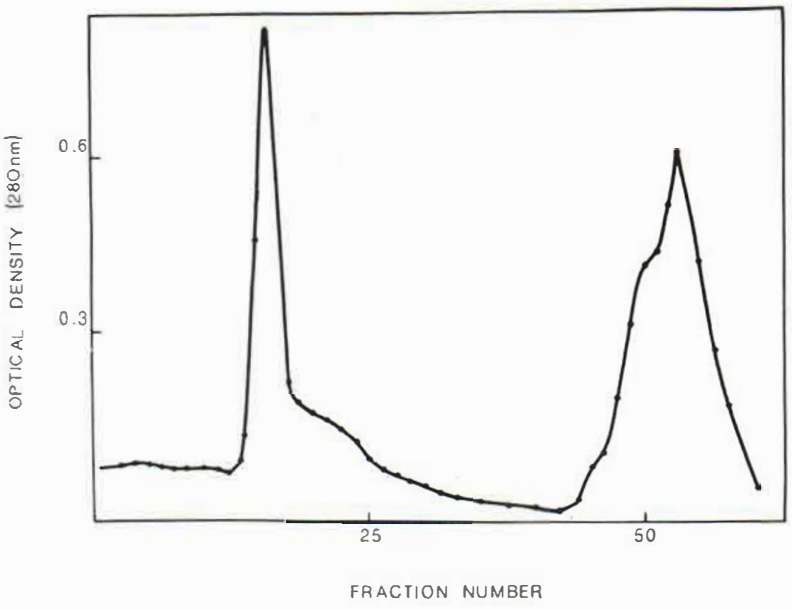


Fig. 1. Sephadex G-100 chromatography of the high-speed supernatant fraction obtained from the cell-free incubation mixture. The column was eluted with TMKES, pH 7.5.

cient in release factors or that portion of the messenger RNA which codes for release may be destroyed by the endogenous ribonucleases in the tissues. In the studies of Steinert & Rogers (15) in which hair roots from newborn animals were studied, release was much more active.

Since our major aim was to characterize the nascent proteins in the incorporation system the results forced us to seek ways to release the incorporated radioactivity from the particulate fraction. The results of such experiments are reproduced in Table II.

Puromycin, serving as a t-RNA analogue, led to the expected increase in solubilized radioactivity but still failed to release 40% of the incorporated radioactivity. Concentrated urea was used at several pH's from 7.0 to 10.0. 8.0 M urea at pH 9.5 was found to be most effective and enabled us to release 88% of the incorporated radioactivity into the supernatant fraction in the epidermal system. In the hair root system alkaline urea released 50% of the labeled polypeptides. Since our aim was to characterize as large as possible a percentage of the labeled material, we chose to do further studies on the alkaline urea fraction. The fact that it contained large amounts of ribonucleoproteins as well as nascent polypeptides was not relevant to our studies since we are only concerned with the newly synthesized labeled material.

Table II. *Release of incorporated radioactivity*

Medium in which experiment was performed	% Incorporated radioactivity localized to fraction	
	Supernatant	Pellet
TMKES (pH 7.5)	18	81
Pyromycin	60	40
Urea (8 M in Tris–HCl, pH 9.5)	88	12

Analysis by molecular sieve chromatography of nascent polypeptides

Fig. 1 represents the elution profile following chromatography on a Sephadex G-100 column of the high-speed supernatant fraction of the cell-free incubation system. The peaks were collected, pooled, and analysed for radioactivity. We found that the initial peak and shoulder, migrating at the void volume, contained 23% of the radioactivity recovered from the column. Seventy-seven percent of the recovered radioactivity was associated with a second peak of material which appeared to be of smaller molecular size and which remained unresolved.

The radioactivity initially localized to the particulate fraction was solubilized in alkaline urea and subjected to chromatography on Sephadex gels of

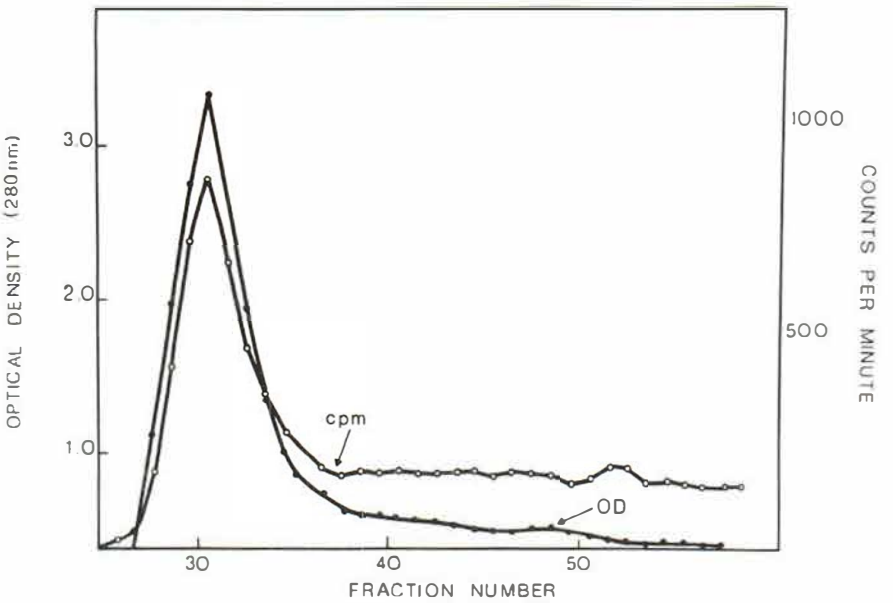


Fig. 2. Sephadex G-200 chromatography of the particulate fraction from the cell-free incubation mixture solubilized in 8 M urea in Tris–HCl, pH 9.5. The column was eluted with 6 M urea in Tris–HCl, pH 9.5.

various exclusion limits. Fig. 2 represents the results of chromatography of this fraction on Sephadex-200 and is representative of all other runs. All the radioactivity and optical density eluted immediately following the void volume and appeared to be of high molecular weight. It is, therefore, apparent that the labeled proteins released from the ribosomes during incubation in the cell-free system are different from those which remain attached to the ribosomal particles, the latter being of higher molecular weight. The data in Table III indicate that we did not recover all of the labeled polypeptides from any of the chromatographic columns when urea-solubilized material was studied. Although 100% of the optical density was recovered from the G-25 columns, in all other cases a portion of the radioactivity was either irreversibly bound to the column or no longer TCA precipitable following chromatography. 100% of the incorporated radioactivity was recovered from the buffer-soluble fraction. This phenomenon may be due to precipitation of the sample on the column.

Table III. *Recovery from Sephadex columns (urea-soluble fraction)*

Sephadex gel	Experiment no.	Optical density, 280 nm (%)	Radioactivity (%)
G 25	1	100	77
	2	99	77
G 50	1	95	100
	2	86	86
	3	95	71
G 100	1	65	46
	2	61	52
G 200	1	60	52
	2	67	56

Acrylamide gel electrophoresis

Disc gel electrophoretic analysis of the buffer-soluble fraction obtained from the incubation mixture revealed multiple protein bands, three of which corresponded to peaks of radioactivity which migrate through the gel (Fig. 3A). The particulate fraction solubilized with alkaline urea migrated in a different manner, as is shown in Fig. 3B. The major portion of the radioactivity did not enter a 7.5% acrylamide gel but remained close to or at the origin. The peak which migrated rapidly toward the anode probably represents small polypeptides. When the gel concentration was lowered to 4% the second pattern shown in this figure resulted. One major peak of radioactivity can be seen with both an ascending and descending shoulder and minor peaks toward the anode. The electrophoretic analysis confirms the chromatographic data, again indicating that the

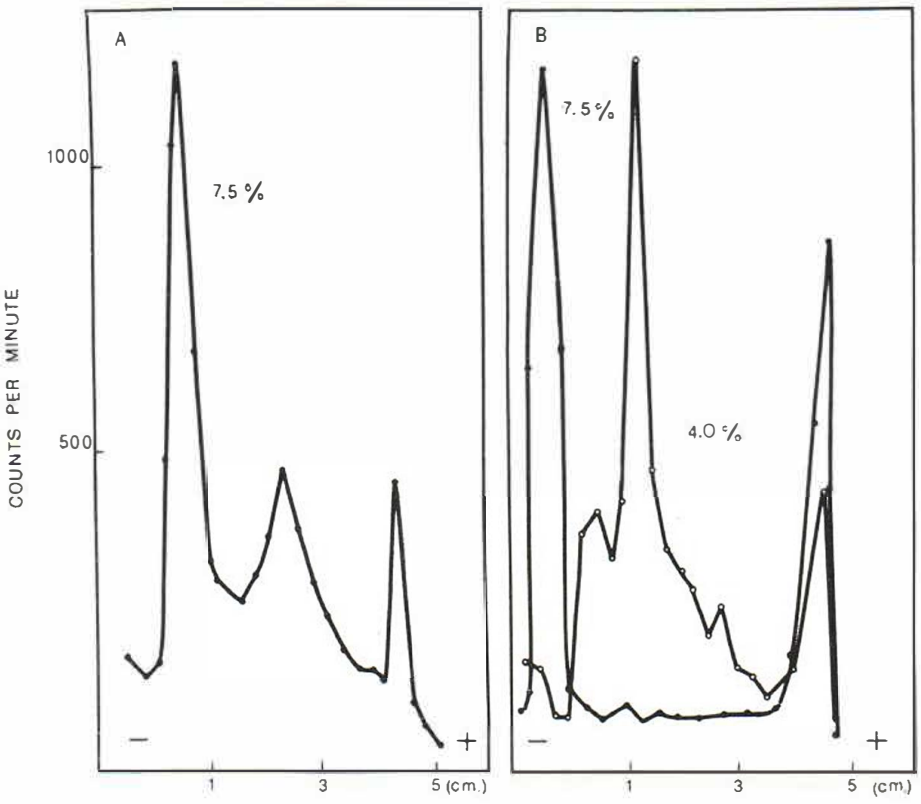


Fig. 3. Disc gel electrophoresis of (A) the supernatant and (B) the urea-soluble fraction from the epidermal cell-free incubation mixture. The supernatant fraction (A) was electrophoresed for 3.5 hours on a 7.5 % acrylamide running gel. The urea-soluble fraction (B) was electrophoresed for 8.5 hours on 7.5 % acrylamide running gel made 6 M with respect to urea and for an equal time on 4.0 % acrylamide made 6 M with respect to urea.

soluble material and that solubilized with urea are different and that the latter is either of higher molecular weight or more negatively charged.

In order to distinguish between these possibilities a series of electrophoretic runs were performed in the presence of either sodium dodecyl sulfate (SDS) or ampholyte, the former leading to differentiation on the basis of size, the latter on the basis of charge. In addition, the pellet was fractionated into urea and DOC-soluble fractions.

During SDS electrophoresis, clear differences were apparent among the three fractions. The buffer and DOC-soluble fractions both yielded multiple species. The urea-soluble radioactive fraction was larger, moving extremely slowly in the gel with a migration velocity slower than that of myosin heavy chain.

By the criteria of Weber & Osborn, therefore, the molecular weights of these three fractions from the epidermal cell-free system are different. The fractions are of various degrees of purity but a major radioactivity labeled band exists in each. The differences in apparent molecular size could account for the differences in migration in polyacrylamide gel electrophoresis noted above.

In addition, Fig. 4 indicates that there are also charge differences among these several fractions. The results of isoelectric focusing runs on the three fractions are displayed. The buffer-soluble fraction (4A) is a complex of 3 major protein species having isoelectric points of 9.5, 8.1 and 5.8. The DOC-soluble fraction (4B) consists of at least two major species with isoelectric points of approximately 6.8 and 4.5. We are unable to state conclusively that other bands which have apparent isoelectric points of 6.1, 4.5 and 4.0 are different species. The urea-soluble fraction (4C) was more homogeneous, consisting of a labeled band whose isoelectric point was approximately 5.0, as well as a shoulder between 5.2 and 6.0.

Although these studies indicate that the cell-free protein synthesizing system which has been devel-

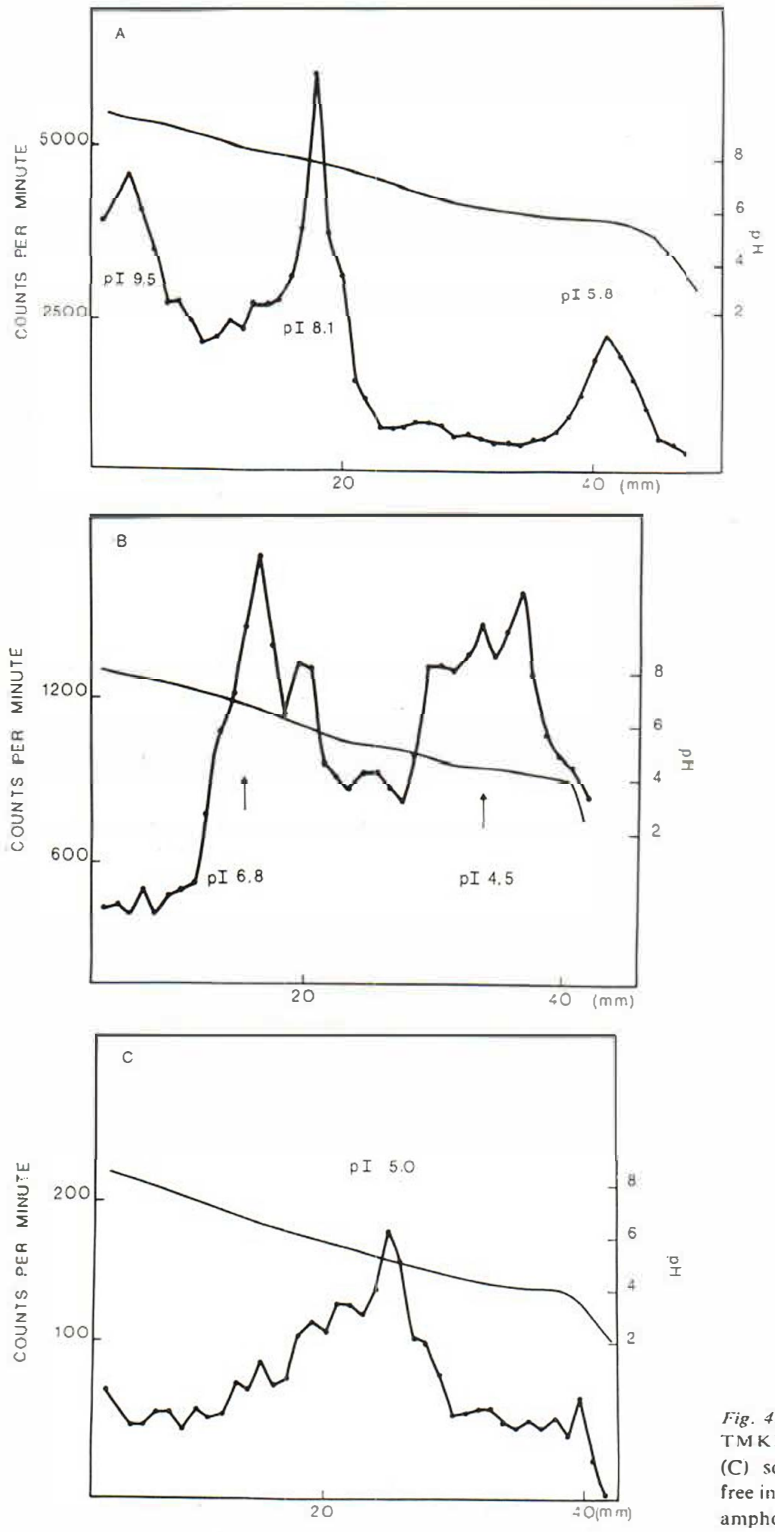


Fig. 4. Isoelectric focusing pattern of the TMKES (A), desoxycholate (B), and urea (C) soluble fractions of the epidermal cell-free incubation mixture. Gels contained 4.0 % ampholine and 6 M urea.

oped using epidermis as starting material does, in fact, synthesize proteins of heterogeneous size and charge, the possibility exists that the material labeled in this cell-free system is not basically related to the proteins, such as keratin and keratohyalin, which are of greatest biologic importance in epidermis. In order to document a clearer relationship between the products of the cell-free system and native epidermal proteins we turned to a tissue slice system which has been more completely characterized.

Tissue slice system

Since the urea-soluble fraction has been shown to consist of the filamentous, helical protein precursors of keratin (2), we chose to examine this fraction from the slice system in detail. The urea-soluble fraction was subjected to polyacrylamide gel electrophoresis and the results are reproduced in Fig. 5. At a gel concentration of 7.5% the labeled material did not enter the running gel but remained at the origin. If the gel concentration was lowered to 2.5%, essentially no resolution occurred and the radioactivity migrated rapidly toward the anode. At a 4% gel concentration, the labeled material entered the gel and three distinct peaks of radioactivity could be resolved.

SDS-acrylamide gel electrophoretograms of the extracts are reproduced in Fig. 6. The results are similar to those for the cell-free system described above. The buffer-soluble fraction contains labeled proteins with molecular weights between 35 000 and 94 000. The DOC-soluble fraction contains material of predominantly higher molecular weight with a major band at 94 000. Again, the urea-soluble fraction had to be run at least twice as long

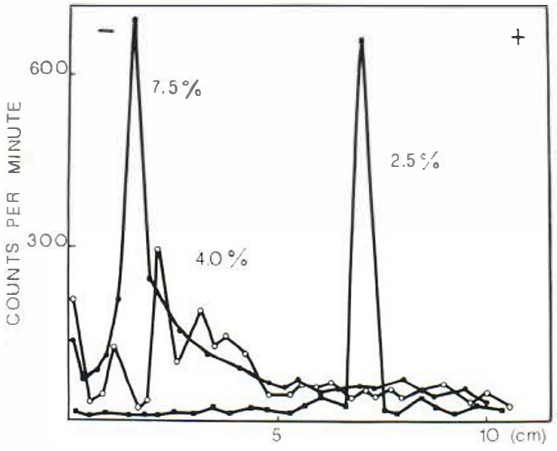


Fig. 5. Disc gel electrophoresis of the urea-soluble fractions obtained from the epidermal homogenate of the tissue slice system. The urea-soluble fraction was electrophoresed on 7.5%, 4.0% and 2.5% acrylamide running gels made 6 M with respect to urea. Electrode buffers contain 6 M urea.

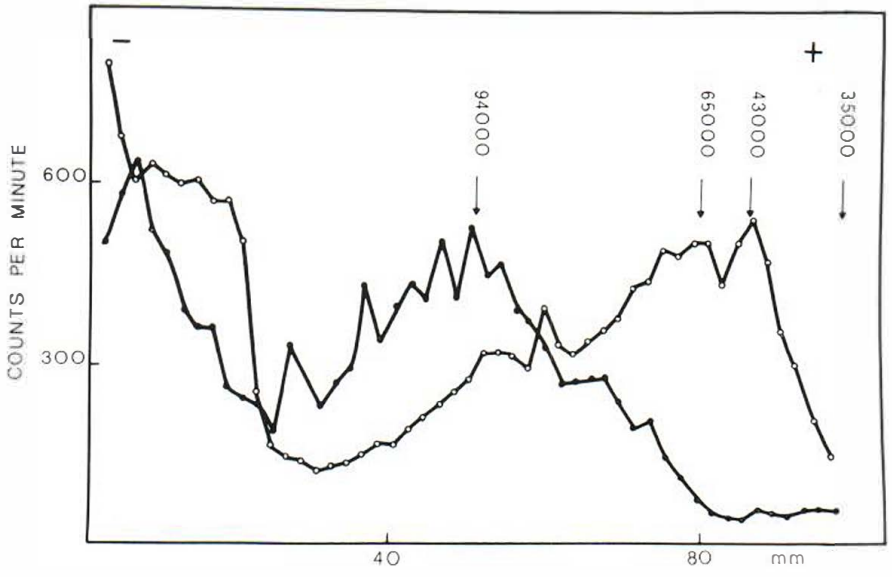


Fig. 6. Sodium dodecyl sulfate–acrylamide gel electrophoresis of the TMKES and desoxycholate-soluble fractions of the epidermal homogenate of the tissue slice system. The gels were 4.0% acrylamide, contained 6 M urea, and were run for 6.5 hours. ●—●, DOC-soluble fraction. ○—○, TMKES-soluble fraction.

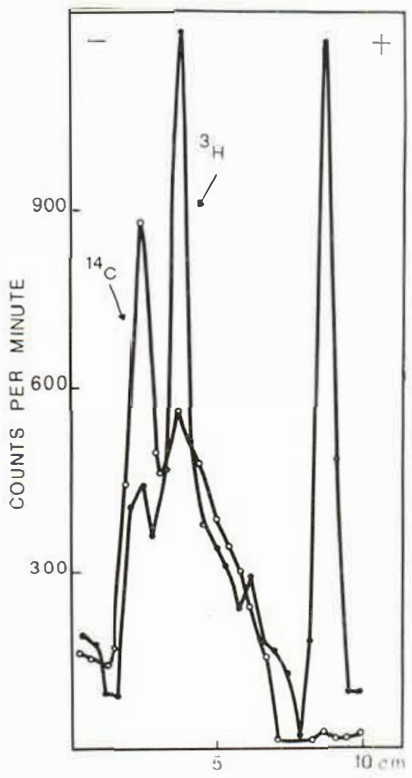


Fig. 7. Disc gel electrophoresis of the ^{3}H-labeled cell-free, urea-soluble fraction and the ^{14}C-labeled tissue slice urea-soluble fraction. The acrylamide concentration is 4.0% and the gels and the electrode buffers contain 6 M urea.

and the electrophoretogram indicated a molecular weight greater than that of the heavy chain of myosin (200 000).

It appears, therefore, that the urea-soluble fraction from the cell-free system is similar in molecular size to that of a filamentous fraction which can be isolated from epidermal tissues slices. To determine whether these two proteins were identical we performed the experiment detailed in Fig. 7. Labeled material was prepared in both the cell-free and tissue slice systems. A tritiated amino acid micture was used as a source of radioactivity in the former and a ^{14}C-labeled mixture in the latter. The urea-soluble fractions from each system were isolated, mixed, and co-electrophoresed on acrylamide gel. Except for the large, labeled peak in the cell-free system which migrates rapidly toward the anode and probably consists of small, incompleted peptides, the major bands in these two systems have identical R_f's.

Immunological characterization of nascent protein

The ultimate goal of the work reported from this laboratory over the past several years is the elucidation of the biochemical pathways of fibrous protein synthesis in the epidermis. In this paper we have shown that although the products of epidermal protein synthesis are heterogeneous in the control-

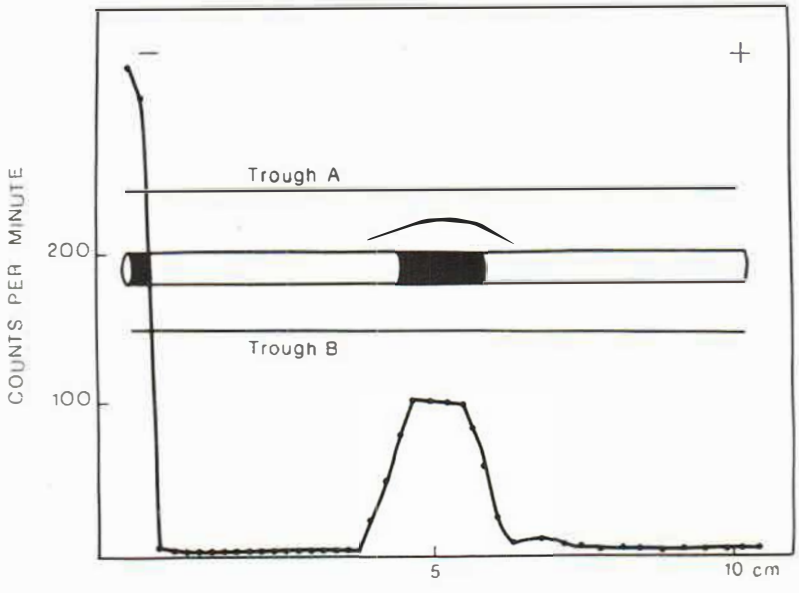


Fig. 8. Immunodiffusion study of urea-soluble fraction from newborn rat tissue slice system. The urea fraction was electrophoresed for 6.5 hours on sodium dodecyl sulfate (4%) acrylamide gels and then subjected to immunodiffusion as described in Methods. Through A contained antibody to newborn rat filamentous protein. Trough B contained non-immune rabbit serum. Following immunodiffusion the plate was stained and subsequently the gel and the surrounding agar were sliced and the radioactivity determined.

lable cell-free system, a protein is labeled which corresponds in many respects to a precursor or component of the fibrous protein. The ultimate proof that the cell-free system directs the synthesis of a keratin precursor will only be available when the keratin molecule is completely characterized. Indirect evidence for such a process is displayed in Fig. 8.

The newborn rat tissue slice system was used, since we have prepared antibody to the filamentous protein from this species (17). Following incubation the urea-soluble proteins were extracted from the tissue and were electrophoresed in the presence of sodium dodecyl sulfate. Following electrophoresis the gel cylinders were placed in agar and used as an antigen source in an immunodiffusion study in which antibody to newborn rat filamentous protein was employed. The figure indicates that the labeled protein, the major protein species evident by staining, and the immunoreactive protein, are found in the same area of the gel. Since the rat antibody does not cross react with guinea pig filamentous protein we are unable to repeat these studies with guinea pig tissue at the present time. The most reasonable interpretation of the results, however, is that the material labeled in the rat slice system is, in fact, the filamentous protein and, by extrapolation, we believe that the material of high molecular weight which was labeled in the guinea pig tissue slice and cell-free systems is of similar origin.

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