Isolation of a Heat-Stable Crystalline Protein from Psoriatic Scales*

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ABSTRACT: A procedure has been developed for the isolation of an acid-stable, heat-resistant, crystalline protein from psoriatic scales. The crystalline protein is extracted from acetone powder preparations of psoriatic scales using 0.25 N H_2SO_4. The dilute acid extracts also contain ribonuclease and lysozyme activities which can be removed by means of carboxymethyl-Sephadex. The crystalline protein, which has been found in every preparation of psoriatic scales which has been studied, had a molecular weight of 27,700 ± 2,000 and was found to be relatively basic on electrophoresis. Antibodies to the crystalline protein could be produced in rabbits. Immunoelectrophoresis demonstrated that the crystalline protein is not a serum protein which could have entered the epidermis by diffusion, as occurs in a variety of inflammatory dermatoses. Immunologic evidence indicates that it is found in the stratum corneum since extracts of callus, which is essentially stratum corneum, reacted identically with antibody to the crystalline protein. The low cystine content indicates that the crystalline protein is not related to keratin.

Previous work in this laboratory has shown that psoriatic scales contain a number of low molecular weight proteins which could be extracted with dilute acid. The purification and characterization of psoriatic RNAase[1] from such extracts was previously reported (Liss and Lever, 1962). This paper describes procedures for the isolation of an acid-stable, heat-resistant, crystalline protein from psoriatic scales.

Experimental Procedure

Materials. Scales were obtained from patients with psoriasis. Yeast RNA was prepared according to the method of Crestfield *et al.* (1955). Lyophilized *Micrococcus lysodeikticus* cells were purchased from the Worthington Biochemial Corp. CM-Sephadex, type C-50 (medium), was a product of Pharmacia Fine Chemicals, Inc., and horse antihuman serum was a product of Immunology, Inc.

Determination of RNAase Activity. RNAase was assayed according to the procedure of McDonald (1955). One milliliter of substrate solution containing 10 mg of yeast RNA in 0.1 M sodium acetate buffer, pH 5.0, was incubated for 15 min at 37° with a suitable aliquot of enzyme solution. The final volume was adjusted to 2 ml with 0.1 M sodium acetate buffer. The reaction was stopped by the addition of 2 ml of a solution consisting of 0.25% uranyl acetate in 2.5% trichloroacetic acid. The optical density of fivefold aqueous dilutions of the supernatant solution was determined in a Beckman DU spectrophotometer at 260 mμ. One enzyme unit is that amount which will liberate 0.1 μmole of nucleotide per incubation mixture. An optical density of 10 at 260 mμ was assumed to be equivalent to 1 mM uranyl acetate soluble nucleotides (Alexander *et al.*, 1961).

Determination of Lysozyme Activity. Lysozyme activity was determined by measuring the rate of lysis of lyophilized *M. lysodeikticus* cells at 450 mμ in a Coleman Junior spectrophotometer according to a modification of the method of Shugar (1952). The reaction mixture consisted of 2.7 ml of a cell suspension (18 mg/20 ml) in 0.067 M sodium phosphate buffer, pH 7.0, and enzyme in a final volume of 3.0 ml. One unit of enzyme is that amount which will produce a decrease in optical density of 0.001/min. The first 5 min of the reaction was used to calculate activity.

Determination of Protein Content. Protein was determined by the method of Lowry *et al.* (1951) or Warburg and Christian (1942).

Starch Gel Electrophoresis. For starch gel electrophoresis, a discontinuous borate buffer system was used (Barrett *et al.*, 1962).

Agar Electrophoresis and Immunoelectrophoresis. Electrophoresis and immunoelectrophoresis were carried out on microscope slides according to a modification of the method of Scheidegger (1955).

Preparation of CM-Sephadex. CM-Sephadex was allowed to swell in water and then was washed with 0.5 N NaOH followed by removal of excess alkali with

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[1] Abbreviations used in this work: RNAase, ribonuclease; DNAase, deoxyribonuclease.

TABLE I: Summary of Purification Procedure.

Step	Lysozyme		RNAase	
	Total Units	Units/mg Protein	Total Units	Units/mg Protein
1. Acid extract	104,630	41	30,620	12
2. $(NH_4)_2SO_4$ fractionation	36,300	133	9,280	34
3. Heat treatment	33,500	250	8,170	61
4. First crystallization				
a. Crystals		30		6
b. Supernatant	3,150	125	4,760	189
5. Fifth crystals		0		0

distilled water. It was next treated with 0.5 N HCl followed by washing with water. The CM-Sephadex was then stirred with 1 M sodium acetate buffer, pH 5.4, filtered, and finally equilibrated by successive treatments with 0.1 M sodium acetate buffer, pH 5.4.

Amino Acid Composition of Crystalline Protein. Samples containing 4.40 mg of protein (dry weight, 16.3% N) were hydrolyzed in 1 ml of 6 N HCl for 20, 40, and 70 hr at 110° in vacuum-sealed tubes. HCl was removed under vacuum (vacuum pump) at room temperature. The residue was dissolved in 0.5 ml of H_2O, and 0.5 ml of 0.2 M sodium phosphate buffer, pH 6.5, was added. The solution was allowed to stand for 4 hr in order to permit air oxidation of cysteine to cystine, and then 0.06 ml of 1 N HCl was added. The solution was quantitatively made up to 5 ml in a volumetric flask using diluent buffer of pH 2.2 (Moore and Stein, 1963). The amino acid composition was then determined on a Beckman-Spinco Model 120-B automatic amino acid analyzer (Moore *et al.*, 1958). Tryptophan was determined with an automatic amino acid analyzer after hydrolysis for 50 hr in 4 N barium hydroxide at 110° in a vacuum-sealed tube (Noltmann *et al.*, 1962), and cyst(e)ine was also determined as cysteic acid after performic acid oxidation (Schramm *et al.*, 1954).

Molecular Weight Determination. Sedimentation equilibrium molecular weights were obtained using the method of Yphantis (1964). Molecular weights were determined at two concentrations, 0.006 and 0.012%, at speeds of 50,740 and 37,020 rpm, respectively. The temperature was 23°.

Rabbit Antibodies to Crystalline Protein. Five milligrams of the crystalline protein was dissolved in 1 ml of sterile saline and then mixed with 1 ml of complete Freund's adjuvant. Rabbits were immunized by injection of 0.5-ml aliquots into each toe pad. The rabbits were bled by cardiac puncture 24 days after immunization and the serum was recovered.

Results

Isolation of Crystalline Protein. A summary of the following purification procedure is given in Table I. The initial acid extract contained, in addition to the crystalline protein, RNAase and lysozyme activities. Both of these enzymes could be completely removed in the procedure leading to the crystallization of the psoriatic protein.

Step 1. Preparation of Acid Extract. An acetone powder was prepared from 42 g of psoriatic scales. The acetone powder was extracted with 800 ml of precooled 0.25 N H_2SO_4 by vigorously stirring the mixture at 4° for 20 hr. The material was centrifuged at 3000 × *g* for 30 min at 4°. The viscous supernatant solution was recovered. The sediment was washed with an additional 200 ml of cold 0.25 N H_2SO_4 and the supernatant solution was recovered after recentrifugation. The supernatant solutions were combined.

Step 2. Ammonium Sulfate Fractionation. The acid extract (880 ml) was brought to 0.65 ammonium sulfate saturation by the addition of 378 g of ammonium sulfate. The material was stirred for 2 hr at 4° and then centrifuged at 3000 × *g* for 30 min. The supernatant solution (1000 ml) was brought to 0.9 ammonium sulfate. The sample was stirred for 2 hr and then allowed to stand in the cold for 24 hr. The material was centrifuged at 3000 × *g* for 30 min and the supernatant solution was discarded. The sediment was mixed with 30 ml of 0.02 M Tris–HCl buffer, pH 7.5, which was 0.1 M with respect to NaCl. The small amount of insoluble material was removed by centrifugation at 3000 × *g* for 15 min.

Step 3. Heat Treatment. The 0.65–0.9 ammonium sulfate fraction was heated in a boiling water bath for 3 min, then immediately cooled by immersion in ice. The flocculent precipitate which had formed was removed by centrifugation at 3000 × *g* for 20 min. The supernatant solution was dialyzed against 2 l. of 0.02 M Tris–0.1 M NaCl buffer, pH 7.5. The dialysate was changed once.

Step 4. First Crystallization. The dialyzed solution (42 ml) was opalescent but contained no sediment. It was next dialyzed for 3 hr against 1 l. of a precooled solution of 0.02 M Tris–0.1 M NaCl buffer, pH 7.5, and ethanol (65:35, v/v). During the dialysis a sediment developed which was removed by centrifugation at

FIGURE 1: Crystalline psoriatic protein magnified 500-fold.

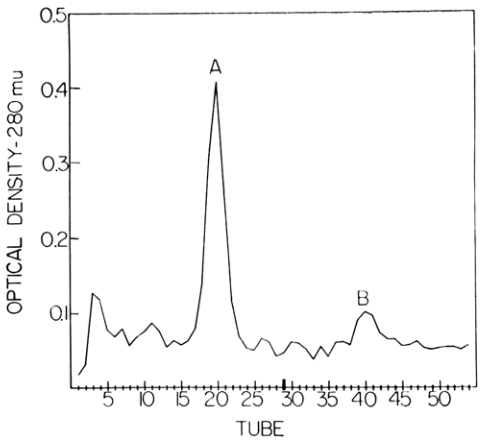

FIGURE 2: Elution pattern of a 1 × 35 cm CM-Sephadex column to which 7 mg of protein was applied. The mixing vessel contained 500 ml of 0.1 M sodium acetate buffer, pH 5.4, and the reservoir vessel 500 ml of 1 M sodium acetate buffer of the same pH for tubes 1–28 (8 ml/tube). Beginning with tube 29, the mixing vessel contained 500 ml of 1 M sodium acetate buffer, pH 5.4, and the reservoir vessel 500 ml of 1 M sodium acetate buffer, pH 5.4, containing 1 M NaCl. Peak A contained crystalline psoriatic protein and peak B contained the RNAase and lysozyme activities.

3000 × *g* for 20 min. The supernatant solution was dialyzed overnight against 1 l. of a precooled solution of 0.02 M Tris–0.1 M NaCl buffer, pH 7.5, and ethanol (1:1, v/v). The dialysate was changed once. The dialyzed sample contained a sediment which was found to be crystalline on microscopic examination. It consisted of a mixture of needles and diamond-shaped crystals. The sample was allowed to stand in the cold for an additional 48 hr in order to obtain maximum crystallization, after which time the sample was centrifuged at 3000 × *g* for 20 min. On prolonged standing in the cold a small amount of additional crystallization occurred.

Step 5. Second–Fifth Crystallizations. The crystalline protein was dissolved in 35 ml of 0.02 M Tris–0.1 M NaCl buffer, pH 7.5, at room temperature and the solution was dialyzed at 4° against 1 l. of a precooled solution of Tris–NaCl buffer and ethanol (3:1, v/v). A small amount of amorphous sediment which formed during dialysis was removed by centrifugation. A second crystallization was performed by dialyzing the supernatant solution overnight against 1 l. of a precooled solution consisting of 0.02 M Tris–0.1 M NaCl buffer, pH 7.5, and ethanol (1:1, v/v). RNAase and lysozyme activity could be removed by three additional recrystallizations from dilute solution by dialysis against 0.02 M Tris–0.1 M NaCl buffer, pH 7.5, and ethanol (1:1, v/v). The five-times-crystallized protein contained no RNAase, DNAase, lysozyme, or proteolytic activity when casein was used as substrate. The crystalline protein consisted of a mixture of needles and diamond-shaped crystals which appeared to be octahedrons (Figure 1).

It can be seen in Table I that, in addition to the crystalline protein, the original extract also contained lysozyme and RNAase activities. The procedure which was used not only resulted in the crystallization of the psoriatic protein from 50% ethanol, it also led to the purification of psoriatic RNAase which was found to be soluble at this ethanol concentration. After a small initial purification, lysozyme activity was progressively lost in the course of the procedure.

Column Chromatography. Alternative procedures for the preparation of the crystalline psoriatic protein were also studied using CM-Sephadex columns. The purification was identical with that previously described through Step 3 (Table I). After the heat treatment step, an aliquot was dialyzed against 0.1 M sodium acetate buffer, pH 5.4, and then applied to a CM-Sephadex column. The column was eluted with a gradient of increasing ionic strength (Figure 2). The column was first eluted with 500 ml of 0.1 M sodium acetate buffer, pH 5.4, in the mixing vessel and 500 ml of 1 M acetate buffer, pH 5.4, in the reservoir vessel. Tubes 18–22 were combined. This fraction did not contain any RNAase or lysozyme activity, but it gave a positive precipitin reaction with rabbit antibodies (see below) to the crystalline protein. The protein was

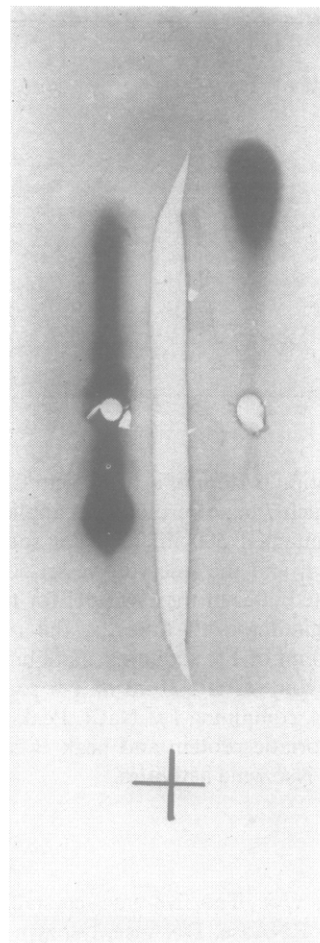

FIGURE 3: Agar electrophoresis (0.1 M Veronal buffer, pH 8.0) of normal human serum (left) and crystalline psoriatic protein (right). The electrophoresis was run on microscope slides for 2 hr using 80 v (constant voltage). The gel was stained with nigrosin.

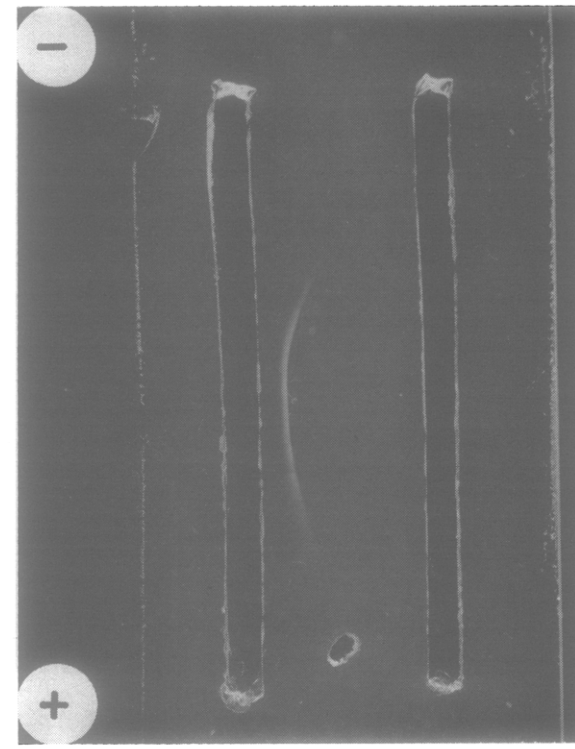

FIGURE 4: Immunoelectrophoresis of crystalline psoriatic protein on microscope slide. The crystalline protein was applied to the center well and submitted to agar electrophoresis for 2 hr using 80 v (constant voltage) and 0.1 M Veronal buffer, pH 8.0. After the electrophoresis, serum from a rabbit which had been immunized with the crystalline protein was placed in the left trough and control rabbit serum from the same animal before immunization was placed in the right trough. The slides were kept at 4° in moist petri dishes for 24–48 hr, at which time the precipitin line became visible.

crystallized by overnight dialysis against 1 l. of 0.02 M Tris–0.1 M NaCl buffer, pH 7.5, and ethanol (1:1, v/v). The protein crystallized exclusively in the form of octahedrons under these conditions. The sample was allowed to stand at 4° for several days in order to get maximum crystallization. The crystals were harvested by centrifugation and dissolved in 0.02 M Tris–0.1 M NaCl buffer, pH 7.5, and then the solution was dialyzed against this buffer overnight. The protein was recrystallized by subsequently dialyzing the sample overnight against 1 l. of 0.02 M Tris–0.1 M NaCl buffer, pH 7.5, and ethanol (1:1, v/v). The sample was again allowed to stand at 4° for several days in order to obtain maximum crystallization. Under these conditions, the protein crystallized in two forms, namely, needles and octahedrons. Therefore, the crystalline forms of the protein appear to be dependent on pH and/or ionic strength.

In order to elute RNAase and lysozyme from the CM-Sephadex column a different eluting-buffer system was used. After tube 28 (Figure 2) the column was eluted with 500 ml of 1 M sodium acetate buffer, pH 5.4, in the mixing vessel and 500 ml of 1 M sodium acetate buffer containing 1 M NaCl in the reservoir vessel. Tubes 39–41 were found to contain both RNAase and lysozyme activities. This fraction also gave a negative precipitin reaction with antibody to the crystalline protein.

Agar Electrophoresis and Immunoelectrophoresis. Although epidermis contains no blood supply it was determined whether the crystalline protein could be a serum protein which could have entered by diffusion, as occurs in a variety of inflammatory dermatoses. Agar electrophoresis of normal human serum and of the crystalline psoriatic protein was carried out on microscope slides using 0.1 M Veronal buffer, pH 8.0 (Figure 3). It was found that the crystalline protein migrated toward the cathode as a single component, and it appeared to be more basic than any serum protein.

Immunoelectrophoresis experiments were also carried out under the same conditions. When horse anti-

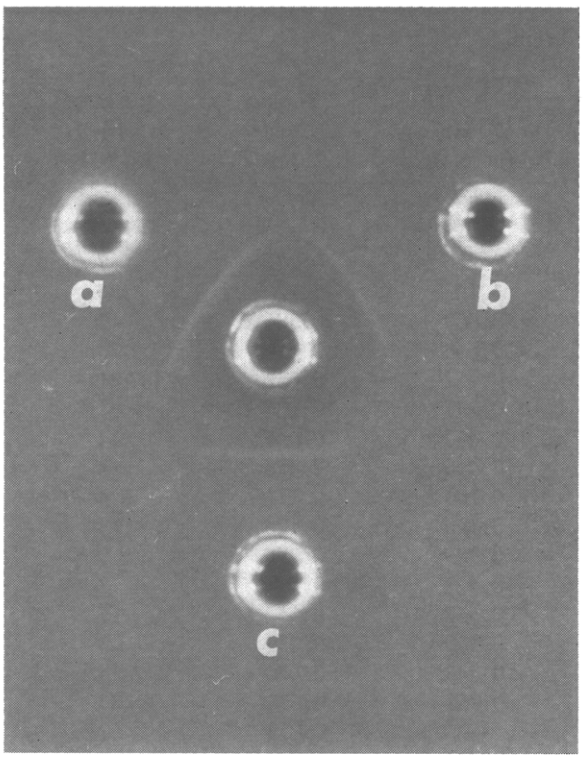

FIGURE 5: Agar double diffusion of (a) crystalline psoriatic protein, (b) extract of acetone powder of psoriatic scales, using 0.02 M Tris–0.1 M NaCl buffer, pH 7.0, and (c) extract of acetone-treated callus using 0.25 N sulfuric acid. Center well contained antibody to the crystalline protein.

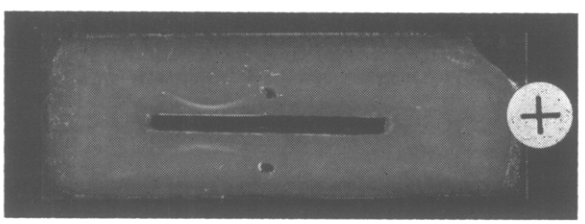

FIGURE 6: Immunolectrophoresis of crystalline psoriatic protein (top) and of callus extract (bottom). The samples were submitted to agar electrophoresis for 2 hr using 80 v (constant voltage) and 0.1 M Veronal buffer, pH 8.0. After the electrophoresis, antibody to crystalline protein was placed in the center trough.

bodies to whole human serum were used, it was found that the crystalline psoriatic protein did not cross react with antibody to any serum protein.

Antigenicity of Crystalline Psoriatic Protein. The crystalline protein was found to be antigenic. The crystalline protein was submitted to agar electrophoresis and immunoelectrophoresis on microscope slides. On immunoelectrophoresis the protein formed

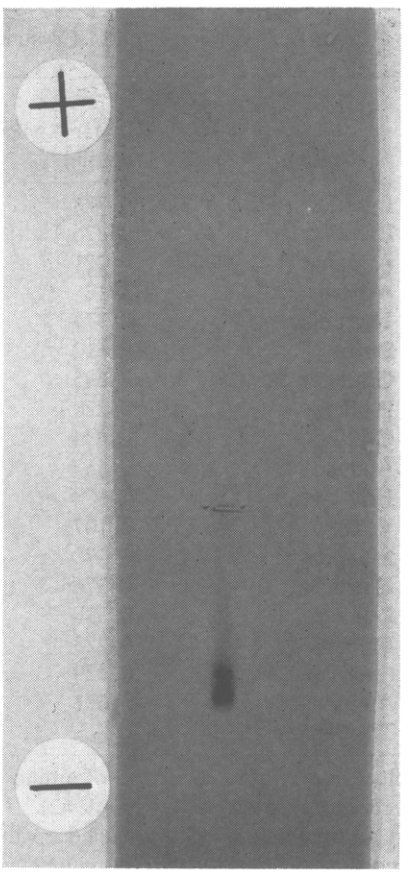

FIGURE 7: Starch gel electrophoresis of crystalline psoriatic protein using discontinuous borate buffer system of pH 8.0. The electrophoresis was carried out for 3 hr on 8 × 12 in. starch plate using 60 ma (constant amperage). The gel was stained with nigrosin.

a single precipitin line with serum from rabbits which had been immunized with the crystalline protein (Figure 4). Concomitant agar electrophoresis of the crystalline protein demonstrated that the migration of the crystalline protein coincided with the area at which the precipitin line was located. In addition, the rabbit antibody did not cross react with normal human serum.

Extraction of Acetone Powders at pH 7.0. In order to determine whether the crystalline psoriatic protein was a derived modified protein product resulting from the rather severe isolation conditions, acetone powder preparations of psoriatic scales were also extracted under much milder conditions. Five grams of an acetone powder preparation of scales was extracted with 18 volumes of 0.02 M Tris–0.1 M NaCl buffer, pH 7.0, at 4° for 20 hr, and the material was then centrifuged at 3000 × *g*. The supernatant solution was recovered and submitted to immunoelectrophoresis on microscopic slides using 0.1 M Veronal buffer, pH 8.0. Antibody to the crystalline psoriatic protein formed a single precipitin

TABLE II: Amino Acid Composition of Crystalline Psoriatic Protein.

Residue	20 Hr (μmole)	40 Hr (μmole)	70 Hr (μmole)	Amino Acid[a] (μmole)	Residues per 27,700 g
Lysine	0.778	0.776	0.778	0.778	25
Histidine	0.226	0.222	0.218	0.229	7
Arginine	0.221	0.222	0.216	0.222	7
Aspartic acid	1.12	1.11	1.12	1.12	35
Threonine	0.377	0.373	0.360	0.386	12
Serine	0.612	0.580	0.540	0.640	20
Glutamic acid	0.645	0.648	0.652	0.652	21
Proline	0.220	0.219	0.237	0.225[b]	7
Glycine	0.444	0.447	0.455	0.455	14
Alanine	0.452	0.456	0.456	0.456	14
Half-cystine	0.108	0.123	0.103	0.111[b,c]	4
Valine	0.107	0.120	0.120	0.120	4
Methionine	0.278	0.280	0.285	0.285	9
Isoleucine	0.376	0.404	0.422	0.422	13
Leucine	0.538	0.545	0.552	0.552	17
Tryosine	0.223	0.239	0.200	0.239	8
Phenylalanine	0.429	0.446	0.443	0.446	14
Ammonia[d]	0.671	0.714	0.753	0.638[f]	20
Tryptophan[e]	—	—	—	0.053	2

[a] Results are expressed as μmoles of amino acid per 0.88 mg of protein extrapolated to zero time hydrolysis or at maximal recovery (Mahowald *et al.*, 1962). [b] Average value. [c] Determination as cysteic acid after performic acid oxidation (Schramm *et al.*, 1954) yielded a value of 0.116. [d] Corrected for ammonia content of reagents. [e] Determined according to procedure of Noltmann *et al.* (1962). [f] Amide ammonia.

line with the extract in an area which coincided with electrophoretic migration of the crystalline protein. The proteins in the pH 7 extract were also concentrated by precipitation at 0.9 ammonium sulfate saturation and the sediment was dissolved in a small volume of 0.02 M Tris–0.1 M NaCl buffer, pH 7.5. The solution was dialyzed against this same buffer and then submitted to Ouchterlony double diffusion in agar (Ouchterlony, 1953). Under these conditions a single precipitin line was formed with antibody to the crystalline protein (Figure 5). Therefore the immunologic evidence indicates that the crystalline protein is also present in extracts which are prepared under milder conditions and, as a result, it can be concluded that it is not a modified protein product.

Presence in Callus. A 0.25 N sulfuric acid extract of 6 g of acetone-treated callus was prepared according to the procedure used for psoriatic scales. The acid extract was brought to 0.9 ammonium sulfate saturation and the sediment was recovered by centrifugation. It was dissolved in 5 ml of 0.02 M Tris–0.1 M NaCl buffer, pH 7.5, and dialyzed against the same buffer. The dialyzed solution was submitted to Ouchterlony double diffusion in agar and to immunoelectrophoresis. On double diffusion in agar, the extract of callus formed a single precipitin line with antibody to the crystalline psoriatic protein (Figure 5). In addition, a single precipitin line was formed on immunoelectrophoresis of the callus extract in an area which coincided with the electrophoretic migration of the crystalline psoriatic protein (Figure 6). This indicates that the crystalline protein is also present in callus and is not specifically found only in psoriatic scales.

Starch Gel Electrophoresis. The crystalline protein was submitted to starch gel electrophoresis at pH 8.0 using a discontinuous borate buffer system. As opposed to the results of agar electrophoresis (Figure 3) it can be seen in Figure 7 that two protein bands were detected, both of which migrated toward the cathode at pH 8.0 and both of which stained with equal intensity with nigrosin (Figure 6). The same electrophoretic results were obtained whether the crystalline protein was prepared by the procedure outlined in Table I or by CM-Sephadex column chromatography. This indicates that the psoriatic protein consists of two molecular species which are closely related chemically.

Molecular Weight Determination. The molecular weight of the crystalline psoriatic protein was estimated by sedimentation equilibrium methods (Yphantis, 1964). The weight-average molecular weight, based on four separate determinations, was calculated to be 27,700 ± 2,000.

Amino Acid Analysis. The results of amino acid analysis of the crystalline psoriatic protein are sum-

marized in Table II. A molecular weight of 27,700 was used for calculation of the amino acid residues. There was a recovery of 95.3% of the weight and 97.5% of the nitrogen. The low content of cyst(e)ine clearly indicates that it is not related to keratin.

Discussion

Epidermal keratinization represents the cellular changes which begin in the basal layer of the epidermis and which result in the formation of the stratum corneum. During the process of normal epidermal keratinization, cytological studies have shown that nuclei, ribonucleoprotein particles, and mitochondria are degraded and, concomitantly, keratin fibrils appear. Psoriasis is a disease manifesting abnormal or incomplete keratinization. As opposed to normal epidermal keratinization, in psoriasis, nuclei and ribonucleoprotein particles are retained.

In studying the relationship of epidermal proteins to normal and abnormal keratinization, psoriatic scales are being used since they represent the product of pathological keratinization. Previous work in this laboratory had shown that psoriatic scales contain a number of low molecular weight proteins (Liss and Lever, 1962). We have now been able to isolate a heat-stable, acid-resistant, crystalline protein from psoriatic scales for which no enzymatic function has been found. The low cyst(e)ine content indicates that it is not related to keratin. The presence of the protein in callus indicates that it is a constituent of stratum corneum, but it may also be present in lower layers of the epidermis. Since the protein could be detected by means of immunologic techniques in extracts which were prepared under milder conditions, it can be concluded that it is not a derived or modified protein product resulting from the rather severe isolation conditions.

The procedure used for the extraction of the crystalline protein from scales also results in the extraction of RNAase and lysozyme. These enzyme contaminants could be completely removed in the course of purification. It was known that psoriatic scales contained RNAase activity in high concentration. The purified enzyme, like pancreatic RNAase, was shown to be of low molecular weight and it had a substrate specificity involving the pyrimidine moieties of yeast RNA (Liss and Lever, 1962).

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