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EPIDERMAL NUCLEASES: PURIFICATION AND CHARACTERIZATION OF RIBONUCLEASE FROM MAMMALIAN EPIDERMIS

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Summary

The major ribonuclease of adult guinea pig epidermis has been isolated and purified over 1000-fold by a combination of ammonium sulfate fractionation, affinity and ion-exchange chromatography, and electrophoresis. The purified enzyme is free from phosphodiesterase and phosphatase activities.

The ribonuclease is optimally active near neutrality in phosphate buffer, with a K_m of 3 μg/ml toward [^{14}C]RNA from Erhlich ascites tumor cells. There are no metal requirements for activity. The enzyme catalyzes the endonucleolytic hydrolysis of high molecular weight yeast RNA and it also hydrolyzes polycytidylic and polyuridylic acids, but not polyadenylic, polyguanylic, and polyinosinic acids. The apparent molecular weight of the active enzyme is 28 500.

Introduction

One of the characteristics of in vitro protein synthetic systems from epidermis or hair roots is a relatively low yield of polyribosomal particles. Sucrose gradient profiles of ribosomal fractions are dominated by monomeric ribosomes over a wide variety of isolation conditions even though polysomes are readily evident in electron micrographs of epidermis or hair roots [1,2]. Since the preparation and homogenization of epidermis is slow and difficult, several processes could occur during homogenization which could account for the low recovery of polysomal material. Among these processes are (1) run-off of ribosomes from the messenger with formation of monomeric ribosomes and subunits, and (2) ribonucleolytic degradation of messenger RNA.

Two types of data seem to favor the second of these hypotheses. Inclusion of cycloheximide during the preparation of ribosomes does not alter the ribosomal pattern on sucrose density gradient centrifugation [1] and addition of antibody directed against pancreatic ribonuclease A increases protein synthesis

in an in vitro system from pancreas which has characteristics similar to those of epidermis and hair roots (Freedberg, I.M., unpublished data).

Epidermis and hair roots are both tissues typified by rapid cellular turnover and differentiation. It is possible that nucleases might play an active part in the biological control mechanisms which maintain the balance between cell multiplication and differentiation. For these reasons we have embarked on a study of epidermal nucleases, reporting in this paper on the purification and characteristics of the predominant ribonuclease of guinea pig epidermis.

Materials and Methods

^{14}C-labeled RNA was extracted from Ehrlich ascites tumor cells (the gift of Dr Edgar Henshaw) grown 24—40 h in the presence of sodium [^{14}C]formate (New England Nuclear; 3.0 Ci/mol). Extraction of the post-mitochondrial supernatant solution was accomplished by the technique of Ravel et al. using hot phenol (85%) containing 0.1% 8-hydroxyquinoline in triethylamine buffer (20 mM, pH 7.5) with 1% sodium dodecylsulfate [3]. Oligonucleotides were removed from the RNA by ethanol precipitation. The resulting polynucleotides had a specific activity of $2.9 \cdot 10^6$ cpm/mg and contained less than 1% DNA as determined by incubation with deoxyribonuclease.

Synthetic polyribonucleotides (polyadenylic acid, polyguanylic acid, polycytidylic acid, polyuridylic acid and polyinosinic acid) were obtained from Miles Laboratories and were used without further purification. Crude yeast RNA was purified by ethanol precipition. Other materials were obtained from the following sources: bovine albumin (Fraction V), α-chymotrypsinogen, cytochrome *c*, myoglobin, ribonuclease A, carbonic anhydrase, phosphodiesterase, alkaline phosphatase, bis-*p*-nitrophenylphosphate and *p*-nitrophenylphosphate from Sigma, special enzyme grade ammonium sulfate and ribonuclease-free ultra pure sucrose from Schwarz/Mann; ovalbumin, soybean trypsin inhibitor and deoxyribonuclease from Worthington Biochemicals; yeast RNA from Nutritional Biochemicals; Sephadex G-25, C-50 and G-75 from Pharmacia; and agarose: 5′(4-aminophenylphosphoryl)uridine 2′(3′)-phosphate from Miles Laboratories.

Enzyme purification

Crude extract. Adult albino guinea pigs were shaved, killed by a blow to the head and skinned. Skin could be stored at this stage without loss of enzyme activity for at least 24 h in the cold in Hanks balanced salt solution containing penicillin (150 units/ml) and streptomycin (150 μg/ml). The skins, dermis side down, were laid flat on a chilled glass plate for epilation with a molten mixture of bees' wax and rosin (4 : 1). Epidermis was removed from 1-cm strips of the epilated skin by stretch-separation [4] and placed immediately in ice-cold 20 mM sodium acetate, pH 5.3 (approx. 10 ml/g epidermis). The epidermal suspension was homogenized in a Potter-Elvehjem homogenizer and centrifuged at 12 000 rev./min and 4° C for 10 min in a Sorvall RC2 centrifuge. Floating debris was removed from the crude homogenate by passage of the supernatant solution over a Millipore prefilter (AP2504200). All further steps of the purification were carried out at 4° C unless otherwise noted. Plastic gloves were worn

throughout the entire purification procedure to avoid introduction of surface nucleases [5] and to minimize the chance of bacterial contamination. Buffers and glassware were routinely autoclaved before use.

Ammonium sulfate fractionation. 20 g of solid ammonium sulfate were added very slowly to every 100 ml of crude extract. The mixture was allowed to stand 30 min before centrifugation at 12 000 rev./min for 10 min. 30 g of solid ammonium sulfate were then added slowly to the supernatant solution obtained from each 100 ml of crude extract and the solution was recentrifuged. The resultant precipitate was suspended in 20 ml of 20 mM sodium acetate, pH 5.3, and dialyzed 24 h against two 1 liter volumes of the same buffer.

Affinity chromatography. A column (0.9 × 15 cm) was prepared from agarose:5′(4-aminophenylphosphoryl)uridine 2′(3′)-phosphate and equilibrated with 20 mM sodium acetate, pH 5.3. The dialyzed ammonium sulfate fraction was diluted to 100 ml with the same buffer and pumped onto the column at room temperature at a rate of 25 ml/h. The column was washed with sodium acetate buffer until the absorbance at 280 nm remained lower than 0.02 for 20 ml. Step-wise elution of the enzyme was accomplished with 20 mM acetic acid followed by 20 mM acetic acid containing 750 mM sodium chloride. Fractions of 3 ml were collected. Activity present in the initial sodium acetate fraction was pooled and rechromatographed. Activity in the acetic acid : salt fraction was pooled and dialyzed for several hours against 20 mM acetic acid and subsequently overnight against 20 mM sodium phosphate, pH 7.15.

CM-Sephadex chromatography. A 0.9 × 14.0 cm column containing Sephadex C-50 was equilibrated with 20 mM sodium phosphate, pH 7.15. The active, dialyzed fraction from affinity chromatography was adsorbed at 15 ml/h, washed with equilibrating buffer and eluted with a 120-ml linear sodium chloride gradient (0—0.3 M). Active fractions were pooled, placed in dialysis bags, concentrated against solid sucrose and stored at —20° C.

Disc gel electrophoresis. Small amounts of highly purified epidermal ribonuclease were prepared as needed by subjecting the concentrated active fractions from the CM-Sephadex chromatography to electrophoresis on polyacrylamide gels [6]. Following electrophoresis, gels were frozen over solid CO_2 and sliced immediately into 1-mm thicknesses. After overnight elution of each disc in 250 µl of 10 mM Tris · HCl, pH 8.1, the active fractions were located by assay. The ascending and descending portions of the active peak were pooled separately, concentrated against solid sucrose and stored at —20° C.

Ribonuclease assays

A. Enzyme purification was monitored by measurement of the release of acid-soluble radioactivity from high molecular weight [^{14}C] RNA. The basic incubation mixture contained 25 mmol of Tris · HCl, pH 8.1, 75 µg of bovine serum albumin, 240 µg unlabeled yeast RNA, 17 000 cpm of ^{14}C-labeled Ehrlich ascites tumor RNA (2.9 · 10^6 cpm/mg) and enzyme in a total volume of 0.35 ml. Incubations were conducted at 37° C in a shaking water bath. Reactions were terminated by addition of 400 µg RNA, 125 µg bovine serum albumin and 100 µl cold 25% trichloroacetic acid to bring the total volume in each assay tube to 0.50 ml. The mixtures were allowed to precipitate at 4° C for 30 min prior to centrifugation for 10 min at 2 000 rev./min and 4° C in an Interna-

tional PR-2 centrifuge. The radioactivity contained in a 300-μl aliquot of the supernatant solution was measured in 5 ml of Bray's solution in a Packard Tricarb liquid spectrometer. 1 unit of enzyme activity is defined as the amount of enzyme required to obtain the release of 1 cpm of acid-soluble ^{14}C radioactivity in a 10-min incubation under the above conditions.

B. Occasional assays on the purified enzyme were performed by measuring the release of acid soluble material with absorbance at 260 nm [7]. Incubation mixtures were similar to those used above except they contained no [^{14}C] RNA.

Phosphodiesterase assays

Assays for phosphodiesterase activity were performed with di-*p*-nitrophenyl phosphate as substrate in Tris · HCl (50 mM, pH 8.1) and in sodium phosphate (50 mM, pH 6.75) [8].

Phosphatase assays

Assays for phosphatase activity were performed with *p*-nitrophenyl phosphate as substrate in Tris · HCl (50 mM, pH 8.1) and in sodium phosphate (50 mM, pH 6.75) [9].

Protein concentration

During early steps of the purification procedure, protein concentration was estimated by the procedure of Lowry et al. with crystalline bovine serum albumin as standard [10]. After chromatographic steps, estimates were made on the basis of optical absorbance at 230 nm with crystalline serum albumin and ribonuclease A as standards. Protein concentrations after electrophoresis were determined by reaction of the acid-hydrolyzed, dialyzed enzyme with ninhydrin, using as standard the Beckman amino acid calibration mixture.

Molecular weight determination

Gel filtration. Sephadex G-75 superfine was used in a 0.95 × 22 cm column which was eluted continuously with 20 mM sodium phosphate (pH 7.15) at a flow rate of 2—3 ml/h. Standardization was accomplished with the following proteins applied in pairs in a total volume of 70 μl: ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, α-chymotrypsinogen, myoglobin, cytochrome *c* and ribonuclease A (the ribonuclease A standard was chromatographed last, after the epidermal nuclease). All protein solutions were dialyzed against the column buffer before application.

Density gradient centrifugation. 5-ml gradients were prepared from RNAase-free sucrose solutions of 7.5 and 15% in 20 mM sodium phosphate (pH 7.15), 100 mM sodium chloride. Protein solutions (60 μl) were layered on top and the tubes centrifuged 24 h at 4° C and 65 000 rev./min in an SW-65 rotor (Spinco L-65 ultracentrifuge). The gradients were scanned at 280 nm on a Gilford spectrophotometer fitted with a flow-cell and fractions of 12 drops collected. Fractions containing cytochrome *c* or myoglobin were read at 418 nm on a Beckman DU spectrophotometer before assay for nuclease activity by Procedure A.

Results

Purification

The data from a typical purification of the major ribonuclease of guinea pig epidermis are presented in Table I. The material eluted from polyacrylamide gels after electrophoresis was purified greater than 1000-fold but only microgram quantities were obtained from a preparation involving the epidermis from 20 adult guinea pigs.

As noted in Methods, homogenates were prepared in sodium acetate buffer (pH 5.2), the buffer of choice for affinity chromatography of beef pancreatic ribonuclease [11]. Although crude extracts of ribonuclease frequently have been prepared in dilute sulfuric acid [12—15], epidermis homogenized in sulfuric acid yielded a ribonucleolytic activity which disappeared rapidly (up to 100% loss in 48 h) while extraction in sodium acetate yielded a stable enzyme. Reextraction with sodium acetate of the crude homogenate pellet produced only minor amounts of additional activity but reextraction with sulfuric acid released substantial amounts of labile activity. This second enzymatic activity could be stabilized by immediate dialysis against sodium acetate but the resultant enzyme had different characteristics during further purification than the one on which we are reporting in this paper.

Affinity chromatography of the crude ribonuclease was originally attempted as described by Wilchek and Gorecki [11]. As expected, most of the activity bound to the resin but release of this activity could not be effected with 200 mM acetic acid. All bound activity did elute if 750 mM sodium chloride was added to the acetic acid. The same elution resulted if the salt was added in 20 mM acetic acid (Fig. 1). Several other authors have recently reported that ribonuclease does not elute from agarose:5′(4-aminophenylphosphoryl)uridine 2′(3′)-phosphate with 200 mM acetic acid and have suggested alternate eluants such as 4 M NaCl in 230 mM sodium acetate (pH 5.2), 200 mM KCl adjusted to pH 2.0 with HCl, or 250 mM phosphoric acid adjusted to pH 3.0 with 1 M NaOH [12—16].

Analytical disc gel electrophoresis of the material eluted from the affinity

TABLE I

PURIFICATION OF GUINEA PIG EPIDERMAL RIBONUCLEASE

Step	mg protein	Units/ml $\times 10^{-3}$	Units $\times 10^{-3}$	% recovery	Spec. act. $\times 10^{-3}$ (units/mg)	Purification
1. Crude homogenate	424	24	3500	100	8.2	1.0
2. Ammonium sulfate	190	79	3400	97	18	2.2
3. Affinity chromatography	4.9	52	3010	86	600	73
4. CM-Sephadex	0.62	714	1820	52	2940	359
5. Disc gel electrophoresis	*	*	*	*	8500	1036

* 50 μg quantities of Step 4 material loaded on to a disc gel yield about 4 μg of purified enzyme. Activity recovery at this stage is variable, depending on how rapidly the sliced gel is assayed for activity and the active fractions pooled. If eluted 18 h before pooling and concentration, activity recovery averages 25%.

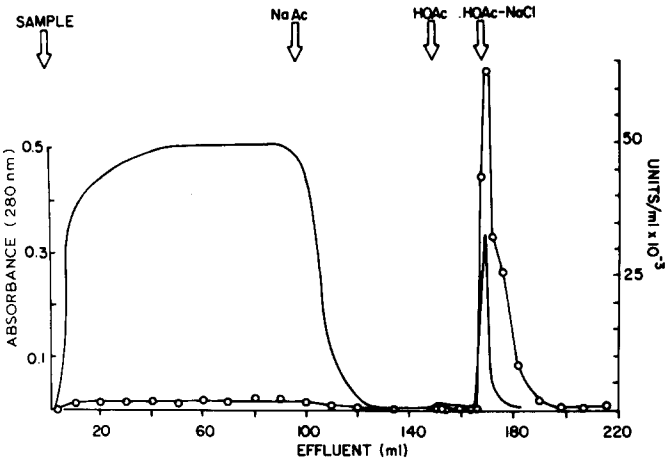

Fig. 1. Affinity chromatography of guinea pig epidermal ribonuclease on agarose: 5′(4-aminophenylphosphoryl)uridine 2′(3′)-phosphate. Column was equilibrated with 20 mM sodium acetate buffer (pH 5.2). See text for details of sample application and elution. ———, absorbance at 280 nm; o———o, ribonuclease activity.

chromatographic resin indicated that the ribonuclease was still impure (Fig. 2). Five or more protein bands were found after staining with Coomassie blue but the ribonuclease activity did not precisely coincide with any of the bands. The enzyme purified by affinity chromatography was therefore dialyzed free of salt, equilibrated with sodium phosphate buffer and subjected to CM-Sephadex chromatography (Fig. 3). Most of the protein did not adsorb to this resin, while approx. 80% of the activity did. Elution with a sodium chloride gradient yielded two peaks of activity at sodium chloride concentrations of 0.17 M and 0.25 M, containing 50% and 10—25% of the applied activity, respectively. The activity eluting at 0.17 M sodium chloride, when concentrated against solid

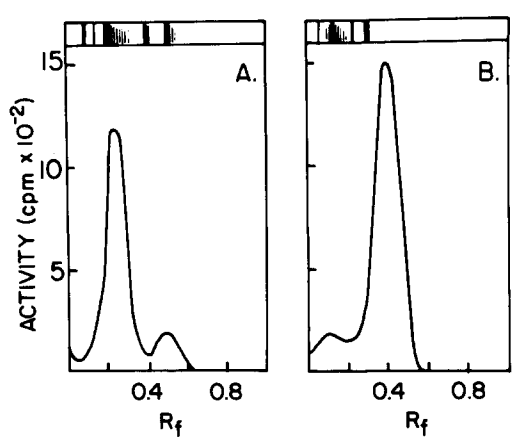

Fig. 2. Disc gel electrophoretic patterns after (A) affinity chromatography on agarose: 5′(4-aminophenylphosphoryl)uridine 2′(3′)-phosphate and (B) CM-Sephadex chromatography. The lower part of each figure is a plot of the eluted activity, the upper part a schematic representation of the protein banding pattern with Coomassie blue. Substantial activity was present in the stacking gel of A.

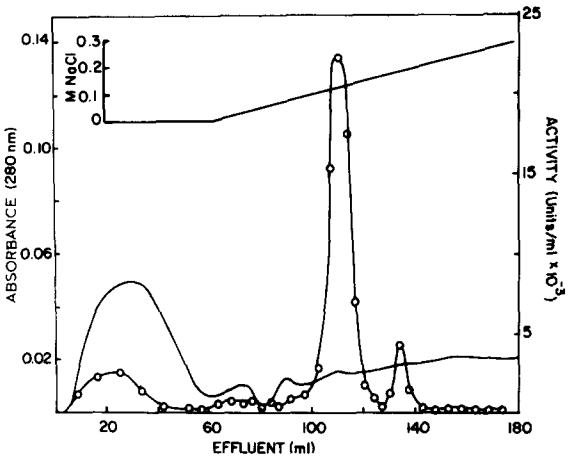

Fig. 3. CM-Sephadex chromatography of guinea pig epidermal ribonuclease. Column was equilibrated with 20 mM sodium phosphate buffer (pH 7.15). After sample application, the enzyme was eluted with a 0—0.3 M NaCl gradient. See text for details. ———, absorbance at 280 nm; ○———○, ribonuclease activity.

sucrose, maintained at least 75% of its activity when stored at −20° C for 6 months.

Small amounts of very highly purified enzyme were obtained by subjecting aliquots of the 0.17 M CM-Sephadex eluate to disc gel electrophoresis (Fig. 2). The enzyme as eluted from the gels is dilute and unstable. Subsequent concentration against sucrose and storage at −20° C yields preparations which are stable (50% activity after 4 months) if not subjected to repeated freezing and thawing.

pH optimum

The epidermal ribonuclease following chromatography on agarose:5′(4-aminophenylphosphoryl)uridine 2′(3′)-phosphate displays a pH optimum of 8.1 in Tris · HCl buffer. Hence, this pH was chosen to monitor the purification process. The most highly purified enzyme, however, is optimally active at pH 6.75 in phosphate buffer (Fig. 4). pH 6.75, therefore, was used to determine enzymatic parameters.

Effect of cations

Inclusion of sodium chloride, potassium chloride, calcium chloride, magnesium chloride or EDTA at 0.5 or 5 mM concentrations in the assay medium did not affect enzymatic activity. Higher concentrations of these compounds (50 mM) slightly depressed activity although it is possible this change reflected a conformational effect on the substrate.

Substrate specificity

The substrate specificity was studied with several natural and synthetic polynucleotides. At concentrations of 0.5 mg/ml, digestion occurred in the following order: polycytidylic acid > yeast RNA > polyuridylic acid (Fig. 5). Polyadenylic, inosinic and guanylic acids were not digested. Sephadex G-25

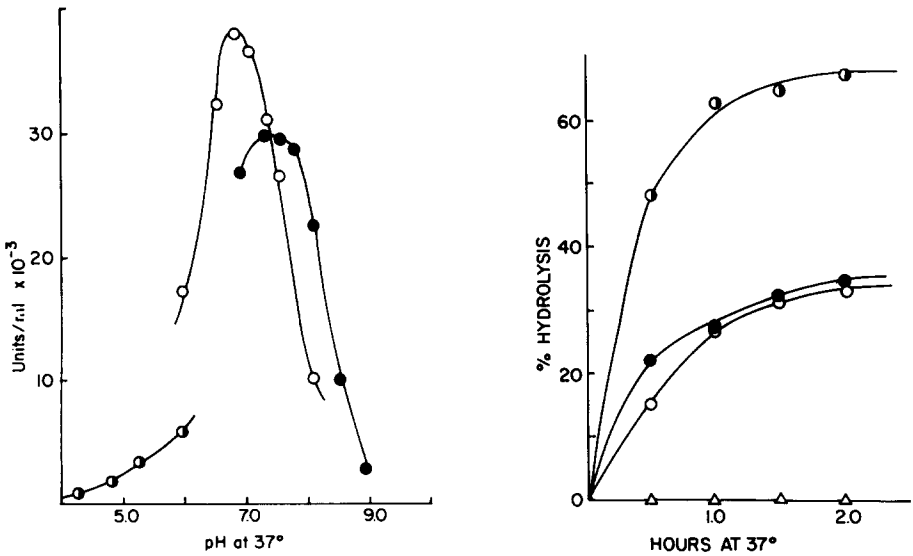

Fig. 4. pH activity curve of guinea pig epidermal ribonuclease. Enzyme = pool of leading edge (anode) of disc gel peak. ●———●, 0.1 M Tris · HCl buffer; ○———○, 0.1 M sodium phosphate buffer; ◑———◑, 0.1 M sodium acetate buffer. pH was varied as indicated.

Fig. 5. Hydrolysis of polynucleotide substrates by guinea pig epidermal ribonuclease. Assays were conducted by Method B as described under Materials and Methods. Each tube contained 7 μg/ml enzyme. ○———○, polyuridylic acid; ◑———◑, polycytidylic acid; ●———●, yeast RNA; △———△, polyadenylic, polyguanylic, polyinosinic acid.

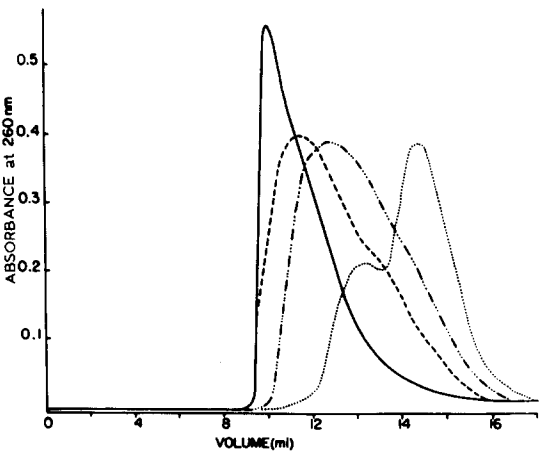

Fig. 6. Chromatography on Sephadex G-25 of partial enzymatic digests of yeast RNA. Following incubation at 37°C for the times indicated, aliquots of the digest were removed, held 5 min at 100°C, chilled rapidly and frozen. The samples were subsequently applied to a 0.9 × 14 cm G-25 column equilibrated with 10 mM ammonium bicarbonate (pH 7.8) and eluted with the same buffer at 30 ml/h. Absorbance at 260 nm was automatically recorded on a Honeywell recorder attached to a Beckman DB spectrophotometer fitted with a flow-cell. ———, 0 h; - - - - - -, 2 h; —·—·—, 4 h; . . . . . ., 0.3 M KOH hydrolyzate (18 h, 37°C) of yeast RNA.

analysis of partial enzymatic digests of yeast RNA indicates a gradual decrease of molecular weight (Fig. 6). A mononucleotide peak was not seen at 6 h, a time at which significant digestion of the substrate had occurred. By 20 h the substrate was reduced to low molecular weight material including mononucleotides. This pattern is compatible with endonucleolytic hydrolysis of the substrate.

Michaelis-Menton constant

The epidermal ribonuclease had a K_m of approx. 3 μg/ml with ^{14}C-labeled Ehrlich ascites tumor RNA as substrate.

Phosphodiesterase and phosphatase activity

No activity was demonstrated towards di-*p*-nitrophenylphosphate or *p*-nitrophenylphosphate at pH 6.75 or pH 8.1.

Molecular weight

The apparent molecular weight of the epidermal ribonuclease as determined either by gel filtration on Sephadex G-75 or by migration in a 7.5—15% sucrose gradient was 28 500.

Discussion

In normal epidermis RNA and DNA are metabolically degraded during differentiation, with only water-soluble products demonstrable in keratinized structures. Since the nucleases which effect this degradation may be central to the overall biological control processes in this tissue, these enzymes have been the subject of much investigation.

Early histochemical studies on normal human epidermis located high ribonuclease activity at the transitional region between non-keratinized and keratinized epidermis and in the upper layers of the stratum corneum [17]. Further histochemical attempts to specify cellular or subcelluar ribonuclease localization have suggested a predominantly perinuclear localization throughout the epidermis, with generally stronger reaction in the granular layer and around hair follicles [18,19].

Ribonuclease activity has been demonstrated biochemically in epidermal homogenates from rat, guinea pig and man, as well as in saline rinses of their skin surfaces [5,20,21]. pH profiles have been determined in crude extracts, and in each case the optimum has been in the alkaline range [8,9] with little acid activity noted [5,20,21].

Ribonuclease activity is elevated in several cutaneous diseases, most notably psoriasis. The enzyme from psoriatic scales, purified 25-fold, displayed several protein bands on starch gel electrophoresis but appeared monodisperse during ultracentrifugation, with a sedimentation coefficient similar to that of ribonuclease A [22]. Double-diffusion studies in agar, however, indicated that immunologically the material was distinct from ribonuclease A [22].

Since there have been no further reports of attempts to purify and study epidermal ribonucleases, we undertook the studies reported in this paper. Affinity chromatography, a technique which can give rapid and quantitative en-

zyme purification [23,25] was attempted and found to be only partially successful for the purification of epidermal ribonuclease. Subsequent chromatography and electrophoresis did yield limited amounts of a highly purified ribonuclease which appeared similar to ribonuclease A except for an apparent molecular weight of 28 500. These findings are similar to those reported for the major ribonuclease of bull semen [26].

The major epidermal ribonuclease which we have extracted with sodium acetate at pH 5.2 displays three peaks of ribonuclease activity when chromatographed on CM-Sephadex. In addition, further extraction of the pellet from the sodium acetate homogenization with dilute sulfuric acid releases activity which sometimes approached in amounts that released initially in sodium acetate. This activity, which is not precipitated by ammonium sulfate at 50% concentration (w/v) and which does not adsorb to agarose: 5′(4-aminophenylphosphoryl)-uridine 2′(3′)-phosphate when applied in sodium acetate (pH 5.2), has as yet not been further analyzed.

It thus seems probable that a number of different ribonucleases exist in epidermis, a situation known to occur in several other tissues. It will be important to isolate the various enzymes, to compare their characteristics, and to determine their cellular and subcellular localization by a technique such as fluorescent antibody staining. However, the amount of purified major enzyme obtained by the procedures reported in this paper at present is too low for the preparation of the requisite antibodies for such a study.

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