Acta derm.-venereol. 49: 468–475, 1969

PROTEOLYTIC ENZYMES IN THE SKIN

II. A comparative study of skin homogenates of five mammalian species

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Introduction

Current knowledge of the peptide hydrolases (E.C. 3.4) in the skin has been recently reviewed (7). It was pointed out that information on the pattern of these enzymes in the skin of any species is far from complete, and that little is known on the exact chemical characteristics of the individual enzymes. While animals of several species have been used as test objects in various studies differences in methods and in experimental conditions have made comparisons between the different species difficult. Directly contradictory results are sometimes found in the literature. Studies between species in which uniform methodology has been used are very few (*e.g.* 1, 4, 5). Furthermore, they are limited either with respect to the number of species studied or to the design of the experiments undertaken. Thus, hydrolysis of each substrate has often been measured at one pH-value alone, based on the assumption that all of the species show a similar pH-profile in the enzymic hydrolysis of the substrates.

In the present study we have measured the hydrolysis of several substrates, commonly used in studies of skin proteolytic enzymes, in a wide pH range so as to obtain a more adequate comparison between the species and to establish data which could explain some apparent contradictions in the findings of earlier investigators.

Material and Methods

Skin samples

Several samples (2–6) of skin from rat, cat, guinea pig and hog dorsum were obtained from freshly decapitated animals. Samples (4) of normal human trunk skin were obtained in the operating room.

The skin was freed from the underlying fascia, muscle and adipose tissue, minced with scissors and homogenized with an Ultra-Turrax homogenizer for one minute in ten volumes (w/v) of 10 mM phosphate buffer pH 8 containing 8 per cent of potassium chloride. The sample tube was kept in crushed ice during the procedure to keep the temperature low.

The crude homogenate was centrifuged at 10,000 g for 20 minutes in an International Refrigerated Centrifuge at +2°C. The supernatant was used in further studies and is referred to as homogenate.

Substrates and buffers

Casein[1] nach Hammarsten was dissolved in 10 mM phosphate buffer pH 8.0 (1 g/100 ml) and denaturated by boiling for 10 min. This stock solution was stored at +4°C,

[1] E. Merck AG, Darmstadt, Fed. Rep. Germany.

This investigation was supported by a research grant from the Sigrid Jusélius Foundation and from the Finnish Medical Council. It forms a part of the research project of the Skin Biology Research Unit (SBRU) in Turku.

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not longer than 5 days. N^{α}-benzoyl-DL-arginine 2-naphthylamide · HCl[2] was dissolved in methanol and diluted with distilled water (1 : 4) to a final concentration of 5 mM. Stock solutions (10 mM) of L-tyrosine ethyl ester · HCl[2] and N-acetyl-L-tyrosine ethyl ester[2] were made in distilled water and were used immediately. These substrates are referred to as casein, BANA, TEE and ATEE.

The universal buffer of Britton and Robinson (2) pH 3.0–12.0 was used throughout the study.

Assay methods

For the measurement of the hydrolysis of the substrates by the skin homogenates the incubation medium consisted of 0.5 ml of buffer solution, 0.5 ml of substrate stock and 0.5 ml of skin homogenate.

With *casein* as substrate the mixture was incubated in a water-bath at 37°C for 60 min, except in assays on the pig skin homogenate with which incubation was carried out for 10 hours. After incubation 0.5 ml of 5 per cent trichloracetic acid was added and the mixture was centrifuged for 15 min at 5000 g. Trichloracetic acid soluble peptides in the supernatant were estimated according to the method of Folin and Ciocalteu (3) reading the optical density at 750 nm. A standard curve for the hydrolysis of casein was prepared from tyrosine solutions. The activity is expressed as the amount of tyrosine liberated by the enzyme preparation in a unit of time (nmole/min/mg protein).

With BANA as substrate incubation time was 3 to 10 hours. Deproteinization was carried out by adding 0.5 ml of 10 per cent zinc sulphate followed by centrifugation. Naphthylamine liberated enzymically was estimated using azo reaction with diazonium salt Garnet GBC[3] and reading the optical density spectrophotometrically at 525 nm (8). Standard curve for the hydrolysis of BANA was prepared by using various amounts of 2-naphthylamine instead of the substrate in the assay solution. The enzymic activity is expressed as the rate of naphthylamine liberation (nmole/min/mg protein).

In the assays with TEE and ATEE as substrate the incubation time was 20–30 min. The proteins were precipitated with zinc sulphate as presented. Hydrolysis of the substrates was estimated by measuring the amount of the residual unhydrolysed substrate according to the principles of Hestrin (6), taking the spectrophotometric readings at 540 nm. Standard curves for the hydrolysis of the esters were prepared from graded concentration series of the substrates. The enzymic activity is expressed as the rate of substrate hydrolysis (nmole/min/mg protein).

The actual pH in the incubation mixtures was measured in the middle of the incubation period with a pH-meter equipped with micro-electrodes. The enzymatic hydrolysis of the substrates was found to be linear in time under the experimental conditions employed. The contribution of spontaneous hydrolysis of the substrates was eliminated by using controls with boiled enzyme.

Protein determinations

The protein concentration of the homogenates was measured according to Lowry *et al.* (9). Bovine serum albumin[4] was used as standard.

Results

Hydrolysis of casein

Rat: The pH-curve for the hydrolysis of casein by the rat skin homogenate is presented in Fig. 1. The curve presents several points of maxima located both at the acid and alkaline side of neutrality. In repeated experiments four separate hydrolysis optima were recognized, *i.e.* roughly at pH 6.0, 7.5, 8.5 and 9.2. The relative height of the individual peaks varied when using skin samples from different animals.

[2] Sigma Chem. Comp., St. Louis, USA. [3] E. Gurr Ltd., London.
[4] Poviet Producten, Amsterdam.

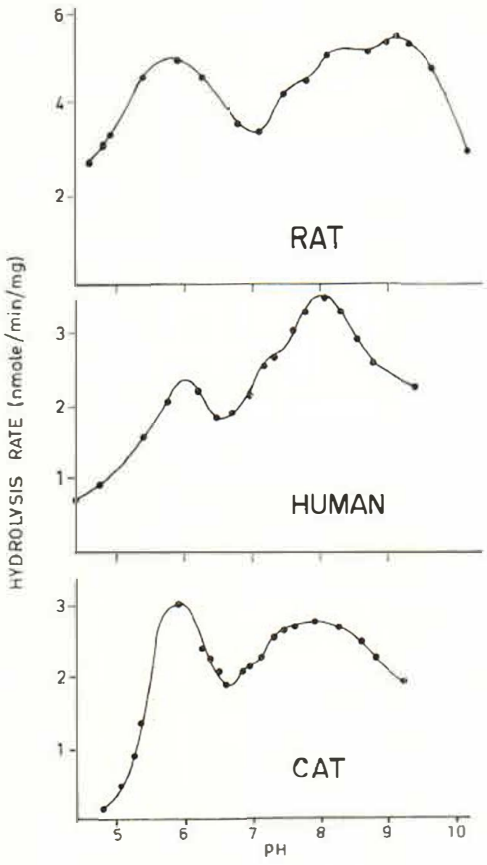

Fig. 1. Hydrolysis of casein by the homogenates of rat, human and cat skin. Experimental conditions as given in Material and Methods.

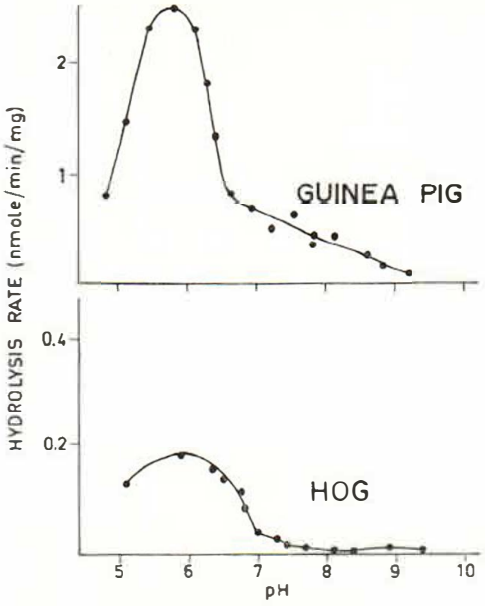

Fig. 2. Hydrolysis of casein by the homogenates of guinea pig and hog skin. Experimental conditions as given in Material and Methods.

Man: The pH-dependence of casein hydrolysis by the human skin homogenate is seen in Fig. 1. The hydrolysis rate at any pH was roughly one half of that found in the case of the rat skin. pH-optima were found roughly at pH 6.0, 7.5 and somewhat above 8.0.

Cat: The hydrolysis rate around neutrality (Fig. 1) was roughly equal to that found in the human skin. A definitive pH-optimum was found at about pH 6. At the alkaline side a broad bell-shaped curve was obtained, with optimum at about pH 8, and no clearcut division into more numerous optima could be observed.

Guinea pig. The pH-curve is presented in Fig. 2. The hydrolysis rate around neutrality was low in comparison to that found in rat, man and cat skin. The hydrolysis of casein took place preferentially at the acid side of neutrality with a prominent peak roughly at pH 6.0. A very low rate of hydrolysis with no distinct optimum was obtained at the alkaline side.

Hog: The rate of hydrolysis of casein by the hog skin homogenate was very low at any pH, roughly one tenth of that found in the guinea pig, as seen in Fig. 2. The form of the pH-curve resembles that found in the guinea pig skin with main hydrolysis at the acid side, around pH 6.

Hydrolysis of BANA

Rat: This substrate was hydrolysed very slowly and an incubation time of even up to ten hours was needed in order to obtain reliable optical densities for the spectrophotometric measurements. The pH-curve obtained with the rat skin homogenate is presented in Fig. 3. The hydrolysis was obtained mainly on the acidic side of neutrality. One peak was recorded at a pH somewhat below 6 and another lower optimum somewhere between pH 6 and 7.

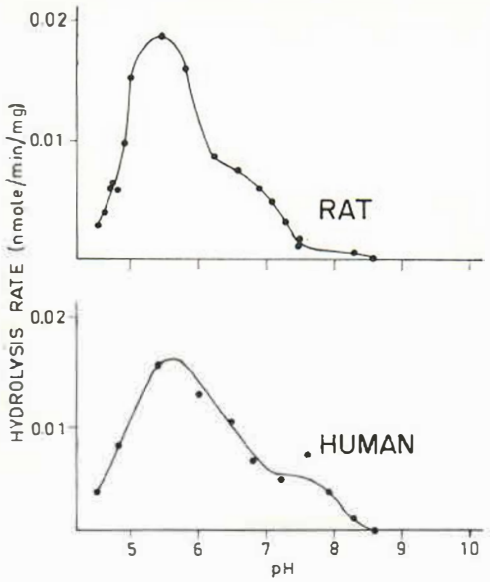

Fig. 3. Hydrolysis of BANA by the homogenates of rat and human skin. Experimental conditions as given in Material and Methods.

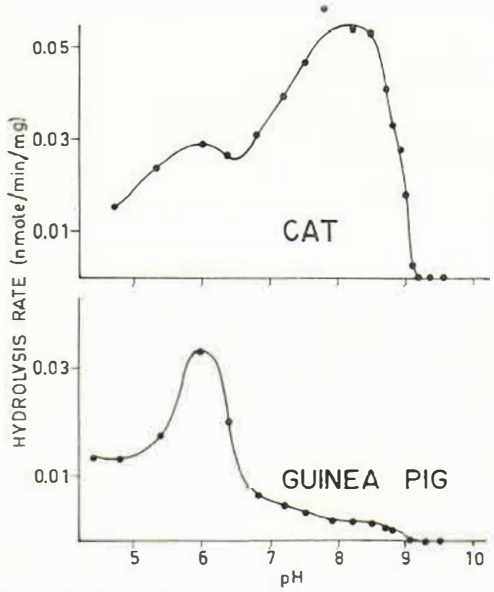

Fig. 4. Hydrolysis of BANA by the homogenates of cat and guinea pig skin. Experimental conditions as given in Material and Methods.

Man: The shape of the pH-curve given in Fig. 3 resembles that obtained from the rat, having a higher maximum at pH somewhat lower than 6 and a more alkaline lower one. In this case, however, the second optimum seems to lie further on the alkaline side, between pH 7 and 8.

Cat: The pH-curve obtained with the cat skin homogenate differs markedly from those of rat and man, as seen in Fig. 4. Two pH optima, one on the acidic side and one on the alkaline side was recognized. The enzyme(s) active on the acidic side had an optimum at about pH 6 and the hydrolysis rate was about equal to that found in the rat and the human skin homogenates at the same pH. The hydrolytic activity at the alkaline side was markedly higher and its pH optimum was somewhere around pH 8.

Guinea pig: The peak of hydrolysis of BANA by guinea pig skin homogenate (Fig. 4) showed hydrolysis at the acidic side of neutrality with a maximum at about pH 6, like the other species. Very little hydrolysis was obtained at the neutral and alkaline range.

Hog: No measurable hydrolysis could be obtained, even after an incubation for 20 hours.

Hydrolysis of TEE

Rat: The hydrolysis of this substrate by the rat skin homogenate was relatively fast, and the pH curve showed two possible maxima, somewhere below pH 8 and around pH 9, as seen in Fig. 5.

Man. Around the neutrality the rate of the hydrolysis by the human skin homogenate was roughly half of that obtained in the rat. The main activity was measured between pH 7 and 9, where a nonsymmetric peak was recorded. A lower activity was observed at higher pH, between 9 and 10 (Fig. 5).

Cat: The hydrolysis of TEE by the cat skin homogenate took place at a clearly wider pH range, i.e. between pH 6 and 10, suggesting optima at pH 7 and 9, as revealed in Fig. 5.

Guinea pig: The guinea pig skin homogenate hydrolysed this substrate more slowly than the homogenates of the other species. A broad pH curve suggested two optima, one around pH 7 and the other around pH 8, as seen in Fig. 6.

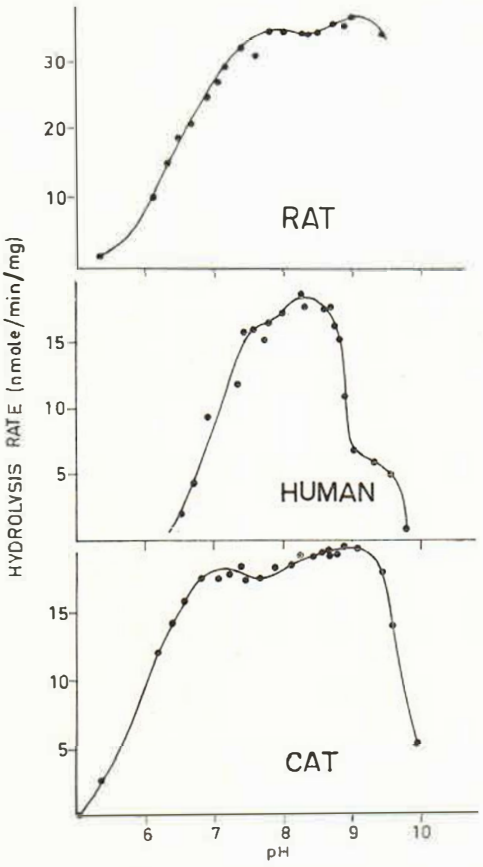

Fig. 5. Hydrolysis of TEE by the homogenates of rat, human and cat skin. Experimental conditions as given in Material and Methods.

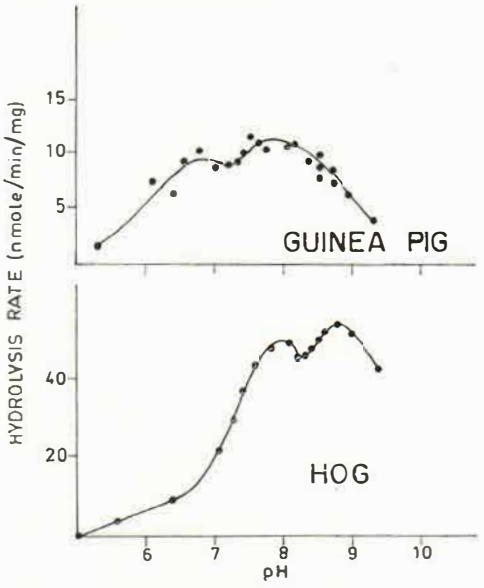

Fig. 6. Hydrolysis of TEE by the homogenates of guinea pig and hog skin. Experimental conditions as given in Material and Methods.

Hog: Hydrolysis of TEE by the hog skin homogenate was obtained almost exclusively above neutrality. Two possible maxima can be detected, one at about pH 8 and the other one at about pH 9, as seen in Fig. 6.

Hydrolysis of ATEE

Rat: Also this substrate was hydrolyzed by the rat skin homogenate mainly at the alkaline side, between pH 7 and 10. Repeated experiments suggested the presence of two alkaline pH optima, one at about pH 7.4–7.9 and another at about pH 8.8–9.2, with fairly intense enzyme activity and a clearly lower activity optimum at about pH 6. The results of one of the experiments is shown in Fig. 7.

Man: The human skin homogenate hydrolyzed this substrate optimally around pH 8 and 9, and practically no hydrolysis was measured at pH 6. The pH curve is seen in Fig. 7.

Cat: The broad pH curve showed two possible maxima, one at about pH 7 and another one at about pH 9 (Fig. 7). The hydrolytic activity was fairly high.

Guinea pig: The hydrolytic activity was centered around neutrality, *i.e.* between pH 6 and 8. A lower activity was observed at about pH 9, but was not recorded in all samples (Fig. 8).

Hog: Good activity was measured between pH 7 and 10, the pH activity curve showing two bell shaped optima, one of them between pH 7 and 8 and the other between pH 9 and 10 (Fig. 8).

Discussion and Conclusions

The skin homogenates of all the species studied showed proteolytic activity. The profiles of the pH curves for the hydrolysis of *casein* were, however, quite differing and the only consistent finding was the presence, in all homogenates, of an enzymic activity operating optimally at about pH 6.

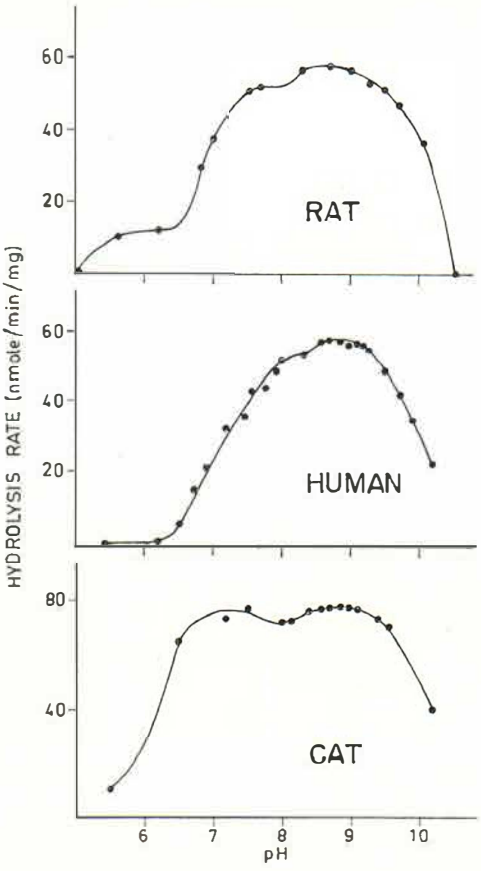

Fig. 7. Hydrolysis of ATEE by the homogenates of rat, human and cat skin. Experimental conditions as given in Material and Methods.

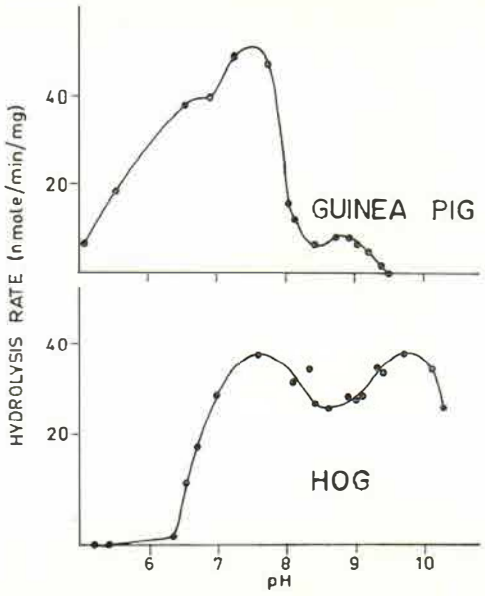

Fig. 8. Hydrolysis of ATEE by the homogenates of guinea pig and hog skin. Experimental conditions as given in Material and Methods.

The hydrolysis rate of casein at this pH was about equal in all of the homogenates studied, except for the hog skin homogenate, which exhibited an activity of only one tenth of that found in the other species. The species with the enzyme pattern most closely resembling that of man were the rat and the cat. On the other hand the pattern of caseinolytic proteinases in the guinea pig and the hog skin is quite different from that found in the human skin.

These findings are in general agreement with the data for the hydrolysis of casein at pH 7.5 by the skin homogenates of several species reported by Golubow *et al.* (5). To our knowledge no extensive species comparisons on the pH dependence of the proteolytic activity of the skin have been reported previously.

BANA, a chromogenic substrate for endopeptidases (proteinases) was hydrolysed by more than one enzymic activity in all of the species studied, except for the hog, which did not hydrolyse this substrate measurably. All of the species showed a hydrolytic peak at around pH 6. This kind of proteolytic activity coincides as to its pH-optimum with that found with casein as substrate at about pH 6, and the same proteolytic enzymes may have acted on BANA and casein. The more alkaline optima at pH 7.5 in the human skin and at about pH 8 in the cat skin do also coincide with caseinolytic optima.

The hydrolysis of BANA, which is a substrate readily hydrolysed by proteases resembling bovine trypsin in their substrate specificity, suggests the presence of proteases of this kind in the skin of the above species. The hydrolysis of BANA by skin homogenates has not been studied earlier even while some earlier investigators have used BAME, BAEE, TAME or TAEE as substrates (10, 13, 14, 15). These substrates are generally split by enzymes attacking also BANA. The latter substrate is, however, to be preferred since it is chemically

more closely related to natural peptides and consequently is a more specific substrate than the corresponding esters.

The ester substrates TEE and ATEE were also hydrolysed by the skin homogenates of all of the different species studied. This is in agreement with the findings of Golubow *et al.* (5) who tested the hydrolysis of TEE at pH 6.5 and that of ATEE at pH 8.0, only. They did not, however, find any activity toward TEE in their human samples and activity toward ATEE was found in only part of the samples.

The pH-dependence of the hydrolysis of these substrates showed marked variations from species to species. These substrates were hydrolysed mainly at the alkaline pH range, the hydrolysis of ATEE by the guinea pig, however, presenting a marked exception. Some of the pH optima appear to coincide with caseinolytic peaks, while some of them did not have any corresponding optimum in the pH curves of caseinolysis. The correspondance between the esterolytic and caseinolytic pH optima was rather good in the rat skin and to some degree observable in the human and the cat skin, while no correspondance whatsoever was found in the guinea pig and the hog skin homogenates in which enzymic proteolysis was optimal at acid pH while the esters were hydrolysed optimally at alkaline pH. The hydrolysis rate of the ester substrates by the hog skin homogenate was equal to that found in other species, even while the caseinolysis was less than one tenth of that found in other species.

These findings point out that TEE and ATEE are possibly hydrolysed in mammalian skin homogenates by some proteolytic enzymes, as reported by Martin and Axelrod for the rat skin (11, 12), but also by several esterolytic enzymes with minimal caseinolytic activity. Therefore, these substrates cannot be used to measure proteolytic activity without prior knowledge of the characteristics of the individual enzymes of the total enzyme pattern. Thus, earlier data collected using these or other esters as substrate cannot be unequivocally interpreted in the terms of proteolytic enzymes.

The diversity and complexity of the skin enzyme pattern in these species, as disclosed by the variability in the profiles of the pH-curves for the hydrolysis of casein, BANA, TEE and ATEE by the skin homogenates forces to *conclude* that meaningful comparisons between species cannot be made by assaying the enzymic activities at only one pH or at a narrow pH-range, as has been often done. Equally, studies at limited pH-ranges can give only incomplete information on the possible changes in the activity of proteolytic or other hydrolytic enzymes in different experimental or pathological conditions.

Our data suggest that the rat is a suitable laboratory animal for studies on skin proteinases since the enzyme pattern in this species, more than in the others, resembles that found in the human skin. On the other hand, the deviations of the other species from the human enzyme pattern render these less suitable for studies intended to clarify chemical problems similar to those encountered in the physiology and pathology of the human skin. The hog, the skin of which resembles grossly as well as microscopically the human skin more than the skin of the other species, deviates more than the other species from the human skin as to the proteolytic enzyme pattern.

It is quite evident that such an enzymic complex as is present, *e.g.* in the rat skin homogenate, cannot be intelligibly studied until specific assay methods for each one of the enzymes are available. Specific assay methods can, on the other hand, be developed only after the separation and thorough characterization of the individual enzymes. Elaborate studies in this line on rat skin proteinases were carried out by Martin and Axelrod (for ref. see 7.) about a decade ago, using the enzymologic methods of that date. Their studies have, however, not been repeated nor their findings verified in other investigations. More efficient chemical separation techniques have since been developed, allowing more extensive purification and thus more thorough characterization of enzymes. Results from studies employing modern techniques of enzyme research applied to the study of rat skin proteolytic

enzymes will be reported by the present authors in later publications.

SUMMARY

The hydrolytic activity of the skin homogenate of man, rat, cat, guinea pig and hog was compared throughout the pH-range 5.0–10.0 with casein, N-benzoyl-arginine 2-naphthylamide (BANA), tyrosine ethyl ester (TEE) and N-acetyl tyrosine ethyl ester (ATEE) as substrates. With casein as substrate, the pH-curve revealed four possible maxima in the rat skin, three in the human and cat skin and only one in the guinea pig and hog skin. All of the species showed a distinct activity peak at about pH 6.0. With BANA as substrate, two optima were revealed in the rat, human and cat skin, one optimum in the guinea pig skin, and no hydrolysis in the hog skin. One of the optima in each species was at about pH 6.0. With TEE and ATEE as substrate, hydrolysis was obtained at the alkaline side of neutrality and 2–3 possible optima, depending on the species, were recorded in the pH-curves. The pattern of the enzymes active toward the substrates used in this study varied markedly from species to species, the enzyme pattern in the rat skin resembling that of the human skin more than did that of other species. Rat skin was suggested to be used in experimental studies on proteolytic enzymes intended to clarify phenomena similar to those encountered in the physiology and pathology of the human skin.

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