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I hereby recommend that the thesis prepared under my supervision by Peter Robert Buechler

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be accepted as fulfilling this part of the requirements for the degree of Doctor of Philosophy

Approved by:

Fred W. Flaherty

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A STUDY OF THE SULFUR DISTRIBUTION IN CATTLE HAIR AND EPIDERMIS

A THESIS

PRESENTED TO THE FACULTY

OF THE

GRADUATE SCHOOL OF ARTS AND SCIENCES

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By

PETER R. BUECHLER

B.S., Fordham University, 1941

M.S., University of Denver, 1946

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ABSTRACT

In unhairing operations, the tanner desires a dissolution of the epidermis with a minimum breakdown of hair and corium tissues. Comparatively little is known about the chemical structure of epidermis, due to practical difficulties in obtaining relatively complete, unmodified, uncontaminated samples of this tissue. Epidermis for this work was obtained from three animals by two methods calculated to yield a minimum of modification. These methods were (1) removal at less than 10°C with dilute sodium chloride solutions, with precautions taken against bacterial action, and (2) removal by the action of distilled water at 60°C. for a period of ten minutes.

These epidermal tissues contained all four cellular layers--germinativum, granulosum, lucidium, and corneum. They contained accessory epidermal structures such as sebaceous glands and sweat gland ducts. Microscopic examination showed them to be free of hair and corium tissues. Hair from the same animals was analyzed simultaneously to permit a comparative evaluation. Quantitative analyses were conducted by semi-micro, micro, and colorimetric methods for the following constituents: Moisture, ash, lipid, total nitrogen, total sulfur, cystine, total disulfide, methionine, sulfur precipitable as sulfate

from the hydrolysates, and total sulfur in the ash. Qualitative tests for H_2S and SO_2, to see if these were evolved during hydrolysis, and Molisch tests for carbohydrate were also conducted.

The total sulfur of the hair can be accounted for as cystine sulfur and methionine sulfur. This is not the case in epidermis. Evidence is presented for the existence of a disulfide other than cystine in epidermis. This may be homocystine, whose existence in nature has been postulated, but which has not heretofore been found.

The analytical values for hair and for epidermis were very different. Epidermis varies not only from hair but also from any keratin so far analyzed, insofar as the constituents determined in this investigation are concerned. This confirms Block's statement that epidermis is not a eukeratin.

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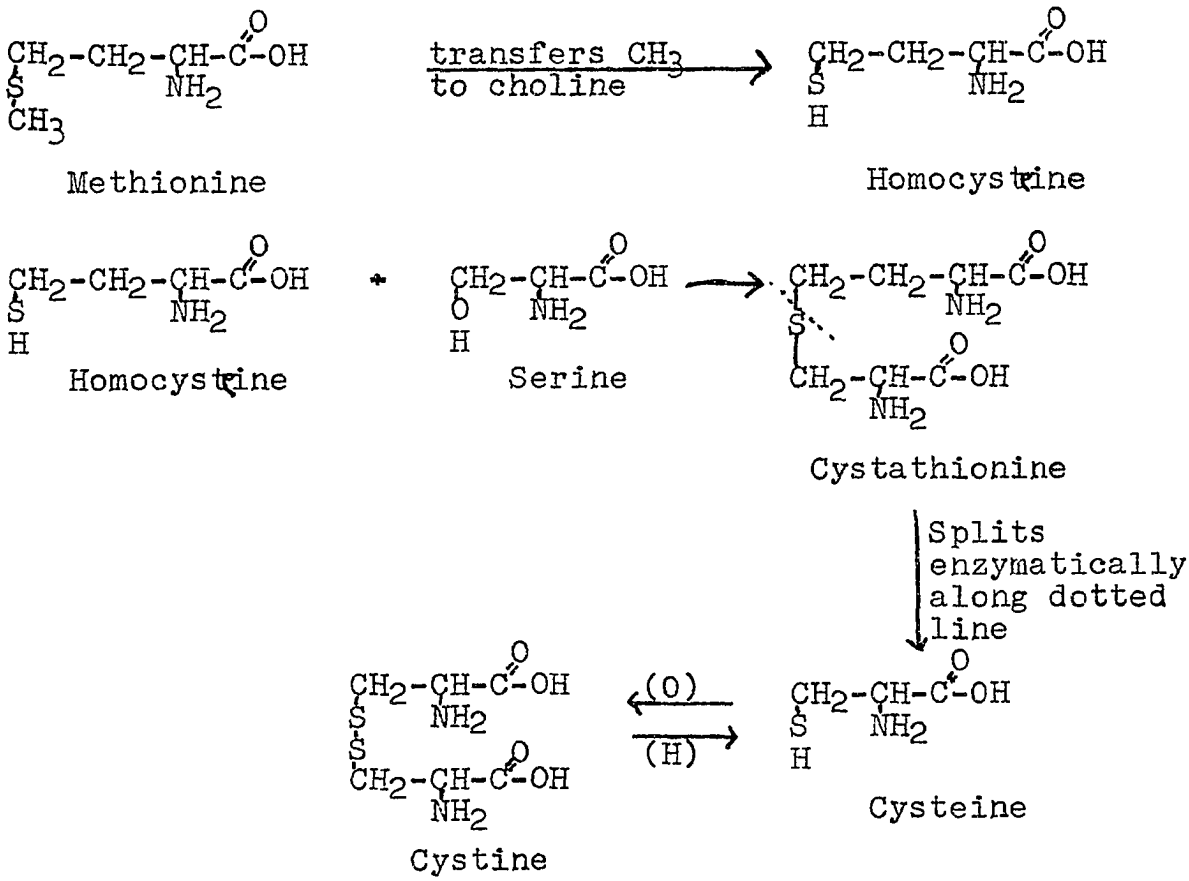
I. INTRODUCTION

The process of unhairing animal hides and skins, as employed by the tanner, is known to involve a chemical modification of the epidermal system. Therefore a knowledge of the chemical composition of the epidermal system is required for a better understanding of the unhairing process. Furthermore, this epidermal system, which represents the body's first line of defense against adverse environmental conditions is very important to the biologist. A survey of the literature (as will be revealed later) shows the inadequacy of our knowledge of the chemical constitution of the components of the epidermal system, particularly of the epidermis itself. In view of this, and since newer techniques have been made available for securing apparently unmodified epidermis, it seemed appropriate to examine the constitution of the so-called keratinous components of the epidermal system, viz., hair and epidermis.

Block and Vickery (1) have stated: "A keratin is a protein which is resistant to digestion by pepsin and trypsin, which is insoluble in dilute acids and alkalies, in water and in organic solvents, and which on acid hydrolysis yields such quantities of histidine, lysine and arginine that the molecular ratios of these amino acids are, respectively, approximately 1:4:12." In addition to these criteria, keratins are usually characterized

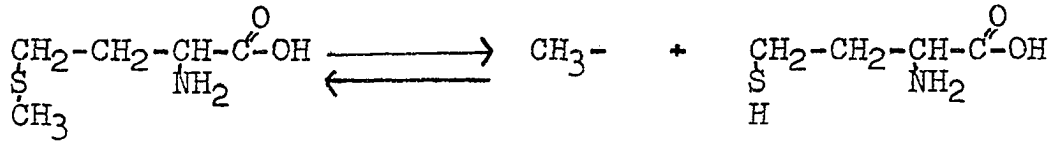
by the ability to assume two characteristic molecular patterns, depending on whether the polypeptide chains are in a stretched or a folded molecular configuration (2), and by a comparatively high sulfur and cystine content, accompanied by a relatively low methionine content (3).

The high cystine and low methionine content of keratins can probably be explained on the basis of the conversion of methionine to cystine during the process of keratinization. DuVigneaud, Kilmer, Rachele and Cohn (4) have pictured the probable mechanism of this conversion as follows:



This postulated mechanism is based on the following facts:

1. Jackson and Block (5), in 1932, demonstrated that methionine could be substituted for cystine in the diet of the growing rat.
2. Tarver and Schmidt (6), in 1939, by the use of methionine containing radioactive sulfur, showed that the sulfur of cystine is derived metabolically from the sulfur of methionine.
3. DuVigneaud and his associates (7), by using deuterium in place of hydrogen in the methyl group of methionine, demonstrated the transfer of this methyl group to choline and to creatine. Feeding experiments wherein a diet rich in choline and synthetic homocysteine was used, showed the process to be metabolically reversible.



Methionine — Labile Methyl — Homocysteine

4. Stetten (8) fed serine containing isotopic nitrogen to animals and demonstrated the presence of large amounts of isotopic nitrogen in the cystine isolated from the tissues of these animals.
5. When homocysteine and serine are incubated with

liver tissue or liver extract, cysteine is formed (9).

6. Synthetic cystathionine undergoes enzymatic cleavage by liver tissue or liver extract to yield cysteine (10,11) and also satisfactorily replaces cystine in the diet (10).

7. Synthetic methionine, containing isotopic sulfur and also isotopic carbon in one of the carbons of the chain, was fed to rats. The cystine isolated from the hair of the rats contained isotopic sulfur but no isotopic carbon (4). Thus the sulfur of methionine, but not its carbon chain, is involved in the formation of cystine.

Cattle hair has been analyzed previously for cystine (12, 13), but apparently not for methionine. However, Block and Bolling (14) have calculated a value of 0.6 per cent methionine for cattle hair from the difference between the values for cystine sulfur and total sulfur as found by Block and Lewis (12) in the hair of adult cows, and Beveridge and Lucas (15) have reported 1 per cent methionine in human hair.

No record has been found of any previous attempt to determine the amino acid composition of cattle epidermis, except for the studies of impure materials conducted by Haugaard in this laboratory and reported by O'Flaherty (16), as a preliminary to this investigation. However, Sullivan, Hess and Howe (17) have reported analyses for whole lamb

skin, and several attempts have been made to analyze human epidermis. Due to the difficulties of obtaining suitable specimens for study, the materials studied have varied widely. The varying nature of the materials has been reflected by the varying nature of the results.

Wilkerson (18, 19) analyzed samples of stratum corneum from a person with dermatitis exfoliativa. Eckstein (20) analyzed the outer layers of human skin, presumably containing some corium material and possible hair roots, after subjecting it to peptic and tryptic digestion. Peptic digestion may possibly have removed most of the corium tissue. However, the high ash content and low nitrogen value which Eckstein found makes it doubtful that he was analyzing only epidermis. Block (21) obtained human epidermis from two sources, namely, cornified epithelium from the sole of the foot and scales from patients with exfoliative dermatoses of various kinds. The same worker also analyzed horse burrs before and after subjecting them to enzymatic digestion (22). Wilson and Lewis (23) have obtained analytical values for human skin, but these authors do not state whether whole skin, or merely epidermis or some layer thereof was used. The values obtained by these workers are tabulated and discussed in a later portion of this thesis in comparison with the values herein determined.

The analyses which are reported in this paper are, it

is believed, the first amino acid analyses which have been obtained from epidermis which contains all four histological layers with a minimum of modification. Furthermore, they are the first to be obtained on bovine epidermis, and they are concerned with those portions of the hide in which the tanner is interested, namely, the areas originally covered by the hair. Epidermal samples were obtained as a result of the observations of Roddy and Jacobs, as reported by O'Flaherty (16), that it was possible to separate the epidermal system from the corium of fresh cattle skin or hide by the action of either 5 per cent sodium chloride solution, at or below room temperature, or of distilled water at 60°C. In this report, data are given which contrast bovine hair and epidermis secured by these separatory techniques which are discussed in the experimental section below.

II. EXPERIMENTAL

A. Experimental Methods

1. Preparation of the Samples

Cattle hides, obtained from a local packer immediately after slaughter, were brought to the laboratory where they were washed, cut up and handled within a few hours as described below. Since the preparations of the materials for experimentation were under way as soon as the body heat was lost, post-mortem changes were minimized.

The separatory procedure employed for Epidermis I was as follows: Pieces of black haired bull hide were unhaired in 5 per cent sodium chloride solution at refrigerator temperature (<10°C.). Sufficient salt solution was used to float the hide pieces in battery jars (about 1:2, skin to solution ratio by weight), and the liquid surface was covered with toluene. Both the toluene layer and the salt solution were changed daily. Removal of epidermis from the pieces was accomplished during the period from the fifth to the thirteenth day. After removal from the remainder of the hide, the epidermis was separated from the hairs by pulling the latter through from the basement layer side of the epidermis. Microscopic examinations by trained histologists checked the completeness of the separations. These microscopic examinations failed to show any alteration of the epidermis, and little opportunity had been given for any significant deterioration. No odor was detected in any stage, either in the solutions or in the tissue. All removed epidermis was quick-frozen in a moist condition and held below 0°C., except during the short periods when it was being freed of hair. When sufficient epidermis had been collected, it was extracted in the Soxhlet apparatus with ethyl alcohol and acetone. This defatted the epidermis and lowered its moisture content so it could be stored for chemical analysis. The hair removed from the epidermis

(including roots) was similarly extracted and stored.

For Epidermis II a red haired steer hide was used. Secured fresh from the slaughter, it was washed, cut into pieces, and quick-frozen. The pieces were individually unhaired immediately after thawing by immersing them in distilled water at 60 ± 2°C. for 10 - 15 minutes. The hair including the roots was loosened and could be pulled out. The epidermis could then be peeled off quite easily from the corium. The few remaining hairs and hair roots were pulled through from the underside of the epidermis. Microscopic examination (45X) by a histologist demonstrated the high efficiency of the separation. Figure 1 presents a photomicrograph of cleared epidermis prepared in this manner. The cleared epidermis was re-frozen, and when sufficient material had been accumulated, it was extracted with alcohol and acetone, air-dried, and stored for analysis. The yield was 3.3 g. of air-dried epidermis from about 30 pounds of hide. Hair from the same epidermis was extracted and stored for analysis as with the first preparation.

Epidermis III was obtained from a red and white haired steer hide. Its treatment was similar to that of Epidermis II. In this case, however, the hair on the hide pieces was cut down to the surface of the epidermis before the warm water treatment. For this purpose, animal clippers, followed by

an electric razor, were used. On removing the epidermis the hair follicles broke in the neighborhood of the sebaceous glands. Consequently, most of the hair roots were left behind in the corium, and less difficulty was experienced in clearing away hair debris under the microscope. Yield, 4.3 g. of air-dried epidermis from about 40 pounds of hide. Hair from this preparation was not analyzed.

2. Moisture Determinations

Thirty-five to fifty mg. samples of hair or epidermis were quickly transferred from the air-tight bottles in which the materials were stored to weighed micro-weighing bottles, stoppered, weighed on a micro-balance, dried to minimum weight in a vacuum oven at 70°C. and the loss computed as moisture. Minimum weight was achieved for both hair and epidermis after twelve hours of drying. After sixteen hours, weight gains were noted, probably due to oxidation. In the case of hair samples, stray hairs tended to escape on opening the bottles. This difficulty was overcome by weighing several small, previously dried porcelain chips along with the bottle and placing these on top of the hair after its transfer to the weighing bottle.

3. Lipid Determination

It was stated under (1) that the lipid of the

epidermis was removed by Soxhlet extraction. The samples are prepared for Soxhlet extraction by drying with cold acetone which is then displaced by cold ethanol. Then Soxhlet extraction, first with ethanol, then with acetone follows. In the case of Epidermis III, each of these fractions was saved, evaporated to dryness on a steam bath and dried at 70°C. in a vacuum oven. In this way, the lipid fractions reported in Table IV were obtained. They were computed to a moisture-free basis.

4. Total Nitrogen Determinations

Due to warnings in the literature about the difficulties of digesting protein materials for micro-Kjeldahl analysis (24, 25), the procedure recommended by Miller and Houghton (25) was at first adopted on a semi-micro scale. However, it was later found that comparable results on our materials could be obtained by a semi-micro modification of the Kjeldahl-Gunning method when digestion is carried out for six hours after clearing. After digestion, the mixture is transferred to the micro-Kjeldahl distillation apparatus, made alkaline and the NH_3 is steam-distilled into 2 per cent boric acid containing a few drops of mixed indicator, made according to the directions of Ma and Zuazaga (26). It was then titrated with fiftieth normal standard hydrochloric acid. Samples were chosen of such a size that the final titration was

15-25 ml.

5. Total Sulfur Determinations

Total sulfur was determined by a semi-micro modification of the Carius method. The sample is oxidized with fuming nitric acid in a sealed tube at 300°C. for a minimum of six hours. The tube is carefully opened and its contents are quantitatively transferred to a 20 ml. beaker. The solution is evaporated to 2 - 3 ml. to remove excess nitric acid, diluted with hot water, two drops of 10 N. HCl are added and the solution again evaporated to 2 - 3 ml. After further dilution, the solution is allowed to come to the temperature of the steam bath. Hot dilute $BaCl_2$ solution is added drop by drop with constant stirring until in slight excess. The mixture is digested for at least an hour on the steam bath and allowed to stand overnight. Next morning, it is heated to the temperature of the steam bath and transferred by the micro-transfer apparatus into a weighed micro Neubauer crucible. The transfer is accomplished by the use of hot, CO_2-free water followed by alcohol. The crucible is dried and ignited, then reweighed and the amount of sulfur computed from the found weight of barium sulfate. Sulfur-containing organic compounds of known composition gave theoretical yields of sulfur by this method as used by us before it was

adopted for use with epidermis and hair.

6. Ash

Ash was determined by ignition in micro-crucibles at 550° - 600°C. until constant weight was obtained.

7. Cystine Determinations

The values of cystine given by us are actually cystine plus cysteine values. Cysteine is oxidized to cystine in the process of hydrolysis. Both the formed and the pre-formed cystine are colorimetrically measured against a cystine standard. For determining the cystine a modification (27) of the Sullivan method was used. The protein was hydrolyzed with 1:1 hydrochloric acid in a sealed tube at 120°C. for eight hours. The hydrolysate was decolorized with a small amount of Norit vegetable charcoal and filtered. The residue was washed with N/1 hydrochloric acid brought quickly to a boil and then with more N/1 hydrochloric acid at room temperature. The filtrate and washings were evaporated to dryness "in vacuo" on a steam bath and the residue was picked up with N/10 hydrochloric acid and tested as follows:

To a 5 ml. aliquot of the solution, in a 25 ml. volumetric flask, 2 ml. of 5 per cent NaCN in N/2 NaOH are added. The solution is then allowed to stand at room temperature (not less than 20°C.) for fifteen minutes. Then 1.0 ml. of 0.1 per cent aqueous sodium

1,2-naphthoquinone-4-sulfonate is added* and the flask is shaken vigorously. Exactly ten seconds later, 5 ml. of 10 per cent anhydrous Na_2SO_3 in N/2 NaOH are quickly added with continuous agitation of the flask. The solution is then allowed to stand for thirty minutes. At this point 2 ml. of a 2 per cent solution of sodium hydrosulfite ($Na_2S_2O_4$) in N/2 NaOH are added. The solution is mixed, made up to volume, shaken vigorously, and then compared in a colorimeter against a cystine standard which was run simultaneously. Both a Duboscq visual colorimeter and a Klett-Summerson photoelectric colorimeter using a number 61 Wratten gelatin filter have been successfully used. In general, the use of the photoelectric colorimeter is to be preferred. Using this instrument, results on known solutions of cystine have been always within 5 per cent and usually within 3 per cent of the known value. Cystine added to hydrolysates has been quantitatively recovered.

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* 0.5 Per cent naphthoquinone, instead of 0.1 per cent naphthoquinone, was employed for the latter half of the cystine determinations on Epidermis II. This was done because it was felt that some of the reagent might be consumed in side reactions because of the large quantities of epidermis involved. The results were essentially the same, so the higher concentration was also used for the analyses of Epidermis III.

8. Determination of Total Disulfide

The determination of total disulfide was performed on Epidermis III and was run on the same three hydrolysates as were used for three of the cystine determinations. Its purpose was to determine whether homocystine was present by determining if a discrepancy existed between the total disulfide and cystine values. The method has been similarly used by Brand, Cahill and Block (28) and by Anslow and Du Vigneaud (11). The latter workers also investigated the possibility of the formation of a mixed disulfide from cysteine and homocysteine which might possibly interfere with the Sullivan reaction. No such interference was found.

The method was first reported by Kassell (29) and later given in considerable detail by Kassell and Brand (30). It was used essentially as reported in the latter reference except for the following modifications:

1. A Beckman Quartz Spectrophotometer, Model DU, was used in place of the Pulfrich Photometer employed by Kassell and Brand.
2. Measurement of the transmission over a wide spectral range showed that the blue color of reduced phosphotungstic acid is caused by a maximum transmission of blue light with a decreasing transmission through the visible red region. However the point of minimum

transmission was at 888 millimicrons, in the infra-red region of the spectrum. Consequently, determinations reported in this paper were made at 888 millimicrons with a slit width of 0.03 mm.

3. The phosphotungstic acid reagent was prepared according to the directions of Folin (31), as advised by Kassell and Brand, except that the final dilution was not made. Under our conditions, linearity of response was lost with the diluted reagent, but Beer's law was followed if the reagent was used in an undiluted form.

4. Though a straight line was obtained by plotting optical density versus molar concentration, indicating that Beer's law was being followed, this line did not pass through the origin. Thus, instead of being of the form --

$$D = \varepsilon C_M$$

it was of the form

$$D = \varepsilon C_M + b$$

where D = optical density

ε = the molar extinction coefficient

C_M = the molar concentration of disulfide

b = the point of intercept with the axis along which D is plotted.

was experimentally found to be 9175 and b had a value of -0.071.

The method was calibrated with known solutions of cystine and of homocystine. It was tested by adding known amounts of cystine, of homocystine, and of mixtures of cystine and homocystine to hair prior to hydrolysis. The added materials were recovered quantitatively in analyses of the hydrolysates.

9. Methionine Determinations

Methionine was determined by the McCarthy-Sullivan procedure (32) as given by Block and Bolling (33) with the exception of a few changes. The hydrolysate is evaporated to dryness "in vacuo" and picked up with N/10 hydrochloric acid instead of adjusting the pH as recommended by Block and Bolling. One ml. of 2 per cent nitroprusside is used instead of 0.3 ml. of 10 per cent nitroprusside, and a number 61 filter is employed in place of a number 54. These minor changes gave us results in conformity with Beer's law for concentrations of methionine from 100 to 200 parts per million. Concentrations of histidine double those of the methionine present did not interfere at all. High cystine concentrations caused a rapid fading of the methionine color as shown by White and Koch (34). In the case of hair samples, this was circumvented by taking readings immediately after acidification as proposed by these authors. The color did not fade in the case of the epidermal samples due to the fact that

the cystine was present in such small amount.

10. Sulfur Determination on the Ash

Samples were ashed in micro-crucibles at 500°C. for 1/2 hr., cooled, and two drops of concentrated HNO_3 were added to the charred remnant. The micro-crucibles were then reheated very gradually to avoid spattering. When the HNO_3 was gone, the crucibles were reheated in a muffle at 600°C. for eight hours. The ash was picked up with dilute nitric acid. The remainder of the procedure was the same as that given for the total sulfur determination.

11. Precipitable SO_4 in the Hydrolysate

Samples of epidermis were hydrolyzed, evaporated to a small volume on the steam bath to remove excess HCl, diluted, allowed to come to the temperature of the steam bath, and precipitated with $BaCl_2$. The remainder of the procedure was the same as for total sulfur.

12. Qualitative Tests

Sealed tube hydrolysates were tested for H_2S and SO_2 immediately upon opening the tubes. Analyses for H_2S were conducted with moistened lead acetate paper. SO_2 was tested for by the benzidine blue test (35). Tests for carbohydrate used were the Molisch test (36), Benedict's test for reducing sugars (37), the Kiliani (38) and the

pine splint (39) tests for desoxy sugar, and the amino sugar test (40).

B. Experimental Results

The experimental results are summarized in Tables I to IV. These will be discussed in order.

Table I shows the analyses of Epidermis I and hair from the same animal. This material was obtained by the salt water treatment. It should be noted that the hair analysis is typical of keratin. The hair has a high total sulfur and cystine content and a low methionine content. The epidermis, on the other hand, has a very low cystine content and a comparatively low total sulfur content for a keratin. Methionine could not be determined on the epidermis because of insufficient material. At the start of the analyses only 400 mg. of epidermis were available.

Results of the analyses of Epidermis II and hair from the same animal are shown in Table II. This material was obtained by the warm water treatment. The hair analysis is again typical of keratin, that is, it has a high total sulfur and cystine content and a low methionine content. The epidermis, however, is still low in total sulfur and cystine and higher in methionine than a keratin would be expected to be. The low proportion of the total sulfur accounted for as cystine

and methionine sulfur made us, at first, doubt the validity of the cystine analyses. The amount of naptho-quinone reagent which we were using for the cystine determination was low, although well in excess of the stoichiometric ratio found by Csonka (41). It was felt that perhaps some of the reagent was being used up in side reactions. Therefore new cystine determinations were made in which the concentration of naptho-quinone was quintupled. Two determinations were made. In one case, a value of 0.17 per cent was obtained, in good agreement with the previous values of 0.21 per cent and 0.17 per cent. In the other case, an error was made in manipulation which subjects the result to question, so that it was not included in the table. However, the result was of the same order, 0.13 per cent. Since the larger concentration of naphthoquinone made no difference, it was also used in the cystine determinations on Epidermis III.

The missing sulfur was still unexplained. Lugg (42) has shown that the reduced form of cystine, cysteine, is adsorbed on the solid humin formed in the hydrolysis of carbohydrate-containing proteins. Therefore, even though hydrolysates previously tested had given a negative response to the Molisch reaction, it was decided to investigate the possible presence of carbohydrates more extensively, particularly since one could logically

expect small amounts of carbohydrate from the nucleic acid of the cell nuclei. Therefore all the carbohydrate tests listed in Section 12 of Part A were conducted on new hydrolysates. None were positive. Since desoxy sugars are unstable in acid solutions and desoxyribose might be an expected sugar, a fairly large amount (about 50 mg.) of solid epidermis was tested by the Molisch reaction. Under these conditions, hydrolysis takes place during the test and the presence of acid-unstable sugars can be detected. In these circumstances, a color was obtained.

New cystine determinations were therefore run on the epidermis which was now hydrolyzed by the method of Brand and Kassell (43) which successfully prevented humin formation. This corroborates the experiences of others (44, 45). The results of these analyses were in good agreement with the earlier ones, as shown in Table II. It must, therefore, be concluded that the amount of carbohydrate present is insufficient to affect the cystine determination.

It was thought that, perhaps, in the process of hydrolysis, changes would occur in the sulfur of the epidermis to change it to other forms. Consequently, sealed tube hydrolysates were tested, immediately after opening the tubes, for H_2S and SO_2. Neither was found. Ethereal sulfates would be hydrolyzed along with the

protein and the sulfate would be precipitable from the hydrolysate. This was tried but did not account for much of the missing sulfur. Inorganic sulfur was sought for in the ash but the amount was very small. Therefore, the missing sulfur was still not explained.

Since the process of keratinization takes place in the epidermis, it was felt that the missing sulfur might be in the form of the sulfur-containing intermediates postulated by Du Vigneaud, as outlined in the Introduction to this thesis. To determine if this was the case, a third sample of epidermis was prepared and tested.

In Table III, the analyses of Epidermis III are shown. This material was prepared by the warm water treatment. The cystine and total sulfur values were again low, and the methionine was again higher than would be expected for a keratin. A value for homocystine was found by the difference between total disulfide and cystine. The methods for these two determinations are discussed in Sections 7 and 8 of Part A.

Although homocystine has never been previously found in nature, it seemed feasible to compute the excess disulfide as homocystine in view of the following evidence.

A discrepancy between total disulfide and cystine in epidermis can exist for the following reasons:

1. There may be a substance in epidermis which causes a repression of the color developed in the Sullivan method for cystine. However, this is very unlikely, because in over twenty years of work with the Sullivan method, no material found in a protein hydrolysate has ever interfered with the Sullivan reaction. This has not been from a lack of attempts to find such materials. Much work was performed in this direction, particularly in the early years of the use of this reaction (46 - 50).
2. The epidermis may contain a large proportion of ascorbic acid, which, according to Kassell and Brand, reacts similarly to disulfides in the disulfide reaction. This seems unlikely because an ascorbic acid content of 0.5 per cent would be required. This is a tremendous vitamin concentration. Some values (51) for ascorbic acid in the richest known materials in this vitamin are:

Fresh turnip greens	--	0.14%
Fresh brussels sprouts	--	0.12%
Freshly dehydrated cabbage	--	0.19%

3. Another disulfide than homocystine may be present. This is not probable, because the only amino acid other than cystine which is capable of forming a disulfide and which has been thus far found in nature is thiolhistidine, which is of vegetable origin (52, 53). This, nevertheless, is a possibility which cannot be eliminated.
4. Homocystine may be present. This is considered the most probable of the possibilities, and extremely likely in view of the strong evidence for the Du Vigneaud mechanism ~~which~~ which was noted in the introduction to this dissertation.

It is to be noted that most but not all of the sulfur can be accounted for. This may be in the form of cystathionine.

The Lipid Analyses for Epidermis III is given in Table IV. Lipid was determined by the method given in Section 3 of Part A. After extraction, the epidermis was air-dried, weighed, and its moisture content determined. By adding the moisture-free weight of epidermis to the weight of the lipid, the lipid fractions were computed to the moisture-free basis given in the table.

III. DISCUSSION

The results obtained would seem to indicate clearly that the hair has the sulfur distribution which is characteristic of a true keratin but the epidermis does not. In this study epidermis has been studied as an intact tissue and histologically much differentiation is observed within this tissue. It is probable that the protein system of the epidermis is not a single protein component but a mixture of such components. One would certainly expect nucleoprotein to be present in the nucleated portions and probably other proteins are present as well. In future studies of this tissue it might be well to determine exactly how much of it is resistant to enzymatic digestion. Block's classification (21) of the epidermis as a pseudokeratin would seem to be justified by the failure of epidermis to show the sulfur distribution characteristic of keratin. However, if one considers a pseudokeratin to differ from a keratin only in the histidine : arginine : lysine ratio as Block's definition (1) would seem to imply, a resistance to enzymatic digestion would be necessary to classify epidermis as a pseudokeratin.

The results obtained in this study indicate the presence of homocystine in epidermis and the failure to account for all of the sulfur would also indicate the possible presence of cystathionine. If the

disulfide material other than cystine is homocystine, this is the first quantitative analysis to determine the natural occurrence in a tissue of this new amino acid whose existence has been previously postulated. It is also, in this case, a corroboration of the theory of sulfur metabolism proposed by Du Vigneaud and his co-workers.

The values for cystine reported in this paper are generally lower than those found in the analyses of previous investigators (See Table V). This can be attributed to several causes. Human hair is richer in cystine than cattle hair (3) so the precursors of the two materials, namely, human and cattle epidermis, could be expected to vary similarly. The samples of epidermis worked on by Wilkerson and by Block were rich in layer corneum. The layer corneum of epidermis is composed of the vestigial remnants of cells which are much closer packed than when they existed in the lower strata of the epidermis. Furthermore the keratinizing process would be expected to be more advanced. Eckstein's material can be expected to contain hair roots, and the skin tested by Wilson and Block, if it is assumed to be whole skin, would contain hair roots, and other cystine containing proteins. Furthermore, all of these investigators used non-specific methods for cystine which would have included homocystine in

their cystine assays. The same kind of objection holds true for Wilkerson's value for methionine. The modified Baernstein method computes methionine from the increase of titratable sulfhydryl groups after treatment with hydriodic acid. However cystathionine would also yield sulfhydryl groups under these conditions.

The low content of disulfide in the epidermis, even when homocystine is included, would seem to indicate that less emphasis should be placed upon disulfide bond reactions as necessary adjuncts to the unhairing process. Unhairing action occurs principally in the epidermis (54, 55) but this material contains comparatively little disulfide. Furthermore, histochemical studies by Jacobs and Roddy (56) and by others (57), indicate that sulfhydryl rather than disulfide predominates in the epidermis. This correlates nicely with the concept of Du Vigneaud wherein the homocysteine, rather than its oxidized form, homocystine, takes part in the keratinizing process.

IV. SUMMARY

Cattle epidermis has been separated from the other components of skin with a minimum of modification and analyzed, particularly with regard to its sulfur distribution. Hair from the same animals was analyzed simultaneously.

Cattle hair was found to have a sulfur distribution

characteristic of keratin. Epidermis was found to vary greatly from this characteristic distribution. This corroborates Block's (21) statement, based on other evidence, that epidermis is not a true keratin.

Evidence has been found for the existence of a disulfide, other than cystine, in the epidermis. This disulfide is presumably homocystine.

The evidence presented indicates that caution should be employed in interpreting the modification of disulfide linkages as a mechanism necessary for the process of unhairing as practised by the tanner.

V. TABLES AND FIGURES

TABLE I

ANALYSES OF EPIDERMIS I AND HAIR

(Obtained by Salt Water Treatment)

(Values given are on a moisture-free, lipid-free basis)

Analysis	Hair		Epidermis	
	Found Values Per cent	Average Per cent	Found Values Per cent	Average Per cent
Moisture	4.40) 4.54)	4.47	7.55) 7.38)	7.47
Total Nitrogen	15.7) 15.7) 15.5)	15.6	16.7) 16.5)	16.6
Total Sulfur	3.8) 3.6)	3.7	1.09) 1.03)	1.06
Cystine	13.4) 13.6)	13.5	0.69) 0.71)	0.70
Cystine N as % Total N		10.1		0.49
Cystine S as % Total S		97		18
Methionine	0.37) 0.44)	0.41		
Methionine N as % Total N		0.25		
Methionine S as % Total S		2.3		
% Total S accounted for as Cystine S + Methionine S		99		

TABLE II

ANALYSES OF EPIDERMIS II AND HAIR

(Obtained by Warm Water Treatment)

(Values given are on a moisture-free, lipid-free basis)

Analysis	Hair: Found Values Per cent	Hair: Average Per cent	Epidermis: Found Values Per cent	Epidermis: Average Per cent
Moisture	8.48 8.23 8.40	8.37	6.02 5.98 5.91	5.97
Total Nitrogen	15.9 16.3	16.1	16.9 17.2 16.8 16.9	16.9
Total Sulfur	4.6 4.4 4.8	4.6	0.76 0.79 0.72	0.76
Ash	0.89 0.89	0.89	0.67 0.69 0.69	0.68
Cystine	16.2 15.4 16.2 15.6	15.8	0.21 0.17 0.17 0.16++ 0.14++	0.17
Cystine N as % Total N		11.5		0.12
Cystine S as % Total S		92		6.0
Methionine	0.34 0.41	0.38	1.33 1.33 1.26	1.31
Methionine N as % Total N		0.22		0.73
Methionine S as % Total S		1.8		37
% Of Total S Accounted for as Cystine S + Methionine S		94		43
Sulfur Precipitable as SO_4 from Hydrolysate				0.07+
S of Precipitable SO_4 as % of Total S				9
Ash S (Based on wt. of original samples)			0.021) 0.013) 0.012)	0.015
S of Ash as % of Total S				2.0
% of Total S Accounted for by all Methods Employed				54

+Larger of two values; based in dry wt. of sample.

++Determined on hydrolysate in which humin formation was prevented.

TABLE III

ANALYSES OF EPIDERMIS III

(Obtained by Warm Water Treatment)

(Values given are on a moisture-free, lipid-free basis)

Analysis	Found Values Per cent	Average Per cent
Moisture	6.29 6.34	6.32
Total Nitrogen	17.43 17.46	17.45
Total Sulfur	0.85 0.85	0.85
Cystine	0.68 0.48 0.55 0.47 0.55 0.55	0.55
Cystine N as % of Total N		0.37
Cystine S as % of Total S		17.2
Methionine	1.61 1.55 1.43	1.53
Methionine N as % of Total N		0.82
Methionine S as % of Total S		38.6
% Of Total S Accounted for as Cystine S + Methionine S		55.8
Homocystine (By difference between total disulfide and cystine)	0.71 0.75 0.77	0.74
Homocystine N as % of Total N		0.44
Homocystine S as % of Total S		20.7
% Of Total S Accounted for as Cystine S + Methionine S + Homocystine S		76.5
Maximum Possible S from Other Sources as Judged from Epidermis II		11
Maximum S Accountable by all Sources		86.5

TABLE IV

LIPID CONTENT OF EPIDERMIS III

(Values Given Are on a Moisture-Free Basis)

Fraction	
I. Cold Acetone Solubles	8.23%
II. Cold Ethanol Solubles	1.11%
III. Hot Ethanol Solubles	2.74%
IV. Hot Acetone Solubles	0.09%
Total Lipid	12.2 %

TABLE V

CYSTINE, METHIONINE AND TOTAL S OF EPIDERMIS

AS DETERMINED BY EARLIER WORKERS

Material	Method	Reference	Nitrogen Per cent	Sulfur Per cent	Cystine Per cent	Methionine Per cent
Stratum Corneum from a Patient with Dermatitis Exfoliativa	Folin-Marenzi and Modified Baernstein	Wilkerson (18,19)	15.25	1.09	2.31 2.38	2.47
idem after Peptic and Tryptic Digestion	Folin-Marenzi and Modified Baernstein	Wilkerson (19)		1.10	2.51	2.60
Cornified Epithelium from the Sole of the Foot and Scales from Patients with Exfoliative Dermatoses of Various Kinds (Pepsin digested)	Folin-Marenzi	Block (21)			3.4	
Horse Burrs	Folin-Marenzi	Block (22)	14.1		Trace	

TABLE V (Continued)

Material	Method	Reference	Nitrogen Per cent	Sulfur Per cent	Cystine Per cent	Methionine Per cent
Outer Layers of Human Skin, Pepsin & Trypsin Digested	Folin-Marenzi	Eckstein (20)	14.2		3.82	
Human Skin I	Folin-Looney	Wilson (23)	11.3	0.70	1.8	
Human Skin II	Folin-Looney	Wilson (23)	12.4		2.1	
Human Skin III	Folin-Looney	Wilson (23)	15.0		2.3	

FIGURE I

Photomicrograph of the Basal Portion of the Epidermis After Removal From Corium by the Warm-Water Treatment

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