CYSTEINE, CYSTINE AND METHIONINE IN THE SKIN OF YOUNG AND ADULT RATS

by

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INTRODUCTION

By observing the swelling of rat skin in salt solutions at a pH between 5 and 7 it was found by GRAUBERT[1], that the skin of young animals (0 to 8 days) swells much more than the skin of adult ones (3 months and older). At a pH below 4 this behaviour is reversed. By comparing this with the swelling phenomena of tendons, cornea and sclera the author came to the conclusion that, while adult skin is made up of collagen predominantly, in young skin mucoproteins are dominating.

The work of KLAUDER AND BROWN[2] drew our attention to the sulfur containing amino-acids of the skin proteins. They found that the normal skin of adult humans contains only 60% of the amount of sulfur present in the skin of infants. Furthermore WILKERSON found the keratinization of skin into nails and hair to be accompanied by an increase in cystine and a decrease in methionine[3], while the ratio of histidine, lysine and arginine remains practically the same[4]. From this work it emerges that maturation and keratinization of the skin are accompanied by changes in the amounts of the sulfur containing amino-acids in the skin proteins. SANDFORD AND HUMOLLER[5] investigated the influence of reducing and oxidizing solutions on the cystine-cysteine ratio in hair by determining total cystine plus cysteine as well as cystine alone. In the last case cysteine was blocked effectively with monoiodoacetic acid.

Therefore it was the object of the present investigation to determine the total cystine (cysteine plus cystine) content, the percentage SS-bonds (cystine content divided by total cystine content) as well as the methionine content in young and adult rat skin to see how these quantities change during maturation. Total cystine content was determined polarographically by BRDIČKA's method[6,7]. Methionine was determined microbiologically with *Streptococcus faecalis* R[8,9]. For the estimation of the percentage SS-bonds the sulfhydryl groups had to be eliminated irreversibly. Therefore an alkylating agent had to be used and like BRDIČKA[10] we chose monoiodoacetate, which does not interfere with the polarographic cystine determination. Contrary to previous authors HALWER AND NUTTING[11] found that the percentage SS-bonds changed during hydrolysis, in agreement with the observation by OLCOTT *et al.*[12] that cystine is reduced to cysteine by tryptophane during hydrolysis. Therefore in our case the intact protein was treated with monoiodoacetate. According to preliminary experiments this reaction proved to be complete within 4 hours under continuous stirring and at a pH of 10. Other control experiments proved that excess iodoacetate is immediately decomposed during hydrolysis without having a change to take away sulfhydrylgroups appearing

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under these circumstances. Finally it was experimentally confirmed that the $-SCH_2COOH$ group withstands hydrolysis.

SLÂDEK AND LIPSCHÜTZ[13] found that several amino acids exert a suppressive effect on the polarographic cystine waves, wich necessitates the use of known additions of cysteine in the total cystine determinations. For the determination of the percentage SS-bonds, however, we omitted the known additions. In that case we used two equal portions of the same skin sample, one of which was treated with iodoacetate; consequently the amino-acid composition of the two hydrolysates was the same and therefore also the suppressive effect on the wave heights. The percentage SS-bonds was found by direct comparison of the wave heights. The correctness of this method was confirmed by control determinations with known additions.

EXPERIMENTAL PART

Preparation of the skin samples

Rats, aged 4 months or older were killed with ether, the dorsal skin was taken off and pinned onto a dissecting board. The hair was removed completely by lathering with shaving-soap and shaving with a safety-razor (depilatories proved to be insatisfactory). The inside was freed of subcutaneous connective tissue. A sample of 3 to 4 g was cut into small pieces and dried for 24 to 40 hrs in a vacuum dessiccator over concentrated sulfuric acid. The dried sample was crushed to a coarse powder in a steel mortar by beating on the pestle with a hammer.

Rats of 2 to 3 days old, who do not yet show any visible hair growth, were killed with ether and the dorsal and abdominal skin was taken off (the maturation of the dorsal skin proceeds more rapidly than that of the abdominal skin, causing a significant difference in development in adult skin but in young skin the difference is negligible[1]). If this was done carefully, no further preparation was necessary. To obtain a sample of 3 to 4 g the skin of 4 animals was required. After cutting into small pieces the sample was dried and pulverized in the same way as described above for the adult skin.

Percentage SS-bonds

With 50 to 100 mg of the dried skin a Kjeldahl determination of the nitrogen content was carried out (at least 50 mg were required to insure a representative sample).

Two equal portions of the sample were brought into 50 ml centrifuge tubes (400 mg of the adult skin and 200 mg of the young skin). To both 30 ml of a borate buffer of p_H 10 were added. One of the tubes received 15 ml H_2O, the other one 560 mg of iodoacetic acid and 15 ml of 0.2 *M* NaOH (a neutral solution of iodoacetate decomposes within a few weeks, therefore we added the acid and an equivalent amount of NaOH separately; the acid we prepared according to the directions given by ABDERHALDEN[14]). Both suspensions were stirred mechanically for 4 hrs.

Then 7½ ml 20% trichloroacetic acid was added to each tube and the tubes were centrifuged, the clear liquid drawn off, trichloroacetic acid added again, centrifuged and the clear liquid drawn off once more. The residues were transferred to two 50 ml flasks. Both received 20 ml 20% HCl and 1 g of urea (HESS AND SULLIVAN[15]) recommend the use of urea to decrease the formation of humin, which destroys cysteine). The solutions were boiled for 8 hrs in a nitrogen atmosphere. The resulting hydrolysates were diluted to 100 ml; the HCl-concentration of each hydrolysate was determined by titration with 0.5 *M* NaOH.

5 ml of each hydrolysate and 10 ml 0.01 *M* $Co(NO_3)_2$ were pipetted into a 50 ml volumetric flask, 1 *M* NH_4Cl and 1 *M* ammonia were added in such quantities as to give — after dilution to 50 ml — a solution 0.1 *M* in NH_4Cl and 0.1 *M* in ammonia (calculated from the previously determined HCl-titer). The polarogram of each solution between −0.8 and −2.0 V. was recorded with a HEYROVSKY-SHIKATA recording polarograph[16] (dropping time 1 sec, galvanometer sensitivity 0.56 μA/mm). The height of the minimum of the cystine wave was measured in each case. For cystine concentrations below $2 \cdot 10^{-6}$ *M* this height is a linear function of the concentration[17] and therefore the wave height of the iodoacetate-treated sample divided by the wave height of the other sample directly gave the percentage SS-bonds. The polarograms were recorded within 20 hrs after the completion of the hydrolysis, as is recommended by SULLIVAN, HESS AND SMITH[18].

Total cystine

The total cystine and methionine content were determined in a separate series of experiments. Again 50 to 100 mg of the samples were used for a Kjeldahl nitrogen determination.

Of the "adult" samples 400 mg and of the "young" samples 200 mg were hydrolyzed as

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described before. After dilution to 100 ml part of the hydrolyzate was reserved for the methionine determination. The HCl titer was determined by titration with 0.5 *M* NaOH. A solution was prepared, containing 3 to 5 ml of the hydrolyzate, 0.002 *M* $Co(NO_3)_2$, 0.1 *M* NH_4Cl and 0.1 *M* ammonia. The polarogram between −0.8 and −2.0 V was recorded. After adding 0.5 ml of a standard solution of 20.0 mg of cysteine hydrochloride (dried in a vacuum dessiccator) in 100 ml 0.1 *M* HCl, the polarogram was recorded again. From the height of the minimum of the cystine wave with and without addition the total cystine content of the hydrolyzate was calculated.

Methionine

The remaining part of the hydrolyzate was extracted with ether to remove fat; the ether remaining in the hydrolyzate was removed by passing carbon dioxyde through the solution. The hydrolyzate was neutralized with 20% NaOH on bromothymol blue and diluted 1:5.

The basal medium used for the microbiological determination was that of STOKES AND GUNNES[8], modified according to Glaxo Laboratories[19, 9] on minor points. The assay was performed with 2 ml of the final medium; samples were analyzed on 5 levels in duplicate. The standard of DL-methionine (30 γ/ml) was assayed on 10 levels in triplicate. The incubating time was 40 hrs, the incubating temperature 33° C. The titration was performed with 0.05 *M* NaOH on bromothymol blue.

RESULTS

The results of the estimation of the percentage SS are given in Table I. There is no correlation between age and percentage SS. As the standard deviation in both groups is about the same, it seems probable that this quantity is kept constant during the whole lifetime. The regulating factor might well be the redoxpotential of the cutaneous tissue, which in its turn might be regulated by the cell metabolism.

TABLE I

Group	Number of animals	Number of determinations	Percentage SS	Standard deviation
Young	36	9	79	6.7
Adult	13	13	80	6.5

In Table II the results of the total cysteine and the methionine determinations are given in percentage of protein (6.25 × Kjeldahl nitrogen).

TABLE II

Group	Number of animals	Number of determinations	% cystine	Standard deviation	% methionine	Standard deviation
Young	40	10	1.23	0.24	1.55	0.21
Adult	10	10	0.54	0.12	1.16	0.12

So there is a very significant variation during the maturation process in both amino-acids (for both differences the probability of insignificance calculated according to FISHER's method is smaller than 10^{-6}). Total cysteine decreases to about 44%, methionine to about 75% of the initial amount. The sulfur content of the skin proteins — calculated from these values — falls to 59% of the initial value, which is in good agreement with the findings of KLAUDER AND BROWN for human skin[2].

From the fact that the percentage SS-bonds remains constant and the total cystine content decreases to 44% of the initial value, it follows that the number of SS-bonds in the skin proteins also decreases to 44% during maturation. Therefore it seems improbable that the rigidity and strength of the cutaneous tissue, which increase considerably during maturation, are a simple function of the number of SS-bonds, as is

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often assumed. The very high total cystine content of hair (about 13% per g of keratin) makes it probable that the cystine lost from the young skin is transferred to the keratin in the hair of the adult animal.

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SUMMARY

The total cystine (cysteine plus cystine) content and the methionine content as well as the percentage SS-bonds (cystine content divided by total cystine content) were determined in the skin of young and adult rats.

While the percentage SS-bonds remains constant at 80%, the total cystine content decreases to 44% (from 1.23% to 0.54% per g protein) and the methionine content to 75% of the initial value (from 1.55% to 1.16% per g protein) during the maturation process.

RÉSUMÉ

Nous avons déterminé la "teneur totale en cystine" (cystéine plus cystine) et en méthionine de même que le pourcentage en liaisons SS (teneur en cystine divisée par la teneur totale en cystine) dans la peau de rats jeunes et adultes.

Tandis que le pourcentage de liaisons SS reste constant à 80%, la teneur totale en cystine décroît jusqu'à 44% (de 1.23% à 0.54% par gramme de protéine) et la teneur en méthionine jusqu'à 75% de la valeur initiale (de 1.55% à 1.16% per gramme de protéine) pendant le processus de maturation.

ZUSAMMENFASSUNG

Der "Gesamtgehalt an Cystin" (Cystein plus Cystin) und der Methioningehalt sowie der Gehalt an SS-Bindungen in Prozenten (Cystingehalt dividiert durch den Gesamtgehalt an Cystin) in der Haut junger und erwachsener Ratten wurden bestimmt.

Während der Gehalt an SS-Bindungen in Prozenten bei 80% konstant bleibt, nimmt der Gesamtgehalt an Cystin bis zu 44% (1.23% bis 0.54% pro Gramm Protein) und der Methioningehalt bis zu 75% vom Anfangswerte (1.55 bis 1.16% pro Gramm Protein) während der Reife ab.

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