HISTOCHEMICAL STUDIES OF SKIN AND HAIR OF MAMMALS WITH REFERENCE TO THE SUBSTANCES REACTIVE TO GLYCOGEN STAINING

HISTOCHEMICAL STUDIES OF SKIN AND HAIR OF MAMMALS WITH REFERENCE TO THE SUBSTANCES REACTIVE TO GLYCOGEN STAINING

By

Hideo TAMATE

Department of Animal Husbandry, Faculty of Agriculture, Tohoku University, Sendai, Japan

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In his previous papers concerning histochemical nature of glycogen in the skin and hair of mammals[9],[10] (Tamate, 1950a and 1950b), the author reported that glycogen existed in the outer root sheath of the hair follicle during entire life of the hair, and that other substances which reacted to glycogen staining were found in Huxley's layer of the inner root sheath, in the hair medulla and in the hair cortex. These substances stained diffusively red with Best's carmine fluid, purple with Bauer-Feulgen's fuchsin sulfite fluid and were resistant to saliva. They also stained red with eosin, and were considered to be something other than pure glycogen.

Bolliger and McDonald[1] (1948) histochemically studied glycogen in the skin and hair of man, sheep, rabbit and phalanger, and stated that the hair root near the papilla frequently stained red with Best's carmine fluid and sometimes contained red globules similar to those seen in the outer root sheath. No further works for this carminophilic substance were made by them.

Lansing and Opdyke[6] (1950) histochemically studied the nipples of the guinea pigs and reported that keratohyalin granules found in *Stratum germinativum* were periodic acid Schiff positive, metachromatic with toluidine blue and stained red with eosin. They concluded that these granules must contain some compound of high polymeric carbohydrates.

The present investigation deals with the histochemical nature of the substances reactive to glycogen staining in Huxley's layer of the inner root sheath, in the hair medulla and in the hair cortex as the third step of investigation concerning histological and physiological studies of glycogen in the skin and hair of mammals.

Materials and Methods

Samples were taken from rats, pigs and cattle during from October, 1950 to May, 1951. Pieces of skin from the belly, back and lips together with the liver as controls were fixed in Toryu's fluid (alcohol-formalin saturated with sodium acetate), embedded in celloidin and sectioned 20 μ thick.

Various stainings were employed as follows:

For glycogen and polysaccharides, Best's carmine stain, Bauer-Feulgen's stain and Hale's method for acid polysaccharides[2] (Hale, 1946); for nucleoprotein and glycoprotein, mucicarmine, Brachet's pyronin-methyl green stain and metachromatic staining of basic dyes such as toluidine blue, thionin and methyl violet; for DNA, Feulgen's nuclear stain; for basic protein, hematoxylin-eosin.

Various enzymes such as saliva, diastase, pepsin, trypsin, pancreatin, ribonuclease and lipase were also employed at 37°C or at 60°C in an incubator before staining. Lipase solution was made from seeds of *Ricinus communis*.

Results

1. *Results Obtained by Using Various Stains.*

A. Best's carmine stain and Bauer-Feulgen's stain.

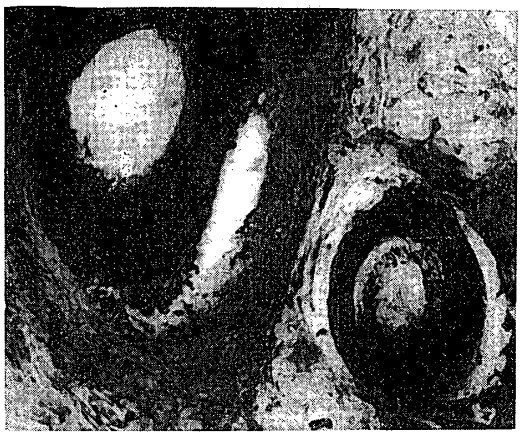
Stained by Best's carmine fluid or Bauer-Feulgen's fluid, red or purple colored substances appeared in Huxley's layer of the inner root sheath of the hair follicle, in the hair medulla and, as globules, in the hair cortex as well as in the outer root sheath where glycogen exists. These colored substances were still observed after salivary digestion except that contained in the outer root sheath (Figs. 1-2).

B. Hale's method for acid polysaccharides.

Stained by this method a dark blue colored substance appeared in the outer root sheath, in Huxley's layer and, less intensely, in the hair medulla and in the hair cortex. The one in the outer root sheath almost disappeared after salivary digestion, while the others did not.

C. Brachet's pyronin-methyl green stain.

Stained by this method according to the procedure recommended by Ichikawa[3] (1950a), a blue colored substance appeared in Henle's layer, the outer layer of the inner root sheath, and a red one in Huxley's layer, in the hair medulla and in the hair cortex. Sections were treated with 0.03 % ribonuclease for 90 minutes at 60°C, or with 0.3 M trichlor-acetic acid for 15 to 20 minutes at 90°C[8] (Pollister and Ris, 1947), and then stained as above. The staining reaction of the sections thus treated was the same as that of untreated ones except the nuclei, although the nuclei were digested by trichlor-acetic acid.



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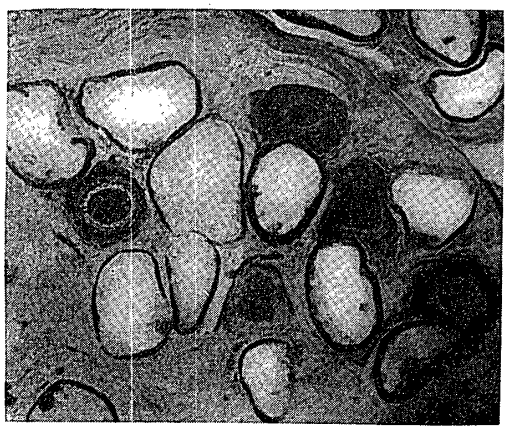

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Fig. 1. Transverse section of pig skin. Best's carmine stain. ×100. The outer root sheath, Huxley's layer, hair medulla and hair cortex contain carminophilic substances.

Fig. 2. Transverse section of cattle skin. Bauer-Feulgen's stain. ×100. The fuchsinophilic substances are found in the portion, where carminophilic substances appear.

D. Mucicarmine stain.

Stained by this method for 30 to 60 minutes, a slight red colored substance appeared in Huxley's layer, in the hair medulla and, as globules, in the hair cortex. Sometimes a deep red colored one appeared in the peripheral layer of the outer root sheath at the level where sebaceous glands connected with the hair follicle. This deep colored substance may be mucin secreted from the sebaceous glands (Fig. 3).

E. Metachromatic stain with basic dyes.

Sections were stained with 0.1 % to 0.5 % aqueous solutions of basic dyes such as thionin, toluidine blue and methyl violet for about 10 minutes. Henle's layer stained blue or bluish purple, while Huxley's layer, hair medulla and hair cortex stained red or reddish purple. With sections treated with 0.3 M trichlor-acetic acid, the nuclei almost lost its stainability to the basic dyes, but cytoplasm did not.

F. Feulgen's nuclear stain.

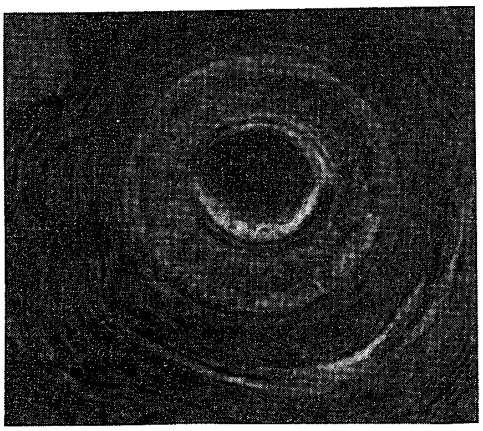
Stained by this method after treatment with 1 N HCl for 25 minutes at 60°C, the nuclei showed intense purple color, but cytoplasm did not (Fig. 4).

G. Eosin stain.

Stained by this method, red colored substances appeared in Huxley's layer, in the hair medulla and, as globules, in the hair cortex as generally known (Fig. 5).

Kelly and Miller[5] (1935) chemically studied the metachromatic staining of dasic byes to distinguish glycoprotein from nucleoprotein and stated that

the former stained reddish, and the latter bluish. If the relation above stated is accepted, the reddish colored portions of the tissue, Huxley's layer, the hair medulla and the hair cortex must contain glycoprotein. Since these portions are Feulgen negative and still stain red with pyronin-methyl green even after treatment with ribonuclease or trichlor-acetic acid, they must contain no nucleic acid in cytoplasm.



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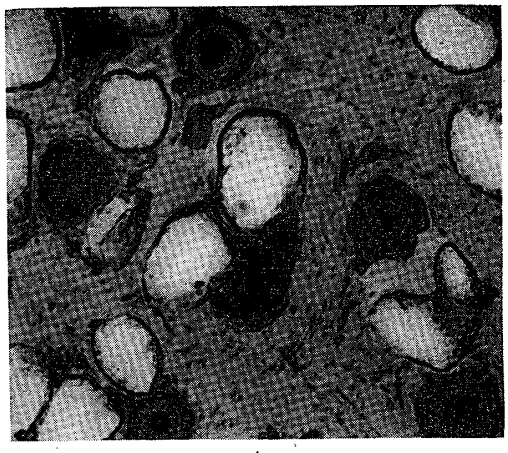

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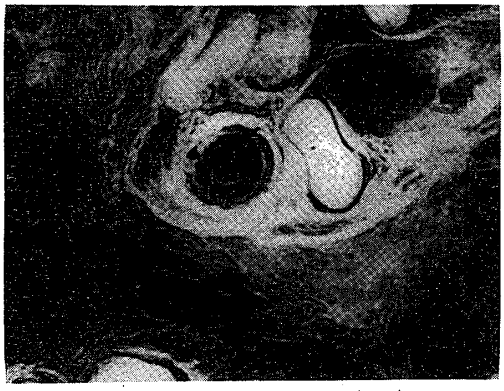

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Fig. 3. Transverse section of rat skin. Mucicarmine stain. ×310 Huxley's layer, hair medulla and hair cortex show positive reaction to mucicarmine.

Fig. 4. Transverse section of cattle skin. Feulgen's stain. ×100. The nuclei show intense reaction.

Fig. 5. Transverse section of cattle skin. Hematoxylin-eosin stain. ×100. The eosinophilic substances appear in Huxley's layer, in hair medulla and in hair cortex.

Fig. 6. Transverse section of cattle skin. Hematoxylin-eosin stain after pepsin digestion. ×100. Eosinophilic substance disappears from Huxley's layer. Henle's layer is partially broken and contains eosinophilic substance.

According to Mori[7] (1949), glycoprotein can be stained red with Best's carmine fluid, and to Hale[2] (1946), acid polysaccharides can be stained blue by his method. Therefore, from the results obtained above, the carminophilic substance found in Huxley's layer, in the hair medulla and, as globules, in the hair cortex must be glycoprotein containing polysaccharides such as glycogen.

2. *Results Obtained dy Using Various Enzymes.*

To further study the histochemical nature of the glycoprotein in the tissue, sections were treated with various enzyme solutions and stained with Best's carmine fluid. The results are shown in Table 1.

Table I. Digestive Power of Various Enzyme Solutions. Best's Carmine Stain.

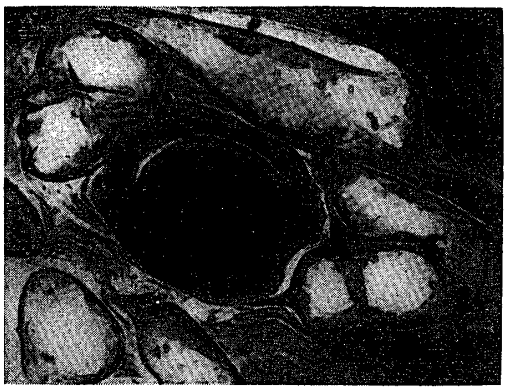
Enzyme solution	pH	Liver in hours	Outer root sheath	Huxley's layer	Hair medulla	Hair cortex	Stratum corneum
Saliva (50%)	7	1--2	3--4	not digested	not digested	not digested	not digested
Diastase (1%)	6	4--5	5--6	〃	〃	〃	〃
Pancreatin (2.5%)	7	4	6--8	〃	〃	〃	〃
Pepsin (1%)	2	not digested	not digested	〃	〃	〃	〃
Trypsin (0.5%)	7	4--6	〃	〃	〃	〃	〃
Pancreatin (2.5%) Saliva (50%)	7	2	4-6	〃	〃	〃	〃
Trypsin (0.5%) Saliva (50%)	7	16	not digested	〃	〃	〃	〃
Pepsin (1%) Saliva (50%)	$\frac{2}{7}$	$\frac{16}{2}$	$\frac{16}{4}$	$\frac{16}{2}$	〃	〃	〃
Lipase solution	7	not digested	not digstede	not digested	〃	〃	〃
Lipase solution Saliva (50%	7	〃	〃	〃	〃	〃	〃

It will be noted that carminophilic substance in the liver and outer root sheath of the hair follicle, the pure glycogen, was easily digested by enzyme solutions containing amylase and not by pepsin or lipase solution. The carminophilic substance in Huxley's layer was digested by co-operation of pepsin and saliva, while that of the hair medulla, the hair cortex and *Stratum corneum* were not by any enzymes used.

Next, sections were treated with pepsin, saliva and pepsin-saliva, and then stained with hematoxylin-eosin or Best's carmine fluid. The results are shown in Table 2.

As will be seen in Table 2, the eosinophilic substance in Huxley's layer disappeared after digestion of pepsin. Eosinophilic substance appeared in Henle's layer partially broken, where the substance was not found formerly (Fig. 6).

Furthermore, the carminophlic substance in Huxley's layer was not digested by pepsin or saliva when they were separately used, but was digested by saliva after treartment with pepsin. The reverse treatment of these two enzymes was also found to be equally effective, but a mixture of them showed no digestive power (Figs. 7-8).



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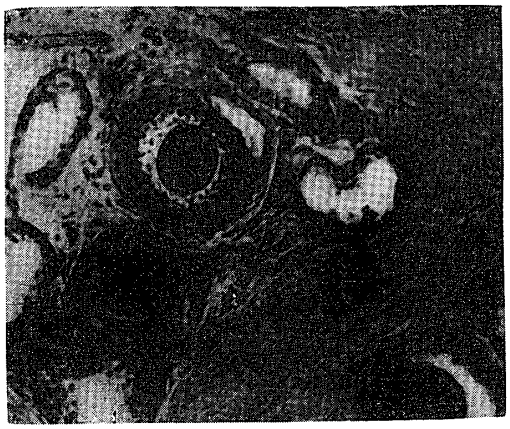

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Fig. 7. Transverse section of cattle skin. Best's carmine stain after pepsin digestion. ×340. The carminophilic substances are not digested in any part of the tissue.

Fig 8. Transverse section of cattle skin Best's carmine stain after digestion of pepsin and saliva. ×340. The carminophilic substance in Huxley's layer disappeared.

From the results obtained above, it will be noted that there are two different components in Huxley's layer, one is stainable with eosin and digestible by pepsin, the other is stainable with carmine and digestible by using both pepsin and saliva separately.

Ichikawa[4] (1950 b) stated that the eosinphilic substance in cytoplasm was some basic protein in general. If the relation is considered in the present investigation the eosinophilic substance in Huxley's layer must be some basic protein combined with the carminophilic substance.

From the results obtained throghout this investigation, it can be concluded that Huxley's layer contain some glycoprotein which consists of basic protein and high polymeric carbohydrates such as glycogen. Lipoid and nucleic acid,

however, were not contained in cytoplasm, because of the fact that lipase, ribonuclease and trichlor-acetic acid showed no digestive power for eosinophilic and carminophilic substances as mentioned above.

Table II. Digestive Power of Pepsin and Saliva. Best's Carmine Stain and Hematoxylin-eosin Stain.

Enzyme solution	pH	Time (in hours)	Best's carmine					Eosin				
			O. r. s.	Hux.	Henl.	H. m.	H. c.	O. r. s.	Hux.	Henl.	H. m.	H. c.
Buffer	7	48	##	##	−	+	+	−	##	±	+	+
Saliva (50%)	7	24	−	##	−	+	+	−	##	±	+	+
Pepsin (1%)	2	4	##	##	−	+	+	−	+	##	+	+
〃 〃	2	6	##	##	−	+	+	−	−	##	+	+
〃 〃	2	16	##	##	−	+	+	−	−	##	+	+
Pepsin (1%) Saliva (50%)	$\frac{2}{7}$	$\frac{17}{2}$	−	−	−	+	+	−	−	##	+	+
Saliva (50%) Pepsin (1%)	$\frac{7}{2}$	$\frac{2}{17}$	−	−	−	+	+	−	−	##	+	+
Pepsin Saliva (mixed)	5	17–24	−	##	−	+	+	−	−	±	+	+

O. r. s. : Outer root sheath ; Henl. : Henle's layer ; Hux. : Huxley's layer ;
H. m. : Hair medulla ; H. c. : Hair cortex ;

Since stainability of carminophilic substance in the hair medulla and in the hair cortex showed good agreement with that of glycoprotein contained in Huxley's layer, these two substance may be the same in their histochemical nature. The difference of digestive power of pepsin and saliva between these substance can be explained as follows: since the carminophilic substance in the hair medulla and in the hair cortex is surrounded by keratin, it resists to all enzymes; on the contrary, however, the carminophilic substance in Huxley's layer is free from keratin and accordingly it is digestible by co-operation of amylase and protease such as saliva and pepsin.

The histochemical nature of basophilic substance in Huxley's layer is yet obscure, but it may be considered to be some compound of hyalin and protein. To support the view just stated, the author noticed the following relation as already mentioned ; after digestion of pepsin the eosinophilic substance ap-

peared in Henle's layer showing that the protein was digested by pepsin and hyalin remained staining with eosin.

As is generally known, keratohyalin granules exist in Huxley's layer and in the hair medulla and they stain reddish with pyronin-methyl green, metachromatic basic dyes and eosin, and are also digestuble by pepsin. According to Lansing and Opdyke[6] (1950), the keratohyalin granules in the pig skin contain some high polymeric carbohydrates. The nature of glycoprotein in Huxley's layer, in the hair medulla and in the hair cortex is identical to keratohyalin granules when the staining reactions and digestive power of pepsin for these two substance are considered.

As to whether keratohyalin and glycoprotein found in the present investigation are the same, it will be discussed in another opportunity.

Summary

The results obtained in the present investigation may be summarized as follows:

1. Stained with Best's carmine fluid or Bauer-Feulgen's fluid, red or purple colored substance appeared in Huxley's layer of the inner root sheath of the hair follicle, in the hair medulla and, as grobules, in the hair cortex as well as in the outer root sheath where glycogen exists.

2. These carminophilic and fuchsinophilic substance are also stained red with mucicarmine, eosin, Brachet's pyronin-methyl green and metachromatic basic dyes. They are also stained blue by Hale's method for acid polysaccharides and therefore it must be glycoprotein.

3. Nucleic acid and lipoid are not found in any part of the tissue examined except DNA in the nuclei.

4. Henle's layer contains protein digestible by pepsin and hyalin stainable by eosin.

5. The glycoprotein in Huxley's layer consists of two components, one is stained red with eosin and digested by pepsin, and the other is stained with Best's carmine and digested by co-operation of pepsin and saliva. The former may be basic protein, and the latter polysaccharides such as glycogen combined with the former.

6. The chemical nature of glycoprotein thus found in Huxley's layer, in the hair medulla and in the hair cortex is identical to that of keratohyalin in *Stratum granulosum*, when the staining reactions and digestive power of pepsin are considered.

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