HISTOCHEMICAL STUDY ON GLYCOGEN METABOLISM IN HAIR FOLLICLES

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By application of Lineweaver-Burk's graphical method to the histochemical system, glycogen phosphorylase and UDP glucose-glycogen glycosyltransferase (glycogen synthetase) activities were studied in hair follicles taken from both the bald and hairy regions of the scalp. The first discernible appearance of the newly formed polysaccharide was recorded at the end point of each point of each enzyme reaction. Although there was no difference between terminal and vellus hair follicles in the distribution of phosphorylase and glycogen synthetase, the activities were higher in the former. In both types of follicles, the bulbar sac was the site of the highest activity of all enzymes except phosphorylase *b*, which showed its highest activity in the lower portion of the anagen external sheath. Phosphorylase *a* activity in the lower external sheath was considerably lower than that in the bulbar sac, but not as low as glycogen synthetase activity. From these data it was concluded that the accumulation of glycogen in the lower external sheath is due to a decrease in phosphorylase *a* activity relative to glycogen synthetase.

It has been suggested that glycogen metabolism participates in such important functions as sweat production, keratinization, and hair cycle (10). Employing histochemical methods, some authors have (2, 3, 13, 17) noted that the accumulation of glycogen is related to glycogen phosphorylase activity in the external hair sheath and have speculated that the glycogen store supplies energy to the hair follicle. Using the highly sensitive fluorometric method in specific areas of hair follicles, Uno et al. (25) found that phosphorylase activity is highest in the external sheath and minimal in the bulbar area, which is the most metabolically active site in the follicle.

In general, glycogen synthesis proceeds through UDP glucose-glycogen glycosyltransferase (glycogen synthetase), and glycogen degradation occurs through phosphorylase (18). Phosphorylase is isolated both in an active and in an inactive form, usually designated as phosphorylase *a* and phosphorylase *b* respectively. The two are interconvertible through the action of a specific, cyclic AMP-sensitive kinase that transfers phosphate from ATP to phosphorylase *b* in the presence of Mg^{++} (5). While phosphorylase *b* requires AMP for catalytic activity, phosphorylase *a* is active without AMP. In order to demonstrate phosphorylase activity in hair follicles, Uno et al. (25) used the incubating medium with added AMP. But the reaction was not that of the active form of phosphorylase. On the other hand, glycogen synthetase exists in 2 different forms, glucose-6-phosphate dependent (D-form) and glucose-6-phosphate independent (I-form) (12). The D to I transformation is mediated by a specific phosphatase and the conversion from I to D is catalysed by

a cyclic AMP-sensitive protein kinase requiring ATP and Mg^{++} (6), which also holds true in the case of cutaneous glycogen (11). Thus, any study of glycogen metabolism in hair follicles requires investigation of both glycogen synthetase and phosphorylase activities. However, glycogen synthetase activity has never been estimated with a quantitative histochemical method. The present study was undertaken in order to analyze glycogen metabolism in the hair follicles with the use of a histochemical quantification method.

MATERIALS AND METHODS

Biopsy skin specimens were obtained from the bald frontal and hairy occipital regions of five patients with male pattern alopecia. As soon as the specimens were excised, they were frozen in small blocks by immersion in isopentane chilled with liquid nitrogen. Sections were cut at $15\ \mu$ in a cryostat maintained at -25°C and were mounted on coverslips. They were dried in air at 20°C for 5 min and kept at 37°C for the predetermined period of time in Coplin jars, each of which contained incubating medium as described below. At the end of each incubation period some sections were taken out, and after being fixed in ethanol for 5 min at 4°C they were immersed for 3 min in Lugol's iodine-iodide solution diluted as much as 10 times with distilled water. They were then dried in air and mounted with glycerin. Thus prepared, the slides were observed continuously with the same binocular microscope, and the first discernible positive reaction was recorded as the end point of enzyme reaction on the given slide. The sites of phosphorylase reaction stain blue to reddish-purple with iodine, those of glycogen synthetase stain reddish-purple to reddish-brown. During the first 3 min of incubation, native glycogen practically disappears from the tissue sections; the little that remains stains brown. Dextran does not stain with iodine.

The following composition was used in order to demonstrate phosphorylase reaction (14, 15): sodium fluoride, 10 mM; ethylenediamine tetraacetic acid, 2 mM; AMP (when present), 2 mM; dextran (with an average molecular weight of 50,000), 60 mg/ml; acetate buffer at pH 5.8, 50 mM; and glucose 1-phosphate in a final concentration of 0.1 to 100 mM. The following medium was used to demonstrate glycogen synthetase reaction (15): ethylenediamine tetraacetic acid, 1 mM; glucose-6-phosphate (when present), 50 mM; dextran (with an average molecular weight of 50,000), 60 mg/ml; Tris-HCl buffer at pH 7.6 (when glucose-6-phosphate present, pH 9.0), 60 mM; and uridine diphosphoglucose in a final concentration from 1 mM to 100 mM. In each case, the final pH was adjusted with 0.1 N HCl or NaOH at 7.2, 7.6 and 9.0, respectively, and the final volume of 5 ml was made up with distilled water.

The dipotassium salt of glucose-1-phosphate, the disodium salt of uridine diphosphoglucose and glucose-6-phosphate, and the sodium salt of AMP from muscle were obtained from Sigma Chemical Co., St. Louis, Missouri, U. S. A. Dextran with an average molecular weight of 50,000 was obtained from Pharmacia, Sweden.

RESULTS

Table 1 shows the relation between substrate concentration and appearance of phosphorylase reaction in specific sites of hair follicles. With the exception of the

TABLE 1. Incubation time necessary for the appearance of phosphorylase reaction in the absence of AMP*

SUBSTRATE CONCENTRATION (mM)	FOLLICLE							
	EXTERNAL HAIR SHEATH				UPPER BULB PORTION			
	upper portion		lower portion					
	T	V	T	V	T	V	T	V
100							21.0±0.84	16.1±0.81
50	10.1±1.16	10.4±0.48					30.3±1.57	27.0±0.87
10	20.2±1.03	21.8±0.55			7.5±0.30	8.5±0.40		
5	46.6±1.55	47.8±0.52	3.1±0.33	3.2±0.25	15.1±0.41	16.2±0.62		
2			6.9±0.33	7.5±0.37	34.4±0.80	41.0±0.71	2.5±0.40	2.7±0.29
1			15.0±0.68	15.1±0.70			5.1±0.35	4.8±0.49
0.5							9.8±0.36	10.8±0.55

* Expressed as minutes. Each figure represents the average of 20 determinations ± standard deviation of the mean. Experimental procedures as described in the text. Abbreviations used as follows: T, terminal follicles; V, vellus.

TABLE 2. Incubation time necessary for the appearance of phosphorylase reaction in the presence of AMP*

SUBSTRATE CONCENTRATION (mM)	FOLLICLE							
	EXTERNAL HAIR SHEATH				UPPER BULB PORTION			
	upper portion		lower portion					
	T	V	T	V	T	V	T	V
10							6.1±0.35	16.1±0.83
5	2.5±0.26	2.6±0.38			2.3±0.22	2.4±0.39	11.5±0.43	28.6±0.84
2	7.5±0.27	6.5±0.46			5.1±0.31	5.6±0.56	32.9±0.30	59.4±0.94
1	15.3±0.46	16.0±1.15	2.5±0.28	3.4±0.38	9.9±0.27	10.5±0.73	2.2±0.29	2.0±0.23
0.5			5.0±0.46	6.0±0.55	20.4±0.55	21.2±0.40	3.7±0.27	3.9±0.23
0.25			9.9±0.36	12.1±0.48			7.4±0.35	7.9±0.30
							14.9±0.48	15.0±0.53

* Expressed as minutes. Each figure represents the average of 20 determinations ± standard deviation of the mean. Experimental procedures as described in the text. Abbreviations used as follows: T, terminal follicles; V, vellus.

TABLE 3. Incubation time necessary for the appearance of glycogen synthetase reaction in the absence of glucose-6-phosphate*

SUBSTRATE CONCENTRATION (mM)	EXTERNAL HAIR SHEATH		FOLLICLE		MATRIX		BULBAR SAC	
	upper portion		UPPER BULB PORTION		T		T	
	T	V	T	V	T	V	T	V
100	33.2±2.33	36.3±3.00			98.1±7.23	108.7±9.02		
50	60.1±1.61	66.1±1.46			125.2±9.13			
10	122.1±9.01	128.0±3.50						
5		12.4±1.06	15.0±3.63	17.7±0.69	19.4±1.25		10.9±1.64	11.7±0.53
2		17.7±1.19	22.4±1.96	43.7±1.25	45.6±0.96		15.6±0.70	20.0±0.82
1		33.6±1.20	37.4±1.79	100.1±2.34	113.6±4.12		29.0±1.78	37.2±1.23

* Expressed as minutes. Each figure represents the average of 20 determinations ± standard deviation of the mean. Experimental procedures as described in the text. Abbreviations used as follows: T, terminal follicles; V, vellus.

TABLE 4. Incubation time necessary for the appearance of glycogen synthetase reaction in the presence of glucose-6-phosphate*

SUBSTRATE CONCENTRATION (mM)	EXTERNAL HAIR SHEATH		FOLLICLE		MATRIX		BULBAR SAC	
	upper portion		UPPER BULB PORTION		T		T	
	T	V	T	V	T	V	T	V
100					43.5±1.06	38.9±1.23		
25			28.1±0.88	21.5±0.61	119.0±2.64	101.6±1.78		
10	10.0±0.76	10.1±0.85	59.4±0.73	43.1±0.80				
5	29.0±1.17	30.6±0.79	120.9±3.62	121.7±2.33				
2	59.1±1.31	61.4±0.71	8.1±0.41	9.5±0.59			7.3±0.36	8.6±0.85
1			19.0±0.70	20.3±0.82			19.0±0.66	19.5±0.80
0.5			43.8±0.91	46.5±0.86			43.7±0.73	45.3±0.48

* Expressed as minutes. Each figure represents the average of 20 determinations ± standard deviation of the mean. Experimental procedures as described in the text. Abbreviations used as follows: T, terminal follicles; V, vellus.

matrix, there was no difference in reaction time between terminal and vellus hair follicles. Reaction time was shortest in the bulbar sac: at a substrate concentration of 1 mM, it was 5.1 (± 0.35) min in terminal and 4.8 (± 0.49) min in vellus follicles. In the lower portion of the external sheath, the reaction appeared in 16.0 (± 0.68) min in terminal and 15.1 (± 0.70) min in vellus follicles. In the matrix, however, the reaction time at 50 mM was 30.3 (± 1.57) min in terminal and 27.0 (± 0.87) min in vellus follicles.

When AMP was added to the incubating medium at a final concentration of 2 mM, the site of the shortest incubation period was the lower portion of the external sheath (Table 2): at 1 mM, 2.5 (± 0.28) min in terminal and 3.4 (± 0.38) min in vellus follicles. In the bulbar sac of terminal and vellus follicles the reaction appeared in 3.7 (± 0.27) min and 3.9 (± 0.23) min respectively. In the matrix, the reaction at 10 mM appeared in 6.1 (± 0.35) min in terminal and 16.1 (± 0.37) min in vellus follicles.

Table 3 shows the relation between the incubation time needed for the appearance of glycogen synthetase reaction and substrate concentration in the absence of glucose-6-phosphate. The time required before the reaction appeared was shorter in terminal follicles than in vellus follicles: in the bulbar sac at 5 mM, 10.9 (± 1.64) min and 11.7 (± 0.53) min for terminal and vellus follicles respectively. In the lower portion of the external sheath, the time at 5 mM was 12.4 (± 1.06) min and 15.0 (± 3.63) min in terminal and vellus follicles respectively.

When the incubating medium contained glucose-6-phosphate in a final concentration of 2 mM, the reaction in the bulbar sac appeared in 7.3 (± 0.36) min in terminal and in 8.6 (± 0.85) min in vellus follicles (Table 4). In the lower portion of the external sheath, the time was 8.1 (± 0.41) min in terminal and 9.5 (± 0.59) min in vellus follicles.

DISCUSSION

Since both phosphorylase and glycogen synthetase are glucosyltransferase, they require an acceptor to which the glucosyl units can be transferred from substrate; glycogen is generally considered to be the best acceptor. In Takeuchi's original method as well as in later, improved methods, glycogen was added to the incubating medium as the primer (4, 9, 20, 21, 22, 23). However, the histochemical reactions of these enzymes depend on an adequate amount of intrinsic glycogen; even massive concentrations of glycogen in the incubating medium can not compensate for inadequate amounts (7, 8, 14). Therefore, these reactions do not necessarily reflect the amount of each enzyme unless the cell has an "adequately large" amount of glycogen. Meijer (8) and Sasai (14) obtained a phosphorylase reaction by using dextran with low molecular weight as the primer in ischemic heart muscle fibers and human apocrine gland respectively. Accordingly, the reactions mentioned above were due exclusively to the activity of phosphorylase or glycogen synthetase.

Several histochemical methods for enzymes presuppose the ultimate formation of a colored product, and traditionally only preparations made at a certain time of incubation with a substrate are observed. However, the formation of the colored product is not always quantitatively related to the activity of an enzyme since it is affected by various other factors, including substrate concentration in the incubating

medium. Benditt and Arase (1), applying Lineweaver-Burk's graphical method of handling the chemical kinetics of an enzyme system to a histochemical diazonium coupling reaction, suggested that the reaction velocity could be estimated by the amount of an end product formed per unit time and that if the amount of an end product is made a constant, the reciprocal of the reaction velocity is directly proportional to the incubation time. They gave prescribed values to various color intensities of an end product, e.g., A, B, and C, and plotted the time required for obtaining the intensity of color represented by C at various substrate concentration. In contrast to the diazonium coupling reaction observed by Benditt and Arase, both the phosphorylase and the glycogen synthetase reactions observed in this study were visualized by directly staining the newly formed polysaccharide in the tissues during incubation. Swanson (19) found that polysaccharide colors with iodine when it has 8 or more glucose units and that the color developed with iodine depends on chain length. On the other hand, another *in vitro* experiment of ours (16) indicated a close connection between the amount of an enzyme and that of the product: i.e., the incubation time necessary for yielding a fixed amount of the product is related to the amount of enzyme. Accordingly, when the minimum amount of reaction product necessary for identifying a colored substance on the tissue section is used as the end product, the enzyme activity can be compared in various sites of the tissue. Thus, the above results can be summarized as follows. 1) There is no difference between terminal and vellus hair follicles in the distribution of phosphorylase and glycogen synthetase, but the activities are higher in terminal follicles than in vellus. 2) In both follicle types, phosphorylase activity is highest in the bulbar sac and lowest in the matrix. 3) The lower portion of the external sheath is the site of the highest activity of phosphorylase *b*. 4) On glycogen synthetase, the activity of the I-form is highest in the bulbar sac and lower in the lower external sheath. 5) The D-form activity is highest in the bulbar sac, and lower in the upper external sheath. 6) Glycogen synthetase activity can not be practically demonstrated in the matrix of either follicle types.

The external sheath of growing hair follicles in man and animals always stores considerable amounts of glycogen. Employing the enzymic method first developed by Passonneau, Uno et al. (24) found a higher concentration in the telogen bulbar sac. The accumulation of glycogen is the result of the disproportion between glycogen synthesis and degradation. In hair follicles from stump-tailed macaques, the activity of glycogen synthetase I-form is highest in the telogen bulbar sac rather than in the anagen external sheath. However, phosphorylase *a* activity is lower in the lower external sheath than in the bulbar sac, whereas phosphorylase *b* activity is high. Therefore, the accumulation of glycogen in the lower external sheath seems to be due to the decrease of phosphorylase *a* activity relative to glycogen synthetase I-form activity in that area.

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