VALIDITY OF IODINE PROCEDURE OF HISTOCHEMICAL METHODS FOR PHOSPHORYLASE IN SKELETAL MUSCLE FIBERS

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Received for Publication June 14, 1970

The iodine procedure for histochemical demonstration of phosphorylase by Takeuchi was improved for demonstration of glycogen metabolic function in muscle fibers of the skeletal muscle. Using this iodine technique, polyglucose synthesized from glucose-1-phosphate by enzymes in skeletal muscle fibers stained with iodine into three types of color, blue, violet and red. The conditions for demonstration of three colors showing the enzyme activities controlled by the intramuscular function of individual fiber were determined. Moreover, it was found that the preservation of iodine color in slides was obtained by using calcium chloride.

Takeuchi and his co-workers reported three types of method for the demonstration of phosphorylase, using the iodine procedure, periodic acid Schiff's reaction and carmine stain techniques. The lead phosphate method proposed by Mölbert (1960)¹⁴⁾ also was tried by Shiraishi, Aoki and Takeuchi (1963)¹⁷⁾ and Hori (1966)⁹⁾, comparing this with their own methods. It has been reconfirmed by their experimental results that the iodine technique for histochemical demonstration of phosphorylase and amylo-1,4→1,6-transglucosidase is the most suitable. Polysaccharides synthesized from glucose-1-phosphate by phosphorylase alone and by both phosphorylase and amylo-1,4→1,6-transglucosidase were different in coloration with iodine, in digestion by α -amylase and β -amylase and in electron-microscopic properties from native glycogen and individual polyglucose in the tissues. (Takeuchi (1959)²⁴⁾, Takeuchi and Sasaki (1963)¹⁶⁾, Shiraishi and Fujimoto (1965)¹⁸⁾).

Recently, Eckner, Riebe, Moulder and Blackstone (1969)¹¹⁾ presented a critical paper questioning the validity of iodine procedure for phosphorylase. However, Takeuchi *et al.*^{30) 31)} maintained the validity of their own technique from chemical, histochemical and electronmicroscopical view points. However, the stainability of these polyglucoses newly synthesized from glucose-1-phosphate by the enzymes with iodine reaction is greatly changeable depending on various conditions of tissue cells and technical matters. In this paper, a suitable condition for demonstration of polyglucose using the iodine procedure was again pointed out and discussed in the skeletal muscle fibers.

MATERIALS AND METHODS

I.) Tissue and Tissue Preparation

Skeletal muscle fibers, mainly abdominal muscles, of normal rabbits and rats

were used for this study. Adult albino rabbits weighing 2000–2500 gm and rats weighing 200–250 gm were beaten on the head, decapitated and then specimens were taken out. All specimens were rapidly frozen by immersing in a liquid nitrogen. Sections of 8μ thickness were cut in a cryostat microtome, then mounted on glass slide and dried under cooler wind at about 15°C for 30 minutes. Then they were kept in a closed coplin jar in a stocker (-20°C) until incubation. Fresh frozen cryostat sections or fixed ones with a cold methanol, a cold ethanol and a cold acetone were used.

II.) Incubating Media and Incubation

The standard medium derived originally by Takeuchi was used, but some modifications and variations of the method under various conditions were tried.

- 1) Soluble starch, Dextran (low molecular weight and high molecular weight) and glycogen were respectively added to the incubating mixture as a primer.
- 2) Some chemicals as activating or inhibiting factors (sodium fluoride, EDTA, AMP, ATP, Mg-ion, glucagon and ethanol) were variously applied.
- 3) P. V. P. (MW 30,000) as protecting agent for the enzyme reaction was used.
- 4) The pH value of the buffer ranged from 4.5 to 7.0.
- 5) Sections were incubated at 37°C for 30, 45, 60 and 90 minutes respectively.

Standard substrate mixture

Glucose-1-phosphate, disodium salt (G-1-P)	100 mg
5-Adenosine monophosphate (AMP)	20 mg
Primer (glycogen)	2 mg
0.1 M Acetate buffer, pH 5.8	25 ml

111.) Iodine Reaction

Variations of iodine concentration and immersion time in Gram's solution were tested. Iodine color preservation was tried by using CaCl_2 .

RESULTS

I. Effect of Fixation

Before incubating for the enzyme reaction, liquid nitrogen frozen sections were immersed in each fixative, namely, a cold methanol, a cold ethanol and a cold acetone. The effect of each fixative for the enzyme reaction is shown in Table 1. Each fixation of these sections prior to the incubation made no appreciable effect with respect to the structure and preservation of newly formed polysaccharides. The longer the fixation was, the less intense the reaction was and the disruption of the cell structure was stronger. In conclusion, the fixation was not useful for the demonstration of the enzyme reaction in skeletal muscle fibers.

II. Effect of Primers

Table 2 shows the effect of some glycosyl acceptors for demonstrating phosphorylase. Two mg of soluble starch, dextrans of molecular weight 70,000 and 197,000 and glycogen were added to each 25 ml of the substrate mixture respectively. However, there were no any remarkable effects on the reaction.

TABLE 1 Effects of Fixation on Phosphorylase Reaction

Fixation	Time (min.)	Intensity	Localization	Cell Structure
Fresh	0	++	G	G
Cold Methanol	1	++	G	G
	5	+	G	G
	10	+	M	M
	10	+	M	M
Cold Ethanol	1	+	M	G
	5	++	G	M
	10	(+)	M	M
Cold Acetone	1	++	GG	G
	5	++	G	G
	10	(+)	M	M

(GG: very good, G: good, M: moderate)

TABLE 2 Effects of Primers

Condition		Reaction Intensity	Iodine Stain		
			blue	violet	red
I	Standard Mixture (St.M.)	++	M	G	G
II	St. M.+Soluble Starch	2 mg	++	G	G
III		5 mg	++	M	M
IV	St. M.+Dextran (M.W. 70,000)	2 mg	++	M	M
V		5 mg	++	M	M
VI	St. M.+Dextran (M.W. 197,000)	2 mg	++	M	G
VII		5 mg	++	G	G
VIII	St. M.+Glycogen	2 mg	++	G	GG
IX		5 mg	++	G	GG

St. M. : G-1-P 100 mg, AMP 20 mg, 0.2 M Acetate Buffer
Distilled Water

III. Effect of Some Chemicals

Some kinds of chemicals were added respectively to the standard substrate mixture, which consisted of 100 mg G-1-P, 12.5 ml of 0.2 M acetate buffer and 12.5 ml distilled water, for regulation of the enzyme reaction.

a) Effect of AMP, ATP, MgCl₂ and Glucagon

There was no necessity of changing the AMP concentration which was recommended originally by Takeuchi. Accordingly, 20 mg AMP addition was used

throughout the present experiment and compared with several concentrations of ATP additions. As shown in Table 3, it was noticed that the enzyme activity was remarkably activated by either AMP or ATP and MgCl_2 . Intensity of the reaction was almost the same as each condition of II, IV, V and VI. Condition VII seemed to bring more stimulative effect to the reaction than the others. However, addition of AMP or $\text{ATP} + \text{Mg}^{++}$ was enough to demonstrate the enzyme activity and to differentiate skeletal muscle fibers with the iodine stain.

TABLE 3 Effects of AMP, ATP, MgCl_2 and Glucagon on Phosphorylase Reaction

Condition	Intensity	Iodine Stain		
		blue	violet	red
I Standard Mixture (St. M.)	(+)	M	M	N
II St. M. + 20 mg AMP	++	G	GG	GG
III St. M. + 5 mg ATP + 2 mg MgCl_2	+	M	G	M
IV St. M. + 10 mg ATP + 2 mg MgCl_2	++	G	GG	GG
V St. M. + 20 mg ATP + 2 mg MgCl_2	++	G	GG	GG
VI St. M. + 20 mg AMP + 10 mg ATP + 2 mg MgCl_2	++	G	G	M
VII St. M. + 20 mg AMP + 10 mg ATP + 2 mg MgCl_2 + Glucagon	+++	GG	G	M
St. M. : G-1-P 100 mg, 0.2 M Acetate Buffer, Distilled Water				

b) Effects of NaF, EDTA and Ethanol

Effects of NaF and EDTA on the phosphorylase activity were histochemically observed. Sections were incubated in various media as shown in Table 4 and stained by iodine reaction. A slightly increased reaction was observed by NaF or both NaF and EDTA addition, compared with control procedures. NaF addition was effective even in the case of the substrate mixture containing AMP. As shown in Table 5, 0.005 M NaF addition was the most appreciable. Ethanol tends to

TABLE 4 Effects of NaF and EDTA on Phosphorylase Reaction

Condition	Intensity	Iodine Stain		
		blue	violet	red
I Standard Mixture (St. M.)	(+)	M	M	N
II St. M. + 0.005M NaF	+	M	G	G
III St. M. + 0.01M EDTA	+	M	M-G	G
IV St. M. + 0.05M NaF + 0.01M EDTA	++	G	G	G
V St. M. + 20 mg AMP + 0.005M NaF	+++	GG	GG	G
VI St. M. + 20 mg AMP + 0.01M EDTA	+++	G	GG	G
VII St. M. + 20 mg AMP + 0.005M NaF + 0.01M EDTA	+++	GG	GG	G
St. M. : G-1-P 100 mg, 0.2 M Acetate Buffer, Distilled Water				

enhance the phosphorylase reaction, but a high concentration of its addition did not significantly stimulate the reaction, in which coloration of the iodine stain tended to be greenish (Table 6). The best reaction was obtained by the addition of 0.005 M NaF and 5% ethanol (see Table 7, Figure 1).

TABLE 5 Effects of NaF on Phosphorylase Reaction incubated in the Medium composed of 100 mg G-1-P, 20 mg AMP, 0.2 M Acetate Buffer and distilled Water

NaF	Intensity	Iodine Stain		
		blue	violet	red
0 M	++	M	M	M
0.001 M	+++	G	M	M
0.005 M	+++	G	G	G
0.01 M	++	G	N	N

TABLE 6 Effects of Alcohol on Phosphorylase Reaction

Alcohol (%)	Intensity	Iodine Stain		
		blue	violet	red
0	++	G	G	GG
10	+++	GG	GG	GG
20	++	G	M(g)	M(g)
30	(+)	M(g)	N	N
40	—	N	N	N

(g) : greenish

TABLE 7 Effects of NaF and Alcohol on Phosphorylase Reaction

Iodine Stain		blue	violet	red	blue	violet	red
Alcohol		5%			10%		
NaF	0.001 M	M	G	GG	G	G	G
	0.005 M	GG	GG	G	GG	G(b)	G(b)

(b) : bluish

c) Effect of PVP as Protective Agent

Addition of some concentrations of PVP to the mixture seemed to give a slightly increased reaction as shown in Table 8. The reaction products in tissue sections, however, tended to be dirty brownish, especially in a red or violet reaction. In conclusion, no appreciable effect on the enzyme reaction could be found from the view point of coloration of iodine stain.

TABLE 8 Effects of PVP Concentration on Phosphorylase Reaction

PVP Concentration (%)	Intensity	Iodine Stain		
		blue	violet	red
0	++	G	GG	GG
2.5	++	G	GG	G*
5	+++	GG	GG	G*
7.5	+++	G	GG*	M*
10	++	M	M*	N
20	+	N	M*	N

* : brownish

IV. PH Difference

The pH values of 0.2 M acetate buffer ranged between 4.5 to 7.0 as shown in Table 9. Sections were incubated respectively to the standard mixture composed of 100 mg G-I-P, 20 mg AMP, 0.005 M NaF, 5% ethanol, 12.5 ml 0.2 M acetate buffer and 12.5 ml distilled water. Each reaction was compared after 30 minutes incubation at 37°C. When the pH value became low, especially among 5.2–6.2, the reaction product seemed to be stained bluish with iodine. However, reactivity diminished apparently when it was very low, especially under pH 5.0. On the other hand, the reddish reaction was observed near the neutral phase of the buffer. Besides, the incubation in a neutral phase made it easy to remove tissue slices from glass slides. From these facts, pH 6.0 was the optimal, in which the better enzyme activity and coloration of iodine stain were observed (Figure 2),

TABLE 9 Effects of pH of 0.2 M Acetate Buffer on Phosphorylase Reaction incubated in the Medium composed of 100 mg G-1-P, 20 mg AMP, 0.005 M NaF, 10% Alcohol and Distilled Water

pH values	Intensity	Localization	Iodine Stain		
			blue	violet	red
4.5	(+)	N	M	N	N
5.0	+	M	M	N	N
5.4	++	G	G	M	M
5.6	+++	G	GG	G	G
5.8	+++	GG	GG	GG	G
6.0	+++	GG	GG	GG	GG
6.2	+++	G	G	GG	GG
6.5	++	M	M	G	GG
7.0	++	N	M	M	G

V. Incubation Time

The serial sections were directly immersed in the mixture containing 100 mg G-I-P, 20 mg AMP, 0.005 M NaF, 5% ethanol, 12.5 ml 0.2 M acetate buffer (pH 6.0) and 12.5 ml distilled water. The longer the incubation time, the stronger the intensity of the enzyme reaction tended to be, as shown in Table 10. However, the diffusion of the reaction products began to occur for longer than 60 minutes. The most suitable incubating time was 45 minutes in skeletal muscle fibers on phosphorylase reaction with iodine method.

VI. Suitable Iodine Concentration and Suitable Immersion Time in Gram's Solution for Coloration

As the iodine stainability of newly formed polysaccharides by phosphorylase was changeable according to the iodine concentration and immersion time in dilute Gram's solution, the best conditions were tested. When tissue sections were immersed in either a high concentration of iodine or for a long time, the reaction color had a tendency to be greenish. The most suitable stainability was obtained by immersing in 10% diluted Gram's solution for 3 minutes (Table 11, 12 Figure 3, 4, 5, 6). A suitable concentration of iodine-glycerin jelly for mounting was 5–10%. When tissue sections of iodine reaction were mounted in Canada balsam or PVP., the coloration of reaction products disappeared following a few hours.

TABLE 10 Effects of Incubation Time on Phosphorylase Reaction

Time (min.)	Intensity	Iodine Stain		
		blue	violet	red
30	++	M	M	G
45	+++	GG	GG	GG
60	+++	GG	G	M
90	+++	GG	M(b)	M(b)

(b) bluish

TABLE 11 Influence of Iodine Concentration for Iodine Stain immersed in Each of the Gram's Solutions for 3 minutes

Gram's Solution (%)	Intensity	Iodine Stain		
		blue	violet	red
1	(+)	M	M	M
5	++	G	G	G
10	++	GG	G	G
20	+++	M(g)	N(g)	N(g)

Gram's Solution (I : KI : H₂O = 1 : 2 : 300)

TABLE 12 Influence of Immersion Time in 10% diluted Gram's Solution on Phosphorylase Reaction

Time (min.)	Intensity	Iodine Stain		
		blue	violet	red
1	(+)	M	N	N
3	++	G	G	G
5	++	G(g)	G	G
10	++	N(g)	N(g)	N(g)

VII. Influence of CaCl₂ on Preservation of the Iodine Stain for the Phosphorylase Reaction

The iodine stain of the phosphorylase reaction has been widely and successfully used to demonstrate newly formed polysaccharides in tissue sections. However, fading of coloration is one of the most unfavourable facts. A durable mounting of the iodine reaction was obtained by immersing in diluted Gram's solution treated with saturated CaCl₂ (Gram's solution: distilled water: saturated CaCl₂ solution= 1 : 3 : 6) and by mounting in iodine glycerin jelly. There was no disappearance of iodine coloration until 4 weeks in high concentrations of CaCl₂ (see Tables 13, 14). Besides, tissue sections mounted even in Canada balsam or PVP after CaCl₂ treatment did not fade completely even after a few days.

TABLE 13 Effects of saturated CaCl₂ Solution added to the Gram's Solution (Gr's)

Condition			Coloration	Preservation			
Gr's	H ₂ O	CaCl ₂		1 day	1 W	2 W	4 W
1	9	0	GG	GG	M	M	N
1	7	2	G	GG	G	G	G
1	5	4	G	GG	GG	G	G
1	3	6	G	GG	GG	GG	G
1	1	8	G	GG	GG	GG	GG
1	0	9	M	GG	GG	GG	GG

TABLE 14 Effects of CaCl₂ after Iodine Stain in the Usual Fashion

Concentration of CaCl ₂	Coloration			Preservation			
	blue	violet	red	1 day	1 W	2 W	4 W
0 M	GG	GG	GG	GG	M	M	N
0.5M	N*	N*	N	GG	M	M	N
1 M	N*	M*	M*	GG	M	M	N
2 M	M*	G	G	GG	M	M	N
3 M	G	G	G	GG	G	G	M
5 M	G	G	GG	GG	GG	GG	G
10 M	G	GG	GG	GG	GG	GG	GG

FIG. 1. The phosphorylase activity in the skeletal muscle of the rabbit (*m. rectus abdominis*) is demonstrated with iodine. Three types of coloration are shown in the individual fiber of the skeletal muscle.

FIG. 2. The phosphorylase activity with iodine stain, in the rabbit skeletal muscle (*m. rectus abdominis*). The large fibers stain dark blue with iodine while the small ones stain red and violet. These colorations depend on the enzyme activities, phosphorylase and amylo-1, 4 $\rightarrow$ 1, 6-transglucosidase.

- FIG. 3. The white skeletal muscle (m. tibialis anterior) demonstrates the phosphorylase activity with iodine, showing three types of color. The blue fibers are predominantly observed in this muscle.
- FIG. 4. In soleus muscle of the rat, the phosphorylase activity is demonstrated in different pattern with iodine stain. The red reaction is predominantly observed in large fibers. It shows a characteristic of the so-called red skeletal muscle.
- FIG. 5. Iodine color for the phosphorylase reaction in the rat gastrocnemius muscle. The upper half of this figure contains the soleus pattern, in which the red stained fibers are predominantly observed in the large.
- FIG. 6. The phosphorylase activity with iodine in the rat gastrocnemius muscle. Three groups of different patterns for the phosphorylase reaction were seen in this section. The intrafusal fibers show the similar reaction to the extrafusal ones.
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DISCUSSION

Takeuchi (1955,²² 1959,²⁴ 1962,²⁶ 1967²⁸) and his co-workers (Sasaki, 1963;¹⁶) Shiraishi & Fujimoto, 1965;¹⁸) Takeuchi & Sasaki, 1970³¹) pointed out histochemically and electronhistochemically that various kinds of polysaccharide synthesized from G-1-P by the phosphorylase and relating enzymes in tissue cells were apparently different from native glycogen. Stainability of these polysaccharides with iodine was different in color respectively. No iodine colorations were observed when G-1-P was omitted from the incubating medium for phosphorylase procedures. It indicated that iodophilic substances were synthesized by the enzyme activity during incubation as reported by Takeuchi *et al.* (1955,²² 1959²⁴) 1965,¹⁸) 1967,²⁸) 1970³¹).

In principle, the action of phosphorylase consists of the addition of α -D-glucosyl units to the non reducing ends of glucosidic chains. Furthermore, according to the chain length of the liner α -D-1,4-polyglucose by the enzyme, the branching enzyme transfers the α -1,4-linkages of chains of glucose units to the α -1,6-linkages.

In the histochemical demonstration of the enzyme activity in tissue sections, the synthesis of polysaccharides may be the result of the combined actions of the enzymes, phosphorylase and branching enzyme. Especially when polysaccharides are stained by the iodine technique, it has also indicated that the change of colors from blue to red of the polyglucoseiodine complex depends on the chain length of the liner α -D-1,4-polyglucose, as has already been established by Swanson (1948²¹). As described in the introduction, polysaccharides synthesized from G-1-P by the enzyme activity in skeletal muscle fiber shows histochemically different colors with iodine. On the basis of several biochemical and histochemical facts, it has been histochemically tried to make suitable and specific demonstration of the enzyme activity with various methodological conditions during incubation in skeletal muscle fibers.

From the histochemical point of view, the structure and enzyme preservation of tissue cells would be most desirable. In cases of dry ice frozen sections, higher enzyme activity and better localization were given by cold acetone fixation for a short time prior to the incubation, which was successfully introduced as the techniques of some cryostat sections. In cases of liquid nitrogen frozen sections, however, any appreciable fixative effects on the enzyme activity and structure preservation were not demonstrable. Freezing and drying would be enough to demonstrate the enzyme activity in skeletal muscle fibers, apart from the observation of wide areas of the tissues.

The histochemical polysaccharide synthesis from G-1-P by the enzyme activities in tissue sections is based on the reversibility of the phosphorylase action *in vivo*. When intramuscular polysaccharide synthesis occurs *in vitro*, the necessity of not only G-1-P as substrate but also of some chemicals such as primer is obvious. Besides, the substrate requirement, pH optimum and activators would give marked effects on the reaction.

The phosphorylase action is dependent on the presence of acceptor molecules as a primer of α -1,4-linked polyglucose chains, and in general, glycogen is considered to be most acceptable as the primer. In Takeuchi's original method as well as in many improved methods, glycogen was added to the incubating medium to serve as the primer. Conversely, some authors showed that glycogen is unnecessary for the histochemical detection of phosphorylase activity. Sotelo (1966)²⁰⁾ especially, suggested that the exogenous glycogen cannot diffuse into the tissues because of its extremely high molecular weight. On the other hand, Meijer (1968¹³⁾) recommended the appreciable findings of the enzyme reaction by using low molecular weight of dextran as a primer in ischemic heart muscle fibers. From his idea, the addition of dextran as a glucosyl acceptor may be considered to be more appreciable than glycogen as in ischemic tissues. As shown in Table 2, however, neither low molecular weight dextran nor glycogen gave remarkable enzyme reaction in skeletal muscle fibers. From these experiments, these additions are not always essential for these glycogen containing tissues, in which native glycogen is a primer in the histochemical procedure.

Some authors (Eränkö & Palkama, 1961²⁾; Godlewski 1960,³⁾ 1963;⁴⁾ Guha & Wegmann, 1959⁵⁾, 1960⁶⁾, 1966⁷⁾; Hori, 1965;⁸⁾ Korsgood & Wulff, 1967;¹¹⁾ Meijer, 1968¹³⁾; Schiraishi *et al.* 1963¹⁷⁾; Takeuchi *et al.* 1958²³⁾, 1961,²⁵⁾ 1968²⁹⁾, 1970³⁰⁾;) have made efforts to establish a suitable histochemical method and proper interpretation of results on the phosphorylase reaction. Increase of the reaction would be attributed to how to stabilize the active form of phosphorylase and how to activate the inactive form. Godlewski (1960)⁴⁾ has previously ascertained the enhancement of phosphorylase activity by NaF addition to the mixture, which would be a suitable approach to detect active phosphorylase. Furthermore, Godlewski (1963)⁵⁾ and Hori (1965)⁸⁾ have histochemically recommended the use of EDTA, which is considered to demonstrate the active form of phosphorylase by preventing the conversion of inactive form to active. In this study, attempts were histochemically made on how to gain the best enzyme activity and the most suitable iodine stain demonstrating different types of skeletal muscle fibers, but were not accurately made on how to differentiate between active and inactive form of phosphorylase. The effect of NaF was evident when comparing the polysaccharide synthesis from G-1-P by spontaneous active phosphorylase and stabilized active one with NaF addition to the medium. On the other hand, the effect of Versene was not evident as seen in Table 4.

Takeuchi (1955)²²⁾ has shown in his original method and his improved one²⁶⁾ 27) that AMP is able to bring about an increased reaction by stimulating the activity of the phosphorylase b-kinase, in which the most suitable concentration of AMP has been ascertained as 20 mg addition to the 25 ml substrate mixture (1958²³⁾, 1961,²⁵⁾ 1962,²⁶⁾ 1966²⁷⁾). Guha and Wegmann (1959⁵⁾, 1960)⁶⁾ demonstrated in their histochemical procedure, applying ATP and Mg-ions, the remarkable possibility of

converting inactive phosphorylase to active. Takeuchi, Hirata and Koya (1961)²⁵⁾ already proved that 3',5'-cyclic AMP with ATP, Mg-ions and glucagon in the substrate mixture stimulated more the phosphorylase reaction *in vitro* than addition of only cyclic-AMP or addition of ATP and Mg-ions. From these observation, some chemicals were applied in the present study. As seen in Table 3, an increased reaction was demonstrated by applying those chemicals of AMP, ATP, Mg-ions and glucagon, apart from iodine coloration. In this case, the phosphorylase activity was too much activated and abundant polyglucose were synthesized, so blue stain with iodine occurred in individual muscle fiber. Therefore, suitable the coloration with iodine for functional fiber types could not be observed in each fiber. For obtaining the differentiation of iodine color types in muscle fibers, other conditions should be selected. As shown in Table 4, addition of 20 mg AMP and 0.005 M NaF to the substrate mixture of 25 ml was most suitable for demonstration of phosphorylase and iodine coloration of intramuscularly synthesized polyglucoses. Although some authors (Eckner *et al.* 1969,¹⁾ Godlewski 1960³⁾, Guha & Wegmann 1959,⁵⁾ 1960)⁶⁾ have pointed out that AMP addition was not enough to demonstrate the activating phosphorylase b-kinase, the medium containing G-1-P, AMP NaF, and ethanol was rather better in this experiment. The effect of alcohol addition, as Takeuchi pointed out in his improved method (1966)²⁷⁾ and Takeuchi and Sasaki's electronhistochemical procedure (1970)³¹⁾, depends on the basis of the observations about inhibition of amylo-1,4→1,6-transglucosidase and protecting agent during incubation. In this experiment, a small amount of alcohol enhanced the reaction for phosphorylase and classified the iodine coloration in the individual muscle fiber. The hydrogen ion concentration of pH 5.8 has been recommended as optimal region for demonstration of phosphorylase by Takeuchi and others,^{16) 18) 22) 26) 30) 31)} while pH 6.5 was considered to be optimal for the histochemical demonstration of amylo-1,4→1,6-transglucosidase.^{23) 27)} Practically, synthesized polysaccharides at the higher pH values have a tendency to stain reddish with iodine, presumably according to coaction of amylo-1,4→1,6-transglucosidase. Moreover, the incubation at higher pH values tended to remove the sections from the glass slide and localization of newly formed polysaccharides became worse. The intense enzyme reaction was observed between pH 5.6 and 6.2. From the view point of iodine coloration, however, the optimal pH in muscle fibers was pH 6.0.

Eränko and Palkama (1961),²⁾ Ohanian (1970)¹⁵⁾ and Kodama (1971)¹⁰⁾ recommended histochemically the appreciable effect of high content of PVP addition to demonstrate the enzyme activity in the tissue and blood cells. So far as using skeletal muscles, PVP had a tendency to stain synthesized polyglucoses brownish with iodine.

When the time of incubation was too long, the reaction products tended to escape from the site of the enzyme activity and the localization also became unsatisfactory. Furthermore, when immersed in the diluted Gram's solution for a long time after incubation the iodine color tended to be greenish. The best reaction for localization, coloration with iodine and intensity was obtained by incubation in the substrate mixture for 45 minutes and by immersion in 10% diluted Gram's solution for 3 minutes.

The problem of the fading of the coloration with the iodine procedure and the difficulty of obtaining permanent slides would be some of the most unfavourable

facts. As tissue sections mounted in the usual fashion, such as the iodized glycerin jelly, faded remarkably in less than one week at room temperature, Takeuchi recommended longer preservation of prepared slides in a stocker at -20°C in darkness and simultaneously the periodic acid Schiff's stain should be combined on another slides. Smith *et al.* (1966)¹⁹⁾ used fortunately Histoclad for the durable mount of slides with iodine reaction. Krisman (1962)¹²⁾ reported chemically that CaCl_2 could preserve the iodine color for a long time. It was found from my experiment that iodine coloration with histochemical procedure was also preserved durably by immersing in a saturated CaCl_2 solution and then mounting with iodized glycerin jelly.

From these results obtained in this study, it was decided that the substrate mixture suitable to differentiate functional skeletal muscle fibers by the enzyme activity is composed of 100 mg G-1-P, 20 mg AMP, 0.005 M NaF, 5% ethanol, 2 mg glycogen, 0.2 M acetate buffer at pH 6.0 and distilled water totalling 25 ml. In this case, it would be better to incubate the section at 37°C for 45 minutes.

According to this method, three types of coloration with iodine procedure for phosphorylase and amylo-1,4 $\rightarrow$ 1,6-transglucosidase were more beautifully observed in individual fiber of skeletal muscles than Takeuchi, Shiraishi and Fujimoto (1965)¹⁸⁾ already pointed out. The deep blue coloration had a tendency to occur in the white fibers which are characterized by their large size in diameter. The red color appeared in the red fibers which are usually small in diameter. The intermediate type of muscle fibers revealed the violet color. The tendency of these coloration was in general observed in the white skeletal muscles such as quadriceps femoris, gastrocnemius, tibialis and peroneus muscles (Fig. 1, Fig. 2, Fig. 3), but it was not always revealed in the other muscles. In the red muscles, such as soleus muscle, the blue reaction occurred in small fibers, while the red one appeared in the large fibers (Fig. 4). Moreover, in the red muscle regions of the usual skeletal muscles the soleus pattern was usually shown (Fig. 5, Fig. 6).

These colorations of polyglucoses synthesized by enzymes presumably depend on the chain length of α -1,4 glucosyl linkage of outer chains which resulted from the activity of enzymes controlled by intramuscular conditions in the substrate mixture. And they presumably assume some conditions of function for glycogen metabolism in the individual fiber. At any rate, it is very important that there are three types of iodine coloration in muscle fibers with iodine procedure for histochemical demonstration of phosphorylase.

ACKNOWLEDGEMENTS

I am grateful to Prof. Dr. Tadao Takeuchi who has critically gone through the manuscript of this paper and given valuable suggestions during this study.

Thanks is given to the staff of his laboratory.

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