HISTOCHEMICAL STUDIES ON PHOSPHORYLASE AND PAS-POSITIVE SUBSTANCES IN THE MUSCULAR PHASE OF TRICHINELLOSIS*

BY

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The behaviour of glycogen in the muscular phase of trichinellosis was studied by several authors (Flury, 1913; Lewis, 1928; Brand et al., 1952; Lewert and Lee, 1954, Richels, 1955; Zarzycki, 1956; Szeky and Nemeseri, 1956; Themann, 1960; Fasske and Themann, 1961; Beckett and Boothryod, 1962; Popow, 1963).

Histochemical methods mostly disclosed an increase in the glycogen level of infected muscles, particularly up to 11 days or between the 10th and 20th day of infection, with its subsequent disappearance from the muscles and accumulation in the larval body (Zarzycki, 1956; Beckett and Boothryod, 1962 and others). As shown by biochemical studies, fully developed muscular larvae are exceptionally rich in glycogen (about 12% w.) constituting energy reserve for rapid growth of parasites during the intestinal phase in a new host, or under other adverse conditions (Brand et al., 1952; Kozar et al., 1966). It has also been suggested that phosphorylating glycolysis does not play a significant role in the respiration of isolated larvae and that the saccharide metabolism is mediated by pentose-phosphorus cycle enzymes (Kozar et al., 1965). The pulp of infected muscles showed between the 10th and 20th day a lower saccharide breakdown rate than that of control muscles, as well as increased amounts of glycogen, whereas after 20 days p.i. the glycogen breakdown of infected muscles was increasing whereby its reserves were undergoing diminution (Karpiak et al., 1963).

As phosphorylase is known to participate in the in vivo glycogenolysis, the present studies were designed to find out their presence and activity in the muscular phase of trichinellosis by means of histochemical methods, in the hope to explain our earlier observations concerning glycogen metabolism and to establish the degree of muscular damage, as the disappearance of phosphorylase from traumatized muscular fibres proved to be a sensitive and early indicator of changes that were

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not recognizable morphologically (Smith, 1965). For better understanding of the occurring processes our material was also examined for the presence of polysaccharides by PAS method using parallely control tests to obtain a fuller picture of their characteristics.

Material and Methods

The material to be examined was taken from normal white mice (control) and infected each with peroral dose of 500 *T. spiralis* larvae; the animals were killed every 2 days in the period beginning from the 5th to 36th day of infection and then at intervals of 1 1/2, 3 1/2 and 4 months after infection. Immediately after death, muscular tissue were taken (m. masseter) which were either frozen in solid CO\(_2\) (in small blocks) and sectioned in cryostat at 20 \(\mu\) or fixed in Carnoy’s fluid and embedded in paraffin.

The cryostat sections were examined for phosphorylase activity by Takeuchi and Kuriaki (1955) method, as modified by Godlewski (1963). Control specimens were incubated with substrate-free fluids (glucose-1-phosphate obtained from the Biochemical Dept., Wrocław University). In view of short-lasting reactions, the preparations were examined immediately and color photographs were taken.

Paraffin block sections were stained by PAS method and with iodine for glycogen detection according to routine method using for control purposes \(\alpha\)-amylase digestion and test with dimedone. Various amylase preparations were used: \(\alpha\)-amylase (Pharmac. Fabrik, Debrecen, Hungary), vegetal diastase (Riedel-De Haën A. G., Seelze, Hannover) or saliva \(\alpha\)-amylase.

Results and Discussion

In histochemical reactions for the detection of phosphorylase, in which glucose-1-phosphate is used in excess glycogenolysis take place and as product of the reaction glycogen is detected or other polysaccharides of glycogen type. If the reaction produces amylosaccharide composed of simple glucoside chains of starch type, the sections take a blue tinge when stained with iodine. If the tissue simultaneously contains a “branching factor” (amylo-1,4-transglucosidase) then simple chains of glycogen are transformed into ramified ones and it takes a brown, mahogany or pink hue with iodine staining.

In addition to phosphorylase “a”, the tissue contain at least one form of the enzyme, referred to as inactive phosphorylase “b”. To activate this form, 3,5 AMP or phosphorylase-beta-kinase active with ATP and Mg\(^{++}\) are necessary. These properties were utilized to develop three histochemical methods allowing for distinction and definition of activities of (1) active phosphorylase “a”, (2) whole phosphorylase “a” + “b”. The whole phosphorylase (“a” + “b”) was determined by activation of phosphorylase “b” by means of AMP or phosphorylase-beta-kinase in the presence of ATP and Mg\(^{++}\).
### Changes in phosphorylase activity, during muscular phase of trichinellosis

<table>
<thead>
<tr>
<th>Localization of reaction</th>
<th>Control animals</th>
<th>Experimental animals</th>
<th>Control animals</th>
<th>Experimental animals</th>
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<tr>
<td>Muscular fibres of control animals and uninfected in experimental animals</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
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<tr>
<td>Infected muscular fibres</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Substance between larva and cyst wall</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>0</td>
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<tr>
<td>Larval body</td>
<td>0</td>
<td>0</td>
<td>+ +</td>
<td>0</td>
</tr>
</tbody>
</table>

- +, ++, ++++, ++++ -- degree of positive reaction
- - -- negative reaction
- 0 -- failure to obtain any data from the given morphologic element and to differentiate between infected and uninfected fibres
The results obtained are compared in Table which requires some explanations. In view of the character of the occurring changes, the muscular phase of trichinellosis was investigated in 3 periods:

1. 5-11 days after infection — penetration of first larvae,
2. 11-25 days after infection — intense changes in muscular fibres,
3. 25 days and later period after infection — cyst formation and stabilization of the processes.

The reactions are discussed separately in:

1. normal muscular fibres of control animals and uninfected muscles of experimental animals,
2. infected muscular fibres in various periods,
3. substance contained between the larval body and cyst wall in later period of infection,
4. larva of parasite in various stages of development.

**Phosphorylase "a"**

In muscular fibres of control animals, the phosphorylase "a" reaction shows mostly an intense brown-violet staining. Stained are almost all (about 95%) muscular fibres. The brown-violet hue of the reaction product indicates the production of glycogen under the effect of phosphorylase "a".

The infected experimental animals exhibited conspicuous changes. In the first period (5-11 days p.i.) the infected fibres are hardly distinguishable from uninfected ones. Larvae are few, but there is already a slightly weaker staining of fibres, reduced in number as compared with control. The change is apparently true for infected fibres, perhaps even for those from the immediate neighbourhood, the more so as in the subsequent period (11-25 days p.i.) when infected fibres were distinct, the reaction in noninfected fibres was less intense than in control (designated by us as ++). After cyst formation (25 days p.i. and later) the intensity of the reaction in these fibres was somewhat stronger, but still weaker than in control.

On the other hand, no phosphorylase "a" activity was seen in the infected muscles and this state persisted until about 25 days after infection.

After cyst formation, the substance contained between the larval body and the cyst wall showed first a trace of phosphorylase "a" activity which then passed into a distinct granular reaction showing a violet hue, which may indicate amylosaccharide synthesis in the larval surrounding (Fig. 1).

In parasites themselves, a brown-violet colour of the reaction was seen only 17 days after infection. The enzyme is localized mostly below the cuticle in the muscular layer. Its activity in larvae tends to increase (Fig. 2) to reach its maximum at about 36 days of infection. When examined 3 1/2 or 4 months after infection, the animals showed a clearcut decrease in the phosphorylase "a" activity in parasites.
2. Phosphorylase "a" activity in encysted larva of *T. spiralis*, 33 days p.i.

Fig. 1. Phosphorylase "a" activity in infected muscular fibres 31 days p.i.
Fig. 3. Phosphorylase "a" + "b" activity in the larval body and in muscles, 31 days p.i.

Fig. 4. Phosphorylase "a" + "b" and beta-kinase activity in muscular phase of trichinellosis 31 days p.i.
The whole phosphorylase "a" + "b"

In control animals, the phosphorylase ("a" + "b") content does not exceed that of phosphorylase "a". It may therefore be assumed that in normal muscles the whole phosphorylase occurs in active form.

Up to the 11th day of infection the intensity of the reaction was unaffected. High activity of the whole phosphorylase was also established between the 11th and 25th day of infection in uninfected muscular fibres. As we already know that infection can produce a decrease in the phosphorylase "a" activity of muscles neighbouring with infected ones, and the addition of AMP to the incubation solution causes activation of phosphorylase "b", it may be admitted that phosphorylase "a" passes into inert form "b".

In muscular fibres affected by parasites, neither active nor inert form of phosphorylase was discovered. In larval body, its neighbourhood and in the cyst wall, the behaviour of the reaction is like that of phosphorylase "a". In larvae, it is localized in muscular cells, being absent in the cyst itself and weak in the substance contained between the larval body and the cyst wall (Fig. 3).

The positive reaction seen in muscular fibres of mice and in muscular cells of the parasite was brown-violet in colour which points to glycogen as being the reaction product. Phosphorylase-beta-kinase, ATP and Mg\(^{++}\) yielded results similar to those obtained within AMP (Fig. 4).

PAS-positive substances

Like other authors, we have found in infected muscular fibres in comparison with uninfected an increase in PAS-positive substances. Changes of granular type reached their maximum at 13 days of infection. Later, during or after formation of the cyst, a stronger reaction occurred in the cyst wall itself and in the substance contained between the larval body and the cyst wall, as compared with the neighbouring muscular fibres.

The performed control tests (various alfa-amylase preparates and dimedone reaction) suggest that PAS-positive substances found to increase in infected muscular fibres are not only glycogen. Iodine reaction for the presence of glycogen was negative both in infected fibres and in the substance in the neighbourhood of larvae contained in the cyst.

In larvae, a strong positive PAS reaction did occur in the layer below the cuticle, in the stichosome cells and in the wall of the alimentary tract. Similar reactions were found by other authors who believed to have discovered the presence of glycogen. Our studies suggest that glycogen is localized in the muscular layer under the cuticle of larvae. However, in the stichosome cells and in the alimentary tract, only a part of PAS-positive substance could be digested with enzymes, hence the assumption that other PAS-positive substance, perhaps neutral mucopolysaccharides, are involved.
Discussion and Conclusions

The present studies imply that *T. spiralis* infection appreciably affects the behaviour of phosphorylase in the muscular tissue. The level of active phosphorylase “a” is considerably reduced. Between the 14th and 21st day of infection the enzyme is absent from the fibres damaged by the parasite and markedly decreased in the neighbouring ones. Slightly different is the behaviour of phosphorylase “b” and phosphorylase-beta-kinase. In *T. spiralis* infected muscles, the activity of the enzyme is entirely abolished, whereas in the neighbouring ones both enzymes are activated under the effect of ATP, Mg++ and AMP.

The decline in the activity of phosphorylase “a” might be responsible for increased glycogen storage in the muscular tissue of infected animals, which would concord with observations of other authors. However, the control tests applied failed to show any increase in the glycogen content, apart from augmentation of other PAS-positive substances. It is therefore possible that the glycogen breakdown is accomplished in another way, which would be consistent with the observation made by Karpiak et al. (1963) who found the infected muscles to contain increased alpha-amylase activity and thereby elevation of glycogen hydrolysis at the expense of phosphorylating hydrolysis.

It should be mentioned that PAS reaction is not invariably a faithful index of the total glycogen content. The Schiff’s reagent only warrants the detection of the soluble glycogen fraction while its insoluble part may increase without being demonstrated by PAS reaction (Kowarzykowa et al., 1963). Decisive are also control tests for glycogen.

After cyst formation, the substance contained between the parasite and cyst wall showed increased phosphorylase activity, but the staining properties of the reaction rather pointed to accumulation of amylosaccharides.

In larvae a distinct phosphorylase activity is mostly localized in the subcuticular muscular layer, where PAS and iodine reactions demonstrated the presence of glycogen. The activity of the enzymes under examination, persisting throughout the experiments, might indicate that the breakdown and synthesis of glycogen includes the participation of the parasite. In the stichosome cells however other polysaccharides seems to increase.

The addition of ATP for the detection of the whole phosphorylase (“a” + “b” + beta-kinase) may also activate UDPG-transglucosilase, but we feel this did nor happen in our case, as the results obtained with the fluid for “a” + “b” phosphorylase were similar to those observed for the activity of the whole phosphorylase + beta-kinase.

Summary

Histochemical procedures (after Takeuchi and Kuriaki, modified by Godlewski) were applied to study the activity of “a”, “a”+“b” phosphorylase and phosphorylase-beta-kinase in the muscular tissue of *T. spiralis* infected mice, examined at various post-infection periods every 2 days between
the 5th and 36th day and then with decreasing frequency up to 4 months after infection. Additional studies involved PAS-positive substances and control tests for glycogen.

*T. spiralis* infection was found to affect distinctly the behaviour of phosphorylase. The phosphorylase "a" disappears from the infected muscles and in the neighbouring ones its activity is considerably reduced as compared with the control. After cyst formation, the substance contained between the larval body and the cyst shows again the presence of phosphorylase which tends to increase and its staining properties indicate the synthesis of amylosaccharides. In the larval body (muscular layer) the phosphorylase appears by the 17th day, increases up to the 36th day to decline in later phases (e.g. 4 months after infection).

The content of the whole phosphorylase ("a" + "b" + beta-kinase) is increased in noninfected fibres of experimental animals (at 11-25 days of infection) this meaning that there the "a" form passes into the inert "b" one.

The decrease in the phosphorylase "a" activity or its lack might indicate augmentation in glycogen reserves of the muscular tissue in infected animals, as postulated by other authors, but the control tests for PAS-positive substances demonstrate that in the infected fibres the increase is true not only for glycogen but also for other polysaccharides. Like in the larval body, the glycogen content is increased exclusively in the muscular layer, whereas in the stichosome cells and in the wall of the alimentary tract glycogen was found to accumulate along with other PAS-positive substances, that are likely to be neutral mucopolysaccharides.

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LITERATURE


BADANIA HISTOCHEMICZNE NA FOSFORYLAZĘ ORAZ SUBSTANCJE PAS DODATNIE W MIĘŚNIOWEJ FAZIE WŁOŚNICY

Z. KOZAR, R. SENIUTA

W tkance mięśniowej myszy zarażonych T. spiralis (badanej w różnych okresach po zarażeniu, między 5 a 36 dniem p.z. co drugi dzień, potem rzadziej, aż do 4 mies. p.z.) oraz myszy zdrowych, kontrolnych, śledzono histochemicznie (metody Takeuchi i Kuriaki w modyfikacji Godlewskiego) aktywność fosforylaz „a”, „a” + „b” oraz fosforylazo-beta-kinazy. Ponadto badano substancje PAS-dodatnie wraz z odczynami kontrolnymi na glikogen.

Okażało się, że zarażenie T. spiralis wpływa wyraźnie na zachowanie się fosforylazi. Fosforylaza „a” ginie zupełnie w włóknach zarażonych i również w włóknach sąsiadujących z nimi aktywność tego enzymu jest słabsza w porównaniu z kontrolą. Po wytworzeniu się torebki w substancji zawartej między larwą a ścianą torebki pojawia się znów fosforylaza, dając z czasem odczyn coraz intensywniejszy, którego zabarwienie świadczy o syntezie amylocukru. W larwach (w warstwie mięśniowej) fosforylaza pojawia się w 17 dniu p.z., jej aktywność wzrasta do 36 dnia p.z., ale w późniejszych okresach (np. po 4 mies. p.z.) widoczny jest spadek aktywności.

Zawartość fosforylazy całkowitej („a” + „b” oraz fosforylazo-beta-kinazy), zwiększa się w włóknach nie zarażonych pasożytu u zwierząt doświadczalnych (w 11-25 dniu p.z.), co świadczy, że fosforylaza „a” przechodzi tam w postać nieczynną „b”.

Spadek aktywności fosforylaz „a” lub jej brak mogą świadczyć o zwiększaniu się zapasów glikogenu w tkance mięśniowej zarażonych, co stwierdzali już inni autorzy. Przeprowadzone jednak reakcje kontrolne na substancje PAS-dodatnie sugerują, że w zarażonych włóknach zwiększają się nie tylko ilości glikogenu, ale i innych wielocukrów. Podobnie i w larwie glikogen wzrasta tylko w warstwie mięśniowej, natomiast w komórkach stichosomu i ściany przewodu pokarmowego gromadzą się prócz glikogenu inne substancje PAS-dodatnie, może Mukopolisacharydy obojętne.