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# THE ROLE OF GLYCOLYSIS IN ENERGY PRODUCTION IN THE ISOLATED SKIN OF THE BROWN FROG (*RANA TEMPORARIA*, L.)

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SUMMARY

1. Total amounts of glycogen and the time course of the disappearance of glycogen were measured in frog skin under aerobic conditions. The calculated maximum energy production from the metabolism of glycogen to $CO_2$ and water was found to be insufficient to account for $Na^+$ transport.

2. Lactate production under anaerobic conditions was correlated with anaerobic $Na^+$ transport. The calculated energy production was found to be large enough to account for the transport recorded.

3. A significant Pasteur effect was observed.

## INTRODUCTION

In the study of regulatory links between metabolism and active transport in the isolated frog skin, it is essential to have quantitative knowledge about the various metabolic abilities of the tissue. LEAF AND RENSHAW[1] measured the production of lactate under anaerobic conditions and found that 3.9–8.6 $Na^+$ were transported per mole of lactate formed. They did not investigate the role of glycolysis under aerobic conditions or the maximal production of lactate under anaerobic conditions. The early experiments of HUF[2], in which he used inhibitors like fluoride and bromoacetate, indicate that catabolism of glycogen *via* glycolysis is supplying the energy for active transport. He also studied the effects of various exogenous substrates. However useful such studies may be, they give no information about the endogenous substrates and the pathway by which they are metabolized. Especially in inhibitor studies, the response will be very difficult to interpret before knowing what is going on in the frog skin at the metabolic level. As there do not exist any quantitative investigations on the metabolic pathways followed in the frog skin, the role of glycolysis has been studied under aerobic as well as anaerobic conditions.

## MATERIALS AND METHODS

The frogs (*Rana temporaria*) were kept partially immersed in tap water at a temperature of about 3° and used without any pretreatment.

*Analysis of glycogen.* 2-$cm^2$ pieces of skin were weighed and dissolved in 500 $\mu$l 1.0 M NaOH on a boiling water bath for 15 min. After cooling the mixture was

acidified with 200 $\mu$l 6.3 M HCl, and glycogen was hydrolyzed at 100° for 2 h. The mixture was then neutralized with 200 $\mu$l of a solution which is 3.5 M NaOH and 1.0 M $NaHCO_3$. The contents of the tubes were then frozen and thawed, which facilitates precipitation of denatured protein, and then centrifuged for 15 min at 10000 rev./min in the Sorvall refrigerated centrifuge. 100 $\mu$l of the supernatant fluid were used for the analysis of glucose by a modification of the glucose oxidase method[3]. The final volume in the glucose analysis was 600 $\mu$l, and the range of the method is 0–33 m$\mu$moles.

*Analysis of free glucose.* The skin pieces were frozen and homogenized at the temperature of liquid nitrogen in the Braun Dismembrator (Braun, Germany). The resulting powder is extracted twice with 1 ml of 0.3 M $HClO_4$ in a small glass homogenizer. The perchloric acid extract is neutralized with a mixture of KOH and Tris, the concentration of which is determined by titration of the perchloric acid, so that the final pH of the neutralized extract is around 7.5. Glucose was then determined as above.

*Short-circuit experiments* were performed according to the method of USSING AND ZERAHN[4] with an automatic voltage clamp setup constructed by HANSEN[5]. Small perspex chambers were used, having a volume of 5 ml on each side of the skin. The skin area was 7 $cm^2$. Anaerobic conditions were produced by running between 100 and 200 ml of oxygen-free Ringer's solution through the chamber. Correlation of anaerobic sodium transport and lactate production was measured in this chamber, the lactate being determined in both the inside and the outside bathing solutions. The total net $Na^+$ transport during the anaerobic period was calculated by graphic integration of the current records.

*Total lactate production* measurements under anaerobic conditions were performed in glass syringes. The Ringer's solution was deoxygenated in the syringe with a stream of nitrogen for about 15 min. The skin was placed in the syringe, which was then closed. Stirring was performed with a steel ball, the syringes being placed on a mechanical table. After 4 h the Ringer's solution was removed, deproteinized with $HClO_4$, neutralized with KOH, and analyzed for lactate according to the method of LUNDHOLM, MOHME-LUNDHOLM AND VAMOS[6]. The lactate method was modified so that the final volume was 575 $\mu$l. A Zeiss spectrophotometer was used. It was possible to measure on volumes down to 350 $\mu$l in ordinary semi-microcuvettes, by keeping the cuvettes in a slightly higher position and using a slit height of 3.5 mm.

## RESULTS

Measurements of glycogen were performed on 68 pieces of ventral frog skin of 34 different frogs. The amount found expressed as glucose units was 234 m$\mu$moles per 100 mg wet weight (S.D. = $\pm$ 68 m$\mu$moles/100 mg). The amount of free glucose was measured in skins from 10 frogs, and was found to be 32.1 $\pm$ 16.9 m$\mu$moles/100 mg wet weight.

An attempt was made to localize glycogen in the various skin layers with the slicing method of HANSEN AND ZERAHN[7]. The epithelium and the chromatophore layer contained together 85 % of the total amount of glycogen, and the results indicated that about half the total amount was found in the epithelium.

The aerobic glycolysis in frog skin was estimated by measuring the rate of dis-

appearance of glycogen in pieces of ventral skin. In these experiments skin pieces of 2 $cm^2$ were incubated in aerated Ringer's solution for various periods of time and then analyzed for glycogen. The results are shown in Fig. 1. Each point represents the average of 4 individual experiments. The rate of disappearance was linear for the first 6 h, after which the curve leveled off. In the first 6 h the rate of disappearance of glycogen was about 18 mμmoles glucose units/100 mg wet weight per h. Under aerobic conditions no lactate could be detected in the bathing solutions.

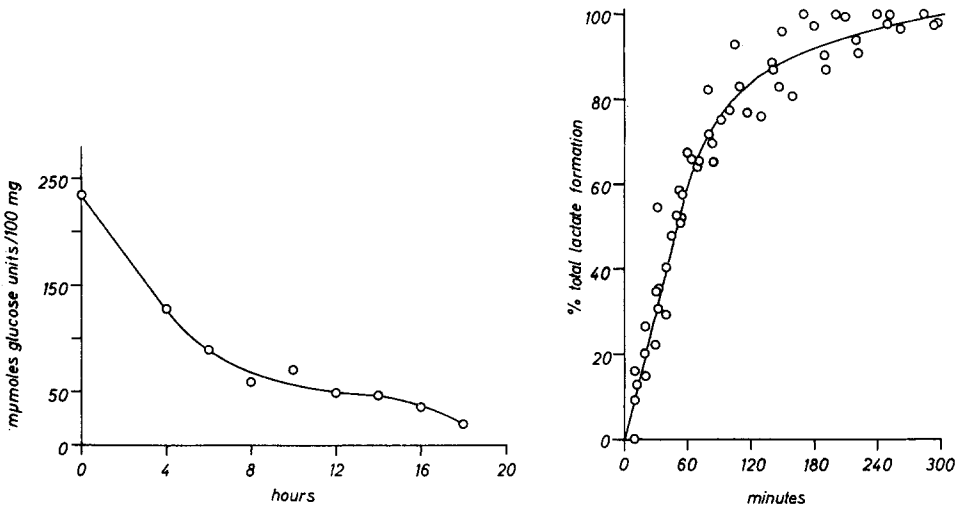


Fig. 1. Aerobic experiments. The amount of glycogen present in frog skin after various times of incubation in aerated Ringer's solution. Every point is the average of 4 different experiments.

Fig. 2. The course of lactate formation by frog skin under anaerobic conditions.

In 5 experiments the lactate production was determined at various intervals over a period of 5 h anaerobic incubation. Most of the lactate is liberated in 4 h. The results of these experiments are shown in Fig. 2.

In 12 experiments 2-$cm^2$ pieces of skin were incubated for 4 h, and the lactate in the bathing solution determined at the end of this period. The amount of lactate produced was 678 ± 36 mμmoles/100 mg wet weight.

TABLE I

THE AMOUNTS OF GLYCOGEN AND GLUCOSE, AND THE PRODUCTION OF LACTATE UNDER ANAEROBIC CONDITIONS

| | *mμmoles/100 mg wet wt.** |
|---|---|
| Glycogen | 234 ± 68 |
| Glucose | 32 ± 17 |
| Lactate produced during 4 h incubation | 678 ± 36 |
| Lactate produced during 1 h incubation | 407 ± 22 |

* Mean ± S.D.

The amounts of glycogen, glucose, and the total lactate production are shown in Table I. Together, glycogen and glucose can account for 79 % of the total lactate formation.

Correlation was made between anaerobic transport and lactate formation in nine experiments, the results of which are shown in Table II. Lactate is preferentially given off to the inside bathing solution. An average of 1.8 $\pm$ 0.8 $Na^+$ are transported per molecule of lactate produced.

TABLE II

CORRELATION OF SIMULTANEOUS LACTATE PRODUCTION AND $Na^+$ TRANSPORT UNDER ANAEROBIC CONDITIONS

The period of measurement starts with onset of anaerobic conditions, and runs until the short-circuit current is zero. The skin area is 7 $cm^2$.

| *$Na^+$ transport ($\mu$equiv)* | *Lactate ($\mu$moles) given off to* | | *$Na^+$ transported per mole lactate* |
|---|---|---|---|
| | *inside solutions* | *outside solutions* | |
| 2.45 | 0.617 | 0.261 | 2.8 |
| 2.49 | 1.204 | 0.241 | 1.7 |
| 0.83 | 1.428 | 0.156 | 0.5 |
| 3.53 | 1.309 | 0.275 | 2.2 |
| 2.87 | 0.897 | 0.096 | 2.9 |
| 1.28 | 0.650 | 0.067 | 1.8 |
| 1.26 | 0.972 | 0.164 | 1.1 |
| 1.15 | 0.469 | 0.130 | 1.9 |
| 2.35 | 1.166 | 0.379 | 1.5 |
| | | | Mean 1.8 $\pm$ 0.8 |

No attempt was made to correlate directly aerobic $Na^+$ transport with glycogen disappearance as the results obtained varied with the duration of the experiment, because frog skins are able to transport for 20 h or more. The total transport capacity was, however, measured in a series of 8 skins. The average total $Na^+$ transport calculated from the current records by graphic integration was 50 $\mu$equiv/100 mg wet weight.

## DISCUSSION

It is possible to determine the role of glycogen as a substrate in frog skin by comparing the calculated rate of ATP formation *via* glycolysis and respiration with the rate of ATP consumption by the active transport. That is, the rate of transport should be compared to the rate of glycogen disappearance.

Under aerobic conditions (Fig. 1) the rate of glycogen disappearance is about 18 m$\mu$moles glucose units/h per 100 mg wet weight. If the pyruvate formed is metabolized to carbon dioxide and water through the citric acid cycle, this would give rise to the formation of 700 m$\mu$moles of ATP per h per 100 mg. ZERAHN[8] and LEAF AND RENSHAW[9] found that 18 $Na^+$ are transported per molecule of oxygen consumed above the basal oxygen consumption in the skin, measured in the absence of $Na^+$ transport. As a P/O ratio of 3 can be expected, 3 $Na^+$ are transported at the expense of 1 ATP molecule. So the aerobic metabolism of glycogen can account for a transport rate of

2.1 μequiv of $Na^+$ per h for 100 mg skin. In 14 experiments the average rate of transport was found to be 4.8 μequiv/h per 100 mg. The maximum rate of ATP formation from the aerobic metabolism of glycogen is too small to account for the rate of transport recorded. In order to test this conclusion another approach was used. The maximum amount of ATP formed from the total amount of glycogen present was compared to the total transport capacity. 234 mμmoles of glucose units generate 9 μmoles of ATP, which would account for the transport of 27 μequiv of $Na^+$. In a series of eight skins, the average total transport was 50 μequiv of $Na^+$ per 100 mg wet weight. This supports the conclusion made above. In addition to the respiration corresponding to $Na^+$ transport, the frog skin has a basal respiration which is of the same order of magnitude as the transport respiration[8]. It must therefore be concluded that glycogen is not the only endogenous substrate in frog skin.

The glycogen content has been measured earlier in this laboratory (H. NIELSEN, unpublished observations). The glycogen was isolated according to the procedure of HASSID AND ABRAHAM[10] and determined with the method of PARK AND JOHNSON[11]. The skin was found to contain 227 ± 50 mμmoles of glucose units/100 mg wet weight, in good agreement with the results presented here.

The total amount of lactate formed by 100 mg skin was 678 mμmoles. Fig. 2 shows that 60 % is liberated at a constant rate during the first hour, giving a rate of production of about 0.4 μmole/h per 100 mg. This is in good agreement with the results obtained on frog skin by LEAF AND RENSHAW[1]. Since more than 70 % of the total lactate production originates from glycogen, it can be calculated that glycolysis is inhibited by about 85 % under aerobic conditions, thus showing a large Pasteur effect. This has not yet been studied in detail.

The $Na^+$/lactate ratio was found to be 1.8 ± 0.8. This value is, however, too small because some of the lactate originates from elsewhere than epithelial cells. An examination of a transverse section of a frog skin reveals[12] that about half the total amount of cells is found in the epithelium. This fact together with the results from the slicing experiments mentioned in the 2nd paragraph of RESULTS may lead to the assumption that the amount of lactate given off by the epithelium may be about half the total amount given off by the skin. Therefore 3.6 $Na^+$ are transported per molecule of lactate formed by the epithelium. If we assume that all cells in the skin contribute equally to lactate formation, we can assume that about 2.4 $Na^+$ are transported per molecule of ATP formed *via* glycolysis in the epithelium, as the formation of 1 molecule of lactate gives rise to the formation of 1.5 molecules of ATP.

From the $Na^+$/oxygen ratio obtained by ZERAHN[8] and by LEAF AND RENSHAW[9] a number of 3 $Na^+$ would be expected to be transported per molecule of ATP. If the mechanism of $Na^+$ transport is the same under aerobic and anaerobic conditions, it is concluded that the energy produced *via* glycolysis under anaerobic conditions is sufficient for the transport of $Na^+$ in the experiments presented here.

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