BBA 75708

GLYCOGEN IN ISOLATED MUCOSAL AND SEROSAL FRACTIONS OF TURTLE BLADDER

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(Received March 8th, 1971)

SUMMARY

1. The amount and behavior of glycogen in isolated fractions of turtle bladder were determined. The mucosal (epithelial) fraction contained approximately 30 % of whole bladder glycogen in both summer and winter. The remainder was contained in the serosal (muscle) fraction.

2. The mucosal fraction made up a significantly greater proportion of total bladder wet weight in August than in January–March.

3. Anoxia and 2,4-dinitrophenol caused rapid depletion of glycogen in both fractions. The substitution of choline for Na^+ in bathing fluids reduced glycogen losses both in the presence and absence of dinitrophenol.

4. In each set of conditions tested, glycogenolysis was more extensive in the mucosal fraction than in the serosal fraction.

5. Although the magnitudes of glycogen losses varied under different conditions, mucosal and serosal losses maintained an approximately constant relationship. It was suggested that glycogenolytic systems with similar characteristics (response patterns) but different intrinsic activities are operative in the two fractions.

INTRODUCTION

Because glycogen is a possible source of energy for ion transport, the relationship of glycogen metabolism to Na^+ transport has been investigated in the Na^+-transporting urinary bladders of turtles and toads[1–4]. However, a complicating factor in the interpretation of the findings is the presence of smooth muscle in these bladders[5]. Muscle contains glycogen, and, if considerable muscle is present, changes in glycogen content of whole bladder tissue cannot be attributed with certainty to the activity of epithelial cells. Experiments on the relationship of glycogen metabolism to ion transport, however, should be concerned primarily with epithelial cells. These cells alone are the active agents in ion transport as shown by the fact that removing them abolishes the transmural potential which the transport process generates[6].

To separate the glycogen metabolism of epithelial cells from that of smooth muscle in the turtle bladder, we have utilized isolated mucosal and serosal fractions

obtained by dissection. This paper reports the effects of anoxia, dinitrophenol, and Na^+ lack on the glycogen levels of the two fractions.

METHODS

Tissues were removed from the turtle, *Pseudemys scripta*, and placed in iced Ringer solution. In experiments where bladder fractions were utilized, the chilled bladder was dissected into mucosal and serosal fractions by blunt dissection[5,7]. The fractions were cut into slices of approximately 0.5–0.75 mm × 1.00 mm with a razor blade.

The mucosal fraction of the bladder contains columnar epithelial cells, connective tissue, small single fibers, and a few small bundles of smooth muscle. The serosal fraction contains large bundles of smooth muscle and connective tissue. As reported previously, the average proportion of epithelial tissue to muscle in whole bladder was 1–2.3 (ref. 5). The behavior of connective tissue glycogen is not considered in the present work since preliminary experiments showed that the amount of glycogen in connective tissue was negligible.

Glycogen was determined by a modified Pflüger procedure of GOOD *et al.*[8,9] using 0.2–0.5 g of tissue. After KOH extraction of tissue, alcohol precipitation and acid hydrolysis of glycogen, glucose determinations were made on duplicate samples of the neutralized hydrolysate using glucose oxidase (glucostat method, Worthington Biochemicals, Freehold, N. J.).

Turtles were obtained from Lemberger Supply Co., Oshkosh, Wisc., and were not fed during the week or two between receipt and use in experiments.

The composition of the Ringer solution in terms of final concentrations was 103 mM NaCl; 3 mM $NaHCO_3$; 3 mM KCl; 2 mM $CaCl_2$; 1 mM $MgCl_2$. Choline chloride was substituted for NaCl and choline bicarbonate for $NaHCO_3$ in Na^+-free Ringer Solution. pH was brought to 7.6–7.8; osmolality was adjusted to 224 mosmoles/kg.

Experiments were carried out at room temperature, approximately 25°.

Statistical analyses were performed according to SNEDECOR[10]. Analysis of variance was calculated on a CDC 6600 computer at Brookhaven National Laboratory.

RESULTS

Glycogen in various muscular and mucosal tissues

Table I presents values of the glycogen content of several turtle tissues. The glycogen content of three types of turtle muscle follows the general pattern reported for mammals. Smooth muscle, represented in this study by intestine serosal fraction, contained much less glycogen than skeletal muscle. A similar observation has been made by BUEDING[11]. Mammalian heart in fasting animals contains more glycogen than skeletal muscle[12]. In agreement, ventricular muscle was richest in glycogen of the three varieties of turtle muscle tested in our study. Intestinal mucosa was relatively glycogen poor.

Distribution of glycogen between mucosal and serosal fractions of bladder

Table II shows the distribution of glycogen between mucosal and serosal frac-

TABLE I

GLYCOGEN CONTENT OF TURTLE TISSUE

Glycogen content is expressed as mg glucose per g wet tissue weight (mean values ± S.E.); *n*, number of experiments. Determinations were carried out immediately after the tissue was removed from the animal. Intestine was kept cold during dissection into mucosal and serosal fractions. Determinations were carried out in the winter months.

	Whole bladder	*Intestine* (mucosal fraction)*	*Intestine* (serosal fraction)*	*Skeletal muscle***	*Heart (ventricle)*
Glycogen	1.97 ± 0.30	1.83 ± 0.30	1.77 ± 0.50	9.83 ± 1.38	12.03 ± 1.97
n	5	12	11	11	11

* Lower small intestine.
** Subscapularis muscle.

TABLE II

GLYCOGEN CONTENT AND WET WEIGHT OF ISOLATED MUCOSAL AND SEROSAL FRACTIONS OF TURTLE BLADDER

First set of values shows glycogen content (mean values ± S.E.) of bladder fractions in winter and summer. Determinations were made immediately after dissection of bladder into fractions. Second set of values shows percent of total wet weight (mean values ± S.E.) contributed by each fraction in winter and summer. Third set of values, percent contribution to whole bladder glycogen content, is derived from the first two sets and is given as a single value without S.E. Numbers in parentheses show numbers of experiments.

	Mucosal fraction	*Serosal fraction*
	Glycogen content (mg/g wet wt.)	
Winter	1.79 ± 0.33 (7)	2.24 ± 0.44 (7)
Summer	2.07 ± 0.14 (12)	3.38 ± 0.25 (12)
P (Winter–Summer)	> 0.4	> 0.05
	% of total wet wt.	
Winter	33.4 ± 1.71 (9)	66.6 ± 1.71 (9)
Summer	42.1 ± 1.32 (6)	57.9 ± 1.32 (6)
P (Winter–Summer)	< 0.001	< 0.001
	Calculated % contribution to whole bladder glycogen	
Winter	28.6*	71.4
Summer	30.8	69.2

* Sample calculation: $[1.79 \times 33.4/[(1.79 \times 33.4) + (2.24 \times 66.6)]] \times 100 = 28.6$.

tions of whole bladder tissue in summer (August) and winter (January–March). To determine the distribution, glycogen content of isolated mucosal and serosal fractions was measured. In a separate set of experiments mucosal and serosal percentages of whole bladder wet weight were found by separating entire bladders into mucosal and serosal fractions and determining the relative proportions of wet weights. From these

parameters, the derived parameter, percent contribution to whole bladder glycogen content, was calculated.

In late summer the glycogen levels of both bladder fractions were higher than those of winter levels but the increases were not significant.

The percent of total wet weight contributed by the mucosal fraction was 33.4 in winter and 42.1 in summer. The difference between summer and winter values was significant ($P<0.001$).

The distribution of bladder glycogen was nearly the same in winter and summer. In winter the mucosal fraction contributed 28.6 % of whole bladder glycogen; the serosal fraction contributed the remainder, 71.4 %. In summer the mucosal fraction contributed 30.8 % of whole bladder glycogen while the serosal fraction contributed the remainder, 69.2 %.

Glycogen loss in mucosal and serosal fractions under aerobic and anaerobic conditions

Glycogen utilization in aerobic and anaerobic toad and turtle bladder tissue has been investigated[1-4], but no distinction between muscle and epithelial glycogen was made in these studies. It was therefore of interest to determine glycogen losses in isolated mucosal and serosal fractions of the turtle bladder after incubation under aerobic and anaerobic conditions.

TABLE III

EFFECT OF ANOXIA ON GLYCOGEN CONTENT

Initial values show glycogen content (mg glucose per g wet tissue weight) of bladder mucosal and serosal fractions before incubation. A portion of each fraction was incubated for 45 min in Ringer solution saturated with the specified gas, following which final values were obtained. Δ, difference between initial and final glycogen content. Values are means $\pm$ S.E. P, significance of difference between paired initial and final values. n, number of experiments. Each experiment utilized 2–4 bladders. Experiments took place throughout the year.

Conditions	n	*Initial content* (*mg/g*)	Δ (*mg/g*)	P	% *Glycogen loss* ([Δ/*initial*] × 100)
Mucosal fraction					
Air (control)	5	1.90 ± 0.20	-0.24 ± 0.04	<0.01	13.2 ± 1.6
N_2	6	2.39 ± 0.13	-1.22 ± 0.09	<0.001	50.6 ± 2.1
				$P_{(air-N_2)}$	<0.001
Serosal fraction					
Air (control)	5	2.83 ± 0.39	-0.13 ± 0.07	>0.1	6.0 ± 2.3
N_2	6	3.23 ± 0.19	-0.87 ± 0.11	<0.001	26.5 ± 2.8
				$P_{(air-N_2)}$	<0.01

Table III presents findings on glycogen losses in mucosal and serosal tissue in air-saturated and nitrogen-saturated Ringer solutions. Duration of incubation was 45 min. Both mucosal and serosal fractions lost glycogen in aerated solutions (controls, Table III). The observed loss under aerobic conditions was probably due in part to glycogen degradation caused by injury to tissues. This is indicated by the fact that bladders incubated as whole sacs show less glycogen loss than bladders incubated as slices (unpublished experiments).

Serosal glycogen loss, 6.0 % of initial, was much less than mucosal glycogen loss, 13.2 % of initial, under aerobic conditions. The serosal fraction (consisting of smooth muscle and connective tissue) thus resembles skeletal muscle in its low rate of spontaneous glycogen loss. In experiments on a series of tissues under aerobic conditions MACLEOD[13] found that glycogen disappearance was slowest in skeletal muscle.

Under nitrogen extensive depletion of glycogen took place in both fractions. As in the aerobic experiments, serosal glycogen loss, 26.5 %, was less than mucosal loss, 50.6%.

2,4-Dinitrophenol-induced glycogen loss in the presence and absence of Na^+

2,4-Dinitrophenol stimulates glycogen breakdown in skeletal muscle[14-15] and in whole turtle bladder tissue[16]. Conversely, the removal of Na^+ from bathing fluids retards glycogen breakdown in toad bladder[2,4]. To gain maximum information on the effects of dinitrophenol and Na^+ removal on turtle bladder mucosal and serosal tissue, a series of experiments involving three factors was set up and the results analyzed by the statistical method of analysis of variance[10]. The three factors were (1) fractions (mucosal and serosal), (2) conditions (presence and absence of Na^+), and (3) treatment (presence and absence of dinitrophenol).

Bladders were dissected into mucosal and serosal fractions and half of each fraction was analyzed immediately. The remaining tissue slices were placed in large volumes of solution (approximately 0.5 g tissue to 300 ml Ringer solution) and incubated for 2 h with gentle stirring and aeration. In dinitrophenol experiments control tissue was not exposed to dinitrophenol; experimental tissue was incubated in the presence of 10^{-5} M dinitrophenol. In Na^+-free experiments choline replaced Na^+ in bathing fluids. Tissue was washed 4 times in choline Ringer before incubation. Osmolality and pH of NaCl and choline Ringer solutions were carefully matched. Sodium concentration in the incubation fluid was determined by flame photometry at the end of the experiment and never exceeded 0.09 mM in Na^+-free experiments. The Na^+ dependence of the response of the two fractions to dinitrophenol was studied by determining the effect of dinitrophenol in the presence and absence of ambient Na^+.

Table IV shows the effects of dinitrophenol and Na^+ replacement on mucosal and serosal glycogen losses. To partition main and interaction effects in these experiments a 2^3 factorial analysis of variance of values for percent glycogen loss was carried out (ref. 10, Chapter 12). The analysis is given in Table V. The conclusions drawn from the analysis follow.

Main effects: (1) Glycogen loss in the mucosal fraction was significantly greater than glycogen loss in the serosal fraction. (2) Glycogen loss in the presence of $Na^+$was significantly greater than glycogen loss in the absence of Na^+. (3) Glycogen loss in the presence of dinitrophenol was significantly greater than glycogen loss in the absence of dinitrophenol.

Interaction effects: (1) No significant interaction between fractions and conditions was evident. In other words, the effect of the presence of Na^+ on glycogen loss was not significantly more pronounced in the mucosal fraction than in the serosal fraction. (2) A mildly significant interaction between fractions and treatment was evident. In other words, the effect of 2,4-dinitrophenol on glycogen loss was more pronounced in the mucosal fraction than in the serosal fraction. (3) A highly significant

TABLE IV

2,4-DINITROPHENOL-INDUCED GLYCOGEN LOSS IN MUCOSAL AND SEROSAL FRACTIONS OF TURTLE BLADDER

Initial values show glycogen content (mg glucose per g wet tissue weight) of bladder mucosal and serosal fractions. A portion of each fraction was incubated for 2 h under the specified conditions, following which final values were obtained. 2,4-Dinitrophenol (DNP) concentration was 10^{-5} M. Δ, difference between initial and final glycogen content. Values are means ± S.E. P is significance of difference between paired initial and final values. n, number of experiments. Each experiment utilized 2–4 bladders. Experiments took place throughout the year.

Conditions and treatment	*n*	*Initial content (mg/g)*	*Δ (mg/g)*	*P*	*% Glycogen loss ([Δ/initial] × 100)*
Mucosal fraction					
NaCl	5	2.53 ± 0.13	−0.50 ± 0.14	<0.02	19.5 ± 3.1
NaCl + DNP	6	1.94 ± 0.10	−1.03 ± 0.11	<0.001	54.0 ± 4.3
Choline chloride	6	2.04 ± 0.32	−0.28 ± 0.10	>0.05	15.5 ± 2.4
Choline chloride + DNP	6	2.24 ± 0.11	−0.51 ± 0.08	<0.01	22.7 ± 3.1
Serosal fraction					
NaCl	5	3.38 ± 0.56	−0.46 ± 0.12	<0.02	13.1 ± 2.3
NaCl + DNP	6	2.77 ± 0.29	−0.78 ± 0.12	<0.01	28.7 ± 4.2
Choline chloride	6	3.79 ± 0.49	−0.32 ± 0.05	<0.01	8.4 ± 0.7
Choline chloride + DNP	6	3.81 ± 0.34	−0.51 ± 0.13	<0.02	12.6 ± 2.3

TABLE V

ANALYSIS OF VARIANCE OF % GLYCOGEN LOSS

Fractions (Fr.), mucosal and serosal; conditions (C), presence and absence of NaCl; treatment (T), presence and absence of 2,4-dinitrophenol. $n = 6$ for each set. In the 2 cases where 5 experiments were performed (see Table IV) the mean of the available observations was inserted as the missing value.

	Degree of freedom	*Sum of squares*	*Mean square*	*F*
Fractions	1	1788.64	1788.64	32.57**
Conditions	1	2349.90	2349.90	42.80**
Treatment	1	2839.92	2839.92	51.72**
Fr. × C	1	160.71	160.71	2.93
Fr. × T	1	358.78	358.78	6.53*
C × T	1	1127.82	1127.82	20.54**
Fr. × C × T	1	190.60	190.60	3.47
Error	40	2196.56	54.91	
Total	47	11012.93		

* Significant at 5% level.
** Significant at 1% level.

interaction between conditions and treatment was evident. Thus, the effect of 2,4-dinitrophenol on glycogen loss was significantly enhanced by the presence of Na^+ (or absence of choline). (4) No significant interaction between all three variables was evident. Thus, the enhancement of 2,4-dinitrophenol-induced glycogen loss due to the presence of Na^+ was not significantly greater in the mucosal fraction than in the serosal fraction.

DISCUSSION

Distribution of glycogen in bladder

The seasonal variation of glycogen in liver and skeletal muscle has been investigated in a few amphibia and reptiles[17–19] but other tissues have received little attention. We know of no data with which to compare our finding that the late summer increase in glycogen of the serosal (muscle) fraction tended to exceed that of the mucosal (epithelial) fraction (Table II). Since our animals were obtained from a supplier and kept in the laboratory without feeding, the variations in glycogen and relative weights of mucosa and serosa shown in Table II may not accurately reflect patterns in tissues of turtles living in natural conditions. However, the findings are meaningful in a quite different context, that of transport studies. Since Table II shows that glycogen in the serosal fraction accounts for approximately 70 % of total bladder glycogen in two seasons of the year, care must be taken to define the behavior of smooth muscle glycogen whenever the relationship of glycogen metabolism to sodium transport is being studied in the turtle bladder. Lactate production also must be interpreted with caution. Glycogen broken down in whole turtle bladder tissue under anaerobic conditions in the absence of substrate has been quantitatively accounted for by lactate production[3,16]. Measurements of lactate production have contributed to the determination of quantitative relationships between Na^+ transport and ATP utilization[3] and to the formulation of models of anaerobic sodium transport by turtle bladder epithelial cells[16,20]. Smooth muscle, however, must have contributed much of the measured lactate. Thus correction for the contribution of smooth muscle is desirable in work of this kind.

Studies on glycogen metabolism and sodium transport in the toad bladder are also subject to ambiguity between epithelial cell and muscle cell metabolism. Published photographs of sections of whole toad bladder[21–23] as well as our own unpublished observations show that the proportion of muscle to epithelial tissue is nearly as great in toad as in turtle bladders. Values for glycogen content of whole bladder tissue are similar in the two animals[1,2,4]; thus it is reasonable to suppose that toad bladder smooth muscle also contains considerable glycogen.

Comparison of glycogen breakdown in mucosal and serosal fractions

In general mucosal and serosal glycogen losses maintained a rather fixed relationship to each other although the mucosal loss was always the greater of the two. The results appear to reflect the responses of glycogenolytic systems with similar characteristics but different intrinsic activities. The rapid mucosal glycogenolysis suggests that phosphorylase activity is greater in mucosal than in serosal tissue.

In addition, differences in the physical and chemical properties of glycogen itself in the two fractions may play a role in the relative rates of glycogenolysis. Nondegraded liver glycogen has been found to have one particularly labile bond per 50000 glucosidic residues[24]. Since glycogen from different tissues differs in molecular weight and other properties[25], it is reasonable to suppose that the proportion of labile bonds also varies.

Glycogenolysis in the presence and absence of NaCl

Glycogen loss was significantly greater in NaCl Ringer solution than in choline

Ringer solution (Table V). In toad bladder, LEAF AND DEMPSEY[2] and HANDLER *et al.*[4] also found sparing of glycogen in Na^+-free Ringer solution and attributed it to the lessening of energetic demands in epithelial cells due to the cessation of sodium transport. The analysis of variance of glycogen loss, however, indicates that the protective effect of Na^+ lack is not related to Na^+ transporting activity in the turtle bladder (Table V). Although one fraction, the mucosal, transports Na^+ vigorously while the other, the serosal, does not, the effect of the absence of Na^+ was similar in both. Furthermore, a depression of phosphorylase a activity in Na^+-free media has been described for rat diaphragm[26]. Thus glycogen breakdown is retarded by removal of Na^+ from bathing fluids in both epithelial and muscular tissues.

2,4-Dinitrophenol-induced glycogenolysis

Dinitrophenol-induced stimulation of glycogen breakdown in turtle bladder may be similar to anoxia-induced stimulation. Dinitrophenol activates mitochondrial ATPase, thus removing phosphorylase-inhibiting ATP and increasing phosphorylase-activating P_i (ref. 15, p. 226–234). Anoxia-induced glycogenolysis also involves these factors [27,28]. Tables III and IV show that the effects of anoxia and dinitrophenol in NaCl–Ringer solution were similar.

The dinitrophenol effect on glycogenolysis was greater in NaCl–Ringer solution than in choline chloride–Ringer solution (Tables IV and V). A possible explanation of this observation is an increase in intracellular pH in tissue incubated in choline Ringer. The biological activity of dinitrophenol (pK, 4.0) is pH dependent, being greatest at low pH values within the physiological range. Although intracellular pH changes were not investigated in the experiments reported here, the interaction of treatment with condition (Table V) suggests that a factor such as pH difference was operating.

ACKNOWLEDGEMENTS

This study was supported in part by National Institutes of Health Research Grants AM 13953 and AM 13037, in part by National Science Foundation Research Grant GB 7764, and in part by the U.S. Atomic Energy Commission. The authors thank Dr.H.R.Wyssbrod, Mount Sinai School of Medicine of the City University of New York, for helpful discussion. The analysis of variance, provided by Dr. Keith Thompson, Biology Department, Brookhaven National Laboratory, is gratefully acknowledged.

REFERENCES

1 A. LEAF, L. B. PAGE AND J. ANDERSON, *J. Biol. Chem.*, 234 (1959) 1625.
2 A. LEAF AND E. DEMPSEY, *J. Biol. Chem.*, 235 (1960) 2160.
3 S. KLAHR AND N. S. BRICKER, *J. Gen. Physiol.*, 48 (1965) 571.
4 J. S. HANDLER, A. PRESTON AND J. ROGULSKI, *J. Biol. Chem.*, 243 (1968) 1376.
5 M. E. LE FEVRE, J. F. GENNARO AND W. A. BRODSKY, *Am. J. Physiol.*, 219 (1970) 716.
6 W. A. BRODSKY AND T. P. SCHILB, *Am. J. Physiol.*, 208 (1965) 46.
7 K. NAKAGAWA, S. KLAHR AND N. S. BRICKER, *Am. J. Physiol.*, 213 (1967) 1565.
8 C. A. GOOD, H. KRAMER AND M. SOMOGYI, *J. Biol. Chem.*, 100 (1933) 485.
9 F. CONSOLAZIO, R. JOHNSON AND E. MAREK, *Metabolic Methods: Clinical Procedures in the Study of Metabolic Functions*, Mosby Publishing, St. Louis, Mo., 1951, p. 161–163.
10 G. SNEDECOR AND W. G. COCHRAN, *Statistical Methods*, Iowa State University Press, Ames, Iowa, 6th ed., 1967.

11 E. BUEDING, *Physiol. Rev.*, 42, Suppl. 5 (1962) 177 (Comment).
12 R. W. LACKEY, C. A. BUNDE AND L. C. HARRIS, *Am. J. Physiol.*, 145 (1946) 470.
13 J. J. R. MACLEOD, *Carbohydrate Metabolism and Insulin*, Longmans, Green and Co., London 1926, p. 151.
14 E. RONZONI AND E. EHRENFEST, *J. Biol. Chem.*, 115 (1936) 749.
15 E. RACKER, *Mechanisms in Bioenergetics*, Academic Press, New York, 1965, p. 236–240.
16 N. S. BRICKER AND S. KLAHR, *J. Gen. Physiol.*, 49 (1966) 483.
17 M. R. MILLER, in A. W. MARTIN, *Comparative Physiology of Carbohydrate Metabolism in Heterothermic Animals*, University of Washington Press, Seattle, Wash., 1961, p. 125–130.
18 H. C. DESSAUER, *Proc. Soc. Exp. Biol. Med.*, 82 (1953) 351.
19 J. C. HALL, K. C. FISHER AND J. R. STERN, *Am. J. Physiol.*, 179 (1954) 29.
20 S. KLAHR, J. BOURGOIGNIE AND N. S. BRICKER, *Nature*, 218 (1968) 769.
21 L. D. PEACHEY AND H. RASMUSSEN, *J. Cell Biol.*, 10 (1961) 529.
22 A. R. KELLER, *Anat. Rec.*, 147 (1963) 367.
23 J. KWON CHOI, *J. Cell Biol.*, 16 (1963) 53.
24 J. MORDOH, C. R. KRISMAN AND L. F. LELOIR, *Arch. Biochem. Biophys.*, 113 (1966) 265.
25 S. A. ORRELL AND E. BUEDING, *J. Biol. Chem.*, 239 (1964) 4021.
26 L. LUNDHOLM, E. MOHME-LUNDHOLM AND N. VAMOS, *Acta Physiol. Scand.*, 75 (1969) 187.
27 H. E. MORGAN AND A. PARMEGGIANI, *J. Biol. Chem.*, 239 (1964) 2435.
28 J. R. NEELY, C. F. WHITFIELD AND H. E. MORGAN, *Am. J. Physiol.*, 219 (1970) 1083.