ANAEROBIC GLYCOGENOLYSIS IN THE MUSCLES OF RANA PIPIENS LIVING AT LOW TEMPERATURES

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When attempts failed (16) to produce glycolytically active extracts from the muscles of winter frogs stored at 4°C., and when lactate production at 0°C. by their gastrocnemii could not be detected (12, 17) we began to study the glycogen metabolism during fatigue of the muscles from such frogs. Previous workers have shown alterations in the carbohydrate metabolism of frog muscles with low glycogen contents, and also seasonal variations in the carbohydrate metabolism of frogs.

If sartorii and gastrocnemii of *R. temporaria* and *R. esculenta* have low carbohydrate reserves, they can contract aerobically on sources of energy only partly carbohydrate (30, 10). When mammalian muscles are rendered low in glycogen by the use of insulin or thyroid, rigor mortis in them is not accompanied by glycogenolysis or by lactate production (15). Olmsted and his colleagues (31, 32) were the first to use insulin convulsions for rendering muscles of *R. pipiens* and *R. catesbiana* nearly free of glycogen; muscles so prepared contracted anaerobically without glycogenolysis or lactate production. Ochoa (30) and Gemmill (10) extended these observations to *R. temporaria* and *R. esculenta*.

It has been known for a long time that there is marked seasonal variation in the carbohydrate metabolism of frogs' tissues. Schiff (36) in 1859 observed that there is little postmortem glycogenolysis at 23°C. by the livers of frogs caught in the winter. Grode and Lesser (13) set up uninjured livers and muscles of frogs for 4 hours at 22-23°C. in Ringer's solution under aerobic conditions. In November and December such preparations showed only very slight decreases in glycogen, despite initial concentrations of 0.75 to 1.24 per cent. At other times of the year, decreases of 15–20 per cent of the initial value were observed. If in November or December the organs were cut or severely injured, marked glycogenolysis took place. Lesser (21) analyzed whole frogs for glycogen. In the summer, anaerobiosis of 2 hours led to marked glycogenolysis. A subsequent 24 hours of aerobiosis led to restitution of most of the glycogen. In November and December, anaerobiosis of 2 hours did not produce glycogenolysis.

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This could be produced by repeated periods of anaerobiosis but aerobiosis did not result in restitution.

Lesser (22) described semiannual periods in a frog's life: the glycogen-stable period of winter, and the glycogen-labile of summer and fall.

Laquer (19, 20) studied lactate production by chopped frog muscles incubated in buffered media, of which phosphate buffers were found to be the most favorable to glycolysis. At 45°C., the muscles of summer frogs produced large amounts of lactate from added hexose diphosphate, starch, or glycogen, but the muscles of winter frogs did not produce lactate from added substrates. When winter frogs were kept alive for several days at temperatures between 22 and 27°C., their muscles gained the ability to produce lactate from added glycogen.

The present experiments were designed to show whether prolonged life at 4°C. altered the glycolytic capacity of frog gastrocnemii, either by depleting carbohydrate reserves, or, more fundamentally, as in the experiments of Lesser (22) and Laquer (19, 20), by altering their glycolytic capacity.

Methods

The experiments were done on two separate batches of frogs in the summers of 1939 and 1940. R. pipiens from northern Vermont, caught in March or April, were kept without food at 4°C. in a few milliliters of tap water that was changed every 2 days. They were stored in this way from 7 to 10 weeks before use. Any frog showing clinical signs of disease, especially "red leg," was discarded.

For each experiment, a frog was allowed to rest alone for several hours at 4°C. One operator then gently held it over a paper cutter and extended its hind legs. The second operator chopped off both its legs with a single stroke at the pelvic girdle, the first laid the legs on ice covered with moist gauze and killed the frog. The second dissected out both gastrocnemii with ice cold instruments, and both set up the muscles in the apparatus for anaerobiosis and stimulation. The usual elapsed time from the stroke that cut off the legs to installation in an atmosphere of nitrogen was 3 minutes.

The apparatus in which the muscle was stimulated consisted of a vertical glass tube 15 cm. long and 4 cm. in diameter, tightly fitted with one-holed stoppers. The muscle was supported in the chamber by a small brass clip at each end, one being firmly applied to the lower stopper, the upper to a fine thread leading to an isotonic myograph,[1] counterbalanced and backstopped. Moist nitrogen, containing less than 0.04 per cent oxygen, was fed from a saturating chamber through a glass tube in the lower stopper, and out through the upper. A thermometer was fastened to the upper stopper in each chamber. The muscles were stimulated directly through the brass clips by make shocks from an inductorium. The interval between stimulations was from 18 to 25 seconds in different experiments. This long interval was used to avoid any possibility of the muscle warming during the experiment. The load was adjusted to give a convenient excursion on the kymograph. The primary voltage was three, and the twitches were kept maximal

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[1] Obtainable from the Harvard Instrument Co.

by adjustment of the secondary coil every 10 minutes. The muscles were stimulated until they would not respond even to the strongest stimulus. This was usually within 2 hours. Control muscles were treated like stimulated in every way but stimulation.

Some of the early experiments were carried out at room temperatures from 25 to 29°C., but the control values were so high, because of heat glycolysis, that all the later experiments were done at 0°C., by keeping both the stimulation chamber and the nitrogen moistener immersed in a wide-mouthed insulated jug filled with cracked ice. The temperature difference between the two chambers that contained the control muscle and the stimulated was never more than $\frac{1}{2}$°C. The muscles did not change in water content in periods up to 2 hours.

When the stimulated muscle was completely fatigued, finely powdered carbon dioxide was poured into the chamber and into the control chamber at such a time that both muscles would have the same period of anaerobiosis. The frozen muscles were then stored in air-tight stoppered tubes surrounded by solid carbon dioxide. The water, glycogen, and lactate content of muscles stored in this way did not change in a week. Samples for estimation of water, glycogen, and lactate were taken by cutting the frozen muscle in a direction parallel with the fibers, into two large pieces and one small piece. The gastrocnemii averaged about 500 mg. in weight. For water determinations, about 50 mg. was used; for glycogen and lactate, about 200 mg.

Water determinations were made by drying the muscle for 24 hours at 110°C.

Glycogen was estimated by the method of Good, Kramer, and Somogyi (11) with slight modification. The tissue was hydrolyzed by 1 ml. of 30 per cent KOH in 15 cc. graduated conical centrifuge tubes fitted at the top with spherical air condensers seated in constrictions, and all subsequent manipulation of the glycogen was done in these tubes. When the glycogen was precipitated in 60 per cent alcohol, the mixture was vigorously stirred by a fine platinum wire fitted to an electric stirrer. This maneuver seemed to improve the precipitation of the glycogen. After the glycogen was hydrolyzed to glucose in 2.2 per cent HCl at 100°C. for 2½ hours, the pH was adjusted to 10 with thymol blue and 5 per cent KOH. The titratable acidity to pH 10.3 of the aliquots was not more than 0.01 cc. of N NaOH, as Forbes and Andreen-Svedberg (8) recommend. Glucose was estimated by the method of Folin and Wu (7). Brominated molybdate was not used, because the reagent blank with unbrominated, even after 2 weeks, was negligible. Each run of glycogen estimations contained suitable reagent blanks and a standard solution of pure glycogen. The blanks were always almost colorless, and recovery of added amounts of glycogen corresponding to the amount in the muscle samples, ranged from 99 per cent to 103 per cent of the amount added.

Muscle extracts for lactate estimations were prepared with trichloroacetic acid by the technique of Newman (29), and aliquots were analyzed by the Edwards (4) modification of the method of Friedemann, Cotonio, and Shaffer (9). Each run of lactate estimations contained suitable reagent blanks and solutions of pure lithium lactate. Recoveries of added lactate in amounts comparable to those present in the samples of muscle ranged from 97 per cent to 103 per cent, and the blanks were of the same order as Wendel's (37).

Control Experiments

Blanks were run on the surgical gauze, solid carbon dioxide, and nitrogen. They were negative for lactate and glycogen. The solid carbon dioxide

used in freezing several fatigued muscles was collected. It was negative for lactate and glycogen. The materials used therefore neither added blanks nor abstracted glycogen and lactate from the muscles.

The water content of 100 of the muscles used in the experiments was estimated. The mean was 80 per cent, S.D. 2.1 per cent. The data are not included, and no correction was made from one experiment to the next, since the water contents were practically the same in all muscles.

The resting value of lactate was estimated for twenty muscles from legs frozen as soon as they were chopped off. The mean value, in milligrams of lactic acid per 100 gm. muscle, was 17, S. D. 13. The average of the control muscles used in 53 experiments run at 0°C. was 25 mg. lactic acid per 100 gm. muscle, S. D. 17. These muscles had all been dissected out, clipped in the apparatus, and exposed to nitrogen for periods up to 2 hours. The control values in 1940 were lower than in 1939, probably because of increased skill in handling the frogs. We concluded that our handling of the frogs and muscles was satisfactory, and that nothing in the methods gave falsely high values for lactate.

The assumption has to be made in experiments such as these that the level of a given substance in the control muscle at any given time may be taken as that which the fatigued muscle would have had at that time if it had not been stimulated. This assumption was shown to be valid for glycogen and lactate by taking both gastrocnemii from each of twenty-five frogs, and treating the pairs exactly alike, in lots of four pairs. Those of the first lot were frozen as soon as they had been dissected out. Those of the second lot were set up in the apparatus and frozen at once. Those of the third lot were set up for 2 hours anaerobically at 0°C. before being frozen. Those of the last lot were set up anaerobically for 2 hours at 25°C. before being frozen. The differences between paired muscles averaged 17 mg. lactic acid per 100 gm. muscle and 62 mg. glycogen per 100 gm. muscle. The differences were independent both of the initial glycogen content and of the previous treatment of the muscle before it was frozen. In three cases the difference in glycogen fell just outside the limits established by Kerly (18) for frogs and by Cori (3) for rats. In Table I, significant changes in glycogen and lactate are considered to be 62 and 17, respectively.

Experiments with Fatigued Muscles

The results of the experiments in which muscles were stimulated anaerobically to complete fatigue (Table I) were variable. About one-half the muscles showed decreases in glycogen and increases in lactate in the range one usually finds with autumn frogs, that is, changes of 100 mg. or more

TABLE 1

Changes in Glycogen and in Lactate during Anaerobic Contraction at 0°C. to Complete Exhaustion

Glycogen is expressed as milligrams of glucose per 100 gm. muscle, wet weight. Lactate is expressed as milligrams of lactic acid per 100 gm. muscle, wet weight.

Experiment No.	Days kept, No.	Contractions, No.	Glycogen		Lactate		Loss in glycogen (control minus twitch)	Gain in lactate (twitch minus control)
			Control	Twitch	Control	Twitch		
A. Eleven experiments of 1939 at 0°C. without much glycogenolysis								
			mg. per cent	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*
20	50	238	494	443	89	120	51	31
28	52	233	142	63	44	55	79	11
29	52	219	346	361	34	117	−15	83
32	52	139	388	378	48	63	10	15
33	53	280	520	520	8	104	0	96
50	22	214	670	685	9	86	−15	77
51	22	160	623	600	68	61	23	−7
60	50	204	370	354	84	82	16	−2
61	50	227	552	540	16	90	12	74
70	57	222	130	105	7	48	25	41
72	57	202	359	320	12	54	39	42
Average.......		213	417	397	38	80	20	42
B. Sixteen experiments of 1940 at 0°C. without much glycogenolysis								
88	25	144	755	790	31	29	−35	−2
89a	74	140	475	465	14	22	10	8
89b	74	140	430	390	20	66	40	46
90	75	288	670	620	20	20	50	0
91	75	153	600	670	40	26	−70	−14
92b	75	144	1160	1090	19	81	70	62
93	76	232	350	310	13	82	40	69
94	76	110	560	620	16	58	−60	42
95	76	140	670	580	14	33	90	19
99a	76	130	750	800	14	38	−50	24
99b	76	109	18	20	14	8	−2	−6
101	77	400	465	425	25	140	40	115
102	77	343	220	195	18	109	25	91
103	77	390	605	640	18	105	−35	87
104	78	360	670	620	13	122	50	109
105	78	207	235	240	16	55	−5	39
Average......		214	540	530	19	62	10	43
C. Twenty-six experiments of 1939 and 1940 at 0°C. with significant glycogenolysis								
Muscle pairs with highest difference			386	720	310	25	176	410	151
Muscle pairs with lowest difference			140	145	50	26	66	95	40
Average for all muscles showing significant glycogenolysis........			249	618	475	22	116	143	94

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per 100 gm. of muscle. About one-half of them showed no significant decrease in glycogen, and about one-seventh of them showed no significant change in either glycogen or lactate; viz., experiments 32, 51, 60, 88, 89a, 90, 91, and 99b. Only the highest, lowest, and average values are given for the experiments that showed significant changes in both glycogen and lactate. The data for the other experiments are given in detail. One explanation for the extreme variability of the results is perhaps suggested by Laquer's work (19) showing that warming winter frogs for a few days restores the ability of their muscle tissue to break down added glycogen. The frogs used in the present experiments were caught by a dealer in Vermont, shipped to Boston, and stored in an animal farm until we bought them. There are no means of telling how much handling and warming they received between their native habitat and our refrigerator.

In agreement with the work of others (18 and 23–27) there was no stoichiometric relation between glycogenolysis and lactate production. Three times as many muscles showed no change in glycogen as showed no significant increase in lactate. This suggests that many of the muscles used preformed carbohydrate intermediaries to produce lactate, but did not break down glycogen, even though it was present.

For each of the experiments of 1939, a calculation was made of the quantity:

$$\frac{\text{(Sum of the heights of all contractions)} \times \text{(weight lifted)}}{\text{(Weight of muscle in grams)}}$$

It gives a rough index of the capacity of a muscle to do physical work. These data are not included, because there was no simple relation between glycolysis and work done. Some of the muscles that did the most work showed no change in either glycogen or lactate, and some of those that did the least showed the most glycolysis. Further, there was no correlation between the capacity of the muscle to do work and its glycogen content. Some of the muscles that did very little work had glycogen contents of over 500 mg. per 100 gm. of muscle, and some of those that did the most work had the least glycogen.

The effect of temperature on these experiments is not clear. Of fifteen experiments at 25 to 29°C., eight showed no extra glycogenolysis during fatigue, over and above the heat glycogenolysis that was proceeding. The control lactate values were so high because of heat glycolysis that all of the later experiments were made at 0°C. Some of the experiments cited by Fletcher and Hopkins (6), Meyerhof (26–28), Hartree and Hill (14), and Peters (34), taken together, imply that the lactate production of fatigue

is less at 0° than at 25°C., but in our experiments at 25 to 29°C. two muscles failed to produce more lactate in fatigue than the lactate produced by heat glycolysis.

The one result reported here that differs considerably from previous work has to do with the relation between glycogen level and capacity to glycolyze. Olmsted and his colleagues (31, 32) found two moribund *R. catesbiana* whose muscles had practically no glycogen and contracted without glycolysis. None of the normal frogs of Ochoa (30) and of Gemmill (10) had muscles that contracted anaerobically without glycolysis, although some of those treated with insulin did so. These three workers and Hoet and Marks (15) consider that a necessary and sufficient condition for anaerobic contraction without glycolysis is depletion of the muscle stores of carbohydrate. Gemmill (10) implied that the capacity of the muscle to do work anaerobically is directly proportional to the carbohydrate level, the important carbohydrate being glycogen. In the present experiments, there was no evident correlation between the glycogen content of the muscle and its failure to glycolyze. Some of the muscles that did not glycolyze had over 500 mg. glycogen per 100 gm. muscle, namely, experiments 51, 88, 90, and 91. Further, even in those muscles that did glycolyze, there was no correlation between the level of glycogen and the amount of change either in glycogen or in lactate. It should be emphasized that some of the muscles twitched 150 times or more, and could do a considerable amount of work with either a high or a low glycogen content. Evidently they had a store of glycogen, but did not use it.

The source of energy for those muscles that did not glycolyze is puzzling. Muscles poisoned with iodoacetate still break down glycogen (25) and split abnormally large amounts of phosphocreatine (25). Ochoa (30) and Gemmill (10) found a few muscles from frogs treated with insulin that did not glycolyze anaerobically and did not split abnormally large amounts of phosphocreatine. Palazzolo (33) first showed that fatty acids disappear when the muscles of frogs and of hibernating hedgehogs become fatigued. There is a similarity between our frogs and Chambers' (2) dogs which, in the third stage of starvation, cannot utilize carbohydrate, even if it is available. Buchwald and Cori (1) have demonstrated a disappearance of fatty acids from frog muscles stimulated to exhaustion. The Meyerhof school (30, 10) say that aerobic contraction by isolated muscles of frogs can take place on sources of energy not all carbohydrate, and suggest fat as a direct source of energy for contraction. Our results indicate that anaerobic contraction under special circumstances can take place without breaking down glycogen.

Addendum.—Unpublished work of Edwards and Dill (5) bears on the present experiments. They went surf fishing on October 11, 1931, in Buzzard's Bay. The water temperature was near 10°C. and there was snow on the ground. They caught a small shark and three skates, pulled them struggling through the surf, killed them, and froze sections of the dorsal muscles in solid carbon dioxide. The blood lactate of the shark was 84 mg. lactic acid per 100 cc., of the skates, 42, 15, and 18. The muscle lactates of these skates were 42, 25, and 31 mg. of lactic acid per 100 gm. muscle. That is, under identical conditions, the blood lactate of the shark was high, but the blood and muscle lactates of two of the skates were at resting levels. These few observations suggest that the muscles of these skates were contracting under conditions that were essentially anaerobic, and yet were not producing much lactate.

SUMMARY

1. A considerable proportion of *R. pipiens* caught in the spring and stored without food for several weeks at about 4°C. had gastrocnemii that did not break down glycogen when they contracted anaerobically to complete exhaustion. A smaller number of the same muscles did not produce lactate.

2. There was no evident relation between failure to break down glycogen and the glycogen content of such muscles, some of which had more than 500 mg. of glycogen per 100 gm. of tissue.

3. The hypothesis of Meyerhof and his followers that aerobic contraction of frog muscles may at times take place with sources of energy other than carbohydrate is therefore extended to include anaerobic contraction.

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