Factors Affecting Normal Carbohydrate Levels in *Rana pipiens*[1]

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Variation in *Rana pipiens* blood glucose levels caused by assay techniques was at least 50% of basal levels. Increases caused by handling and anesthesia were as great as 200% for glucose and 800% for lactate. Dietary status induced variations as great as 100% in blood glucose and muscle glycogen and 600% in liver glycogen. Maximal seasonal differences (March to August) of approximately 50% for blood glucose and tissue glycogen concentrations were observed. Frogs from different geographic areas showed differences in October glycogen reserves as great as 25 to 50%.

In amphibians, as well as other poikilotherms, physiological levels of carbohydrates and related substances are subject to considerable variation (Table 1). As a result, it is often difficult to measure the effects of hormones or other agents with high precision or repeatability. Moreover, since the sources of this variation are often unknown or not taken into consideration by investigators, it is often not possible to assess the reliability or significance of variation that has been reported both within and between species. The purpose of this study is to identify some of the factors that can lead to variations in measured carbohydrate levels and suggest ways whereby the variation can to some extent be controlled or standardized. Factors taken into consideration in this study include the reliability of analytical procedures, effects of handling, anesthesia, activity, fasting, and seasonal variation.

MATERIALS AND METHODS

Animal source and maintenance. Adult male *Rana pipiens* were obtained from the Nasco Company, Oshkosh, Wisconsin, having been collected from the Red River valley of North Dakota, or they were collected locally from Whitmore Lake, Michigan. They were kept in running tap water in large covered tanks at a water temperature of 15–17° and air temperature of 19° throughout the year. The tanks were illuminated with fluorescent lights on a 12-hr light:12 hr dark cycle.

The frogs were force fed 1–2 g of beef liver twice a week, supplemented with crickets (*Acheta domesticus*) weekly when not in experimental use.

All frogs were kept under laboratory conditions for at least 2 weeks before experimental use, unless otherwise specified.

Anesthesia. Except in some of the experiments dealing with the effects of anesthesia, the frogs were anesthetized by immersion in 0.5% tricaine methanesulfonate (Finquel, Ayerst Laboratories) until they lost reflex response to a pinch of the toe, usually 10–15 min.

Tissue samples. Blood was collected from the ventricle of the heart in a finely drawn glass pipet inserted through a small incision in the skin. No anticoagulant was needed. For glucose and lactate determination two 50-μl aliquots of blood were transferred to tubes of deproteinizing solution. Glycogen was determined on midportions of the right gastrocnemius muscle and on the tips of three liver lobes. Tissue samples were removed, blotted, weighed, and placed in hot 30% KOH for digestion within 30 sec after the sampling procedure was begun.

Analytical methods. Blood glucose was determined by the glucose oxidase method (Glucostat, Worthington Biochemical Corp.). Total blood sugar was determined by the Folin–Malmros method as modified by Horvath and Knehr (1941). Total reducing carbohydrates were assayed by the phenol–sulfuric acid method of Dubois *et al.* (1956). Lactate was analyzed by the lactate dehydrogenase method of Scholz *et al.* (1959) using reagents supplied in kit form from Boehringer–Mannheim Corporation. Glycogen was determined on muscle and liver samples using the method of Good *et al.* (1933) to isolate the glycogen.

TABLE 1

NORMAL BLOOD SUGAR OR GLUCOSE LEVELS OF SEVERAL ANURAN SPECIES

Species	Blood sugar [mg% ± SE (Range)]	Number of animals	Assay method	Method of blood collection and immobilization	Reference
Rana pipiens	16.3 ± 1.3	18	Glucose oxidase	From tongue of Finquel-anesthetized frogs	Mathews and Zaentz, 1963
Rana pipiens	24.5 ± 3.0 (52, winter–60, fall)	151	Glucose oxidase	From severed neck	Hutchinson and Turney, 1975
Rana pipiens		(30–43)	Folin–Malmros	From heart of pithed frogs	Mizell, 1965
Rana pipiens	(65–85)	—	Folin–Malmros	From conus of pithed frogs	Seiden, 1945
Rana catesbeiana	13.5 (0–70)	154	Nelson	From conus of pithed frogs	Wright, 1959
Rana catesbeiana	6.5 ± 0.5 (0–20.9)	61	Glucose oxidase	From cannula in sciatic artery	Herman, 1977
Rana temporaria	38 ± 1.4	60–70	Hagedorn and Jensen	From heart of pithed frogs	Smith, 1954
Rana tigrina	33	25	Nelson–Somogyi	From conus of pithed frogs	Rangnekar and Sabnis, 1964
Bufo bufo	22.1 ± 1.4 / 13.7 ± 1.1	10 / 10	Glucose oxidase, hexokinase	From severed neck	Hermansen and Jørgensen, 1969

The glycogen pellet was then dissolved in 50 ml of distilled water, and aliquots containing $10-50$ μg of glycogen were assayed by the phenol–sulfuric acid method (Montgomery, 1957).

All biochemical assay methods were used essentially as described in the references. The details of our procedures are described in Farrar (1972).

The accuracy of the assays in recovering glucose, glycogen, and lactate was determined by adding known quantities of high and low standard solutions of these substances to frog blood or liver homogenate and assaying six replicate samples of these preparations by the standard procedures. Mean recoveries as a percentage of the theoretically expected values were as follows: glucose, by glucose oxidase—high standard 97%, low standard 94%; lactate—high standard 91%, low standard 92%. It may thus be assumed that the values reported in this paper are within at least $5-10\%$ of the true levels of the substances in the samples assayed.

Liver slice techniques. Livers were rapidly removed from frogs whose spinal cords had been transected, placed in iced amphibian Ringers, and sliced free hand. The slices were blotted, weighed, and incubated at 25° for 30 min in 25-ml flasks containing 2 ml of amphibian Ringers buffered with 0.1 M sodium bicarbonate of pH 7.8. Glucose was analyzed on 50 μl of deproteinized supernatant using the glucose oxidase method.

Statistics. The Mann–Whitney U test (Siegel, 1956) was used for sample sizes less than 20; Student's t test was used for samples larger than 20. In tests of significance a probability of 5% or less was considered to be significant.

RESULTS AND COMMENT

1. Comparison of the Results of Different Assay Methods for Blood Sugar

It is probable that a significant amount of the variation in carbohydrate values reported in the literature is due to the use of different assay procedures. This is particularly true for glucose in view of the possibility that sugars, other than glucose, and other reducing substances could contribute significantly to measurements presumed to reflect primarily blood glucose.

To obtain a sense of the magnitude of this potential source of variance in blood glucose levels in frogs, aliquots of pooled blood samples were analyzed by three different methods: the glucose oxidase method for glucose, the Folin–Malmros method for reducing substances, and the phenol–sulfuric acid method for reducing carbohydrates. Two pools of blood, each from six frogs, were used. Six aliquots of both were analyzed by the glucose oxidase method; for comparison six aliquots of one were also analyzed by the Folin–Malmros method, and six aliquots of the other by the phenol–sulfuric method.

The results (Table 2) obtained by the glucose oxidase and the phenol–sulfuric methods were similar. However, the values obtained by the Folin–Malmros method were 50% higher.

The higher values obtained by the Folin–Malmros method cannot be due to reducing carbohydrates other than glucose, since the phenol–sulfuric acid method would have detected all reducing sugars, oligosaccharides, polysaccharides, and derivatives with free or potentially free reducing groups (Dubois *et al.*, 1956). It is probable that other non-sugar-reducing substances such as glutathione are released during the relatively harsh tungstic acid deproteinization procedure employed in the Folin–Malmros method and give spuriously high "blood sugar" measurements with this method (Fales *et al.*, 1961). Because real blood glucose levels are low, the percentage error introduced by such substances may be relatively large. In this regard we may note that two reports of blood "glucose" values over 50 mg% in *Rana pi-*

TABLE 2

COMPARISON OF THREE ASSAY METHODS FOR BLOOD GLUCOSE OR SUGAR IN POOLED FROG BLOOD

	Glucose oxidase	Folin–Malmros	Phenol–sulfuric acid
Blood glucose or sugar	23.6 ± 0.9 (6)	36.8 ± 3.2* (6)	—
[mg% ± 1 SE (n)]	27.6 ± 2.0 (6)	—	29.1 ± 2.0 (6)

* $P = 0.001$.

piens both used the Folin–Malmros method (Mizell, 1965; Seiden, 1945; see Table 1). Values based on methods utilizing tungstic acid or other harsh deproteinizing procedures in blood glucose analysis are suspect.

Although the enzymatic methods of glucose determination utilizing hexokinase and glucose oxidase were not compared in this study, the hexokinase method has been found to yield consistently lower blood glucose levels in toads (Hermansen and Jørgensen, 1969; Table 1) and various teleost fish (Chavin and Young, 1970; Lewis *et al.*, 1977). While both enzyme systems are specific for glucose the hexokinase method may give the best estimates of "true glucose." Glucose oxidase when purchased as Glucostat reagent frequently has amylase, maltase, and invertase activity (Fales *et al.*, 1961). If significant amounts of polysaccharides are present falsely high blood glucose values could be obtained. Our work has not ruled out the presence of such compounds. The use of Glucostat Special, containing a more purified enzyme preparation, could alleviate this problem.

2. The Effect of Handling, Pithing, and Anesthesia on Blood Glucose and Lactate

It is probable that disturbance of frogs during experimental manipulation results in changes in blood glucose and lactate and liver glycogen. This has occasionally been noted to be true in some other ectothermic vertebrates (Chavin, 1964; Smith, 1954). The purpose of the experiments reported in this section was to evaluate the effects of some common experimental manipulations, including handling, pithing, and induction of anesthesia, upon blood glucose and lactate.

(a) Comparison of blood glucose levels of pithed and Finquel-anesthetized frogs was done as follows: Six frogs were doubly pithed and blood was drawn from the heart within 30 sec. Six other frogs were anes-

TABLE 3
COMPARISON OF BLOOD GLUCOSE LEVELS OF PITHED AND FINQUEL[a]-ANESTHETIZED FROGS

|	Pithed	Anesthetized
Blood glucose [mg% ± 1 SE (*n*)]	17.9 ± 4.8[b]	14.6 ± 0.9

[a] Tricaine methanesulfonate.
[b] n.s. ($P = 0.47$).

thetized by immersion in 0.5% Finquel for 10 min, after which blood was drawn from the heart. The mean blood glucose values (Table 3) are not significantly different ($P = 0.47$) but variation is much greater following pithing. Consequently, individual values obtained from pithed animals may not be reliable, and pithing should be avoided as a method of immobilization.

(b) Serial blood sampling, requiring repeated or continuous anesthesia and handling, is a convenient, if not absolutely essential, practice in studies of the effects of hormones or other agents on tissue levels of carbohydrates. Unfortunately, we have found that this practice leads to distinct increases in blood glucose and lactate, even in placebo-injected animals.

Figure 1 shows the result of an experiment in which the frequency and interval of anesthetization and/or blood sampling were varied. One group of frogs was anesthetized in 0.5% Finquel at 0, 1, and 3 hr, and blood taken at each interval. A second group was anesthetized at each interval, but blood drawn only at 3 hr. A third group was initially anesthetized in 0.5% Finquel and kept in a continuous state of anesthesia in 0.06% Finquel for 3 hr, at which time a blood sample was taken.

Blood glucose levels of all three groups were two to three times higher than at zero time, indicating that anesthetic alone can cause hyperglycemia, whether applied repeatedly or continuously. Blood withdrawal or arousal are not necessary for this effect. Consequently, continuous or repeated anesthesia must either be avoided or
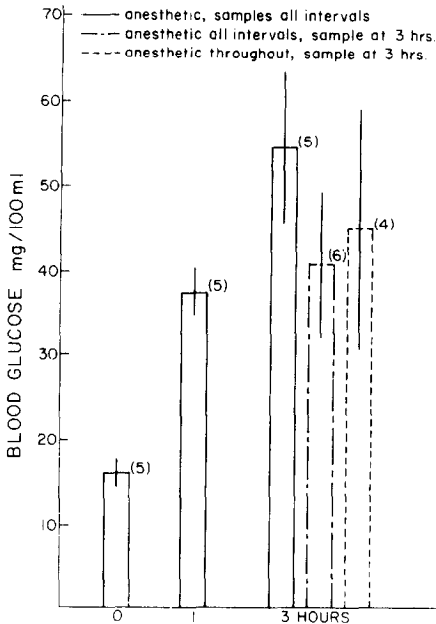
FIG. 1. Effect of frequency and interval of anesthetization and/or blood sampling on blood glucose. Numbers of animals in each group are indicated parenthetically. All groups are significantly different from initial controls. $P \leq 0.05$.

very carefully taken into account in experiments involving serial sampling.

(c) In order to evaluate the effects of repeated capture and handling upon the hyperglycemic response of frogs during repeated sampling, an experiment was done in which frogs were anesthetized without handling. Frogs were placed in containers with measured amounts of water several hours prior to the experiment and Finquel was added to a concentration of 0.5% to the water without disturbing them. The results (Fig. 1) show that repeated anesthetization without handling can induce a threefold rise in blood glucose in 3 hr.

Lowering the concentration of Finquel to 0.25 or 0.125% (the lowest effective concentration) failed to eliminate the hyperglycemic response to repeated anesthesia (Fig. 2). Blood lactate was not determined in all these experiments or at all time intervals, but in one experiment repeated anesthetization in 0.5% Finquel at 0, 1, and 3 hr produced an increase in blood lactate from an initial level of 19.5 ($\pm$ 2.5) to 49.3 ($\pm$ 8.0) mg% at 3 hr.

(d) The effects of other anesthetics, including Nembutal and Equithesin (which contains chloral hydrate and pentobarbital) injected intraperitoneally were tested and compared with those of Finquel. Neither was as effective an anesthetic as Finquel. A minimal effective anesthetic dose of Equithesin (4.2 ml/kg body weight) administered at 0, 1, and 3 hr induced a level of hyperglycemia (49.5 $\pm$ 6.4 mg%) equivalent to that caused by 0.5% Finquel (55.3 $\pm$ 3.9 mg%) after a similar 3-hr serial treatment. This suggests that hyperglycemia does not result from toxic effects of tricaine, such as its acidic nature in water (Ohr, 1976), exerted during immersion. Because several
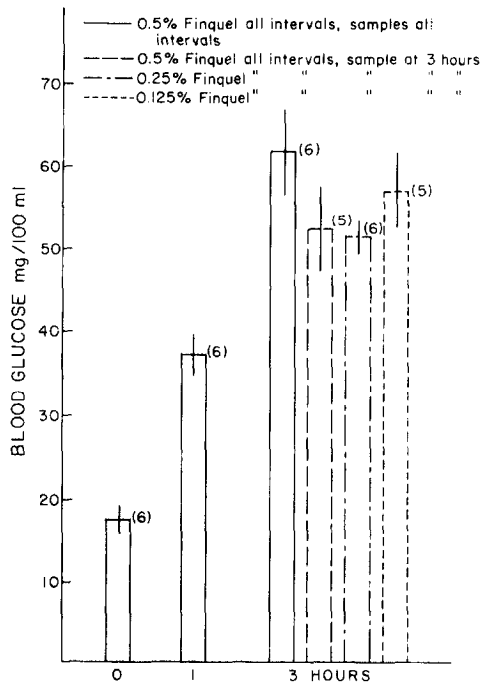
FIG. 2. Effect of repeated anesthetization with various doses of Finquel on blood glucose. Numbers of animals in each group are indicated parenthetically. All groups are significantly different from initial controls. $P \leq 0.05$.

	Saline controls	0.06% Finquel	0.03% Finquel	0.015% Finquel
Mean glucose release [μg/mg/30 min $\pm$ 1 SE (n)]	1.4 ± 0.5 (16)	1.5 ± 0.2 (5)	1.8 ± 0.3 (5)	1.6 ± 0.1 (5)

[a] Tricaine methanesulfonate.

different types of anesthetics all cause hyperglycemia, the anesthetized state itself may be characterized by high blood glucose. If so, insufficient ventilation and anaerobiosis may be occurring.

(e) The possibility that Finquel could induce hyperglycemia by directly stimulating hepatic glycogenolysis was tested by incubating liver slices *in vitro* with the anesthetic. Finquel concentrations of 0.015, 0.03, and 0.06% were prepared in the amphibian Ringers incubation medium. Control flasks were identical except Finquel was omitted.

After 30 min of incubation at 25° no effect of Finquel on glucose release rates was seen (Table 4). Adrenalin and glucagon induce maximum glucose release from frog liver slices incubated in this medium and at this temperature (Farrar, 1972; Tindal, 1956) so we know the glycogenolytic system is capable of responding to stimulation under these conditions. We do not know, however, whether other doses of Finquel might have been effective, but it seems unlikely that the internal concentrations of Finquel could exceed 0.06% following immersion of frogs in concentrations of 0.125–0.5%, the levels that induced hyperglycemia. It is likely that the hyperglycemic effect of Finquel is indirect, possibly through an adrenergic or other neurogenic effect on liver.

(f) The possibility that Finquel induces hyperglycemia by eliciting adrenalin release was tested by the use of two blocking agents: dihydroergotamine, a β-adrenergic blocking agent that inhibits adrenalin-induced hyperglycemia in rats, dogs, cats, and rabbits (Himms-Hagen, 1967) and

hexamethonium, a ganglionic blocking agent (Himms-Hagen, 1967).

Dihydroergotamine was injected into three groups of frogs at doses of 1, 10, or 25 mg/kg, hexamethonium at doses of 25, 50, or 100 mg/kg, intraperitoneally 30 min before initial anesthesia. The animals were anesthetized in Finquel at 0, 1, and 3 hr, and blood samples taken at 0 and 3 hr for glucose and lactate determination.

Neither dihydroergotamine nor hexamethonium prevented Finquel-induced hyperglycemia or hyperlactacidemia. This could be due to an incorrect choice of doses of the inhibitors, incorrect timing of the treatments, species differences in response to the inhibitors, or the possibility that the Finquel-induced hyperglycemia/hyperlactacidemia is not adrenergically induced.

3. Effects of Activity on Blood Glucose and Lactate

In experimental situations frogs may be excitable, and they often exhibit episodes of vigorous jumping in their containers for brief periods. Such activity might be expected to be accompanied by adrenalin release and accelerated muscle and liver glycogenolysis. Glucose utilization, on the other hand, might also be increased. Since the level of activity is uncontrolled and variable, this could be an important source of uncontrolled variance in carbohydrate levels in experimental situations.

Blood was carefully removed from five quiet frogs which were anesthetized in 0.5% Finquel for 10 min. The frogs were sutured and revived in their containers. Twenty-four hours later the same frogs were forced to hop continually for 5 min in a moist con-

TABLE 5
EFFECT OF ACTIVITY ON FROG BLOOD GLUCOSE AND LACTATE

Mean blood glucose [mg% ± 1 SE (n)]		Mean blood lactate [mg% ± 1 SE (n)]	
Before activity	After activity	Before activity	After activity
15.7 ± 2.1 (5)	33.0 ± 2.5 (5)	20.5 ± 4.3	186 ± 16

tainer. At this time they were fatigued and would not hop further. Blood samples were then drawn again, and both sets of samples were analyzed for glucose and lactic acid.

The results are shown in Table 5. Blood glucose levels were doubled by the enforced activity and blood lactate increased ninefold.

Data obtained from animals that have been physically active therefore do not accurately represent basal levels of glucose or lactate, and during experimental studies animals must remain undisturbed and quiescent. Even so, slight activity, unnoticed by the investigator, may cause changes in lactate and glucose levels. Tissue glycogen levels, especially muscle glycogen, would also be expected to be appreciably influenced by activity.

4. Effects of Fasting and Feeding on Carbohydrate Values

Dietary status is extremely important in studies of mammalian carbohydrate metabolism and is always carefully controlled and specified. This has not always been true in work with amphibians. Frogs and toads are generally not fed at biological supply houses (Gibbs et al., 1971) where they are stored for many months, and feeding and fasting practices in experimental laboratories are not standardized. The following experiments were done to determine the effects of feeding and fasting on liver and muscle glycogen and blood glucose and to suggest possible guidelines for experimental work.

Male frogs were collected at Whitmore Lake, Michigan, as they emerged from winter hibernation. One group of six ani-

mals was used for assays immediately upon arrival in the laboratory and served as the initial control group. Six animals were fasted in the laboratory for 9 days before assays were done, and six others for 2.5 months. A group of 18 frogs was fed liver and crickets for 4 months, then divided into three subgroups of six animals each which were killed for assays at 3 hr, 3 days, and 5 days, following their last force-fed meal.

Frogs emerging from hibernation had large liver and muscle glycogen stores (Table 6, initial controls) even though they had no food in their digestive tracts and presumably had fasted since entering the lake in early November.

During 9 days of fasting there was a significant decrease in blood glucose (Table 6), but it is not known whether this was an effect of fasting or due to the period of quiescence and reduced stress in the laboratory. Liver and muscle glycogen levels did not change significantly during this period.

After 2.5 months of fasting the frogs had significantly lower body weight, liver weight, and liver and muscle glycogen. Blood glucose levels were not lower than after 9 days of fasting.

After the 4-month feeding program, carbohydrate levels were not increased over the levels present at the time of capture (Table 7). Compared with animals assayed 3 hr after feeding, animals fasted for 3 or 5 days showed no significant change in muscle or liver glycogen. Following feeding blood glucose is elevated during the absorptive phase, but declines to a stable postabsorptive level by 3 to 5 days and shows no further decrease when frogs are fasted for intervals ranging from 5 days to

TABLE 6
EFFECTS OF LONG-TERM FASTING ON ENERGY RESERVES

Treatment	Body weight (g)[a]	Liver weight (g)	Liver glycogen (g/100 g liver)	Total liver glycogen (mg)	Muscle glycogen (g/100 g muscle)	Blood glucose (mg%)	Fat body weight (mg)
Initial controls[b] (6)[c]	25.3 ± 0.9	0.65 ± 0.07	14.6 ± 2.6	94.9 ± 18.1	1.12 ± 0.09	27.6 ± 2.0	9.3 ± 0.8
Fasted 9 days (6)	26.3 ± 1.4	0.73 ± 0.07	10.3 ± 1.1	75.2 ± 12.8	1.28 ± 0.10	21.8 ± 1.5*	21.3 ± 7.3*
Fasted 2.5 months (4)	20.6 ± 0.9*	0.38 ± 0.03*	2.3 ± 0.4*	8.8 ± 1.4*	0.61 ± 0.17*	22.8 ± 1.5	0.0*

[a] Mean ± 1 SE.
[b] Controls were sacrificed within 24 hr of collection and not fed. Animals had just emerged from hibernation in March.
[c] Number of frogs per group.
* $P \leq 0.05$.

TABLE 7
EFFECTS OF SHORT-TERM FASTING ON ENERGY RESERVES

Treatment	Body weight (g)[a]	Liver weight (g)	Liver glycogen (g/100 g liver)	Total liver glycogen (mg)	Muscle glycogen (g/100 g muscle)	Blood glucose (mg%)	Fat body weight (mg)
Initial controls[b] (5)[c]	25.3 ± 1.1	0.84 ± 0.07	10.3 ± 1.7	86.5 ± 21.4	1.03 ± 0.26	32.6 ± 4.0	19.2 ± 9.0
Fasted 3 days (5)	29.9 ± 2.5	0.97 ± 0.16	12.7 ± 1.0	123.2 ± 26.0	1.20 ± 0.25	15.8 ± 3.0*	54.7 ± 21.5
Fasted 5 days (5)	23.7 ± 3.0	0.69 ± 0.10	10.6 ± 0.9	74.1 ± 6.5	0.63 ± 0.04	19.6 ± 1.7*	30.1 ± 20.3

[a] Mean ± 1 SE.
[b] Controls were fed for 4 months after collection and sacrificed 3 hr after feeding.
[c] Number of frogs per group.
* $P < 0.05$.

2.5 months (Tables 6 and 7). Since blood glucose is at postabsorptive levels and liver and muscle glycogen stable between 3 and 5 days after fasting, a fasting interval of 3 to 5 days is recommended in experiments with frogs.

Compared with mammals, it is obvious that frogs have a remarkable capacity to maintain liver and muscle glycogen during prolonged fasting. Even after 2.5 months of fasting liver and muscle glycogen concentrations are not greatly different from those of nonfasted rats. This comparison is misleading to some extent, however, because fasted frogs also have greatly reduced liver size (Table 6).

The capacity of the frog to maintain relatively high levels of blood glucose and liver and muscle glycogen during prolonged fasting is probably due to gluconeogenesis from tissue protein. This assumption is supported by the following observations and calculations: (a) the daily caloric requirement of a 25-g frog at $18-25°$ is approximately 0.17 kcal, assuming an oxygen consumption of 0.42 liters/day, respiratory quotient of 0.82, and a caloric equivalent of 4.7 kcal/liter of oxygen consumed (Fromm and Johnson, 1955; McNabb, 1969); (b) assuming 50% of the body weight to be skeletal muscle, the total muscle glycogen is 0.14 g, and the total liver glycogen is 0.095 g (Table 6, Controls), giving a total body glycogen content of about 0.235 g; (c) assuming a caloric content of 4.2 kcal/g of carbohydrate, there would be 0.99 kcal in stored glycogen.

With a daily caloric requirement of 0.17 kcal, a 25.3-g frog contains only enough glycogen to last approximately 5 to 6 days. Yet, no drop in glycogen is seen in 5 or 9 days. Since the fat bodies were very small in frogs in this study (averaging 9 to 21 mg), and since a 20% decrease in body weight occurred after 2.5 months of fasting, gluconeogenesis from tissue protein must have been a major energy source, sparing glucose and glycogen to a very significant extent.

5. Seasonal Variation in Carbohydrate Reserves

Although the occurrence of seasonal variations in blood glucose and liver and muscle glycogen has previously been reported for several species of amphibians (Byrne and White, 1975; Mizell, 1965; Smith, 1954), we felt it important to reexamine this important variable in the present study. In addition to examination of this phenomenon in freshly captured *Rana pipiens* we have attempted to determine whether seasonal changes occur under relatively constant laboratory conditions.

(a) *Freshly captured frogs.* Assays were done on North Dakota frogs in March, August, and October. The animals used in March were captured as they migrated to hibernation sites the previous October and were maintained under natural environmental conditions in outdoor tanks until March when they were shipped. The animals used in August and October were shipped immediately after capture. The assays were done on postabsorptive frogs within 1 week after their arrival. Each group consisted of six animals.

The results are given in Table 8. In August all parameters were significantly different from values measured in March or October. Liver and muscle glycogen were lower whereas blood glucose was highest at this time. Liver glycogen varied from 6.3 g% and 54 mg total in August to 12.5 g% and 199 mg total in October. Muscle glycogen varied from 0.7 g% in August to 1.6 g% in October. Blood glucose showed a seasonal increase of over 45% between March and August. No significant differences were found in carbohydrate levels between March and October animals.

(b) *Laboratory-maintained frogs.* One group of North Dakota frogs received in September was kept in the laboratory under conditions described under Materials and Methods until the following August, when assays for blood glucose and liver glycogen were done. A control group was assayed upon receipt in September, and a second

TABLE 8

SEASONAL CHANGES IN ENERGY RESERVES OF NORTH DAKOTA FROGS

Date of sacrifice	Body weight (g)[a]	Liver weight (g)	Liver glycogen (g/100 g liver)	Total liver glycogen (mg)	Muscle glycogen (g/100 g muscle)	Blood glucose	Fat body weight	
							mg	mg/g body wt
March 4 (6)[b]	45.9 ± 2.6	1.75 ± 0.16	10.4 ± 1.0	184 ± 28	1.5 ± 0.2	14.2 ± 1.2	32.4 ± 2.9	0.8 ± 0.1
August 7 (6)	33.0 ± 2.1*	0.85 ± 0.10*†	6.3 ± 1.3*†	54 ± 14*†	0.7 ± 0.1*†	20.7 ± 2.4*	279.6 ± 46.8*†	8.5 ± 1.3*†
October 13 (6)	39.7 ± 3.2	1.59 ± 0.09	12.5 ± 0.8	199 ± 21	1.6 ± 0.1	18.5 ± 2.8	125.5 ± 22.2*	3.3 ± 0.6*

[a] Mean ± 1 SE.
[b] Number of frogs sacrificed.
* Significantly different from same parameter in March. P < 0.05.
† Significantly different from same parameter in October. P < 0.05.

control group of freshly caught frogs was assayed in August simultaneously with the laboratory-maintained group.

Blood glucose levels were not significantly different in any of the three groups, but liver glycogen was much lower in freshly caught frogs in August than in either freshly caught frogs in September or those kept in the laboratory for 11 months (Table 9). Seasonal cyclicity of liver glycogen content was apparently halted under long-term laboratory conditions (Table 9). We did not do the necessary experiment to determine whether the late winter decline in blood glucose occurs under long-term laboratory conditions.

According to Mizell (1965) in Rana pipiens blood glucose levels are lowest in winter, rise during the breeding season in the spring, and remain high during the summer and fall. Glycogen is highest in fall and winter and falls to lower levels in the summer. Our results, limited to three points in the annual cycle, substantiate his observations, and further demonstrate a parallel pattern of fluctuation in muscle glycogen levels.

A number of workers have confirmed a similar seasonal pattern in wild amphibians (Mizell, 1965; Smith, 1954; Hermansen and Jørgenson, 1969), but no agreement exists concerning whether seasonal changes continue in laboratory-acclimated amphibians. Bartell (1969) observed monthly differences in blood glucose levels of Ambystoma tigrinum kept in the laboratory under uniform conditions for various periods. Bergerhoff and Hanke (1967) found seasonal fluctuations in blood glucose in Rana temporaria stored in a cold room for 3 to 4 weeks before use. On the other hand, Hermansen and Jørgenson (1969) observed no differences in blood glucose levels of summer-collected frogs that were kept at 20–22° for days or weeks. Compiling all of our data for North Dakota frogs kept in the laboratory for at least 2 weeks, we find that blood glucose levels are lowest in the winter (Table

TABLE 9

CARBOHYDRATE LEVELS OF FROGS[a] RECENTLY COLLECTED AND FROGS MAINTAINED
IN THE LABORATORY

Conditions	g/100 g liver [mean ± 1 SE (n)]	
Group I. Collected in September		
A. Sacrificed in September	12.5 ± 0.8 (6)	18.9 ± 1.5 (12)
B. Sacrificed in August, after 11 months in the laboratory	11.7 ± 1.7 (5)	17.9 ± 1.6 (5)
Group II. Collected in August. Sacrificed in August with Group IB.	6.3 ± 1.3 (6)*	20.7 ± 2.4 (6)

[a] North Dakota *Rana pipiens*.
* Significantly different from Group IB. $P = 0.015$, P is probability of a higher U in the Mann–Whitney U test.

10). However, the difference between seasonal means is only half as great as that seen seasonally in freshly collected frogs (cf. Table 8), and as noted above seasonal variation in glycogen levels may be abolished altogether under certain laboratory conditions. One can conclude that some seasonal variation occurs in laboratory-maintained animals, but it is not as great as in animals under natural environmental conditions.

6. *Variation in Carbohydrate Reserves between Different Geographic Populations*

Rana pipiens is widely distributed geographically across the northern United States (Pace, 1974). Although appreciable differences in levels and seasonal patterns of carbohydrate reserves might be expected to occur in adaptation to geographic differences in environment, whether such differences actually occur has not been in-

vestigated in this species. Seasonal changes in glycogen content of the liver vary considerably with latitude in *Rana temporaria* (Lagerspetz, 1977).

In order to examine this possibility, glycogen and blood glucose levels of frogs from North Dakota and Michigan collected and assayed in October were compared. No significant difference was seen in blood glucose between the two geographic groups (Table 11). However, the levels of liver and muscle glycogen were significantly higher than in North Dakota frogs.

We have not determined whether this difference is due to a difference in the peak levels of glycogen stored prior to hibernation in the fall, or whether it reflects a shorter season and earlier onset of hibernation in North Dakota than in southern Michigan. It is clear, however, that at a given time of the year very appreciable differences in stored energy reserves can occur between two different populations.

TABLE 10

MEAN BLOOD GLUCOSE CONCENTRATIONS FOR NORTH DAKOTA FROGS
SACRIFICED THROUGHOUT THE YEAR

	September to January	January to March	July to August
Blood Glucose [mg% ± 1 SE (n)]	19.9 ± 2.4 (69)	16.0 ± 0.5 (27)	19.5 ± 1.5 (11)
Range	10.8–34.5	8.5–24.5	12.7–30.1

TABLE 11

COMPARISON OF NORTH DAKOTA AND WHITEMORE LAKE *Rana pipiens* DURING OCTOBER

Collection site and date[a]	Body Weight (g)[b]	Liver weight (g)	Liver glycogen (g/100 g)	Total liver glycogen		Muscle glycogen (g/100 g)	Blood glucose (mg%)	Fat body weight	
				mg	mg/g body wt			mg	mg/g body wt
North Dakota, October 13, n = 6	39.7 ± 1.4	1.59 ± 0.20	12.5 ± 0.6	199.0 ± 3.0	5.0 ± 0.3	1.6 ± 0.2	18.5 ± 1.0	129.5 ± 3.2	3.3 ± .5
Whitmore Lake, Michigan, October 3, n = 6	30.9 ± 0.8*	0.87 ± 0.20*	7.0 ± 0.9*	65.7 ± 2.7*	2.1 ± 0.5*	0.64 ± 0.1	17.9 ± 1.1	63.0 ± 3.2	1.9 ± 0.6
Whitmore Lake, Michigan, October 31, n = 6	27.4 ± 1.0*	0.94 ± 0.12*	9.5 ± 0.7*	86.0 ± 1.9*	3.1 ± 0.4*	—	15.1 ± 1.0	19.3 ± 1.2*	0.7 ± 0.2*

[a] Mean ± 1 SE.
[b] Date of sacrifice, collected 3 days to 1 week earlier.
* $P \leq 0.05$, Michigan compared with North Dakota.

Therefore, suppliers should be asked to specify the geographic source of frogs and to keep frogs from different localities and seasons separate. Investigators cannot assume this is done unless specifically indicated.

SUMMARY AND CONCLUSIONS

1. Blood glucose values vary according to the methods used in obtaining blood and measuring the sugar. Enzymatic methods of glucose determination using glucose oxidase and hexokinase give lower absolute values than those assays that also detect other reducing substances, especially where tungstic acid deproteinization is used. *The variation caused by differences in assay technique may be greater than 50%.*

2. Variations in the use of anesthetics, and including prolonged or repeated anesthesia, can cause hyperglycemia and hyperlactacidemia. Activity and excitation are also important causes of elevated glucose and lactate values. *Increases caused by methods of handling and anesthesia may be as great as 200% for glucose and 800% for lactate.* These values probably represent upper extremes, but unless great caution is used we would expect these factors to frequently cause spuriously high values and increased variance, possibly of 50% or more.

3. Blood glucose and liver and muscle glycogen remain remarkably high in *Rana pipiens* during short- or long-term fasting. Even so, many weeks of fasting may reduce muscle glycogen to 50% and liver glycogen to 20% of initial, immediately postabsorptive values. Blood glucose is even more stable than glycogen during prolonged fasting, showing no change after 2.5 months. However, blood glucose may increase 100% in the absorptive phase following feeding. Thus, although frogs exhibit a remarkable tolerance of fasting, *dietary status may induce variations as great as 100% in blood glucose and muscle glycogen*

and 600% in liver glycogen. Standardized dietary regimens should be followed, and the practice of storing frogs indefinitely without feeding should be avoided.

4. Normal resting levels of glucose and glycogen vary seasonally in *Rana pipiens.* Between the three intervals examined by us *the maximal differences observed* (March to August) *were approximately 50% for blood glucose and liver and muscle glycogen concentrations.* It is probable that some of the seasonal transitions, particularly between summer and fall, occur rather rapidly. Under certain laboratory conditions seasonal changes may be moderated (glucose) or abolished altogether (liver glycogen).

5. In October frogs from North Dakota have liver glycogen levels 25–40% higher and muscle glycogen 50% higher than Michigan frogs. Blood glucose values did not differ significantly between frogs of the two regions. Frogs collected from different geographic areas and at different seasons may be expected to show differences in glycogen reserves as great as 25–50% due to these factors.

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