SOME PHYSIOLOGICAL DIFFERENCES BETWEEN THE BLOOD

OF FROGS AT HIGH AND LOW TEMPERATURES

By

William DeMott Stull

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

As early as 1775 John Hunter (1837) experimented with the effect of temperature on living animals. He performed numerous experiments in which he subjected animals to freezing temperatures. He made three important discoveries; that poikilothermic (cold blooded) animals give off heat, that warm blooded animals produce more heat than cold blooded animals and that living tissues show a super-cooling effect. Since the time of Hunter the work on the effects of temperature has centered mainly on four general problems: hibernation in mammals, acclimatization to both high and low temperatures, effect on the rate of reactions, and temperature control in birds and mammals.

Early in the nineteenth century the first studies on hibernation in mammals were made by Spallanzani (1803), Mangili (1807), Saissy (1811), and others. Since this pioneer work there has accumulated a great mass of data which has not explained the causes underlying hibernation nor given a full understanding of the mechanism which in some mammals, as the woodchuck and ground squirrel, permits the ready reversion during hibernation to a poikilothermic condition. A review of the subject containing many references to the early as well as modern works has been made by Johnson, 1931.

The literature on acclimatization of protoplasm to temperatures outside the normal range begins, according to Davenport (1896), with the report of Dutrochet in 1837 of acclimatization of Nitella to high temperature. Davenport concludes that change in water content is probably the cause underlying acclimatization. He bases his conclusion on the facts that the coagulation temperature of egg albumin rises as the water

content is diminished and that heat resistant spores contain less water
than the vulnerable vegetative forms. This theory was again put forward
by Hatai (1924) who showed that temperatures lethal to various regions
in the earthworm are in direct proportion to the water content of the
tissue. Stier and Taylor (1939) have brought forth evidence from which
they conclude that in the frog acclimatization is due to changes in
metabolism rate produced by changes in pituitary activity.

A theory proposed by Jaques Loeb (1892) is interesting in this
connection. He says:

> Raising the concentration of the salt solution in which an
> animal or tissue lives has qualitatively and quantitatively
> the same effect as lowering the temperature; lowering the
> concentration has qualitatively and quantitatively the same
> effect as raising the temperature.

Greely (1901 a and b), a student of Loeb, has substantiated this conten-
tion and points out further that cells lose water both when placed at
low temperatures and when immersed in hypertonic salt solutions.

No attempt is made here to discuss temperature as it affects
the rate of biological reactions. Most of this work has been concerned
with attempts to fit the observations to mathematical formulae. Hoagland
(1935) and Bělehrádek (1935) have written excellent reviews on this
subject.

Temperature control in homoiothermic animals has been studied
principally by subjecting the animal to such severe cold that it cannot
produce heat rapidly enough to maintain its normal internal temperature.
In all this work the writer has been unable to find a comparison of the
effects of lowering the temperature in mammals with the effects produced
in poikilothermic animals. Such a comparison would be helpful by
pointing out which changes are due to temperature alone and which are

due to the animal's attempt to maintain a homeostatic condition with regard to body temperature.

The seasonal changes in the physiology of poikilothermic animals has been studied by a number of workers. There has been some attempt to determine whether changes attributed to seasonal variations are merely adjustments to temperature or are linked with other factors of the animal cycle. Holzapfel (1937), in her recent review, has covered much of the literature on seasonal variations in cold blooded animals, particularly the frog, and has added observations of her own. She considers the changes to be "an intrinsic function of the animal itself, and in _Rana pipiens_ and _Rana catesbeiana_, not dependent upon environment". The seasonal variations in blood gases, blood sugar, and nitrogen distribution in the alligator have been reported by Hopping (1923).

Van der Heyde (1921) determined the temperature coefficients for excretion of urea, ammonia and uric acid in frogs. He found the average values of Q_{10} for these substances to vary from 1.6 between 1° and 21° C. to 3.6 between 21° and 31° C. It has since been shown (Adolph, 1927) that the rate of formation of urine doubles or trebles for each 10-degree rise in temperature and at 0° C. almost no urine is formed after twenty-four hours. Difference in the excretion of urine in summer and winter frogs have been reported by de Haan and Bakker (1921). They found that summer frogs excrete more urine than winter frogs at the same temperature. Since this difference is slight, they attribute most of the difference in excretion under natural conditions to temperature. They found temperature to have no effect on the urea concentration in the plasma or on the ratio between the urea concentration in the plasma and the urine. Later, in an attempt to account for

the greater excretion in summer frogs, de Haan (1927) found that the plasma protein content, as indicated by the refractive index, is almost the same in the winter frogs as in the summer frogs and that the protein content of the plasma rises when hibernating winter frogs are warmed. Similar work was done on the turtle by Campbell and Turner (1937) who were primarily interested in the changes which occur in the colloid osmotic pressure. They found that turtles exhibit a lowering of 75% in colloid osmotic pressure after three and one-half months in the cold, while that of those maintained at room temperature does not change. In neither group was there a change in the protein nitrogen, but in both there was a slight lowering of the refractive index. Austin, Sundermon, and Cramack (1927) found in alligator serum an increase in protein content of from 4.28 grams per 100 cc. at 7^0 -11^0 C. to 5.56 grams per 100 cc. at 33^0 -38^0 C. The animals were kept at each temperature for three days before the determinations were made.

While working with bull frogs to determine the effect of insulin on cold-blooded vertebrates, Olmsted (1924) observed that at room temperature the average blood sugar per 100 cc. was 12 mg., at 28^0 C. it was 56 mg., and at 34.5^0 C. 117 mg. The same thing has been found to hold true for the alligator (Austin et al, op. cit.) in which the blood sugar concentration of 115 milligrams per 100 cc. at 9^0 C. rose to 193 milligrams per 100 cc. at 35^0 C. Seasonal variations in the glycogen content of the muscles and liver as well as in the blood glucose of the South American toad, Bufo arenarum, have been recorded by Mazzocco (1938). He found that glycogen is at a minimum during the season of sexual activity in spring. This is followed by a slow rise during the summer to a maximum in mid-winter. The blood sugar was at

a maximum in the summer and decreased at the beginning of fall to a low during winter and spring. No relationship, either direct or indirect, with the glycogen was observed. Robertis (1938) found a great decrease in the liver glycogen in winter toads kept at 32° C. for twenty days.

The changes in rate of the activities of frogs with changes in temperature are well known. As early as 1911 Hill made careful observations on the heat production in frogs at various temperatures. He reports a production of .92 calories per cc. per hour at 28.6° C. and only .21 calories per cc. per hour at 15° C. By extrapolation to 37° C., average mammalian temperature, he shows that at that temperature the frog, theoretically, would produce 2.1 calories per cc. per hour, a metabolic rate of the same order as found in mammals such as man or the ox. The average Q_{10} obtained was 2.5. Adolph (1929) found 2.7, or practically the same Q_{10}, for oxygen consumption in frog skin.

A difference in the heart rate has been observed in summer and winter frogs both by Barcroft and Izquierdo (1931) and Holzapfel (op. cit.). The former report that in winter frogs there is a linear relation between the log of the frequency of heart beat and the reciprocal of the absolute temperature between 5° and 20° C. while in summer frogs there is a direct linear relationship between the rate and the temperature.

A number of authors have noted that frogs when maintained at low temperature, gain weight due to intake of water (Ott, 1924, Barthelemy, 1926, Adolph, 1927, and Holzapfel, 1937). Ott has shown that when frogs are transferred from a low to a high temperature water is lost progressively for three to five days. According to his work, at low temperature the tissue gained little or no water

leading him to the conclusion that the water accumulates in the lymph spaces rather than in the tissues.

The present study is an effort to interpret some of these phenomena. To this end the changes in red blood cell numbers, white blood cell numbers, hemoglobin concentration, water concentration in the blood and muscle, blood sugar, urea, and the rate of water absorption were determined in the bull frog, *Rana catesbeiana*, and the grass frog, *Rana pipiens*, under various temperature conditions.

METHODS

Mature male frogs were used in all experiments. The bull frogs were an average of seven inches in length. They were obtained from a supply house in Louisiana. The grass frogs averaged three inches in body length and approximately seventy-five grams in weight. These were obtained from a dealer in Ohio.

It was originally planned to use the bull frogs for at least two determinations at each temperature. This was accomplished with only one frog because of the high mortality of these animals under the conditions at which it was necessary to keep them. The use of this form, therefore, was abandoned in favor of the smaller and less expensive grass frogs. The small size of grass frogs made continued removal of sufficient amounts of blood impossible, but their low cost made it practicable to sacrifice them in order to obtain enough blood. With these, ten individuals were used for each series of determinations at each temperature.

The blood for determinations was drawn from the ventricle of the heart with a Luer type glass syringe fitted with a 3/4 inch, number 26 hypodermic needle. In the case of the bull frogs the blood was drawn from living, unanesthetized individuals in the following manner. The animal was laid ventral side up on a board and its legs were securely tied to prevent movement. The needle was inserted through the skin just left of the center of the sternum and pushed toward the head, making approximately a thirty-degree angle with the surface. When the heart was reached the needle was given a quick thrust to pierce the wall of the ventricle. Somewhat over one cubic centimeter of blood was slowly

drawn from the heart each time. The same frog was used for more than one determination. A week was allowed between drawings to permit the replacement of the lost blood.

The grass frogs were sacrificed when blood was obtained. The brain and spinal cord were pithed, and the frogs were clipped ventral side up on a Harvard frog board. The heart was exposed by cutting away the over-lying skin and body wall, care being taken to avoid the larger blood vessels. The pericardium was opened and the blood drawn from the ventricle. It was possible, in some cases, to draw as much as two cubic centimeters by this method. The blood was drawn quickly to lessen the likelihood of including extravascular fluid which would enter the blood system when the bleeding reduced the pressure and to lessen the amount which would be drained from the organs as this blood would be apt to have a composition slightly different from that in the direct circulatory system.

The two experimental temperatures used were 26 ± 1^{0} C. and 8 ± 2^{0} C. In all cases, except the work on the rate of change in water content at low temperature, the frogs were kept at least a week at each temperature. In order to make all conditions, except the temperature, as identical as possible all of the animals to be used for a set of determinations were placed in a Freas electric incubator at 26^{0} C. and were fed for five days on ground beef and beef liver. The food was placed in the mouth by hand. They took the food quite readily, and there were few cases of regurgitation. After two days of starvation, following the feeding period, the animals on which measurements were to be made at low temperatures were transferred to an electric refrigerator which was maintained as nearly as possible at 8^{0} C. The determinations were made

on the high temperature group of frogs thirty-six hours after the low temperature group had been placed in the refrigerator. Those on the low temperature group were made seven days after they were placed in the refrigerator. Hill (1911) has shown, as mentioned in the introduction, that the heat production of the frog has an average Q_{10} of 2.5. From this it may be calculated that the total heat production of a frog over a period of seven days at 8° C. is equivalent to that produced at 26° C. in thirty-six hours. Thus, when the frogs at low temperature were removed for collection of blood they were, as nearly as possible, at the same nutritional level as those which had remained the additional thirty-six hours at high temperature.

Where an anticoagulant was needed powdered potassium oxalate was generally used. Frog blood, in common with that of other cold-blooded vertebrates, has a long coagulation time (Howell, 1928, pp. 467, 473), and no difficulty was encountered from coagulation in the syringe if the instrument was clean. In the series in which the rate of change in per cent of water, the number of red cells, and the per cent volume of red cells were determined, coagulation was prevented by cooling all instruments to 0° C. by packing them in crushed ice. The drawn blood was immediately emptied from the syringe into a vial packed in ice. This proved to be a satisfactory method of preventing coagulation without producing any change in osmotic pressure of the serum, as is the case with added chemical anticoagulants. In the series in which the rate of change in water content alone was measured there was no necessity of preventing coagulation.

All blood counts were taken by the use of a standard Spencer Bright-line improved Neubauer hemocytometer. The blood was diluted one

to one hundred in a blood dilution pipette. The diluting medium used was 1% acetic acid deeply colored with crystal violet. As both the red and white cells of the frog's blood are nucleated the use of this fluid made possible the counting of both types from the same drop. Because of the low number and large size of the red cells the large corner squares of the counting chamber, which are ruled sixteen to the square millimeter, were used. One hundred and twenty-eight of these square millimeters were counted in each determination. A sixteen-millimeter objective and a number ten ocular proved adequate in almost all cases. In a few doubtful instances the identity of a cell was determined under a four-millimeter objective.

The hemoglobin was measured colorimetrically with a Hellige-Wintrobe glass wedge haemometer, calibrated for human hemoglobin. As the interest here was in comparative rather than absolute values no attempt was made to recalibrate it for frog hemoglobin.

To determine the water content of the blood a sample of approximately one gram was placed in a previously weighed covered vial. It was immediately weighed and was then dried to a constant weight in an oven at 70° C. in a pressure reduced to 160 mm. of mercury. Muscle water was determined on the gastrocnemius muscles. Immediately following the removal of blood, the legs were severed at the knees. The gastrocnemius muscles were then dissected from the legs. The fluid on the surface of the muscles was removed with filter paper, and the muscles were placed in vials. After the gross weights of the muscles were determined they were frozen in the freezing chamber of the refrigerator, a treatment which facilitates drying. The initial water content was determined by drying to constant weight in the vacuum oven.

Blood sugar was determined by the iodometric method of Shaffer and Somogyi (1933). All determinations were run immediately after the blood was drawn.

Urea determinations were made following the procedure of Folin and Svedberg (1930). A fresh extract of jack bean meal was used as urease.

Two series were run to observe the rate of change in the water content of frogs when transferred from high to low temperatures. The first series included ten individuals. All of these frogs were placed in the oven at 26° C., were fed for five days and then starved for three. Following this two were removed. Determinations were made on these of the per cent water content of the blood and muscle, the number of red blood cells, and the per cent volume of the red cells. The remaining eight were placed in water at 8° C. and transferred to the refrigerator. Determinations were made on these at intervals over a period of ten days. The per cent volume of the red blood cells was determined by placing a sample in a Van Allen hematocrit and centrifuging it ten minutes at 3000 revolutions per minute. The Van Allen hematocrit is calibrated in one hundred divisions. If the hematocrit is filled exactly to the one hundred mark before centrifuging, the height of the column of packed red cells after centrifuging gives a direct reading of the per cent red cells. The second series included twelve frogs. Again two were used at 26° C. while the remaining ten were cooled to 8° C. and run at intervals over a period of approximately fifty-four hours. In the second series, the per cent water in the blood and muscle were the only factors measured.

RESULTS

Cell Counts. In table I are shown the red cell counts in the
bull frog, R. catesbeiana, at 8° C. and 26° C. These counts have direct
relationship to the temperatures to which the frogs were conditioned.
The average count at the lower temperature is 296,000±10,000 cells per
cubic millimeter and at the higher temperature 399,000±25,000 cells per
cubic millimeter. The measure of error used in these and in all subse-
quent calculations is the mean square error as calculated according to
Bessel's equation#, this being essentially the standard error. A dif-
ference between the average values of two groups of determinations is
taken as significant when this difference is twice the sum of the mean
square errors of these averages.

In R. pipiens, the grass frog, the red cell counts (Table II)
show a similar decrease with a lowering in temperature. In this species,
the red cell count at 8° C. is 370,100±12,700 and at 26° C. is
527,400±8,000. This difference is highly significant, while in the
bull frog, where there are greater individual variations, the error is
much larger. The difference within the species, however, is of the
same order, there being from 26° C. to 8° C., a drop in average counts
of 25.8% in the bull frog and 29.9% in the grass frog.

A similar relationship between temperature and white cell
count in the grass frog is shown in table II. The average values are
11,660±540 at 8° C. and 15,500±1,500 at 26° C. The difference in the
average values of each temperature group is 24.8% of the average count

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\# $\mathcal{M}$ (mean square error) $=$ $\sqrt{\dfrac{d^2}{n(n-1)}}$

Table I

Effect of temperature on

Red blood cells in R. catesbeiana

Frog No.	Erythrocytes per mm.^{3}	
	8°C.	26°C.
10	320,000	471,000
	252,000	540,000
14	322,000	338,000
	266,000	
16	362,000	356,000
	248,000	386,000
17		365,000
18	304,000	382,000
19	302,000	354,000
Average	296,000 ±10,000*	399,000 ±25,000*

*Mean square error (Bessel) used in all calculations.

Table II

Effect of temperature on

Blood cells in R. pipiens

	8°C.			26°C.	
Frog No.	Erythrocytes per mm.^{3}	Leucocytes per mm.^{3}	Frog No.	Erythrocytes per mm.^{3}	Leucocytes per mm.^{3}
25	357,000	12,500	35	532,000	16,000
26	388,000	9,500	36	558,500	12,500
27	334,500	10,600	37	542,000	39,000
28	374,000	15,000	38	443,000	11,500
29	356,500	10,000	39	547,500	18,000
30	383,000	11,500	40	535,000	14,000
31	395,500	10,500	41	467,500	11,500
32	295,000	17,000	42	589,500	9,500
33	333,000	9,500	43	529,500	16,000
34	484,500	10,000	44	526,500	7,500
Average	370,100 ±12,700	11,660 ±540	Average	527,400 ±8,000	15,500 ±1,500

at 26° C. This per cent difference is somewhat lower than the per cent difference in the red cells for the same frogs as shown above, but when the greater error of the averages of the white cell counts is taken into consideration these percentages are quite comparable.

Hemoglobin. The hemoglobin concentrations in the bull frog (Table III) are 8.08±.87 grams per 100 cubic centimeters at 8° C. and 10.09±.92 grams per 100 cubic centimeters at 26° C. The difference between these average concentrations is not significant because of the wide individual variations which are due probably to the loss of blood in drawing the samples. These variations range from 4.9 to 12.5 grams per 100 cubic centimeters in the 8° C. group and from 7.5 to 14.4 grams per 100 cubic centimeters in the 26° C. group. Exceptionally low concentrations would be expected if the frogs had failed to replenish the blood cells, while high values might be expected due to the stimulating effect of the loss of blood on the blood building activities. In the grass frog, where the blood was drawn but once and the animal sacrificed, there is a variation of from 8.8 to 13.2 grams per 100 cubic centimeters in the 8° C. group and from 13.5 to 18.3 grams per 100 cubic centimeters in the 26° C. group. The averages for these groups are 10.56±.56 at 8° C. and 15.54±.30 at 26° C., the difference between these averages being significant.

Per cent Water in the Blood. In table IV, it may be seen that the water content of the blood varies inversely with the temperature; this is in contrast to the cells and hemoglobin content which vary directly. There is a difference between the two species of frogs in the average percentages of water content of the blood. In both species there is approximately 4% more water in the blood of frogs at 8° C. than

Table III

Effect of Temperature on

Hemoglobin concentration

in grams per 100 cc.

R. catesbeiana			R. pipiens			
Frog No.	8°C.	26°C.	Frog No.	8°C.	Frog No.	26°C.
10	7.2	14.4	30	11.0	35	16.5
	7.1	13.4	31	10.6	36	14.9
14	4.9	7.5	32	8.8	37	15.3
	7.4		33	9.2	38	15.2
16	8.6	7.9	34	13.2	39	14.0
	6.2	11.9			40	16.2
17		9.9			41	13.5
18	12.5	8.9			42	16.5
19	10.7	8.8			43	18.3
					44	15.0
Average	8.08±.87	10.09±.92		10.56±.56		15.54±.30

Table IV

Effect of temperature on

Per cent water in blood

R. catesbeiana			R. pipiens			
Frog No.	8°C.	26°C.	Frog No.	8°C.	Frog No.	26°C.
10	89.10	81.69	25	85.75	35	81.27
14	89.54	81.69	26	84.76	36	81.35
	88.10		27	87.06	37	81.30
16		83.99	28	87.26	38	81.34
17	88.55		29	85.89	39	82.36
18	87.42	84.03	30	85.01	40	80.95
19	89.16	86.05	31	85.63	41	81.48
			32	88.19	42	81.19
			33	87.34	43	80.77
			34	83.00	44	82.69
Average	88.65±.33	84.52±.91		85.99±.47		81.47±.018

in those at 26° C. In the bull frog there is 88.65±.33% water at

8° C. and 84.52±.91% water at 26° C. In the grass frogs the values

are 85.99±.47% at 8° C. and 81.47±.018 at 26° C.

Progressive Changes in Per Cent Water in Blood and Muscle,

in Erythrocyte Count and in Per Cent Volume of Erythrocytes. Tables

V and VI give the progressive changes which occur in the water content

of the blood and muscle when frogs previously acclimatized to 26° C.

are subjected to a temperature of 8° C. It will be seen that these

changes are not constant, nor do the values rise to a level and remain

there. In the series in table V, the per cent water in the muscle

rises from an average of 79.37% to a maximum of 80.96% during the first

fourteen hours and then falls, reaching a minimum of 78.81% in fifty

hours. After 131 hours the water is relatively constant at an average

of 79.86%. The value of 80.65% at 204.5 hours is to be disregarded

since this frog showed abnormal values for all determinations made upon

it. After fifty hours the data are meager, making the true course of

the changes uncertain. The changes occuring during the first fifty

hours are paralleled by the second series of frogs in this experiment

as shown in table VI where the greatest water content (80.93%) is reached

in nineteen and one-half hours and the lowest level (77.79%) is reached

in approximately forty-eight hours. At fifty-four hours the water con-

tent is again of the order of that at 26° C.

The per cent water in the blood presents a somewhat similar

picture. However, the total changes are greater and the time relations

are different than in muscle. The greatest change in the water content

of the muscle is from 77.79% to 80.96% or a range of 3.17%. The water

content of the blood, on the other hand, varies from 82.38% to 89.44%,

19

Table V

Variation with time in per cent water of blood and muscle,

and in erythrocyte count and per cent volume

in R. pipiens originally at 26°C.

Frog No.	Hours at 8°C.	%H2O Muscle	%H2O Blood	Erythrocytes Per cmm.[3]	% Volume Erythrocytes
151	0.0	79.23	84.97	317,000	39.5
152	0.0	79.51	83.74	400,500	46.0
153	12.0	79.78	83.62	548.500	47.0
154	14.0	80.96	88.20	318,000	30.5
155	26.5	79.38	89.44	244,000	26.0
156	50.0	78.81	86.79	387,000	35.5
157	131.0	79.91	87.06	330,500	32.0
158	133.0	79.67	88.39	290,000	29.5
159	194.0	80.00	89.18	282,000	26.5
160	204.5	80.65	85.47	521,000	40.0

Table VI

Variation with time in the water content

of muscle and blood of R. pipiens

originally at 26°C.

Frog No.	Hours at 8°C.	%H20 Muscle	%H20 Blood
161	0	79.16	83.17
162	0	78.57	82.38
163	5.5	78.13	84.24
164	12	80.10	87.81
165	19.5	80.93	87.88
166	24.5	79.41	88.52
167	29.75	80.04	89.43
168	34.5	79.09	85.12
169	42.75	78.76	85.73
170	47.75	77.79	86.73
171	53.75	79.23	85.28
172	59.5	79.37	89.05

a range of 7.06%. The maximum in the water content of the blood occurs later than does the maximum in muscle. In table VI, the maximum of 89.43% water is reached by the blood in approximately thirty hours or ten hours after the maximum is reached by the muscle. In table V, this lag amounts to twelve and one-half hours. The maximum value for blood water in this group is 89.44% and occurs at twenty-six and one-half hours. These two maxima are remarkably close in value, there being but .01% difference between them.

The erythrocyte counts and the per cent volume of erythrocytes recorded in table V show an inverse relation to the water content of the blood. The maximum red cell count of 548,500 cells per cubic millimeter and the maximum per cent volume of erythrocytes of 47.0% accompanies the minimum water content and conversely the minimum red cell count of 244,000 cells per cubic millimeter and the minimum per cent volume of erythrocytes of 26.0% accompanies the maximum water content.

Blood Sugar. As shown in table VII, there is a pronounced direct relationship in frogs between the blood sugar and the temperature. The average value for the bull frogs at 8° C. is 33±1.5 milligrams per 100 cubic centimeters, and at 26° C. is 59±1.3 milligrams per 100 cubic centimeters. The average values are lower in the grass frog. At 8° C. the values, in all but one individual, are below the limit of the method used. This limit is 21 milligrams per 100 cubic centimeters. The one value obtained is 26 milligrams per 100 cubic centimeters which is close to the limit. At 26° C., the grass frogs have an average blood sugar level of 45.4±3.4 milligrams per 100 cubic centimeters. At this temperature they exhibit a wide individual variation.

Table VII

Effect of temperature on

Blood sugar in milligrams per 100 cc.

R. catesbeiana			R. pipiens			
Frog No.	8°C.	26°C.	Frog No.	8°C.	Frog No.	26°C.
20	34		71	_*	62	62.2
21	29	56	72	-	63	44.0
22		62	73	-	64	29.2
		58	74	-	65	34.2
23	36	58	75	-	66	36.3
24	33		76	-	67	73.2
			77	-	68	52.2
			78	-	69	50.4
			79	26	81	36.3
			80	-	82	43.4
					84	38.1
					85	50.8
					86	40.2
Average	33±1.5	59±1.3				45.4±3.4

*Where no value is given the blood sugar value was below the limit of this method.

Urea. There is no significant difference in the urea content of the grass frogs at high and low temperatures as shown in table VIII. The .39 milligram of urea nitrogen per 100 cubic centimeter difference that was found is not as great as either of the errors.

Table IX is a general summary of the differences in blood composition between frogs conditioned at 8° C. and 26° C.

Table VIII

Effect of Temperature on

Urea nitrogen in milligrams

per 100 cc. in R. pipiens

Frog. No.	8°C.	Frog No.	26°C.
112	2.1	116	1.2
113	4.6	117	4.6
114	4.0	119	3.2
123	5.4	120	2.9
124	2.2	122	1.9
125	2.2	131	3.8
127	5.2	132	2.6
129	5.5	133	5.2
130	1.4	134	3.9
115	1.9	135	1.3
Average	$3.45 \pm .52$		$3.06 \pm .43$

Table IX

Averages for each group of determinations

taken from Tables I-IV and VII-VIII

	8° C.	26° C.
Erythrocytes per mm³		
R. catesbeiana	296,000 ± 10,000	399,000 ± 25,000
R. pipiens	370,100 ± 12,700	527,400 ± 8,000
Leucocytes per mm³		
R. pipiens	11,660 ± 540	15,500 ± 1,500
Hemoglobin in gm. per 100 cc.		
R. catesbeiana	8.08 ± .87	10.09 ± .92
R. pipiens	10.56 ± .56	15.54 ± .30
Per cent water		
R. catesbeiana	88.65 ± .33	84.52 ± .91
R. pipiens	85.99 ± .47	81.47 ± .018
Blood sugar in mgm. per 100 cc.		
R. catesbeiana	33 ± 1.5	59 ± 1.3
R. pipiens	#	45.4 ± 3.4
Urea nitrogen in mgm. per 100 cc.		
R. pipiens	3.45 ± .52	3.06 ± .43

Values for the blood sugar in R. pipiens at 8° C. were in all but one case below the limit of the method employed.

DISCUSSION

White Cell Concentrations. At both temperatures used in these experiments there are greater individual variations in the white cell counts of the grass frog than in the red cell counts (Table II). This is especially true in the frogs at 26° C. where the counts range from a low of 7,500 cells per cubic millimeter to 39,000 per cubic millimeter. In mammals, the number of leucocytes is intimately related to the physiological condition of the animal as brought about by exercise and emotional states (Zoethout and Tuttle, 1940, p. 160). If this holds true for the lower vertebrates, the wider variation in the active high temperature group as compared to the dormant low temperature group would be expected. This dependence upon the physiological condition of the animal holds true in the frog, for the determinations reported here show that the white cell count besides varying more widely than the red cell count varies independently of it. This conclusion is based on the very low correlation between the counts of the two types of cells within a temperature group. The coefficient of correlation between the red cell counts and the white cell counts of the individual grass frogs in the 8° C. group is -.23 and in the 26° C. group is .19 (see Table II). If the abnormally high white cell count (39,000 per mm.[3]) of frog number 37 of the 26° C. group is discarded, the coefficient of correlation in this group falls to the extremely low figure of .021.

Hemoglobin. As an index of the concentration of hemoglobin in the cells, the grams of hemoglobin per 100 cubic centimeters of blood is divided by the number, in thousands, of the red blood cells per cubic millimeter. When this is done we find that in the grass frog the average index at 8° C. is .0286 and at 26° C. is .0295. The difference between

these indices is well within the error. This close agreement shows that there is no significant change in the hemoglobin concentration in the cells themselves. The change in hemoglobin concentration of the blood thatoccurs is due to a change in the cell content of the blood.

Holzapfel (1937) has shown an annual cycle in hemoglobin concentration in grass frogs kept at 0° C. which was independent of the cycle in erythrocyte concentration. The hemoglobin content was lowest in February when the cell count also was lowest and rose slowly during the spring and summer to a peak in November. The cell count on the other hand rose sharply to a peak in April and fell again to its February level in May. This was followed by a new rise throughout the summer to a new peak in November. She has, however, reported no determinations on frogs at high temperatures over this same period of time.

Since the hemoglobin concentration of the blood is reduced with a lowering of temperature, presumably the oxygen-carrying power of the blood also is reduced. This, however, is an incidental change as will be shown later, produced by dilution of the blood rather than a change in the concentration of hemoglobin in the cells.

The Relationship Between the Water Content of the Blood and the Erythrocyte Count. The water content of the blood varies inversely with the cell count and the hemoglobin concentration. The wide and consistent variation in the water content and blood cells in R. pipiens at 8° C. as shown in tables II and IV permit the construction of a formula expressing the relationship between water content and the cell count. This, when done by the method of least squares and when three significant figures are retained, gives the formula

$$C = 3200 - 32.9W$$

where C is the cell count in thousands and W is the per cent water.
If the use of this formula under all conditions gives theoretical
values which approximate actual counts, this is evidence that the cell count
is more dependent upon water content than on any other factor. When
we substitute the average per cent water at 26° C., in R. pipiens for
W we obtain a theoretical value of 500,000 for the blood cell count
which is not significantly different from that obtained experimentally.
From this it would appear that the water content is the chief component
that is directly affected by the temperature and that the change in
red cell count and therefore the change in hemoglobin concentration is
due only to hemodilution.

Evidence from two other sources may be used to substantiate
this conclusion. Although the bull frog is a distinct species, we find
that this formula is as applicable to the blood of the bull frog as to
the grass frog. When the average values for the water content of bull
frog blood (Table IV) are substituted in the formula, we obtain a value
for C which is not significantly different from the observed counts.
For 8° C. bull frogs the theoretical value, according to this formula,
is 283,000 as compared with the observed count of 296,000±10,000. At
26° C. the theoretical count is 419,000, as compared with 399,000±25,000,
values closer than that obtained in the grass frog from which species
the formula was derived.

The other source of evidence is taken from the values of the
progressive change in water content and red cell numbers as shown in
table V. These observed counts, corresponding theoretical counts, and
the deviations of these counts are given in table X.

Table X

Application of the formula

expressing the relationship between

per cent blood water and erythrocyte count

Frog no.	% H_2O blood	Observed cell count	Theoretical cell count	Deviation
151	84.97	317,000	404,000	+87,000
152	83.74	400,500	445,000	+44,500
153	83.62	548,500	449,000	-99,500
154	88.20	318,000	298,000	-20,000
155	89.44	244,000	257,000	+13,000
156	86.79	387,000	346,000	-41,000
157	87.06	330,500	336,000	+ 5,500
158	88.39	290,000	292,000	+ 2,000
159	89.18	282,000	266,000	-16,000
160	85.47	521,000	388,000	-133,000

As all of the observations on frog number 160 are widely
different from the others of the group, it would seem probable that
this frog was not normal and its high blood count is a symptom of some
physiological maladjustment and hence the figure is discarded.

The algebraic sum of the deviations of the remaining normal
frogs is -24,500, which when divided by the number of frogs in the
group, amounts to only -2,722 cells per frog.

De Haan (1927) recorded that frogs hibernating, presumably
at 0° C., when brought to a temperature of $23-25^\circ$ C. show an increase
in protein content of the plasma and per cent volume of the red cells.
He made no attempt to correlate this with loss in water content of the
blood. From the evidence presented here, these changes may have been
reflections of hemoconcentration.

There is no work recorded in the literature which indicates
that changes in the frog in blood composition as brought about by low
temperatures are due to hemodilution. In fact, Holzapfel (op. cit.)
states that with the approach of hibernation, which presumably is
simultaneous with a reduction in environmental temperature, there
occurs a reduction in the blood volume which by February amounts to
eighty per cent. Accompanying this, there is a reduction in the total
number of blood cells. This would not imply hemodilution.

The results shown in tables I-IV are from determinations
made on frogs in July, and those in table V are from determinations
made in February. During the observations made by this writer, no
attempt was made to measure the total blood volume of the frogs but
had the volume decreased to the extent recorded by Holzapfel, there
would have been difficulty in obtaining blood. The frogs used by

Holzapfel evidently were emaciated. The frogs of eighty-millimeter body length used by her weighed but twenty-five grams, while those of similar length in this experiment weighed fifty grams. Perhaps this difference in the condition of the frogs accounts for the difference in the results.

Thus it is concluded that many of the changes in concentration of blood components that accompany changes in temperature are due to hemodilution or hemoconcentration.

Progressive Changes in Water Content of Blood and Muscle as Effected by Temperature. Progressive changes occur in the water content of the blood and muscle of frogs transferred from 26° C. to 8° C. as shown in figure 1. The water contents of both increase during the first twenty-four hours and decrease during the second twenty-four hours. The peak in the case of muscle is between 14 and 20 hours, while that for blood is at approximately 30 hours. In the first and second series run (Tables V and VI), the muscle water reaches a low at approximately 48 hours at which time it is lower than the values for these same series at 26° C. The water content of the blood rises to a peak more slowly and decreases to a low more rapidly than does the water content of muscle. It never drops as low at 8° C. as it was at 26° C. In both blood and muscle, the water contents begin to rise at approximately fifty hours. This rise is sharp in the case of blood water where it eventually approaches the height attained at thirty hours. The rise in the muscle is slower and the water content does not exceed the level at 26° C. After this rise, the water contents in both blood and muscle appear to have reached an equilibrium for they remain quite constant.
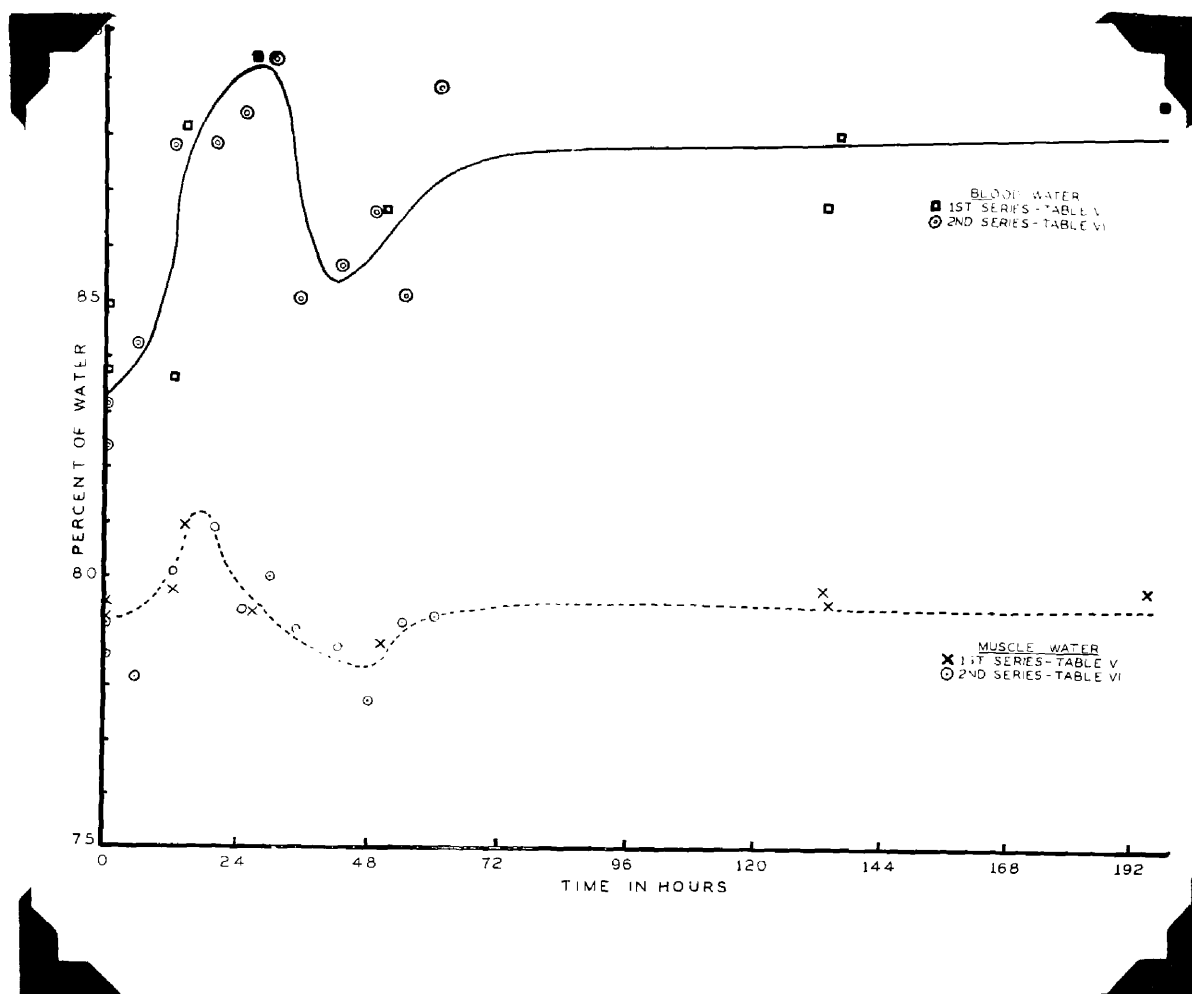
Figure 1.   Progressive changes occurring with time at 8°C.
in the per cent water in the blood and
muscles of frogs previously conditioned
to 26°C.

In attempting to explain the above described changes in water content of muscle and blood in frogs transferred from a high to a low temperature, several factors suggest themselves. Adsorption, imbibition, osmotic pressure and blood pressure have been used to explain such water transfers and appear to be factors which independently or jointly have brought about the water shifts observed here.

Adsorption, a physical factor with a negative temperature coefficient, tends to increase total water content in the frog at low temperature. This change in adsorption is an effect of temperature directly on a physical factor and is not a secondary effect brought about by a change in the metabolic activity.

Adsorption is often given as the underlying cause of imbibition. Imbibition has a negative temperature coefficient. The effects of imbibition are inseparable from those of adsorption. Increase in the imbibition of water at low temperature has been shown by Hauberriesser and Schönfeld (1913) in mammalian ligament and by Lichtwitz and Renner (1914) in mammalian muscle and the renal cortex. The latter found, however, that the water content of the renal medulla decreased with decrease in temperature. Also, Adler (1921) found that the water content of frog muscle decreases with a decrease in temperature. Bělehrádek (1935) is of the opinion that since all of the experiments excepting Adler's were performed on isolated tissue that perhaps the decrease in water content of muscle at low temperature indicates a regulatory mechanism in the frog as a whole. However, this decrease reported by Adler was not found in the experiments performed by the writer.

Osmotic pressure has a positive temperature coefficient. Thus, increases in osmotic pressure at low temperature causing increases in water absorption in tissues are secondary effects produced by changes in metabolism. Scott, Herrman and Snell (1917) have shown that in man hemoconcentration occurs during muscular exercise. This factor conceivably could operate here because the frogs at 8° C. are dormant and exhibit no movement unless disturbed. The active movements in the high temperature group produces metabolites which raise the osmotic pressure within the muscle. The increase in osmotic pressure draws water into the muscle cells from the surrounding lymph, thereby increasing the osmotic pressure of the lymph. This increased osmotic pressure of the lymph in turn causes an increased amount of water to pass at the capillaries from the blood to the lymph, thus reducing the per cent water of the blood.

The fact that some tissues imbibe more water at low temperature than at high has been reported by a number of workers. Jacobs (1928) states that it has long been known that "low temperatures tend to increase the swelling of erythrocytes." He suggests that this is probably due to a change in the osmotic pressure brought about by a shift in base within the cell between hemoglobin and other acids.

Blood pressure, another factor possibly affecting the water content of the blood in frogs, has been shown by Scott (1917) to be inversely proportional in man to the water content of the blood. Frogs placed at low temperature show a great decrease in heart rate (Barcroft and Izquierdo, 1931; Taylor, 1931). If this decrease in heart action reduces the blood pressure, it would thereby unbalance the blood pressure-colloid osmotic pressure relationship of the blood and cause a shift of water from the lymph to the blood.

The hydrostatic pressure of the blood in the capillaries determines by its relation to the osmotic pressure of those colloids for which the capillary wall is impenetrable, the direction and rate of exchange of water (isotonic salt solution) between the tissue spaces and the blood. When the capillary pressure is lower than the osmotic pressure any excess of fluid in the tissue spaces will be absorbed [into the blood.] (Krogh, 1929, p. 293)

The reduced muscular activity and the reduced blood pressure if active in the dormant frogs, would have parallel effects on water content. The water would be drawn from the tissues into the blood. This would produce an increase in the per cent water in the blood and a decrease in the muscle water. There would be no gain in the total amount of water in the frog.

It has been shown here (Tables V and VI and Fig. 1) that the muscle does not exhibit a decrease in water content correlated with an increase in the blood water. Also, a gain in weight in the whole frog when placed in cold water has been observed by Ott, 1924; Barthelemy, 1926; Adolph, 1927 a; and Holzapfel, 1937. Changes in muscular activity and in blood pressure then do not explain adequately the changes in water content described here. They may, however, play a part in the balance.

Adolph (1927 a) has observed that frogs gain 8% in weight when they are taken from an environment of $20°$ C. and placed in one of $1°$ C. This change was complete in twenty-four hours. Figure 1 shows the water exchange to come to an equilibrium in the blood and muscle in between fifty and sixty hours. As shown in figure 1, the changes in the per cent water are not absolutely parallel. The maximum is reached by the muscle at approximately seventeen hours and the water content is decreasing at twenty-eight hours, the time at which the maximum is reached by the blood. It would be supposed that the increase

in water content in the muscle is merely a reflection of the increase in the blood. If this is the case, the regulation of water content appears first in the muscle and is more effective there for although there is a slight tendency for the water content of the blood to return to its level at 26° C. it remains well above it while the muscle effects a complete return.

A number of investigators (van der Heyde, 1921; de Haan and Bakker, 1924; and Adolph, 1927 a) have shown that very little urine is excreted by frogs at low temperatures. Adolph has shown that after six to twenty-four hours at temperatures near zero, almost no urine is formed. This would preclude the possibility of the kidneys being the controlling structures. Adolph (1925, 1927 b, 1930) has presented evidence which he claims proves the skin to be the sole controlling factor in water balance. He has also claimed (1925) that the water migration is dependent upon the potential in the skin and that sodium is the chief factor controlling this potential and, therefore, water migration. Rubenstein (1935) has shown that when the inside of frog skin is bathed in Ringer solution and the outside is bathed in water, stimulation of the skin nerves decreases the passage of water from the outside to the Ringer solution on the inside. This would indicate that there is a nervous control of water passage through the skin.

Frog's skin is well supplied with blood vessels in order to carry on its respiratory functions. If, then, the full control of the water content of the frog were in the skin, any regulation in this content would first be shown in the blood rather than in the muscle.

It is of some interest to compare the above results with those reported on mammals. In the hibernating woodchuck (Rasmussen, 1916)

there is an increase of 5% in erythrocytes accompanied by a decrease in leucocytes, and an increase in hemoglobin and specific gravity. The blood picture does not become normal in the awakened animal until after it has eaten and drunk. This hemoconcentration is probably due to a loss of water by evaporation which cannot be replaced by the dormant animal. Barbour and his coworkers (Barbour and Tolstoi, 1921; and Barbour et al., 1924-25) have shown that there is an initial hemodilution of 2% when the body temperature of dogs is raised by placing them in a bath at 40° C. and an initial concentration of 2% when the body temperature is lowered by baths at 10° C. These changes are transitory, lasting only a few minutes, and the normal water content is soon regained although the abnormal temperature is maintained.

Blood Sugar. The direct variation in the frog's blood sugar content with variations in temperature was incidentally noted by Olmsted in 1924, while working on the relation of temperature effects of insulin on the blood sugar level of bull frogs. He inadvertently allowed one frog to reach a temperature of 34.5° C. He found the blood sugar in this individual to be 117 mgm./100 cc. as compared with 12 mgm./100 cc. in the frogs which had been maintained at room temperature. In frogs kept at 28° C. for two days, he found an average of 56 mgm./ 100 cc. This is very close to the value reported here for 26° C. At 8° C., however, the value for bull frogs is much higher than that reported by Olmsted at room temperature (presumably 20° C.). The great individual variation he found in the blood sugar at 28° C. (32-112 mgm./ 100 cc.) was due probably to the fact that he exposed the animals to this high temperature for only two days whereas a longer exposure might have resulted in more uniform values. Warren (1940) working with

R. pipiens, found that the blood sugar of fifteen frogs kept at room temperature varied from 36 mgm./100 cc. to 128 mgm./100 cc. He did not accurately control the temperature in his experiment and the high variations in the results render his figures useless for comparison. In the results reported here and shown in table VII, the variation in blood sugar at temperature controlled to within a range of 2° C. is only one-half that which he found. The variations which he attributes to individual variations are doubtless due in a large measure to variations in the temperature of the frogs at the time the determinations were made.

An annual cycle in the blood sugar level has been shown in the South American toad, Bufo arenarum, by Mazzocco (1938). He found the concentration to be maximum during summer. With the beginning of fall, the level began to decrease and reached a minimum during the winter and spring. The liver glycogen cycle was related to the sexual cycle since the glycogen content was lowest during the spring and rose slowly to a maximum in mid-winter. On the basis of the results reported here on frogs, the glucose cycle would appear to be controlled by temperature since the blood sugar level varied directly with the annual temperature changes.

Urea. The lack of any significant differences in the concentration of urea in the blood confirms the work of de Haan and Bakker (op. cit.) who found no significant difference at 0°, 10°, and 26° C. The temperature coefficient for metabolism is 2.5 (Hill, 1911) and for the formation of urine (Adolph, 1927 a) is 2-3. Since these are of the same order the urea production decreases at the same rate as elimination, thus keeping the urea concentration in the blood constant.

Cold blooded animals, in contrast to warm blooded animals, have virtually no control of their body temperature and hence must adjust themselves to relatively sudden daily changes in temperature and to cyclic seasonal changes. Hibernating mammals, normally homoiothermic, are known to lose temperature control and hence become essentially poikilothermic with the approach of hibernation and remain as cold blooded animals throughout this period. In autumn with its warm days and cold nights, chipmunks are known to hibernate at night and become active again during the day, showing a diurnal alternation between the homoiothermic and the poikilothermic state. These changes in temperature relations evidently are quite similar in both groups of animals.

Obviously, with temperature changes, temporary or cyclic, certain metabolic adjustments are necessary within the animal. The work reported here and that recorded in the literature to date, suggests that the chief modifications are changes in blood composition, activity of endocrine organs, changes in metabolic rate accompanied by changes in excretory and secretory activity of glands, and histological and cytological modifications.

Before an adequate explanation of the significance of temperature adjustment is found, more work on all of these factors is necessary.

SUMMARY AND CONCLUSIONS

Under the conditions of the experiment, that is the maintenance of frogs at two widely separate temperatures, 26° C. and 8° C., the following general conclusions are justified:

1. The blood cells, both erythrocytes and leucocytes, and the hemoglobin content of the blood decrease with a decrease in temperature.

2. The number of leucocytes in the blood of frogs varies more widely with the physiological conditions of the individual than do the erythrocytes. This variation is not correlated with the individual variation in the erythrocytes.

3. There is a lowering in the per cent hemoglobin in the blood when frogs previously conditioned to high temperature are subjected to a low temperature for one week. This lowering is caused by a corresponding drop in the erythrocyte count and not to any change in the hemoglobin content of the individual cells.

4. There is a four per cent increase in blood water of frogs previously conditioned to 26° C. and subjected to a temperature of 8° C. The lowerings in cell count and hemoglobin concentrations are solely the effects of this hemodilution.

5. Frogs placed at 8° C., after being conditioned one week at 26° C., show progressive changes in water content of the muscles and blood. These changes occur in the following order: first, a relatively rapid rise, an equally rapid fall, and, finally, a gradual rise to a constant level. The fall in water content of muscle begins before the initial rise in water content of blood has been completed. At final equilibrium which is reached in about sixty hours of exposure to 8° C., the water content of muscle is approximately the same as at 26° C.,

while the water content of blood is appreciably higher than that of blood at 26° C. These observations lead to the conclusions that there is a regulatory mechanism controlling the water content of muscle and that such a mechanism does not exist for the blood.

7. The glucose content of the blood is directly related to the temperature. The difference in glucose content of frogs maintained at 26° C. and of those at 8° C. is approximately 50%, a change too great to be accounted for by the extent of hemodilution observed in the low temperature frogs.

8. There is no significant difference in the urea concentration in frogs maintained at 8° C. and those maintained at 26° C. This is because the rate of production of urea and the rate of elimination of urea vary in the same direction at the same rate with temperature changes.

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