Physiol. Chem. &Physics 1 (1969)

STUDIES ON IONIC DISTRIBUTION IN LIVING CELLS
I. LONG-TERM PRESERVATION OF ISOLATED FROG MUSCLES

GILBERT N. LING and GRACE BOHR

Department of Molecular Biology, Division of Neurology, Pennsylvania Hospital, Philadelphia, Pennsylvania

(Received November 3, 1969)

SUMMARY

A method was described for the ***in vitro*** preservation of adult frog muscle tissues at 25°C for up to **8** days, as judged by the criteria of resting potential, the K^+ and Na^+ contents, and mechanical contractility.

Since the earliest days of biology, frog muscle has been a highly useful experimental material. Yet this muscle and many other types of isolated tissues cannot usually last for experimental purposes for more than a few hours at room temperature. While this length of time is sufficient for kinetic and other short-term experiments, there are experiments which require a longer period of observation. The steady level of K^+, for example, is not reached until after seventy-two hours.[1,2] Cooling prolongs the survival time; it also correspondingly slows down the time to reach a new equilibrium. A method for the long-tern~ preservation of isolated frog muscle in an artificial medium beyond 72 hours, aside from meeting this particular need for longer-term experiments, also opens the way toward future studies on the long-lasting effects of hormones and drugs on mature, normal cells in a controllable manner.

This paper reports the successful development of such a method for the preservation of isolated frog muscle at 25°C for at least 8 days.

MATERIALS AND METHODS

I. Materials

All the experiments under consideration here were performed on the sartorius muscles of leopard frogs ***(Rana pipiens,*** Schreber), which were force-fed hamburger meat every **3** or 4 days in an aquarium provided with running water maintained at room temperature the year round.

All chemicals used were of C.P. grade. Sodium penicillin G and streptomycin sulfate U.S.P. were obtained from E. R. Squibb and Sons, New York: GIB medium, a chemically defined medium, hitherto unreported, is given in Appendix A.

II. Compositions and Preparations of Incubation Media

Solutions A, B, C, and D (Table 1) were prepared from the following stock solutions: NaCl (5 x 0.118 M), KCl (0.118 M), $NaHCO_3$ (5 x 0.118 M), NaH_2PO_4 (0.118 M), Na_2HPO_4 (0.118 M), $CaCl_2$ (0.0845 M), $MgCl_2$ (0.118 M), and glucose (5 x 0.236 M). They were sterilized by autoclaving at 20 lbs/sq in: A, B, and C for 30 minutes, and D for 15 minutes to prevent decomposition. After cooling to room temperature, A and B were bubbled with 5% CO_2-95% oxygen mixture until equilibrium was reached (about 20 minutes). Solution E, not further sterilized. was obtained by mixing potassium-free GIB medium, and sterile distilled water with penicillin (Na) and streptomycin sulfate. In most of the experiments reported in this paper, the GIB medium also contained 10% fetal calf serum. This serum-GIB medium contained 0.15 to 0.24 mM K^+ ion. To make Solution I, 44 ml of C and 94.8 ml of D were added to 2233 ml of A. For Solution 11, 23 ml of C and 42.4 ml of D were added to 113.6 ml of B. (The solutions should remain perfectly clear.) Finally, 111 ml of E were added to each liter of Solutions I and II. The final mixtures I (A+C+D+E) and II (B+C+D+E) contained, respectively, 0.2 (or less when no calf serum was included) and 10.0 mM of K^+ ion. The total Na^+-ion concentration in both mixtures was 100 mM. Experimental solutions containing 2.5 mM K^+-ion concentrations were obtained by mixing Solutions I and II.

III. Sterile Tissue Dissection and Incubation

Sartorius muscles of leopard frogs were isolated under sterile conditions. After dissection, the muscles were routinely examined for possible damage: those showing signs of injury were discarded.

As a rule, 4 or 5 sartorius muscles were incubated in a 250 ml Erlenmeyer flask with a screw cap (Bellco, Vineland, N. J.) containing 110 ml of incubating medium. After the introduction of the muscle CO_2-O_2 mixture (5%:95%) was allowed to flow over the surface of the medium for a few seconds to flush out air in the flask; then the screw cap was tightly closed. The caps and necks of the flasks were wrapped with Parafilm to insure air tightness for many days. The phenol red in the GIB medium served to monitor the pH. A salmon-pink color indicated a proper pH of about 7.2. The flasks were shaken either in an Aminco constant-temperature bath maintained at 25°C ± 0.05°C at a rate of 100 excursions per minute, each full excursion measuring 1 inch. or in a constant-temperature room maintained at 25°C ±1.0°C.

Table 1. Composition of stock solutions used in making up incubation solutions. Solutions A, B, C, D, and E are combined as described in the text.

Solution	A	B	Solution	C
NaCl	296.8	148.4	$CaCl_2$	40
KCl	0	100		
$NHCO_3$	69.2	34.6	$MgCl_2$	48
NaH_2PO_4	40	20		
Na_2HPO_4	24	12		
H_2O	1803	821.6		
Total Volume (ml)	2233	1136.6		88

Solution	D	Solution	E
Glucose	**120**	**GIB medium (K-free)**	**383.2 ml**
		H_2O (sterile)	**67.6 ml**
		Penicillin	**500 mg**
		Streptomycin	**500 mg**
Total Volume (ml)	**120**		**450.8**

IV. Methods of Resting Potential and Contractility Measurements

The resting potentials of single muscle fibers were measured according to the method described by Ling and Gerard.[3] Saturated KCl-filled microelectrodes were used. Isometric and isotonic contractions were recorded on smoked drum in the conventional manner. Electrical shocks from a Grass stimulator were applied through a pair of platinum wire gauze sheets separated by a gap 1 cm wide in which the sartorius muscle was suspended in a normal Ringer phosphate solution (ref. 4, p. 566).

V. Methods of Ion Extraction and Analysis

Two new methods were used for the extraction of alkali-metal ions from muscle tissues. Both of these methods satisfy the requirement for assaying a large number of samples accurately, simply, and economically.

1. Cold HCI extraction: a muscle sample weighing around 100 mg was soaked in 3 ml of 0.1 N HCI overnight (4°C).
2. Hot HCI extraction: a muscle sample was projected into 3 ml of 0.1 HCI in a 15 ml polypropylene centrifuge tube (Nalgene), which was heated in a boiling water bath for 20 minutes. (Shaking, which may homogenize the tissue, was avoided).

One ml aliquot of the cold or hot HCI extract was diluted with 3 ml of 1/8 M NaCl for K^+-ion assay; another 1 ml aliquot was diluted with 3 ml of 1/8 M KH_2PO_4 for Na^+-ion assay. K^+-ion and Na^+-ion standards all contain similar "radiation buffers." The K^+-ion and Na^+-ion contents were assayed on a Beckman DU spectrophotometer with a flame attachment. By substituting the blue-sensitive photomultiplier tube with an R 136 wide-range multiplier tube (Hamamastu TV Co., Ltd., c/o Kinsho-Mataichi Corp., 80 Pine St., New York, N. Y.), the K^+-ion sensitivity was increased.

Table 2 shows a comparison of the cold and hot HCI methods with the methods of dry-ashing in platinum crucibles in a muffled furnace at 550°C (Ernst and Barsits, 1929; Fenn and Cobb, 1934) as well as with a fourth method (Extraction with 0.05 M LiOH). There are no significant differences between the K^+-ion and Na^+-ion concentration assayed with

Table 2.

Experiment Number		No. of Samples	$[K^+]_{tis}$ (μmoles/g)	P	$[Na^+]_{tis}$ (μmoles/g)	P
1	Ashing	4	83.5 ± 4.5	>0.6	26.0 ± 2.0	>0.9
	cold 0.1 N HCI extraction	4	80.7 ± 5.9		25.5 ± 2.0	
2	cold 0.1 N HCI extraction	5	90.6 ± 2.1	>0.05	23.6 ± 1.8	>0.1
	0.05 N LiOH	5	83.5 ± 1.1		26.3 ± 0.9	
3	Ashing	8	80.2 ± 0.9	>0.1	26.3 ± 1.9	>0.7
	cold 0.1 N HCI extraction	8	78.7 ± 0.8		23.1 ± 1.9	
4	Hot HCI	8	82.1 ± 3.1	>0.05	33.5 ± 2.6	>0.5
	Cold HCI	8	79.1 ± 3.3		32.1 ± 1.4	

cold HCI or with the hot HCI extraction method, in comparison with dry-ashing. A difference between the cold HCI and LiOH extraction for K^+-ion is not significant at the $p = 0.01$ level but is significant at the $p = 0.05$ level.

In most experiments performed before 1967, the cold HCI extraction method was used; later experiments utilized the hot HCI extraction.

RESULTS

I. Resting Potential

We incubated sartorius muscles for up to 10 days at 25°C in a medium containing a normal K^+-ion (2.5 mM) and a normal Na^+-ion concentration (100 mM). On each day the resting potential of 4 muscles from one sample flask was measured in a bath containing the same medium in which it was incubated. At least 16 individual fibers were studied on each muscle. The data are shown in Figure 1. The overall average and standard error for the 10 sets of resting potential measurements is 87.4 ± 1 mv, in comparison with 88.0 ± 3.3 from 16 measurements of freshly isolated muscles presented here and 88 ± 1 mv previously reported by Nastuk and Hodgkin.[5] In other series of experiments, we found the average resting potentials to become lower and the individual values more scattered beyond the 10th day. The muscles remained fully excitable before the 10th day (see below).

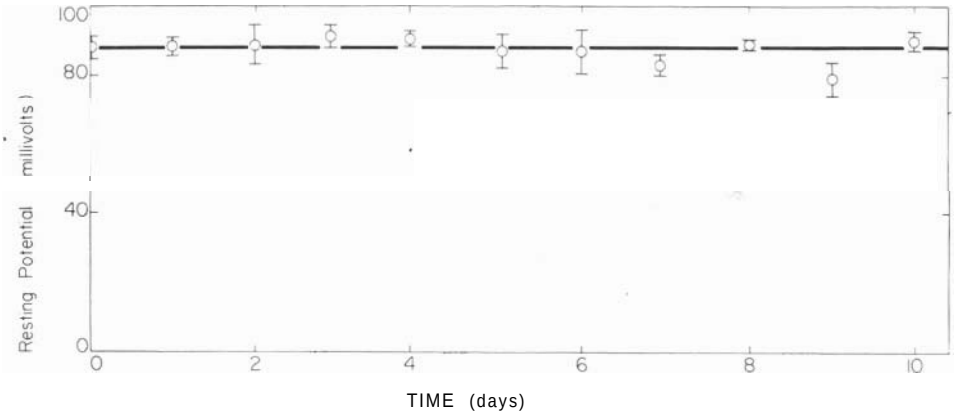


Figure 1. The resting potential of isolated frog sartorius muscles preserved *in vitro* at 25°C. Each point represents the mean of at least 16 measurements on 16 different muscle fibers in 4 different muscles.

II. The K^+-ion and Na^+-ion Content

On each day 4 muscles were analyzed for K^+ and Na^+ contents. The mean and standard errors are presented in Figure 2 in terms of micromoles per gram of fresh tissue. No significant deviations were observed until the 8th day. The overall average for muscles kept from 1 to 8 days for the K^+-ion is 86.4 ± 1.6 micromoles/g fresh tissue; for Na^+ it is 32.4 ± 2.1 μmoles/g. These figures compare with 78.5 ± 4.4 μmoles/g for K^+ and 32.4 ± 2.1 μmoles/g for Na^+-ion in freshly isolated muscles.

The average figure for the K^+-ion content from selected data in the literature according to certain statistical criteria for K^+ was 85.8 μmoles/g.[4] The Na^+-ion content of fresh as well as preserved muscles here reported is somewhat higher than the literature values. A possible explanation is the fact that we routinely used fed frogs in our experiments, while some data from other investigators might have been obtained from starved ones. Another possible cause is the greater effectiveness and reproducibility (in our hands, at least) of the hot HCl extraction method used.

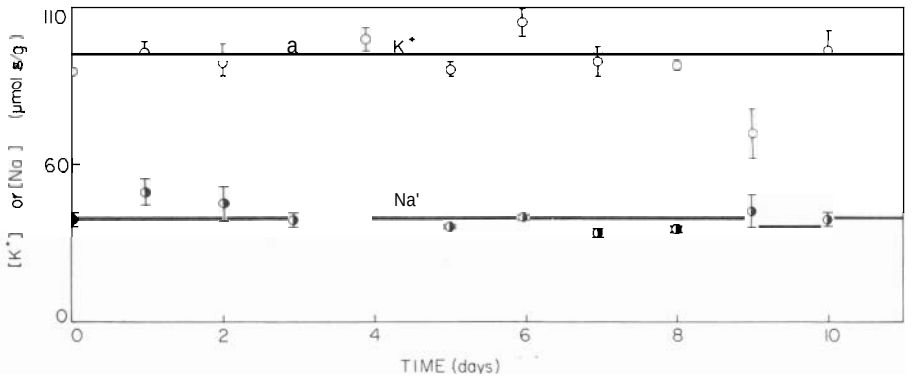
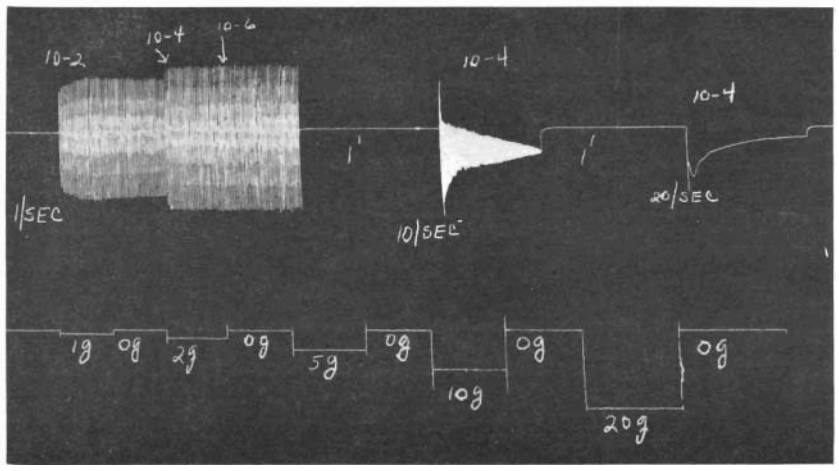


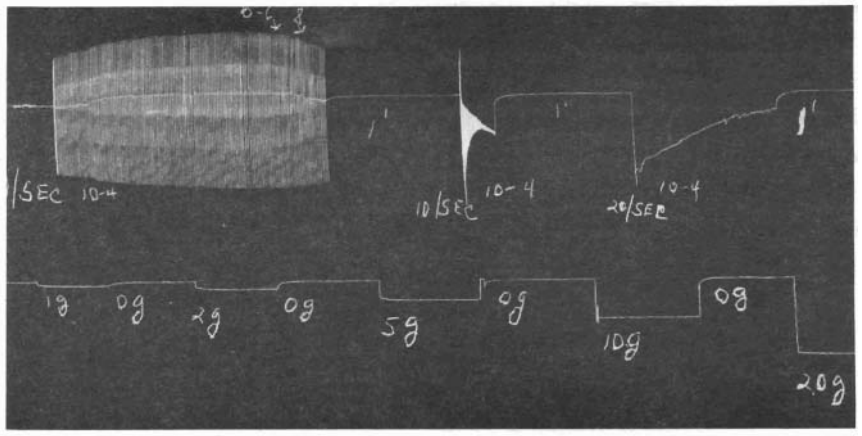
Figure 2. The K^+-ion and Na^+-ion contents of isolated frog sartorius muscle preserved *in vitro* at 25°C. Isolated sartorius muscles were preserved in a medium containing 100 mM Na^+ and 2.5 mM K^+-ion. Each point represents the mean K^+-ion or Na^+-ion concentrations in μmoles per gram of fresh muscle tissue. In this and following figures the distances between the horizontal bars represents twice the standard error of the mean.

III. Contractility

We recorded both isotonic and isometric contractions of preserved muscles. However, only the tracing of isotonic contractions after 8 days of preservation is shown (Fig. 3A), in comparison with a freshly isolated sartorius muscle treated in an identical manner (Fig. 3B). The record shows 3 groups of contractions in each, with the stimulation frequencies respectively at 1/sec, 10/sec, and 20/sec. 10-2, 10-4, and 10-6 refer to increasing voltage of the stimulus pulses. The lower tracing in each chart shows the calibration of the isometric level in grams of loads. From these and similar graphs we observe no significant differences in the work performances of the normal muscle and that of muscle after 8 days of preservation. In isotonic measurement, the preserved muscle as a rule gave a somewhat stronger contraction to a specified stimulus and lever ratio.



A



B

Figure 3. The isometric contractility of sartorius muscles after 8 days of preservation **(A)** and of a freshly isolated control muscle.
1/sec, 10/sec, and 20/sec refer to frequency of stimulation. 10-2, 10-4, and 10-6 refer to 20, 40, and 60 volts of the stimulus strength read off the stimulator; actual voltage across the muscles was much less due to the high resistance of the Ringer solution bathing the muscles.

DISCUSSION

Judging by the criteria of resting potential, K^+- and Na^+-ion content, and mechanical contractility, the method of in ***vitro*** preservation described is capable of preserving isolated frog muscle in a physiological state close to normal for at least 8 days.

An important factor in this preservation is the use of the antibiotics, for frogs obtained from the dealers are frequently infected. As an answer to this problem, Gibbs et al. have suggested treatment of frogs with tetracycline: and by soaking frogs for a few hours in a tetracycline solution, we did significantly reduce the number of contaminated flasks. We discontinued this procedure, however, on finding a significant pharmacological response to tetracycline—the treated frogs no longer responded to insulin in the same manner as untreated frogs.'

Occasionally frogs are obtained infected with fungus. Inclusion of nystatin checks fungal growth. In most cases, however, muscles are free of fungus and can do well without the use of fungicide.

Fetal calf serum represents the chemically undefined part of the medium. It can be deleted in experiments where the medium is close to that of normal Ringer solution. Indeed, we have successfully preserved muscles in a fairly good condition in simple sterile Ringer-bicarbonate solution with the antibiotics for 4 days. Earlier, Lubin and Schneider reported preservation in Ringer solution containing tetracycline of frog muscles for 3 days at 25°C.[8] The fetal calf serum and other vitamins and amino acids in the GIB medium, however, are important aids in prolonged preservation under the more stressful conditions entailed by most of the experiments to be described in succeeding papers of this series.

As it stands, the method cannot preserve muscle much beyond 8 days, although at times we were able to keep muscle for a few days longer. So far, we have not attempted to improve the preservation method further, as 8 days is long enough for the experiments for which it was designed.

ACKNOWLEDGMENT

This investigation was supported by the National Institute of Health Research Grant 2 RO1-GM11422-05 and -06, and to a lesser extent by a grant from the John A. Hartford Foundation, Inc., New York. The senior investigator was also supported by Public Health Research Career Development Award K3-GM-19,032.

APPENDIX A

COMPOSITION OF GIB MEDIUM

Components	Grams/Liter	Components	Grams/Liter
NaCl	6.8000	L-threonine	.050
$Na_2HPO_4 \cdot 7H_2O$	2.642	L-tryptophan	.010
$Ca(NO_3)_2 \cdot 4H_2O$	.030	L-valine	.050
$MgSO_4 \cdot 7H_2O$	.200	L-glutamine	.300
Oxalacetic acid	.150	L-cystine	.025
Glucose	1.200	L-tyrosine	.035
β-alanine	.030	Ascorbic acid	.052
L-arginine	.100	P-aminobenzoic acid	.00035
L-asparagine	.035	Choline	.003
L-cysteine	.080	Cyanocobalamine	.00005
L-aspartic acid	.035	i-inositol	.005
L-glutamic acid	.080	Pyridoxal HCI	.00055
L-glutathione (reduced)	.012	Niacin	.000125
Glycine	.015	Nicotinamide	.0025
L-histidine	.035	Ca Pantothenate	.0025
L-isoleucine	.050	Pyrodoxine HCI	.002
L-leucine	.050	Riboflavin	.00055
L-lysine	.060	Thiamine HCI	.005
L-methionine	.012	Folic Acid	.002
L-phenylalanine	.035	Biotin	.00015
L-serine	.020	Phenol Red	.010

REFERENCES

1. G. N. Ling and M. M. Ochsenfeld, **J. *Gen. Physiol. 49,*** 843 (1966).
2. G. N. Ling and G. Bohr, Studies on Ion Distribution in Living Cells. 11. Cooperative Interaction Between Intracellular K^+ and Na^+ Ions (in press).
3. G. N. Ling and R. W. Gerard, **J. *Cell. Coinp. Physiol. 34.*** 383 (1949).
4. G. N. Ling, ***A Physical Theory of the Living State: Association-Induction Hypothesis***, Blaisdell. New York, 1962.
5. W. L. Nastuk and A. L. Hodgkin, **J. *Cell. Coinp.*** *Physiol. 35*, 39 (1949).
6. E. L. Gibbs, T. J. Gibbs, and P. C. Van Dyck, ***Lab. Animal Care 16,*** 142 (1966).
7. G. N. Ling, M. C. Neville, P. Shannon, and S. Will, ***Physiol. Chem. Phys. 1***, 42 (1969).
8. M. Lubin and P. G. Schneider, ***J. Physiol.*** 138. 140 (1957).