In vitro Techniques for Avoiding Edge Damage in Studies of Frog Skin

Abstract. *Frog skins mounted between chambers without compression, with a liquid insulator and a tissue adhesive, were studied in vitro. Since voltage and electrical resistance per unit area were unchanged by a tenfold decrease in area of skin studied, it is concluded that these mounting techniques do not produce edge damage and consequently provide a means for study of the skin in its absence.*

In conventional in vitro techniques used to study the transport characteristics of frog skin, the tissue is compressed between fluid-filled chambers in order to achieve mechanical and electrical isolation of the solutions bathing the inner and outer surfaces of the skin. It is implicitly assumed that compression of the skin has no significant effect on measurements of the transport properties of the skin. However, Dobson and Kidder showed by systematically varying the ratio of perimeter length to surface area of their chambers that, indeed, "edge damage" produced by skin compression leads to a lowering of both measured potential difference and resistance (*1*). Their findings support the view that edge damage produces a low resistance shunt in parallel with the more centrally located undamaged tissue. Consequently, edge damage leads to underestimation of both potential difference and resistance, especially in chambers of small cross-sectional area where the edge-to-surface ratio is relatively large. More recently, Walser reported the existence of edge damage in toad urinary bladder mounted for in vitro study (*2*).

In the present studies, techniques were developed for mounting frog skin without compression between chambers of large (6.4 cm^2) and small (0.64 cm^2) cross-sectional area. Since the measured potential difference and resistance were found to be independent of the edge-to-surface area ratio of skin studied, it is concluded that the techniques described below provide an in vitro means for study of the skin that does not produce edge damage.

Two techniques were devised for mounting abdominal skin of *Rana pipiens* (*3*) without compression between Lucite half chambers. In the first technique, skins were mounted between gaskets made of cured Sylgard 184 (*4*). The skin did not rest directly on the gaskets, but was separated from them by thin films of liquid Sylgard 184. Although this technique proved adequate for the small chambers, the liquid Sylgard films did not remain stable in the large chambers. Consequently, a second technique was developed which gave results identical to those obtained when Sylgard 184 was used in the small chambers, and in addition proved satisfactory for use in the large chambers.

In the second technique, the tissue adhesive isobutyl-2-cyanoacrylate (IBC-2, Ethicon, Inc., Somerville, N.J.) was used to glue the skin to Lucite gaskets. The gaskets were sealed in recesses in the faces of the Lucite chambers with liquid Sylgard 184 (Fig. 1). After the IBC-2 made contact with the skin, the adhesive polymerized rapidly to form a uniform, continuous plastic bond between the skin and the gaskets. Since the results obtained in small chambers with IBC-2 seals were identical to those with liquid Sylgard 184, and since no changes in either electrical resistance or open-circuit voltage were observed when IBC-2 was added in large amounts directly to the solutions bathing the skin, it is concluded that these sub-

Table 1. Summary of electrical parameters of frog skin, determined between 23 January and 4 March 1970 from the voltage-current relationships of 19 abdominal skins of *Rana pipiens*. The mean ratio of R_1 to R_2 was 1.50, and of R_2 to R_3 was 1.55. The symbols are defined in Fig. 2.

	Parameter						
	V_{oc} (mv)	I_{sc} (μa/cm^2)	R_1 (ohm·cm^2)	R_2 (ohm·cm^2)	R_3 (ohm·cm^2)	E_1 (mv)	E_2 (mv)
Mean	77.6	25.5	6583	4430	3047	127.7	43.0
S.E.	± 4.9	± 2.0	± 760	± 507	± 384	± 5.1	± 4.7
Range	(43.6–128.0)	(11.0–44.7)	(3149–15,300)	(1926–7376)	(1433–5907)	(91.0–164.3)	(10.5–79.1)

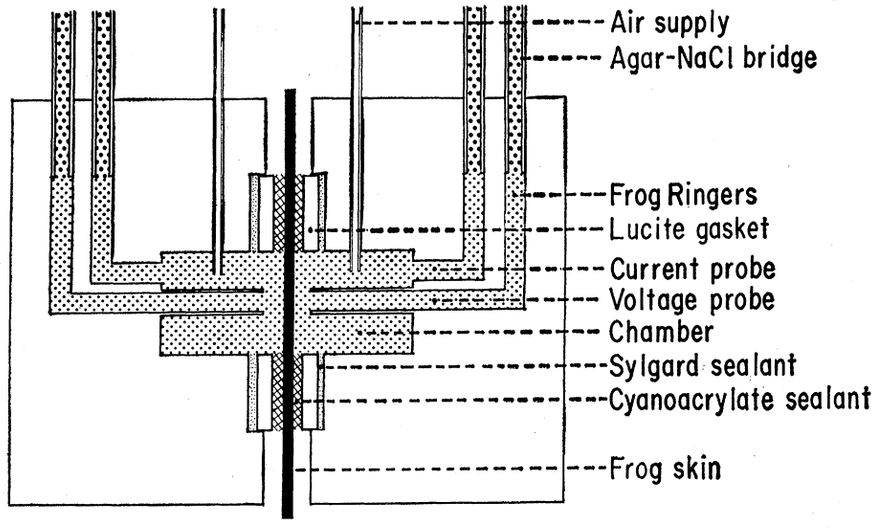

Fig. 1. Arrangement for mounting skin without compression, with the use of the tissue adhesive isobutyl-2-cyanoacrylate, circular Lucite gaskets, and Sylgard 184. Aeration and mixing of the bathing solutions was done by bubbling 100 percent oxygen through 32-gauge tubing. Polyethylene bridges filled with 3*M* NaCl-agar connected the bathing solutions to the external electronics via AgCl electrodes. The tips of the voltage probes were permanently fixed 0.5 mm from the surface of the skin. When pulses of current were passed through the chambers without a skin in place, the resistance of the Ringer solutions between the voltage recording electrodes was found to be insignificant in comparison with skin resistance (< 1 percent). Consequently, no correction was made for the series resistance of the solution in calculating skin resistance.

stances exerted no pharmacologic effect on the electrical parameters of the skin.

In order to determine the electrical resistance of the skin, constant-current pulses several hundred milliseconds in duration were passed through skins bathed on both sides with the same Ringer solution (*5*), and the resulting voltage deflections were recorded with a Tektronix 564B storage oscilloscope. With small current pulses (ΔI) which produced voltage deflections (ΔV) less than 20 mv, both hyperpolarizing and depolarizing current pulses produced identical voltage deflections, indicating that skin resistance ($\Delta V/\Delta I$) was linear in the immediate vicinity of open-circuit voltage (V_{oc}). However, when larger currents were used, the voltage response to hyperpolarizing current was greater than that observed for depolarizing current of the same magnitude. This latter observation, reported by Finkelstein in 1964 (*6*), showed that the resistance of the skin was not linear for all voltages. More recent studies by Candia (*7*) and Pierpont and Dennis (*8*) also show that the frog skin exhibits rectifying properties.

In order to characterize more completely the relationship between steady-state voltage (V) and current (I), I-V plots were made as follows. When the open-circuit voltage had been stable for at least 30 minutes, a voltage clamp was used to deliver a series of hyperpolarizing and depolarizing step voltage pulses in 10- or 20-mv increments, and the resulting current flow was recorded as before with the oscilloscope. The circuitry of the voltage clamp was arranged to deliver a pulse 600 msec in duration which began and ended at open-circuit voltage. After an initial current transient, the current responses reached a plateau and the current at 600 msec was taken to be the steady-state current. When the skin was voltage clamped with the inside above +200 mv or below −50 mv (voltage measured with respect to the outside), stable currents were not observed in most skins. Thus, we have limited the present studies to the voltage range where steady-state current values were observed.

A typical I-V plot is shown in Fig. 2. In the vicinity of open-circuit voltage, slope resistance is linear, a finding which supports the observation, noted above, that skin resistance measured with small constant-current pulses is linear. It was also apparent on inspection of the I-V plots that skin resistance was not linear over the entire voltage range investigated. The data points characteristically fell into what appear to be three regions of differing slope resistance, labeled R_1, R_2, and R_3 in Fig. 2. After the data points (usually three to nine) were assigned to their respective regions, the slope resistances were calculated by the method of least squares, and the voltages E_1 and E_2 were found by solving for the points of intersection of the slope resistances. In this way it was possible to characterize the "resistance" of the skin and, in addition, to provide a means of comparing the electrical parameters of a skin mounted first in a large and then a small chamber. A summary of the electrical parameters determined in 19 frog skins is given in Table 1.

Table 2. Ratios, ± standard errors, of values of electrical (I-V) parameters determined in small chambers to values of the same respective parameters determined in large chambers. $N = 11$. A ratio of unity would indicate no difference. The symbols are defined in Fig. 2. Since ratios are presented, the values are dimensionless quantities.

V_{oc}	I_{sc}	R_1	R_2	R_3	E_1	E_2
1.04	0.95	1.01	1.06	1.06	1.01	0.99
± .04	± .04	± .06	± .02	± .04	± .06	± .08

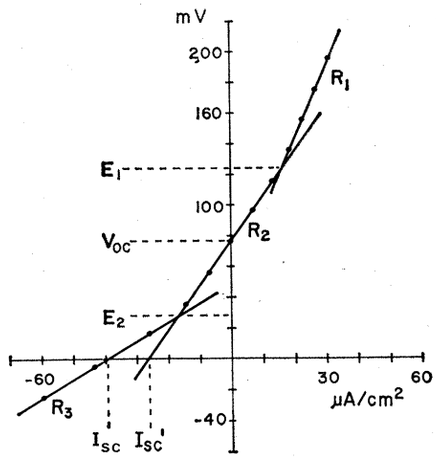

Fig. 2. The I-V relationship of a frog skin determined with the use of a voltage clamp. Slope resistances R_1, R_2, and R_3 for this particular skin were calculated to be 4903, 2913, and 1190 ohm · cm², respectively. The open-circuit voltage, V_{oc}, is 76 mv; E_1 and E_2 (123.5 and 27.0 mv) are the calculated voltages at which the regression lines R_1, R_2, and R_3 intersect. Short-circuit current ($I_{sc} = 39.7$ μa/cm²) is the current which flows in the external circuit when transepithelial voltage is clamped at zero. Extrapolation to the abscissa of the slope resistance R_2 obtained with small current pulses gives an "apparent short-circuit current," $I_{sc'}$. Since slope resistance is not linear between open-circuit voltage and short-circuit conditions, $I_{sc'}$ underestimates I_{sc}. In 19 studies, $I_{sc'}$ was found to be 79.7 ± 3.2 percent of I_{sc}. This finding confirms the discrepancy first noted by Linderholm in 1952 that the error in d-c resistance values is of the order of 10 to 20 percent, owing to the nonlinearity between current and voltage in the region of 0 to 50 mv (*10*).

By varying the ratio of edge-to-surface area (E/S) of the exposed skin, Dobson and Kidder (*1*) showed that both open-circuit voltage and resistance fell as the E/S ratio increased, a finding which we have confirmed (*9*). This finding supports the view that the damage at the edge creates a parallel path through which current can be shunted around the undamaged skin. Thus, electrical measurements made in the presence of edge damage would not be properties of the undamaged skin alone. In small chambers, this effect would be more pronounced than in large chambers, owing to the greater contribution of the resistance of damaged tissue to the measured resistance. In the absence of edge damage, both open-circuit voltage and resistance per unit area should be independent of skin area studied (or the E/S ratio). We tested for the presence of edge damage in 11 skins mounted with IBC-2 by comparing open-circuit voltage and resistance of the *same* skin, determined first in 6.4-cm² chambers ($E/S = 1.4$), and then in 0.64-cm² chambers ($E/S = 4.5$). After open-circuit voltage had reached a stable value in the large chambers, the I-V relationship was determined. Then the skin with the large gaskets still attached was remounted on small gaskets in chambers having one-tenth the area of the large chambers. After an additional incubation period of 30 to 90 minutes, the I-V relationship of the skin was determined again and compared with the previous I-V relationship. The results of these studies show that the open-circuit voltage and slope resistances R_1, R_2, and R_3 are indepen-

dent of the E/S ratio (Table 2). Thus it is concluded that by avoiding compression of the skin by using the techniques described, it is possible to study the properties of the skin in vitro without edge damage.

It was of interest to determine the degree to which edge damage could alter the measured electrical parameters. This was done in small chambers by determining the electrical parameters before and after compression of the same skin between the chambers. In 11 skins V_{oc} fell to 34 percent of the control value. Resistances R_1, R_2, and R_3 fell to 18, 27, and 39 percent of control values, respectively, and the I-V plots appeared to be essentially linear and showed little, if any, rectification. This observation would be consistent with the view that in the presence of edge damage to the degree produced in these studies, the measured electrical resistance would be a measure primarily of the damaged edge.

The effect of edge damage on tracer urea-^{14}C flux was also determined in seven studies and found to increase 763 percent. These data taken together show that edge damage in frog skin can be quantitatively significant and should be taken into account in the interpretation of data obtained from skins conventionally mounted with compression.

SANDY I. HELMAN
DAVID A. MILLER

Department of Physiology,
Tulane University School of Medicine,
New Orleans, Louisiana 70112

References and Notes

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3. The electrical studies reported here were done with frogs (*Rana pipiens*) obtained from Lemberger Corp., Oshkosh, Wis., during the months of August 1969 through March 1970. Since completion of these studies, frogs from Vermont (J. M. Hazen Co., Alburg, Vt.) have also been used and identical results obtained.
4. Sylgard 184 (Dow Corning, Midland, Mich.), a silicone resin with added hardener, was allowed to cure in plastic petri dishes to form flat sheets 1 mm thick, from which circular gaskets with a 5-mm difference between outside and inside diameters were cut. These fit into recesses 0.5 mm deep in the faces of the chambers.
5. Bathing solution: 100 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 0.625 mM NaH_2PO_4, 2.5 mM Na_2HPO_4, pH adjusted to 7.4 with dilute NaOH.
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Polymorphism of Soluble Glutamic-Pyruvic Transaminase: A New Genetic Marker in Man

Abstract. *Soluble glutamic-pyruvic transaminase (GPT) has three common phenotypes, each representing the homozygous and heterozygous expression of two alleles,* Gpt^1 *and* Gpt^2 *at an autosomal locus. The frequencies of these alleles vary considerably from one population to another.*

Glutamic-pyruvic transaminase (GPT), also known as alanine aminotransferase (E.C. 2.6.1.2), catalyzes the reversible conversion of L-alanine and α-ketoglutarate to L-glutamate and pyruvate. GPT resembles glutamic-oxaloacetic transaminase (GOT), malate dehydrogenase (MDH) and isocitrate dehydrogenase (ICD) in having two distinct molecular forms: one cytoplasmic (soluble) and the other mitochondrial (*1*). Genetic variation of both forms of GOT and MDH have been described in man (*2*), but there are no previous reports of such variation in either form of GPT.

Soluble GPT is particularly abundant in liver and heart (*3*); red cells also have considerable activity (*4*). The enzyme has been purified from rat liver and pig heart. The molecule, which has a mass of about 100,000 daltons, appears to consist of two subunits (*5*). Various factors, including nutritional state, pregnancy, corticoid activity, exercise, and drug administration are associated with marked changes in hepatic GPT concentration (*6*). The serum level of GPT is a very useful indicator of liver cell damage (*7*).

To demonstrate the isozymes of soluble GPT, we prepared red cell hemolyzates (*8*) and subjected them to vertical starch-gel electrophoresis at 8 volt/cm for 18 hours at 4°C in 0.1M tris-citrate buffer, pH 7.5. The GPT stain we developed was based on the chemical reactions in the spectrophotometric assay, which measures the oxidation of reduced nicotinamide adenine dinucleotide ($NADH_2$) accompanying the conversion of pyruvate to lactate by lactate dehydrogenase (*9*). Under ultraviolet light, GPT activity is detected in the gel as bands of defluorescence, representing the sites of $NADH_2$ oxidation to NAD (*10*).

Three different electrophoretic patterns were observed; they were designated GPT 1, GPT 2-1, and GPT 2 (Fig. 1). The single bands of GPT 1 and GPT 2 migrated anodally about 6 cm and 8 cm, respectively. The GPT 2-1 pattern consisted of three bands; two with the mobilities of GPT 1 and GPT 2, and a third with intermediate mobility. The GPT 2 band usually stained less intensely than the GPT 1 band.

Family studies (Fig. 2) demonstrated that the electrophoretic patterns represent the phenotypic expression of two autosomal allelic genes, Gpt^1 and Gpt^2 at the structural gene locus of GPT. The homozygous phenotypes are GPT 1 and GPT 2; the heterozygous type is GPT 2-1.

The triple band pattern of the heterozygote suggests that GPT has a dimeric structure, in agreement with previous physicochemical measurements (*5*). Thus GPT 1 and GPT 2 are dimers, each composed of two identical subunits. In the heterozygote, the slow and fast bands represent these two dimers, while the intermediate band is a hybrid dimer containing both kinds of subunits. Preliminary linkage data obtained from family studies show that the GPT locus is not closely linked with the loci of the ABO, MNS's or Rh blood groups, the haptoglobin serum group or the enzymes acid phosphatase and phosphoglucomutase-1. On the other hand, in two small families

Table 1. The GPT phenotypes and the Gpt^1 gene frequencies of blood donors from three different ethnic groups.

Ethnic groups	Number tested	GPT types			Gpt^1 frequency
		1	2-1	2	
Caucasian	253	59	133	61	0.496
Afro-American	220	146	66	8	0.814
Oriental-American	215	71	115	29	0.598