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BIOSENSING TECHNICS FOR HUMAN DETECTION

I. THE FROG SKIN TRANSDUCER: PRELIMINARY EXPERIMENT

Interim Report

By

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With the technical assistance of

Paul C. Montgomery

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U. S. ARMY LIMITED WAR LABORATORY

Aberdeen Proving Ground, Maryland 21005

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ABSTRACT

Isolated frog skin used as a transducer whose bioelectrical potential is measured as a function of chemical species and concentration, is shown to offer large, reproducible, reversible changes to a variety of organic substances of low molecular weight, over a concentration range of at least five log units. A high degree of variability of response between frog skins, and a lack of data on ultimate sensitivities at usefully low levels for selected substances, are major problems that remain to be examined.

FOREWORD

TABLE OF CONTENTS

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

I. INTRODUCTION

An earlier evaluation of possible technics using living material as sensors suggested a number of approaches offering promise, including the use of isolated living membranes such as skin or gut.[1] In such preparations exposure to certain substances would be expected to alter the electrical potential across the membrane, because of changes induced in metabolic activities and transmembrane transport of materials. This brief paper describes preliminary experiments designed to examine the utility of frog skin as a chemical input – electrical output transducer, through the screening of responsiveness to a selected group of compounds.

Frog skin has offered an excellent tool for studies in which bioelectric measurements have led to insights and generalizations as to selective transport processes in various membranes, specific ion transport, metabolic relationships, and enzyme control, including the role of cholinesterase. This work was pioneered by Ussing and associates in 1951.[2] Current speculation about the actions of substances to modify the electrical properties of frog skin includes consideration of such possible mechanisms as potential shunts in the skin, lowered resistance to ion transport, enzyme rerouting, use of energy stores otherwise earmarked, upgrading of substrates to high energy forms, etc.

II. METHOD AND APPARATUS

Small green frogs, Rana pipiens, were obtained from a Vermont supplier and stored at $5^{\circ}C$ to $10^{\circ}C$. For an experiment, a frog was decapitated and the spinal cord was destroyed. Belly skin was gently and rapidly removed, rinsed in frog Ringer solution and immediately mounted in a test cell. The Ringer solution pH was routinely adjusted to 7.0 with HCl in all the experiments.

Test cells were designed to allow mounting the skin as a membrane barrier between two liquid chambers. Two cell configurations are illustrated in Figure 1. The cell used in early experiments (A in Figure 1) consisted of 2 polyethylene chambers, each with a liquid volume of about 35cc, separated by the frog skin. The area of skin exposed to liquid in the two chambers was about $2.5 cm^2$. In later experiments, a single-chambered cell, fabricated from a 10cc polystyrene pill vial was used (B in Figure 1). The center was cut out of the separate polyethylene snap-top cap, which then was used to clamp a piece of skin over the open end of the vial. About $1 cm^2$ of skin was thus left exposed. The inside of the vial was filled with frog Ringer solution. One electrode was brought through the
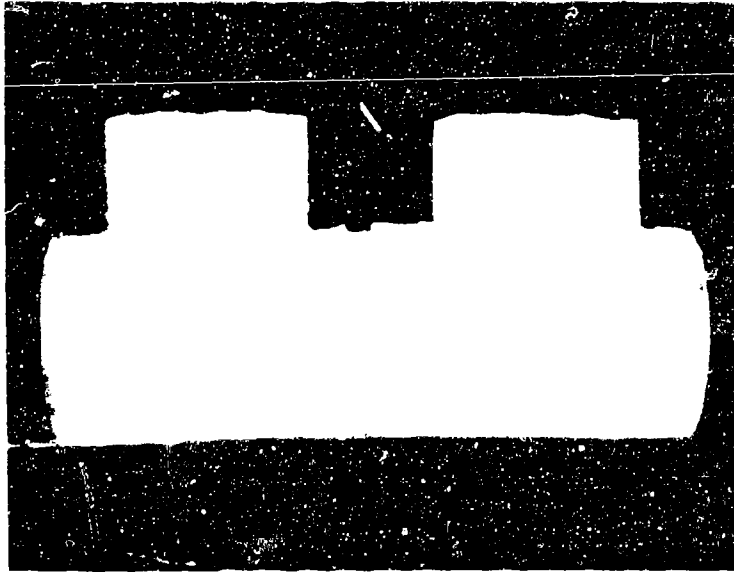
A.
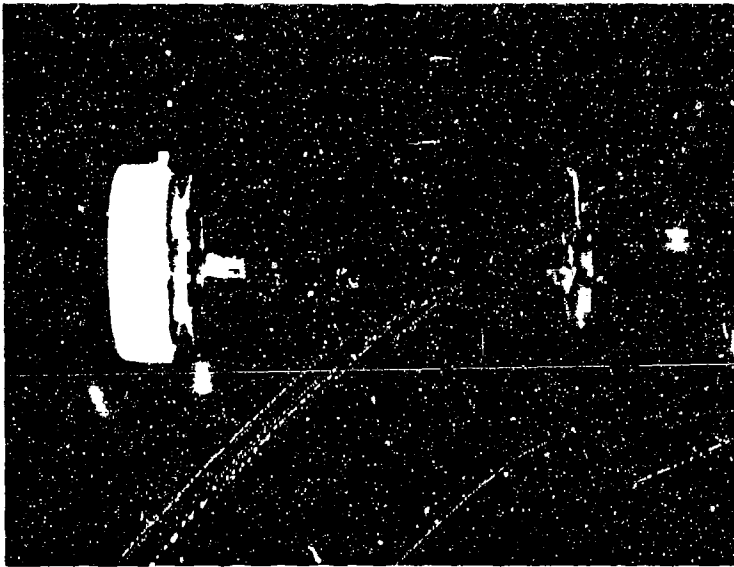
B.

Figure 1. Cell configurations. A. Two-chambered cell. B. Single-chambered cell.

back of the vial to a position in the cell close to the skin. For a test, the skin, mounted on the end of the vial, was dipped in the test solution contained in a 25 cc beaker. A second electrode was held in the outside solution near the skin.

In experiments in which double-chambered test cells were used, the cells were submerged in a water bath in which the temperature was maintained at $34° \pm 1°C$. About 5 minutes were allowed before readings were made for temperature equilibration. In most cases air was bubbled into the solutions in both cell chambers at a rate of about 25 cc per minute. There was no attempt to regulate the solution temperature in tests in which single-chambered cells with end-mounted skin were used.

The electrodes used with both types of cells consisted of polyethlene tubes of 1/8 in. diameter, filled with 3M KCl in agar. Each tube was connected to a separate, sealed bottle of 3M KCl solution. A Beckman Ag-AgCl reference electrode was immersed in each sealed bottle. A conductor from each of the Ag-AgCl reference electrodes led to the measurement circuit. No special shielding was used. The electrode conductors were connected to a Keithley model 600-B electrometer-amplifier, which served the dual function of indicating the biopotential level and amplifying it for recording. Most readings on the Keithley were made on the 0 to 100 millivolt scale, with the instrument adjusted for high input resistance. Instrument drift was checked periodically and the zero was reset as needed. Prior to each experiment, the electrodes were immersed together in Ringer solution and the residual (asymmetry) potentials were noted. The residual potentials were generally under 1 millivolt.

Signals from the Keithley were recorded with a Varian G-11 stripchart recorder set at 100 millivolts full scale. A chart speed of 1/2 in. per min was used in most of the experiments. The complete experimental set-up, consisting of water bath (used with double-chambered cells only), electrodes, electrometer-amplifier and recorder, is shown in Figure 2.

In a typical experiment, the magnitude of the potential across the frog skin, in millivolts, was first measured with both skin surfaces bathed in plain Ringer solution at pH 7.0. One surface of the skin was then exposed to the test substance dissolved in Ringer solution. With end-mounted skins, exposure was accomplished simply by dipping the cell in the solution. With the double-chambered cells, Ringer solution was removed by suction from one chamber and replaced by the test solution.

Skin response was recorded as the maximum change in voltage occurring within 5 minutes, relative to the voltage seen immediately preceding the stimulus.
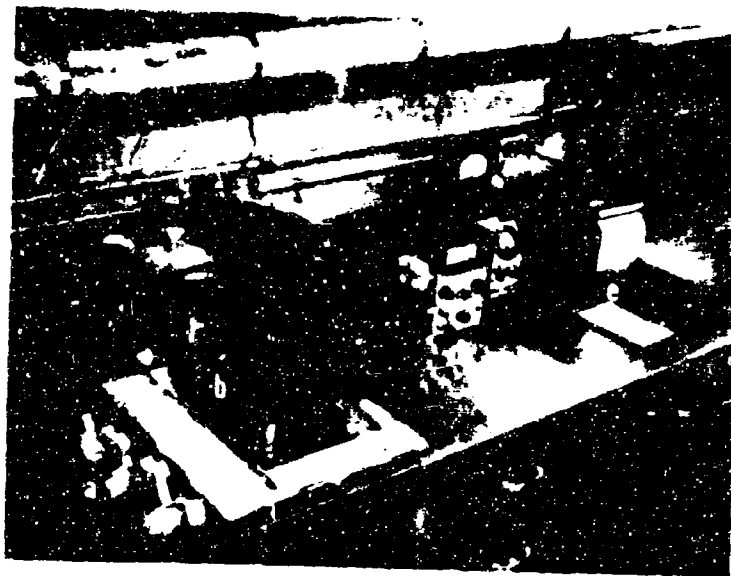
Figure 2. Experimental set-up showing
a. cells, b. water bath, c. electrode
assemblies, d. electrometer e. recorder.

A change, if any occurred at all, was usually seen within 1 to 2 minutes. The stimulated side of the skin was then rinsed in fresh Ringer solution, and reversal of the effect, with return of the voltage to some steady state level, was awaited. When a steady state was judged to have been attained, a new substance, or a different concentration of the first one, was applied. If the voltage dropped rapidly, threatening to fall below about 10 millivolts, indicating possible irreversible deterioration of the skin, the stimulus solution was immediately replaced with fresh Ringer solution.

Duration of the experiments varied from 1/2 to 6 hours with successful preparations, using one or more substances at one or more concentrations.

The results that are recorded here represent a total of 200 observations using 16 different substances. These substances included materials found in human body products. Graded concentrations of 6 of the substances were tested, including lysine, indole, skatole, hexanoic acid, urea and epinephrine. Initial tests utilized concentrations appropriate for gross screening. The ultimate sensitivity of active substances was not determined. Table 1 lists all of the substances tested and the number of tests made with each.

III. RESULTS

Frog skin responded to all of the test substances in the concentrations used. Response magnitude measured as maximum steady state open-circuit voltage change varied from -55 to +15 millivolts. Changes of more than 1 to 1.5 millivolts were regarded as significant. The recorded responses were not simply momentary voltage changes, but were observable on the records as continuous, smooth, systematic deviations from a reference level.

A wide range of skin sensitivity to a variety of substances is shown on the composite graph in Figure 3. As shown by the graph, the sensitivity extends over 5 orders of magnitude (10^5:1) for concentrations of the various substances of 0.1 mg per cent to 10 gm per cent, and over 3 orders of magnitude (10^3:1) for specific single substances. No chemical analytic technic is known with this long range that does not require scaling, filtering or diluting technics.

Skin response was a direct function of the concentration of test substance. Change in electrical output was generally negative at higher concentrations, perhaps involving some e ent that inhibited biopotential production. This change became less negative at lower concentrations, and even became positive for many of the substances at some relatively low concentration. Change in a positive

TABLE I

Substances used for stimulation of isolated frog skin.

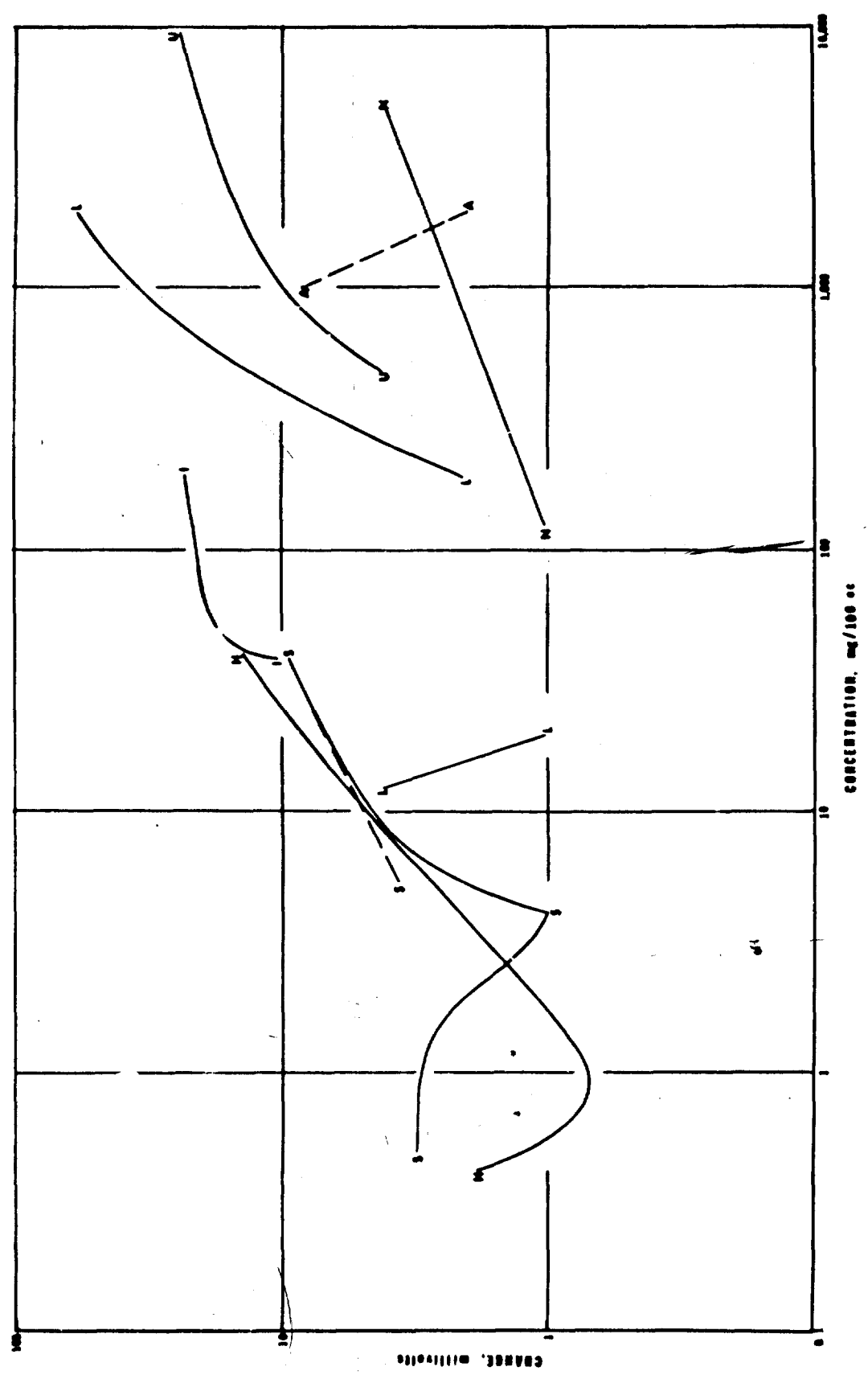| Substance	No. of Readings	No. of Skins	Surface exposed to test substance*
Urea	46	17	I, O
Lysine	33	10	I, O
Indole	39	5	I, O
Skatole	21	3	I, O
Hexanoic Acid	10	2	O
Pitressin	9	6	I, O
Epinephrine	8	3	I, O
Taurine	5	4	I, O
Dextrose	6	4	I, O
Na citrate	5	4	I, O
4-Aminobutyric acid	5	3	I, O
Nembutal	34	3	I, O
Menthol	3	2	I, O
Squalane	2	1	I, O
Maltose	2	1	I
Ephedrine	2	1	I, O

* I = inner surface of skin, O = outer surface skin.

Figure 3. Composite graph showing sensitivity of frog skin to various substances.
A = aminobutyric acid, C = Na citrate, E= epinephrine, I = indole, L = lysine,
M = maltose, N = nembutal, O = menthol, S = skatole, T = Taurine, U = urea.
Only negative changes are represented.

d: *ion may have been indicative of an excitatory or stimulating process. In a few stances more complex response patterns were observed, involving other reversals of the response. The possibility exists of distinguishing various compounds by the shape, position and slopes of their stimulus-response curves.

IV. DISCUSSION

In order to explore further and to predict the interesting properties of frog skin as a chemical transducer, rigorous controls must be applied in future experiments to reduce, if possible, the variability of response. This variability is probably a composite of many factors. Until exact limits for these controls are established, the following points should be considered:

The temperature should be controlled within $0.5^{\circ}C$. The pH of all solutions should be kept within 0.5 unit. Glucose, and perhaps insulin, should probably be added to the Ringer solution to enhance durability and viability. Meticulous care should be observed in the preparation of solutions, skin and apparatus in order to minimize random contamination of the system. Inert plastic materials should be used wherever possible.

Voltage measurements should be started as soon as possible after the frog skin is mounted. The chemical stimuli should be applied in such a manner that no physical or chemical change occurs except that resulting directly from contact of the test substance with the skin. It is probably desirable to alternate exposure of the skin to test substances and to plain Ringer reference solutions. A standard test material, such as urea can be used for calibration purposes. At the end of an experiment, residual voltage should be obtained following a terminal treatment with NaCN.

Ultimate sensitivity of frog skin toward the various substances tested was not determined. Dilutions should be carried out to trace levels (below 1 microgram/cc) of test substances in replicate experiments. Gain of the electrical measuring equipment can be increased, other electrical variables of skin can be sampled, and additional control can be established over conditions of the chemical and physical environment in order to obtain measures of ultimate sensitivity. It is possible that the sensitivity limits of frog skin to a given substance can be modified (increased) by selective treatment of the skin with compounds such as dimethyl sulfoxide, certain organo-phosphates and/or fluorides, various hormones, and possibly by electrical stimulation. At the present time it appears worthwhile to explore some of these possibilities, recognizing that the goal is an increase in sensitivity of several orders of magnitude over that observed in the experiments reported here.

9
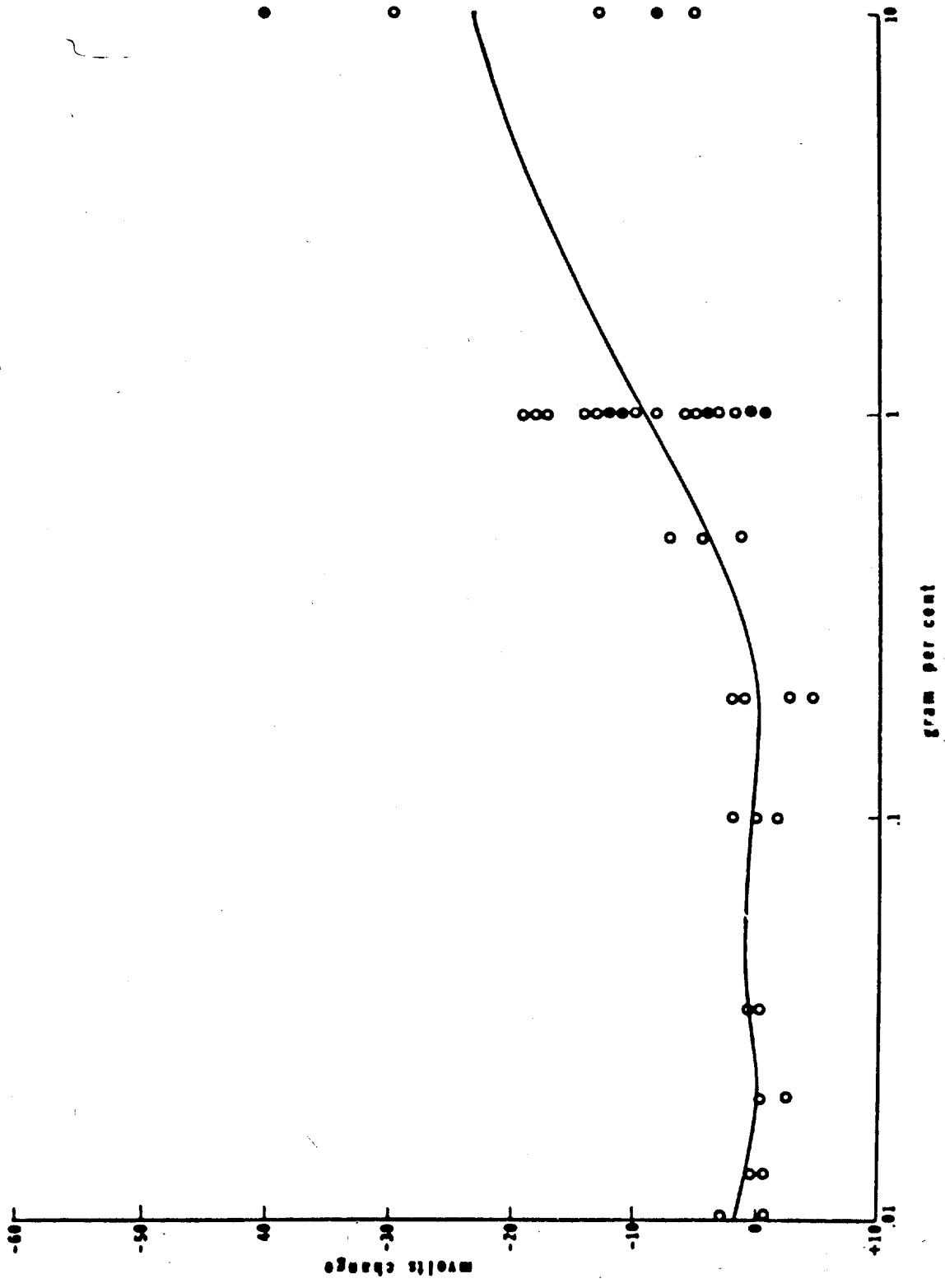
Figure 4. Sensitivity of frog skin to urea. Open circles = outer surface of skin exposed, closed circles = inner surfaces of skin exposed. The line is drawn approximately through means of the data points.

Figure 5.. Sensitivity of frog skin to lysine. Graph construction as in
Figure 4. Broken line = region of uncertainty due to insufficient data.

Figure 6.   Sensitivity of frog skin to indole.
Graph construction as in Figures 4 and 5.
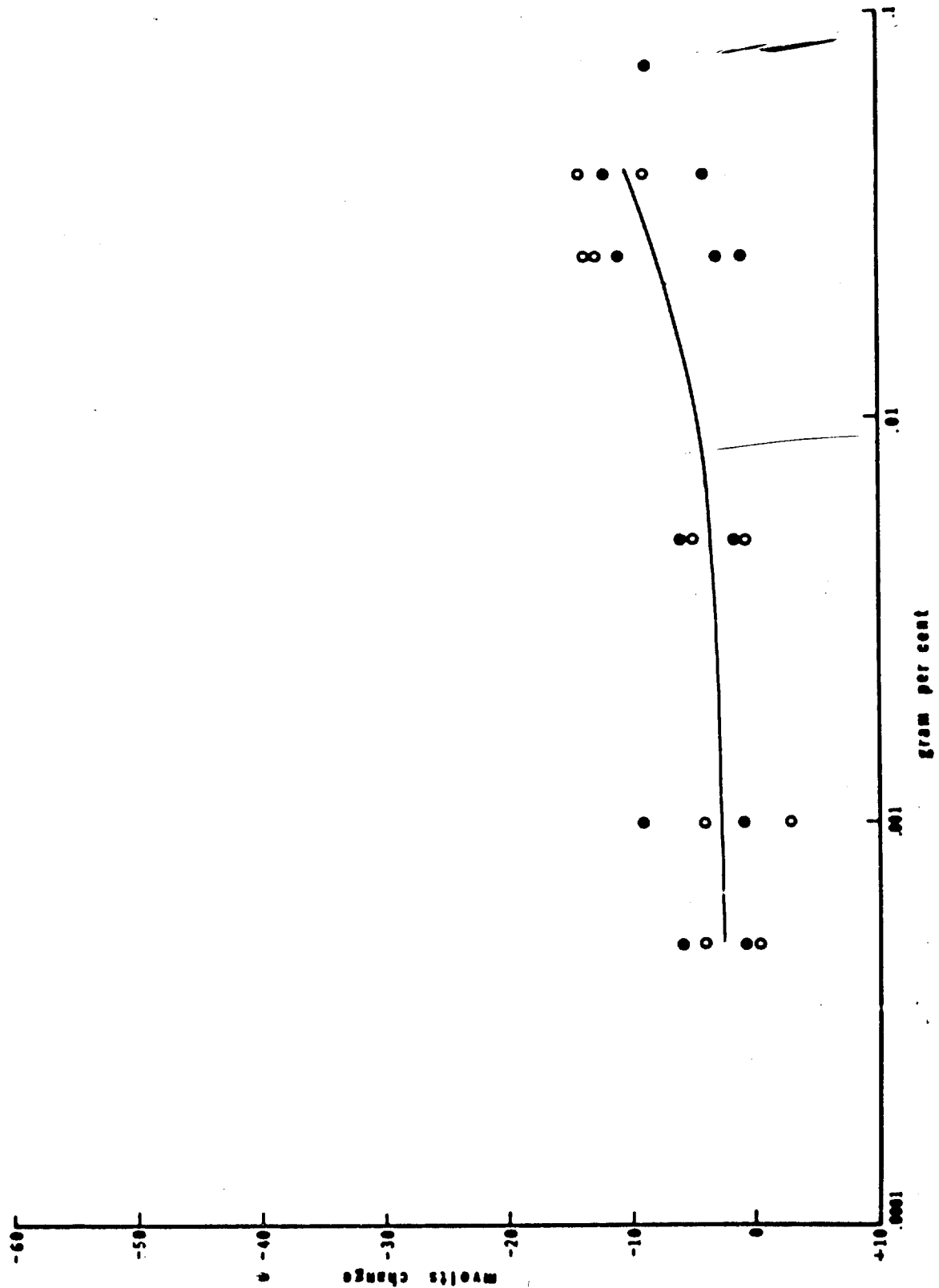
Figure 7. Sensitivity of frog skin to skatole.
Graph construction as in Figures 4, 5 and 6.

V. SUMMARY

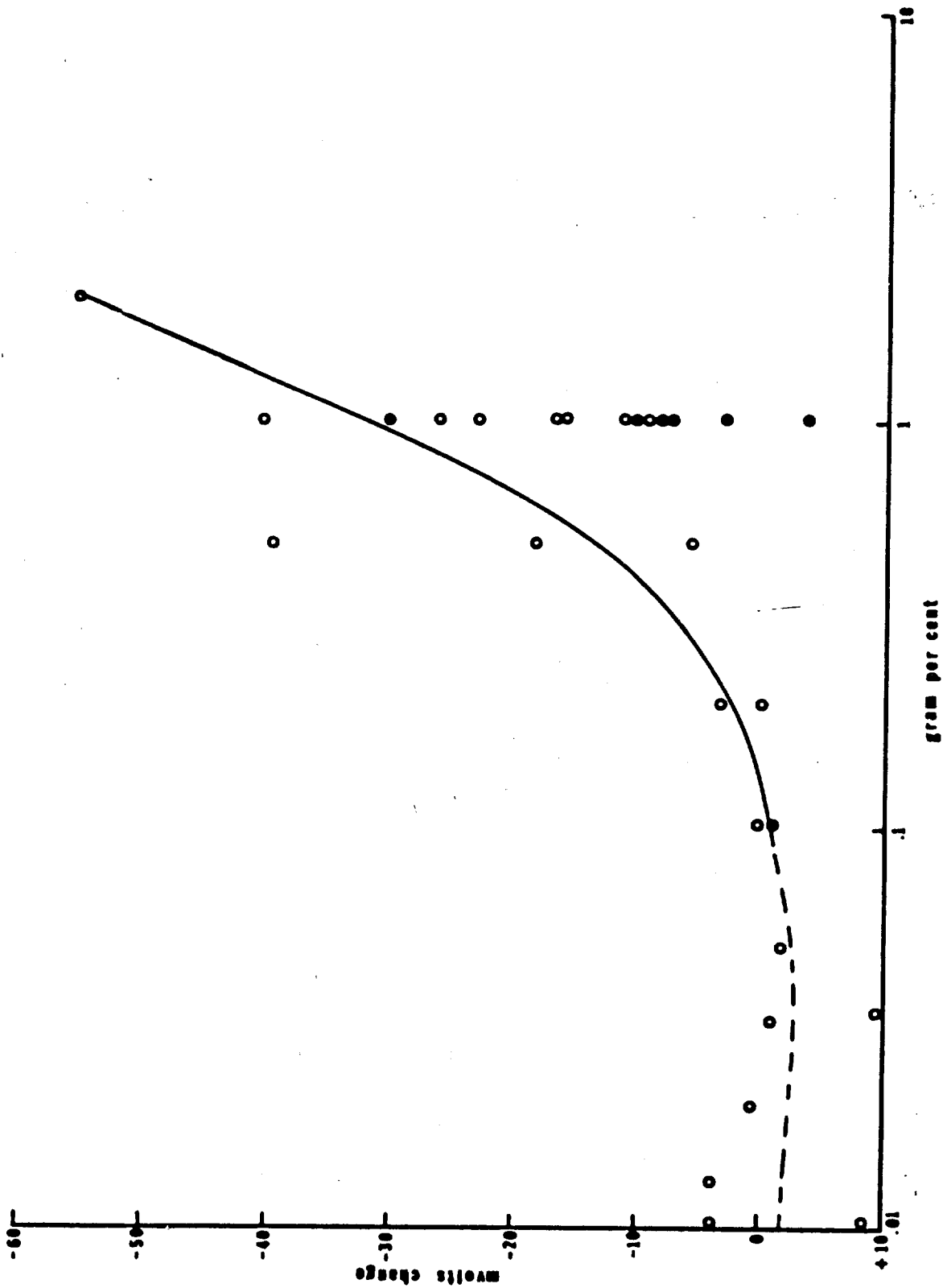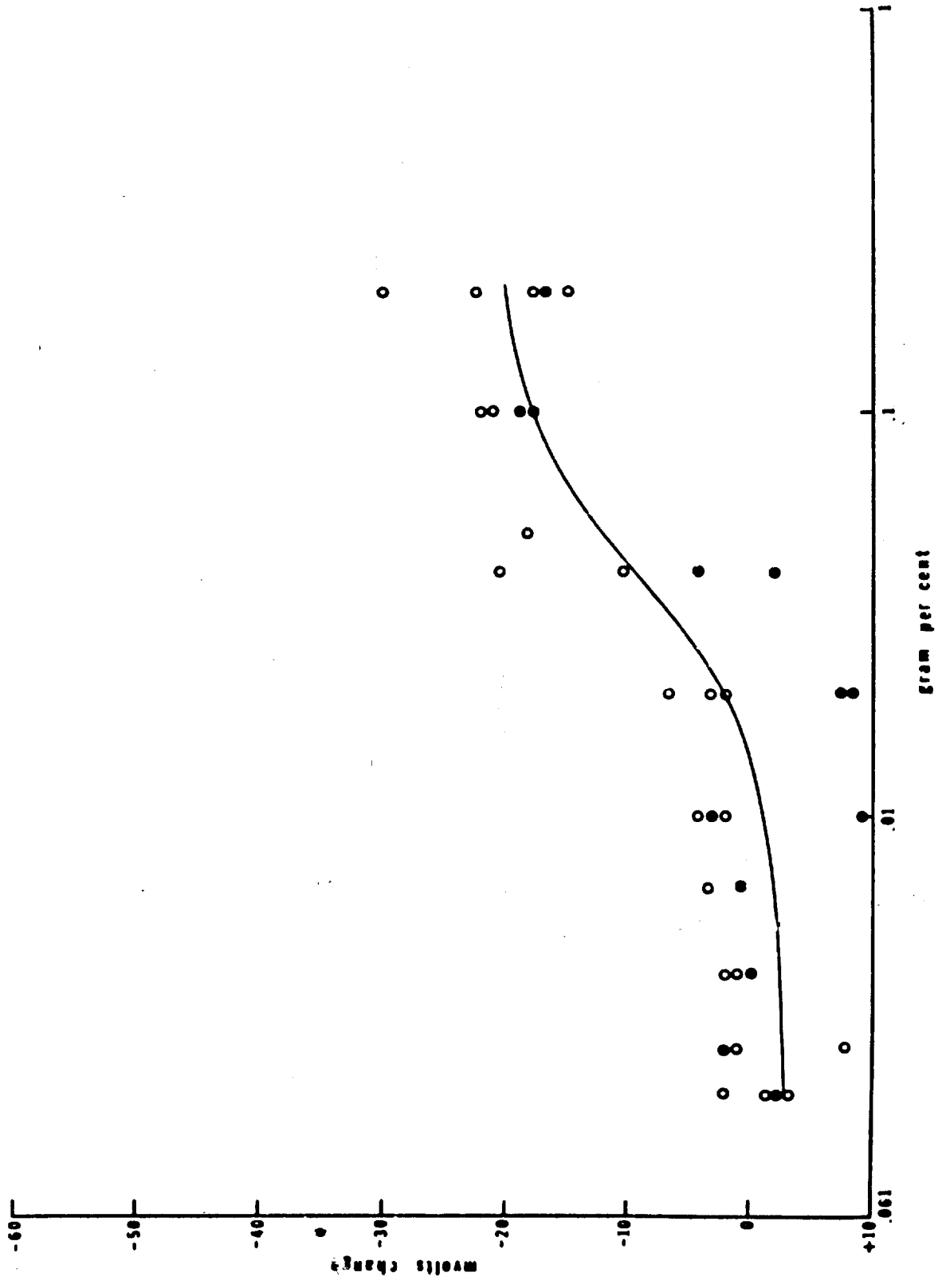1. Reversible changes in bioelectric potential of isolated frog skin exposed to a variety of different substances of low molecular weight occur over a concentration range of at least 5 log units.

2. A high degree of variability between frog skins, and a lack of data on ultimate sensitivities at usefully low levels for selected substances, are major problems that remain to be examined.

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2. Ussing, H., Diffusion Through Biological Membranes, in "Ion Transport Across Membranes," H. T. Clarke, Ed. Academic Press, New York, 1954.

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