BIOELECTRIC POTENTIALS AND ACTIVE TRANSFER IN FROG SKIN

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ABSTRACT

The vibrating probe voltmeter for the measurement of bioelectric potentials by Blüh and Scott has been used in an improved form for measurements of frog skin potential differences.

In good agreement with earlier findings the observed frog skin potential differences were found to be of the order of 100 millivolts, and the polarity such that the inside of the skin was positive relative to the outside.

Bioelectric potential measurements were made during the influx of sodium chloride and amino acids in aqueous solutions into frog skin in either direction. Characteristic potential changes were observed for different substances and opposite directions of flux, and have been used to demonstrate the asymmetry of frog skin permeability.

Transfer mechanisms for sodium chloride and amino acids have been advanced from the standpoint of the assumption that an electrical field exists in the frog skin membrane.

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INTRODUCTION

Asymmetric diffusion and active transfer of substances through living membranes present fundamental biological problems. Various explanations and "mechanisms" have been invoked to account for the phenomena. One of these assumes that a bioelectric potential difference across the membrane plays a role in active transfer or at least regulates asymmetrical diffusion of electrolytes or charged particles.

Bioelectric potentials of membrane surfaces have been the subject of several investigations, undertaken to determine the potential, as to sign and magnitude, by various methods. The cause of the bioelectric potentials has frequently been discussed and has been associated with metabolic activity.

The influence of a bioelectric field on the diffusion of electrolyes through living membranes has been under investigation, as mentioned before, but no experimental

work has been done in which bioelectric potential measurements were made during diffusion processes. The reason for the neglect of experimental research in this direction is due to the fact that the usual methods of bioelectric measurements require the use of an electrolytic contact of some kind or another with the surface, so that the observation of bioelectric potential changes in the membrane may be disturbed by potential changes produced by the electrodes. Liquid contact can be a particularly disturbing factor in the study of permeability of membranes showing irreciprocal diffusion, i.e., exhibiting asymmetry in their permeability with respect to direction in the membrane.

Measurements, undisturbed by the above mentioned factors, of bioelectric potentials of permeable membranes during diffusion processes have become possible through the use of Blüh and Scott's vibrating probe voltmeter. By the use of this instrument, no material contact with the living surface is required.

In the present investigation, the vibrating probe voltmeter was used successfully for the study of steady surface potentials and their variation in time and during diffusion processes. This paper is an account of measurements of membrane potentials of frog skin and the study of the diffusion of sodium chloride and amino acids through frog skin, a membrane known to exhibit the property of

asymmetric permeability for these substances. The original apparatus of Blüh and Scott was modified and improved upon, and special apparatus designed for the determination of frog skin potential differences on the living animal, and for the measurement of potential changes during the diffusion of solutions through isolated sections of frog skin.

A discussion of the results in the light of earlier diffusion experiments is given, and a new mechanism for the active transfer of amino acids suggested.

A. DIFFUSION PROCESSES IN BIOLOGICAL SYSTEMS

a. Ordinary diffusion.

The passage of material into cells through the cell walls and through membranes presents an important biological problem. Free or simple diffusion is one process by which substances pass through membranes. Free diffusion represents the net transfer of material through a given cross-sectional area of a medium where the amount of material transferred per unit time from one region to another is proportional to the concentration gradient between the two regions. The property of a membrane in permitting the passage of material through it is called permeability. The transport of a series of polyhydric alcohols through rat intestine (9) and of organic nonelectrolytes through the alga Beggiatoa mirabilis (10, p. 232), are examples of simple diffusion in biological systems.

The differences in the permeability of biological membranes have been found to have some correlation with the structure of the membrane. Theories of simple diffusion are mainly based on two concepts: (a) diffusion through a cell structure which is comparable to a mechanical sieve, and which segregates diffusing particles according to their size, and (b) the dissolving of the particles in a fat-like substance, which represents the intermediate between two solutions on either side of the boundary. The theories, known as the Sieve - theory and the Lipoid - theory respectively, (cf. 10, p. 229 ff.), do not explain all the facts of simple diffusion through living membranes, either independently, or in combination; other theories, including those postulating an electric membrane potential, have been brought forward and found satisfactory to a greater or lesser degree.

b. Active-transfer.

While simple diffusion explains the presence of ions and molecules in various parts of a biological system, it does not account for the transfer of material against a concentration gradient, or more generally, an activity gradient. According to Höber (10, p. 525):

"Active transfer usually is manifested by the

> establishment of an unbalance in concentrations. Solute or solvent molecules are shifted 'up-hill' against a diffusion gradient; they are 'accumulated' as the result of osmotic work, enabled by the liberation of metabolic energy."

There is no doubt that a great many cases exist where such active transfer takes place through membranes; e.g. amino acids through the intestine of rat (9), hydrochloric acid through the gastric mucosa. Quoting from Crane, (6, p. 116), we note that:

> "The high concentration gradients against which active secretion can take place make it one of the most impressive phenomena in the physical chemistry of biological tissues. For instance, gastric hydrochloric acid is secreted by oxyntic cells as a solution whose H^+ ion concentration is several million times as great as that in the cell, and this concentration difference is maintained across a distance not greater than one micron, and probably much less..."

Brooks and Brooks (3) review some ion-accumulation mechanisms, all of which assume the production of an electrolyte in the interior of the cells, and in which the diffusion of the electrolyte outward causes external ions to accumulate within the cells. It is assumed that membranes have a mosaic structure in which adjacent parts have specific cation or anion permeability. The structure, together with the ion exchange mechanism leads to an accumulation of both anions and cations within the cells.

A possible mechanism for active transfer is one that assumes a special carrier system in the membrane. In this case one has to postulate that a compound is formed between some mobile constituent of the membrane and the substance being transferred. The absorption of glucose in kidney tubules (10, p. 561 ff.) and intestine (10, p. 547 ff.), is believed to be due to glucose phosphates being formed and subsequently hydrolyzed at the region of the blood stream. Ussing (17), in explaining the fact that the influx of Na^+ is greater than the outflux in isolated frog skin, suggests that sodium permeates as an uncharged complex, but becomes a free sodium ion on the inside.

The active transfer of amino acids through membranes has been observed, and must be considered to have great significance, since amino acids are physiologically very important substances. Thus Höber (10, p. 551) writes:

> "It is conspicuous from old observations of Overton concerning the osmotic properties of frog muscle, that amino-acids, like glycine or alanine, enter with great slowness, if at all, although, because of their smaller molecular size, one could expect them to exceed, for instance, erythritol. Still more unexpected is their inertia in penetrating the cell surface of the sulfur alga Beggiatoa, which is remarkable for its outstanding behavior as a molecular sieve and in which the permeation rates of several amino-acids have been found to be abnormally low. The reason for this slowness is not of a physiological nature, since the slowness is evident also in diffusion experiments with collodion membrane, and can be explained as being

> due to the empholyte character of the amino-acids ....which, probably due to the formation of a shell of water dipoles around the ampholyte ions, brings about an enlargement of the molecular volume. Consequently, a porous membrane, such as the intestinal wall...would be expected to be passed comparatively slowly also. But the contrary is true. In comparison with acid amides, with erythritol, with xylose, the amino-acides actually pass the intestinal wall much faster than was anticipated from their diffusion rates."

Höber and Höber, (9), in experimenting with exicsed isolated intestinal loops (rats), found that the percentage amount of amino acid absorbed per unit time decreases with increasing concentration in the loop within a certain range of concentrations. A similar effect occurs in the active reabsorption of amino-acids by the kidney tubules (10, p.562). A suggestion can be made that there is present a driving force or carrier mechanism, transporting these substances across the tissue membranes.

c. Diffusion and electric forces.

With reference to membrane permeability and in particular to that of active transfer, Teorell (15, p. 939), has suggested that only two kinds of forces are significant for the passage of matter through biological membranes: osmotic forces, caused by concentration gradients and electric forces, produced by electric potential gradients, which may exist across the diffusion boundary or membrane.

Both forces may act simultaneously on particles present in the diffusion layer. In biological systems, most substances carry an electric charge, either because they are ions, or become electrically charged by an appropriate transformation or complex formation (e.g. phosphorylation of glucose on the combination of fatty substances with bile acids). The passage of substance per unit time through a cross-sectional area normal to the direction of flow (called flux) is given by the expression:

Flux of a substance = mobility X concentration X force.

$$\frac{dN}{dt} = u \times a \times \left(\frac{RT}{a}\frac{da}{dx} \pm n F \frac{dV}{dx}\right),$$

where N is the number of particles or amount of substance, u is the mobility of the particle, V is the potential, a is the activity of the particles, T is the absolute temperature of the system, R is the universal gas constant, F is the Faraday constant, n is the charge or valency, and x is the distance in the diffusion layer measured in the direction of flow.

The flow of material tends to continue across the membrane until a steady state is reached where

$$\frac{dN}{dt} = 0$$

$$\text{i.e., } \frac{RT}{a}\frac{da}{dx} = \pm n F \frac{dV}{dx}$$

The minimum amount of osmotic work which must be done is equal to the electrical work:

$$RT \ln \frac{a_i}{a_o} = nFV,$$

where the activity on the side to which the substance is moved is a_i and the activity on the side from which the substance is moved is a_o. The emf. for doing such osmotic work should be measurable, providing appropriate instruments with high input impedance are used.

d. Asymmetric permeability.

In the above mentioned experiments, (A,b) active transfer through membranes is suggested by the variation of the rate of diffusion with the concentration gradient. Another approach to the study of active transfer is the observation of diffusion in different directions through the membrane. A difference in the diffusion rates would suggest that in one preferential direction an "active transfer" of the diffusible substance is taking place. A study using frog skin in which an "irreciprocal permeability" was observed, is due to Wertheimer (19). He found that the living frog skin is permeable to chlorine ions (in NaCl) from outside to the inside of the skin, but is practically impermeable in the direction from inside to outside. Water passes easier from the inside to the outside. For amino acids, polypeptides and peptones, the diffusion transport

is better from the outside to the inside, usually much better than in the opposite direction. The size of the molecule does not seem to have any relation to the permeability. Carbohydrates showed practically no diffusion from outside to inside under conditions where amino acids passed easily and vice versa. For dead frog membrane, in contrast to the living membrane, all fine differentiations of permeability disappear.

In the case of frog skin several investigations have been made to determine the bioelectric potential across the membrane, and good agreement has been found between different observations. Such an electric field introduces into the membrane cross-section an asymmetry which may be responsible for irreciprocal or asymmetric diffusion. For this reason it appears promising to attempt a correlation between the bioelectric field gradient and the asymmetric transport of salts and amino-acids through frog membrane. A study of this problem is the subject of the present research.

B. BIOELECTRIC POTENTIALS

a. Electric potential differences in membranes.

The existence of bioelectric potentials is well established, although their maintenance and functions are still giving opportunities for scientific considerations. A book by Lund (13) contains reviews of the work of his school, and brings, in an appendix, a valuable comprehensive bibliography. An article by Crane (6) gives a table of reliable data on bioelectric potentials.

The table shows that the steady bioelectric potentials developed across living membranes are usually of the order of tens of millivolts, and in some cases reach values of over 100mv. The potentials of cell membranes show a considerable range, from a few mv. to 140mv., whereas the specific electric organ of various fishes attain values of several hundred millivolts. Several

investigations have found for frog skin values from 10 to 80 mv., and for frog gastric mucosa 10 to 40 mv. Lund (13, p.237) summarizes the electrical properties of frog skin as follows:

> "General agreement is found on the following facts which apply to the isolated tissue: (1) In cell layers of the frog skin, the E.M.F. is oriented so that the outside is negative to the inside as measured in the external circuit; (2) The potential is quite variable, ranging from 10 or less to 250 millivolts; (3) The time course of the potential is roughly divided into a preliminary period of fluctuation lasting about an hour, followed by a period of slow decrease for two to twelve hours, succeeded finally by a more rapid decrease until electrical zero is attained in more than twenty hours; (4) Measurements in vivo agree with measurements made on the isolated skin during the first two hours; (5) Where pieces of less than 2 square centimeters of area are used, large variability is observed in different regions of skin from a single animal; (6) The inherent E.M.F. is located in the epithelial layer of the skin; (7) The E.M.F. increases with rising temperature, the effect being reversible between limits of 0°C. and 40° C.; (8) Variations in O_2 tension, respiratory accelerators such as dinitrophenol, respiratory inhibitors such as KCN, when applied within the E.M.F. simultaneously and generally in the same direction, although quantitative relations have not been established".

As to the location of the source of emf. in frog skin, Meyer and Bernfeld (14, p. 377) suggest that,

> "It (frog skin) is composed of at least four layers of different permeability, one of which is specifically permeable to H^+ ions and is very likely identical with the 'basal membrane' situated between the stratum germinativum and the corium. The major part of the resting potential

of the skin is located across this membrane and is due to the difference of H^+ concentrations on both sides of the membrane".

The next section deals with the methods of bioelectric potential measurements, and the concomitant difficulties involved, together with the possibility of their removal.

b. Measurement of bioelectric potentials.

The measurement of potential differences of biological origin presents the main difficulty of maintaining the normal undisturbed conditions in the living system. Short circuiting and polarization of the biological source of potential have to be avoided by reducing currents drawn to values less than 10^{-8} amps. Polarization of the electrodes. In the older methods electrodes are brought in contact with the membrane often producing injury of the surfaces thereby causing injury potentials.

The usual experimental method of measuring small potentials employs D.C. amplifiers, whose high input impedance ensures that polarization is reduced to a minimum. In this method, contact with some kind of an electrode is necessary and one has to consider that the electrode materials - usually electrolytes - can affect

the surfaces whose potentials are being measured, by (1) shortcircuiting (although this may be reduced through the use of microelectrodes) and (2) by the introduction of diffusion potentials. The latter effect is a particularly disturbing factor when the membrane exhibits asymmetric permeability (A, d), and when the variation of bioelectric potential during diffusion processes is under investigation.

In order to circumvent the difficulty connected with the contact electrodes, Blüh and Scott (1) have suggested the use of a vibrating probe voltmeter. In this instrument, no direct contact is made by the electrode on the membrane surface, and contact by a reference electrode is made at a considerable distance from the membrane. The feasibility of the method for bioelectric potential measurements was tested by Blüh and Scott in preliminary experiments, and has recently been confirmed by Jones, Flowers, and Pomeroy (11a), who used it to measure bioelectric potentials of plant tissues and seeds.

In the present paper, the vibrating probe voltmeter has been used for the measurement of frog skin potentials and the study of diffusion processes through frog skin by means of observations of the bioelectric potential changes.

C. THE VIBRATING PROBE VOLTMETER METHOD

a. Apparatus.

The principle of the vibrating probe method consists of a capacitative coupling between the electrically charged surface, S, and a metallic vibrating probe, P, (Fig. 1). The probe is set in oscillation with the help of a telephone T which is driven by an electrical oscillator (Sylvania Model) O. The probe undergoes sinusoidal mechanical oscillations in a direction normal to the surface, so that the probe's capacitative coupling varies according to the equation:

$$C = C_o (x) + C_1 (\Delta x, x) \sin wt,$$

where C_o is the capacity of the coupling, x is the distance from the probe to the surface at rest, and C_1 is the maximum change in capacity due to the maximum displacement Δx, of the probe. Further $w = 2nf$, where f is the

frequency of the probe, and t is the time at which the capacitative coupling has the value C. The variation in capacity is responsible for the induction of an alternating current in the probe. This signal is transmitted to an electrometer tube of a pre-amplifier stage, PA, of low input capacity; the amplifier signal is transferred to a narrow band high gain amplifier A (Fig. 1). The amplifier is tuned to the probe frequency. The output of the amplifier is fed into a cathode ray oscilloscope, C.R.O.

The signal from the probe can be compensated by a reverse potential applied to the surface S, with the help of a potential divider PD and an electrode E, in contact with the surface. The potential divider is used also to reduce initially the probe potential to zero or to a minimum, in order to null any undesirable potentials present in the circuit.

The vibrating probe arrangement is shown in Fig.2. The mechanical oscillations of the probe are produced by a telephone T (connected to an oscillator O, Fig. 1) which sets in vibration a small iron tube through which passes a flat glass rod (7 cm. long, 1.5 mm. wide and 0.3 mm. thick). The glass rod is fixed at points f_1

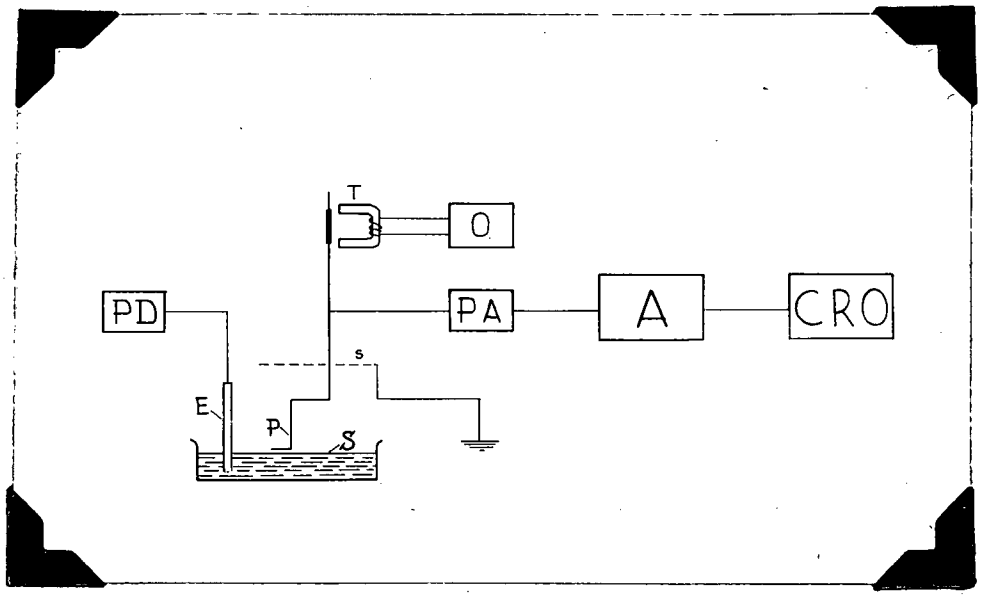

Fig. 1. Block diagram of vibrating probe method

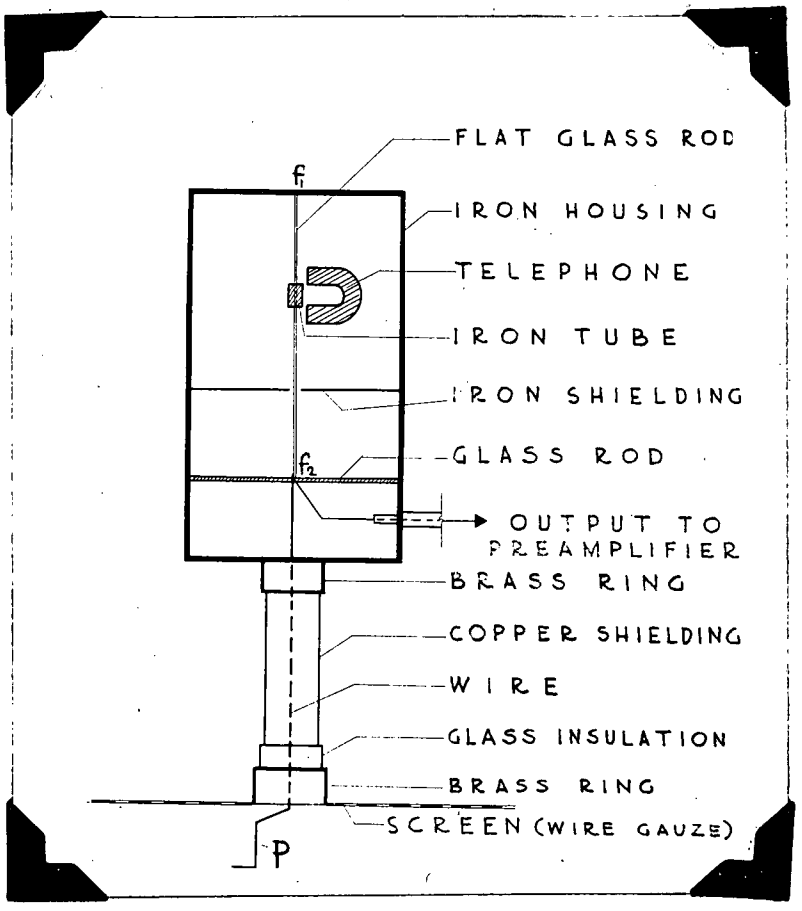

Fig. 2. Cross-section of the vibrating probe voltmeter

and f_2 and has a rectangular cross-section, which tends to limit its oscillations to a single plane (the plane of the paper as shown in Fig. 2). In this way variations in signal output due to changes in the plane of oscillation of the probe are avoided. The probe, an iron wire of circular cross-section (diameter 0.72 mm.) is attached to the glass rod at point f_2; the end of the probe is bent at right angles, and vibrates with its natural frequency 250 c/s. It is useful to drive the probe with the least amount of power supplied to the telephone, since in spite of shielding, some of the A.C. signal affects the probe and is liable to be amplified. At the natural frequency the probe oscillations are more closely sinusoidal, and for a given amplitude more amplification can be applied and hence greater sensitivity results. A thin, shielded, wire soldered to the probe at f_2, conducts the potentials induced in the probe to the preamplifier stage PA.

The preamplifier PA, consists of an R.C.A. tube 959, placed in a metal box close to the probe arrangement (cf. Fig. 5). The signal from the probe is applied across a very high resistance R(10^4 Mohms) to the control grid g_3 of the tube, (Fig. 3).

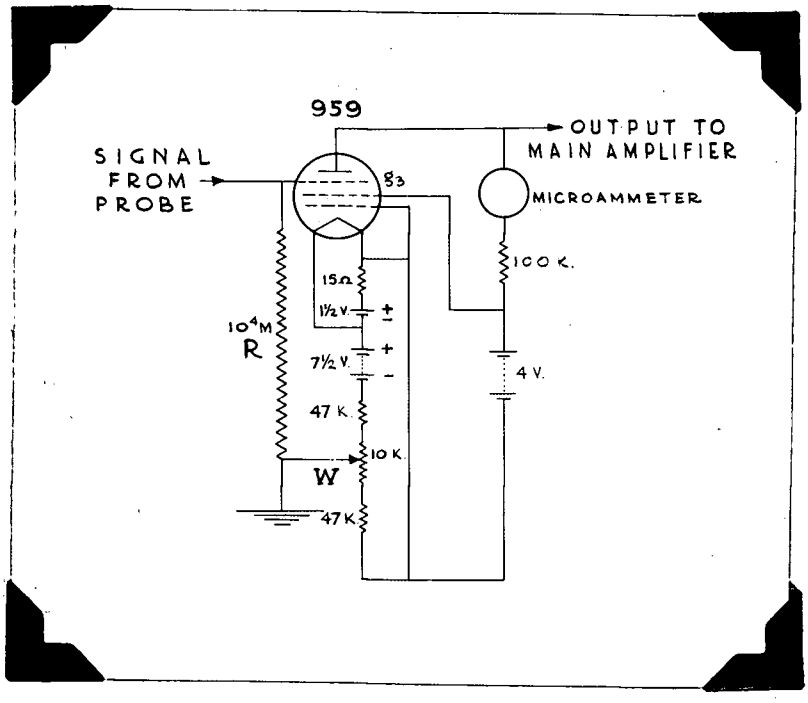

Fig. 3. Pre-amplifier circuit

The 959 electometer tube operates satisfactorily with the plate voltage : 3 volts, screen voltage : 4 volts, plate current : 12 microamps. Fine variation of the negative bias of the tube is obtained by a 10^4 ohm. wire wound variable resistance W between two 47K fixed carbon resistors. (cf. Fig. 3). A 10^5 ohm. variable carbon resistor, previously used in the bias circuit, produced sporadic changes in the output signal (because of poor contact). A battery of 7.5 v. supplies the bias circuit and a 1.5 volt battery serves for filament heating.

The grid leak resistance and glass envelope of the 959 tube must be carefully freed from impurities (by washing with alcohol or ether) otherwise variations in the output signal are likely to occur from breakdown leakages across the surfaces.

The output of the preamplifier is conducted by a shielded cable to a narrow band high gain amplifier (Fig. 4). The circuit is that of the amplifier described by W. Wilson (20). To avoid 60 c/s modulation, the filaments are heated by a 6 volt storage battery. Plate voltage (300 volts) is supplied by an independent regulated power supply (Lambda Electronics Corporation; Model 25), capable of delivering 125 m.a. for D.C. voltage settings from 200-325 volts.

The amplifier, built to provide sufficient gain and selectivity at the probe frequency of 250 cps., produces maximum signal to noise ratio, and shows fast response to changes of the probe signal.

The signal from the preamplifier is fed into a 1620 high gain pentode tube TI through a resistance capacitance coupling network. A cathode follower T2, in the second stage, provides sufficient power so that the signal may be further amplified with minimum distortion.

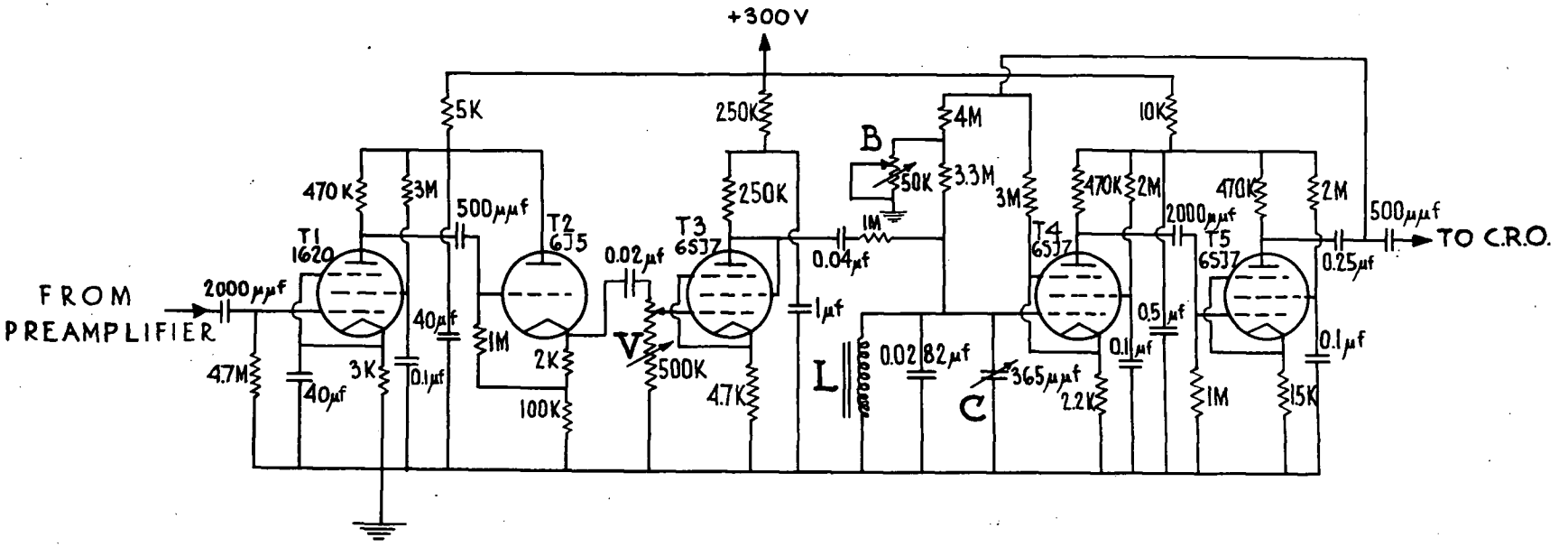

Fig. 4. Main amplifier circuit

A 6SJ7 amplifier tube T3 connected as a triode separates the gain or volume control V from the selective section of the amplifier.

An LC circuit and two stages of high gain, pentode connected 6SJ7 tubes T4 and T5 form the selective section. This prevents the selectivity from being affected by the gain setting. High stability and the removal of tube noise is achieved by means of a negative feedback network connected to the 2,200 ohm. cathode resistor of T4. The selectivity provided by the LC circuit is controlled by a change of resistance B, that is, through the amount of positive feedback delivered. The LC circuit is thus loaded by a controllable negative resistance so that the selectivity can be increased. A toroid coil L of 20 henries and 610 ohms (DC) keeps the resistance of the LC network at a minimum and improves selectivity. So by advancing B, the band width can be varied from 50 cps. down to 0.5 cps. The frequency to which the amplifier is tuned can be varied by changing the variable capacity C of the L-C-circuit. At the probe vibration 250 cps. and at maximum selectivity, the gain of the amplifier is approximately 10^6.

The output of the amplifier is fed into a Sylvania cathode ray oscilloscope, C.R.O. (cf. Fig. 1).

The horizontal gain is set at zero, and only the vertical gain is used. The trace on the screen therefore appears as a line. The change in the length of the trace for a given gain setting and probe distance is proportional to any change in potential induced in the probe by its capacitative coupling with the potential surface S.

The total arrangement of the apparatus is shown in Fig. 5. On the right hand side the two metal housings are respectively the vibrating probe arrangement and the preamplifier. On the table, appearing next to the preamplifier, is the Sylvania oscillator; on its left, the potential divider and the main amplifier are shown, while the cathode ray oscilloscope can be seen in the background.

b. Measurement of frog skin potentials.

An attempt was made to measure the potentials on both sides of living frog skin with reference to a distant reference electrode. The frog (rana pipiens) was pithed and thus made immobile. The animal was fitted to a stand as seen in Fig. 6. A flap of abdominal skin was secured between two lucite plates, attached to a small bridge (cf. Fig. 7), holding the frog in place. The lucite plates have circular openings of 1.4 cm. diameter, so that the vibrating probe can be brought close to the plane of the

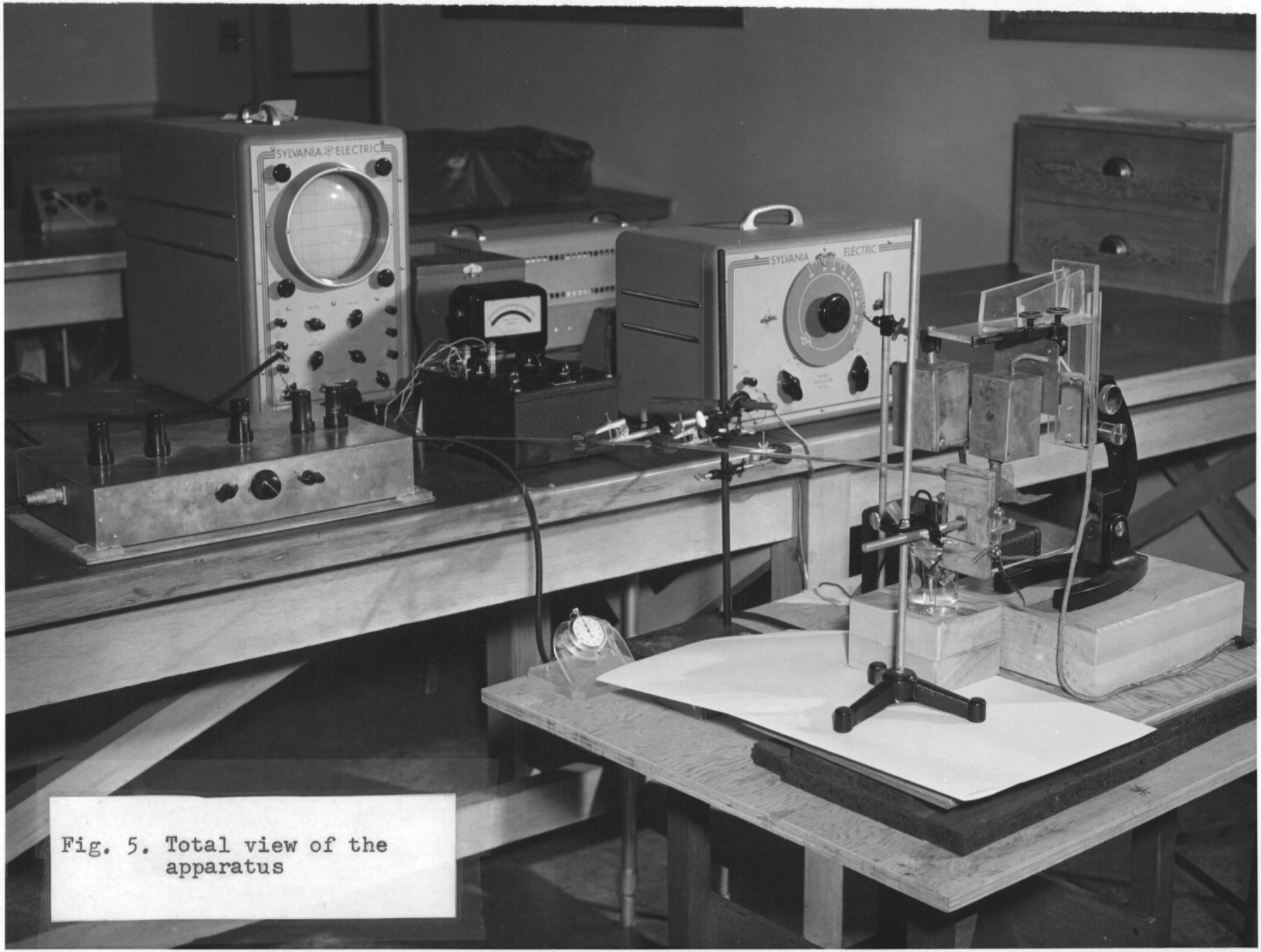

Fig. 5. Total view of the apparatus

membrane surface from both sides. Since the membrane is secured through the lucite clamp, the respiratory movement of the frog does not affect the probe distance.

The wooden board to which the frog is fastened can be rotated through 180°, so that either the epidermis (outside) or the corium (inside) faces the probe (OFP or IFP respectively).

A reference point for potential is established by immersing one leg of the frog into saline contained in a beaker (Fig. 6 and 7). A calomel electrode is introduced into the solution to make contact with the potential divider supplying the nulling potential. At all times the skin of the frog must have sufficient conductivity to guarantee the transfer of potential to the membrane.

Fig. 7. shows the relative position of the lucite holder and frog, and the vibrating probe voltmeter. During the measurement, the probe is placed in the centre of the opening in the lucite (visible in the photograph), and brought close to the membrane surface with the aid of the micrometer screw of the microscope stand.

The procedure of measurement used is as follows:

(1) The oscillator is tuned to the probe frequency in the following way. The probe is brought close to

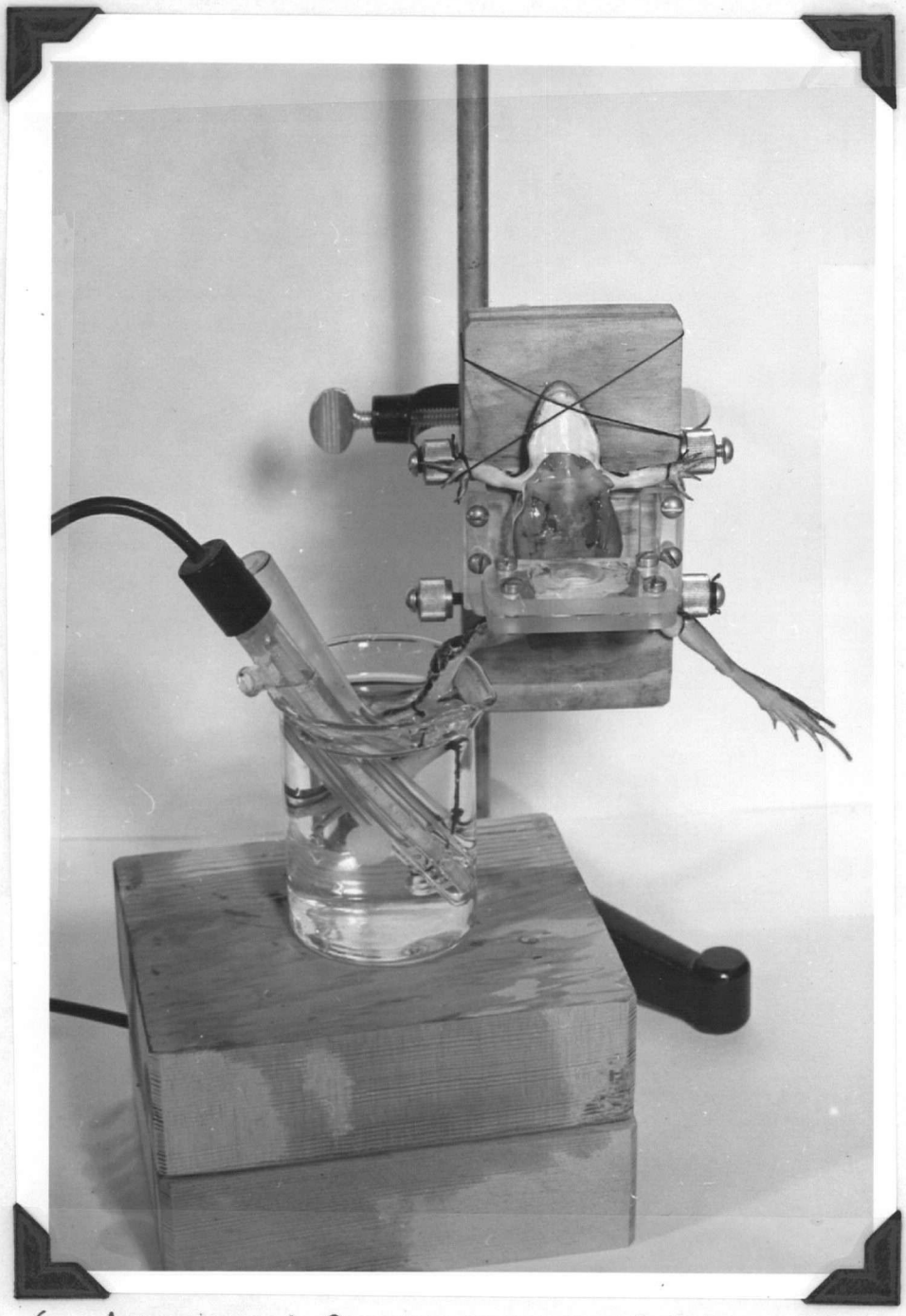

Fig. 6. Arrangement for measurement of frog skin potentials.

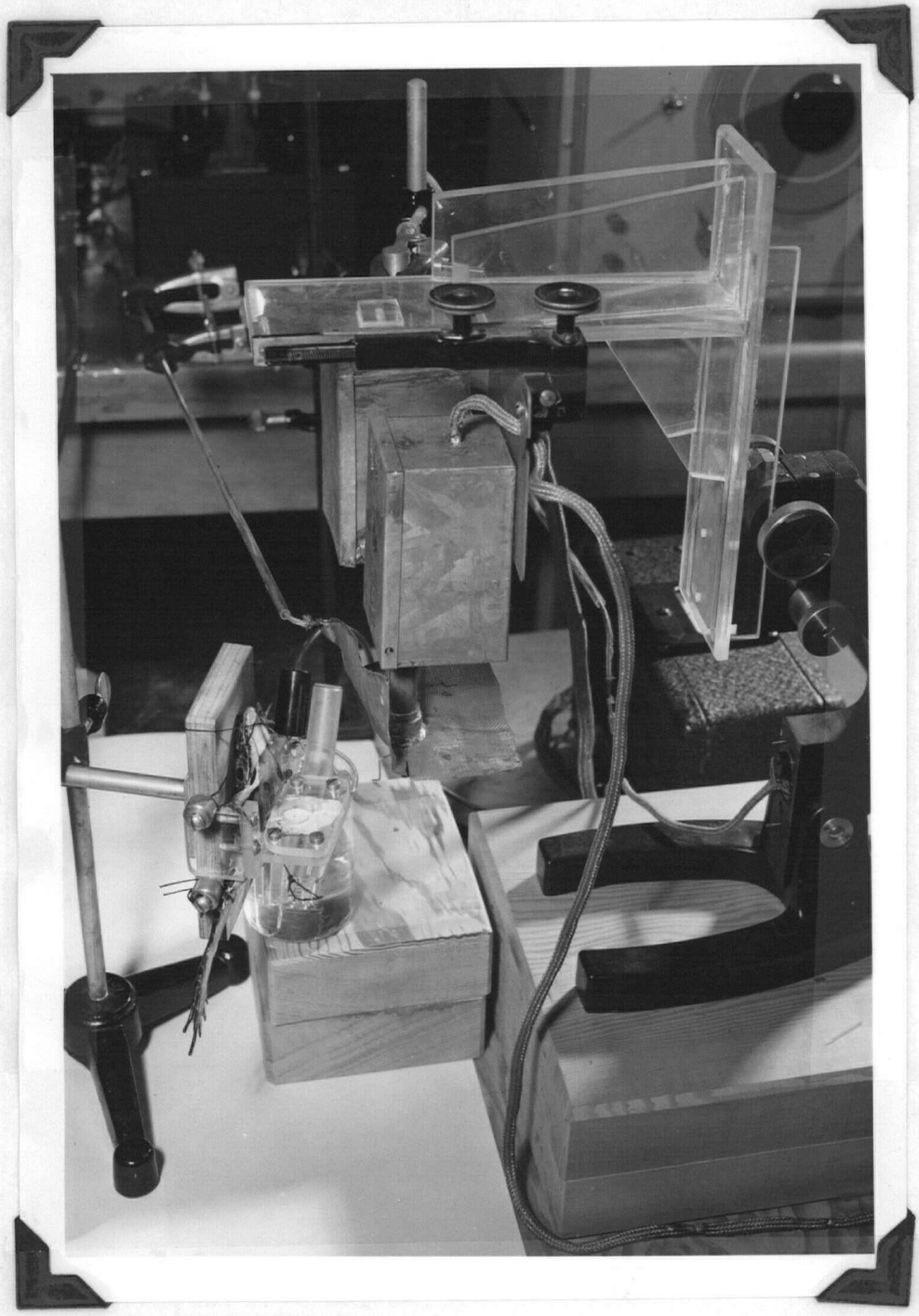

Fig. 7. Frog holder placed under vibrating probe

the surface which has a definite potential. The band width of the amplifier (control B; Fig. 4) is made wide, and the volume control V is set so that a convenient trace length appears on the CRO screen. The oscillator frequency is varied until a maximum trace length is obtained.

(2) Tuning of the amplifier to the probe frequency is achieved as follows. The volume control is set to zero, and the band width control B advanced until the CRO trace has a barely noticeable length. With V again set to obtain a convenient trace length, the capacity control C is varied until the signal is a maximum.

(3) The membrane is connected to the potential divider circuit through the electrode E, as shown in Fig. 7 (left hand side of the figure). A double throw switch allows the application of potentials of different polarity. A resistance of approximately 10^5 ohms is in series with the decade dial box from which the compensating potentials are drawn. The potential is supplied from six No. 6 batteries.

If the trace on the screen has a convenient length, the resistance of the potential divider is varied until the trace length is a minimum; this indicates that the surface potential has been compensated. In order to get a measure of sensitivity, one can observe the change in trace length caused by a given change in potential on either side of the minimum position. E.g., when a change

of 1000 ohms is effected with an appropriate choice of probe distance, the deflection of the trace on the screen can be made to be two inches. A sensitivity of this order — expressed as x inches per 88mv. — was considered sufficient for the measurements in question; however much higher sensitivity can be achieved with the present apparative arrangement.

Since the sensitivity depends on probe distance, as was found in preliminary experiments, the same sensitivity was maintained for a set of measurements by varying the probe distance (the amplifier gain was kept constant).

(4) Equal changes of trace length produced by equal potential variations to either side of the minimum position assure (more accurately than the observation of the minimum trace length itself) that the minimum position has been attained indicating the complete compensation of the surface potential. The resistance R of the decade dial box set for the minimum trace length is read off, and the corresponding potential V calculated from the formula:

$$V = \frac{R}{10^5 + R} \; 8.8 \text{ volts},$$

where 10^5 (actually 94,000) is the number of ohms of the fixed carbon resistor in series with the decade dial box

(Fig. 8).

(5) Measurement of membrane potential differences involve the measurement of the difference in potential required to compensate the surface potential of each side of the membrane, relative to the same reference point and at a given sensitivity.

With the frog membrane, first the inside, and then the outside (or vice versa) of the skin was facing the probe, (IFP and OFP), and independent measurements of the potentials made. The change in the position of the frog membrane, since it does not make contact with the vibrating probe, does not affect the contact potentials unavoidably connected with the combination of various metals, etc., in the measuring instrument.

c. Measurement of frog skin potentials during diffusion processes.

The diffusion of salts, amino acids, etc. through living membranes is probably, as pointed out previously, connected with the electric field gradient existing in the membranes, and a correlation can be attempted between the rate of diffusion observed in special diffusion experiments, and the measured bioelectric potential across the membrane. It is, however, of interest to investigate

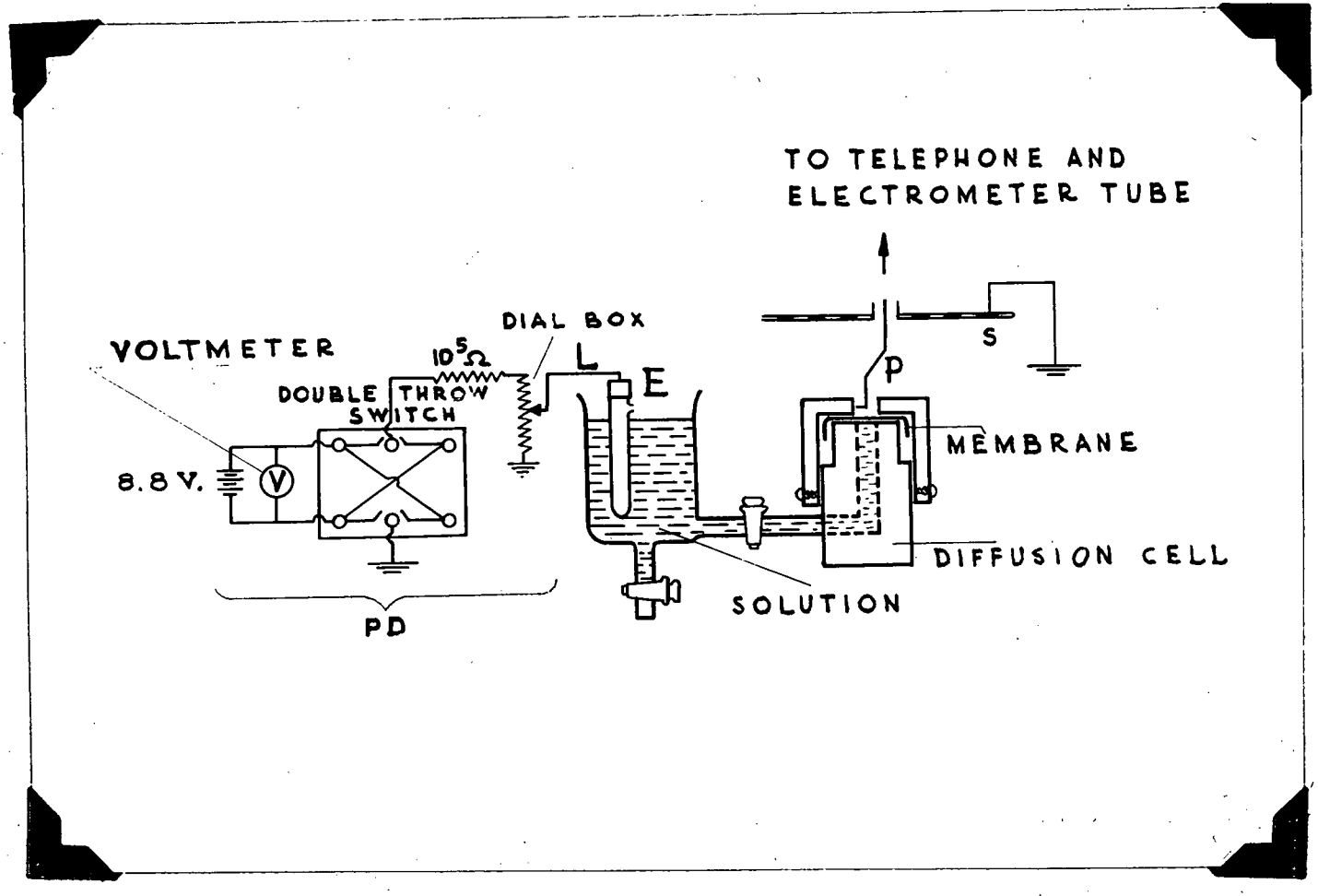

Fig. 8. Compensating potential divider (left) and Diffusion cell (right).

whether the diffusion of substances into (and through) the membrane affects the bioelectric potential. This was done in experiments described in this section.

A special cell, made of lucite, was built (Fig. 8, right side), which allows the measurement of potentials of frog skin surfaces which face the vibrating probe, whereas the other side of the skin is in contact with a solution of the salt, etc. This solution is connected through a glass tube to a reservoir, filled with the same solution. The beaker holds the electrode E, connected to the potential divider (Fig. 8, left hand side). The electrode E permits the application of a compensation potential.

The membrane is held between a lucite sleeve and a lucite cylinder. An opening (diameter 1.6 cm.) in the top of the lucite sleeve allows the probe to be positioned over the surface.

In preparing the diffusion cell for a potential measurement, the following procedure was used:

(1) A solution of the diffusing substance is poured into the reservoir (the conducting tube has a stop cock permitting isolation of the reservoir and diffusion cell).

(2) The membrane to be studied is placed over the top of the main body of the diffusion cell and the lucite sleeve is fitted over it, holding the membrane in place.

(3) The inverted diffusion cell is filled with the solution, and connected to the glass tube in the upright position through a tapered glass-lucite joint. One introduces the calomel electrode into the reservoir solution, and equalizes the levels of the solution in the reservoir and the cell.

(4) The probe is brought sufficiently close to the membrane surface through the opening at the top of the diffusion cell. With an appropriate gain setting of the amplifier and sufficiently small probe distance one applies the nulling procedure described in the preceding section, (C,b,3).

(5) In some instances, a small volume of water covering the membrane, was placed in the opening of the diffusion cell below the probe, and the potential changes observed resulting from the diffusion of materials through the membrane into the liquid layer.

The diffusion cell arrangement was used in experiments with cellophane membranes (preliminary measurements) and with frog skin obtained from the back and abdomen. Skin with an area of 4 cm^2, was removed from the frog, and used either immediately, or after preserving it in water for a few hours.

D. RESULTS OF EXPERIMENTS

(a) Potential measurements.

As explained in a previous section (C,b), a value for the membrane potential results as a difference between the potentials of the two surfaces of the skin measured relative to a reference point.

In general, it has been found that these differences are of the order of 100mv. (50 - 200), and that normally the inside (IFP) is positively charged relative to the outside (OFP). The results were tabulated, for example as follows:

No. 2. Back.				
Position	Time minutes	Potential mv.	Mean mv.	Potential difference D(I+) in mv.
OFP	33:00	205		
	35:20	196		
	37:00	192		
	38:30	188	193	
				63
IFP	47:40	255		
	48:50	255		
	52:00	257		
	54:50	256	256	
				60
OFP	66:15	201		
	68:10	197		
	72:05	193		
	74:45	193	196	

The abbreviation OFP represents "Outside (of skin) faces probe", and IFP: "Inside facing probe". The difference "D" between the outside and inside potentials is given as D(I+) or D(O+), where the plus sign indicates that I or O is positive relative to the corresponding opposite O or I, respectively.

In the preceding table, the inside is found positive relative to the outside, the potentials being D(I+) = 61.5mv.

Higher membrane potentials up to 200 mv. were observed; a reversal of potential occurred after the frog was kept for 3 hours preceding the experiment in a dry container.

Some of the results are exhibited in the form of graphs (c.f. Fig. 9, 10). Fig. 9 shows that after 3 hours the skin potential of initially 95 mv. is reduced to a small value, and is even reversed to D(O+) = 9mv.

In Fig. 10, the results of potential measurements with No. 7 are given. In this case, the potential difference is practically constant over a period of 1 hour. The drifts of the potential levels (IFP and OFP) occur nearly parallel to one another and depend upon changes in contact potentials, etc. After one hour a small volume of 0.7% NaCl solution was placed on the outside skin (OFP), allowed to be absorbed, and the remainder drained off.

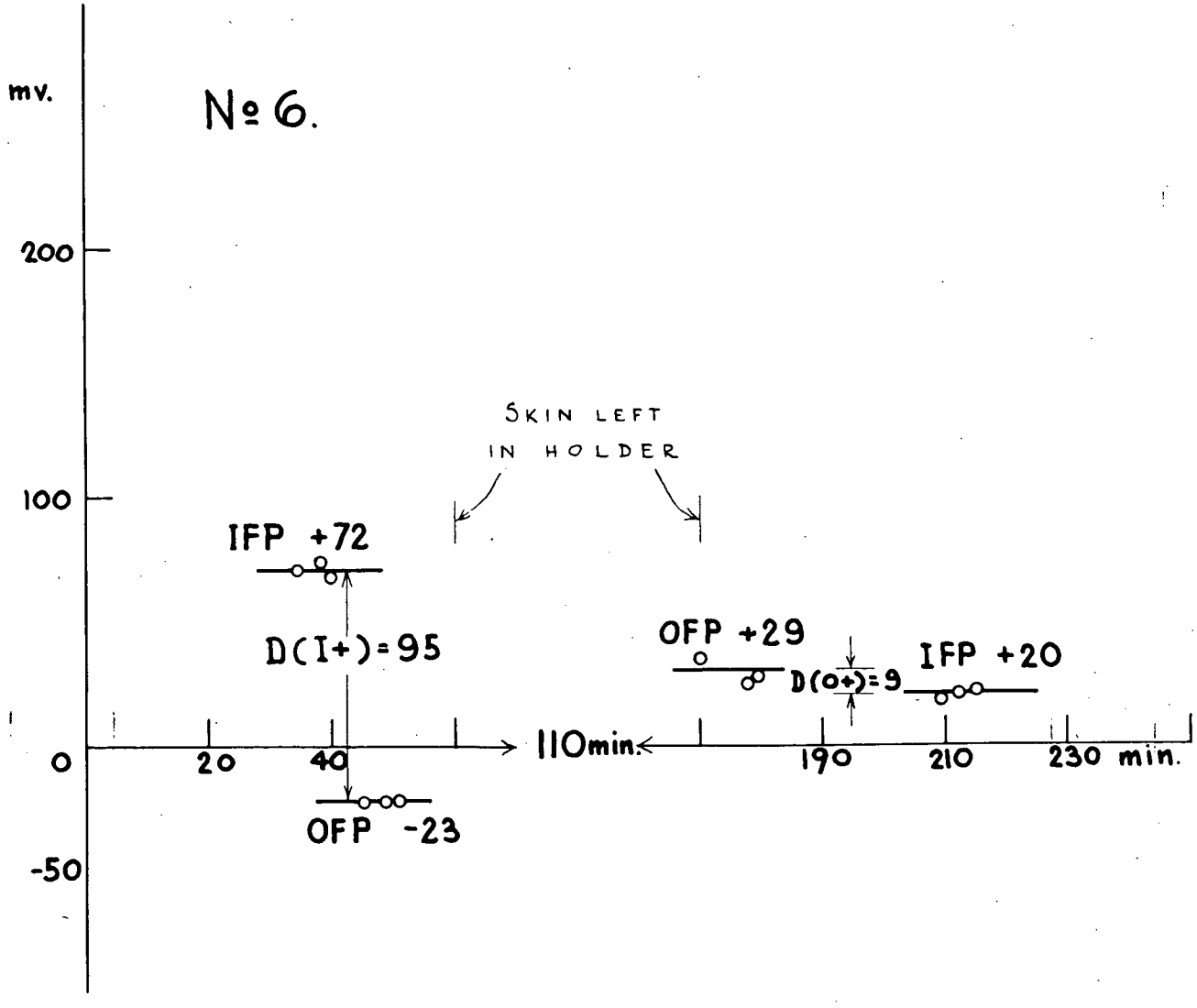

Fig. 9. Potential differences of frog skin

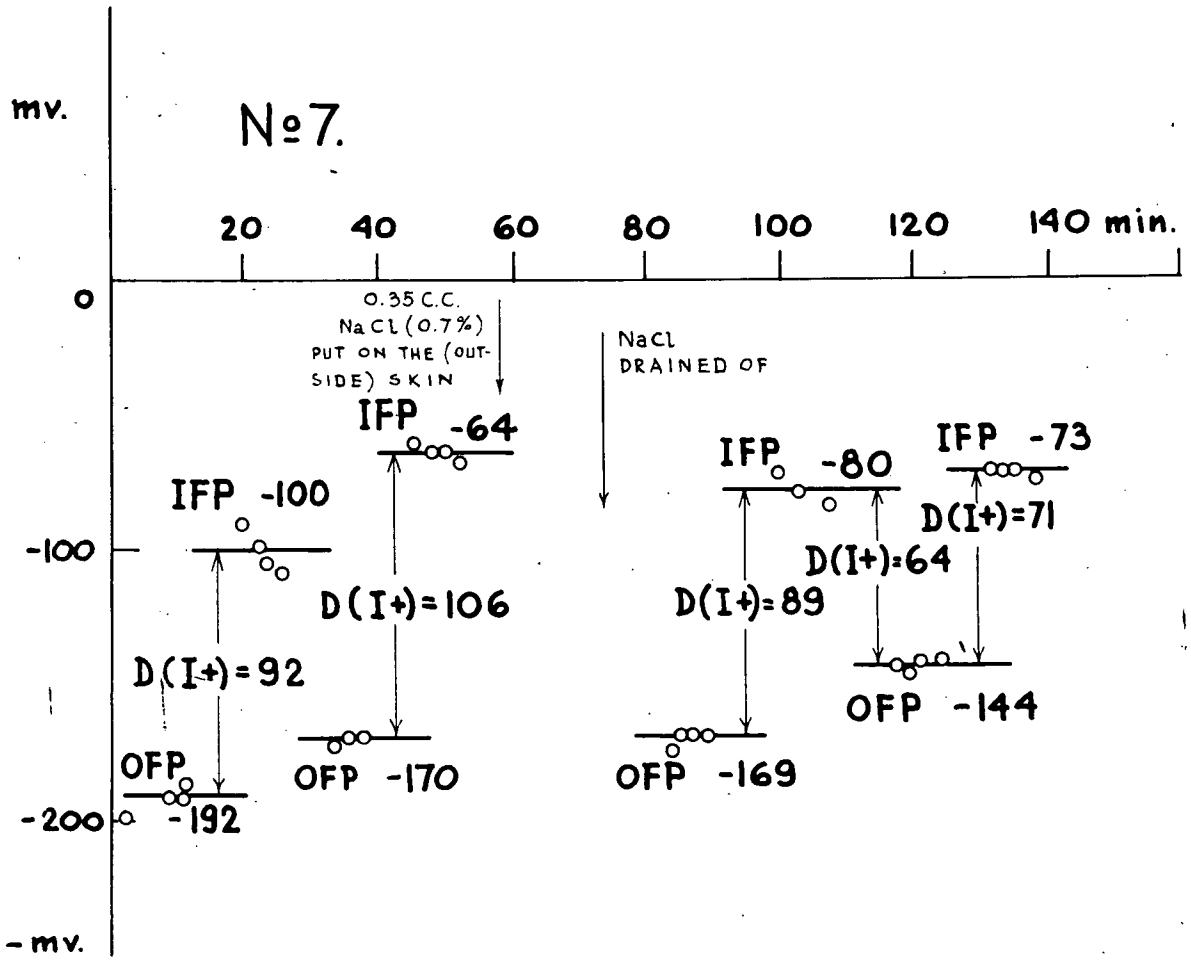

Fig. 10. Potential differences of frog skin

Following the absorption of sodium chloride from the side of the epidermis, the potential falls slightly to $D(I_+) = 89$ mv., and later to $D(I+) \equiv 71$ mv., with a tendency of reaching a zero value after about 3 hours, similar to the case shown in Fig. 9.

The general picture of measurements is in good agreement with the main points of the statements quoted in section B, a.

b. Diffusion experiments.

As has been mentioned before, frog skin shows irreciprocal permeability for the diffusion of sodium chloride and of a few amino acids, as found by Wertheimer (19). It would have been of interest to repeat Wertheimer's diffusion experiments on frog skin, and to measure directly, by analytic chemical methods, the rate of transport of solutes, etc. Such measurements were planned, but could not be undertaken for lack of time.

As stated above (C,c) an attempt was made to measure the potential changes during diffusion through frog skin, and to bring the results of these experiments in general agreement with Wertheimer's results.

A few preliminary experiments were performed with

cellophane membrane, soaked in distilled water, into which the diffusion of water, HCl, and glycine was observed. These experiments served mainly to get one familiar with the apparatus and with the procedure of measurements. In the case of water in contact with the lower surface of cellophane, relatively little change in potential of the upper surface was observed. With hydrochloric acid, changes of potential in both directions were found in different experiments. This is understandable since the original polarity of the membrane surface potentials are undefined, depending obviously on spurious effects. The diffusion of glycine through water-soaked cellophane is relatively slow, and produces only small potential changes (approximately 10 mv.).

The main results of the potential measurements with frog skin, into which NaCl (0.7% solution) diffuses, are shown in Fig. 11 and 12. In both figures the units of the co-ordinates are <u>changes</u> in potential (in mv.) and <u>changes</u> in time (in minutes). Several curves of different experiments have been represented in one graph for closer comparison. The first recorded point, on the left side of the curve, was normally measured about 10 minutes after contact was made between the frog skin and the solution. The initial stage of a given diffusion process could therefore not be observed, but the rate of diffusion in the

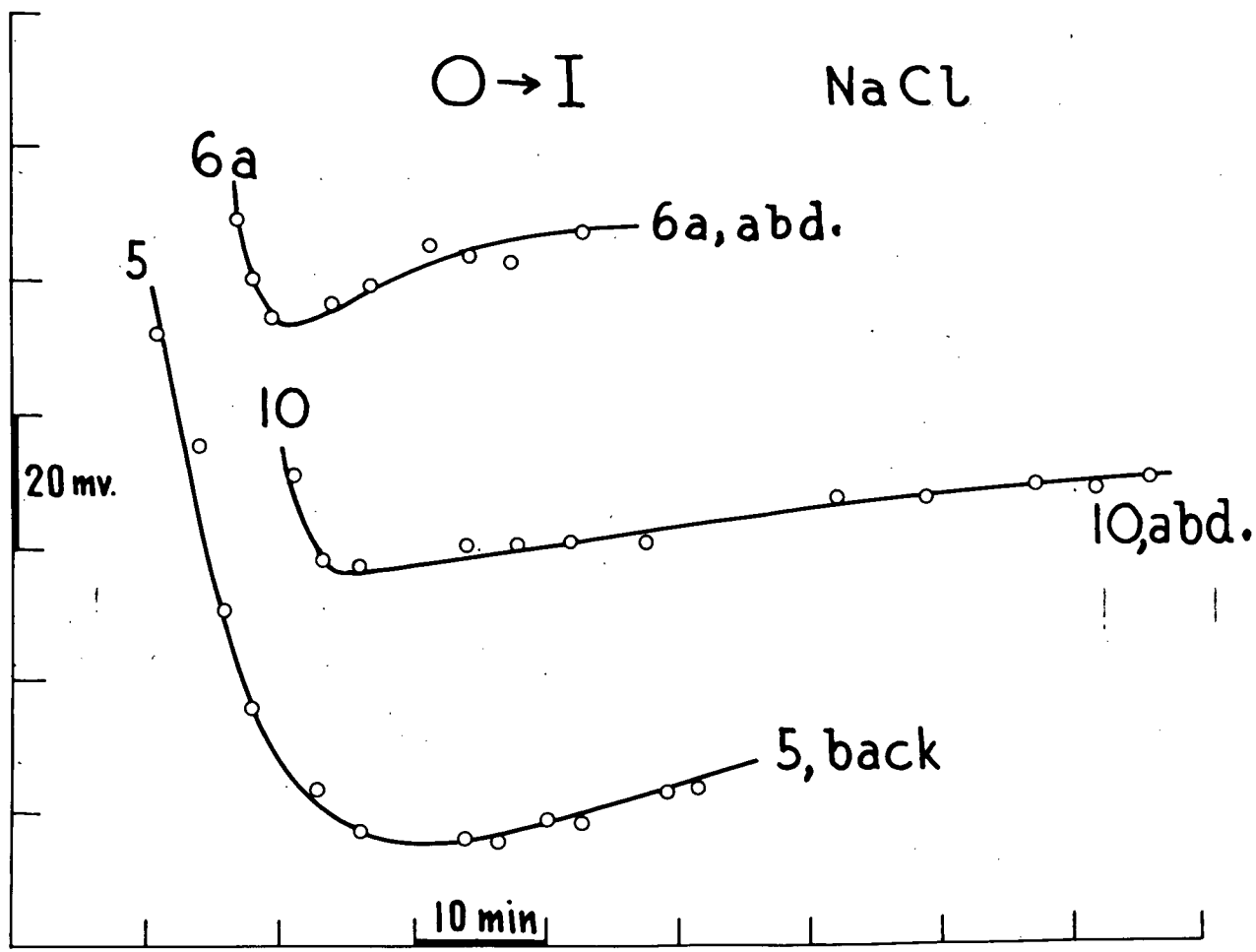

Fig. 11. Potential changes during diffusion process

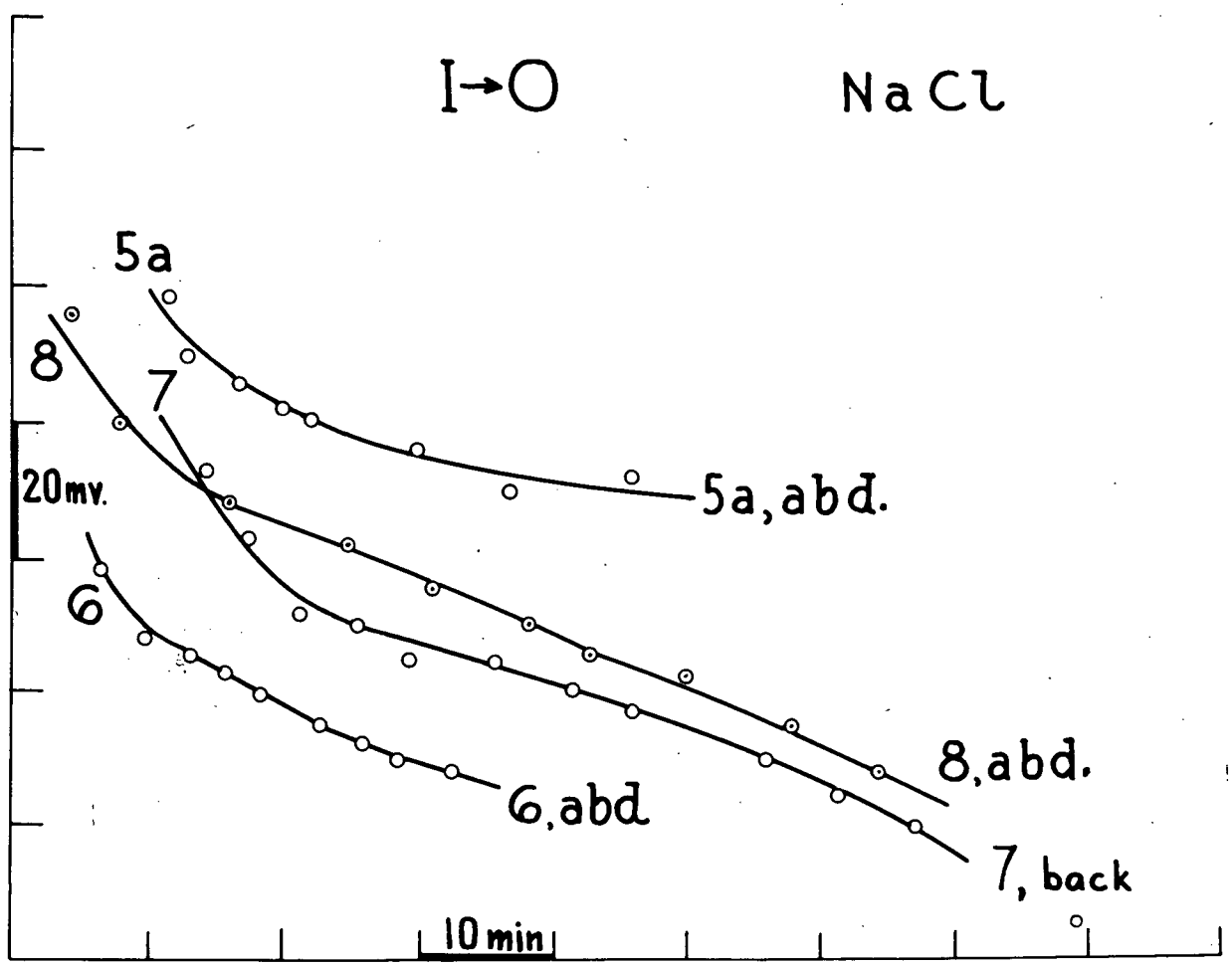

Fig. 12. Potential changes during diffusion process

initial and later stages can be deduced from the slopes of the curves.

Referring to Fig. 11, we observe a sharp fall of potential for diffusion in the direction outside to inside (O→I), where the inside of the skin faces the probe (IFP). After about 20 minutes from the beginning of the diffusion process, the curves reach a minimum, and thereafter show a slight increase.

Fig. 12 gives the results of NaCl diffusion under the condition (I O) and OFP. In this case, no sharp potential decrease was found in the first part of the diffusion process and no minimum was reached.

For the diffusion of glycine and alanine through frog skin under the condition, O→I and IFP (Fig. 13), there is a rapid fall of potential at the beginning, and a somewhat slower fall at the end. In Fig. 14, the potential changes during the diffusion of amino acids through frog skin is shown for the case I→O and OFP. For glycine, the potential variations are relatively small and show fluctuations of the order of 40 mv. in 20 minutes, becoming smaller with increasing observation time. Alanine, however, gives a curve for the condition, I→O and OFP, (Fig. 14), which resembles initially the alanine curve in Fig. 13, for (O→I) and IFP. The curve under the condition, (I→O) and OFP, does differ, however, from that under the condition,

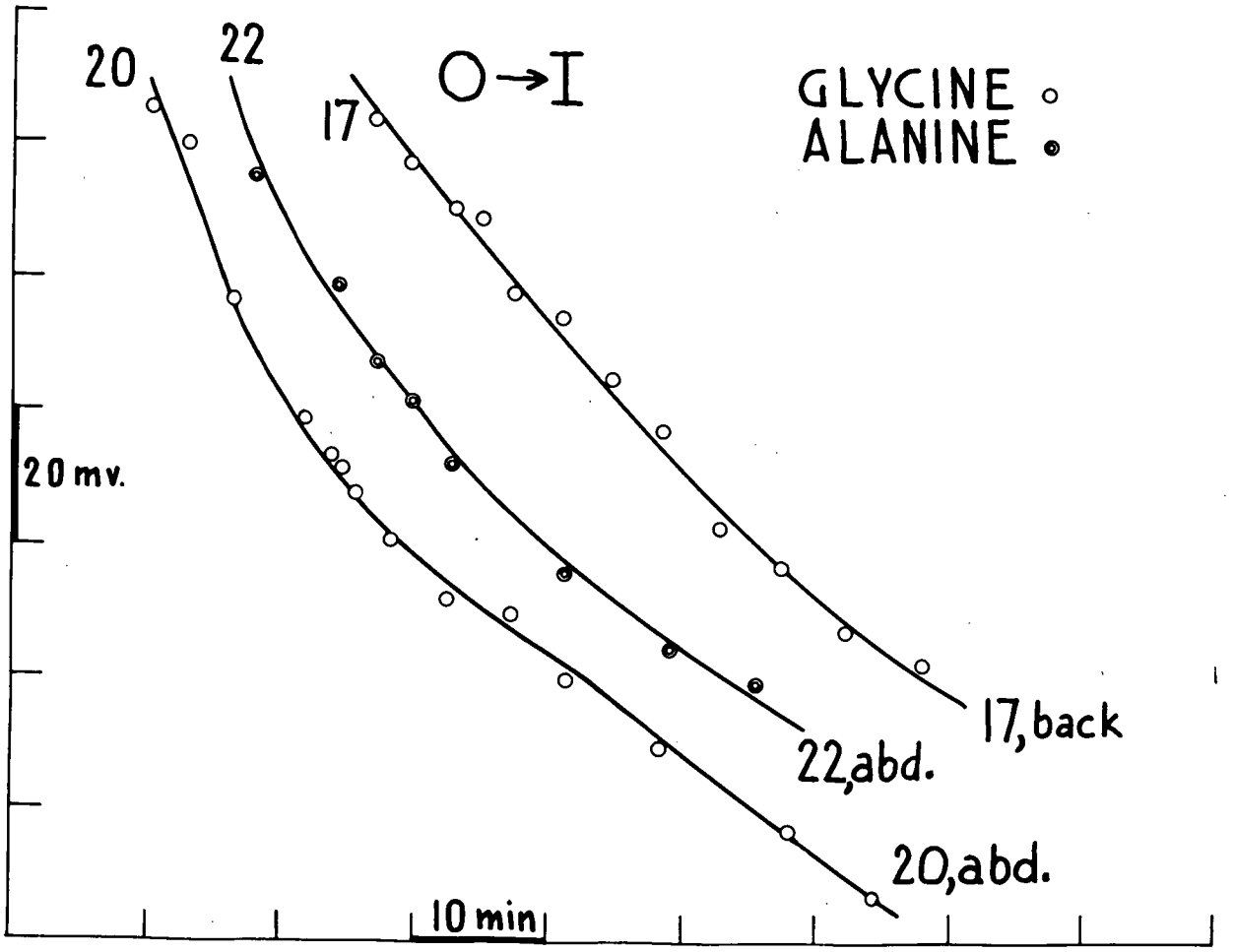

Fig. 13. Potential changes during diffusion process

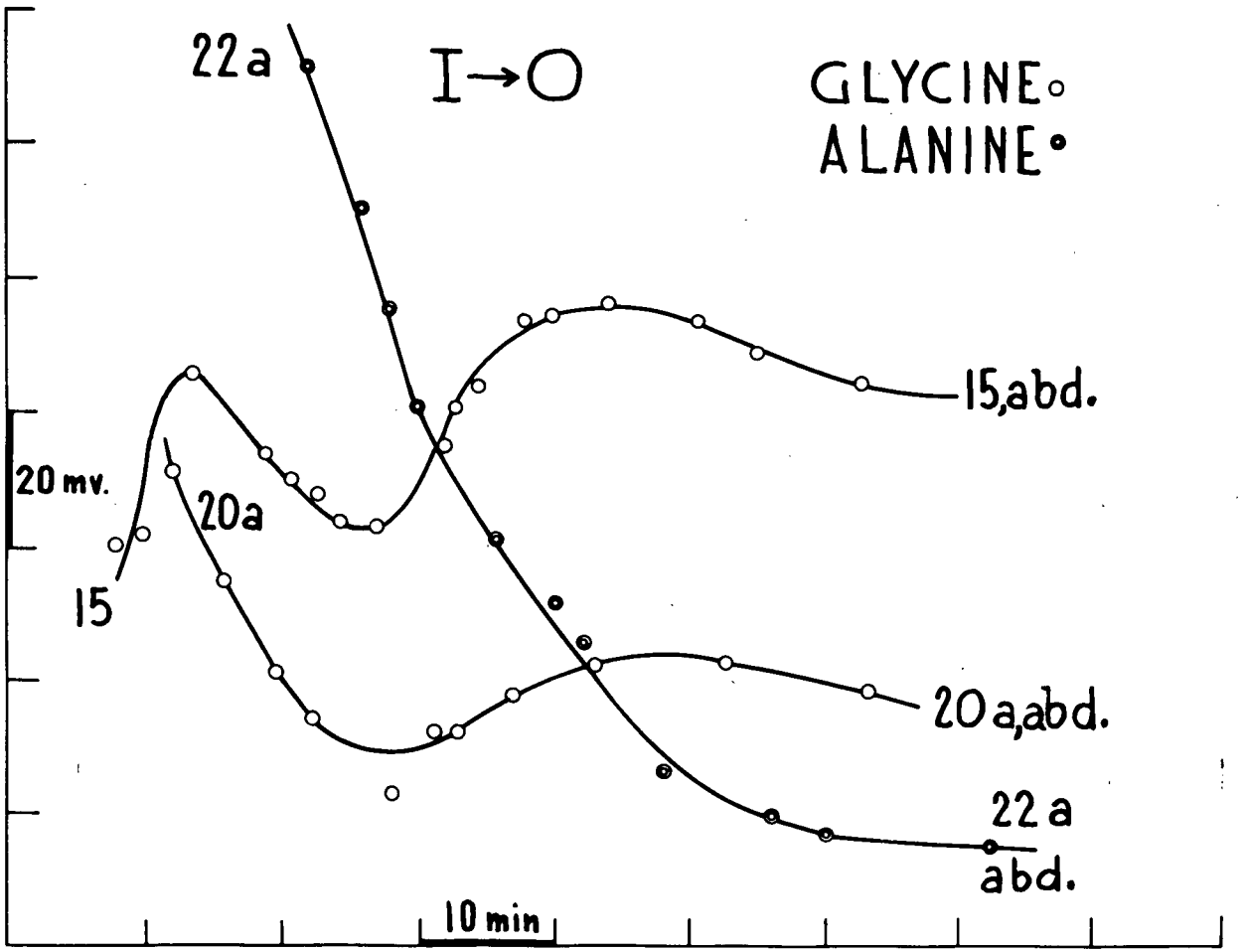

Fig. 14. Potential changes during diffusion process

($I \to 0$) and IFP, in that the former reaches a minimum value, whereas the latter continues to decrease slowly.

In all cases, the rate of potential variation indicates a corresponding rate of influx of solute into the membrane. The correlation between the measured rates of potential changes with the observed actual diffusion rates will be made in the following chapter. It should be kept in mind, however, that in our experiments we deal not with true through-diffusion processes, but only with influx of material into the frog skin.

E. DISCUSSION OF THE RESULTS

a. The structure of frog skin and the location of the electric field.

The general result of the potential measurements with frog skin membrane (D,a) was the existence of a potential difference of about 100 mv. (Fig. 9 and 10). This is in agreement with earlier investigators (6, 8, 13, 14, 17).

In answer to the question of the values which the electric field in the membrane may reach, histological evidence has to be examined. The skin consists mainly of two layers, denoted as the mesodermal corium and the ectodermal epithelial layer. The corium, which consists of connective tissue containing blood vessels and muscle cells, is expected to have no specific property of permeability. The total thickness of the frog skin is 0.1 to 0.2 mm. The epithelium consists of a large number of layers, distinguished as stratum squamosum, epithelium, stratum germinativum, and membrana basilaris; the latter is about 60 micron from the skin surface. The highest metabolism and cell division takes place in the stratum germinativum,

consisting of cylindrical cells (10 - 20 microns long and 7 microns thick), which are oriented perpendicular to the basal membrane.

According to several investigations, the bioelectrical potentials in frog skin are affected by the oxygen supply and decrease with falling oxygen tension. The location of the greater part of the membrane potential in or close to the stratum germinativum is suggested. Meyer and Bernfeld (14) conclude that it is the thin basal membrane, separating the epithelial layer from the corium, which is the actual seat of the layer part of the skin potential. This homogeneous membrane has a thickness of the order 1 micron, or 10^{-4} cm. and if we assume that a potential difference of 100 mv. = 10^{-1} v. exists across such a membrane, the electric field across the membrane is of the order 10^{3} v./cm. Across thinner parts of the basal membrane the field could reach much higher values. It might also be possible to assume that the potential difference is located between the highly metabolic stratum germinativum cells, and the basal membrane. In this case, a thickness of the interstice of 10^{-6} - 10^{-7} cm. may exist, and under this assumption, electric fields of intensities 10^{5} - 10^{6} v. /cm. can be expected. The stratum germinativum cells in normal frog skin, would have to be of negative charge, and the basal membrane of positive charge.

b. The irreciprocal permeability of frog skin for sodium chloride.

The experiments of Wertheimer (19) on the diffusion of solutes through frog skin were performed with skin of the hind legs of frogs, a fact, which, one may assume does not hunder a comparison with the present measurements in which skin of the abdomen and the back were used.

In his NaCl-diffusion experiments, Wertheimer analyzes the concentration of the chlorine ion only, and finds that there is a much greater transport of chlorine from outside to inside (O→I) than in the reverse direction (I→O); in fact, the skin showed a more or less perfect impermeability for chlorine ions in the direction inside to outside (I→O) when the diffusion took place from Ringer's solution instead of from NaCl solution.

A later paper by Huf (11) shows that isolated frog skin transports chloride ions from O→I, even when both sides are bathed with Ringer's solution. Krogh (12) observed that living frogs absorb salt in the direction O→I from a 0.01 millimolar solution. More recently, Ussing (17) studied the transport of chlorine with the use of the isotope Cl^{38}, and finds that the chlorine influx parallels the influx of sodium observed with the help of

the isotope Na^{24}. It is to be expected that the transfer of both chlorine and sodium ions is similar, since a separation of these ions could not take place except to a very small degree because of strong electrostatic attraction between the ions. Ussing found that the chlorine influx is somewhat smaller than that of sodium.

Knowing the direction of the electric field in the frog membrane, one can understand that chlorine ions are carried electrostatically from O(-)→I(+). According to our findings (Fig. 11), when the potential of I is being measured (IFP), a rapid fall of potential occurs initially in consequence of the influx of NaCl in the direction O→I. The fall of potential on the inside can be explained by the transfer of the negative charges of the chlorine ions. The minimum, and the following slight increase in the curve, should then be due to the positive sodium ions which, having a smaller mobility, accumulate in the membrane at a slower rate than that of the chlorine ions. Its smaller mobility may be due to a hydration layer or to complex formation (see below), and to the fact that free sodium ions are hindered by the direction of the electric membrane field.

In Fig. 12, we have a slow decrease of the potential of the outside of the skin (OFP), which is negative relative

to the inside. Obviously, in this case the electric field in the membrane opposes the shift of the negative chlorine ions, and favours the motion of the positive sodium ions. We see, however, in Fig. 12, that the potential on O(-) decreases, indicating an increase in negative charge. The excess of negative charge can be explained through the assumption that the diffusion of sodium ions is hindered in the direction I→O because of a special unidirectional mechanism operating in the direction O→I. It might well be that the original surface potential of the frog skin is considerably lower than the first observed potential value; then an initial influx of free positive sodium ions increases the potential of the surface, and a subsequent transfer of the chlorine ions produces the slow decline of potential. The increase of potential, taking place in the first few minutes, could not be observed because of the time required to adjust the measuring instrument.

The result of Fig. 11 shows that at the beginning of diffusion, the chlorine ion has the greater mobility, but the sodium ion accumulation follows it closely, and apparently in time, reverses the effect of chlorine on the potential. The accumulation of ions is a consequence of the fact that the diffusing material cannot pass through the skin into a liquid medium.

According to Ussing (17), a special mechanism of sodium ion transport operates in frog skin in the direction O→I, where the sodium ion diffuses through an electric field as an electrically neutral complex through combination with a larger anion.

In conclusion: The greater rate of change of potential from O→I (Fig. 11), as compared with the case I→O (Fig. 12) indicates an irreciprocal transfer mechanism in frog skin for NaCl in the direction O→I. This active transfer of NaCl can be explained by the shifting of the negative chlorine ions produced by the electric field, and by the transport of the positive sodium ions through a mechanism involving complex formation.

c. The active transport of amino acids.

Wertheimer (19) has studied the diffusion of amino acids through frog skin in experiments similar to those with NaCl (E,b). The results of his measurements have been stated as follows (19, p. 389):

> "It can be followed from all experiments on amino acids, polypeptides and peptones that the permeability is better from outside to inside, mostly much better than in the opposite direction. M ost of the substances are practically impermeable from the inside to outside....There is little doubt that for the single amino acids there are qualitative

> differences in permeability. One extreme is tyrosine which does not pass in both directions as was shown by Millon's reagent. On the other side stands glycine, where the difference in permeability in both directions was smallest. In intermediate position are the other amino acids, polypeptides, and peptones, which pass from outside to inside easily but in the other direction, hardly at all. One cannot see how to explain these differences. There seems to be no effect of the size of the molecule."

Comparing Wertheimer's findings with the observations of potential changes of frog skin, shown in Fig. 13 and 14, one can see that the relatively great diffusion rate for the case O→I (as found by Wertheimer) is parallel to the relatively large and continuous change in potential for O→I shown in Fig. 13. Referring to Fig. 14 (I→O), we observe for glycine that a steady state is reached after some fluctuations of potential have taken place. For alanine there is first a considerable fall of potential, but later a steady state also seems to be reached (differently from the behaviour of the alanine in Fig. 13). This is in agreement with the fact that Wertheimer finds little diffusion in direction I→O. One would therefore assume that the potential changes give a clear picture of the asymmetric permeability of frog skin, in agreement with the established irreciprocal permeability found by Wertheimer, and others.

The irreciprocal permeability of frog skin suggests an active transfer of amino acids through it, that is, the

existence of a mechanism in the membrane which actively shifts amino acids in one direction, comparable to a pumping mechanism, and which hinders the passage of amino acids in the other direction (Cf. Sec. A,b). Such active transfer of amino acids has been observed by Höber and Höber (9), experimenting with intestinal loops of rat, and more recently has been suggested by Christensen et al. (4).

> ".....who have made comparisons in the rate of amino acid concentrations in extracellular and intracellular fluids, and have found them to be consistently higher in the latter. They have found the intracellular amino acid concentration to be particularly high in fetal guinea pig tissues and associate the rapid growth of the fetal tissue with this finding. They have been led to postulate the presence of an active amino acid concentrating mechanism in the cell membrane". (Quotation from (21)).

The same difference in amino acid concentrations might be expected between normal and cancerous growth, and therefore an analogous amino acid active transfer mechanism may exist in carcinoma.

It is obvious that the understanding of the active transfer phenomena, particularly for amino acids, is important to the understanding of the physiology of transport and growth processes. Several suggestions have been made about transport mechanisms effective in membranes, though none, to our knowledge, has paid special attention to find an explanation for the irreciprocal diffusion of

amino acids. Some of the mechanisms assume that a special carrier complex is present in the membrane; and that an ion or molecule is attached to that complex on one side of the membrane, and is liberated on the other side, the carrier complex being confined to the membrane. The conditions regulating the attachment on the one side, and the liberation on the other side of the membrane have remained more or less obscure, but have been explained under the assumption of differences in hydrogen concentrations in the two liquids in contact with the membrane, or through differences in chemical composition and reactivity (3). The effect of an electric field in the membrane, as we saw before (E,b), can be used to explain the transport of one type of ion.

In the case of amino acids, the larger number of molecules present in solution exist in form of neutral particles, so-called dipolar ions. Their unidirectional transport through a membrane might be open to explanations along the lines of the active transport mechanisms referred to above.

A possible mechanism of active amino acid transport not requiring any ad hoc assumptions — suggested to the writer by Dr. Otto Bluh -- is offered in the following.

The dipole molecules, as electrically neutral particles, diffuse unhindered into a membrane even if the membrane possesses a strong electric field. Once they have entered the field, however, they undergo dissociation as a direct effect of the electric field on the dipole molecules. This dissociation is known under the name of the "Wien dissociation effect" (cf. 2), and takes place to an appreciable degree only if the electric field intensity is of the order of 10^4 to 10^6 volt/cm. The dissociation effect for amino acids was first investigated by Blüh and Terentiuk (2), who observed considerable increases (20 percent) of electric conductivity of amino acids (and of proteoses, etc.) in electric fields of the order 10^5 volt/cm. The rise in conductivity observed in amino acids (and certain other weak electrolytes) can be explained on the assumption of the removal of a proton from the dipolar ion, thus producing temporarily a negative amino acid particle.

In section (E,a) it was shown tentatively that the electric field intensity in living membranes may attain values of 10^5 or 10^6 volt/cm. From the histological and physiological evidence, it can be assumed that the field is located in the thin basal membrane or close to it, and that it may reach the field intensities required for the dissociation effect. As has been suggested before, certain thin parts of the basal membrane may be the foci of high

electric fields, and serve as "portals" for the unidirectional entry of amino acids. These points could be different from the points or areas responsible for the transfer of negative ions present in strong electrolytes. For other membranes, as those of marine eggs, studied by Cole (5), thinknesses of the order of 10^{-6} to 10^{-7} cm. have been found with the help of capacity measurements.

If we assume the existence of a strong electric field in the frog skin membrane, the amino acid molecule conceivably would dissociate upon entering the field space, lose its positive charge, and thus be shifted as a negative particle in direction towards the positive side of the membrane, irregardless of the side from which the molecule enters. In case of frog skin we know that diffusion of amino acids takes place more or less exclusively from outside to inside, that is from the negative to the positive side. The electric field thus actively shifts amino acid molecules as negative ions in the direction $O \rightarrow I$, and prevents the transport in the opposite direction. A transport of positive charge, either of protons, or of other positive ions, will have to take place simultaneously, but the negative amino acid will recombine, upon reaching the field-free space, with proton of the surrounding medium.

This sort of pumping mechanism for amino acids may be assumed to operate in all cases where an active transfer or unidirectional diffusion of amino acids has been observed, e.g. in the intestine of the rat, in fetal growth etc. The Wien dissociation effect might also play a role in the membrane diffusion of weak electrolytes in general. Correlations between irreciprocal diffusion and the direction of the electric field in the membranes concerned, could be easily established, whereas the question of the intensity of the field will likely always remain debatable. The mucosa of the intestinal wall of frog, e.g. has been found negatively charged relative to the charge of the deeper layers, in agreement with the direction of net diffusion flow. The hypothesis of active transfer of amino acids, here presented, may prove to be of heuristic value in the investigation of growth processes, and particularly those of carcinomatous growth.

F. SUMMARY

The vibrating probe voltmeter for the measurement of bioelectric potentials by Blüh and Scott has been used in an improved form for measurements of frog skin potential differences.

In good agreement with earlier findings the observed frog skin potential differences were found to be of the order of 100 millivolts, and the polarity such that the inside of the skin was positive relative to the outside.

Bioelectric potential measurements were made during the influx of sodium chloride and amino acids in aqueous solutions into frog skin in either direction. Characteristic potential changes were observed for different substances and opposite directions of flux, and have been used to demonstrate the asymmetry of frog skin permeability.

Transfer mechanisms for sodium chloride and amino acids have been advanced from the standpoint of the assumption that an electrical field exists in the frog skin membrane.

G. ACKNOWLEDGEMENTS

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