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Capacitance changes in frog skin caused by theophylline and antidiuretic hormone

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1. Impedance loci for frog skins have been calculated by computer analysis from voltage transients developed across the tissues.

2. Attention has been paid to simultaneous changes in conductance and capacitance of skins treated either with antidiuretic hormone (ADH) or with theophylline. These drugs always caused an increase in conductance and usually the skin capacitance also increased. However, changes in conductance were not correlated with capacitance changes.

3. Changes in capacitance caused by the drugs may represent pore formation in the barrier to water flow, since both drugs increase hydro-osmotic flow in epithelia. If this interpretation is correct, then 0·14% of the membrane area forms water-permeable pores in response to a maximal dose of ADH. This value is somewhat less than the value obtained previously (0·3%) by graphical analysis.

4. A theoretical account is given of the relative accuracy of the computer method and the graphical method for voltage transient analysis.

Antidiuretic hormone (ADH) increases the conductance and capacitance of the outward facing membranes of isolated frog skin (Cuthbert & Painter, 1969). It was suggested that the conductance increase was due to an increase in sodium permeability, while the capacitance increase resulted from the creation of water-filled pores in the membrane. Theophylline also increases skin conductance, but the increase in permeability is to chloride ions rather than sodium ions (Cuthbert & Painter, 1968a, b). In this paper the effects of theophylline on membrane capacitance are reported.

An important question is whether the conductance and capacitance changes are obligatorily linked, which in turn raises the important question of whether ions and water molecules move through common channels in the membrane. To answer these questions attention has been paid to any correlation existing between conductance and capacitance increases in skins treated with ADH and with theophylline. Previously (Cuthbert & Painter, 1969), analysis of the experimental data was by a lengthy and tedious graphical method which precluded a rigorous examination of these problems, but here analysis has been carried out using a computer.

Methods

The experimental methods were identical to those described previously (Cuthbert & Painter, 1969). Theophylline (as theophylline hydrate, B.D.H.) was dissolved in Ringer solution and a small volume was added to the solution bathing the inside of the skin to give the appropriate final concentration.

Theoretical

The a.c. capacitance can be calculated from the formula

$$C = 1/2\pi\ Rfo \quad \ldots\ldots (1)$$

where C is capacitance, R is the difference in resistance at zero and infinite frequency, and fo is the fundamental frequency, that is the frequency at which the reactance is minimal (note reactance is negative). In Fig. 1 resistance and reactance are plotted in the complex plane to give an impedance locus. The impedance, Z, at a particular frequency, f, is given by the vector of length l, and phase angle ϕ. The computer programme was designed to print out values of l and ϕ for frequencies of 0–500 c/s in intervals of 10 c/s. Thus a particular impedance locus was derived from fifty points, compared with only four or five points obtained by the graphical method. The value of fo, the fundamental frequency, could be readily and accurately determined by interpolation. As it was not possible to know fo with great accuracy, in our previous paper we were not able to determine the a.c. capacitance, but only the average a.c. capacitance over the range covered by the four or five experimental points. The accuracy with which individual impedance values could be determined was much increased by computer analysis (see **Appendix**).

Results

Effects of theophylline

The effect of theophylline (1 mM) on the impedance loci of frog skin is shown in Fig. 2. The points were obtained by computer analysis and the loci represent ideal curves. Before addition of the drug the locus was almost a perfect semicircle with a polarization angle of 2°, indicating that the polarizable element was behaving as

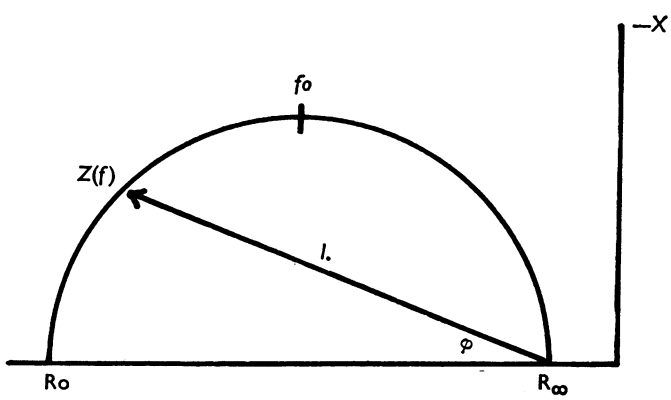


FIG. 1. Impedance locus diagram.

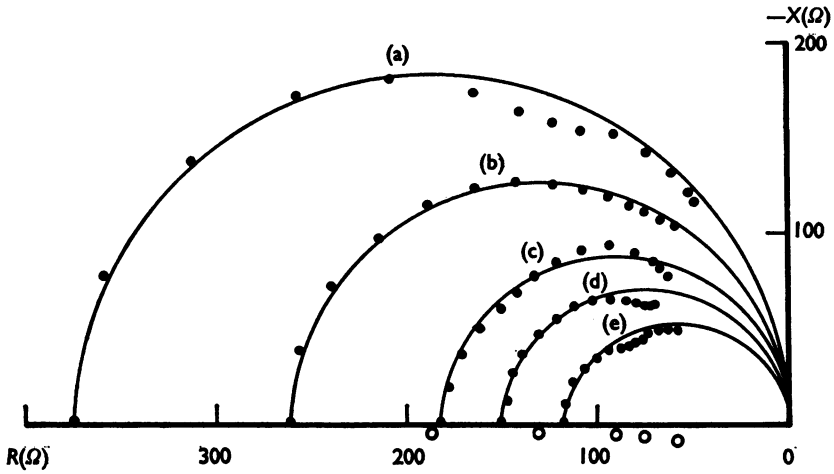


FIG. 2. Impedance loci for a piece of frog skin (4·5 cm²) before (a) and 5 min (b), 10 min (c), 15 min (d) and 35 min (e) after addition of 1 mM theophylline to the inside surface of the skin. Closed circles show the values for 0 to 130 c/s in steps of 10 c/s. Open circles show the centres of the impedance loci.

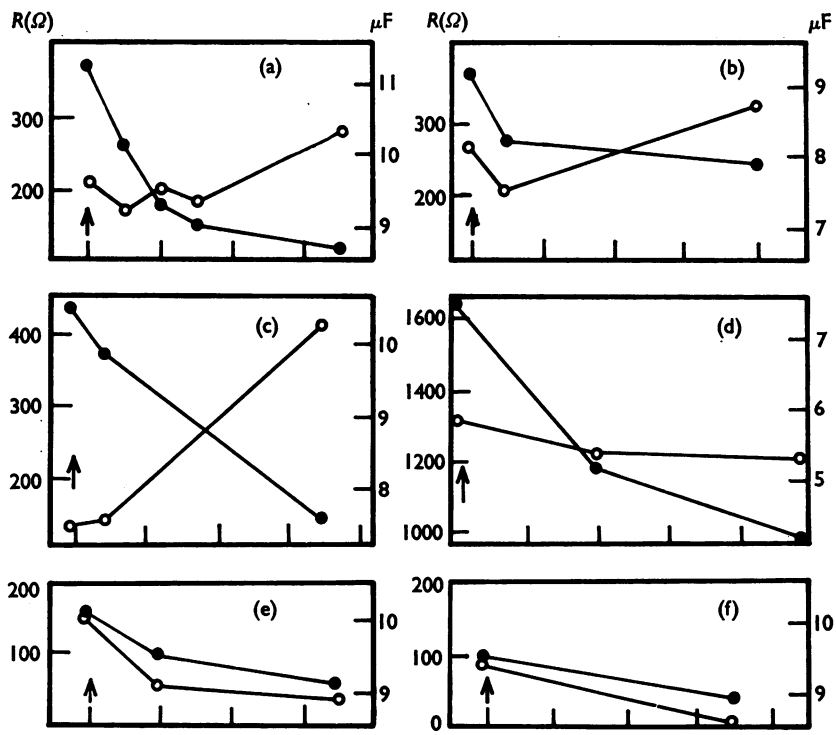


FIG. 3. Changes in resistance (●) and capacitance (○) in pieces of frog skin (4·5 cm²) after addition of 1 mM theophylline to the inner bathing solution. The drug was added at the time indicated by the arrows. The time intervals on the abscissa are 10 min.

a simple condenser. The difference between the resistance at zero and infinite frequency was 374 Ω. This resistance (called *Rp* in our previous paper) is believed to represent the cell membrane resistance and does not include the resistance of the electrodes, Ringer solution or cell cytoplasm. Experiments described in our previous paper (Cuthbert & Painter, 1969) showed that the greater part of the skin resistance was located at the outer facing membranes of the skin. Theophylline increases the conductance of these membranes because 35 min after addition of theophylline (1 mM) the resistance had fallen to 120 Ω (representing a 68% fall in resistance) while the polarization angle increased progressively to 8°. In this situation the polarization element no longer behaved as a simple condenser but as a frequency dependent impedance of constant phase angle. Using equation (1) (Cole formula) the a.c. capacitance of the skin was calculated for before and after treatment with theophylline. Before theophylline was added the fundamental frequency,

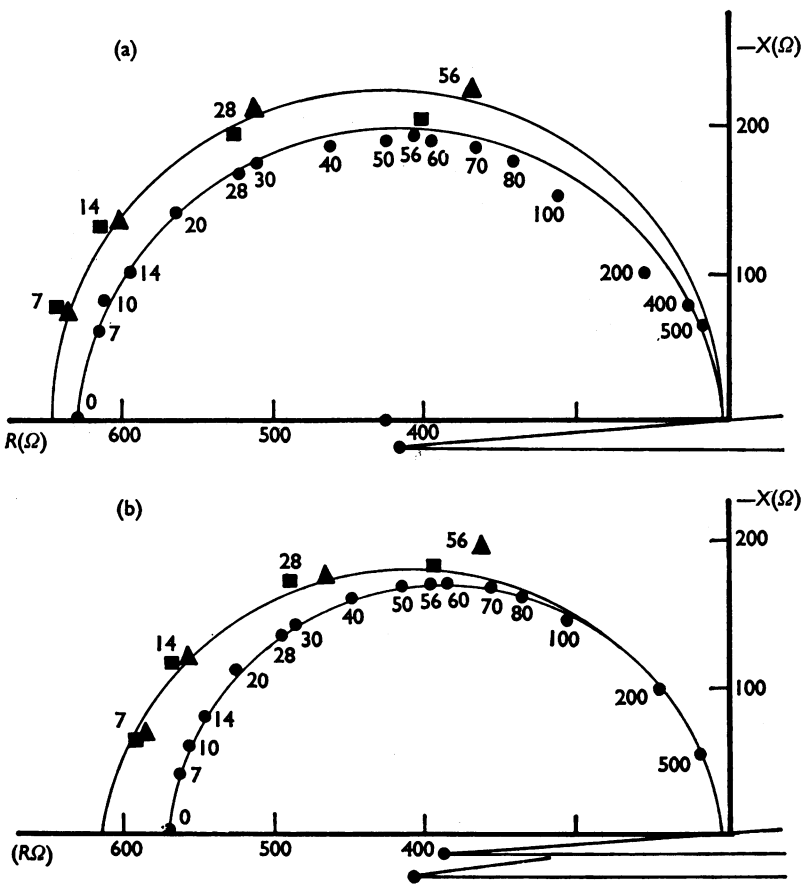


FIG. 4. Impedance loci for pieces of frog skin (4·5 cm²) before (a) and 30 min after (b) treatment with ADH (300 m-u./ml.) added to the inner bathing solution. In (a) and (b) the smaller semicircle indicates values obtained by computer analysis. Frequencies (c/s) are indicated by the points. The larger semicircles indicate values calculated from graphical analysis according to Teorell (1946) (■) and according to Cuthbert & Painter (1969) (▲). In this experiment the resistance at infinite frequency has been plotted so as to include the resistance of the electrodes, Ringer and cell cytoplasm. The resistance of the latter was approximately 10 Ω.

fo, was 44·5 c/s, corresponding to an a.c. capacitance of 2·14 μF/cm^2. After 35 min the fundamental frequency had risen to 128·3 c/s and the a.c. capacitance had increased to 2·30 μF/cm^2. Figure 3a shows the relation between the resistance fall and the capacitance increase in this experiment. Similar plots for five other experiments in which theophylline (1 mM) was added to the inside of frog skins are shown in Figs. 3b–f. Although the capacitance increased in three experiments after theophylline was added the increase was not correlated with the resistance fall. In Figs. 3a and 3b it can be seen that the conductance increase was almost complete before the capacitance increased. In three experiments (Figs. 3a, b and e) the capacitance initially fell when theophylline was added and in three of the six experiments there was an overall fall in capacitance. In one of these latter where the initial skin resistance was very high (Fig. 3d) an enormous conductance change was accompanied by only a minor change in capacitance.

In these six experiments theophylline caused a rapid fall in skin potential, as was reported previously (Cuthbert & Painter, 1968b). Capacitance changes were not correlated with either changes in potential or polarization angle. Statistical analysis of these six experiments showed that capacitance increase caused by theophylline was of very low significance ($P=0{\cdot}25$).

Effects of ADH

Figure 4 shows impedance loci for a piece of frog skin before and after treatment with ADH added to the inner surface of the skin. This figure compares the loci obtained by graphical construction (both by the method used by Teorell (1946) and by the slightly modified method used previously by us (Cuthbert & Painter, 1969) and by computer analysis. The graphical results differ from the computer solutions in the way described in **Appendix**. The average a.c. capacitance increased from 1·81 μF/cm^2 to 1·95 μF/cm^2 according to the loci obtained by graphical analysis. The actual a.c. capacitance increased from 1·60 μF/cm^2 to 1·69 μF/cm^2 from the loci obtained by computer analysis. Both methods show similar percentage capacitance increases, 7·9% and 5·7% respectively for the graphical and computer values. The polarization angle remained at 5° according to the computer derived loci, whereas the graphically derived loci indicated an increase from 0° to 9°.

TABLE 1. *A.C. capacitance of frog skin before and after treatment with ADH* (300 *m-u./ml.*). *ADH was allowed to act for approximately* 30 *min.*

Experiment	Capacitance μF/cm^2		Percentage increase in capacitance	Percentage increase in conductance
	Before ADH	After ADH		
203	1·67	1·69	1·2	36
303	1·69	1·85	9·3	73
309	1·41	1·44	2·2	24
365	2·00	2·26	12·9	83
366	1·60	1·69	5·7	17
375	1·94	2·14	10·7	23
389	1·60	1·94	21·4	123
390	2·30	2·36	2·9	102
394	2·18	2·25	3·2	52
396	2·47	2·53	2·3	50
414	2·10	2·18	3·3	28
Average values	1·905	2·030	6·8	—

ADH caused a statistically significant ($P=0{\cdot}0025$) increase in skin capacitance.

Since it has been shown previously (Cuthbert & Painter, 1969) that ADH does cause a statistically significant ($P=0{\cdot}0025$) increase in capacitance, when the average a.c. capacitance over the frequency range covered by the graphically determined values were considered, it was thought to be worth while to recalculate the data from previous experiments by computer. Table 1 shows the a.c. capacitance of skins for eleven experiments in which ADH was added. The data can be compared with Table 1 in our previous paper. The values obtained are very similar to those obtained before and analysis shows that the capacitance increase due to ADH

TABLE 2. *Correlation indices*

Experiment	% resistance change / % capacitance change	% conductance change / % capacitance change	Conductance change (mhos) × 100 / Capacitance change (μF)
203	25·0	30·0	0·875
303	4·5	7·8	0·173
309	8·8	10·9	0·497
365	3·5	6·4	0·110
366	2·6	3·0	0·099
375	1·7	2·1	0·177
389	2·6	5·7	0·243
390	17·4	35·1	0·710
394	10·7	16·2	0·581
396	14·7	22·1	1·104
414	6·7	8·4	0·158

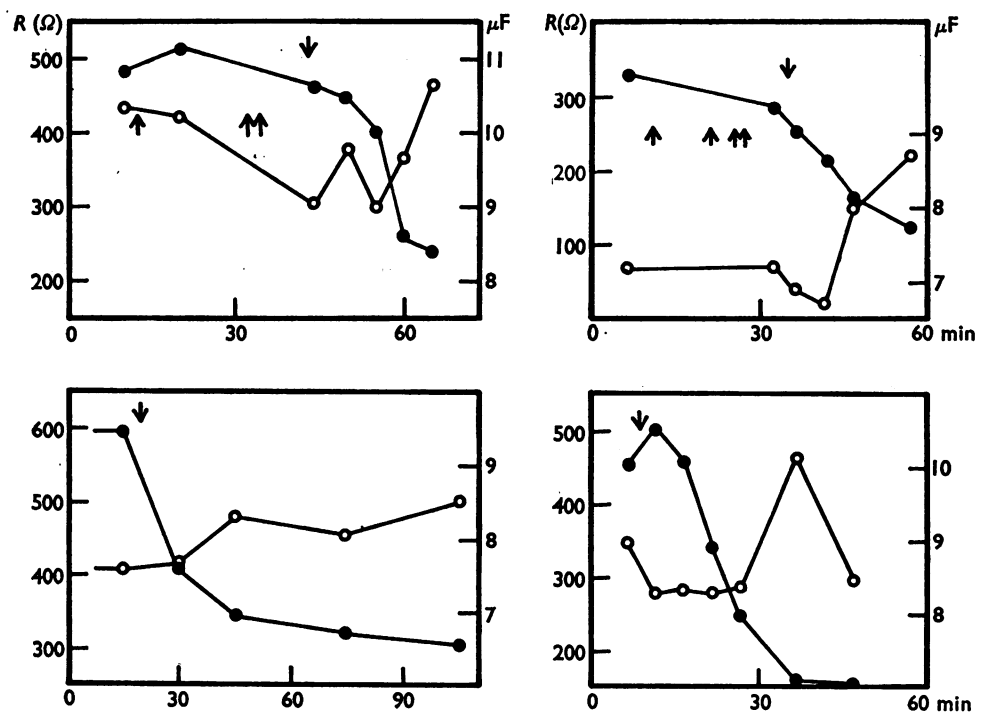


FIG. 5. Changes in resistance and capacitance of pieces of frog skin (4·5 cm²) after addition of ADH (300 m-u./ml.) to the solution bathing the inside of the skin. Addition of chlorbutol (5×10^{-4}M) is indicated by each upward arrow and ADH by downward arrows.

is statistically significant at the $P = 0{\cdot}0025$ level (using comparison of paired observations by *t*-test, Goldstein, 1964).

In addition, since we now have accurate values for the a.c. capacitance we have tried to correlate the increase in capacitance caused by ADH with the conductance (or resistance) change which also occurs after hormone treatment. Table 2 shows the values of three different correlation indices. These are the ratios of percentage resistance change to percentage capacitance change, percentage conductance change to percentage capacitance change and absolute conductance change to absolute capacitance change. The values of the indices vary over a 15-fold, 14-fold and 11-fold range respectively. If the changes in conductance were linked with capacitance changes then these indices would be expected to be constant. The variation in the indices would suggest that these two events are not obligatorily linked.

We have also used the computer programme to derive impedance loci for intermediate times between addition of ADH and its maximal effect. The experimental data for this were available from earlier experiments, but analysis was deferred until now due to the tedium and inaccuracies associated with the graphical method. Four experiments were chosen, three showing a large and one a minor capacitance increase. Intermediate resistance and capacitance values for these four experiments are shown in Fig. 5. It is clear from these that a capacitance increase and resistance fall occur after ADH treatment, but that in general the major resistance changes precede the major increases in capacitance. Since commercial ADH (Pitressin, Parke Davis) contains chlorbutol as a preservative we were concerned that the effect on capacitance may have been due to this substance. Accordingly in two of the experiments illustrated in Fig. 5 the skin was exposed to an excess of chlorbutol before the hormone was added. Chlorbutol had only minor effects on resistance and capacitance.

Discussion

It is clear from the results presented in this paper that conductance and capacitance increases in frog skin caused by drugs are not concomitant events. Even though ADH causes a statistically significant ($P = 0{\cdot}0025$) increase in capacitance this was not correlated with the increase in skin conductance, as shown by the variation in the various correlation indices and the discrepancies between the time courses of the two events. The increase of skin capacitance associated with the action of theophylline was only of low significance, and in one experiment there was a massive conductance increase accompanied by a slight decrease in capacitance. Furthermore in experiments where both the conductance and capacitance increased in response to treatment with drugs the time courses of the increases were dissimilar.

ADH and theophylline are known to increase sodium transport across and water flow down an osmotic gradient in various epithelia (Leaf, 1967). ADH and theophylline differ in their actions on sodium transport in that ADH acts by increasing the permeability to sodium ions (Civan, Kedem & Leaf, 1966) while theophylline increases the permeability to chloride ions (Cuthbert & Painter, 1968a, b). In an earlier paper (Cuthbert & Painter, 1969) we suggested that the conductance and

capacitance increases in frog skin caused by ADH were due to the formation of water-filled sodium permselective pores, with the proviso that the capacitance increase may have been due in part " to the formation of additional pores in the barrier to water flow." The introduction of water into the cell membrane seems to be the most probable way to account for the increase in skin capacitance. Considering the formula for a parallel plate condenser

$$\frac{C=4\pi\ AK}{d} \qquad \text{.....(2)}$$

where C is capacitance, A is area, d is the thickness of the dielectric and K the dielectric constant, then a 7% increase in area or decrease in dielectric thickness would be necessary to explain the capacitance increase caused by ADH, that is on average 1·905 to 2·03 μF/cm^2. However, if water-filled pores were introduced into the membrane these would represent regions of high permittivity, since the dielectric constant of water (80) is high compared with membrane lipids (3–5). Only 0·14% of the membrane area would need to be occupied by such pores to account for the average capacitance increase of 0·12 μF/cm^2. This value is lower than our previous estimate of 0·3% of the total area, which was based on average capacitance values determined by the graphical method.

There are a number of pieces of evidence in favour of a coupled movement of ions and water through biological membranes. Water movement across the gall bladder (Diamond, 1962 ; Diamond & Harrison, 1966) and the intestine (Smyth & Wright, 1966) is associated with streaming potentials due to a drag on cations. Alternatively cations moving through membranes may drag water molecules along with them, causing electro-osmotic effects as shown by frog skin (House, 1964) and nerve (Stallworthy & Fensom, 1966). In addition some small uncharged solute molecules can move across membranes by solvent drag as was shown for urea in the urinary bladder (Leaf & Hays, 1962) and for acetamide and thiourea in toad skin (Andersen & Ussing, 1957). Evidence against the idea for a coupled movement of water and ions is that the effects of ADH on water and sodium movement can be dissociated by removing calcium from the bathing fluid (Petersen & Edelman, 1964). Also the polyene antibiotic amphotericin stimulates sodium transport across the toad bladder without affecting water flow (Lichtenstein & Leaf, 1965). It seems clear from this work that the paths for water and ions which are opened by ADH and theophylline are not common, although there may be other drug insensitive pathways which can accommodate water and ions and so explain electro-osmotic and streaming potential effects. The failure of theophylline to increase capacitance in some experiments may represent a failure of these skins to show an increase in water flow. Water flow down an osmotic gradient in frog skin is small (about 7 μl./cm^2 per hr with a gradient of 200 mosm., unpublished observations) and is not much increased by ADH. We can find no data about the effects of theophylline on hydro-osmotic flow in frog skin, and would particularly like to know if it regularly increased water flow, in view of the failure in three experiments to measure any increase in skin capacitance after treatment with this drug. Experiments are now in progress with other epithelia to test the hypothesis that the capacitance increase seen in frog skin represents the formation of pores in the barrier to water flow. To do this we are examining the effects of drugs on streaming potentials and making simultaneous measurements of capacitance and water movement.

Appendix

In this appendix an account is given of the increased accuracy which results from computer analysis compared with the graphical method. Sine wave analysis of voltage transients is performed by considering a stepped wave which approximates to the curve to be analysed. Figures 6a and 6b show two stepped waves which approximate to the real curve. Figure 6a represents the type of stepped wave employed by Teorell (1946), while Fig. 6b shows the type used by us in our previous paper (Cuthbert & Painter, 1969). (Our method was similar to that employed by Bedford & Fredendall (1942) and did not employ the correction for phase shift recommended by Teorell (1946), but usually gave loci passing through $R\infty$. We considered this essential since $R\infty$ was the most accurately determined point.) In the analysis consecutive steps of the approximate wave were added vectorially to give the final voltage vector, which was then converted to an impedance vector. The accuracy of the final vector is determined by the number of steps into which the transient is divided, that is the timing frequency, *tf* (number of steps/sec). The changes caused by increasing the timing frequency are illustrated in Fig. 7. An experimental voltage transient (that for the experiment illustrated in Fig. 4a) was divided up into a number of stepped waves corresponding to various timing frequencies. The heights of the steps were added vectorially at the appropriate angles to give the voltage vectors for a frequency of 20 c/s; these are shown in Figs. 7a–d. The voltage vectors were converted to impedance vectors and plotted in the complex

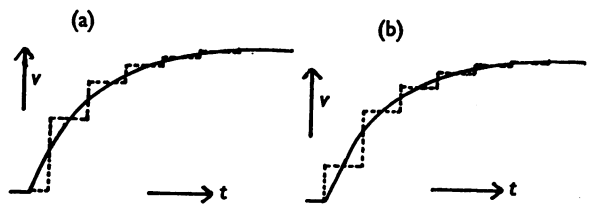
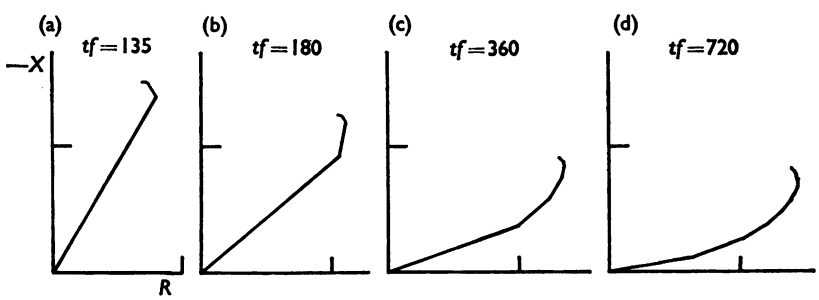


FIG. 6. Diagram showing the ways in which voltage transients can be represented by stepped waves which approximate to a real wave. (a) Stepped wave considered by Teorell (1964). (b) Stepped wave used previously by Cuthbert & Painter (1969).



FIGS. 7a–d. Graphical constructions of the impedance vector for a frequency of 20 c/s using various timing frequencies (*tf*). The voltage transient was taken from the experiment illustrated in Fig. 4a. The graduations represent 250 Ω.

plane in Fig. 8 (curve b) together with the computer solution for 20 c/s. At this frequency the graphical values for resistance approached the computer solution while the reactance values always remained too large. The procedure was repeated for two other frequencies, 7 c/s and 56 c/s. At the lower frequency (7 c/s) graphical values of the resistance eventually exceeded the computer solution at high *tf*s while reactance values were again consistently too large. At frequencies higher than 20 c/s, for example, 56 c/s, graphical resistance values were smaller than the computer solution while the reactance values were in excess of the computer solution unless very high *tf*s were used.

Normally, when the graphical method was employed one timing frequency was used throughout. Table 3 shows the deviation between the computer values and the graphical values when a *tf* of 1,000 was used for calculating impedance vectors at 7, 20 and 56 c/s. It can be seen that the error increases with increasing frequency, giving loci which are too large. Graphical values therefore vary in a complex but systemic way from the computer solutions. This inaccuracy is because it is impossible to divide the transient into sufficient steps for graphical solution. It is worth pointing out that the graphical values shown in Fig. 8 represent many hours of drawing and calculation, and even though the graphical method was used to its

TABLE 3. *Comparison of computer and graphical data*

	7 c/s	20 c/s	56 c/s
Graphical values	426, 77	351, 175	172, 227
Computer values	416, 60	363, 146	206, 188

All values shown are in ohms, the first figure in each pair refers to resistance and the second figure to reactance. The timing frequency (*tf*) for the graphical values was 1,000.

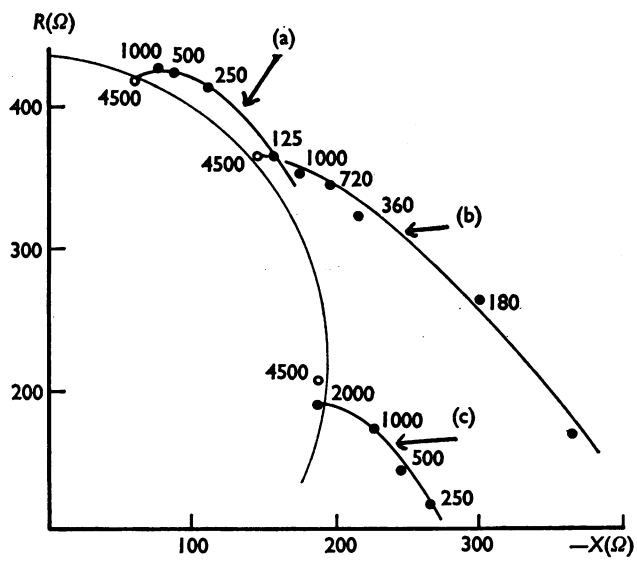


FIG. 8. Graphical (●) and computer (○) solutions for frequencies of (a) 7 c/s, (b) 20 c/s and (c) 56 c/s for the voltage transient from the experiment illustrated in Fig. 4a. The timing frequencies are shown against each point.

practically feasible limit, the values obtained were inferior to those from computer analysis. Reference to two papers by Teorell (1946, Fig. 10, and 1954, Fig. 5), the previous paper by these authors (Cuthbert & Painter, 1969) and to Fig. 4 in this paper shows that the graphical method gives impedance loci which are too large and explains, in part, why they fail to pass through the resistance axis at *Ro*. In this work a programme (Autocode) was developed which used a *tf* of 2,000 to 12,000 (depending on the transient to be analysed), whereas the highest *tf* used for graphical analysis was 1,000. The analysis was carried out on the Titan Computer, Mathematical Laboratory, University of Cambridge.

Our thanks are due to Dr. A. G. Robiette for devising the computer programme. One of us (E. P.) was in receipt of an M.R.C. Scholarship for Training in Research Methods.

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