THE UNIVERSITY OF ASTON IN BIRMINGHAM

TEST METHODS FOR ASSESSING TOPICAL FORMULATIONS

by

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SUMMARY

Three different techniques have been studied to evaluate the effect of topical formulations on human skin.

Measurement of electrical skin impedance as a non-invasive technique for the in vivo assessment of skin hydration is reassessed, with the aim of evaluating the moisturising effects of various therapeutic treatments. Monitoring of baseline untreated forearm skin suggests wide inter- and intra- subject variations. The equivalent circuit appropriate for modelling the skin has been elucidated. For the electrode configuration commonly used short-circuiting of the electrodes occurs during product assessment.

The performance of laser Doppler flowmetry for monitoring cutaneous vasodilation has been evaluated using various nicotinic acid esters as model compounds. Results indicate that the time taken for the nicotinates to elicit a response by the flowmeter, is the most reproducible parameter. This response varies with the concentration of nicotinate applied. The importance of the physicochemical properties of the products is emphasized.

The final technique examined here has been developed to assess the irritation potential of surfactants. The concentration of surfactant required to elicit human red blood cell lysis has been used as an index for measuring surfactant irritation and the results compared with those obtained by human patch and rabbit eye tests. For a series of surfactants and surfactant mixtures containing different number of moles of ethylene oxide, the results suggest, the higher the concentration required to induce haemolysis, the lower will be the irritation.

KEY WORDS:- skin impedance - skin moisturising
laser Doppler flowmetry - percutaneous absorption
surfactant irritation - haemolysis

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To Sulaxana and Shanved

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CHAPTER 1.0: INTRODUCTION

1.1. JOHNSON WAX

The Company was founded in 1886 by Samuel Curtis Johnson when he bought a parquet flooring business in Wisconsin, U.S.A. The old methods of keeping them clean ruined the surface, so, two years later, he started making wax pastes to keep them in good condition.

Today, Johnson Wax is a world wide organisation with fifty or so operating companies around the globe. It is privately owned, one of the largest of its kind. The Company produces virtually every consumer home caring product - for furniture, floors, carpets, silver and brass, ovens, laundry and air fresheners. It has products too for the car and personal care.

In Britain, the Company's production, warehousing, and administration are based at Frimley Green, Surrey. The Research and Development Centre, which covers Europe, Africa and the Near East, is situated at Egham, in Surrey. All new product development as well as existing product refinement in the region is carried out here. The entire R&D

operation is divided into four separate groups, each with its own manager, all of whom come directly under the control of the Regional Director of R&D. Three of the groups are subdivided into separate departments consisting of teams of chemists and technicians working on specific product categories. The fourth group is concerned with product evaluation and consumer perception.

1.2. PROBLEM STATEMENT

Johnson Wax is a company which operates in a fiercely competitive market, where flexibility and speed of reaction count for much. Product development and launch is highly consumer orientated, resulting in extensive market research.

One particular area which is very sensitive to consumer perception is personal care, particularly application of therapeutic treatments such as lotions, creams, soaps and pharmaceuticals on the skin. The assessment and development of these products is often highly subjective depending on the consumers' and chemists' perception.

Market surveys can be extremely expensive, even though they are a necessity before any launch of a skin care product. However, often during product development the chemist is faced with various raw materials which may or may not be acceptable to the consumer. Rather than carrying out extensive consumer research for each variable, scientific methods for optimizing various products in the laboratory, are considered to be more cost-effective.

The ultimate objective would be to generate data within the laboratory that would correlate with consumers' perception. The data generated could also be used to provide a scientific and quantitative basis for claims substantiation in advertising.

1.3. PROBLEM INVESTIGATION

The assessment of the physicochemical effects of topically applied medication and cosmetics on the skin, presents a number of difficulties. In order to study these effects there are a number of methods available. Animal models are often used, particularly for novel formulations, but are usually inadequate because of marked inter-species

variation. In vitro methods, especially those using excised human skin are adequate models for diffusional properties, provided trans-follicular transport is not important. In vivo methods are problematic due to the large variation in the physical properties of human skin owing to body region, age and sex. However, in vivo methods are extremely useful, especially if non-invasive, to study the effects of formulations under realistic conditions.

The current project attempts to evaluate the performance of a topically applied product by asking three questions:

a) How can the purely epidermal effects of a product be assessed? An answer is sought by investigating impedance measurements as a technique for monitoring moisturising efficiency. (Chapters 2 - 5).

b) How can the vascular effects or sub-stratum corneum effects of topically applied agents be monitored. Laser velocimetry is investigated for this purpose. (Chapters 6 - 10).

and

c) How can the effects of individual components of the formulation be quantified. Here lysis of erythrocytes by surfactants, which are common components of cosmetic formulations is studied. (Chapter 11).

CHAPTER 2.0: SKIN IMPEDANCE - REVIEW

2.1. INTRODUCTION

The surface of our skin is covered by 15-20 layers of flat dead cells known as the stratum corneum (Plate 1, Appendix). Although it is a thin membrane, being about 20-30 μm thick in most areas, it plays a crucial role in the maintenance of our life in the atmosphere and it is the main target of cosmetic treatments (1). The primary use of these moisturising cosmetics is to prevent the skin from drying out and to reduce surface roughness, thus improving general appearance. The end benefit is attributed to the addition or retention of moisture to the skin and formation of an oily layer to produce an occlusive effect (2).

2.2. WATER AND THE SKIN

It was Blank (3) who first showed that the water content of the stratum corneum is an important factor in maintaining the flexibility of this

layer. Beneath the stratum corneum lie fully hydrated living tissues and it is mainly these that provide the corneum with water. In addition, the sweat glands are also a source of moisture (4).

For normal skin water is lost to the environment particularly at low relative humidity, high temperatures and high air flow near the skin surface. This often results in chapped skin (5). Furthermore, in the presence of certain skin diseases or following attack by solvents, surfactants and other irritants, water loss increases markedly (4).

The stratum corneum is claimed to be the main "barrier" to water loss for the organism (3,6). This is generally accepted to be due to the slow diffusion of water through the entire stratum corneum (7). This diffusion increases markedly when the stratum corneum is damaged resulting in dry and brittle skin and even though there is always an adequate supply of water from the underlying tissues, the rate of moisture loss from the skin surface may exceed the rate of water replenishment. It is under these circumstances that cosmetic creams and lotions may be of value.

Hygroscopic water-soluble substances are responsible for much of the water binding within the stratum corneum (8). These substances are surrounded by a lipid film which retains the water within the stratum corneum. However, transfer of water through the film occurs by osmosis. Application of a solvent may remove the lipid film allowing the dissolution of the substances which in turn reduces the extensibility of the stratum corneum (4,8).

2.3. MEASUREMENT OF MOISTURE CONTENT OF SKIN

There is a need for an accurate method to measure the water content of skin. Such a technique would be useful not only to determine the efficacy of moisturising agents, but, more importantly, to explain the mechanisms of action of such products. The term "moisturiser" is a neologism of the cosmetic industry and the best definition is perhaps that of Kligman (9):

".....a topically applied substance or product that overcomes the signs or symptoms of dry skin".

The human finger is perhaps the most sensitive, cheapest and easiest instrument to use. However, the results it generates are not quantitative or reproducible.

Roughness and scaling of the stratum corneum are associated with among other factors a decrease in water content, therefore any assessment technique, apart from being reproducible and quantitative, should either measure this decrease or directly measure the stratum corneum water content.

In vitro techniques cannot be considered very useful since isolation of the stratum corneum must alter its interaction with moisturisers. However, some such methods are still frequently used for fundamental research and are reviewed elsewhere (10, 11).

In vivo techniques are generally indirect and monitor changes in the physical properties of skin such as mechanical, optical and electrical (10, 11, 12, 13, 14). Methods that measure cutaneous excretions such as transepidermal water loss are also available (15). Finally, there are methods that measure the surface changes in skin such as its coefficient of friction (16), surface contours (17) and visible features (18).

2.4. HISTORY OF ELECTRICAL CHARACTERIZATION OF THE SKIN.

The electrical properties of skin have been used by many investigators to indicate the condition of the skin or its underlying tissues. The earliest attempts used direct current or low frequency alternating current (19, 20). These methods suffer from polarization currents which mask the true electrical resistance of the tissues. Gildemeister (21) overcame this by using higher frequency alternating currents.

Most of the original studies examined the relationship between body impedance and thyroid function (22). A number of authors tried to separate the impedance of the inner tissues to that of skin. However, their techniques tended to be very cumbersome (22, 23, 24).

Recent rapid development in electronics and instrumentation has led to renewed interest in skin impedance as a technique for studying the structure and function of skin. This can also be attributed

to the need for non-invasive in vivo methods as alternatives to animal models. Even though electrical apparatus can be expensive, they have found a number of recent applications in the assessment of moisturising products and diseased skin. Some of these methods will be discussed in greater detail particularly with relevance to their accuracy and reproducibility.

2.5. GENERAL DIFFICULTIES WITH IN VIVO SKIN IMPEDANCE MEASUREMENTS.

Over the last two decades numerous researchers have tried to solve the technical and theoretical problems involved with skin impedance measurement. Some of the problems are largely due to the fact that the technique requires the application of electrodes to the region of measurement. This may result in a "contact impedance" between the electrode and skin surface.

In the case of "dry" electrodes, i.e. those that have no electrode gel to promote electrical contact between the surfaces, results may be influenced by

the amount of pressure applied on the electrodes to improve contact with the skin. For "wet" electrodes the influence of the electrode gel on the skin must be considered.

The application of any electrode, be it wet or dry, will interfere with the normal fluid exchange between the skin surface and its environment, thus modifying the moisturisation of the stratum corneum.

An additional factor which must be considered is the choice of electrical measurement. As previously mentioned direct current and very low frequency measurements result in the polarization of the electrodes and so have long been abandoned. Alternating current measurements have therefore been used, some at a single frequency, while others over a range of frequencies. However, the choice of frequency or frequencies is of importance depending on the information being sought.

There are also more fundamental problems. There is a lack of knowledge of the precise mechanism of electrical conductivity in the skin and also the distribution of the field lines inside the skin, i.e. the precise anatomical structures involved in the measurement are not known.

The keratin in the skin may be considered as a dielectric medium, i.e. one of weak electrical conduction. However, Leveque and de Rigal (25) have proposed three possible mechanisms by which water increases the conductivity of the skin:

(i) the keratin chains, which have a dipolar moment, are made more movable by the plasticizing effect of water;

(ii) the intercellular spaces contain ions which are more mobile under an electrical field, especially if the viscosity of their environment is reduced by water;

(iii) water molecules themselves may dissociate into hydronium and hydroxyl ions allowing the exchange of protons.

If the assessment of moisturising products is to be made correctly these fundamental and technical problems must be addressed first. However, it must also be remembered that in vivo skin measurements are susceptible to internal and external changes. The properties of cells are related to changes in the metabolic, physiologic and psychologic state of

the parent organism. Also skin being an external surface is changed by environmental conditions particularly temperature and humidity. Therefore if skin impedance measurements are to be made in vivo the influence of these factors must be accounted for as far as is practical.

2.6. METHODS OF SKIN IMPEDANCE MEASUREMENT.

There are several methods cited for the measurement of skin impedance. Early methods were associated with some of the technical problems discussed above and more recently methods which attempt to overcome them have been developed. Brazier (22) and Barnett (23) were the first to show that impedance measurements made at high frequencies reflected the dielectric properties of the inner body tissues rather than those of the skin.

Plutchik and Hirsch (26) showed that skin impedance varied depending on the frequency of measurement. They found that the magnitude of the impedance decreased nearly tenfold from a frequency of 1 to 1000 Hz, suggesting this to be a good range for assessing the skin's electrical characteristics.

Tregear (27) studied the influence of electrode skin contact at a single frequency measurement of 1.5Hz. He found that dry electrodes gave very much higher impedance values than those using agar gel or saline as a contacting medium. Furthermore, immediately after an electrode was placed on the skin surface impedance was very high, taking up to ten minutes for it to drop to a constant value for the dry electrode and a lot less for the wet electrode.

The various techniques proposed in the literature are discussed below with particular emphasis on methods that have been used to assess skin diseases or to investigate the moisturising effects of various products. Those techniques concerned with the electrical properties of skin per se are only briefly considered here. A detailed review is given by Salter (28).

2.6.1. LOW FREQUENCY METHODS

The dielectric properties of human skin depend on the frequency of measurement. There are three main zones of frequency, each considered to represent different anatomical structures

(29). The low frequency range known as α - relaxation (10-100Hz) is thought to originate in the stratum corneum. β - relaxation (10-100kHz) is characteristic of most biological tissues such as the lungs, liver, and muscle, while the highest relaxation zone γ is due to the dipolar relaxation of free water.

Clar et al (13, 30) have proposed that since the stratum corneum is the main object of study for moisturisation, only α - relaxation should be used.

Various parameters can be obtained from the measurements of impedance at different frequencies, however, this can be time consuming. The authors suggest that the impedance and an intrinsic parameter known as the relaxation time at a single frequency of 25Hz, is sufficient for rapid measurements.

These authors have developed an electrode junction that is claimed to reproduce the atmospheric vapour pressure near the skin surface. This junction is made up of a mixture of polyethylene glycol, 0.1M NaCl and water with relative concentrations depending

on the temperature and humidity of the test environment. The authors have shown, in vitro, that the electrode does not affect water loss from rat skin surface. However, they have not illustrated the possible advantages of this electrode system to human in vivo testing.

Using their experimental technique, Clar et al have demonstrated the contribution of the stratum corneum to the impedance and relaxation time. Furthermore, they suggested that an increase in the relative humidity of the environment at constant temperature resulted in a decrease in impedance due to an increase in moisture content of the stratum corneum. They have also measured the effect of moisturising creams both in the short and long term (13, 30).

These authors also found large inter- and intra- subject variability in impedance values. However, symmetrical sites gave very similar values provided the subject had been allowed to relax before being measured.

Serban et al (31, 32) have also used low

frequency measurements to assess the effects of various moisturisers. Their electrode system consisted of a stainless steel wire mesh applied to the skin under partial vacuum. The authors claim that repeated application of this electrode gave fairly constant conductance and capacitance values. However, the electrode was held gently on the skin surface for only 15 seconds for measurements at 1592Hz. Only one electrode was used at the test site while an aluminium foil was placed in the mouth as the reference electrode.

A number of moisturising lotions were tested in the above work and it was claimed that minute changes in the water content of the stratum corneum could be detected. They also used sodium lauryl sulphate to dry the skin and showed that electrical measurements could monitor the increasing visible irritation of the skin.

Isherwood (33) designed an electrode that consisted of a block of pins through which dry air flowed to remove any surface moisture. The electrode is held on the skin surface using a bellows type arrangement which allows application at constant pressure. This system, though simple, is susceptible to surface conditions of the stratum corneum. Using this type of electrode Isherwood found an increase in capacitance of the stratum corneum with an increase in water content. However, no studies were undertaken to assess the moisturising effects of cosmetics, probably because of the qualitative nature of the data produced.

Thiele and Malten (34, 35) have developed an electrode to measure the damage caused by alkaline solutions to the skin in vivo. In their experiments they measured the skin impedance at 25Hz using platinum electrodes immersed in the alkaline solutions being tested. As a control they used saline solutions and found these to have little effect on the skin impedance.

In order to assess the sensitivity of impedance as a measure of skin irritation, Malten and den Arend (36) applied dimethyl sulphoxide at various concentrations in saline and for a variety of exposure times. They compared the impedance results with clinical changes and water vapour loss. Impedance measurements tended to give a response well before visible changes were noticed or any water loss was perceived.

Skin impedance has also been used to monitor the effect of heat, surfactants and dimethyl sulphoxide on the permeability of excised human skin (37), with measurements carried out at 1.5Hz, using stainless steel mesh electrodes applied at constant pressure.

Simultaneous water permeability and impedance measurements of skin treated with various solvents showed a good correlation (37). The various solvents applied are thought to increase the hydration of the skin thus leading to increased water permeability and impedance. In vitro results such as these are useful for understanding the more fundamental

principles involved but are difficult to relate to in vivo measurements.

2.6.2. HIGH FREQUENCY METHODS

As stated earlier, the low frequency range of 10-100Hz is useful for characterising the α -relaxation of the stratum corneum, whereas higher frequencies provide information on deeper layers of the skin (13). However, penetration of the electrical waves into the skin does not solely depend on frequency but also on the configuration of the electrodes (25).

If the electrodes applied to the skin are placed at a distance inferior to the thickness of the layer being assessed, in this case the stratum corneum, the electrical field lines will uniquely pass through this layer. (25, 38).

Tagami et al (38, 39) have developed an instrument that automatically measures conductance and capacitance at a high frequency of 3.5MHz. They used two concentric

brass electrodes of 1mm and 4mm external diameter separated by an insulator. In order to minimise occlusion of these dry electrodes they are only applied for very short periods at a constant pressure.

Even though the separation of the electrodes was far greater than the average stratum corneum thickness of 20-30 μm, the authors demonstrated that the hydration detected by their method seemed to be in the outer-most portion of the stratum corneum.

Tagami et al went on to show data on the state of moisturisation of the stratum corneum. They studied the effect of various skin lesions on skin impedance.

Lawler et al (40) measured the impedance by balancing the skin electrically with an equivalent circuit containing variable resistors and capacitors. They used stainless steel disks as electrodes with normal saline as contacting gel. The authors carried out most of their measurements at 4000Hz and found impedance to increase with stratum corneum thickness as well as electrode size.

Determinations on a large population of 104 subjects showed no significant difference in skin impedance between the sexes. Stripping the stratum corneum showed its contribution towards the total skin impedance and the authors proposed impedance as a technique for studying diseased skin (40).

2.6.3. OTHER METHODS

So far the methods proposed have usually used two electrodes on the test site, but some methods use one electrode on the test site while the other is applied at a distance as the reference (32). In addition, Campbell et al (41) proposed measurements using four electrodes in order to eliminate the problems associated with electrode skin contact.

The operating principle is similar to that of high frequency measurement. As already discussed, if the electrodes applying current to the skin are placed at a distance inferior to the thickness of the stratum corneum, the

field lines will pass uniquely through this layer (25). By placing two other electrodes in between the first ones, the values of the local potentials can be read and if these are measured at a very high frequency, contact pressure is claimed to have a negligible effect on the reading (41).

Campbell et al used four microelectrodes 25 μm wide and 35 μm apart to validate their approach for the measurement of skin impedance of plantar skin, which is of course thicker than skin from most other areas of the body.

Untreated samples showed a relationship between the impedance and stratum corneum water content. The effects of exposure to formaldehyde, urea and lipid extraction which, it is proposed, affect the ratio of bound and unbound water in the stratum corneum were also shown.

Jackson et al (42) propose a similar system which involves measurement of the phase lag between current and voltage at 2kHz in vivo. However, the electrode spacings are far greater than those used by Campbell et al and

any substantiation is based on in vitro measurements only. This instrument is available commercially and has the great advantage that it is portable. However, the precise anatomical structures being measured are not known.

2.7. A.C. IMPEDANCE

2.7.1. A.C. IMPEDANCE DEFINITIONS

A.C. impedance studies of any system involve the application of a potential or current which is time dependent. When a potential which is time dependent in a sinusoidal manner, i.e. $\Delta E \sin\omega t$ (where ΔE is small) is applied between two electrodes situated on either side of a test substrate, a current $\Delta i \sin(\omega t+\theta)$ will flow as a consequence. Note that ω equals $2\pi f$ where f is the frequency of the sinusoid in Hertz (Hz). In addition current will also flow at angular frequencies of 2ω, 3ω, etc.

One can define an impedance z as having a magnitude given by:

$$|z| = \frac{\Delta E}{\Delta i} \qquad [1]$$

and a phase angle, θ , which corresponds to the phase difference between the applied sinusoidal potential and the resultant sinusoidal current. An impedance is therefore a vector quantity, since it has both magnitude and direction. Impedance can be represented as a point in a plane (Figure 1) where it can be characterised by $|z|$ and θ or by its real and imaginary components, z' and z" projected on the ordinate and abscissa respectively.

If the test substrate consists of a pure resistor of magnitude R ohms, then z=R and θ = 0. A pure resistance is represented by a point on the ordinate for any frequency as shown in Figure 2(a).

FIGURE 1: COMPLEX PLANE REPRESENTATION OF IMPEDANCE

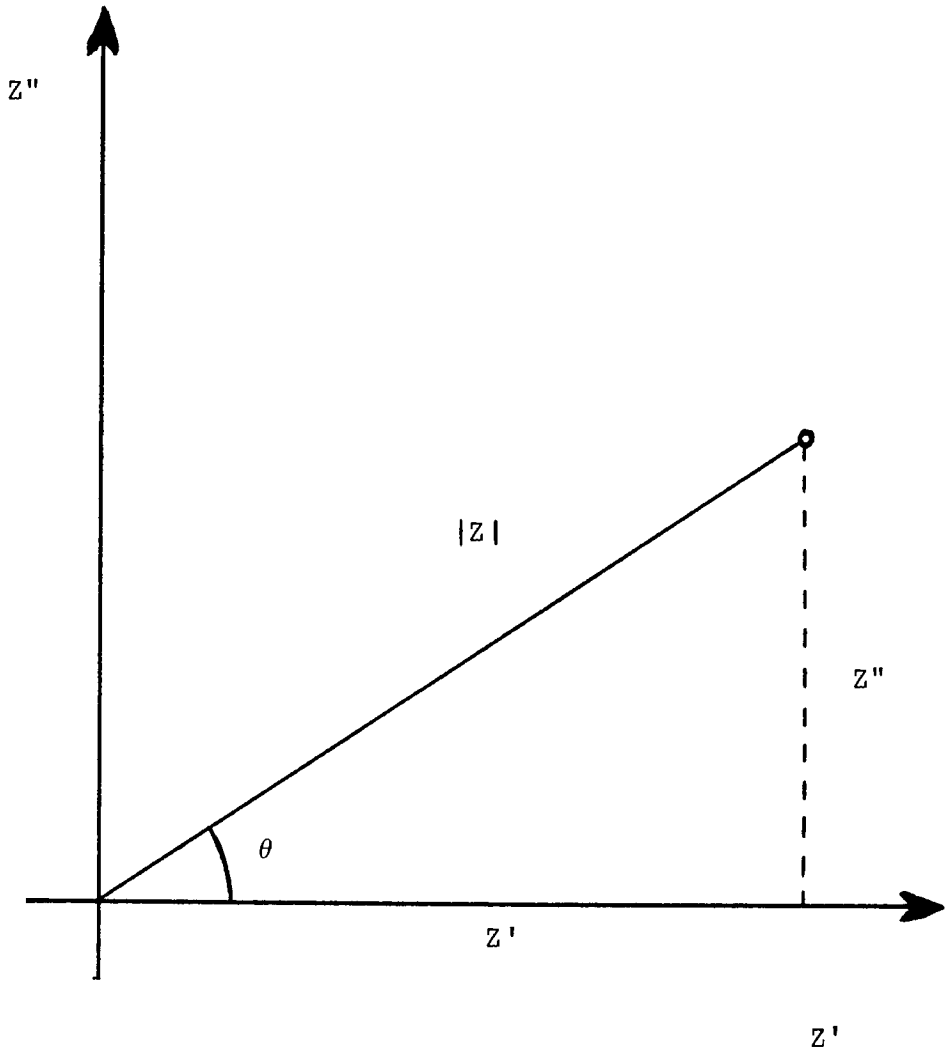
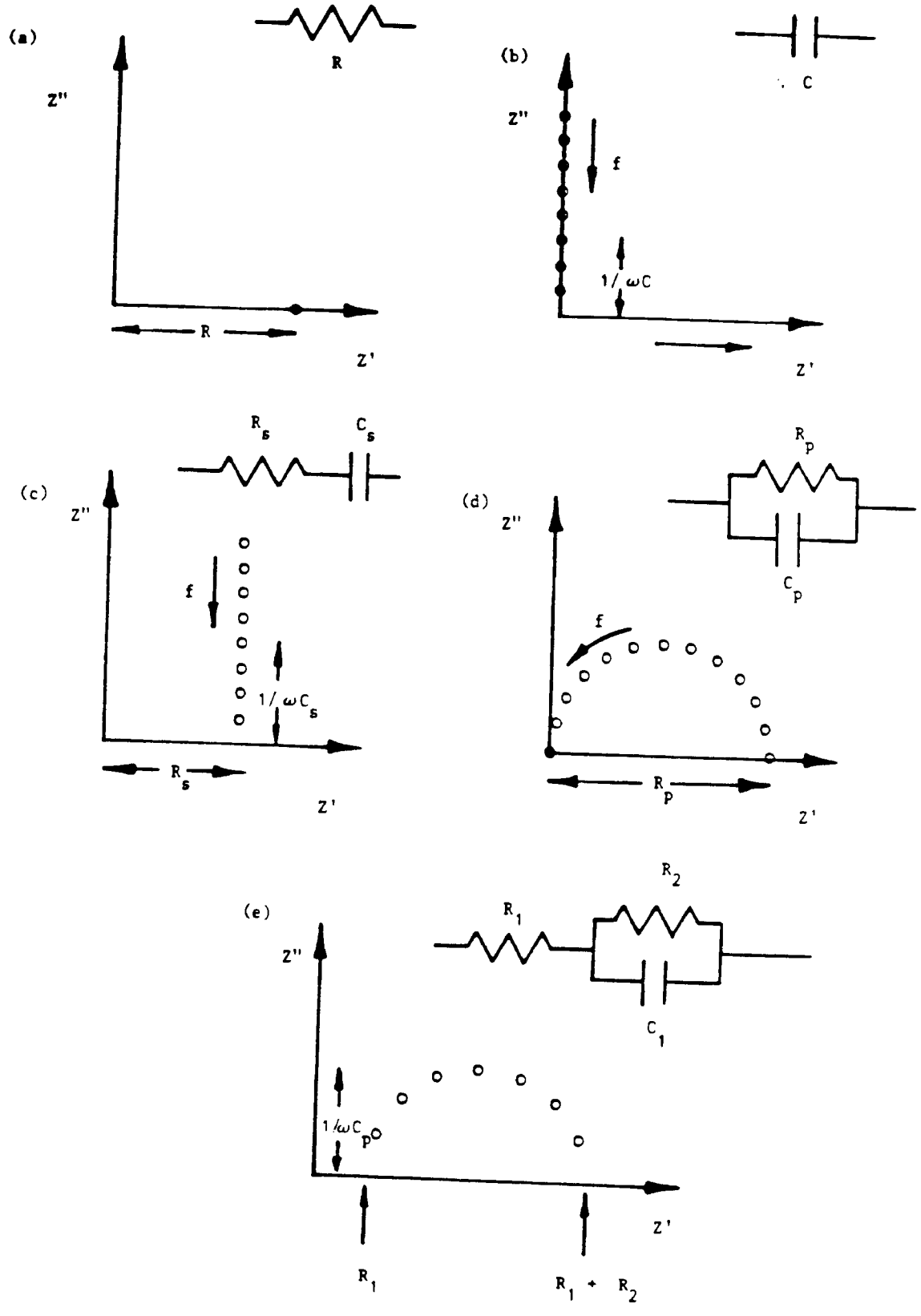


FIGURE 2: COMPLEX PLANE IMPEDANCE SPECTRA WITH THEIR ASSOCIATED EQUIVALENT CIRCUITS



However, when a pure capacitance, C Farads, is substituted for the cell the situation becomes somewhat more complicated. In this case θ = 90^{o} but z is frequency-dependent through the relationship:

$$z = 1/\omega\ c \qquad [2]$$

As the frequency of the sinusoidal potential is varied, the representative point also varies as in Figure 2(b). Figures 2(a) and 2(b) are the simplest form of complex-plane impedance spectra, which are representations of the impedance being measured as a function of frequency. Such plots are also referred to as Argand diagrams, Nyquist plots or Cole-Cole plots.

The test substrate is usually far better represented by a more elaborate network of resistances and capacitances known as the equivalent circuit. These show a more complicated behaviour in the impedance plane. For example a resistance and capacitance in series gives the impedance spectrum shown in Figure 2(c).

For this case

$$z' = R_s \quad \text{and} \quad z'' = 1/\omega C_s$$

and since

$$z^2 = z'^2 + z''^2$$

then $$z^2 = R_s^2 + (1/\omega C_s)^2 \qquad [3]$$

θ can take all values between 0° and 90° depending on the measurement frequency. From equation [3] one can derive an equation for the impedance:

$$z = R_s - j/\omega C_s \qquad [4]$$

where $j = \sqrt{-1}$

If instead of a series combination of resistance and capacitance we have a parallel circuit, the impedance spectrum is quite different (Figure 2(d)) and in this case the impedance is given by:

$$z = (1/R_p + j\omega C_p)^{-1} \qquad [5]$$

This may be represented as the admittance, Y, which is given by:

$$Y = 1/z = 1/R_p + j\omega C_p \qquad [6]$$

It is possible to interconvert admittance and impedance and also show the relationship between R_p and R_s and C_p and C_s (43).

For a given impedance spectrum one can calculate the components of an equivalent circuit of resistances and capacitances responsible for it (Figure 2(e)). With measurements on experimental systems, such as skin, it is more usual for the investigator to measure the impedance of the test site and subsequently to try to find the appropriate equivalent circuit and then identify the physical significance of the various components. Results can then be compared with theoretical models.

2.7.2. INSTRUMENTATION

The simplest experimental arrangement for measuring the impedance of a biological system

involves the use of an a.c bridge. Here the test cell, which is in one arm of the bridge is balanced against variable decade capacitance and resistance boxes in another arm. If at the balance point of the bridge, the components of the impedance are R_s and C_s then $z' = R_s$ and $z'' = 1/\omega C_s$. However, since a balance point takes several minutes to obtain, this arrangement is time consuming for measurements over a wide frequency range.

A considerable improvement can be obtained by directly measuring the in phase and quadrature components of the potential and the current using phase sensitive detectors. This gives a d.c. output that is related to both the amplitude and the phase of the sine-wave input. By suitable phase shifts one can obtain the real and imaginary parts of the impedance. In this case no balancing is involved, but to obtain an impedance spectrum with approximately five points for each decade in frequency down to 1Hz may take two hours or more. Furthermore the data obtained generally needs to be processed numerically before the impedance can be obtained.

A more sophisticated experimental arrangement is based upon the Solartron 1170 and 1250 series of Frequency Response Analysers (Solartron Electronics Group, Farnborough, Hampshire, U.K.). This system removes a great deal of the tedium involved in single impedance measurements and allows a large frequency range to be covered more quickly and usually more accurately (44).

The Frequency Response Analyser (FRA) consists of a programmable generator which provides the perturbing sinusoidal signal, a digital correlator to analyse the response of the system, and a display to present the results.

The FRA analyses the response of a system to a sine wave at frequency f. It also rejects any harmonics present and minimises the effects of random noise. Harmonics occur due to the non-linearity of the system on test and take the form of sine waves at integral multiples of frequency f. A single measurement at a particular frequency can be made by programming the generator with the required frequency and signal amplitude. The generator can also be programmed to sweep through a

large frequency range by choosing suitable values for the maximum frequency, the minimum frequency and the number of points per decade at which measurements are to be taken.

The instrument can take measurements sequentially in either direction, at equally spaced intervals, either on a logarithmic or a linear scale over the required range. The response is given once a measurement has been completed and can be displayed in any of three possible notations:

i) amplitude (A) and phase angle (θ) relative to the input signal,

ii) log A and (θ) or

iii) the real and imaginary parts of the impedance.

It is possible for the FRA to be interfaced with a computer so that a real-time plot of the measured data can be displayed on the visual display unit (VDU). The data may be stored in an appropriate mass storage device,

from where it may be recalled for further manipulation. The Solartron FRAs perform rapid measurements obtaining a complex plane impedance spectrum over a wide frequency range, within a matter of seconds. Measurements are slower at frequencies below 1Hz, since each single measurement is dependent upon the period of the signal which gets larger and longer as frequency decreases.

2.7.3. EQUIVALENT CIRCUITS FOR SKIN

The electrical properties of skin are very complicated. The most commonly used method to describe them involves the use of equivalent circuits. Alternative attempts to model skin are based on the theories of solid state physics. Since the electrical pathways through skin are so numerous, the models used to interpret the impedance behaviour use lumped parameters. Essentially these parameters consist of simple resistors and capacitors that electronically give the same results as does skin.

If impedance measurements of skin are taken over a wide frequency range the complex plane diagram will take the form shown in Figure 3. This is essentially a circular arc with its centre depressed below the real axis and offset from the origin. For a given amplitude of sinusoidal stimulation the same behaviour can be assumed to obey the empirical Cole equation (45):

$$Z = R_s + jX_s = R_\infty + \frac{R_o - R_\infty}{1 + (\omega j \tau_\rho)^{1-m}} \qquad [7]$$

An equivalent circuit for skin impedance can also be derived as shown in Figure 4.

As can be seen from Figure 3 the resistance of skin to alternating current is dependent on Frequency. Figure 4 shows the presence of two resistors as does equation [7], because at very low frequencies the impedance of skin is very high tending towards the direct current impedance which is equal to R_o. On the other hand, at very high frequencies, the reactance of human skin becomes negligible and so the impedance tends to R_∞.

FIGURE 3: RESULTANT IMPEDANCE PLOT FOR IN VIVO SKIN MEASUREMENTS

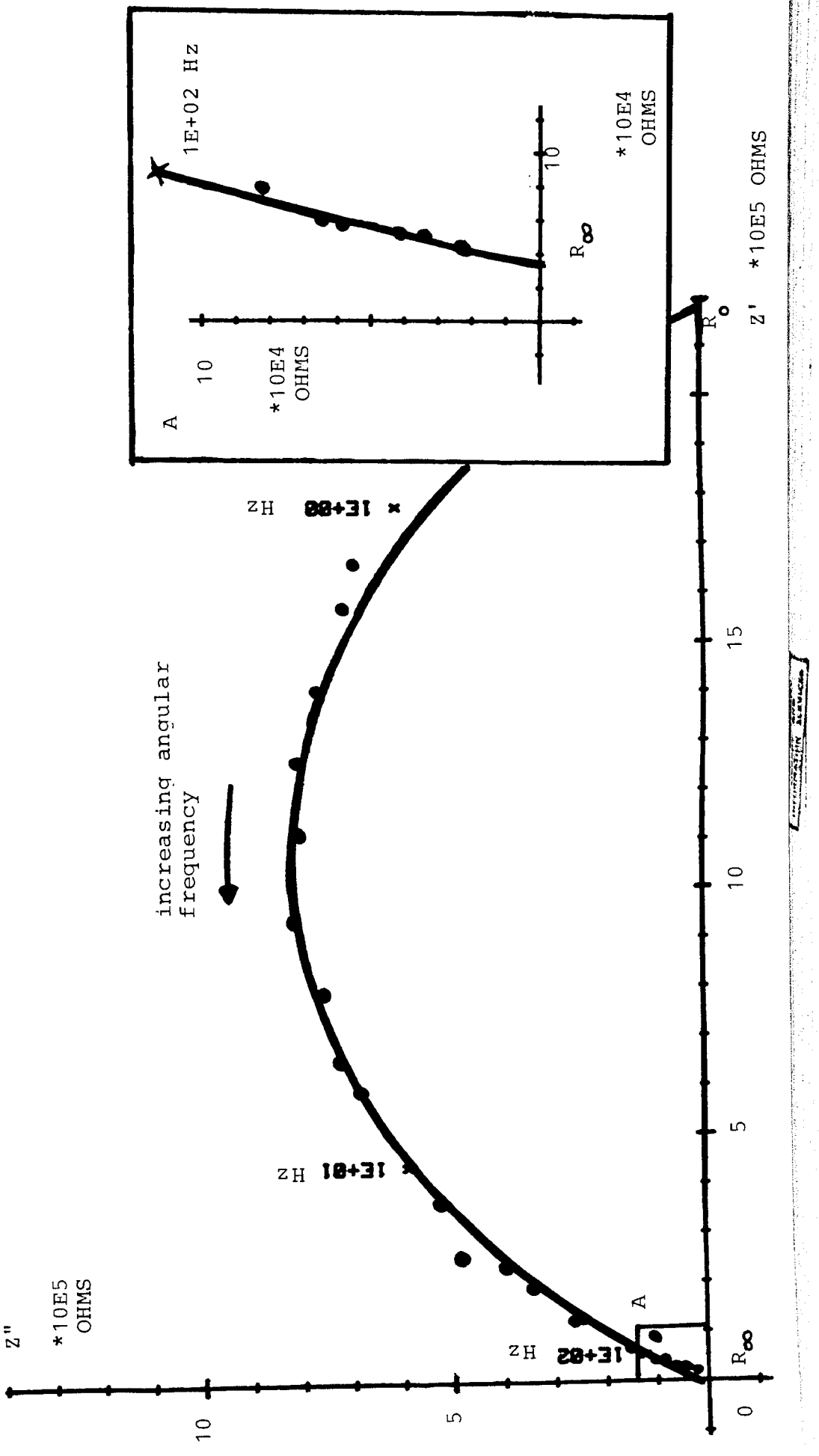
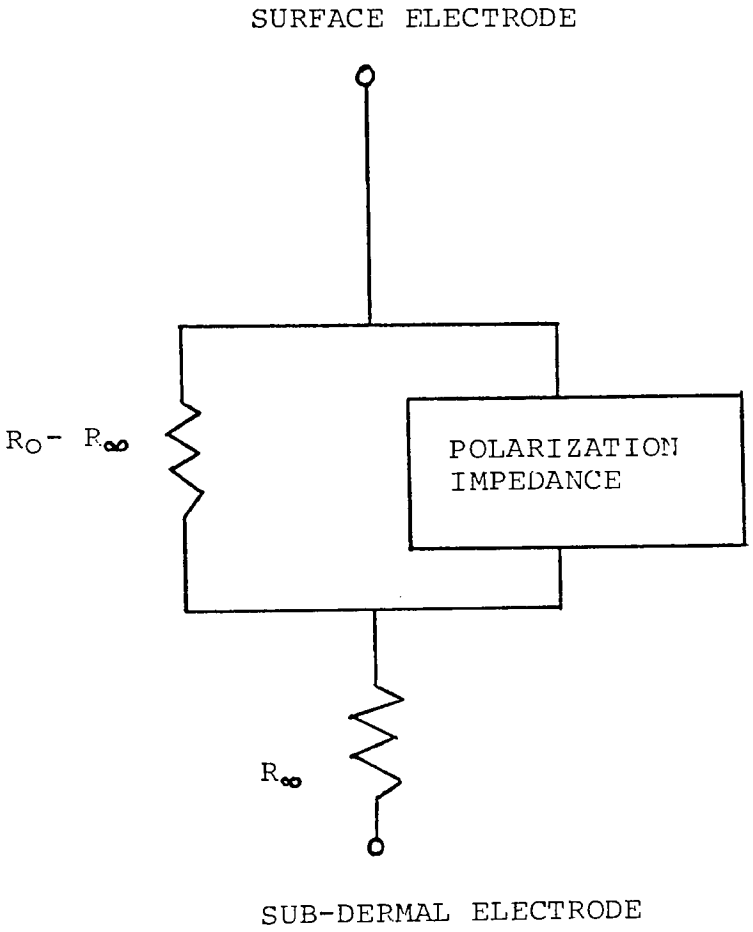


FIGURE 4: EQUIVALENT CIRCUIT FOR SKIN IMPEDANCE

(NB This represents the situation for one surface electrode and one sub-dermal electrode)



As already mentioned, ω is the angular frequency and is equal to $2\pi f$ where f is the frequency in Hertz. The parameter m and τ_p are related to the polarization impedance. This is a variable impedance element of constant phase angle employed to account for the inhomogeneity within the skin (23, 47).

There are a number of sources for this charge (46). At a high potential the orbits of the electrons around individual atoms may be distorted giving electronic polarization. Also molecules may distort giving opposite charges within the same structure even though remaining electrically neutral. Movement of these charges is termed atomic polarization. In ionic solids displacement of the individual ions may give rise to ionic polarization.

If an alternating current is applied rearrangements of the molecular dipoles will give a net increase in the current flow between one electrode and the other. Furthermore the magnitude of the polarization will vary with frequency since rearrangements will take a finite time. At sufficiently high

frequency the material will no longer respond by reorientation and so polarization will diminish. Each reorientation process can be characterised by a constant termed the relaxation time.

In a material such as skin the response to alternating current will be difficult to interpret. There are many different complex molecules present and these are surrounded by water molecules associated with varying numbers of ions. These will result in several different relaxation processes. Thus the parameter τ_p in equation [7] represents a mean relaxation time and m is related to the width of the distribution of relaxation times present.

The actual mechanisms that produce the complex skin impedance profile are difficult to describe. In the model proposed by Barnett (23), the phase angle remained constant whereas the impedance was inversely related to a power of the frequency.

Tregear (47) modified this by having a chain of relaxing elements in series. Each relaxing element consists of a resistor and capacitor in parallel which are meant to represent the local resistivity and permittivity respectively of a layer of cells in the stratum corneum. The resistors are claimed to change relative to changes in the hydration of a cell layer.

Salter (48), in his model, proposes that for the arrangement of proteins found in skin, a type of semiconduction is produced. Proteins in pure form have a large energy gap between their valence and conduction bands. Electrons may be thermally excited across the gap increasing the conductivity of the material by many orders of magnitude. These states of intermediate energy are known as localised states. Salter derived the following equation for impedance and found that it held down to very low frequencies:

$$Z = \frac{R_o}{1 + (j\omega\tau_p)^{1-m}} \qquad [8]$$

2.7.4. ASSESSMENT OF STRATUM CORNEUM HYDRATION

The previous section discussed the various theoretical models used to describe the empirically derived skin impedance behaviour. One of the important points for consideration is that due to the complicated electrical nature of skin, a full complex plane impedance diagram should be used for all measurements. Previous authors (13, 32, 38) who have used skin impedance to assess the moisturising effect of cosmetics and pharmaceuticals have relied on single frequency measurements, mainly due to the slow rate of measurement of their instrumentation. The recent development and use of the FRA improves the rate of measurement tremendously allowing full impedance profile studies with relatively little effort.

The models discussed above yield empirical values for impedance using various equations. For purposes of electrical characterization, the skin may be divided into two regions: the highly conductive inner tissue and the more resistive stratum corneum. $R_o - R_\infty$ from

Figures 3 and 4 may be considered to represent the stratum corneum resistance, whereas R_∞ is the inner tissue resistance.

Since the assessment of the effects of moisturisers mainly concerns the stratum corneum, the $R_o - R_\infty$ term is of prime importance. In practice these values can be obtained by subjecting the complex impedance measurements to a least squares fit. The value for the stratum corneum resistance can then be obtained from the intercepts of the semi-circle on the ordinate. Thus provided a wide frequency spectrum is being used any changes in the hydration state of the stratum corneum should be easily reflected by the parameter $R_o - R_\infty$.

CHAPTER 3.0: SKIN IMPEDANCE - EXPERIMENTAL

3.1. MEASURING ASSEMBLY

Impedance measurements were made using a Solartron 1174 Frequency Response Analyser (Solartron Electronics Group, Farnborough, Hampshire, U.K.) As mentioned before, the FRA consists of a programmable generator which provides the perturbing sinusoidal signal, a digital correlator to analyse the response of the system and a display to present the results (44).

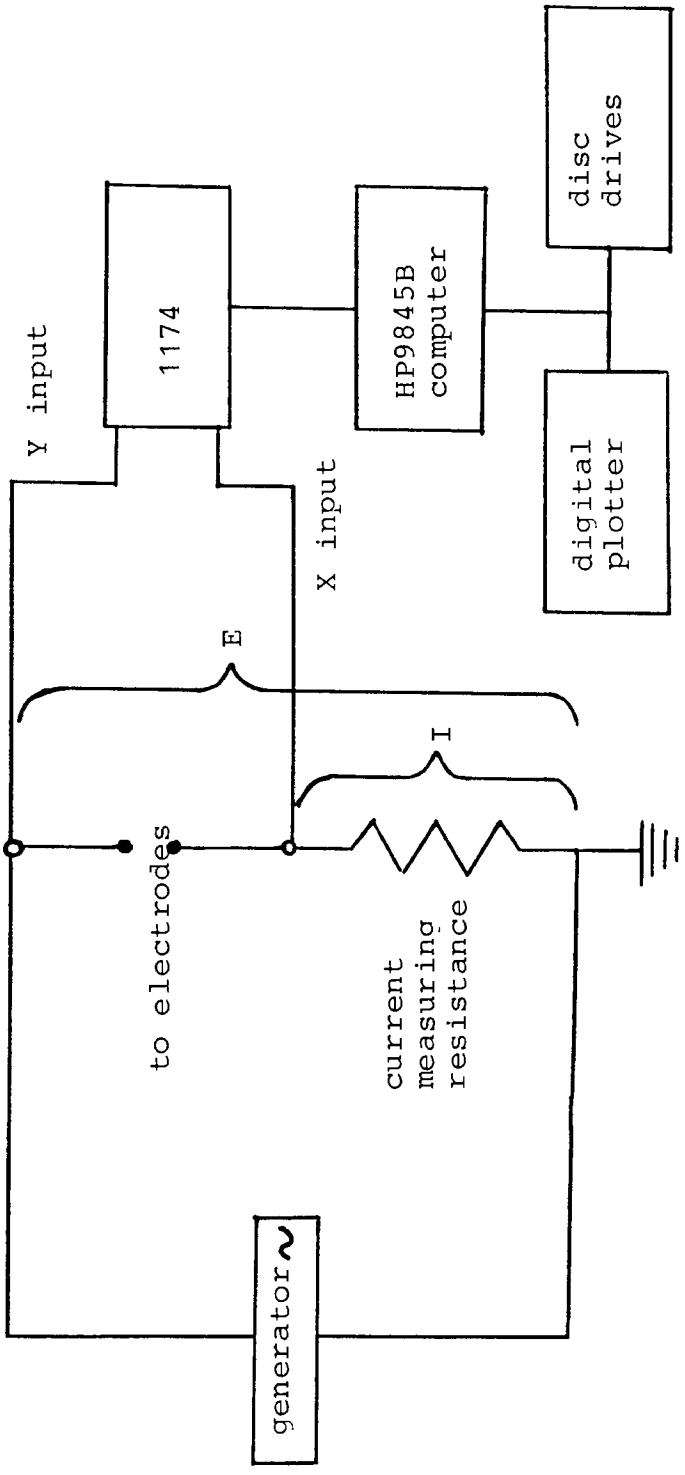
The Solartron 1174 was linked to an HP 9845B (Hewlett Packard, Colorado, U.S.A.) desk top computer and associated peripherals. This facility allowed the complex plane impedance diagram to be displayed on the VDU of the computer as the measurements progressed. Furthermore, the data was rapidly stored on flexible discs for subsequent manipulation. (PLate 2, Appendix).

Figure 5 shows a typical experimental arangement using the Solartron 1174 linked to the HP 9845B.

In this case, the generator provides a perturbing sinusoidal voltage across the measuring electrodes. A resistance of similar magnitude to skin ($1.0*10^6$ ohms) is placed in series with the electrodes to obtain a value for the current flow. The correlator analyses the response and gives an output in terms of the real and imaginary parts of the impedance.

The frequency range chosen was 1-500Hz. This allowed the measurement and storage of a complex impedance spectrum in less than two minutes. Furthermore, as can be seen from Figure 3, the spectrum adequately covers the region considered representative of the skin. For frequencies above 500Hz very little information can be obtained on the skin. However, for frequencies below 10Hz the time for a single measurement is equal to the period of signal, e.g. 10^3 seconds at 10^{-3}Hz. This consideration is true for all techniques. In this experimental arrangement a minimum of 1Hz was used so as to provide adequate information on the skin spectrum without slowing down the rate of measurement too much. The generator was programmed to take sequential measurements from 1Hz to 500Hz, employing a logarithmic scale with 10 points per frequency decade being measured. This gave 28 points for every impedance spectrum.

FIGURE 5: SCHEMATIC DIAGRAM OF THE EXPERIMENTAL ARRANGEMENT FOR AUTOMATED IMPEDANCE MEASUREMENTS



The actual perturbing signal applied was 100mV RMS. This was found adequate to give a response by the FRA and not too high to cause undue perturbation of the system.

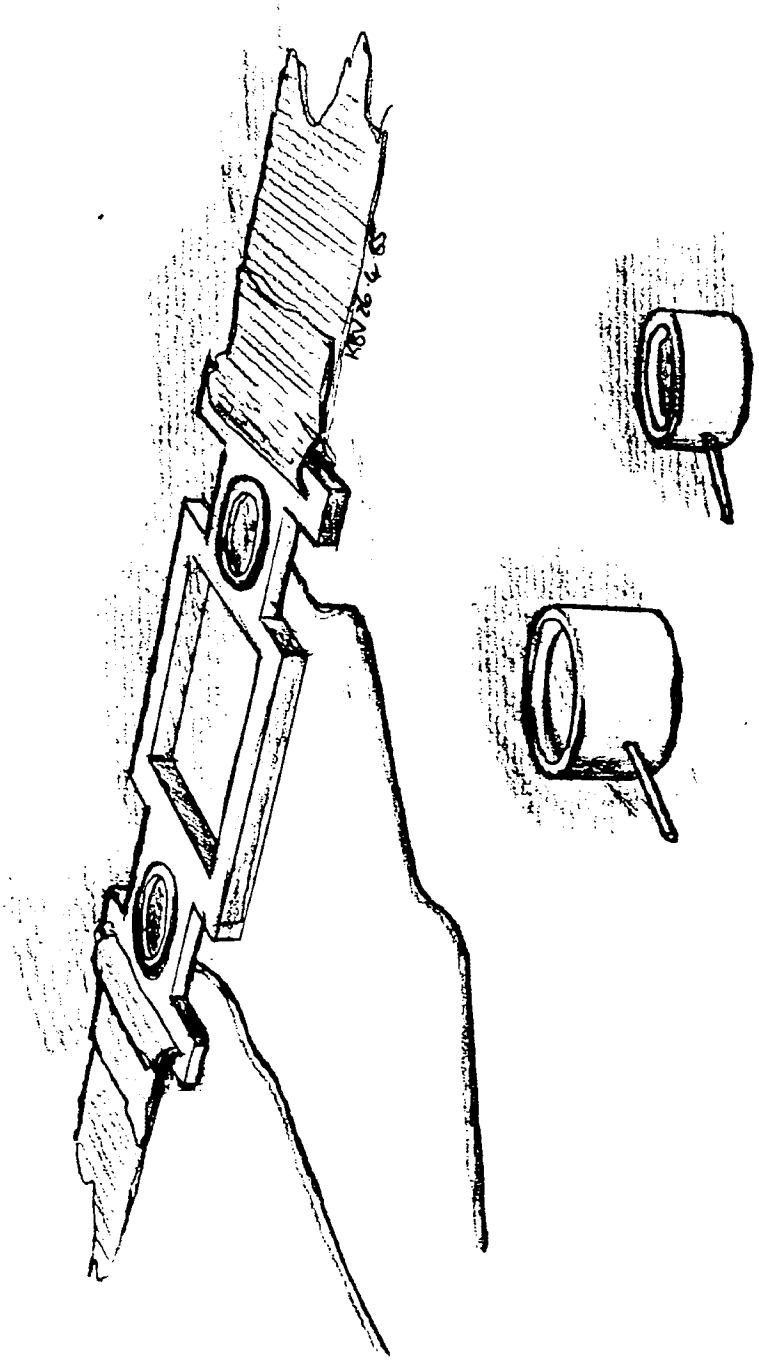
3.2. ELECTRODES

The use of both dry and wet electrodes is fairly common for moisturisation assessment of the skin (13, 32, 36, 38). The various problems associated with electrode skin contact have already been discussed. One of the main reasons for using skin impedance is to obtain a method that is not only accurate, but also easy to use in practice.

Dry electrodes need to be applied at constant pressure either continuously or intermittently. This often means the subject needs to remain very still for long periods of time. This leads to discomfort for the test subject and possible interference with the results.

Those electrodes that are claimed to take care of the water vapour losses either by placing junction fluids or using partial vacuums can be very

FIGURE 6: ELECTODES AND ELECTRODE ASSEMBLY
FOR IMPEDANCE MEASUREMENTS



cumbersome as well. For this reason the electrodes chosen here had a simple salt free contact gel (Spectra 360, In Vivo Metric Systems, Healdsburg, California, U.S.A.) to ensure good contact with the body surfaces, without being influenced by the subject's movements.

Ag - AgCl electrodes were used (E223, In Vivo Metric Systems) with an 8mm diameter sensor. The electrodes were mounted in a solid epoxy housing with a cavity 2mm deep. Figure 6 shows the type of electrodes used and the complete electrode assembly. Two electrodes were held 39.5mm apart from their centres, in an adjustable elastic bracelet. This ensured comfortable and secure contact with the skin (Plate 3, Appendix).

3.3. PROCEDURE

Johnson (49) found significantly higher skin resistances in Negro subjects than in Caucasians. However, no differences were found between the sexes or over a wide age range for the same racial group. Hence, in this study, the subjects chosen were all Caucasians, of both sexes, over the age range of 20-26 years.

In order to minimise any psychological and physiological effects on skin impedance, and also to prevent discomfort, the tests were conducted with the subject in an adjustable reclining chair. Before commencement of any measurement the subjects were left to "equilibrate" for a period of 30 minutes.

Skin impedance has also been reported to be affected by the temperature and relative humidity of the environment (13). Therefore all measurements were conducted in an air-conditioned room and the relative humidity closely monitored. The temperature of the room was maintained at $22 \pm 2^{\circ}C$ while the humidity was within 40-60% during the period of experimentation.

The application of moisturisers, especially cosmetics, tends to be mainly on the hands. However, these have a large concentration of sweat pores which may still be active at low temperatures, especially when the subject is under stress (1). It is also very difficult to apply electrodes securely on the hand. All measurements were therefore conducted on the volar or ventral areas of the forearm since it provides a sufficiently large surface area for electrode application.

CHAPTER 4.0: SKIN IMPEDANCE - RESULTS AND DISCUSSION

4.1. INTRODUCTION

In the electrode configuration used here, the hypothetical equivalent circuit should be as shown in Figure 4 with an additional $R_o - R_\infty$ resistance and polarization impedance parallel element arising from the second electrode. The main parameter chosen for analysis in this project is the diameter of the semi-circular plot depicted in Figure 3, i.e. $R_o - R_\infty$. This is hereafter referred to as the impedance of the skin. In fact it is considered to be representative of the impedance of the stratum corneum and refers to the two resistance elements in parallel with the polarization impedances.

4.2. IMPEDANCE VARIATION WITH TIME

Figure 7 gives an example of the time-dependence of the computed parameter. The subjects were rested for 30 minutes, prior to application of the electrodes followed by immediate impedance measurement. The measurements were then repeated every 2 minutes for nearly an hour.

Immediately after electrode application all subjects gave a sharp decrease in the impedance values, followed by reasonably steady values after about 10 minutes. This effect has already been demonstrated by Tregear (27) and is claimed to be due to build up of transepidermal water loss under the electrodes (41).

The same subjects were tested under similar conditions, using the same test site, 48 hours and 96 hours after the initial measurements. As can be seen from Figures 8 and 9, even though the impedance-time profiles were similar, the values were not. For example, subject C gave a mean steady state value, i.e. from 10 to 60 minutes of measurement, of $7.48*10^6$ ohms. However, 48 and 96 hours later the values had changed to $4.88*10^6$ ohms

FIGURE 7: VARIATION OF IMPEDANCE WITH TIME

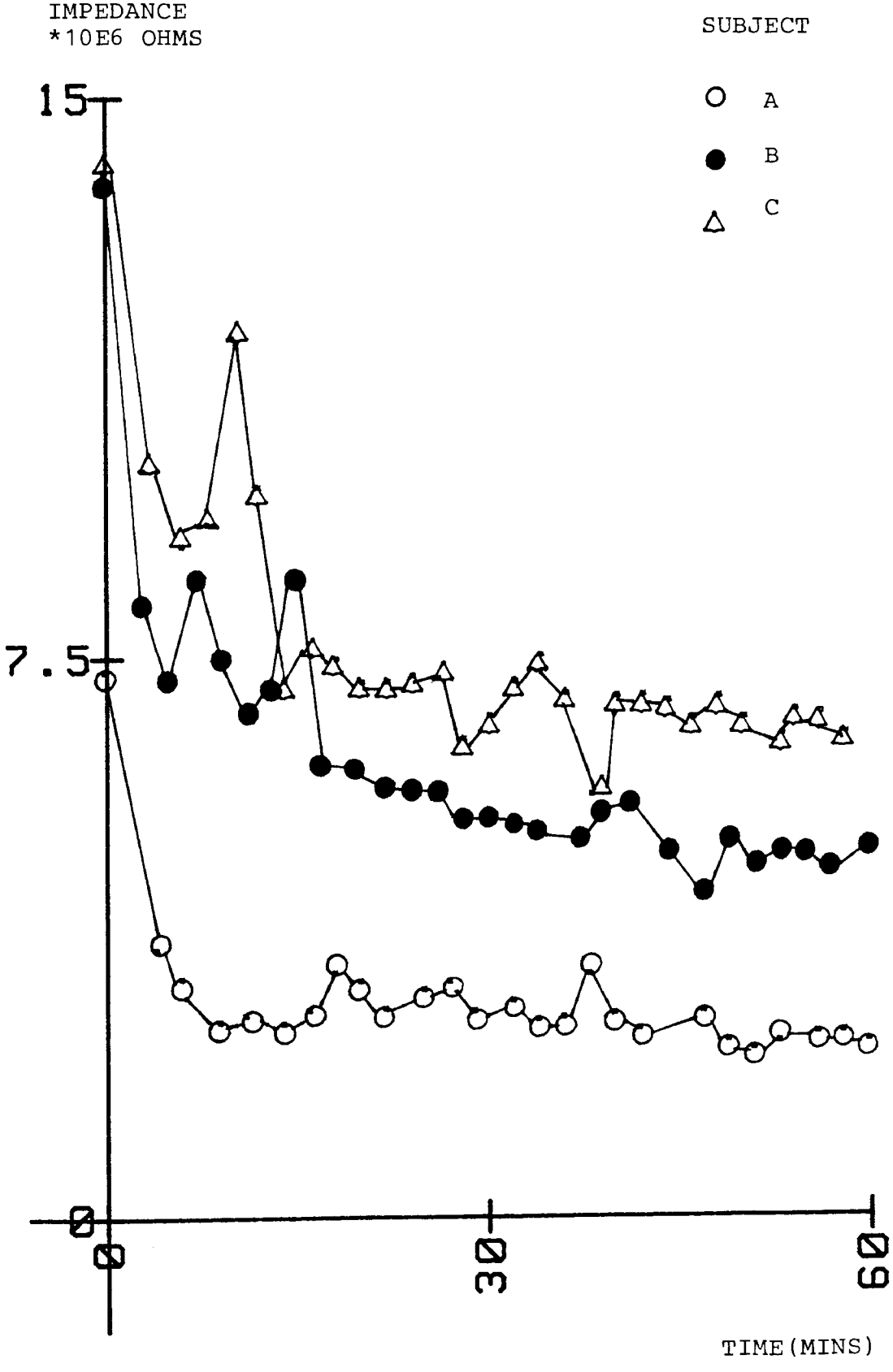


FIGURE 8: VARIATION OF IMPEDANCE WITH TIME 48 HOURS AFTER THE FIRST MEASURMENT

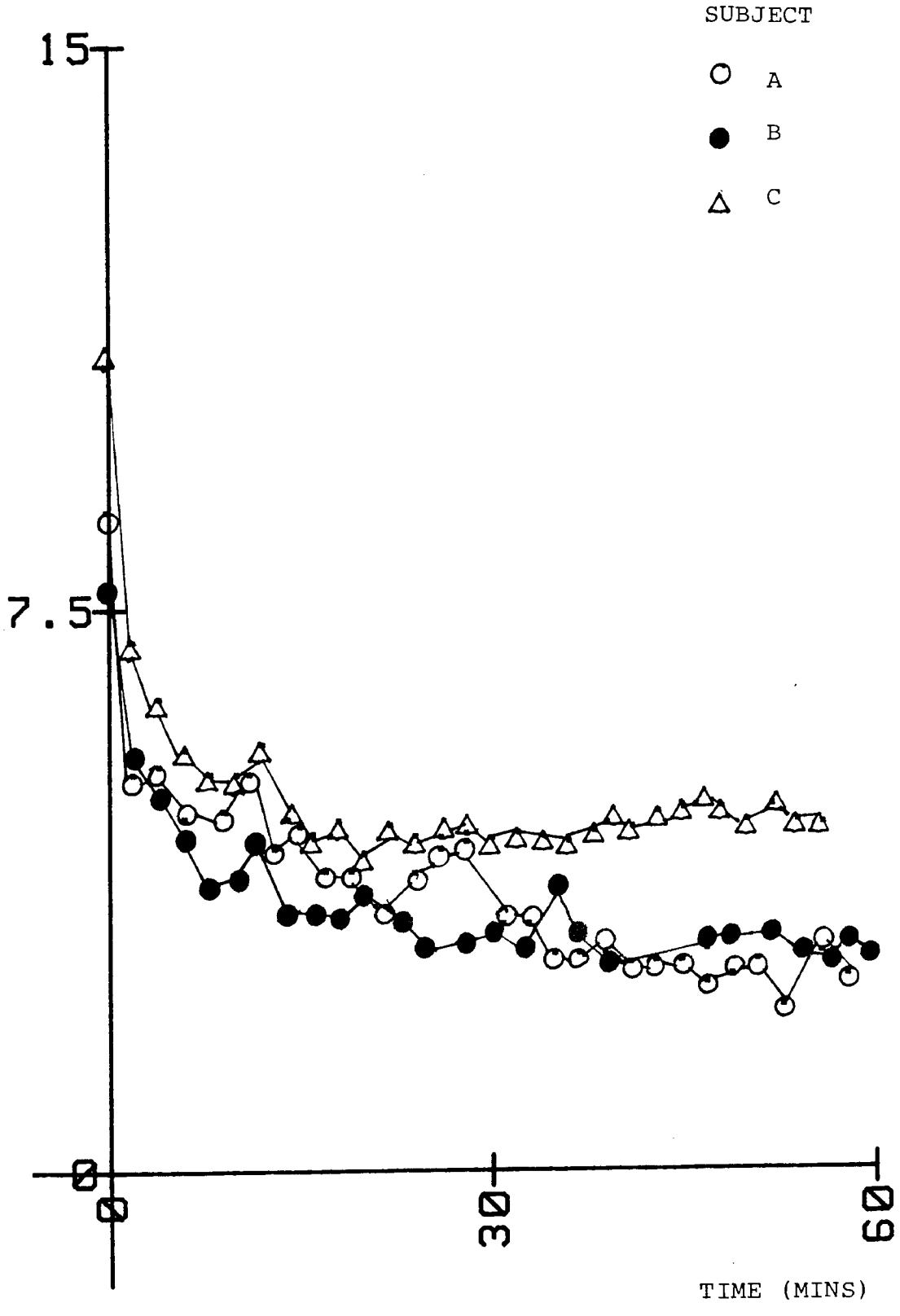


FIGURE 9: VARIATION OF IMPEDANCE WITH TIME 96 HOURS AFTER THE FIRST MEASUREMENT

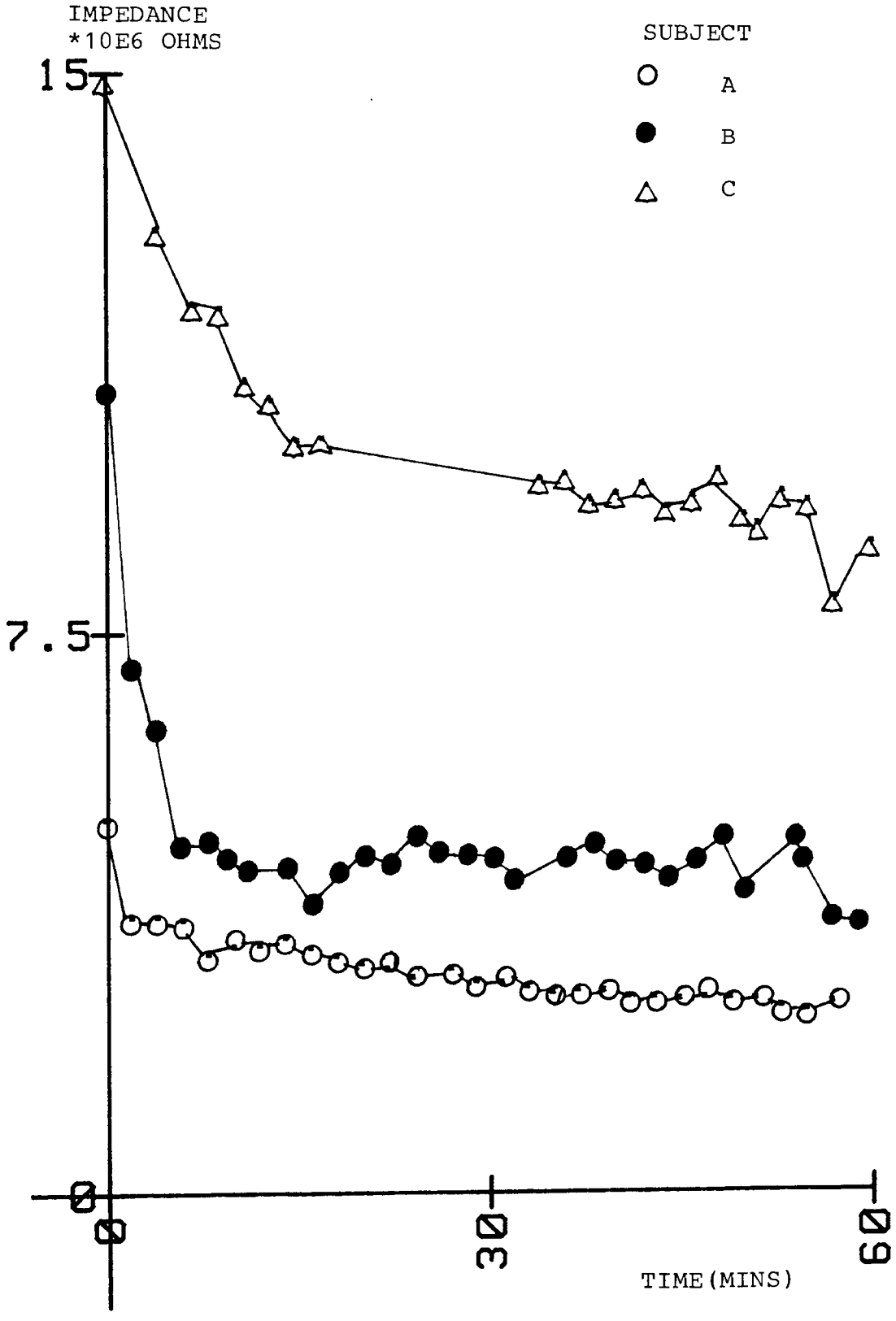
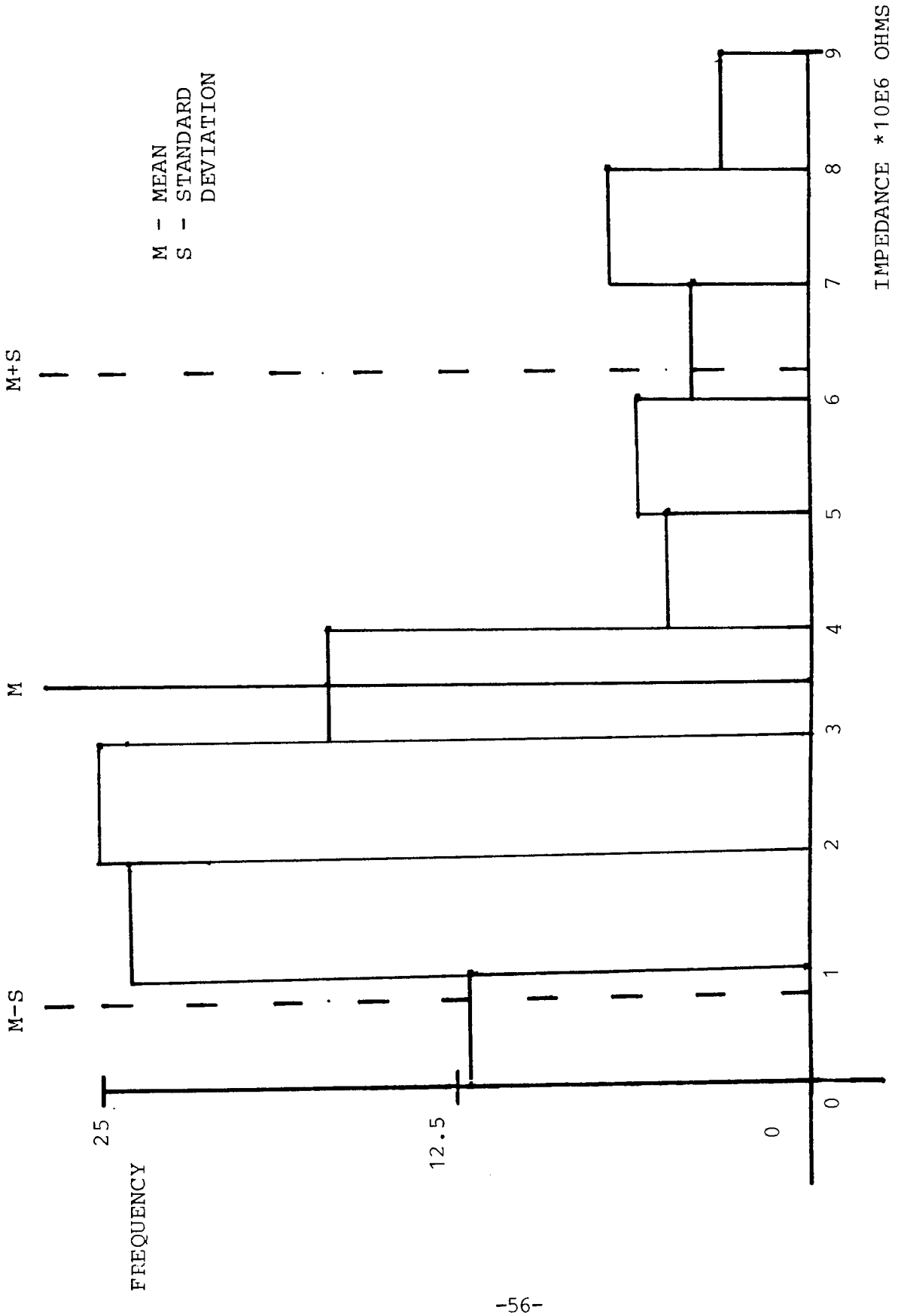


FIGURE 10: VARIABILITY OF MEAN SKIN IMPEDANCE
IN HUMAN VOLUNTEERS



and $10.18*10^6$ ohms respectively. This should be expected since the state of the skin changes continuously depending on the physiological and psychological state of the subject (13).

4.3. INTER-SUBJECT VARIATION

The time-dependence of the computed parameter, both hourly and daily, also shows a wide inter-subject variability as seen in both the rate of decrease of impedance after electrode application and the mean steady state impedance values. Figure 10 shows the extent of variability of the impedance for values averaged from 10 to 40 minutes of measurement. For untreated Caucasian forearm skin, impedance was found to vary from about $0.22*10^6$ to $15.18*10^6$ ohms.

4.4. EFFECT OF ELECTRODE SPACING ON SKIN IMPEDANCE

It is possible for the electrodes to introduce a contact or interfacial resistance of their own. This may contribute to the overall impedance and perhaps give a higher value than would be expected from skin alone (50). This contribution is separate from the influence of the occlusive effect of electrodes, which leads to build up of transepidermal water loss (27).

In order to check this theory, impedance measurements were made at two different electrode separations. If the influence of the electrodes is far higher than that of skin, the resultant impedance for the two separations should be similar, whereas if skin impedance is a major factor, doubling the electrode spacing should double the impedance.

Two pairs of electrodes, at 35mm and 70mm separation from their centres, were applied to the skin. The resultant impedance plots are shown in Figure 11. Generally the mean steady state impedance values for the 70mm separation are 1.5 to 3.5 times those of the 35mm separation. This would suggest that the influence of the skin is far greater than that of the electrodes.

FIGURE 11: VARIABILITY OF IMPEDANCE WITH ELECTRODE SPACING

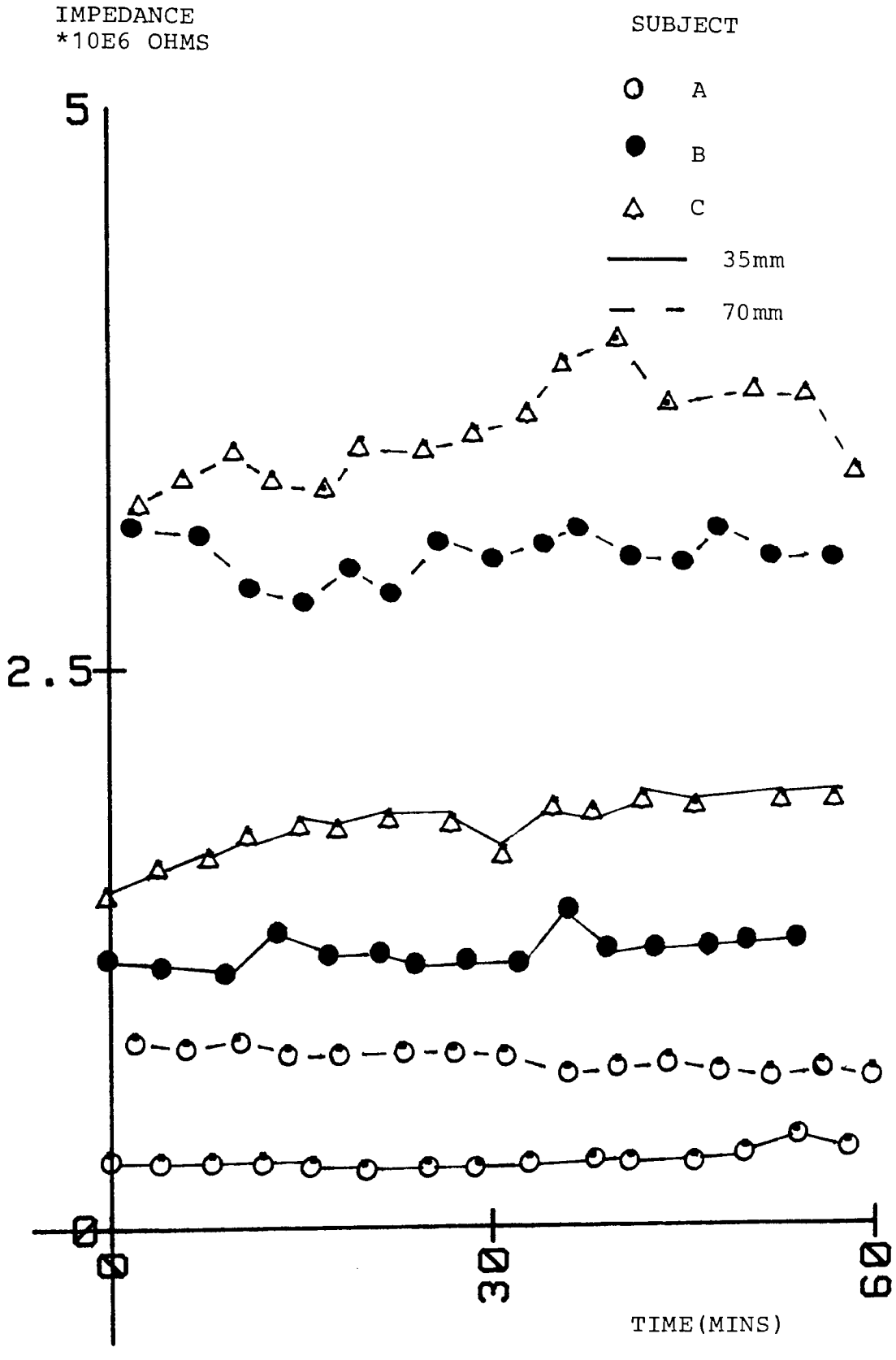
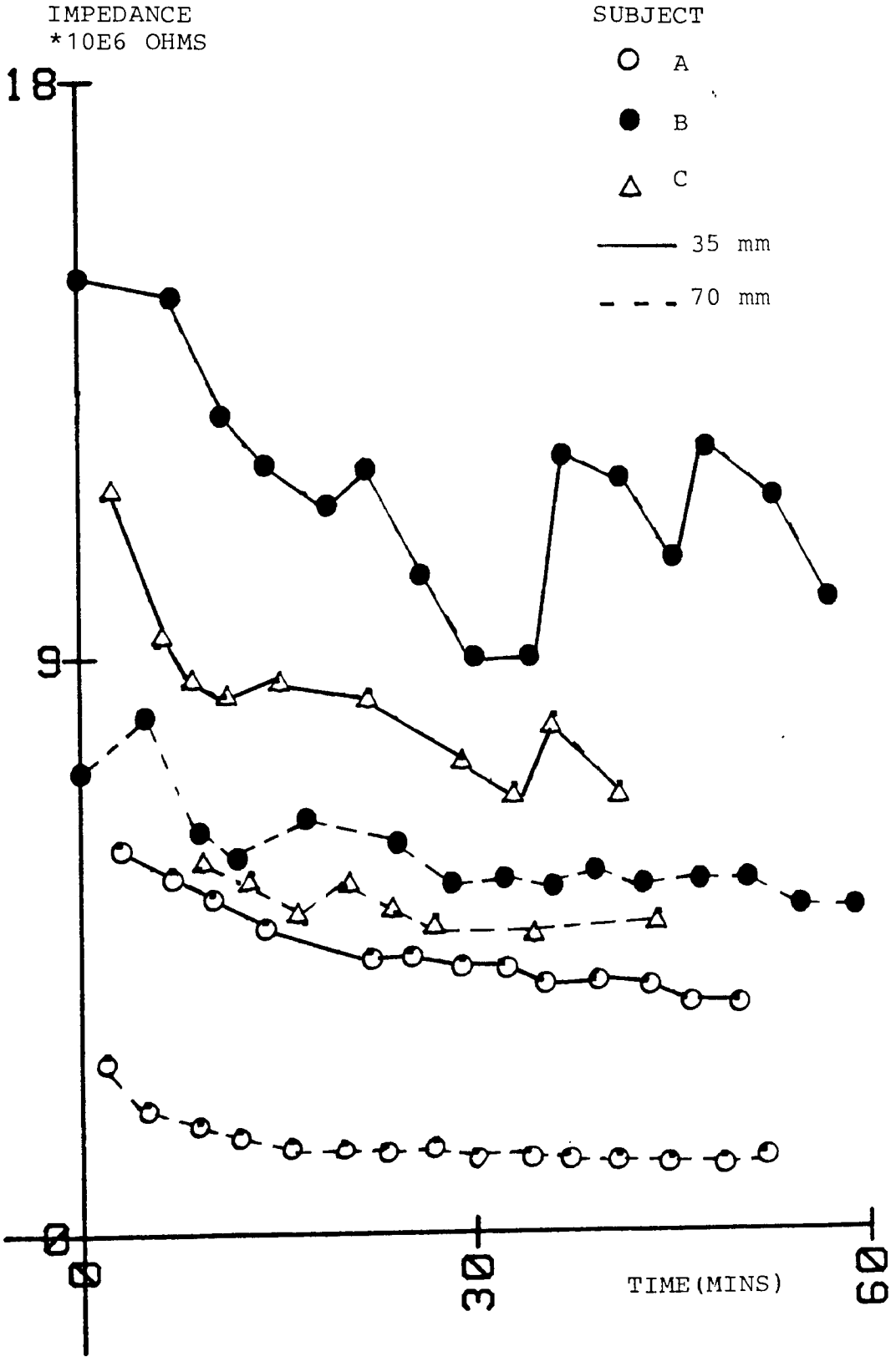


FIGURE 12: STUDY OF IMPEDANCE VALUES FOR ALTERNATIVE ELECTRODE SPACING



The experiments were repeated with the location of the electrodes reversed. Previously, the 35mm separation electrodes were applied on the volar side of the forearm and 70mm on the ventral side. When these positions are reversed, the steady state mean impedance values are also reversed (Figure 12). Now the 35mm separation gives a far larger value, suggesting that it is the location of the electrodes rather than their separation that is of importance.

4.5. INTRA-SUBJECT VARIATION

Various authors have reported considerable variation in the impedance of skin from one region of the body to another (13, 38, 48). The results obtained in this study suggest that impedance also shows marked variation with site in the same region of the body. In order to confirm this, measurements were conducted on adjacent sites of the forearm. The two neighbouring sites chosen were on the volar and ventral areas. Each site was measured consecutively and the measurements repeated for nearly an hour. Thus the values obtained were congruous in order to eliminate any physiological changes in the subject. Figure 13 shows that consistently higher impedance values were obtained from the ventral site.

FIGURE 13: EFFECT OF ELECTRODE LOCATION ON IMPEDANCE VALUES - VOLAR/VENTRAL SITE

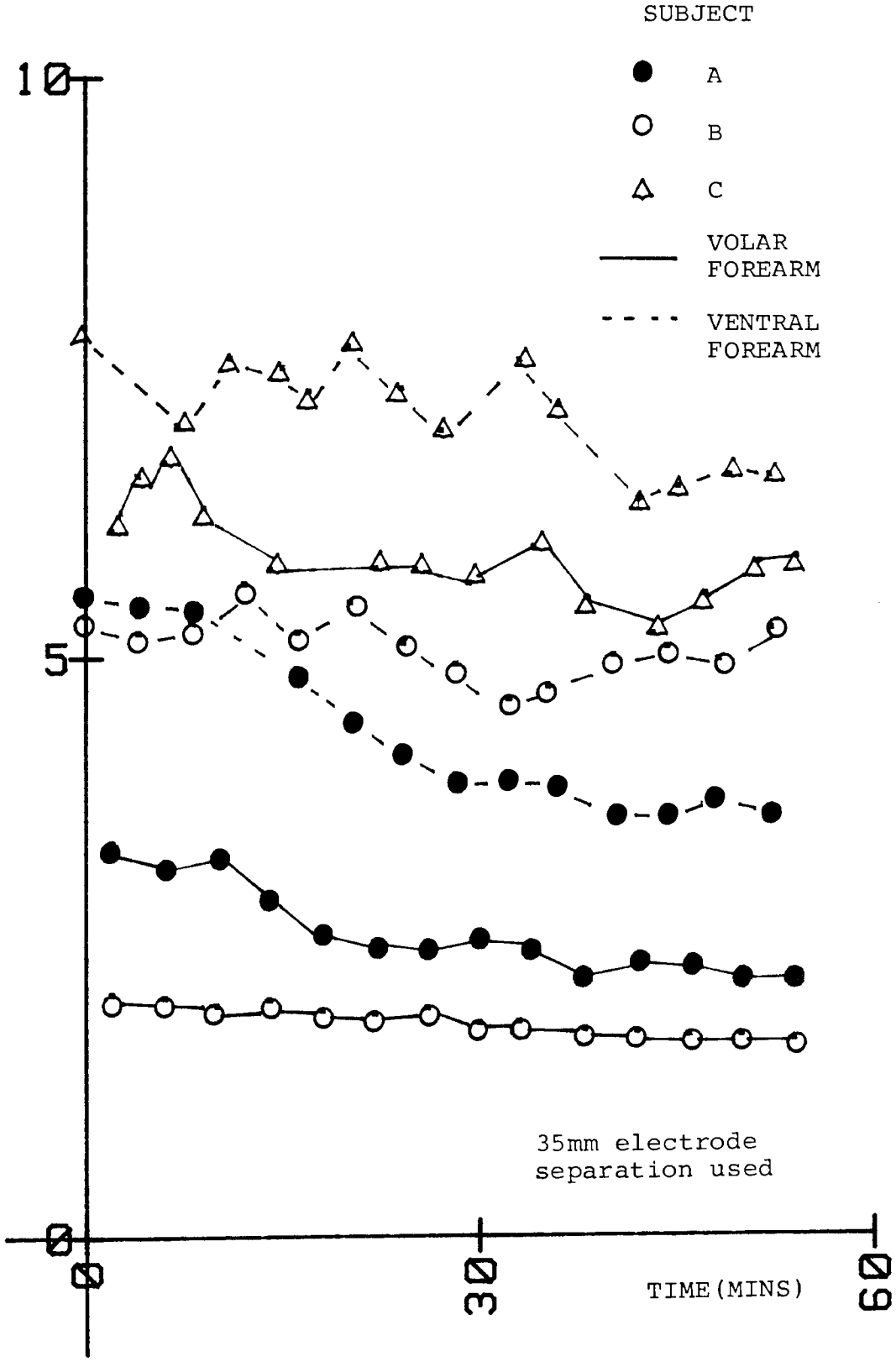
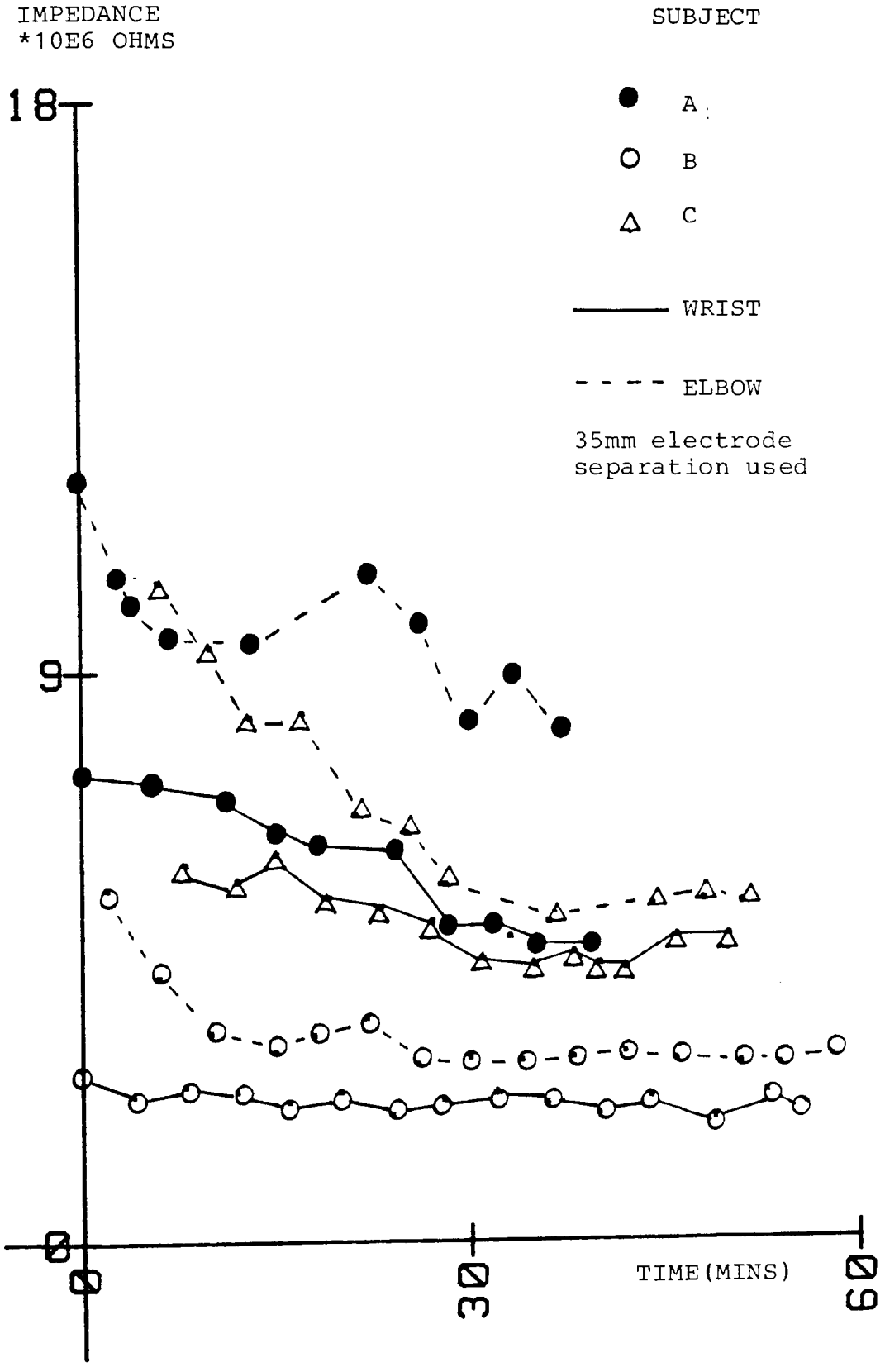


FIGURE 14: EFFECT OF ELECTRODE LOCATION ON IMPEDANCE VALUES - WRIST/ELBOW SITE



Further comparisons between the regions proximal to the wrist and elbow on the volar forearm were also made. The results gave consistently higher values for the elbow site (Figure 14).

Tagami et al (38) reported that the extensor surface of the forearm gave much higher impedance values, at a single frequency, than the flexor surface. Regional variation in skin impedance may be attributed to differences in skin structure such as thickness and concentration of the cutaneous appendages. However, various other physical techniques give more consistent local values, allowing adjacent sites to be used as adequate controls for test sites (10, 11).

The data presented here indicate wide intra-subject variation on adjacent sites of the forearm. This may also be attributed to local differences in skin structure, but no data have been found in the literature to confirm this.

4.6. IMPEDANCE MEASUREMENT ON SYMMETRICAL SITES

In order to assess the moisturising attributes of a product, both the test and control sites should give reasonably similar impedance values, before treatment. The data presented so far suggest that the wide inter- and intra-subject variations seen make skin impedance measurements unsuitable for assessing moisturising products.

Several authors have used symmetrical sites for product assessment and have reported very similar skin impedance values before treatment (13, 31, 32, 38). In order to confirm this, impedance measurements were made on symmetrical sites of the forearm. Each site was measured consecutively and the measurements repeated for 15 minutes.

The data obtained are recorded in Table 1 and assessed for significant differences between measurements, for each subject, on each forearm. Five of the six subjects tested gave significant differences in impedance values between the forearms.

This discrepancy with the reported data may be attributed to their measurements being less accurate since only single measurements were made for a given site and only at a single frequency (13). Furthermore, overall mean values were used rather than just the individual mean values as in the present study.

TABLE 1: ASSESSMENT OF IMPEDANCE VALUES FOR SYMMETRICAL FOREARM SITES MEASURED FOR FIFTEEN MINUTES.

Test Method : t - Test by Difference (110)

$$t = \frac{\Sigma D}{\sqrt{\frac{N \Sigma D^2 - (\Sigma D)^2}{N - 1}}} \qquad N = 4$$

SUBJECT	LEFT FOREARM Z_1 E6	RIGHT FOREARM Z_2 E6	D $=Z_1 - Z_2$	D^2	t	SIGNIFICANT DIFFERENCE
1	3.76 3.51 3.39 3.56	4.09 3.41 3.47 3.53	-0.33 0.10 -0.08 0.03	0.109 0.01 0.006 0.0009	0.74	NO
2	3.28 3.40 3.27 3.31	2.83 3.04 2.78 2.68	0.45 0.36 0.49 0.63	0.203 0.129 0.24 0.397	8.59	P<0.01
3	1.43 1.35 1.29 1.29	1.34 1.27 1.26 1.22	0.09 0.08 0.03 0.07	0.0081 0.0064 0.0009 0.0049	5.13	P<0.05
4	1.01 1.03 1.07 1.01	1.63 1.50 1.47 1.55	-0.62 -0.47 -0.40 -0.54	0.384 0.221 0.16 0.29	10.95	P<0.01
5	4.08 3.96 3.56 3.24	2.69 2.36 2.23 2.05	1.39 1.60 1.33 1.19	1.93 2.56 1.77 1.41	16.87	P<0.01
6	1.67 1.68 1.72 1.75	2.66 2.45 2.35 2.23	-0.99 -0.77 -0.63 -0.48	0.98 0.59 0.39 0.23	6.62	P<0.01

4.7. EFFECT OF ELECTRODE REMOVAL AND REAPPLICATION ON SKIN IMPEDANCE

The use of the same site, both as a pre-treatment control and test area, provides a further possibility for product assesssment using skin impedance. Before this can be examined the effect of removal and reapplication of the electrodes on the readings must be monitored, especially since a rapid initial drop in impedance has already been attributed to accumulation of transepidermal water in the stratum corneum below the electrodes (27, 41).

Measurements were conducted with the electrodes already in position for 30 minutes (Figure 15) or immediately after application (Figure 16). Once steady state was reached, the electrodes were removed, the electrode gel gently swabbed off and the electrodes reapplied, on the same site with new gel. In addition, a pair of electrodes was applied adjacent to the test site to serve as a control to monitor any dramatic changes in the psychological and physiological states of the subject, which may affect the impedance values.

FIGURE 15: EFFECT OF ELECTRODE REMOVAL AND REAPPLICATION ON IMPEDANCE VALUES.

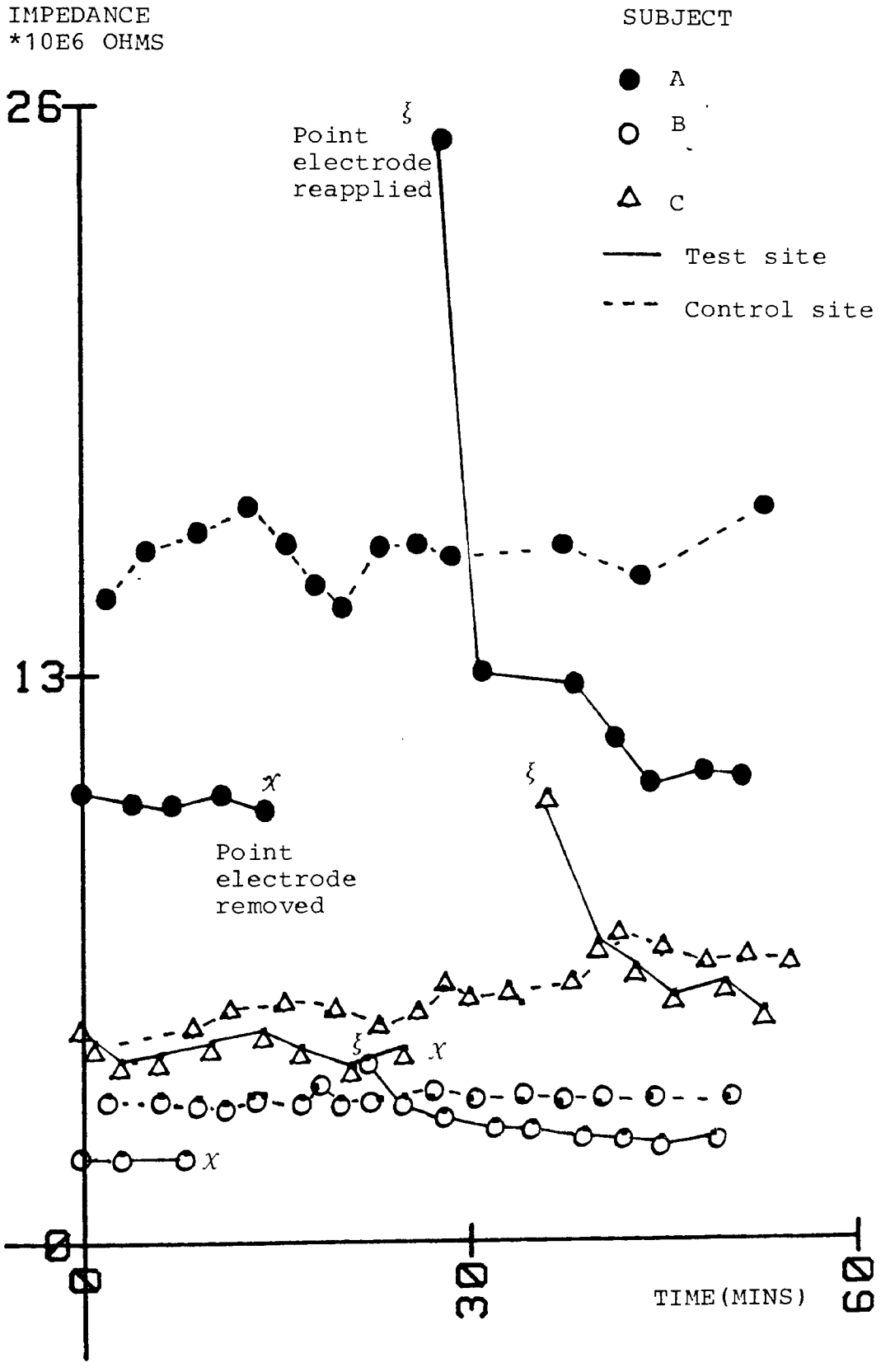
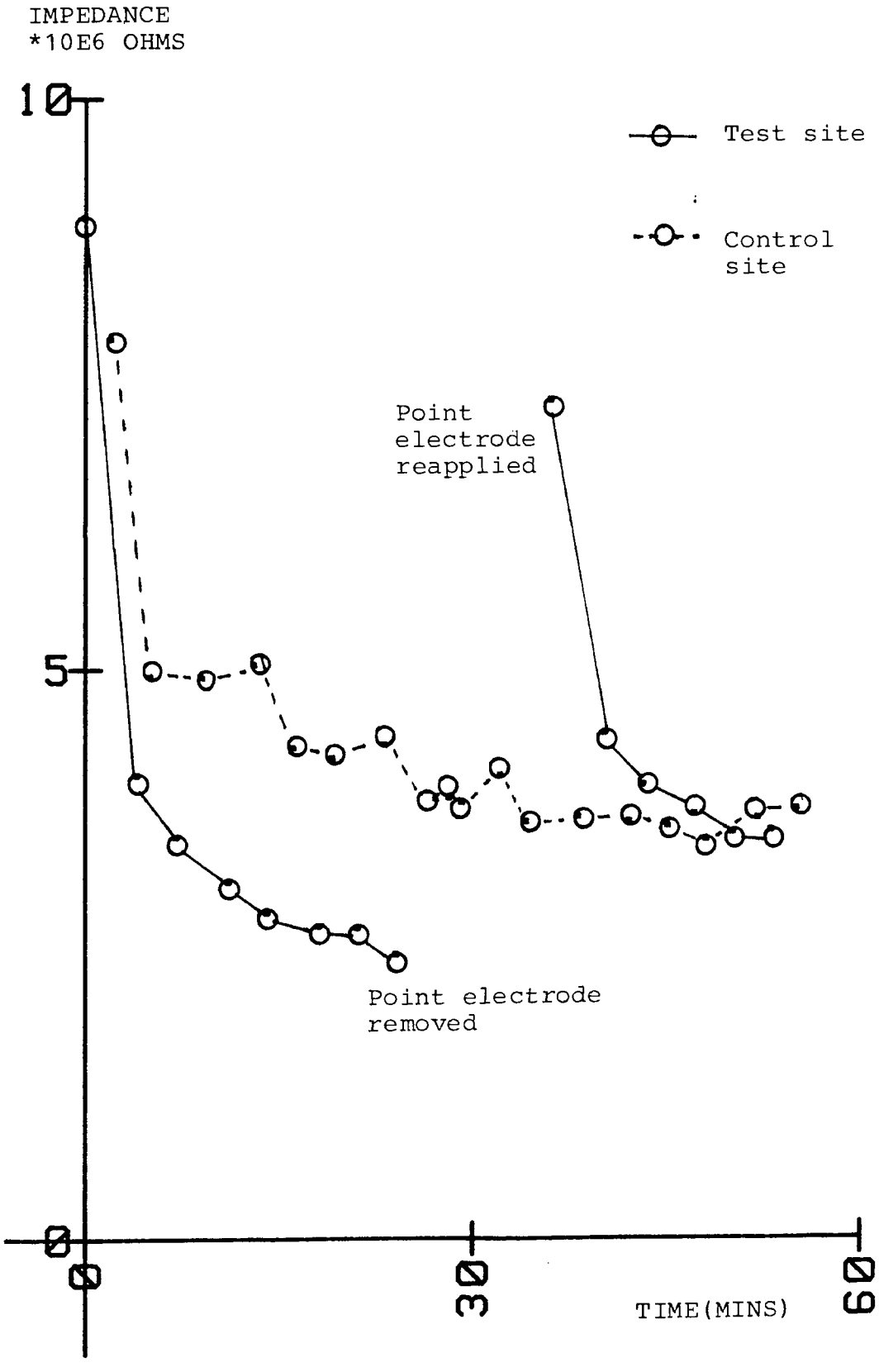


FIGURE 16: COMPARISON OF INITIAL IMPEDANCE DROP WITH ELECTRODE REMOVAL AND REAPPLICATION



Irrespective of the approach used electrode removal led to an increase in the impedance values followed by a rapid decrease after reapplication. This suggests that steady-state impedance is a function of electrode-skin equilibration rather than just moisture equilibration within the stratum corneum on its own. If the latter was the cause, one would anticipate a much more rapid equilibration upon reapplication of the electrodes. However, this was not the case.

4.8. ELECTRICAL FIELD LINES PROFILE FOR SKIN

The distribution of the field lines inside the skin, i.e. the precise anatomical structures involved in the measurement are not known. Low frequency measurements are claimed to be representative of the stratum corneum (13, 29) and as previously discussed, measurements were conducted over 1 - 500Hz frequency range.

In order to identify the electrical field lines profile for the electrode configuration used here, the skin was stripped using Sellotape. Wolf (51) first suggested stripping the stratum corneum to study the epidermal cells. This was followed by a

number of authors who demonstrated that the barrier properties of the skin resided in the stratum corneum (47, 52).

Using Sellotape, the stratum corneum was stripped 15 times below each electrode (Figure 17) and in a separate experiment it was stripped 16 times between each pair of electrodes (Figure 18). This number of strippings should have resulted in adequate removal of the stratum corneum, especially since the skin was beginning to glisten with moisture and the subject was also beginning to suffer from increasing discomfort. Again a pair of electrodes was applied adjacent to the test site,as untreated control, to monitor any dramatic changes in the mean steady state impedance.

Sharp impedance drops were obtained when the stratum corneum was stripped below the electrodes, whereas no significant changes were observed when the region between the electrodes was stripped.

The data presented so far suggest that in the case of untreated skin, the stratum corneum is the main contributor to the impedance being measured. Furthermore, the deeper, more conductive epidermal layers are being measured in the area between the

FIGURE 17: EFFECT OF STRIPPING THE STRATUM CORNEUM BELOW THE ELECTRODES ON SKIN IMPEDANCE

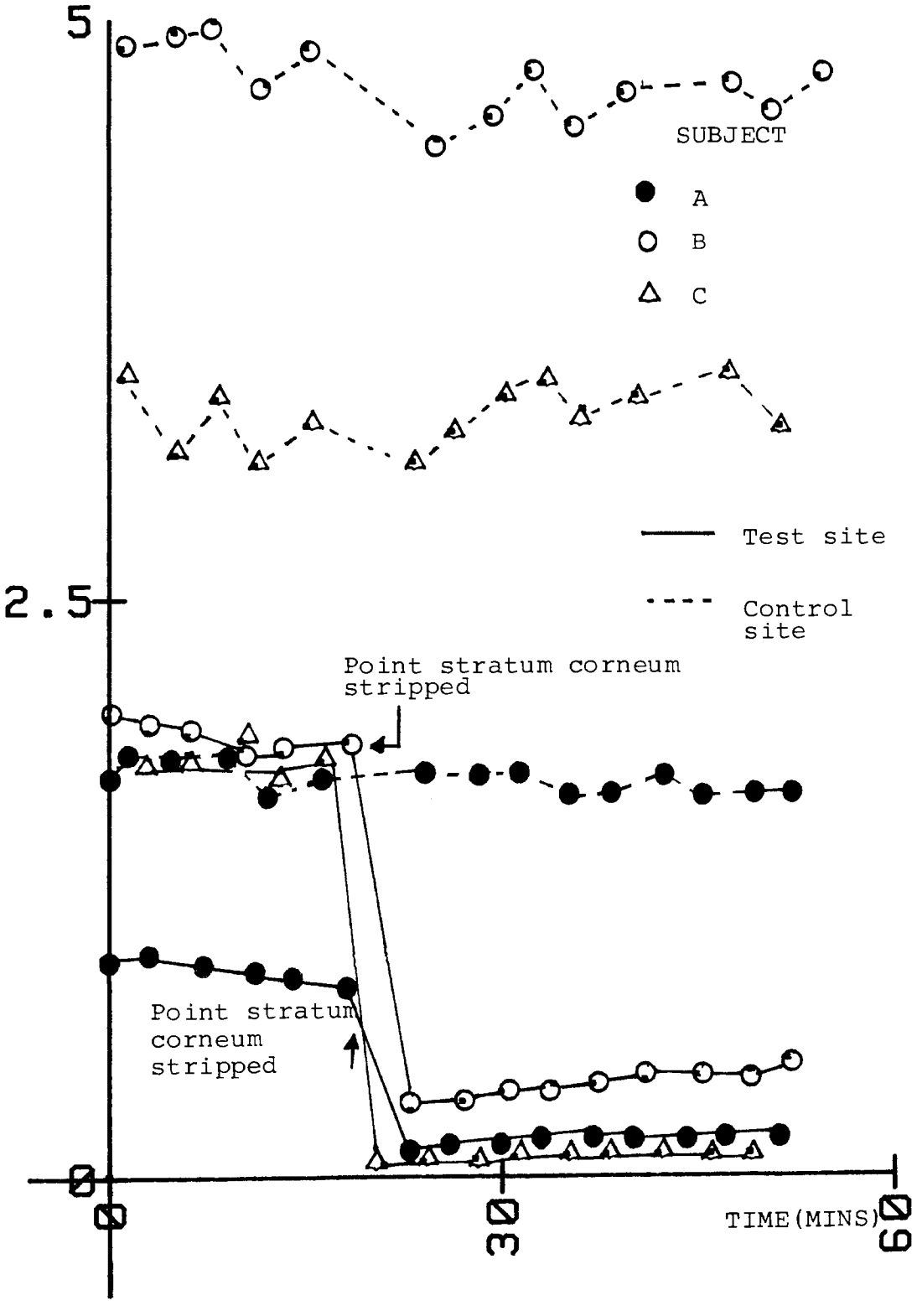
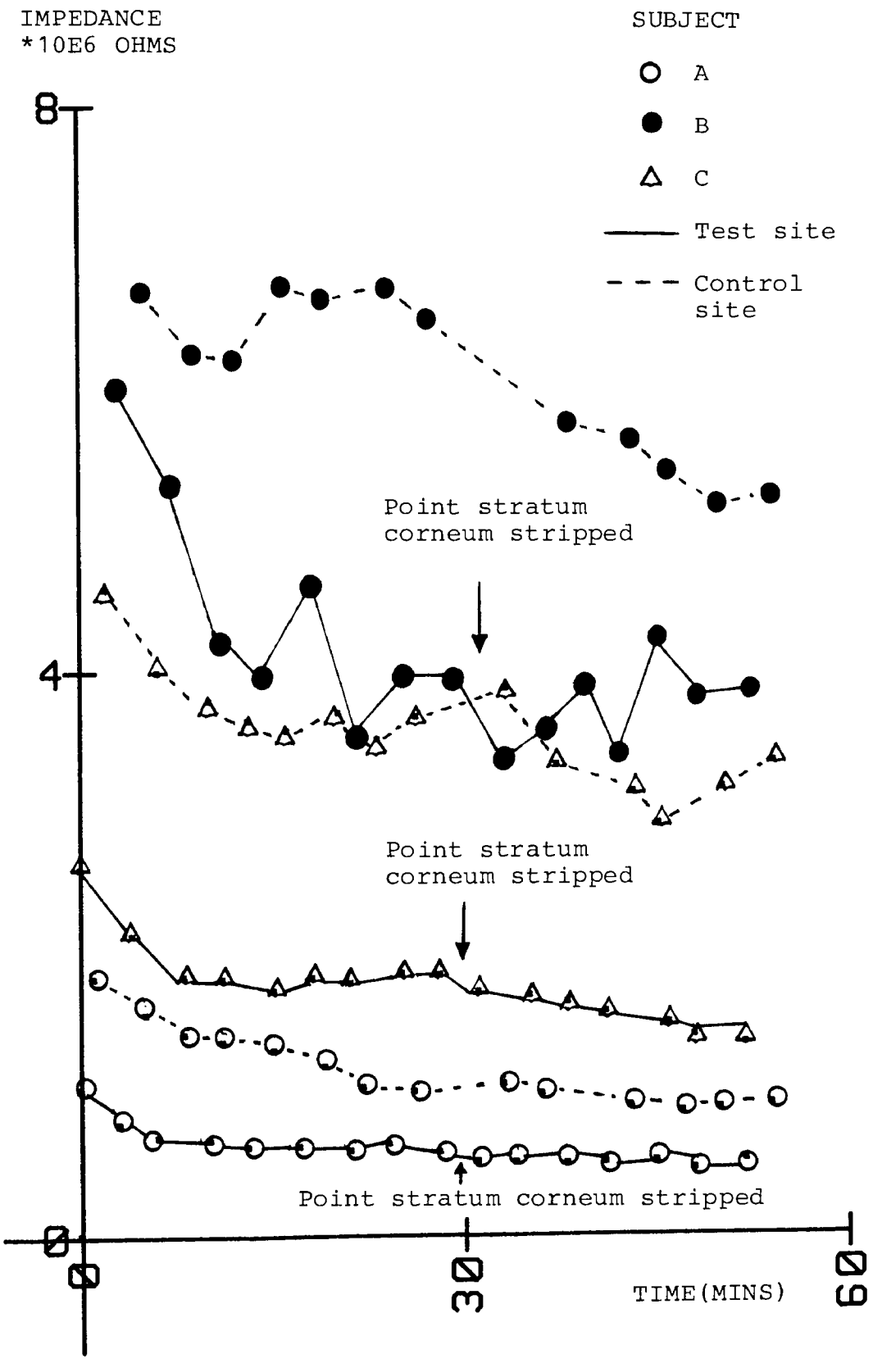


FIGURE 18: EFFECT OF STRIPPING THE STRATUM CORNEUM BETWEEN THE ELECTRODES ON SKIN IMPEDANCE



electrodes and the stratum corneum contributes to the impedance only in the region immediately below the electrodes.

This further suggests that if the electrode separation, in this case 39.5mm, far exceeds the stratum corneum thickness, the electrical field lines will not traverse the entire stratum corneum between the electrodes. However, it does justify the use of the semi-circular diameter (Figure 3) as the main parameter representative of stratum corneum impedance.

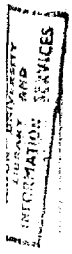
4.9. ASSESSMENT OF TOPICAL PRODUCTS USING SKIN IMPEDANCE.

4.9.1. INTRODUCTION

The data discussed so far shows the wide variability of the resting hydration levels of the stratum corneum in vivo. This makes it difficult to use adjacent and symmetrical sites on the body for the assessment of topical products. However, various authors have already shown skin impedance to be a useful technique for skin hydration measurement (13, 31, 38).

The electrode set up here leaves two possible methods for topical product assessment. Either the product may be applied below the electrodes or between them. In the case of product application below the electrodes, the untreated control should be the same site before electrode removal.

Consideration, however, must be given to the fact that when electrodes are removed, there results an initial unsteady state impedance. Nevertheless, it is proposed that the moisturising properties of a product will be such that not only would it counteract the unsteady state impedance, but also give a decrease in the overall untreated steady state impedance.



If the product is applied between the electrodes, it is proposed that the stratum corneum will be sufficiently hydrated for the electrical field lines to pass through it, thus giving a decrease in the overall impedance. Again the untreated control would have to be the same site measured prior to treatment. However, since the electrodes need not be disturbed, no consideration has to be

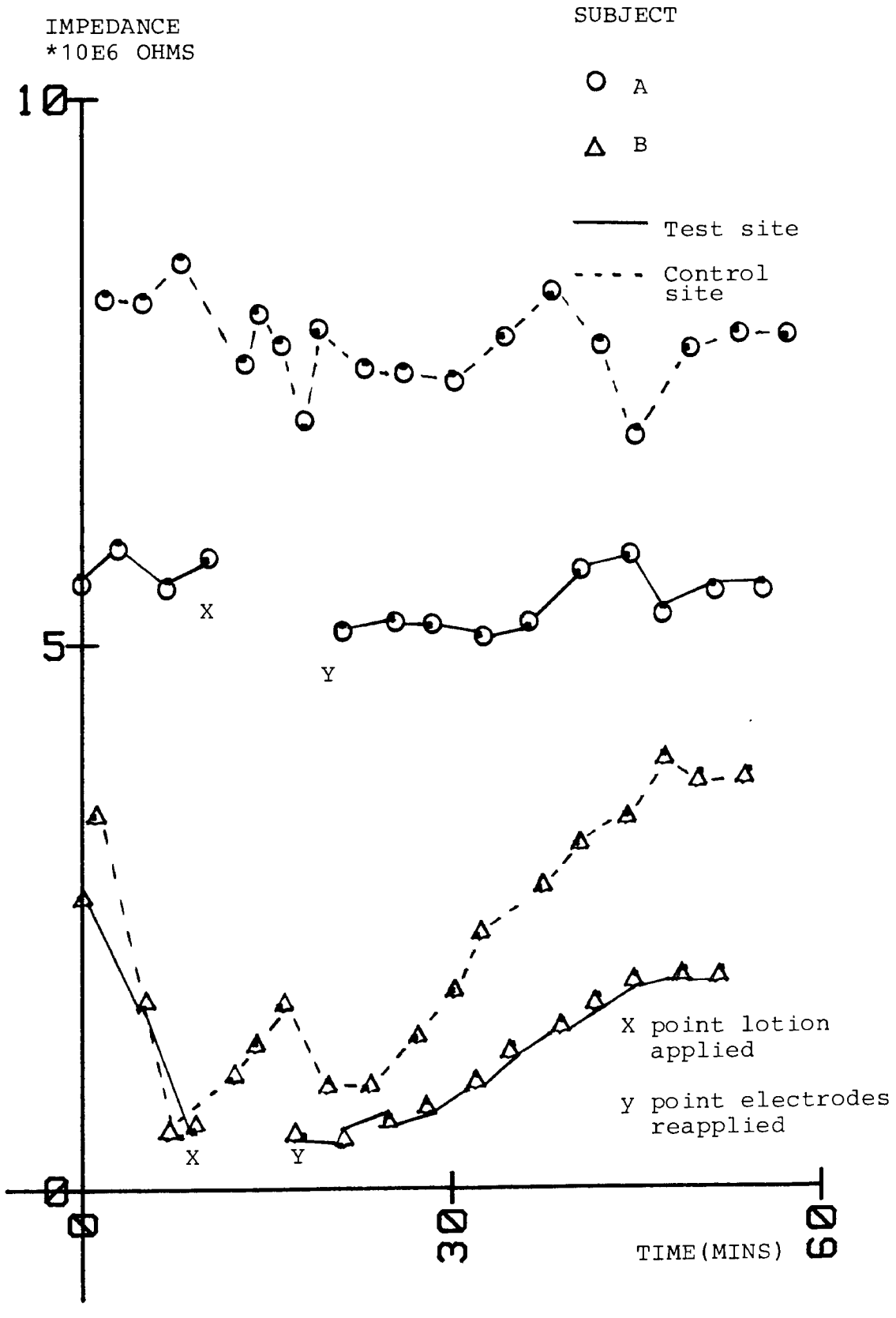
given to the initial unsteady state impedance values.

4.9.2. APPLICATION OF TOPICAL PRODUCTS BELOW THE ELECTRODES

Two pairs of electrodes were applied on adjacent sites of the volar forearm. The subject was allowed 30 minutes of equilibration followed by 10 minutes of impedance measurement on both sites. One pair of electrodes was removed and a commercial oil-in-water based skin lotion applied. Excess lotion was gently rubbed in for a minute on both the electrode sites only. No product was applied between the two sites. The electrodes were then reapplied using fresh contact gel.

From Figure 19, it can be seen that the impedance values remain constant before and after treatment. Also they remain steady for another 45 minutes after product application. Meanwhile, the adjacent control site shows no dramatic changes either, suggesting the subject's natural physiological steady state

FIGURE 19: APPLICATION OF SKIN LOTION BELOW THE ELECTRODES



impedance measurements also remain constant, during the period of experimentation.

Previously, when the electrodes were removed and reapplied without treatment (Figures 15 and 16), the impedance returned to an initial high value followed by a rapid decrease. The effect of applying a lotion seems to eliminate this initial unsteady state impedance. Since there is a wide inter- and intra- subject variability in both the magnitude and rate of decrease of this unsteady state impedance, it is therefore not possible to objectively analyse the lotion by application below the electrodes. Furthermore, if the result obtained was entirely due to the moisturising attributes of the lotion, the steady state values obtained after treatment, should vary from subject to subject and not be as consistent as found.

Nevertheless, it is interesting to note that, previously, the time taken to achieve a steady state, after electrode application, was suggested to be due to not only moisture equilibration within the stratum corneum, but also to electrode-skin equilibration.

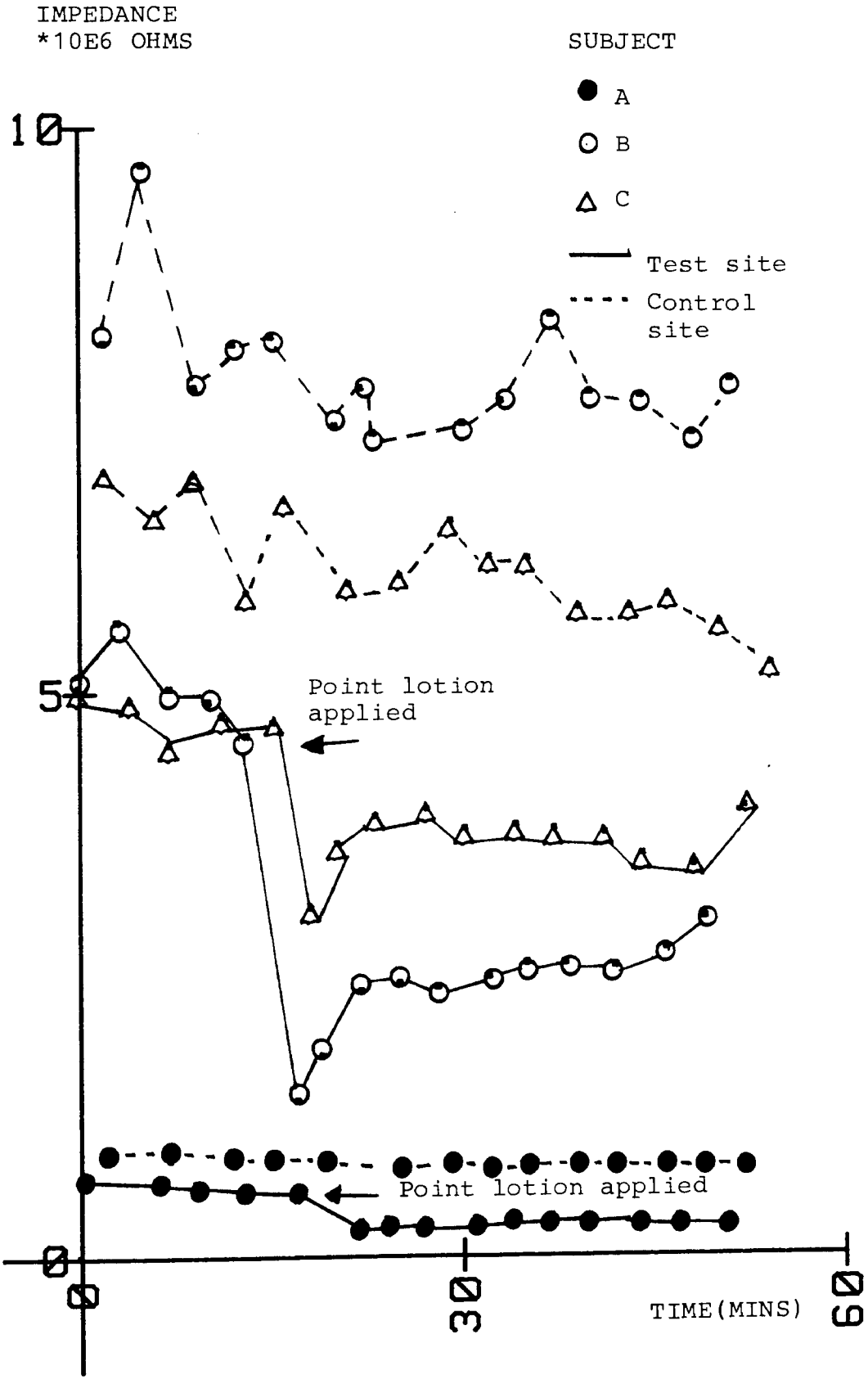
The fact that the lotion maintains the steady state suggests both causes are of similar importance. The lotion probably prevents moisture loss as well as enhances electrode skin contact.

4.9.3. APPLICATION OF TOPICAL PRODUCTS BETWEEN THE ELECTRODES

An alternative assessment proposal is the application of the topical product between the electrodes. The same skin lotion used previously was gently rubbed in for a minute and excess swabbed off. The data for different subjects is recorded in Figure 20. There is a sharp decrease in the steady state values immediately after product application, followed by a new, albeit lower, steady state.

The magnitude of decrease was calculated as a percentage change in the steady states. The data varied tremendously from subject to subject and for the same subject on repeat measurements. For the lotion used here, the drop in steady state impedance was anywhere between 30% and 90%.

FIGURE 20: COMPARISON OF UNTREATED SKIN WITH LOTION TREATED USING IMPEDANCE VALUES



Since the skin lotion tested had a vast array of constituents, single components with known hydration effects were examined, in order to enable more meaningful conclusions to be drawn.

Dimethyl sulphoxide (DMSO), at concentrations above 50% aqueous, has been found to swell or expand the protein fibres in the stratum corneum and thus enhance percutaneous absorption (53, 54). Allenby et al (37) found sharp decreases in the impedance of skin within minutes of DMSO application. Furthermore, Malten and den Arend found decreases of impedance in the range 60% to 90% DMSO aqueous, with progressive increase in the strength of the solution and duration of exposure (36).

Methanol and ethanol are also claimed to damage the epidermis and increase its permeability (55). It is possible that this alteration may also result in a change in the impedance of skin.

Two pairs of electrodes were applied on adjacent volar forearm sites. One pair was used as a control to measure the subject's natural physiological impedance. This ensured that any changes in the test site could be attributed to the products only. Both sites were measured for about 10 minutes to obtain untreated steady state values. After this, 60% DMSO aqueous or pure ethanol were applied between the test electrodes. The products were applied in soaked filter paper, which was left on the test site for the duration of the experiment.

Figure 21 gives the data for the 60% DMSO and Figure 22 for the ethanol. Both products gave decreases in the impedance values immediately after product application. However, these decreases tended to vary widely between subjects and on repeat measurements on the same subject.

The possibility of short circuiting of the electrical field lines via the product on the skin surface, between the electrodes, was also considered. A thin film of white soft paraffin was applied to the area immediately

next to the electrodes, as shown in Figure 23 to act as an insulator. The paraffin was applied first to ensure that it did not affect the mean steady state untreated values. The solvents were then applied, as above, soaked in filter paper and the impedance continuously monitored. Figure 24 supports the short-circuiting hypothesis since in this case no drop in impedance occurs on application of the test material.

FIGURE 21: COMPARISON OF UNTREATED SKIN WITH 60% DMSO AQUEOUS SOLUTION

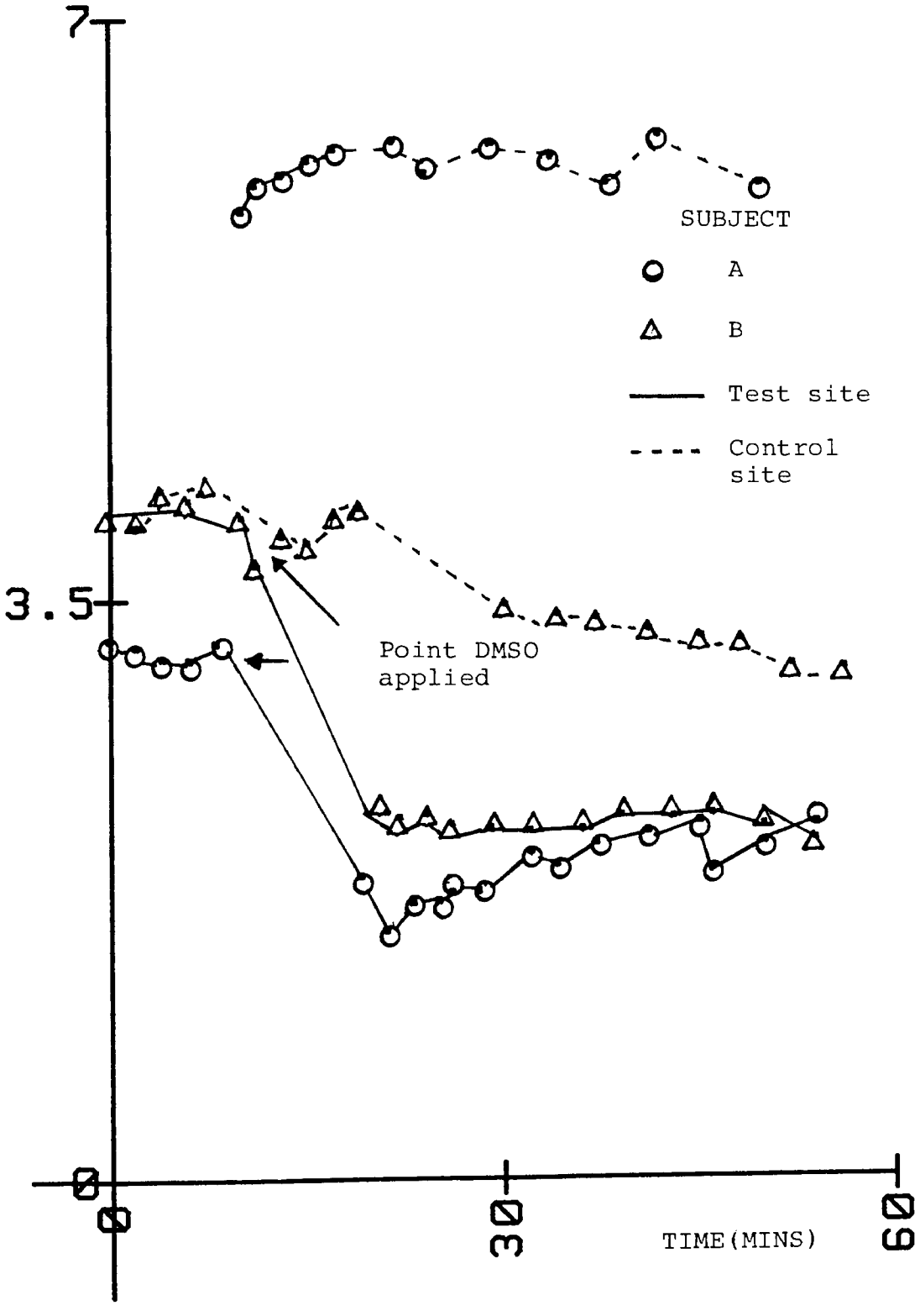


FIGURE 22: EFFECT OF ETHANOL ON SKIN IMPEDANCE VALUES

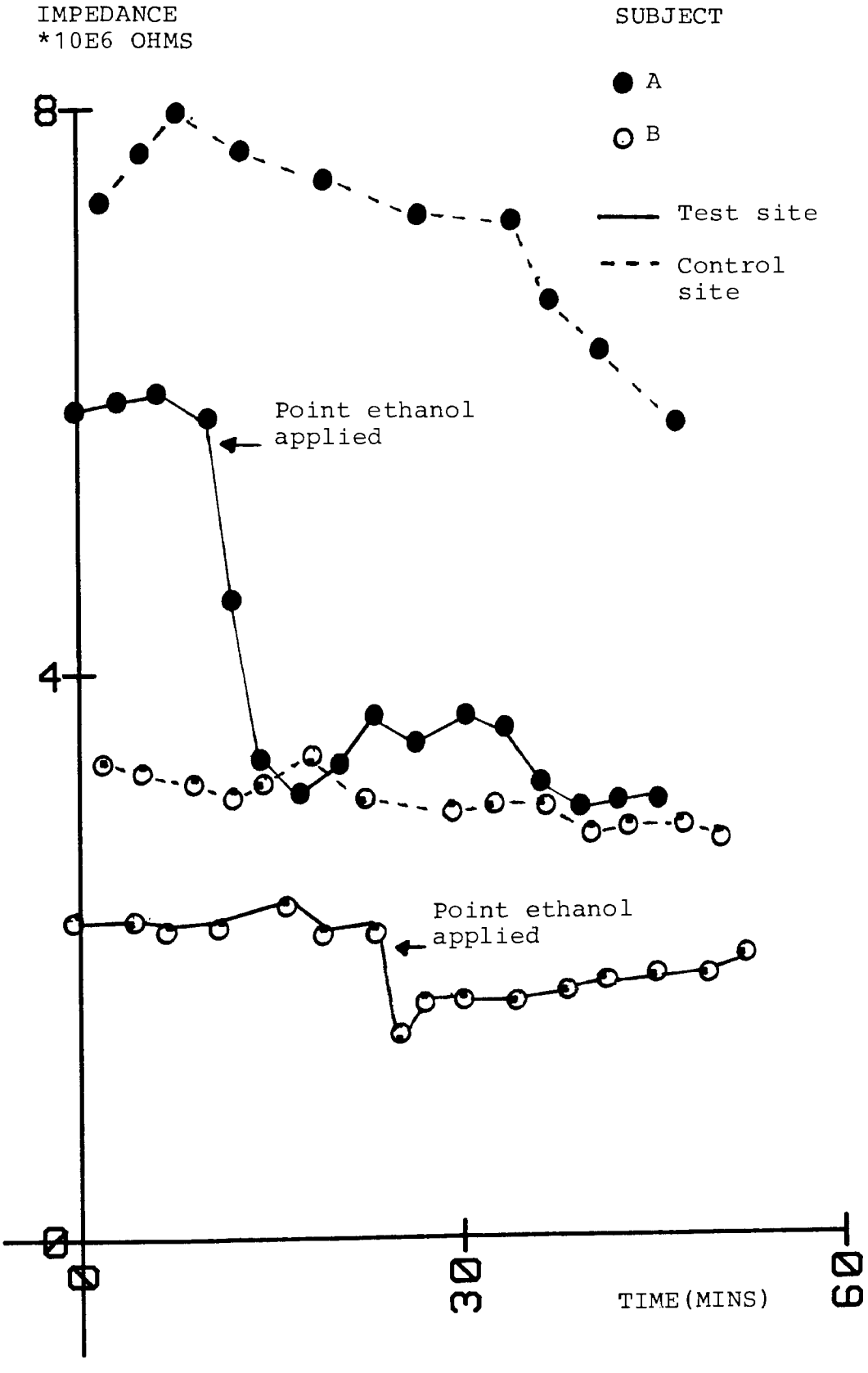


FIGURE 23: DIAGRAM SHOWING ELECTRODE SHIELDING WITH WHITE SOFT PARAFFIN

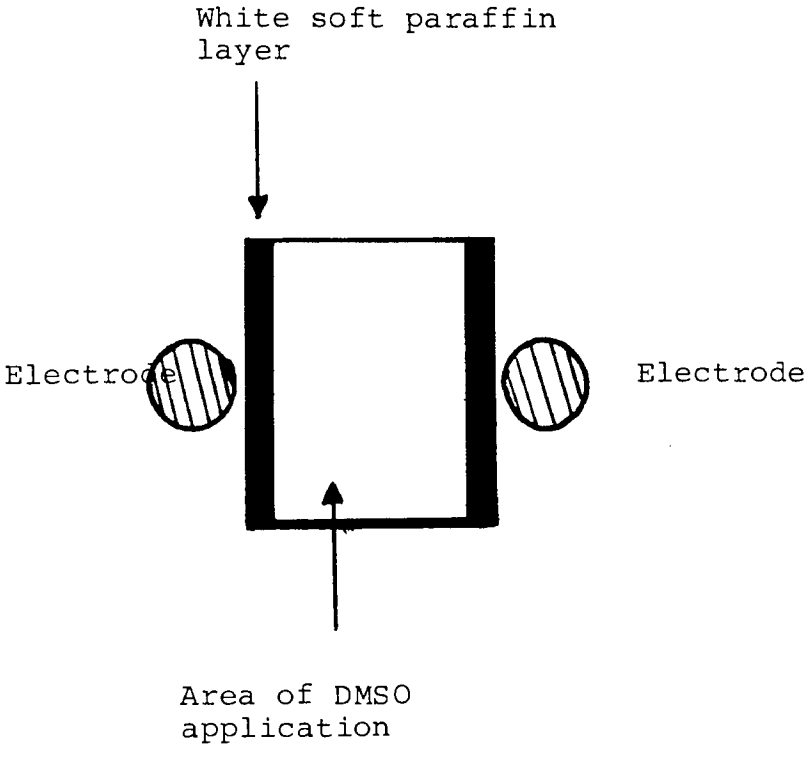
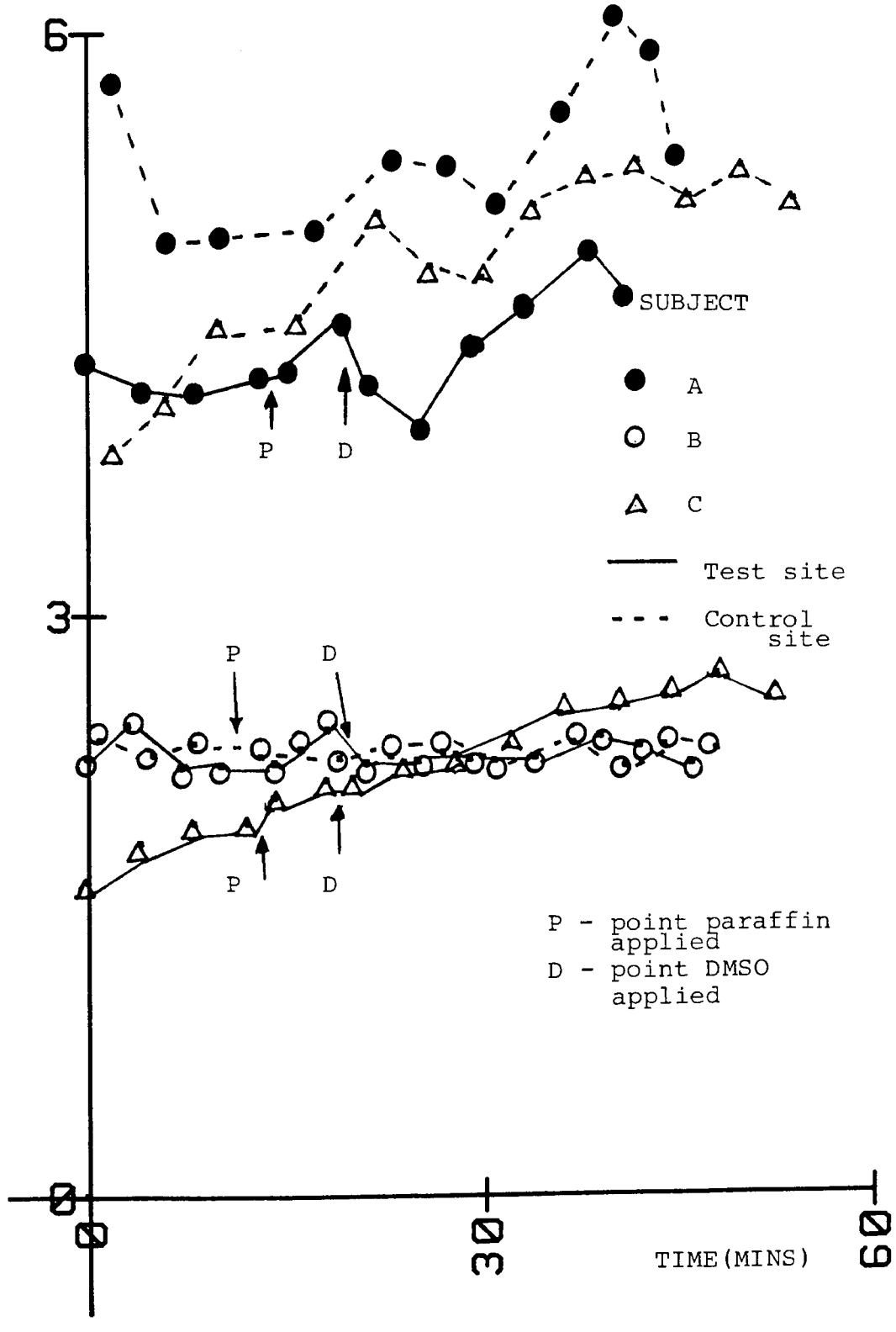


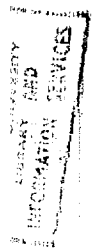
FIGURE 24: EFFECT ON IMPEDANCE OF ELECTRODE SHIELDING USING WHITE SOFT PARAFFIN PRIOR TO APPLICATION OF 60% DMSO AQUEOUS SOLUTION



CHAPTER 5.0: SKIN IMPEDANCE - CONCLUSION

This investigation has considered the application of electrical impedance measurements to monitor the hydration state of the skin. The method has been previously proposed for the in vivo assessment of the moisturising effects of both cosmetic and pharmaceutical formulations, in a non-invasive manner.

The apparatus used here allows the rapid assessment of skin impedance over a wide frequency range. Skin has been modelled using equivalent electrical circuits to determine the anatomical structures contributing to the impedance.

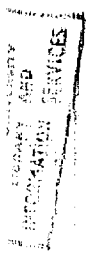


Two pairs of electrodes are used, applied on the skin using electrode gel. This ensures good contact and an adjustable elastic bracelet containing the electrodes, ensures the ease of use of the apparatus for clinical measurements.

By Sellotape stripping experiments, it has been shown that the parameter of analysis is

representative of the stratum corneum impedance and thus justifies the use of the apparatus and model of the skin. For the electrode set up used here the area of stratum corneum below the electrodes rather than that between the electrodes determines the value of impedance obtained.

Monitoring of baseline untreated forearm skin values suggests a wide inter- and intra- subject variation. Symmetrical sites of the forearm also give extreme differences in impedance values. These data make it difficult to objectively assess the moisturising attributes of topical products.



The possibility of using the test site as its own untreated control, for product assessment has also been examined. However, this has been eliminated due to short circuiting of the electrodes and changes in the steady state impedance on removal and reapplication of the electrodes.

CHAPTER 6.0: LASER DOPPLER - REVIEW

6.1. INTRODUCTION

Cutaneous blood vessels have the ability to regulate the metabolic, haeomodynamic and thermal state of the individual.

The complex nature of the blood supply to the skin makes its measurement difficult. The total blood supply may be of interest to some, whilst others may wish to know the flow in specific vessels. On the other hand, those measuring pharmacological effects require instant measurement of the changes in blood flow. Often these measurements need to be continuous to study the time course behaviour of drugs.

There are various methods available for the measurement of cutaneous microcirculation (56, 57, 58, 59). Thermal-conductance methods rely on heating the skin and detecting the clearance of this heat by the blood passing through the tissues.

However, since skin is not a good conductor of heat, a lag period exists between blood flow heating the skin further from the test site and the time taken for the warmth to permeate the surface (56).

The rate of disappearance of a radioactive isotope is another common method for studying skin blood flow. This method does, however, give a temporary increase in blood flow due to the trauma of application of the isotope by injection (59).

Plethysmography measurements are useful for recording blood volume per se or changes in blood volume. Some measure the absorption of light by haemoglobin, whilst others measure tissue dilation under venous occlusion (58).

Most of these methods are invasive and do not give continuous measurements. Recently, a technique utilising the Doppler effect on coherent light (laser waves), has been advocated as non-invasive, accurate and easy to use for the continuous measurement of vascular perfusion (67, 68, 69).

The nicotinic esters have been used to study various physicochemical factors effecting topical drug formulations (60,61). They rapidly penetrate the skin (62), dilating the blood vessels at the dermal-epidermal interface (63). In this study, laser Doppler flowmetry has been applied to monitor changes in blood flow due to the percutaneous absorption of various nicotinates in alternative solvents.

6.2. HISTORY OF LASER DOPPLER BLOOD FLOW MEASUREMENT

Yeh and Cummins (64) first described the laser Doppler technique for studying flow patterns in fluids. In order to form a dilute colloidal suspension, they dispersed polystyrene spheres in a large diameter tube and were able to measure velocities as low as 0.007cm/second. Their method uses optical heterodyning, which involves the mixing of a reference beam with light scattered from the moving target. The two beams are applied on an optical detector, where they "beat" together and produce a frequency proportional to the Doppler-shifted frequency. This frequency is regarded as representative of the velocity of the target.

The technique has expanded into many different fields of science and technology where determination of velocity is desired. The most common practical limitation is the difficulty sometimes encountered in directing the incident light into and the scattered light out of the region of interest.

The use of the laser Doppler technique for blood flow measurements was first reported by Riva et al (65). They measured blood flow through 200 μm - diameter capillary tubes and demonstrated a linear relationship between the maximum frequency shift of the laser and the observed flow. They then went on to measure the flow in the retinal artery of a rabbit and subsequently, Tanaka and Benedek (66) measured the flow velocity in the vein of a rabbit using a fiberoptic catheter.

Stern (67) was the first to consider using this technique to measure the cutaneous microcirculation, followed closely by Holloway and Watkins (68) who developed a portable instrument capable of clinical measurements.

The laser Doppler techniques using lasers of very low power, have the important advantage of being rapid, safe and non-invasive. Recently, several have become available commercially. They essentially work on the same principle, although their processing systems may be different (68, 69, 70, 71).

6.3. PRINCIPLE OF TECHNIQUE

Skin consists of a system of capillary loops based in the dermis (Plate 1, Appendix). A capillary loop is supplied by a terminal arteriole in the subpapillary plexus and returns to a venule. The actual loop lies mainly perpendicular to the surface of the skin and usually has a 7.5-17 μm external diameter. The concentration of capillary loops varies from site to site, with about 60-70 capillaries per mm^2 (72).

The outermost layer of the skin, the epidermis, does not contain any capillaries and it is quite translucent. Light is therefore incident upon the red blood cells from multiple angles, due to scattering in the epidermis and also the random orientation of the capillary loops.

When coherent laser light is applied to a medium which is in internal motion, the light which is back-scattered from the moving particles, in this case red blood cells, will undergo a shift in frequency by the Doppler effect.

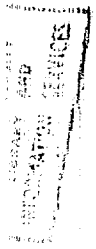
Usually a low powered (2-5 mW) helium-neon laser is employed as a light source. The light is transmitted through an optical fibre to the skin surface where it is reflected by the non-moving tissues and the moving red blood cells, the latter causing a Doppler shift in the beam. The reflected light is then transmitted back to where it can be analysed.

Since the red cells in the capillaries are moving at different velocities and at random angles of orientation, this results in the single laser output frequency being shifted to a spectrum of different frequencies. The actual flow velocity measured therefore tends to be an average value of the broadening of this spectrum.

Watkins and Holloway (73) use a 5mW, continuous wave, helium-neon laser as a light source. They transmit this through a quartz fibre to the skin

surface. The reflected light, consisting of both Doppler shifted and nonshifted signals is transmitted to the face of a photodiode where the two signals are optically heterodyned (mixed) and "beat" together at a frequency proportional to the Doppler shift. The photodetector outputs a current which is proportional to the beat-frequency spectrum. To obtain a single output value for this spectrum, the root mean square (RMS) value is used. This RMS value contains a signal for the nonshifted backscattered light, which has to be deducted to give a true blood flow reading.

The main problem with this method of analysis is the occurrence of external noises which influence the blood flow signal. These noises may be due to the environment, photodetector (shot-noise) and the laser (mode-interference and wide band noise). Nilsson et al (69) suggest that these noises are so high that in low blood flow rates (e.g. forearm blood flow), they mask the true readings. They have therefore devised a detector system to overcome this problem.



Essentially their instrument gathers light from two adjacent scattering areas of the tissue. The light

is then transmitted to two photodetectors where the beat signals produced are high-pass filtered and normalised in the same way in both channels. The two signals are passed through a difference amplifier where common components such as noise from the laser and the environment are suppressed. This tends to give a higher signal to noise ratio, making it easier to measure blood flow velocities.

6.4. ANALYSIS OF TECHNIQUE

6.4.1 COMPARISON WITH OTHER KNOWN TECHNIQUES

The laser Doppler technique has been widely tested to ascertain its performance for transcutaneous measurements of vascular activity. Comparisons have been made with other measuring techniques. Holloway and Watkins (68) compared the laser Doppler with the xenon 133 radioisotope clearance technique. The test site was irradiated with ultraviolet light 24 hours prior to measurement, to induce erythema and increase blood flow. The authors found a linear correlation between the two techniques, with a regression coefficient of 0.89.

The radioisotope technique reflects blood flow at the region below the depth of xenon injection. Furthermore, "trauma of injection" is found to effect the initial blood flow values, giving inflated measurements. Laser Doppler velocimetry, on the other hand, does not directly measure flow and the actual depth of measurement is variable depending on factors such as epidermal thickness. The low correlation coefficient obtained in this analysis may also be accounted for by the fact that adjacent skin sites were used and inter-subject cutaneous circulation was not accounted for.

Johnson et al (74) on the other hand, carried out measurements using occlusion plethysmography and laser Doppler flowmetry, comparing results for each individual. They obtained a slightly higher correlation coefficient range of 0.94 to 0.98. Their method consisted of increasing skin blood flow by raising the whole-body temperature to 39^{o}C, with water-perfused suits. Measurements were made on the same site of the forearm using the two techniques, thus eliminating intra-subject variability. The correlation coefficient was calculated for each subject.

6.4.2. COMPARISON WITH A FLUID MODEL

Nilsson et al (75) suggest that the correlation between the results obtained by laser Doppler flowmetry and many other methods for studying skin blood flow is not very good, due to the fact that the different methods react in different ways to changes in blood flow. To overcome this the authors designed a mechanical fluid model.

The geometry of the model allows the red cells to be assessed during radial movement over a wide cell velocity distribution. Furthermore, the average red cell velocity can be varied using a pump and the cells are diluted in physiological saline, thus allowing control over their concentration. Finally, the degree of oxygen saturation in the red cell suspension can be controlled.

The fluid model simulates some of the properties of microcirculatory flow of the skin in a fixed geometry. The authors assessed the laser Doppler flowmeter response

for different red cell concentrations and velocities. As the red cell volume fraction, i.e. concentration of red cells in the channels of the model, increases, the output signal increases. Furthermore, for a given red cell volume fraction, as the average velocity of the cells increases, the output signal also increases.

If the flowmeter output signal is plotted against the product of red cell volume fraction and velocity, the response is very linear. This product is the flux of the red cells and the linear relationship will hold regardless of red cell concentration or velocity. Even though Nilsson et al's fluid model only simulates microvascular flow optically, rather than anatomically, the result it produces gives a linear and unique relationship between the flowmeter and the flux of the red cells.

The authors also examined the dependence of haemoglobin oxygen content on flowmeter response. They found that changing the oxygen pressure altered the flowmeter reading to a minor extent. A relative change in the flowmeter reading of about 1% per 20 mm Hg increase in oxygen pressure was observed (76).

6.4.3. ASSESSMENT OF WELL-KNOWN VASCULAR RESPONSES TO PHYSIOLOGICAL STIMULI

In addition to the fluid model, Nilsson et al (75) have demonstrated the performance of the flowmeter in measurements of some well-known vascular responses to physiological stimuli. These include the influence of increased transmural pressure on skin circulation as well as the effect of vascular response to changes in local skin temperature in terms of thermal vasodilation or cold vasoconstriction.

The overall in vivo physiological assessment shows that the laser Doppler technique rapidly and continuously measures changes in the microcirculation. This technique would appear to be useful for the non-invasive assessment of the time course behaviour of drugs applied topically to the skin.

6.5. ASSESSMENT OF HUMAN SKIN BLOOD FLOW

The morphological picture of the cutaneous vasculature is complex and differs widely from area to area. These differences have been attributed to: (a) the kinds of skin the blood vessels perfuse, (b) the thickness of the various dermal and hypodermal layers, (c) the types and number of appendages present and, (d) the specific relation of the skin to the bones and muscle fascias under it (77).

Recently, laser Doppler flowmetry has been used to further understand the microcirculatory blood flow. Tenland et al measured the spatial and temporal variations in blood flow, especially for areas of the skin that may be regarded as homogeneously perfused (78). They have also examined day-to-day variations in forehead and forearm blood flow.

The results obtained by these researchers showed a wide difference in blood flow values for adjacent sites of the forearm. This only further emphasizes the complex nature of the skin blood flow circulation even for sites measured as close as 2.5mm apart as in this case. Additionally, the

laser Doppler instrument used by Tenland et al (78) is claimed to measure a hemisphere with a radius of 1mm (75). This is a fairly large volume in microvascular terms, where 60-70 capillaries may be present per mm^2 of skin surface (72).

For instruments that have three fibres it is important to consider the orientation of the probe (75). The fibres are of 0.6mm diameter placed adjacent to each other in a triangular pattern. Due to this asymmetrical arrangement of the fibres in the probe, a 90° rotation moves the end of the illuminating fibre approximately 0.5mm. Thus changing the orientation of the probe may give blood flow values for a different region of the skin.

Tenland et al (78) considered this hypothesis and found that some individuals gave different readings if the probe was rotated by 90°, indicating the importance of careful localization and angular orientation of the probe when results of repeated recordings are compared.

One of the problems with other microvascular measuring systems is that they disturb the natural state of the test area. Even the laser Doppler is

subject to this problem, though not to any large extent. If continuous measurements are performed without disturbing the test site or probe, stable flow values are obtained. However, as Tenland et al (78) found, removing and reapplying the probe every 30 seconds results in unstable values. These authors also found large day-to-day variations for the same skin site.

Sundberg (79) also examined long-term variation using a similar instrument. Their results showed very little variation in the mean blood flow values for measurements made on five different days over a 2-3 week period. Sundberg also found that the forehead gave much higher mean blood flow values than the forearm; however, the forehead values varied to a lesser extent than those from the forearm.

It is difficult to explain this discrepancy in the day-to-day readings especially since both authors used similar instruments. However, the protocols used were slightly different. For example, Sundberg made measurements at a probe temperature of 34^{o}C, whereas Tenland et al recorded at 25^{o}C. Furthermore, Tenland et al placed greater control over their subjects by having a 30 minutes rest

period prior to testing and no food or drink intake was allowed 3 hours prior to the experiment. It is possible that food intake results in a reduction of blood flow to the skin as a result of an increase in the gastrointestinal tract.

Tur et al (80) carried out measurements of skin blood flow using the laser Doppler and photo-pulse plethysmography, as a function of anatomic position. Their results suggest that a collection of regions; the fingers, palms, face and ears have much higher cutaneous perfusion.

A variety of vessel types in the microcirculatory bed are subject to spontaneous rhythmic contractions. However, due to the lack of human skin transparency, most of the studies have been by dissection or of species that have transparent vascular beds such as the rabbit ear (81). Recently, the laser Doppler technique has been used to further understand rhythmic vasomotion in human skin in vivo (82).

The results obtained so far tend to reflect mean blood flow values. There is a wide inter- and intra- subject variation in rhythmical activity and this has been attributed to changes in the diameter

of the blood vessels at a local level. However, the frequency of these contractions decrease at the more distal parts of the body.

The laser Doppler technique does not measure the blood flow in individual vessels. It is also difficult to know which part of the skin vascular system is being assessed. Since a good correlation between the laser Doppler and the xenon clearance technique has been shown, it may be inferred that the laser Doppler method also monitors cutaneous blood flow (68).

Nilsson et al (75) suggest that for their instrument the area measured is a hemisphere of 1mm radius. However, the depth measured will depend on the concentration of the capillary loops and the thickness of the skin.
Light that misses the loops will penetrate far deeper thus measuring flow in the deeper vessels. Consideration must also be given to the dependence of the measured blood flow on the haematocrit.

Gush et al (83) found that by placing a physical barrier of increasing thickness using Dermalite tape (a translucent white adhesive paper about 0.16mm thick), blood flow values of the epidermis

as measured by the laser Doppler method decreased gradually. These authors also investigated the effect of variation of the probe fibre separation. By changing the separation of the transmitting and receiving laser fibres, the Doppler shifts obtained varied. For probe fibre separations less than 2mm it was found that the laser Doppler measures flow of blood in the papillary loops and the uppermost dermis. In order to assess blood flow in the deeper dermal tissues, the authors suggested probe separations greater than 3mm. However, the intensity of the detected light rapidly decreases with increased separation thus requiring more powerful lasers.

The results discussed above suggest that a number of factors must be taken into account before any experiments on human skin blood flow using the laser Doppler technique can be initiated. It is important not to disturb the probe during an experiment and to ensure the same orientation when reapplying the probe, if removed. An area that still requires clarifying is that of long term variations in blood flow for the same site.

However, the quick response time of the instrument is well suited for experiments where rapid changes in tissue perfusion are of interest.

6.6 APPLICATIONS OF LASER DOPPLER FLOWMETRY

6.6.1. INTRODUCTION

The development of the laser Doppler technique has led to an increase in the study of areas where microvascular flow measurement is of importance. These investigations have considered the effects of various types and degrees of stress on basal flow values, rather than quantification of skin blood flow in absolute terms.

Recently, the laser Doppler has been used to assess (a) the irritancy of various chemicals and formulated products (84-91), (b) the severity of vascular disease and burns, and to monitor recovery of cutaneous circulation following plastic surgery (92, 93, 94) and (c) the percutaneous absorption of vasodilators (95, 96, 97).

6.6.2 IRRITATION

Assessment of the irritant potential of chemicals and formulated products is usually undertaken by a subjective visual examination of the exposed skin sites, using different scores for the degree of erythema, oedema, scaling and papules. The increase in redness, erythema, is usually a manifestation of an increase in cutaneous blood flow following vascular dilation. Thus it should be possible to study skin irritation reactions more objectively using the laser Doppler method.

Nilsson et al (84) were the first to show the value of the laser Doppler velocimeter as a quantitative and objective tool for assessing skin irritation. They exposed the skin to various concentrations of the surfactant, sodium lauryl sulphate, under occlusion for 24 hours. The test sites were graded visually and assessed with a laser Doppler at 26, 48 and 72 hours after product application. A good relationship was observed between the applied doses of sodium lauryl sulphate, the measured blood flow values and the visual scores. Some

of their data implied that the naked eye might be unreliable in comparison to the laser Doppler for the assessment of skin irritation.

Skin irritation studies are normally undertaken by applying the test material in an occlusive patch for several hours. Repeated readings are recommended usually 20 minutes, 24 hours and 48 hours after the patch has been removed (98). To date it has been difficult to ascertain the irritant effect of not only the test material, but also the vehicle, test patch, and tape. The normal practice of accounting for these "extra effects" is to wait for a while after the patch has been removed, before assessment and also to use a patch without irritant as a control.

Wahlberg and Nilsson (85) assessed the irritant effect of propylene glycol using the laser Doppler technique. Single and repeated exposure without occlusion did not cause an increase in blood flow. Occlusion, on the other hand, gave increased blood flow values as well as detectable erythema by the naked eye. This obviously increases the sensitivity of the test method, but may be criticized for not mimicking actual exposure conditions.

Wahlberg and Wahlberg (87) assessed the irritant effect of the patch test per se by monitoring blood flow values immediately after patch removal for sites without irritant and a standard battery of irritants. The patches were removed 48 hours after application and further measurements made at 72 and 96 hours after initial application.

Their results showed that the combination of test patches, irritant, vehicles and tape will cause an increase in local blood flow. However, the increase due to the dry patch test lasts only for 30 minutes, thus the practice of waiting for some time after removal of the patches and repeating the readings is justified.

The erythematous response from topical administration of various chemicals has been quantified objectively using laser Doppler flowmetry. On occasions, complaints may be obtained of skin irritation where no signs of erythema or eczematous may be seen (88, 89).

Lachapelle et al (88) found that an anhydrite paste based on calcium sulphate gave increases in measured blood flow compared with untreated control. There were no visible signs of irritation even after 5 successive applications of the paste in patch chambers; yet the laser Doppler gave increased blood flow values. This paste had been used by Belgian miners to shore up metallic beams and they had complained of itching or burning sensations, even though no reactions were seen.

Wahlberg (86, 89) tested a number of solvents and alkaline solutions using laser Doppler flowmetry. Varying the exposure time, mode of administration (single or repeated) and concentration of the test substances effected the measured blood flow values. An oil used in industry and claimed to cause rashes, even though no visible erythema was present, gave a gradual increase in blood flow recorded by the laser Doppler.

The laser Doppler technique cannot be regarded as a replacement for the traditional patch testing technique, since it

does not measure scaling, oedema, papules and so on. It may be regarded as being more sensitive and reliable than the naked eye, particularly in cases of marginal irritancy. However much work is still required to define the limits of application of the method for irritancy studies. Outstanding problems are biological variation and high sensitivity required to separate pathological from physiological responses.

Drouard et al (90) have examined the effect of UV erythema and the protection given by sunscreens using the laser Doppler. Their results showed that the laser Doppler readings correlated well with those based on visual erythema assessment. The lotion vehicle had no protective effect whereas the sunscreen with its active ingredient had a significant effect. These results were obtained after single UV exposure, but when real-use situations were mimicked using repeated exposure, the results were only further emphasised. The laser Doppler may thus be used to quantify UV exposure and also assess chemicals that prevent UV induced changes.

6.6.3. DISEASE AND SURGERY

Kristensen et al (92) have applied the laser Doppler flowmeter for the assessment of microvascular disease. They made digital blood flow measurements on patients with Raynaud's phenomenon and compared the results with those regarded as normal subjects.

In patients with Raynaud's phenomenon fingertip blood flow tended to react differently when cooled. The cooling gave lower blood flow values and took much longer to rewarm, when compared with normal subjects. These workers propose a defective function of the arteriovenous anastomoses as an explanation for the deviation from normality. Overall, these results suggest that the ability of the laser Doppler method to make continuous measurements, allows the classification of vascular diseases.

In the study and treatment of burns, the classification of burn depth is required for

prognosis. For superficial burns where parts of the stratum germinativum are preserved, healing takes place within 10-14 days, since the microvascular bed is maintained. For deep dermal burns the entire epidermis is destroyed and so are parts of the nerve elements and the microcirculation.

Superficial dermal burns are usually best treated by exposure to air, whereas the deep dermal burns require early excision and grafting (99). Traditionally, clinical and histological diagnosis are used to determine the type of burn but the former tends to rely on experience and the latter is invasive.

Since the microcirculation plays an important part in the regeneration of skin, Alsbjorn et al (93) have used laser Doppler flowmetry to diagnose burn type. They made blood flow measurements in conjunction with clinical and histological assessments. Their protocol gives a schedule for using the laser Doppler method in such a way that different burn depths may be classified. However, this schedule can only be used as a guideline to improve the diagnosis of the depth of a burn-wound,

since there is no strict patho-anatomical border between the different burn depths.

6.6.4 PHARMACODYNAMICS

There are various drugs available that alter skin blood flow when applied topically. The laser Doppler technique has been used to further understand the pharmacodynamics of various vasodilators.

Guy et al (96) have extensively examined the local pharmacodynamic response to the topically applied vasodilator, methyl nicotinate. They have considered the penetration, residence and elimination of the drug. The laser Doppler method gives a measure of the initial onset of erythema and the time course of the response.
Dose-response behaviour as a function of time was monitored for a concentration range of methyl nicotinate in distilled water and also by varying the drug application time and administration area.

Initial results suggest that as the drug concentration is lowered, the onset of erythema is delayed and the maximum increase in blood flow, as measured by the instrument, decreases. Furthermore, the period during which elevated microperfusion exists compared with pretreatment, is also reduced (95).

Further data suggests that for methyl nicotinate concentrations greater than 10-25mM, the magnitude of the response is saturable and the effect is progressively prolonged for higher dosages (96). The results above have been generated for the products applied at a constant area and a given time. When the product application time is increased the magnitude of the response and the time the response lasts increases as well. However, no significant changes were found when the application area was varied. This is probably due to the application area of the product being similar to or greater than the area measured by the laser Doppler.

Guy et al (97) have also measured the effect of age and racial differences in the response of human skin to methyl nicotinate.

Generally the data suggests a remarkable similarity in the response across a wide range of skin types. This similarity is difficult to explain, especially since the vascular pattern is claimed to change considerably with age (72).

Sundberg (79) has measured changes in forearm skin blood flow after administration of sublingual nitroglycerin. A rapid and transient increase was observed which peaked after 3-4 minutes and returned to pretreatment values 10-15 minutes later.

Overall the results obtained by using vasodilators, especially when applied topically, show that the laser Doppler method may be used to objectively optimise formulations for percutaneous absorption.

CHAPTER 7.0: LASER DOPPLER - METHOD FOR STUDYING PERCUTANEOUS ABSORPTION

7.1. INTRODUCTION

Various substances have been applied to the skin since the beginning of time, for belligerent, religious, cosmetic and medicinal purposes. This practice continues today in the cosmetic and pharmaceutical fields. In order to optimise these topical formulations, there is often a need to know the extent and rate of absorption of components of the formulation.

Cosmetics are normally applied to intact skin and the main region of interest is the epidermis. For certain substances such as sunscreen agents, accumulation of the product is required on the outer regions of the epidermis only. However, in the case of some pharmaceuticals, percutaneous absorption allows the delivery of the drug directly into the systemic circulation (54, 100). There are a number of benefits in using transdermal drug delivery systems. They eliminate variables such as changing stomach pH, which may influence gastrointestinal absorption.

Furthermore, input of the drug may be controlled. If a drug passes through the liver before entering the systemic circulation, its concentration may be drastically reduced by metabolism and variability in this process leads to different amounts entering the systemic circulation.

The pharmaceutics mentioned above are for systemic delivery. There are also a wide variety of skin disorders which are normally alleviated by topical drug application. However, it is important to remember that the percutaneous absorption will vary if the skin is diseased, normally allowing transfer of material across the skin more rapidly.

The penetration of any active material will depend on two main factors. An active will normally be applied in a vehicle and therefore its rate of release from the formulation is important. Secondly, the exact transport rate of the active through the skin will also be of importance (100).

Various methods have been proposed to study these factors and to understand the physicochemical factors affecting the release and diffusion of the active molecules in the topical base and in various layers of the skin.

7.2. ANALYSIS OF PERCUTANEOUS ABSORPTION

Skin forms a barrier to the ingress of foreign materials and also maintains the body fluid balance. The highly complex nature of skin affords a variety of routes for the transfer of material to the lower layers of the epidermis and to the cutaneous vasculature. However, it is difficult to ascertain the exact route of penetration for a given molecule.

The main barrier to percutaneous absorption is claimed to reside in the outermost layers of the epidermis, the stratum corneum (3, 7, 52). However, there are two possible routes within this layer. The active may diffuse through the whole thickness of the corneum i.e. by intracellular diffusion or it may diffuse intercellularly (100).

The viable epidermis is much more highly permeable than the stratum corneum and the blood vessels situated in the epidermal-dermal region usually remove and transfer the drugs which diffuse through very rapidly so that diffusion from the epidermis is essentially into a sink. In most cases these

layers have no effect on the overall transfer rate of the active compounds (101).

Another possible route for molecular transfer across the stratum corneum are the various appendages which traverse the skin. However, these routes are not regarded as very important since they only contribute a very small portion to the overall surface area available. Also appendages such as eccrine glands have a current of fluid moving towards the skin surface, which may act against any downward transfer. The hair follicles have also been suggested as a possible route, especially for larger molecules in the presence of surfactants (101).

7.3. METHODS FOR PERCUTANEOUS ABSORPTION MEASUREMENTS

There are various techniques available for the study of percutaneous absorption. The choice of method will depend on whether the rate of release from a topical formulation or the diffusion across skin of the active is required.

In vitro methods are used to measure the release of the active from the vehicle into the skin. They

may also be used to measure diffusion rates across excised or model skin. In order to measure the release rate, a simple diffusion cell may be used (102). The cell consists of a donor and receptor phase separated by a membrane.

The topical product is applied in the donor phase and the rate of transfer of the active to the receptor phase can be measured. This cell is limited to semi-solid formulations and is usually adequate, provided the membrane is not rate-limiting.

Diffusion cells may also be used to simulate in vivo conditions. The diffusion rates of the active may be measured to evaluate the effect of formulation changes under controlled conditions (103). If excised skin is used, it is often difficult to obtain it intact and to get reproducible results. Model membranes are easier to obtain and experiment with, but suffer from being distant from real systems.

Recently, mathematical models have been proposed for the assessment of percutaneous absorption (100, 104). The problem with this approach is that skin diffusion is complex and requires solutions to unsteady-state conditions. Furthermore, metabolism

within the system complicates the diffusion equations. The advantage with mathematical models is that the effect of various physicochemical factors, such as partition coefficients, on the diffusion rates may be quantitatively assessed using model compounds.

Close correlation between experimental and model data for aspirin, caffeine and salicylic acid have been found (54). The experimental data in this case was obtained from urinary excretion of the above products following topical application, as measured over a given time period.

Histological studies may be used to examine the route a compound takes, but usually it is difficult to assess the permeation rates. There are two common methods for studying in vivo percutaneous absorption.

The first method for studying in vivo penetration is to monitor the appearance of the applied drug in the circulation or urine. Radiolabelled tracers are often used (105). The rate at which the labelled drug leaves the surface and disappears into the skin can be measured by placing a counter immediately above the application site. This may

be used as a measure of the diffusion rate through the stratum corneum. However, it does not include an estimation of the quenching of the signal by the skin. The permeation rate may also be measured by analysing the urine or blood. Radio tracer techniques are not usually able to distinguish between parent drug and metabolites. This shortcoming can be overcome by using more selective assay techniques such as chromatography.

A common method for assaying percutaneous absorption in vivo in humans is to measure a physiological response after topical drug application. One such method has been to assess the blanching response from corticosteroids, to examine the effect of formulation changes on penetration rates (106). Evaluation of the effect is achieved by scoring the degree of pallor, but this is a subjective assessment. Instrumental methods, such as photometric integration may, however, overcome some of the problem.

Another method of inducing a physiological response is to use the esters of nicotinic acid (62). These compounds rapidly penetrate the stratum corneum and dilate the blood vessels at the dermal-epidermal interface (63). The onset of erythema may be used as a measure of nicotinate penetration and the

effect of formulation changes on the absorption, may also be observed (60, 61).

This technique is useful for the study of processes within the skin and the vehicle. Most of the original work has been by subjective assessment of erythema. Recently, photopulse plethysmography and laser Doppler flowmetry have been advocated as more objective tools for assessing the rates of response (95, 96). Furthermore, it may be possible to follow the response-time curve in a more quantitative manner, allowing the analysis of results using mathematical models.

7.4. MECHANISM OF ACTION OF NICOTINIC ACID ESTERS

Rubefacients have been used for their therapeutic effects for a long time. The discovery of the nicotinic acid derivatives as potent vasodilators has led to their use for the measurement of percutaneous absorption. However, the complex nature of skin and the microvascular bed makes it difficult to derive information regarding the route of penetration or the mechanism of action of the nicotinates.

Fulton et al (63) were the first to investigate the probable mechanism of action in situ. Since man has limited visible microcirculatory beds, these workers used the semi-transparent cheek pouch of the Syrian hamster for their studies. They applied the rubefacients using micropipettes and studied the responses under microscopy.

The results obtained showed that the extent of the vasodilator response exceeded the portion of the vessel exposed to the rubefacient. Furthermore, the rate of this dilation was far greater than that expected by diffusion alone. Tissue injury with a resultant release of histamine has been attributed as a possible mechanism. However, these authors found that although histamine produced vasodilation, extremely high quantities were required, unlike the very dilute solutions of nicotinate needed to give a response.

Fulton et al suggested that the mechanism of action is due to local nerve conduction. This is possible since there is present a secondary nerve plexus that parallels the blood vessels.

Even though these authors applied the various esters of nicotinic acid they did not quantify

their results. Fountain et al (107) have measured the effect of one ester, methyl nicotinate on the erythematous response. Under constant conditions the time of onset of erythema is reproducible. Furthermore, the magnitude of the erythematous area depends on the concentration of methyl nicotinate applied.

Guy and Maibach (108) have also evaluated the radial increase in the erythematous area as a function of methyl nicotinate concentration and application time on human skin. They found that the rate at which the erythematous area increases responds well with the concentration and application time of the ester. Methyl nicotinate took 10 minutes to give a radial increase of 1cm of erythema. This suggests a diffusion coefficient of $10^{-3}cm^2/s$, which is far too fast compared with an ordinary aqueous diffusion of $10^{-6}cm^2/s$. This would give a time of 6 days to obtain a similar increase in area (108).

The mechanism proposed by these authors is that the blood flowing through the capillaries carries the nicotinate rapidly to other parts of the vascular bed, before eventual removal from the site of action.

Stoughton et al (62) compared the rates of penetration of various nicotinic acid esters as well as their routes of penetration. The latter is more difficult to quantify, but initial erythema tended to be near the follicles, suggesting a possible primary route.

By using intradermal injection of serially diluted nicotonic acid and derivatives in saline, these workers found all the products applied gave a visible erythema down to 10^{-9}M concentration, but not at 10^{-10}M.

From topical applications in 95% alcohol they measured the minimum concentrations required to elicit erythema.

These concentrations reflected well with the relative and absolute solubilities of the nicotinates in water and ether, suggesting the importance of water and lipid solubility for percutaneous absorption.

7.5. APPLICATIONS FOR METHYL NICOTINATE PERCUTANEOUS ABSORPTION

Since the nicotinic esters rapidly penetrate the skin and produce a visible physiological response, they have been used to measure the percutaneous absorption of various compounds and particularly the effect these vehicles have on this penetration.

Hadgraft et al (60) examined the effect glycerol has on the percutaneous absorption of methyl nicotinate from an aqueous vehicle. Glycerol is commonly used in cosmetic and dermatological preparations for its humectant properties. It is claimed to form an adherent film on the skin surface and is said to prevent evaporation of the aqueous phase of creams on storage (113).

These workers measured the rate of onset of erythema due to methyl nicotinate from aqueous vehicles containing different concentrations of glycerol and compared the results with changes in various physicochemical parameters.

For concentrations of glycerol above 60% a sharp decrease in the rate of onset of erythema occurred.

When these results were compared with the partition coefficient and diffusivity of the nicotinate a close correlation was found. Both the partition coefficient of the nicotinate between skin and the vehicle and diffusivity within the vehicle decreased as the glycerol concentration increased.

Hadgraft et al (61) also examined the thermodynamic activity of the nicotinate. They found that for solutions of glycerol and water of equal thermodynamic activity, the rate of penetration of the nicotinate was similar.

Fountain et al (107) have studied the surface concentration effects on the rate of erythema observed. They applied methyl nicotinate in polyethylene glycol 300 and found that after the initial erythema had subsided, this could be restored by agitating the fluid on the skin surface. This suggests the greater importance of the rate of diffusion of the nicotinate within the vehicle, rather than the partition coefficient.

The possibility of using the nicotinates as a marker compound to assess the efficiency of penetration enhancers has also been studied.

Hadgraft (100) found that for an oily cream, addition of 10% urea increased the penetration rate of hexyl nicotinate through the skin by nearly a third.

7.6. LASER DOPPLER MEASUREMENT OF THE PERCUTANEOUS ABSORPTION OF METHYL NICOTINATE

The nicotinic acid esters have been used for the study of percutaneous absorption for over two decades (60, 61, 62, 63). The main parameter of analysis has been the time of onset of erythema. Recently two techniques have been proposed for the objective non-invasive assessment of the percutaneous absorption of the nicotinates in humans. These are laser Doppler flowmetry and photopulse plethysmography.

Photopulse plethysmography is claimed to measure changes in cutaneous blood volume. It depends on the percentage of IR radiation absorbed by the haemaglobin in the blood. The laser Doppler technique, as mentioned previously, measures the flux of the cutaneous blood i.e. is a measure of velocity and concentration of red blood cells. This study is mainly concerned with laser Doppler measurements.

To date most of the work seems to be concerned with methyl nicotinate from an aqueous vehicle. Guy et al (95) were the first to utilise laser Doppler flowmetry and photopulse plethysmography to study the topical application of this vasodilator. They compared the instrumental response of both techniques with visual observation of erythema onset and found a good correlation. The instruments were also sensitive to changes in the concentration of the nicotinate.

Guy et al (96) went on to study the local kinetics of percutaneous absorption of methyl nicotinate in more detail. They monitored the dose-response behaviour as a function of time over a wide concentration range and also by varying the drug application time and administration area.

These workers used the following three parameters to describe the time course behaviour and extent of the response: (a) the magnitude of the maximum response, (b) the area under the response-time curve and (3) the time required for the response to return to 75% of the maximum value.

For a given application time and administration area, increasing the concentration of nicotinate, gave an increase in the above parameters. This was also reflected by increasing the application time for a given concentration and administration area.

Laser Doppler flowmetry has been proposed as a technique for the measurement of the transcutaneous kinetics of methyl nicotinate in a non-invasive and in vivo manner.

In this review, laser Doppler "flowmetry" has been used synonymously with laser Doppler "velocimetry". Guy et al (109) distinguish the terminology by applying it to two different commercial instruments.

CHAPTER 8.0: LASER DOPPLER - EXPERIMENTAL

8.1. MEASURING ASSEMBLY

Skin blood flow measurements were carried out using a Periflux PF1d laser Doppler flowmeter (Perimed KB, Stockholm. Sweden). The instrument uses a 2mW helium-neon laser operating at 632.8nm. Light is brought to the skin surface via a single optical fibre. The backscattered light, containing both a Doppler shifted and unshifted signal, is picked up from two adjacent sites and brought to impinge on the surface of two photodetectors.

Figure 25 schematically represents the operating principle of the Periflux. The beat signals produced in the photodetectors are high-pass filtered, amplified and normalized in an identical way in two separate channels. Common components such as noise from the laser and the environment are suppressed in a difference amplifier. The output signal from the instrument is claimed to be a measure of the blood cell flux, which is a product of the number of red cells moving in the measuring volume and the mean velocity of these cells.

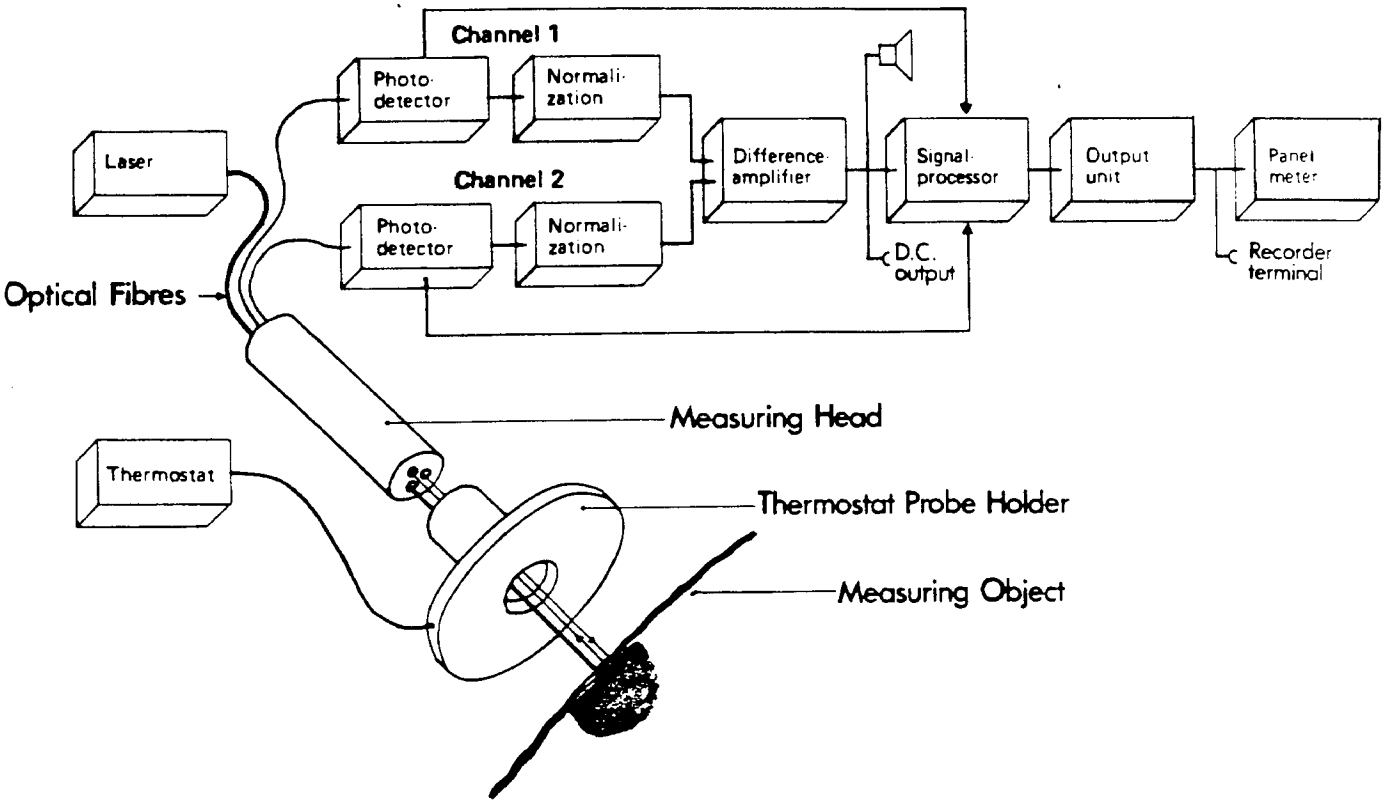


FIGURE 25: OPERATING PRINCIPLE FOR THE PERIFLUX LASER DOPPLER FLOWMETER - PF1d

Pamphlet PF001, March 1981

PERIMED, STOCKHOLM. SWEDEN

This output signal was recorded on paper using a pen recorder (LKB 2210 Recorder, Bromma, Sweden). Blood flux is obtained as an analogue output of 0-10V, rather than an absolute volumetric unit. This was represented on the recorder as a range of 0-100%. Recorder speed was set at 0.1mm/s for real time blood flow measurements.

The probe head is held in a plastic holder attached to the skin surface with double sided adhesive tape. A thermostat probe holder is also available and allows measurement from 26^{o} to 40^{o} in $2^{o}C$ steps. The plastic holder attachment restricts the area illuminated by the laser to approximately $28mm^{2}$. In order to eliminate skin compression and improve the depth sensitivity of the measurement, the probe holder retains the probe face 0.8mm above the skin surface.

Due to the large variation in the average blood flow from site to site, the signal-to-noise ratio may be improved by limiting the upper frequency of the high-pass filter within the instrument. In this work recordings were made on the forearm where the degree of perfusion is fairly low. Thus the recordings were made at 4 kHz upper frequency limit, a 3 second time constant and a X10 gain.

8.2. PROCEDURE

The subjects chosen were of both sexes in the age range 20-26 years. They were placed in a room in an adjustable reclining chair where measurements were carried out on the volar and ventral areas of the forearm (Plate 4, Appendix).

The measurement room was air-conditioned and the relative humidity closely monitored. The temperature of the room was maintained at 22$\pm$ 2^{o}C, while the humidity was measured to be 40-60% during the period of experimentation. Daily humidity changes were found to be minimal.

Prior to any measurement the laser Doppler was switched on for at least 15 minutes to ensure the laser was adequately warmed. After the warming up period, both the laser Doppler and the pen recorder were adjusted to zero, using their own zeroing adjustments.

Since skin blood flow is claimed to vary from site to site, the test site was also used as the

pretreatment or untreated control. A plastic probe holder was applied to the volar or ventral area of the forearm and measurements made for 5 minutes (Plate 5, Appendix). If steady values were not obtained, the subject was left to rest. Once steady values were attained, the probe was removed from its holder and the product applied on the skin surface through the probe holder.

The thermostat holder was not used, thus all measurements were conducted at room temperature. Unless otherwise stated, 5 μL of test material was applied using a syringe. Once the substance had been applied the test site was left undisturbed. Since the probe head remained 0.8mm above the skin surface, this ensured no occlusion of the applied material. In order to ensure the same region of skin was being measured, the orientation of the probe head was maintained whenever it was removed and reapplied.

CHAPTER 9.0: LASER DOPPLER - RESULTS AND DISCUSSION

9.1. INTRODUCTION

In order to characterize and test the laser Doppler technique, various nicotinic acid esters were applied topically to the skin. The nicotinates examined were the methyl, ethyl and hexyl esters, each in various vehicles.

The effect these solvents had on blood flow was ascertained prior to addition of the nicotinates. Among the solvents used as vehicles were a range of aliphatic alcohols, glycerine, monopropylene glycol (MPG), deionised water and kerosine (grade of petroleum over distillation range 200-240^{o}C).

9.2. PARAMETER OF ANALYSIS

When 5 μl of kerosine was applied through the probe holder onto the forearm skin, no changes were found in the blood flow value even after 70 minutes of continuous measurement. On removal of the probe holder some kerosine still remained on the skin surface, but no visible erythema was detected on

the actual test site. Slight redness was noticed on the area below the probe holder, but this was attributed to local disturbance on removal of the adhesive tape, between the probe holder and the skin.

Figure 26 gives a typical response, as measured by the laser Doppler, to 5 μL of 0.1M hexyl nicotinate (Sigma Chemical Co., St. Louis, Mo. U.S.A.) in kerosine. In this sample profile the pretreatment value measured continuously for 5 minutes, is slightly higher than the value observed immediately after product application. This was not always the case, but may be due to disturbance of the test site on removal and reapplication of the laser probe.

Immediately after product application no significant change was found in the blood flow value. This was followed by a sudden and sharp increase, after which the blood flow value remained at an elevated steady state. Finally the blood flow returned slowly to pretreatment values.

This profile suggests it should be possible to objectively assess the response of the microcirculation to stimulus by hexyl nicotinate.

Furthermore there is the possibility of measuring the percutaneous absorption of nicotinates in an objective manner. In order to characterize the profile obtained a number of parameters have been evaluated as shown schematically in Figure 27. These parameters may be considered to represent the initial rate of absorption of the product and the subsequent increase and eventual decay of the microcirculation blood flow.

The parameters used include:

a) The time during which the steady state blood flow is maintained after product application - t_o;

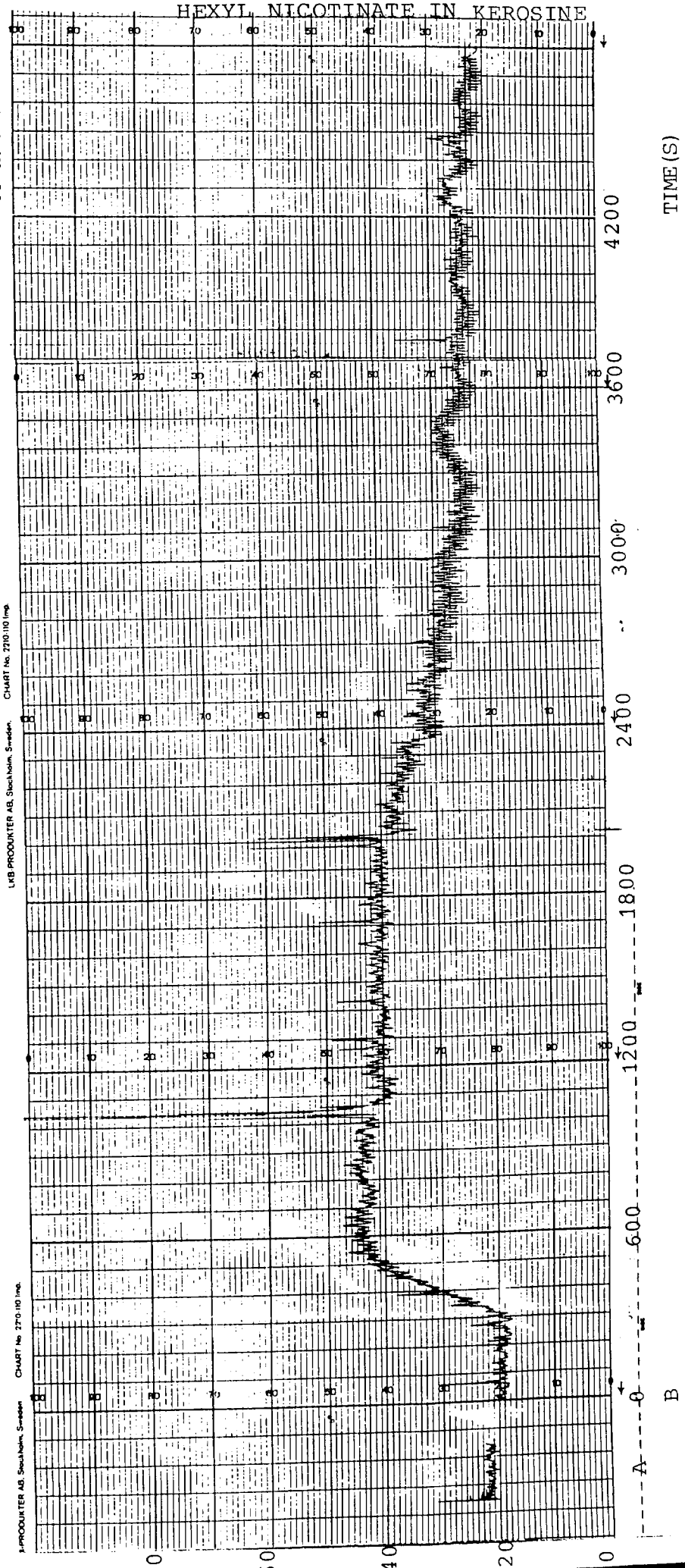
b) the time taken for the sharp increase in blood flow - t_r;

c) The time during which the steady state blood flow was maintained - t_p,

and

d) the time for the blood flow to return to 75% of the maximum value - t_d.

FIGURE 26: TYPICAL RESPONSE CURVE AS MEASURED BY THE LASER DOPPLER TO 5μL OF .1M HEXYL NICOTINATE IN KEROSINE



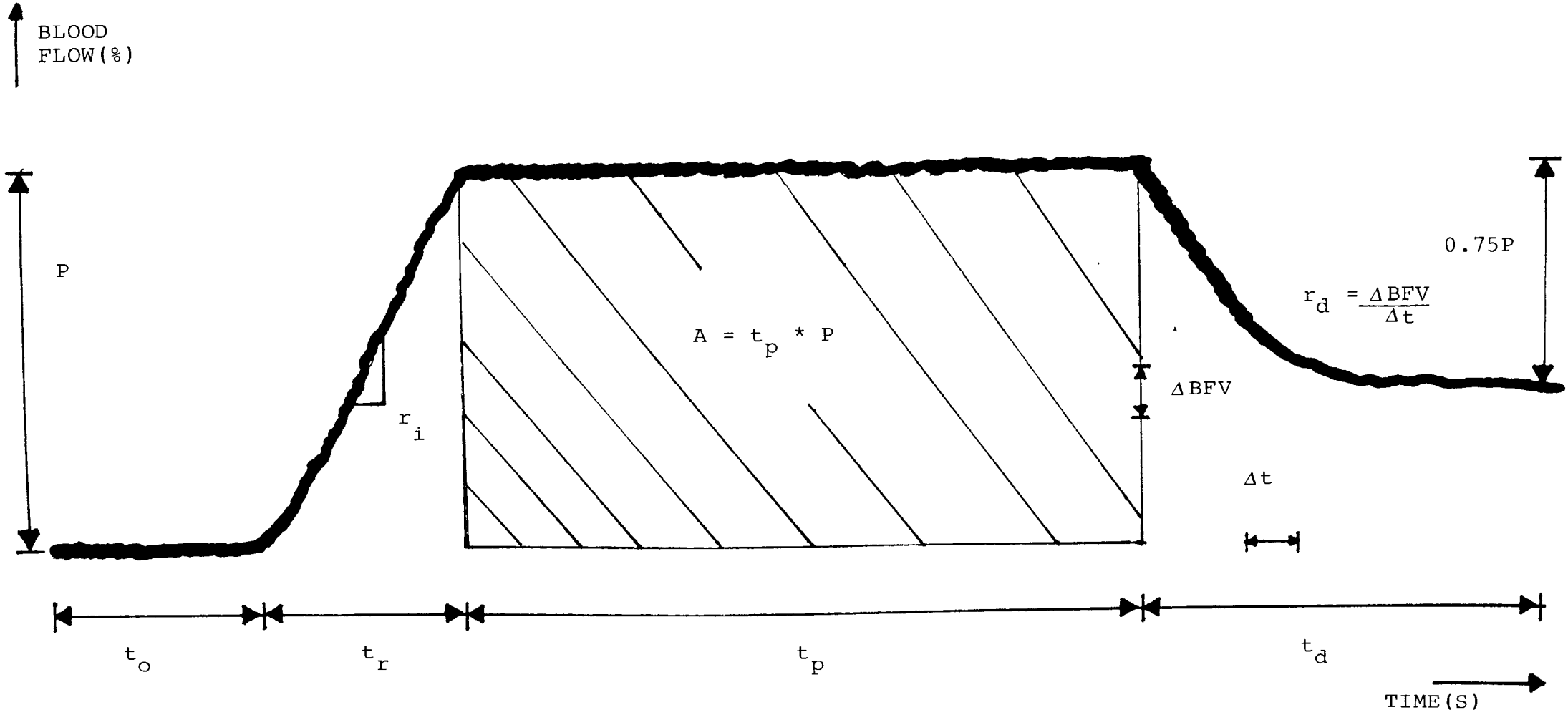


FIGURE 27: PARAMETERS USED FOR LASER DOPPLER PROFILE ANALYSIS

The other parameters considered were:

a) The maximum increase in the blood flow - P%;

b) area under the elevated steady state portion of the curve - $A = t_p.P$;

c) the increase in blood flow per unit time - r_i

and

d) the log decrease in blood flow per unit time - r_d.

9.3. MEASUREMENT OF HEXYL NICOTINATE ABSORPTION FROM KEROSINE.

The variation of parameters listed above was tested for a concentration of 0.05 and 0.10 M hexyl nicotinate in kerosine. 5 μL of each product was applied through the probe holder. This ensured a constant area of application. Once the product had been applied, the test site was not disturbed.

Table 2 shows the results obtained for these two concentrations. The only parameter that varies significantly is t_o, the time taken for the instrument to respond. Guy et al (95), when applying methyl nicotinate to forearm skin, found a good correlation between visually assessed erythema onset and onset of response by the instrument - t_o. They also found t_o was sensitive to concentration of methyl nicotinate in the distilled water vehicle.

Since only t_o varied for 0.05 and 0.10M hexyl nicotinate in kerosine, a wider concentration range was tested. The parameters examined were t_o, t_r, r_i and P as depicted in Figures 28 to 31. The concentration range evaluated was from 0.00625 to 0.2M hexyl nicotinate in kerosine. Again only t_o, the rate of erythema onset could be consistently correlated with concentration, decreasing with an increase in hexyl nicotinate concentration.

Figure 28 also suggests that above approximately 0.075M hexyl nicotinate, the rate of onset remains fairly constant. Whereas if the other parameters are considered no significant differences are found around this concentration. For example P, the

TABLE 2: ANALYSIS OF LASER DOPPLER PROFILES OF HEXYL NICOTINATE IN KEROSINE ON FOREARM BLOOD FLOW (REFER TO FIG. 27).

CONCENTRATION (M) / PARAMETERS	0.10 MEAN ± S.E.	0.05 MEAN ± S.E.	
t_o (S)	234 ± 38	638 ± 60	*
t_r (S)	214 ± 34	323 ± 59	
r_i (S^{-1})	0.269 ± 0.098	0.0765 ± 0.02	
P (%)	35 ± 5.0	44 ± 6.5	
t_p (S)	2178 ± 184	2447 ± 234	
t_d (S)	1836 ± 118	1907 ± 122	
r_d (S^{-1})	0.269 ± 0.093	0.0132 ± 0.001	
A (S)	78454 ± 14675	72971 ± 19526	
N	5	6	

* - $P<0.01$ using t - test for two independent samples (111)

N - number of measurements.

FIGURE 28: VARIATION OF LASER DOPPLER RESPONSE (t_o) WITH CONCENTRATION OF HEXYL NICOTINATE IN KEROSINE

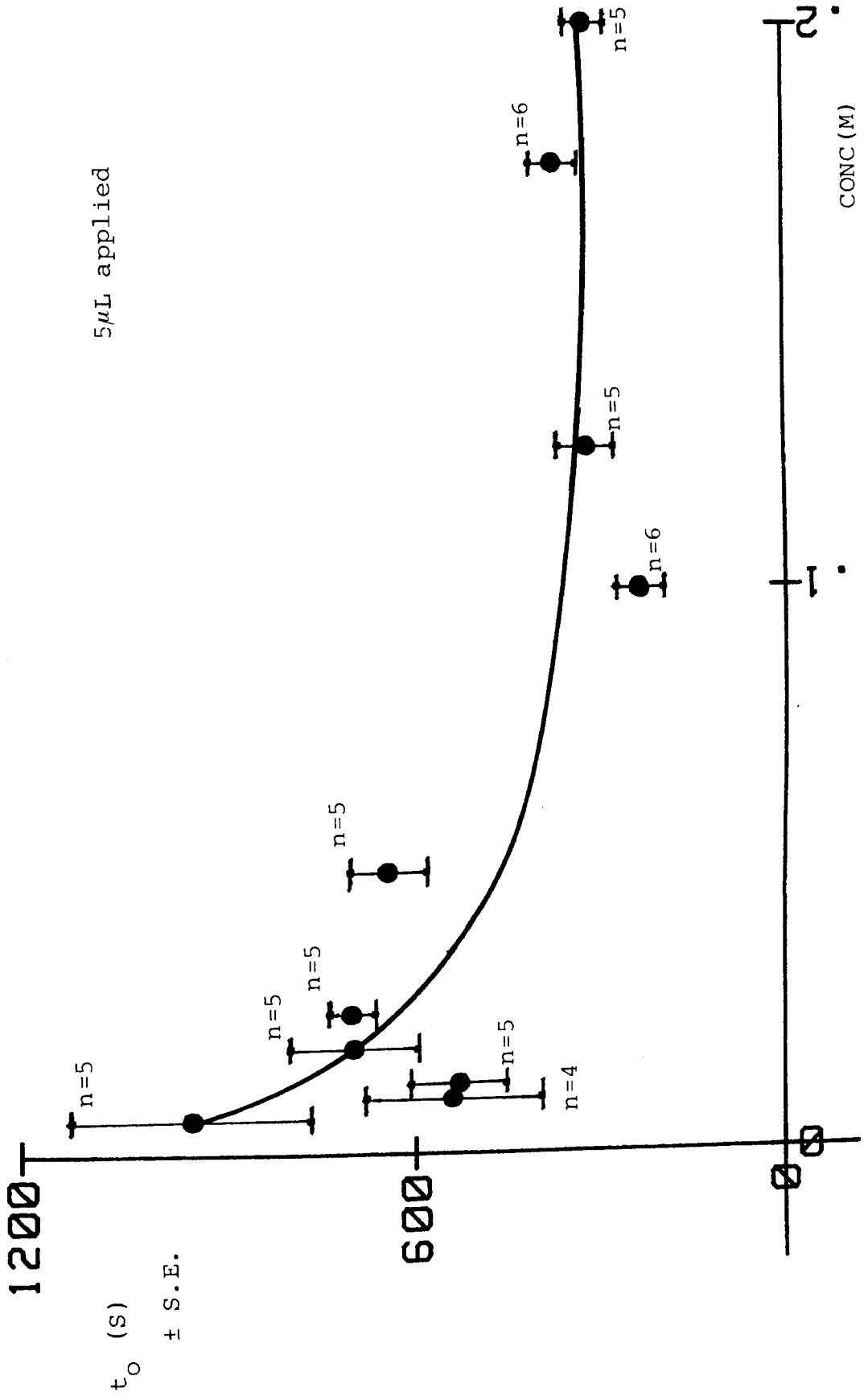


FIGURE 29: VARIATION OF t_r VALUES WITH CONCENTRATION OF HEXYL NICOTINATE IN KEROSINE

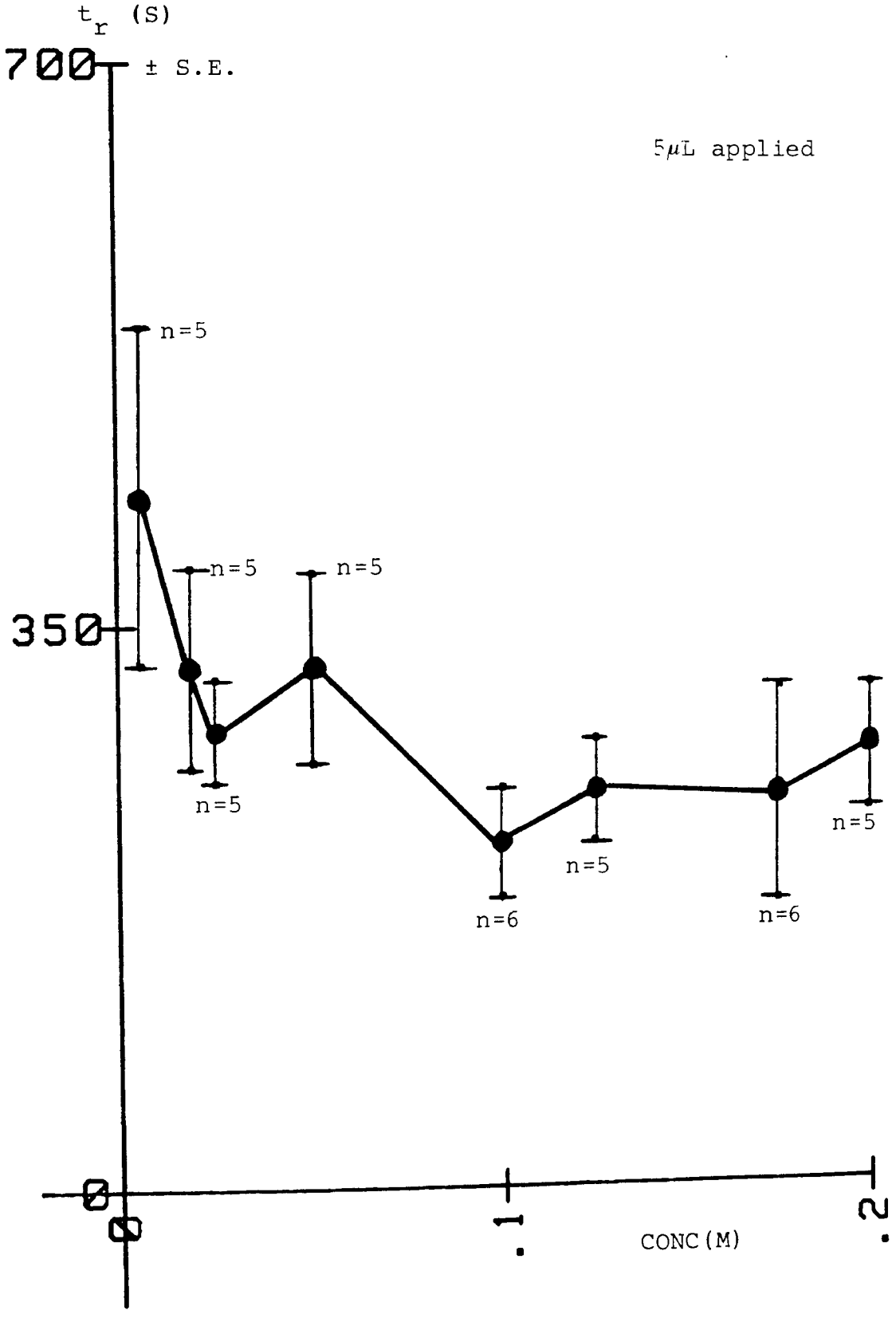


FIGURE 30: VARIATION OF r_i VALUES WITH CONCENTRATION OF HEXYL NICOTINATE IN KEROSINE

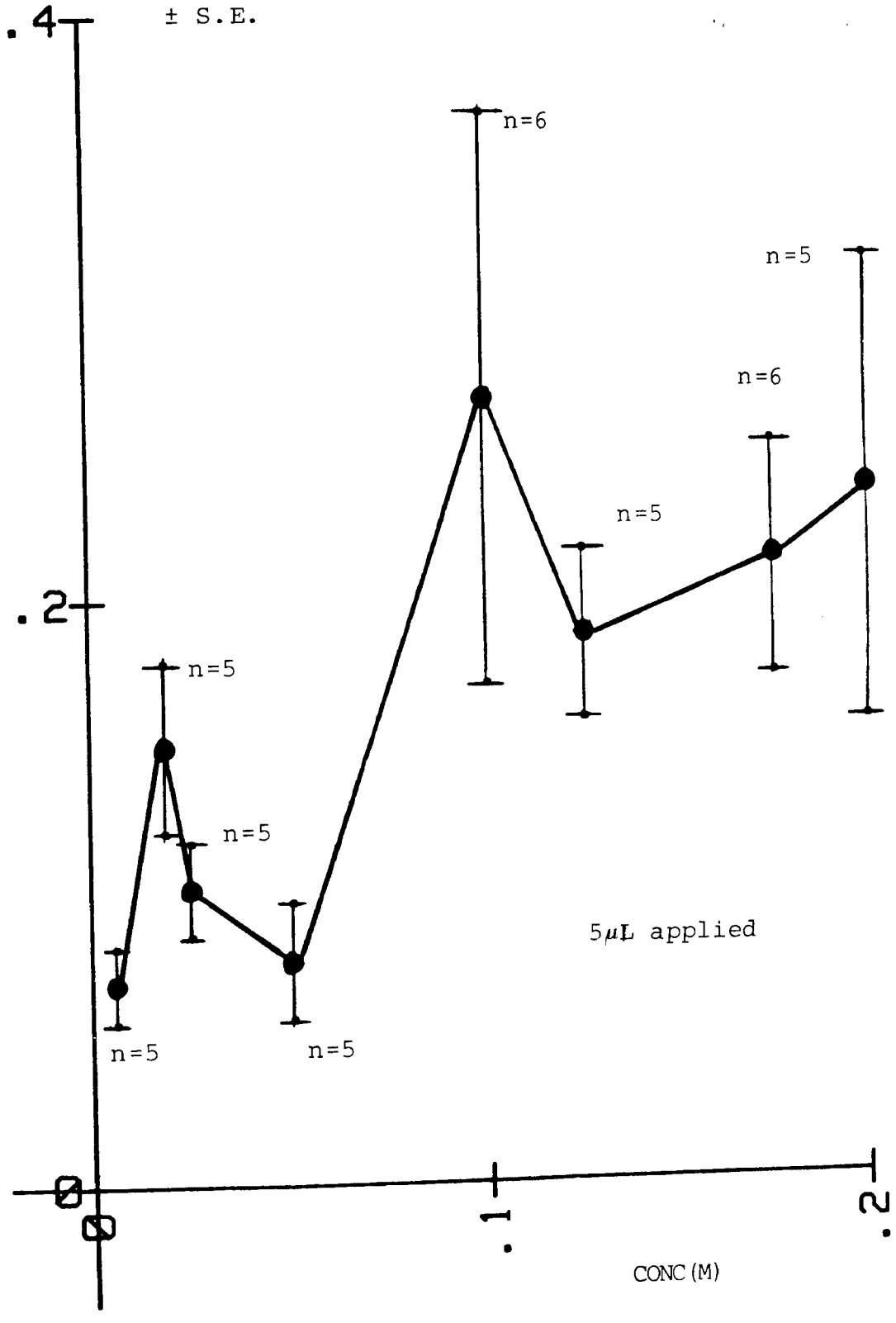
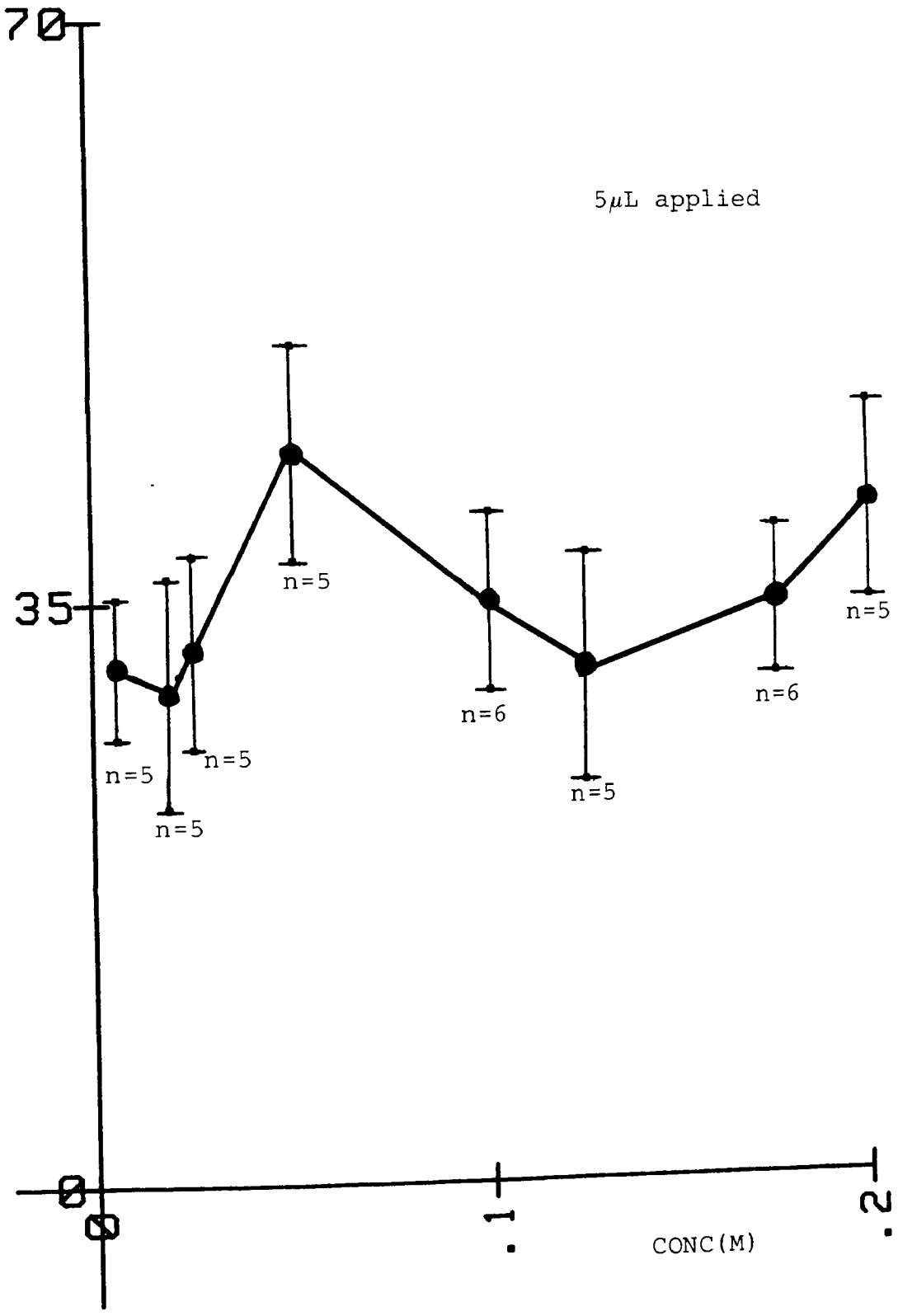


FIGURE 31: VARIATION OF P VALUES WITH CONCENTRATION OF HEXYL NICOTINATE IN KEROSINE



maximum instrument response remains fairly constant over the concentration range measured. In fact P is larger at 0.05M hexyl nicotinate than 0.10M.

Guy et al (96) have used P as well as the area under the response-time curve and the time taken for the response to decay to 75% of the maximum value to assess methyl nicotinate percutaneous absorption. Their results indicate a dose-response relationship for the above three parameters. Furthermore, for concentrations above 0.01 to 0.025M methyl nicotinate, the parameters do not vary significantly, suggesting a saturation of the pharmacodynamic effect.

The results for maximal response obtained here show consistent saturation of response. The rate of onset of erythema however changes with concentration (Figure 28) at the lower values. Once the microcirculation has responded, the rate of absorption and removal of the nicotinate seems fairly similar, regardless of concentration applied. Further work is required at much lower concentrations to determine the sensitivity of the microcirculation. The concentration range measured here may be above the point of saturation for the parameters considered.

The main finding of these results and those of Guy et al (96) is that the rate of penetration of the nicotinate must be considered separately from the response of the microcirculation to this material. The two parameters will give different points of saturation. Even though the nicotinate and vehicle used here are different from that of Guy et al, care must be taken if the microcirculation is to be studied in real-time.

For subsequent work carried out in this study t_o has been used as the main parameter of analysis, since it gives more quantifiable data.

9.4 ASSESSMENT OF ALTERNATIVE NICOTINATES

Stoughton et al (62) considered the percutaneous absorption of nicotinic acid and its various derivatives. They applied topically, serial dilutions of these compounds in 95% alcohol, on the volar surface of the forearm. The minimum concentration required to elicit visible erythema was used to indicate the relative rates of penetration of the products. Generally their results indicated that as the molecular weight of the ester increased the concentration required to elicit a response also increased.

In this study the rate of onset of erythema as determined by the laser Doppler method and the maximum response by the instrument was studied for methyl, ethyl and hexyl nicotinate. 5 μL of a 0.01M solution of each nicotinate in kerosine was applied through the probe holder onto the volar or ventral areas of the forearm.

From Figure 32 it can be seen that the rate of penetration or erythema onset depends on the ester used. Table 3 shows data for the maximum response of the instrument, for the various nicotinates applied. As can be seen no significant differences occur in P for the different nicotinates. This only further emphasizes the sensitivity of t_o as a parameter of analysis for percutaneous absorption.

FIGURE 32: MEASUREMENT OF ONSET OF LASER DOPPLER RESPONSE FOR ALTERNATIVE NICOTINATES IN KEROSINE

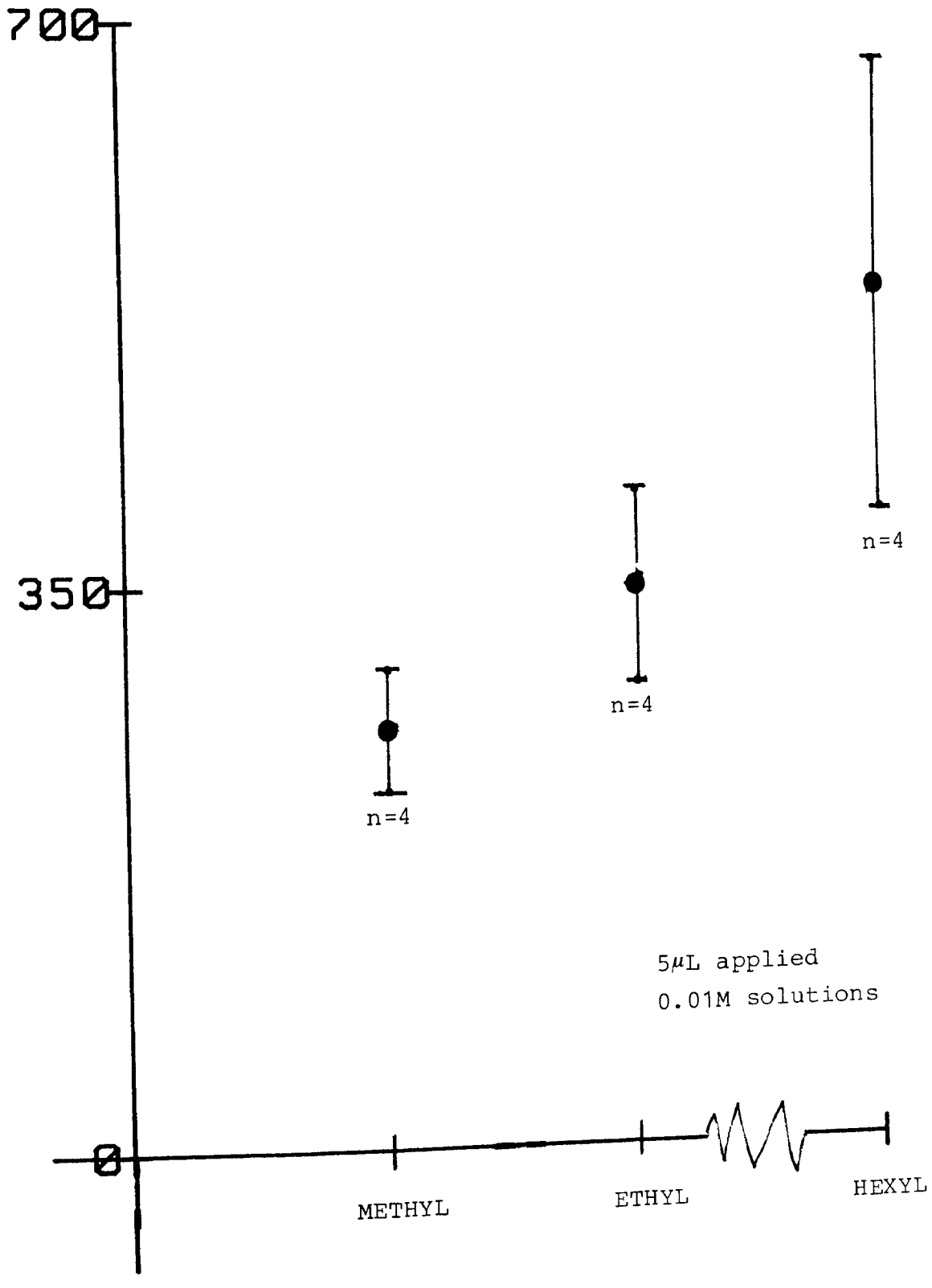


TABLE 3: **MEASUREMENT OF MAXIMUM RESPONSE P% FOR 5 μL OF 0.01M NICOTINATE IN KEROSINE BASE**

	Mean P%	± S.E.
Methyl Nicotinate	44.3	2.9
Ethyl Nicotinate	43.7	9.4
Hexyl Nicotinate	39.3	15.8

Stoughton et al (62) with results for the minimum concentration required to elicit erythema, found that methyl nicotinate penetrated nearly 5 times faster than hexyl nicotinate. The results produced here only show a 2 fold difference. These relative rates of penetration are fairly wide between the two test methods. Even though different vehicles were used, the relative rates of penetration were determined from the same vehicle, so the physicochemical parameters affecting absorption should remain consistent.

These differences in results could be attributed to the increased sensitivity of the laser Doppler technique, particularly since the latter is claimed

to detect erythema not visible to the naked eye (87, 88). However, Guy et al (95) have found a good correlation between onset of visible erythema and instrument response, suggesting that this discrepancy cannot be entirely due to the technique of measurement.

Another possible reason for the difference in the relative rates of penetration is that Stoughton et al measured follicular and perifollicular erythema as their end-point. The area detected by the laser Doppler will contain some follicles, but the method monitors the whole of the cutaneous vasculature.

9.5 MEASUREMENT OF HEXYL NICOTINATE ABSORPTION FROM MPG

So far kerosine has been used as a vehicle for the various nicotinates tested here. Fountain et al (107) assessed the rate of absorption of methyl nicotinate using different vehicles. Their technique involved measuring the area and duration of erythema produced for a given concentration of nicotinate from different vehicles.

The results produced by these workers showed that the vehicles play an important part in the degree and duration of erythema produced. For example, propylene glycol gave a much reduced initial mean diameter of erythema than water, but the effect lasted much longer.

The main problem with their technique is that it is subjective and does not measure the extent of erythema. Their assumption is that the mean diameter of the erythema is a measure of the response. For example, their results produced for methyl nicotinate in water at different concentrations, showed a change in the mean diameter of erythema, but not in the onset and duration of action.

Results produced here have shown the sensitivity of erythema onset times for hexyl nicotinate at different concentrations in kerosine (Figure 28). However, the duration of action was similar for the 0.05 and 0.10M concentrations. The same process was repeated under similar conditions with an alternative vehicle, monopropylene glycol (MPG).

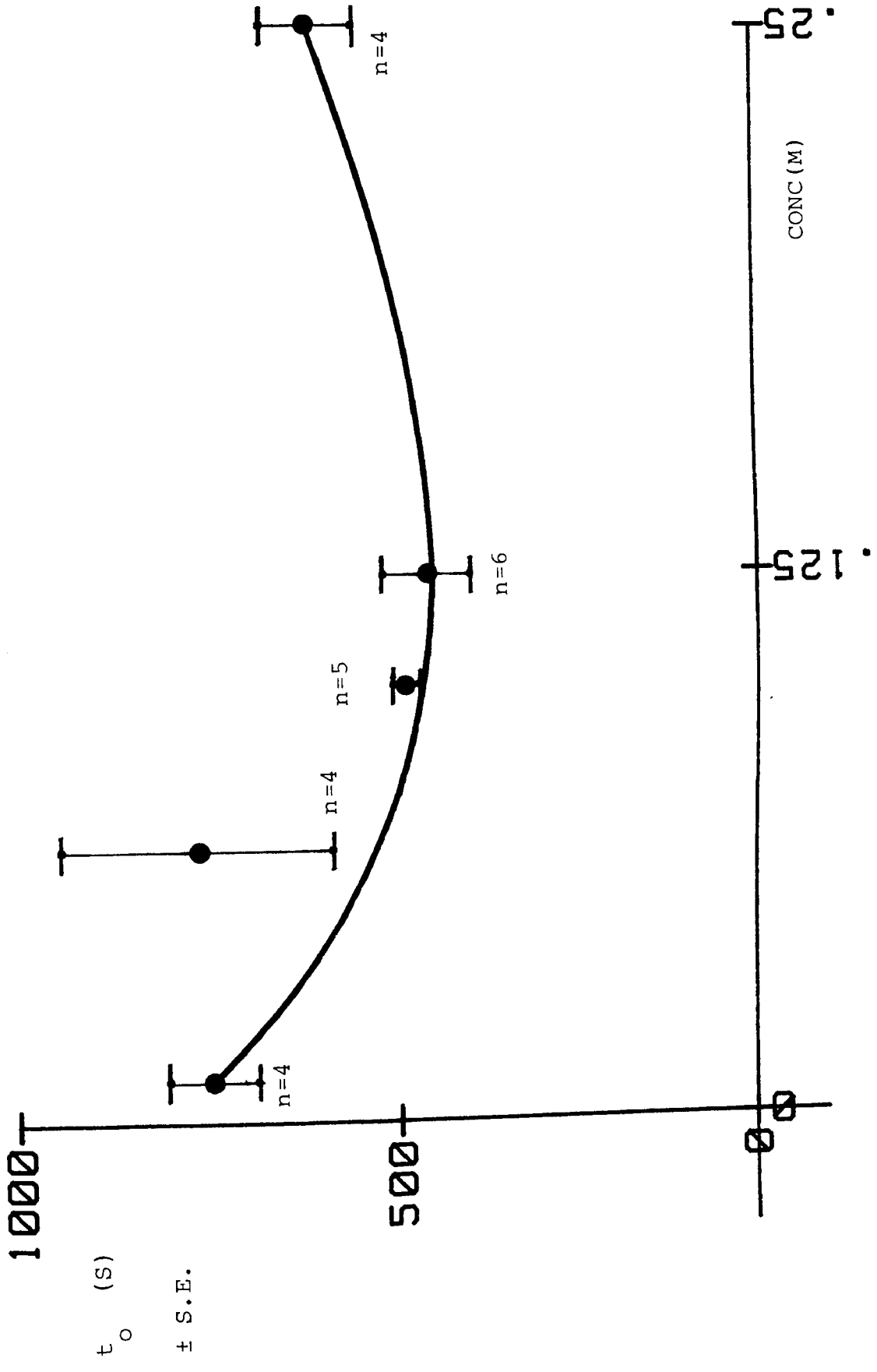
Figure 33 shows the variation in the onset times with changes in hexyl nicotinate concentration and Table 4 gives values for the maximum measured change in blood flow.

TABLE 4: **MEASUREMENT OF MAXIMUM RESPONSE P% FOR HEXYL NICOTINATE IN MPG.**

Concentration (M)	P%	
	Mean	± S.E.
0.25	34	± 4.2
0.125	39	± 5.2
0.10	41	± 3.0
0.0625	21	± 1.75
0.01	45	± 1.45

The actual hexyl nicotinate concentrations applied varied from 0.01 to 0.25M. However, for MPG, applied neat, no changes in blood flow were detected even after 60 minutes of continuous monitoring. Figure 33 shows that t_o is fairly sensitive to concentration changes, whereas P remains stable at most of the concentrations measured, except for 0.0625 M (Table 4).

FIGURE 33: VARIATION OF LASER DOPPLER RESPONSE t_o WITH CONCENTRATION OF HEXYL NICOTINATE IN MPG



If the hexyl nicotinate data is considered at a given concentration for the two vehicles, the erythema onset values show significant differences. For example at 0.10M, t_o is 234 $\pm$ 38 (S) from kerosine and nearly twice as high from MPG, 487.5 $\pm$ 17 (S). Furthermore, as can be seen from Figures 28 and 33, this is reflected throughout the concentration range measured.

The maximum response P however shows no significant differences between the vehicles (Tables 2 and 4) again reflecting the greater sensitivity of t_o.

9.6 PHYSICOCHEMICAL FACTORS AFFECTING PERCUTANEOUS ABSORPTION.

9.6.1. INTRODUCTION.

In order to predict the exact transport rate of a product through the skin, it is necessary to study various physicochemical properties of the compound (54). Formulation changes may be used to improve the rate of release of a given compound from its vehicle. Once this active has entered the skin surface, the rate of diffusion will normally be controlled by the

barrier properties of the stratum corneum. In certain circumstances the diffusion through the skin may be increased by using penetration enhancers such as urea, surfactants and sulphoxides (54, 100).

The rate of release of an active from a vehicle will be determined by two major factors. These are the thermodynamic activity of the diffusant (54) and the micro-viscosity of the vehicle (100). Both these factors may be altered by formulation changes, but usually modifying one affects the other.

In order to analyse the laser Doppler data produced here, the partition coefficients and viscosity of the various nicotinates and vehicles were considered.

9.6.2. DETERMINATION OF PARTITION COEFFICIENTS.

The partition coefficients were determined for methyl, ethyl and hexyl nicotinate using kerosine and MPG as the vehicles. Originally the partition coefficient of methyl nicotinate

between cadaverous skin and ethanol was considered. However, sensitivity problems were encountered.

The skin phase was therefore represented using isopropyl myristate (IPM). This has been previously used in the investigation of percutaneous absorption, since it is claimed to be representative of the high proportion of monoesters in skin lipids (60, 112).

The initial work carried out here showed that kerosine and IPM were miscible, so the partition coefficients were determined between MPG/kerosine and IPM/MPG for the three nicotinates. The partition coefficients for IPM/kerosine were then calculated from the above data. It may be more accurate to determine the solubilities of the nicotinates separately in IPM and kerosine. However, this is difficult because of the very high solubilities expected.

In order to ensure accurate partitioning, mutually saturated solvent solutions were prepared and the partition coefficients determined over a range of concentrations. Saturated solutions were prepared by adding a given quantity of one solvent to 2.5 times the quantity of a second solvent. These were then mechanically shaken for 5 hours.

The nicotinate solutions were prepared as follows:

a) First 50ml of a 0.05M nicotinate in MPG saturated kerosine solution was made.

b) Using 25ml solutions at a time 0.025, 0.0125 and 0.00625M nicotinate in kerosine solutions were prepared by serial dilution.

c) To each of these 25ml solutions, 25ml of kerosine saturated MPG was added.

d) All the solutions were then mechanically shaken for 20 hours.

This process was repeated for the IPM\MPG mixture, using the same quantities and concentrations. The analysis of the nicotinate concentration within the solvents was determined as follows:

a) The solvent mixtures were allowed to separate.

b) An initial comparison of the UV spectra showed an overlap in the absorption spectra of the solvents and the nicotinates. The nicotinate was therefore separated and assayed by HPLC using acetonitrile as eluent (HPLC Grade, Fisons Scientific, Loughborough. Leics.) and reversed phase HPLC column (PXS - 1025 ODS - 2, Whatman Ltd., Maidstone, Kent). Column dimensions were 4.6mm x 25cm.

c) The peaks were then quantified by UV spectro-photometry at 265nm.

For the nicotinate concentration range tested here, the UV absorbance data gave a linear

response. For example for hexyl nicotinate in IPM\MPG:

$y=0.975 + 5484x$ and $r=0.99$ where y=peak height (mm)and x=concentration (M).

Table 5 gives the partition coefficients for methyl, ethyl and hexyl nicotinate in the various solvent systems.

9.6.3. EFFECT OF PARTITION COEFFICIENTS ON PERCUTANEOUS ABSORPTION.

Hadgraft et al (60) have found that the onset of erythema was delayed when glycerol was added in increasing concentrations to a methyl nicotinate aqueous solution. This delay correlated well with changes in the partition coefficient and diffusivity of methyl nicotinate in the vehicle, suggesting the mechanism of methyl nicotinate percutaneous absorption to be by lipid rather than an aqueous pathway.

If kerosine is used as the vehicle, there is a

TABLE 5: PARTITION COEFFICIENTS OF THE VARIOUS NICOTINATES IN DIFFERENT SOLVENT SYSTEMS

NICOTINATE ESTER FUNCTION	SOLUTION	PARTITION COEFFICIENT
METHYL	MPG/KEROSINE	11.67/1[b]
	IPM/MPG	0.36/1
	IPM/KEROSINE[a]	4.18/1
ETHYL	MPG/KEROSINE	6.33/1
	IPM/MPG	0.53/1
	IPM/KEROSINE[a]	3.35/1
HEXYL	MPG/KEROSINE	1.01/1
	IPM/MPG	2.67/1
	IPM/KEROSINE[a]	2.70/1

a = Calculated values.
b = The partition coefficients are a ratio of the peak height of nicotinate in each solvent layer.

good correlation between erythema onset and the partition coefficients of methyl, ethyl and hexyl nicotinate in IPM\kerosine base. As shown in Figure 32, the time of erythema onset increases with increasing molecular weight of the nicotinate.

Hexyl nicotinate takes much longer to elicit erythema than the ethyl ester which in turn takes longer than the methyl ester. The decrease in the partition coefficients of the nicotinates between IPM and kerosine (Table 5)is reflected by a change in t_o. The results here suggest that the rate of penetration of the various nicotinates is dependent on the rate of release of the ester into the skin, from the vehicle.

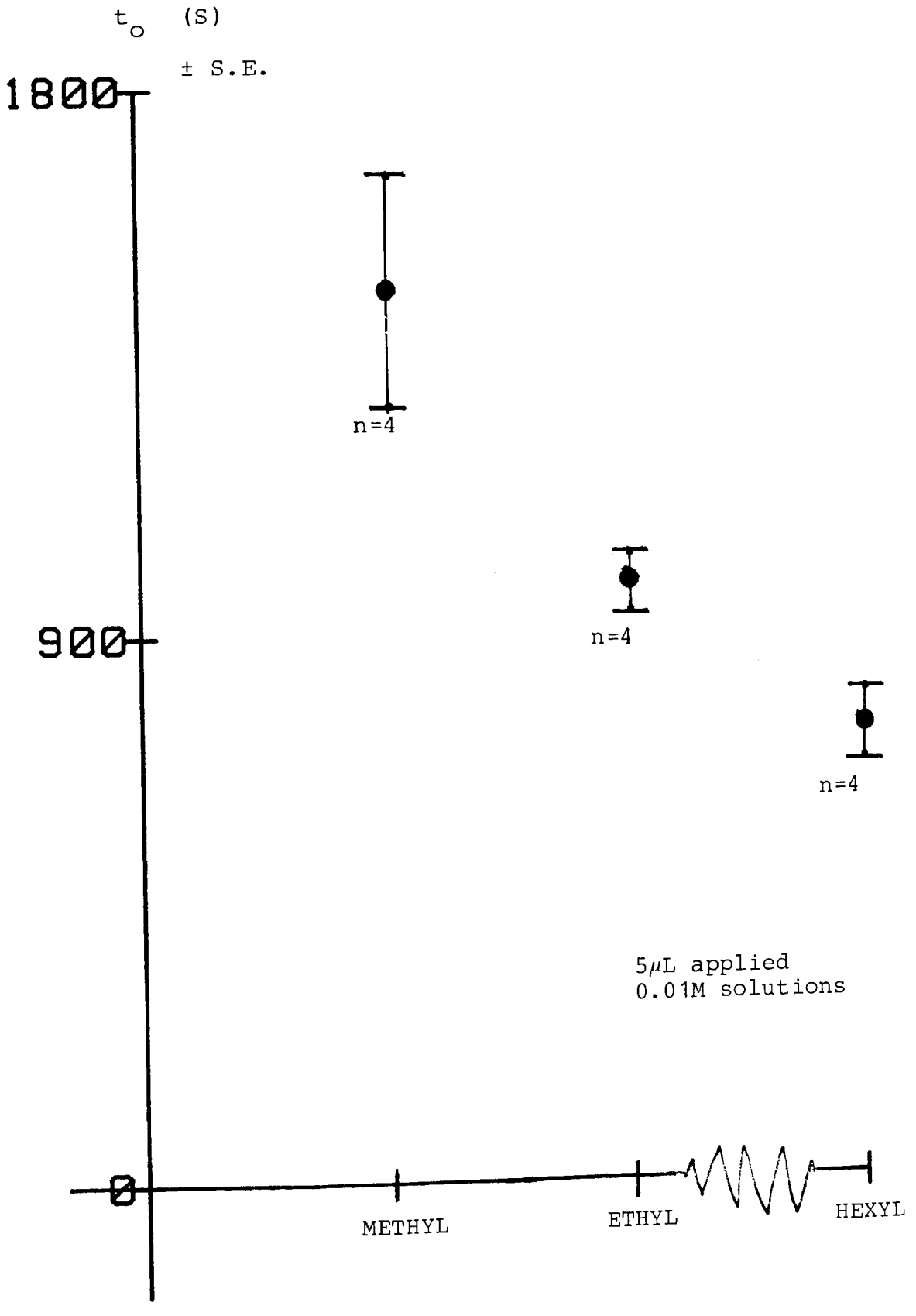
Stoughton et al (62) found a similar change in the penetration rates to nicotinic acid and its derivatives. The ability of these compounds to penetrate into the skin could be related to their relative and absolute solubilities in water and ether. The penetration rate decreased with increasing molecular weight of the nicotinate when 95% alcohol was used as the vehicle.

When MPG was used as the vehicle, an entirely different effect was obtained. The partition coefficients for the three nicotinates, between IPM and MPG, showed an increase with an increase in molecular weight of the ester (Table 5). Thus hexyl nicotinate has a higher partition coefficient than ethyl and methyl respectively.

When the partition coefficients are considered with relevance to the rate of erythema onset, as depicted in Figure 34, the results are congruous. As expected the rate of erythema onset increases as the partition coefficients increase.

In the case of kerosine the decrease in the partition into the skin may be due to slower diffusion of the nicotinates in the vehicles as their molecular size increases but reported data indicated that this effect is likely to be small (62). However for MPG, since the opposite effect occurs, molecular diffusion cannot be the explanation.

FIGURE 34: MEASUREMENT OF ONSET OF LASER DOPPLER RESPONSE FOR ALTERNATIVE NICOTINATES IN MPG



So far the data suggests that the partition coefficient of the nicotinate is the main factor controlling its rate of penetration through skin. If the partition coefficients for hexyl nicotinate in IPM\MPG and IPM/kerosine are considered, it can be seen from Table 5 that these are very similar. Thus the rate at which hexyl nicotinate penetrates the skin should be fairly similar from either vehicle. However Figures 28 and 33 suggest that this is not the case. As previously mentioned at 0.10M hexyl nicotinate concentration t_o is nearly twice as high from MPG as it is from kerosine. This would suggest that the MPG and the kerosine may be affecting the permeability of the skin to different extents. It seems unlikely that this discrepancy is due to the higher viscosity of MPG compared with kerosine. However problems with calculated partition coefficients must be taken into account.

Fick's Law has been applied to study the behaviour of methyl nicotinate permeation through in vivo skin (60). It may be simply

described as:

$$J = - K \Delta C \qquad [9]$$

Where J - steady state flux of nicotinate

(moles cm^{-2} hr^{-1})

K - permeability constant for the nicotinate

(cm hr^{-1})

ΔC - concentration difference of nicotinate across the skin (moles cm^{-3})

The permeability constant, K, will depend on the thickness of the skin, the diffusion of the nicotinate across the skin and the skin: vehicle partition coefficient of the solute. In this case the permeability constant could be assumed to be the same for hexyl nicotinate from either kerosine or MPG, especially since they have similar partition coefficients. Thus from equation 9 the rate of penetration of hexyl nicotinate across the skin should depend on its concentration gradient.

For the hypothesis proposed and results obtained, when kerosine is used as a vehicle there must be a rapid loss of this solvent from the skin surface, thus increasing the concentration gradient of hexyl nicotinate across the skin. This loss may be by evaporation and absorption into the skin. No data have been found on the rate of permeability of kerosine, however MPG is claimed to form a film on the skin surface and is used in cosmetic creams to influence skin texture and condition (113). Thus the concentration gradient of nicotinate may decrease more rapidly when MPG is used as the vehicle.

9.6.4. EFFECT OF VEHICLE VISCOSITY ON PERCUTANEOUS ABSORPTION.

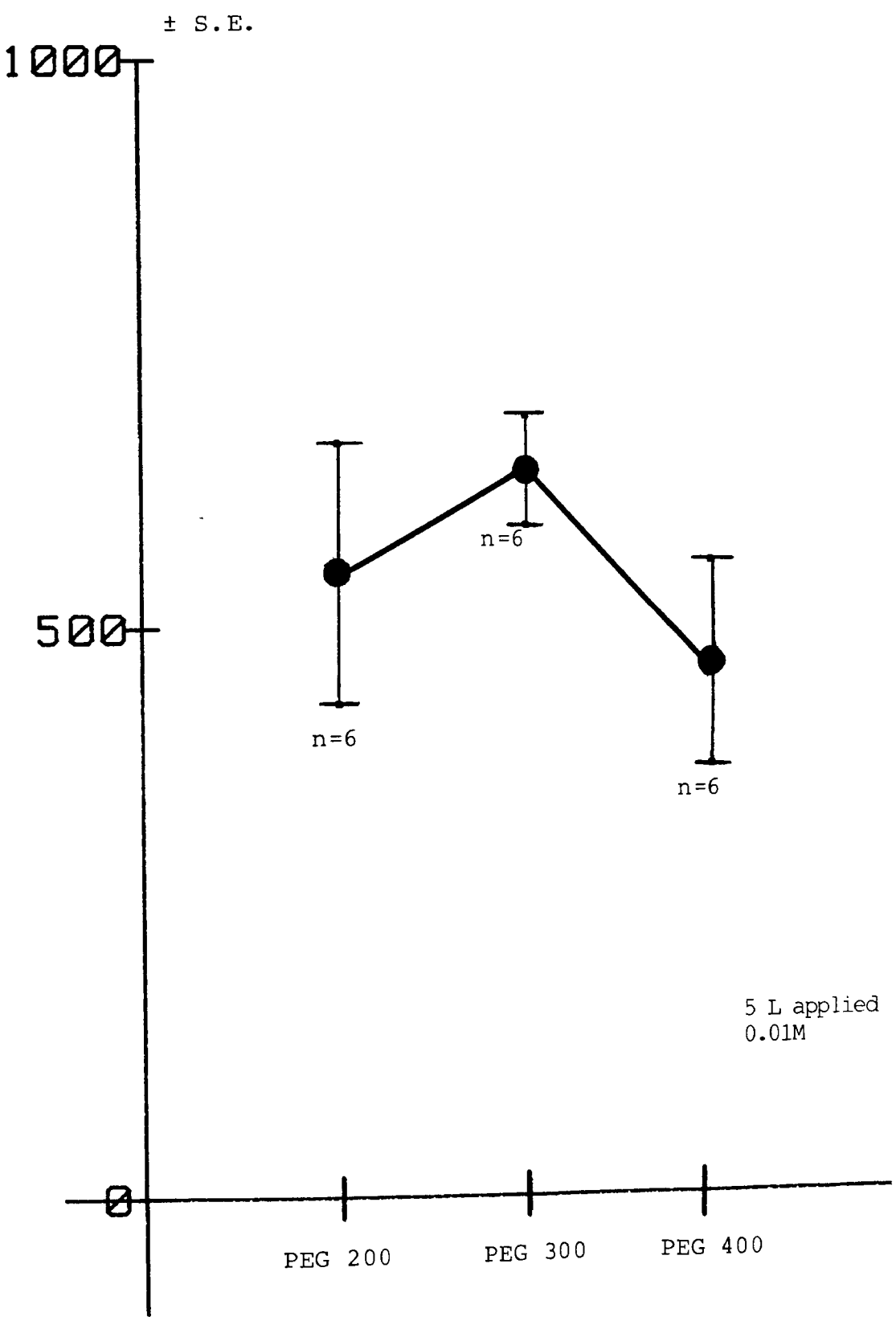
Fountain et al (107) found that the diffusion of methyl nicotinate through polyethylene glycol 300 is sufficiently slow, that it becomes rate limiting. Similarly Hadgraft et

al (60) have shown the rate of diffusion of methyl nicotinate in a glycerol aqueous vehicle correlates well with both the partition coefficient and rate of erythema onset.

In order to test the importance of vehicle viscosity, hexyl nicotinate was applied in a range of polyethylene glycols (PEG). The glycols chosen were PEG 200, PEG 300 and PEG 400, representing a viscosity range from 53 to 105 cs. 5 μL of a 0.01M hexyl nicotinate in PEG solution was applied to the skin surface.

As can be seen from Figure 35, no significant differences were found in the rate of erythema onset for the different glycols, suggesting viscosity may not be a crucial factor. However it is possible that the viscosity range chosen was too narrow to show any variation. Furthermore only t_o has been used as the parameter of analysis. The degree of or time period for which erythema lasts may also be important (107).

FIGURE 35: EFFECT OF VISCOSITY ON THE RATE OF LASER DOPPLER RESPONSE USING HEXYL NICOTINATE FROM POLYETHYLENE GLYCOL



It is interesting to note that for the same hexyl nicotinate concentration applied in the glycols, similar values were obtained from MPG. This suggests a much closer relationship between the vehicles, than that with kerosine.

9.7 ASSESSMENT OF METHYL NICOTINATE IN A SERIES OF ALIPHATIC ALCOHOLS.

Various authors have measured the percutaneous absorption of a series of homologous primary alcohols (55, 101). However, this work has been in vitro, using diffusion cells to study absorption of the alcohols from aqueous and nonaqueous vehicles.

In this study the effect of increasing chain length of the vehicle was examined on methyl nicotinate penetration, using a series of primary alcohols (C_2 - C_{10}). Prior to applying the methyl nicotinate solutions, 5 μL of each alcohol was applied on the forearm and blood flow values monitored. Some of the alcohols tended to elicit a response. However, this was not reproducible even for the same subject. Furthermore the point of

increase in blood flow, t_o was not always as sharp as that obtained for the various nicotinates applied previously from kerosine and MPG.

Table 6 represents the mean onset values for those alcohols that gave a response. Butanol, nonanol and decanol gave no response even after 30 minutes of continuous monitoring. These results are fairly widespread making it difficult to draw any conclusions. Deionised water was also applied to the skin surface and this gave a response for some of the subjects. Nilsson et al (84) have also found an increase in irritation when distilled water was applied in an occlusive patch for 24 hours.

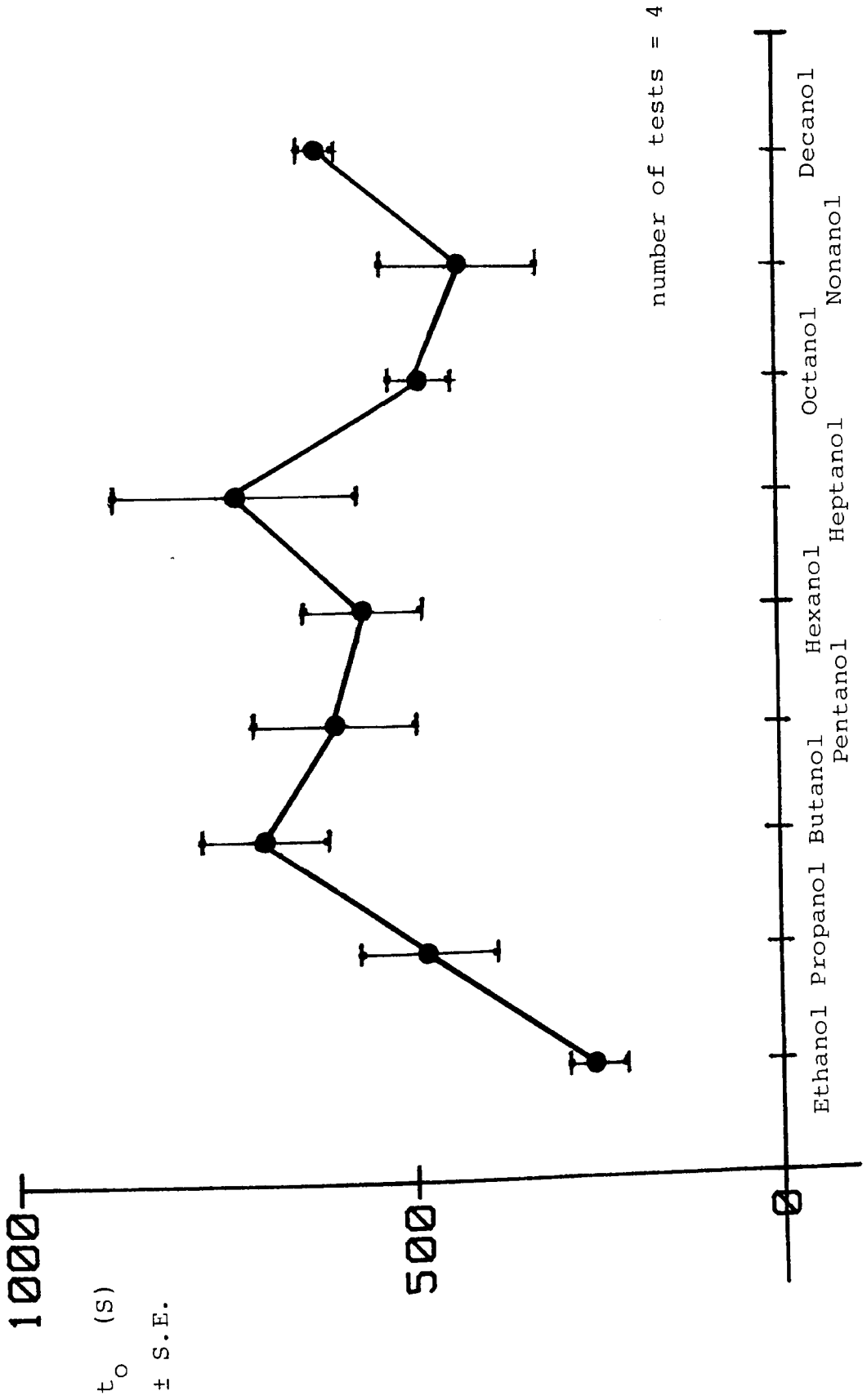
5 μL of a 0.01M methyl nicotinate in alcohol solution was applied to the volar and ventral areas of the forearm. After product application, blood flow was continuously monitored without disturbing the test site. Figure 36 shows data for the time taken by the instrument to respond to an increase in blood flow. This data shows very little differences between the various alcohols. Only ethanol seems to give a faster response time.

TABLE 6: THE RESPONSE TIME BY THE LASER DOPPLER (t_o) TO VARIOUS SOLVENTS BY CERTAIN SUBJECTS.

SOLVENT [a]	t_o (S)	± S.E. (S)	NUMBER OF SAMPLES
ETHANOL	473	57	7
PROPANOL	1133	185	3
PENTANOL	923	195	4
HEXANOL	660	160	6
HEPTANOL	290	46	3
OCTANOL	900	300	2
DEIONISED WATER	493	67	10

a = 5 μ L of each solvent applied.

FIGURE 36: MEASUREMENT OF ONSET OF LASER DOPPLER RESPONSE TO 0.01M METHYL NICOTINATE IN A SERIES OF ALCOHOLS

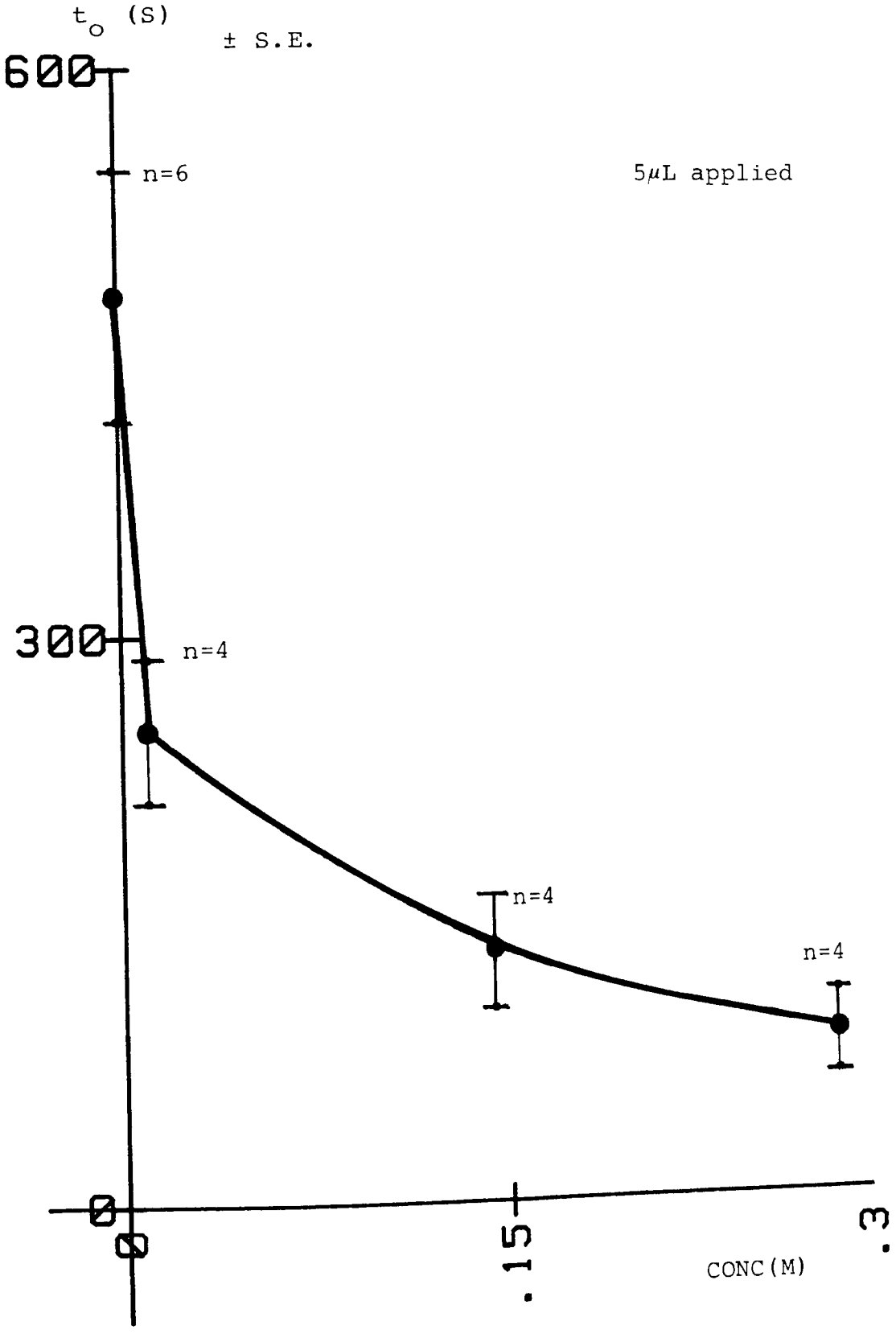


From the in vitro work (55) it was found that the alcohols applied as pure liquid systems behave in a number of different ways. The lower alcohols tended to permeate the epidermis far more rapidly whereas the higher alcohols (C_5 - C_{10}) being good lipid solvents took much longer. This should be reflected by a faster onset time for methyl nicotinate for the lower alcohols (C_2 - C_4), assuming dissolved methyl nicotinate diffuses along with the solvent. However as can be seen from Figure 36 this was not the case, apart from ethanol.

Methyl nicotinate has been reported to penetrate the skin via partitioning and diffusion across a lipid membrane (60). Thus when applied from the higher lipid solubilizing alcohols (C_5 - C_{10}), the time taken for permeation should be much longer than for the lower less lipid solubilizing alcohols. The results reported here suggest that the mechanism is far more complex, particularly since some of the alcohols may also permeate the skin.

The effect of changing methyl nicotinate concentration in ethanol was measured using the t_o values. From Figure 37 it can be seen that t_o varies, with possible saturation at the higher concentrations.

FIGURE 37: EFFECT OF METHYL NICOTINATE CONCENTRATION IN ETHANOL ON ONSET OF LASER DOPPLER RESPONSE



CHAPTER 10.0: LASER DOPPLER - CONCLUSION

The laser Doppler technique has been used to assess the percutaneous absorption of the nicotinic acid esters from various vehicles. When hexyl nicotinate is applied using kerosine as the vehicle, the time taken for the instrument to respond is dose dependent upto a concentration of about 0.05M. This may be regarded as the time for penetration of sufficient nicotinate to elicit a vasodilator response. The interdependence of vasodilator response and hexyl nicotinate concentration was less obvious when MPG was used as the vehicle.

The magnitude of increase in blood flow due to the hexyl nicotinate concentration in kerosine and the subsequent changes in the microcirculation have also been monitored. No significant changes were found over the concentration range studied indicating perhaps the complexity of the kinetics of drug depletion from the site of action (Table 2).

For hexyl nicotinate in kerosine, concentrations above 0.075M give a fairly constant onset of laser Doppler response. This indicates there is a maximum diffusion rate through the skin, which can be attained at a sufficiently high dose of drug applied.

The time of erythema onset has also been used to assess the percutaneous absorption of methyl, ethyl and hexyl nicotinates using kerosine and MPG as the vehicles. The time taken for erythema onset correlates well with their partition coefficients between skin, represented by IPM and the vehicle.

For kerosine a decrease in IPM/kerosine partition coefficient occurs with increasing molecular weight of nicotinate giving an increase in erythema onset time. When MPG is used, the effect is reversed, i.e. the higher nicotinates penetrate and partition off far more rapidly than the lower nicotinates.

The erythema onset time does not depend on the partition coefficient of the nicotinate only. Hexyl nicotinate has similar partition coefficients from kerosine and MPG, but different rates of permeation from these vehicles. This may be due to loss of kerosine from the skin surface, which results in a higher concentration gradient of nicotinate across the skin in the kerosine case, than when MPG is employed as the vehicle.

Application of methyl nicotinate in a series of homologous alcohols, propanol to decanol, showed no significant differences in the erythema onset time. Onset time was shorter with ethanol vehicle.

Methyl nicotinate has significant water solubility and the shorter onset time from ethanol, compared to higher alcohols, is approximately the same as that from water (Table 6). This does not accord with importance of vehicle/skin partitioning in the penetration of topically applied substances. One implication is that the higher alcohols are modifying skin barrier function.

In order to elucidate this observation, further work is required using alternative solvents and different nicotinates, with particular emphasis on their physicochemical parameters.

CHAPTER 11.0: SURFACTANT IRRITATION ASSESSMENT

11.1 INTRODUCTION

The use of surfactants is well established in cosmetics and toiletry products and falls into five main areas depending on the surface-active properties required (113): a) Detergency, b) Wetting, c) Foaming, d) Emulsification and e) Solubilization. These properties are not mutually exclusive, but their very nature may result in undesirable biological effects such as skin irritation.

Surfactant skin irritation is a term that encompasses a vast array of dermatological effects including erythema, oedema, scaling, eschar and dryness. In order to evaluate these properties a number of techniques have been developed which have also helped to elucidate the mechanisms of interaction between the skin and surfactants (115 - 128).

Various toiletries such as liquid soaps , shower gels and foam baths use both natural and synthetic

surfactants as their main active materials. There is a need to develop "milder" products and towards this end a new cheap and rapid method for evaluating the irritation potential of surfactants is proposed.

This method essentially involves the measurement of surfactant concentration, in saline, required to lyse a solution of blood cells in saline. The concentrations obtained for various surfactants have been qualitatively compared with irritation values obtained from more conventional test methods.

The mechanisms by which surfactants irritate the skin will be discussed later, however it is on the basis of epidermal cell damage that the method proposed here is considered. It is difficult to directly quantify disruption of epidermal cells, however the easy availability and change in colour on lysis, makes blood cells more useful and practical.

11.2.REVIEW

11.2.1. METHODS FOR SURFACTANT IRRITATION ASSESSMENT

In the safety testing of substances applied to the skin, a number of tests are employed to evaluate the final product or its constituents. These include in vitro tests which may be useful for understanding the more fundamental principles of irritation and in vivo animal models which may allow closer simulation of the corresponding human response. Human in vivo testing is perhaps the best, but even here laboratory tests may not adequately simulate "in-use" application of the final product by the consumer.

A number of tests have been adapted to measure the irritation potential of surfactants per se or in various preparations. The traditional and probably most widely used technique is that of the patch test (98). This involves single application of the test substance to the skin, using gauze patches or aluminium discs, secured with adhesive tape. The patches are usually left on for a period of 24

hours after which scores are allocated according to the degree of erythema, oedema and various other formations. A combined score may then be calculated to give an irritation index for the product. Usually a standard surfactant is added to act as a marker.

Patch testing may be applied to various animals such as albino rabbit and guinea pig (114) as well as humans (115). The main disadvantages with this technique are its subjective nature during assessment and lack of correlation with actual "in-use" evaluation. Laser Doppler flowmetry may be used to quantify surfactant irritation by patch tests, however it only measures erythema and none of the other reactions (84 - 86).

The actual occlusive nature of the test has also been shown to modify the response and therefore lead to misinterpretation (87). It is possible to apply the product in an open patch, but on a cumulative basis (85). This may be regarded as more relevant to "in-use" conditions, but it is also more time consuming.

Even though there are various limitations to the patch test method, it is still an adequate and reproducible technique for screening irritating substances (115).

In order to predict the potential for damage on the human eye by various cosmetic products, an eye test has been developed. This test, commonly known as the Draize test, uses albino rabbits as the test subjects (114). The procedure involves application of the product to one eye, using the other as control. The eyes are examined at various intervals for ulceration of the cornea, inflammation of the iris and swelling of the conjunctiva. Again the test may be altered and different reactions studied.

There are methods which are claimed to be more directly relevant to the "in-use" situation, but still applicable in the laboratory. One such method involves soaking of the hands in a surfactant solution, usually for an hour over a number of successive days. The scaling caused by this immersion test (116) may be

graded subjectively and the differences between surfactants evaluated from hand to hand. The problem with this test is that it requires a fairly large panel of subjects and can also be cumbersome and time consuming.

Imokawa et al (117) propose a more rapid method to evaluate skin roughness that requires fewer subjects and correlates well with the immersion technique. Essentially they pump a 1% surfactant solution at 40^{o}C for 10 minutes on the inner surface of the forearm. Three adjacent sites are used to compare alternative surfactants. This test is carried out daily for 3 or 4 successive days after which skin roughness is subjectively evaluated.

A number of methods have been developed to characterize the effect of surfactants after subject panels have been exposed to the products under realistic conditions. Seitz et al (118) have produced a series of photographic standards to quantify various grades of dry skin. By using these photographs a reproducible reference scale is

available for any future surfactant evaluation.

In the method proposed by Prottey et al (119) the effect surfactants have on acid phosphatase, a lysosomal enzyme of the stratum corneum, is quantified by a spectrofluorometric procedure. Exposure conditions to the surfactants are made as real as possible and the acid phosphatase is obtained using tape strips. The acid phosphatase activity correlates well with visually observed changes.

In order to make surfactant irritation testing more objective, various physical measurements have also been employed. As previously mentioned laser Doppler flowmetry may be used to quantify erythema (84). Van der Valk et al (120) have assessed irritancy based on measurements of water vapour loss from the skin. Exposure to surfactants was by patch test for a period of 24 hours. When water vapour loss measurements were compared with visual assessment of the skin, a good correlation was obtained.

In vitro evaluation methods largely rely on the ability of surfactants to denature epidermal protein. This ability has been found to correlate well with the irritation potential of surfactants. A number of methods have therefore been developed to evaluate the degree of protein denaturation. These include measurement of the amount of sulfhydryl groups liberated (116), change of specific rotation (117), the degree of the inhibition of invertase (121) and quantitative analysis with gel-permeation chromatography (122). A further area using this principle is the measurement of the permeability of human epidermis in vitro, after exposure to various surfactants (123).

11.2.2. MECHANISMS OF SURFACTANT IRRITATION

Many factors have been reported to influence the ability of surfactants to irritate the skin. These include removal of skin surface lipids (124), loss of naturally occurring hygroscopic materials in the stratum coerneum (125), protein denaturation (116) and epidermal lysosomal injury (119).

Physicochemical factors such as surface activity and lipophilic properties have been found to be of secondary importance (126, 127).

Imokawa and Mishima (128) found that the adsorption of the surfactant molecules on the skin correlated well with their ability to induce roughness. Furthermore cumulative insult resulted in the persistence of adsorbed surfactant molecules, that were difficult to remove even after repeated rinsing.

The following mechanism of surfactant irritation is therefore proposed:

a) Cumulative insult results in adsorption of surfactant molecules (128) leading to

b) denaturation of epidermal keratin and rupturing of the biomembrane system (116, 126) which

c) results in a breakdown of the permeability of the horny layer allowing further adsorption into the skin (123, 128).

The removal of skin surface lipids, loss of naturally occuring hygroscopic materials and epidermal lysosomal injury may be regarded as part of the adsorption and disruption process.

11.2.3. MECHANISMS OF HAEMOLYSIS BY SURFACTANTS

The mechanism of haemolysis by surfactants has been researched mainly to increase an understanding of biological membrane molecular architecture. In order to obtain a better understanding of surfactant haemolysis, the basic features of the rupture of the cell membrane have been compared with various physicochemical properties of the compounds. The main properties examined are the critical micelle concentration, the hydrophile-lipophile balance, the nature of the charged head group and the length of the surfactant alkyl chain (129-132).

Erythrocytes from various species have been used for haemolysis studies. The degree of interaction between the site of action and the mode of interaction depends on whether the haemolytic agent is anionic, cationic or nonionic.

Cationics for example have a much higher ability to remove phospholipids from the cell membrane prior to lysis than anions. Furthermore, the longer the alkyl chain of cations, the lower the concentration required for haemolysis. Generally an increase in concentration of erythrocytes requires a higher surfactant concentration, to give a similar degree of haemolysis (129).

The haemolytic capacity of nonionic surfactants decreases with increasing polyoxyethylene chain length and gives a maximum for an alkyl chain length of 12 carbon atoms. Zaslavasky et al (130) suggest the hydrophile-lipophile balance determines the capacity of nonionics to bind to the erythrocyte membranes, but the actual lysis depends on the size of the lipophile group.

Various models have been proposed to simulate surfactant haemolysis. Generally they are based on (131):

a) The affinity of cell membranes to "pick-up" surfactant molecules and

b) the uptake required for lysis.

Azaz et al (132) propose that for nonionic surfactants, haemolysis occurs due to the formation of peroxides rather than any surface activity. The mechanisms of haemolysis by surfactants are therefore still not very well understood, requiring further research.

11.3. EXPERIMENTAL

Human blood was freshly obtained and a 0.75% ($^{wt}/_{wt}$) blood in saline solution prepared. The saline solution (0.9% NaCl $^{wt}/_{wt}$) ensured the blood remained whole and did not coagulate. The solution was stored at $5^{o}C$ when not in use.

The surfactants used were AKYPOs and AKYPOSALs supplied by ChemY, Emmerich, West Germany. AKYPOs are a series of alkyl polyglycol ether carboxylic acids, whereas AKYPOSALs are a series of fatty alcohol sulphates. Only natural alcohols (C_{12}-C_{14}) were used here. Table 7 gives a list of the various surfactants used with both the product and generic names.

BLE 7: VARIOUS SURFACTANTS USED IN LYSIS EVALUATION.

PRODUCT	GENERIC NAME
AKYPOSAL E020	SLES (2EO)
AKYPOSAL E030	SLES (3EO)
AKYPO RLM38	SLEC (3.8EO)
AKYPO RLM45	SLEC (4.5EO)
AKYPO RLM100	SLEC (10EO)
AKYPO RLM160	SLEC (16EO)

SLES - Sodium lauryl ether sulphate

SLEC - Sodium lauryl ether carboxylate

EO - No of moles of ethylene oxide

Surfactant solutions were also prepared in saline with pH adjusted to 5.8-6.2 using NaOH or Citric Acid. Initial studies showed the point of haemolysis occured when the cloudy reddish blood solution became a clear red liquid.

A rough value for the concentration of surfactant in saline required to give lysis was first obtained by serial dilution. The accuracy was improved upon by adding approximately 0.1gm of surfactant solution to 10gm of the blood solution and shaking the mixture. If lysis had not occurred the process was repeated until the clear red liquid was obtained. If lysis had not occurred after the addition of a gram of surfactant solution, a stronger surfactant solution was used with a fresh sample of blood solution.

The objective of this study was to qualitatively compare the concentration of surfactant required to give lysis, with in vivo irritation data. The data used here was provided by ChemY and included 24 and 48 hour human patch scores as well as Draize scores on the irritation of the cornea, iris and conjunctiva of albino rabbits.

In the case of human patch test scores, test and control materials were applied to the upper outer arm under occlusion for an initial period of 24 hours. Patches were then removed and various skin irritation reactions assessed, using a numerical scoring system, an hour later. Immediately after assessment an identical fresh patch was applied to the same site for a further 24 hours and skin reactions examined one hour after removal (133).

For the Draize eye test the procedure used was that set by the Code of Federal Regulations, U.S.A. (114). The test material was placed in the lower lid of one eye of the animal. The lids were gently rubbed together and the animal released. Six albino rabbits were used for each test material and the eyes were scored at 24, 48 and 72 hours. Total scores were obtained for the cornea, iris and conjunctiva (133).

11.4. RESULTS AND DISCUSSION

Figures 38 to 40 graphically represent the concentration of surfactant required to give lysis and these are compared with various irritation

scores. The assumption here is that the higher the concentration of surfactant required to give lysis, the less irritant it is. However, since for the irritation scores, the higher the value the greater the perceived irritation, the reciprocal of the scores have been plotted to give better representation of the data.

Each lysis concentration represented on these graphs is the mean of three different tests, using the same bulk blood solution and surfactant solution. When some of these results were repeated under similar conditions, but using a freshly prepared blood solution, the results were fairly reproducible. However, it may be important to add a surfactant marker if alternative blood supplies are used.

As can be seen from Figure 38 there is a close correlation between the lysis concentration and the human patch test scores at 24 and 48 hours. Sodium lauryl ether sulphate (SLES) with two moles ethylene oxide is found to be very irritant and this is reflected by the low concentration required to give lysis.

When a series of sodium lauryl ether carboxylates (SLEC) is examined, again a close trend is obtained between the concentration of lysis and human patch scores (Figure 38). There is a discrepancy at the higher ethylene oxide values. SLEC (10E0) is found to be less "irritant" than SLEC (16E0) by the lysis method, but this is not reflected by the irritation scores. SLEC (4.5E0) is claimed to be more irritant than SLEC (3.8E0) and this is also shown by the lysis method.

Figure 39 shows the lysis data for some SLESs and SLECs compared with Draize scores. The scores presented here represent ulceration of the cornea and swelling of the conjunctiva. SLES (3E0) is less irritant than SLES (2E0) and therefore requires a greater concentration to produce lysis. A corresponding discrepancy is found between SLEC (10E0) and SLEC (16E0) as found in the human patch data, except for the conjunctiva scores which are similar.

Since toiletries often contain a mixture of surfactants, the concentration of lysis was measured for various ratios of SLES (2E0): SLEC (10E0) and SLES (2E0): SLEC (16E0). From Figure 40

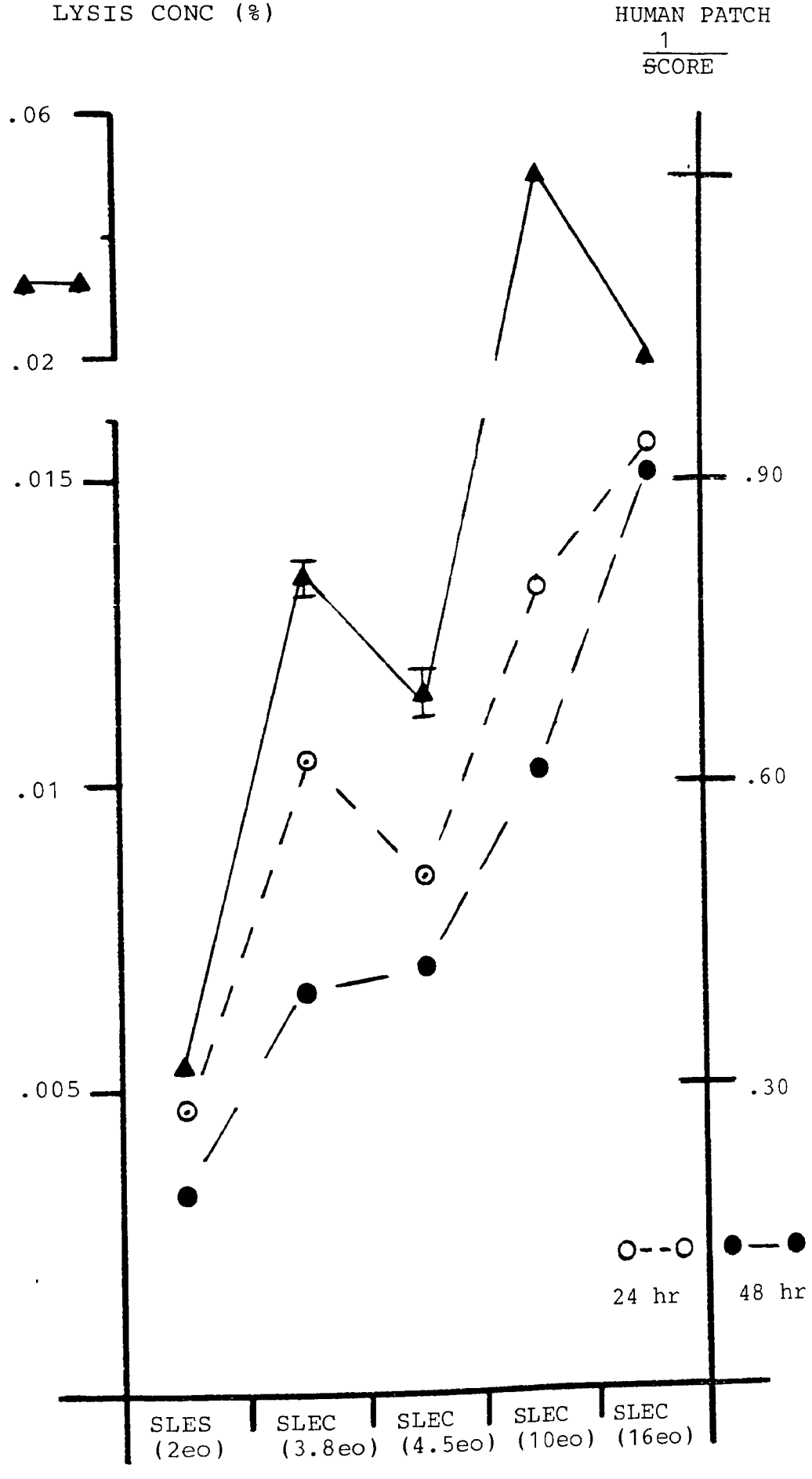
it can be seen there is a good correlation between the trends of the Draize scores and lysis concentrations. As the concentration of SLES (2E0) decreases in either mixture, the irritation score also decreases and the concentration of lysis increases. This is to be expected as SLES (2E0) is far more irritant than SLEC (10E0) or SLEC (16E0) as shown in Figures 38 and 39.

There is a discrepancy in the data produced. From the irritation scores the mixture containing SLEC (16E0) should be less irritant and therefore give a higher concentration of lysis when compared with the mixture containing SLEC (10E0). However, this was not the case suggesting a peak lysis concentration value for the SLEC's. This seems to occur whether SLEC is used in the pure form or in a mixture.

Different salts of lauryl sulphate were also evaluated. The salts gave very similar lysis concentration values as shown in Table 8. The literature suggests magnesium to be far milder than the sodium and triethanolamine salts (134). However, it must be noted that since the surfactant solutions are applied in saline, it would theoretically be difficult to find differences

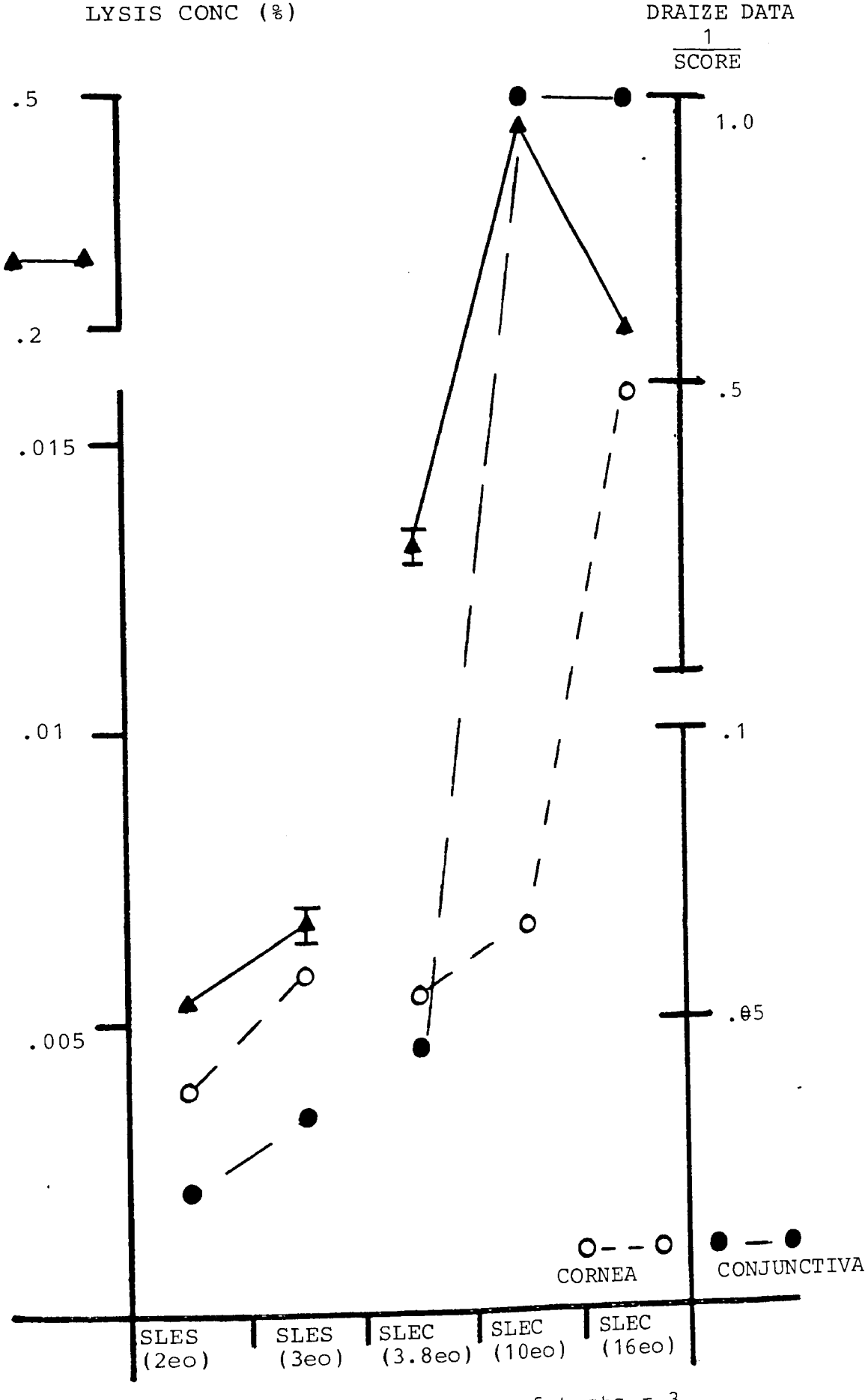
between the various salts especially at low concentrations.

FIGURE 38: SURFACTANT LYSIS CONCENTRATION FOR A SERIES OF SLEC's COMPARED WITH HUMAN PATCH TEST DATA



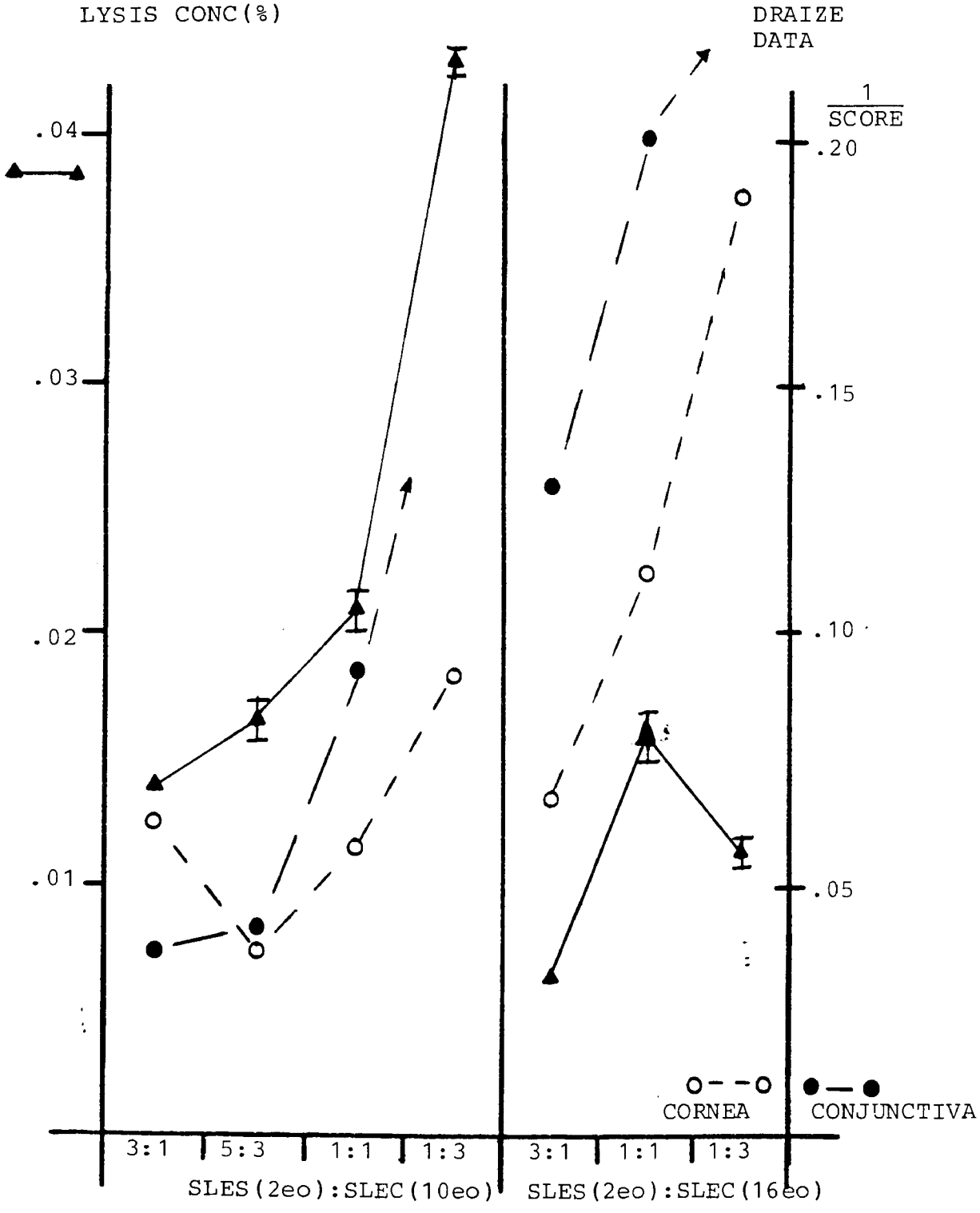
number of tests = 3

FIGURE 39: SURFACTANT LYSIS CONCENTRATION FOR VARIOUS SLES's AND SLEC's COMPARED WITH DRAIZE DATA



number of tests = 3

FIGURE 40: SURFACTANT LYSIS CONCENTRATION FOR A MIXTURE OF SLES's AND SLEC's COMPARED WITH DRAIZE DATA



number of tests = 3

TABLE 8: **LYSIS CONCENTRATION FOR VARIOUS SALTS OF LAURYL SULPHATE**

LAURYL SULPHATE	LYSIS CONCENTRATION (wt/wt %)
SODIUM	0.0043
MAGNESIUM	0.0048
TRIETHANOLAMINE	0.0056

11.5.CONCLUSION

A new in vitro method has been developed to measure the irritation potential of surfactants. The technique involves the measurement of the concentration of surfactant required to elicit blood cell haemolysis. The results suggest the higher the concentration of surfactant required to induce lysis, the lower will be the irritation produced as asssessed by human patch and Draize eye tests.

The main advantage with this method is that it is cheap and easy to use. The results produced so far suggest the lysis method may be used to evaluate the irritation potential of surfactants with alternative number of moles of ethylene oxide. The lysis method may also be used to measure the effect of mixtures of surfactants.

Due to the nature of the method, it is difficult to measure the variation in the hydrophilic group of the simple lauryl sulphate surfactant. Further work is still required to assess the effect of different types of surfactants and also the importance of the length of alkyl chain.

CHAPTER 12.0: SUMMARY AND FUTURE WORK.

The overall aim of this project was to develop laboratory methods for assessing the efficacy of topical products. These studies were to be used for supporting other aspects in the Johnson Wax skin care product development programme.

The first stage involved investigating the purely epidermal effects of topically applied products. Owing to previous experience and work at Johnson Wax, the research effort was initially concentrated on the development of alternating current impedance methods for monitoring the moisturisation of various therapeutic treatments. During the first stage the equivalent circuit appropriate for modelling the skin was elucidated and those components of the skin impedance that could be measured were established.

Monitoring of baseline untreated skin showed wide inter-and intra-subject variabilities and the application of products using the previously used electrode configuration resulted in the

short-circuiting of the electrodes.
Wide day to day variability was also observed.

To study the sub-stratum corneum effects of topically applied agents, the vasodilator response was examined. Nicotinate esters were chosen as model active compounds and laser Doppler flowmetry used to monitor the response. If such a response could be precisely monitored, then the effect of moisturisers on the skin could perhaps be evaluated by assessing their indirect effects on the permeability of marker compounds such as the alkyl nicotinates. An increase in hydration would be expected to alter drug permeabilities.

In order to apply such an indirect method it was essential to evaluate the performance of laser Doppler flowmetry in assessing nicotinate induced vasodilation. Results reported here indicate the time taken for the nicotinates to elicit a response by the flowmeter is the most reproducible parameter. The importance of the physicochemical

properties of the products is emphasized.

Further work is still required to understand the mechanisms and routes of penetration through the skin. However the laser Doppler method may be regarded as an objective in vivo technique for monitoring percutaneous absorption of compounds which exert a vasodilator response.

The final technique examined here is for assessing the irritation potential of surfactants. The results suggest the concentration of surfactant required to induce red blood cell haemolysis is closely related to the irritation produced as assessed by human patch and Draize eye tests. This technique is simple and easy to perform. However it requires further evaluation for a range of surfactants and surfactant mixtures in order to establish its full potential.

PLATE 1: GENERALIZED SECTION THROUGH SKIN

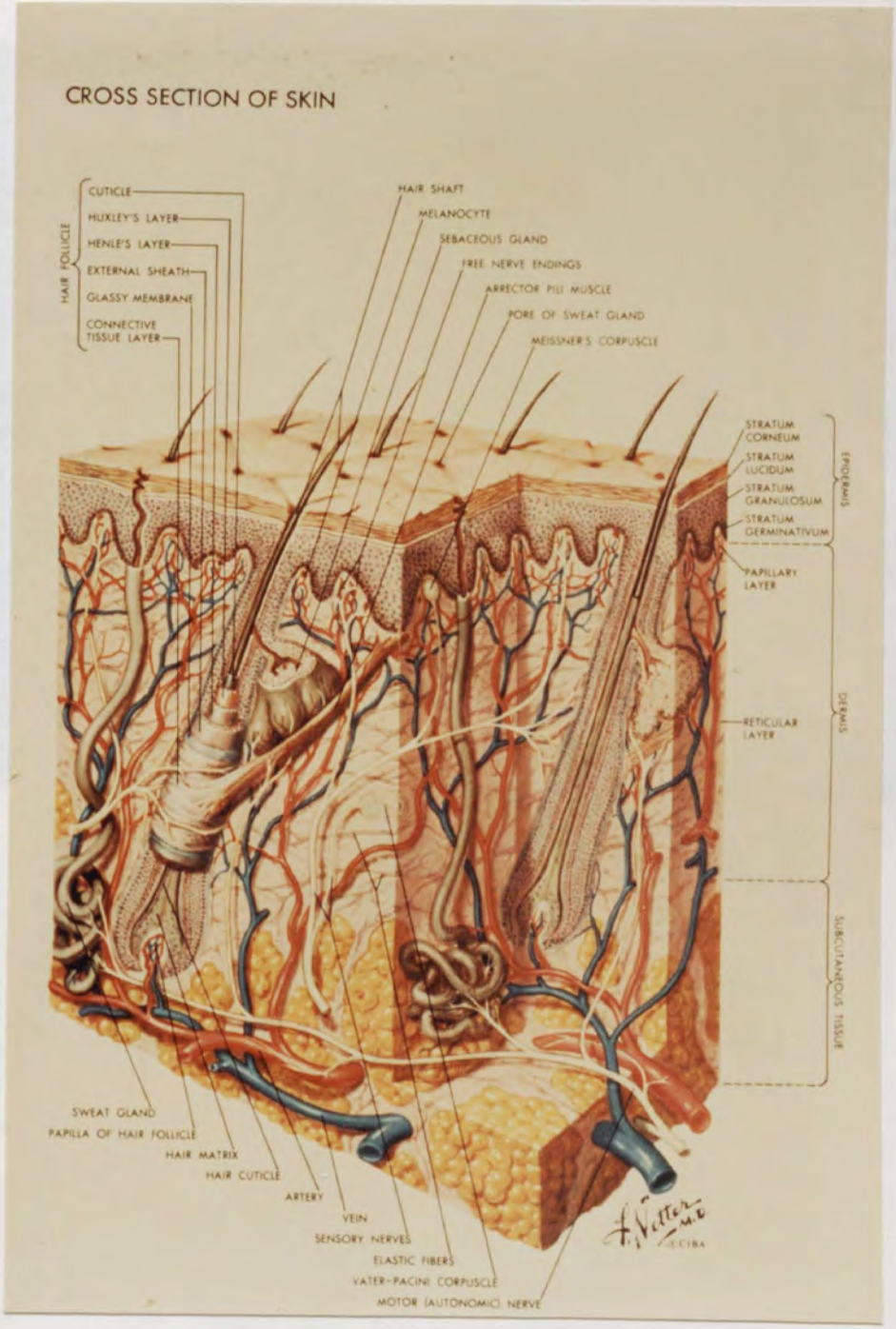


PLATE 2: SKIN IMPEDANCE EQUIPMENT AND MEASUREMENT

PLATE 3: ELECTRODES USED FOR SKIN IMPEDANCE MEASUREMENTS

PLATE 4: LASER DOPPLER EQUIPMENT AND MEASUREMENT

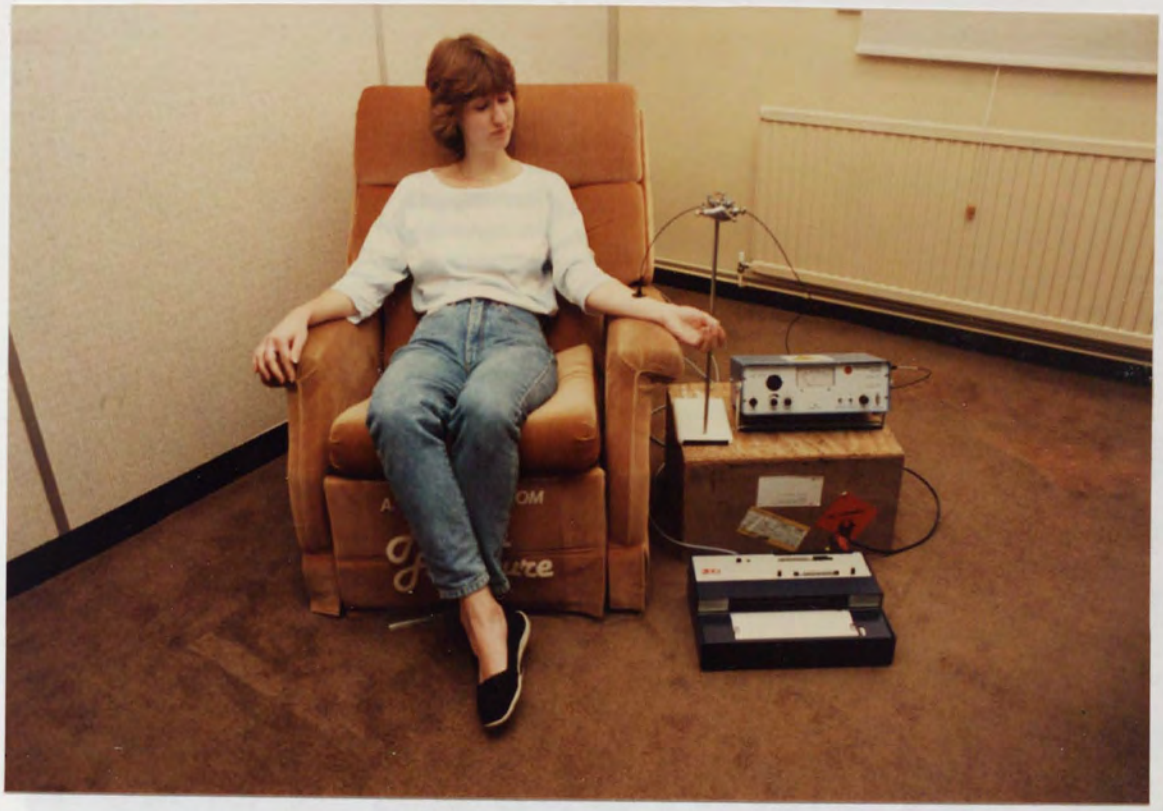


PLATE 5: PROBE HOLDER USED FOR LASER DOPPLER MEASUREMENTS

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