TECHNIQUES IN THE INVESTIGATION OF ACNE

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There are several major features to be considered in the pathogenesis of acne and these are:

1) An increased rate of sebum excretion.
2) Obstruction of certain of the pilosebaceous ducts with highly keratinized protein.
3) Abnormal microbial physiology on the skin surface and in the pilosebaceous duct.
4) Factors that are responsible for the mediation of inflammation.

It is impossible to discuss all relevant techniques and so I will limit myself to techniques for measuring (1) sebum excretion, (2) sebaceous gland metabolism which is related to sebum excretion, and (3) techniques for sampling bacteria. In addition I will describe a method for measuring surface lipid free fatty acids, a technique which gives an indirect measurement of bacterial function. A summary as to the availability of the materials is at the end of the paper, as is a comment on clinical trials.

1. SEBUM PRODUCTION

Sebum excretion rate and acne are significantly correlated (1). The indications for measuring sebum excretion rate are two-fold. To investigate sebaceous gland physiology and secondly to screen anti-androgens as there is evidence that drugs which significantly reduce sebum excretion will help acne. The two most widely used techniques for measuring sebum excretion rate are the gravimetric technique (2, 3) and the light transmission technique of Schaefer (3, 4).

a) Gravimetric method. In this technique an area on the forehead is delineated with elastoplast, over this is then placed six sheets of absorbent paper (Fig. 1) held in position with gauze and an elasticated bandage fixed at the back of the head with nylon mesh. After ten minutes the paper in contact with the skin is removed. On holding this up to light one will see a mass of grease; this paper is discarded and the procedure is repeated every 10 minutes for three or four occasions. After 30—40 minutes it can be seen that the grease pattern on the paper has changed to a spotty pattern, each spot representing sebum emerging from small groups of pilosebaceous ducts. When this so-called follicular pattern has been achieved a new set of cleaned absorbent papers are placed on the forehead for three hours. The time of collection is noted accurately. The lipid is extracted from the paper with an organic solvent such as ether; equipment such as a rotary evaporator is then used to evaporate the sample to dryness. The sample is weighed in a previously weighed tared glass flask. Thus by knowing the weight, the time and the area, the rate of sebum excretion can be determined.

The problem with this technique is that attention to detail must be given otherwise errors easily creep into the results. One such problem is that micro-condensation can affect the results, so we ensure that all relevant equipment is dried by using phosphorus pentoxide as a dessicant.

b) Transmission technique. In this technique the principle depends upon the fact that when fat is placed upon an opaque glass surface there is an increase in the amount of light which will pass through it. For this technique ground glass samplers, the so-called platelets, 1.5 × 1 cm., are required as is an instrument such as a spectrophotometer, which will read

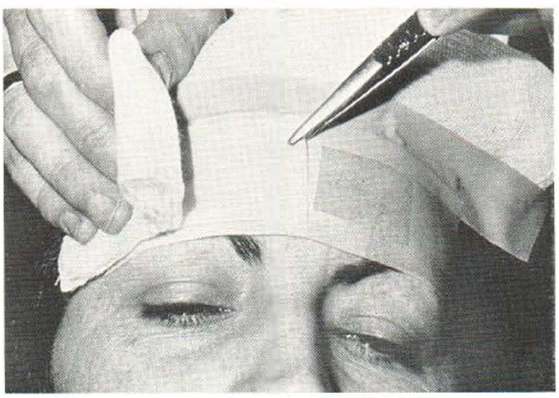

Fig. 1. Shows the absorbant papers being placed in position.

changes in light transmission. A follicular pattern is prepared as in the absorbent paper technique. The clean platelets are applied to the forehead for 20 seconds with firm pressure using double-sided Sellotape® (Fig. 2a-b). The platelets are then placed in the spectrophotometer and the amount of light which passes through is read at 460 nm. The patient is told that he must not touch the forehead until the end of the time period, usually 3 hours. After 3 hours the platelet is then re-applied to the forehead and reread in the spectrophotometer. The difference in light transmitted represents the amount of lipid collected and the absolute value of sebum collected can be determined by using a callibration curve of change in light transmission against weight of sebum. The problem with this technique again revolves around attention to detail. Inadequate pressure will produce a low sebum collection. It is absolutely necessary that the platelets not be touched and for this reason they are handled with double-sided Sellotape® . It is also easy not to correctly align the glass platelet in the spectrophotometer; this would produce a false result. We have had to adapt our spectrophotometer so that the platelet is precisely at 90° to the light beam.

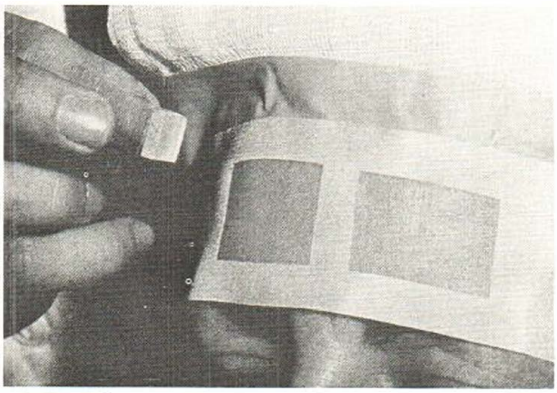

Fig. 2a. Shows the absorbant glass about to be applied to the forehead using double-sided Sellotape.

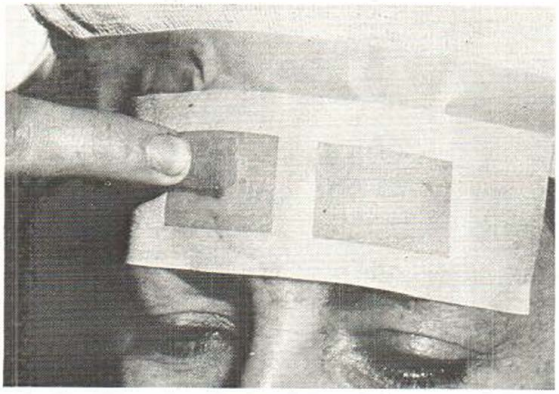

Fig. 2b. Shows the absorbant glass being pressed firmly onto the forehead.

Physiological variables affecting sebum production. With either technique certain physiological and pathological variables are known to affect the results and these must be remembered, particularly when investigating anti-androgens. It is known that there is a diurnal variation in the amount of surface lipid collected (Fig. 3) (5). The external temperature and the patient's body temperature will also modify the amount of lipid collected by either technique (Fig. 4) (6, 7). If the

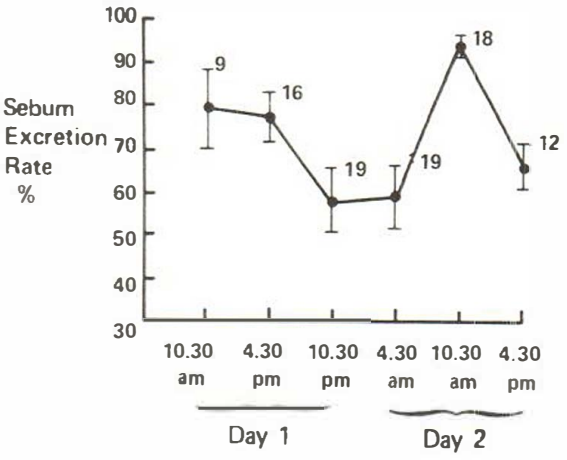

Fig. 3. Shows the diurnal variation of sebum excretion rate.

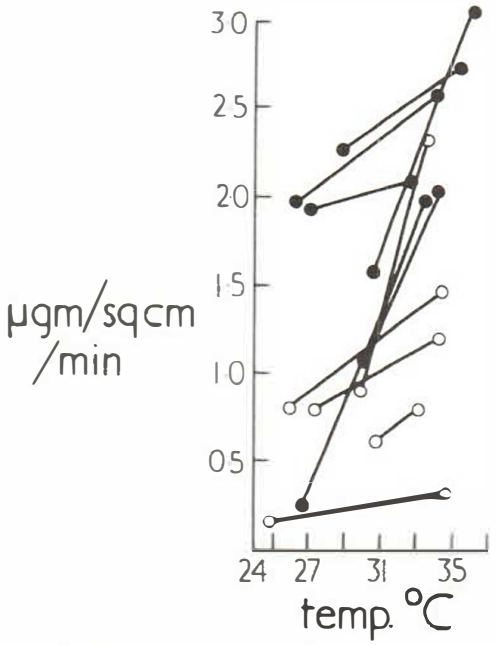

Fig. 4. Shows the variation of sebum excretion rate with skin surface temperature.

patient is sweating excessively, this certainly will reduce the amount of lipid picked up in the gravimetric technique. An individual with a somewhat bumpy skin due to severe acne will have pockets of lipid which, by either technique, will not be picked up because of the surface irregularities; this will again produce an erroneous result.

Comparison of the two techniques. Several authors have shown that the gravimetric technique is reproducible both on the same patient on different days and from the right to the left side of the same individual (2, 3). Schaefer has shown that the transmission technique is also reproducible. He has also shown that it compares satisfactorily with the paper technique.

A right to left side comparison of the paper technique shows a good correlation (Fig. 5). A right to left side comparison of the platelet technique also shows a good correlation ($r = 0.81$) (Fig. 6). In our laboratory we have also compared the two techniques and this shows a significant correlation ($r = 0.75$) (Fig. 7). Thus, both techniques are reproducible. Our advice to anyone interested in measuring sebum excretion rate is to choose either of the two techniques and prove to their own satisfaction that the technique is reproducible in their own department. The choice will depend on the availability of pre-existing techniques and equipment. It is possible that the transmission technique is less satisfactory at high levels of sebum production (7).

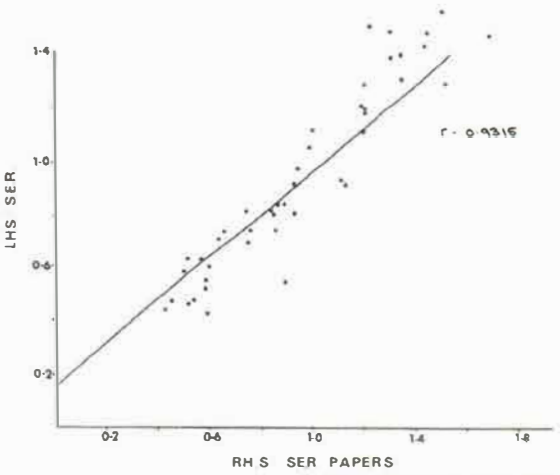

Fig. 5. Shows the correlation between the sebum excretion rate on both sides of the forehead as collected by the absorbant paper technique.

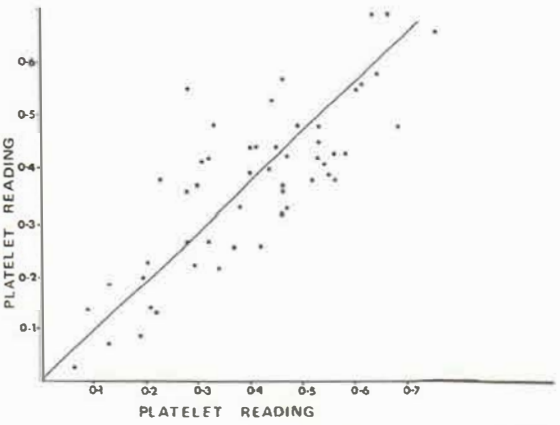

Fig. 6. Shows the correlation between sebum excretion measured on both sides of the forehead using the light transmission technique.

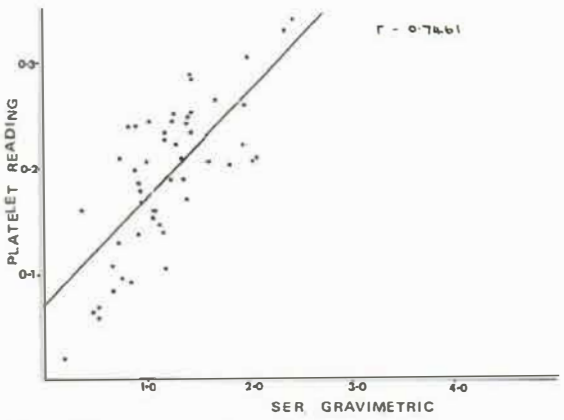

Fig. 7. Shows the correlation between excretion rate of sebum collected on the forehead; from one side the sebum was collected by the transmission technique and from the other side by the ahsorbant paper technique.

2. RADIOACTIVE INCORPORATION STUDIES

Another technique for assessing sebaceous gland production is to measure the incorporation of radioactive precursors such as a glucose or acetate into skin biopsies. Before discussing this aspect, two additional points should be brought up. To develop techniques one often requires adequate supplies of skin and patients do not give skin away readily, thus we now collaborate with a local hair transplant clinic. Admittedly the skin is not completely normal but our radioactive studies have shown that this skin is not vastly different from adjacent forehead skin as far as sebaceous gland biosynthesis is concerned. Each week we can obtain up to 100 × 4.5 mm biopsies. The second point is that it may be necessary to isolate the pilosebaceous units from the dermis and this technique merits additional discussion.

(a) Isolation of sebaceous glands. In 1966 a technique

was developed which allows the separation of the epidermis from the sebaceous glands and these in turn from the dermis (8). In this technique, chemically pre-treated skin facilitates micro-dissection of the structures. Several methods of chemical pre-treatment can be used, we prefer 1 molar calcium chloride; the biopsy being left for 2 hours in this solution at room temperature. After 2 hours the biopsy is removed and under dissecting microscope observation, the epidermis, using dissecting forceps can be peeled off the dermis; this epidermal fraction also contains the upper pilo-sebaceous ducts (Fig. 8). I stress the word 'upper' as it will be shown in the next section that the technique described by Kellum, only removes 50% of the pilosebaceous units — 50% being left in the dermis (9). Using micro-dissection scissors and forceps with practice and patience one can separate the sebaceous glands, the pilosebaceous ducts and the epidermis from each other.

The trick for separating the deeper sebaceous glands from the dermis is simple (10). The dermis is squeezed with micro-dissection forceps and out pop the many deeper sebaceous glands. By now, these deeper sebaceous glands are somewhat macerated but quite satisfactory for biochemical studies. The practical point to stress is that three factors are necessary for the isolation of these individual structures. A good dissecting microscope, perfect micro-dissecting equipment and practice. Kellum's technique can also be performed on animal skin but we find human skin more easy to manage; perhaps this is related to our lack of practice with animal skin. I now wish to return to radioactive incorporation studies.

For several years people have investigated sebaceous gland lipid metabolism. We are interested in this also but we are extending our studies to protein biosynthetic pathways. For these radioactive metabolic investigations the biopsy is transferred from the patient with the minimum of delay into the incubation fluid which is normally glucose-enriched Krebs Ringer bicarbonate buffer containing either 2 mM (u ^{14}C) glucose or acetate. The incubation is carried out for 3 hours at 37°C. The buffer contains antibiotics and is purged with 95% oxygen and 5% CO_2. The sample is then processed by the modified micro-dissection technique. Each anatomical fraction is homogenized in micro-grinders and each fraction is then assayed for whatever the researcher is investigating. One can determine the radioactive incorporation into each anatomical part and we can determine the incorporation into the various lipid classes (10). Sebum is a complex mixture of lipids. It contains lipids, cholesterol, cholesterol esters, wax esters, triglycerides, free fatty acids and squalene. It is known that sebum contains predominantly triglycerides, squalene and wax esters and that lipid derived predominantly from epidermal structures contains less squalene and wax esters but more cholesterol and cholesterol esters. We have shown that the "squeezed sebaceous gland", removed by the modified micro-dissection technique are definitely sebaceous in origin, and that the squeeze technique doubles the amount of radioactive label in the sebaceous fraction (10). Such metabolic investigations allow us to investigate the controlling effect of physiological factors such as hormones on sebaceous gland function and the technique can also be used in the screening of anti-androgens. These metabolic studies can also be used in *in vivo* studies by injecting the radioactive precursor before biopsy. This can allow the investigator to conduct time course studies, but injection of radioactive labelled precursors also raises ethical problems.

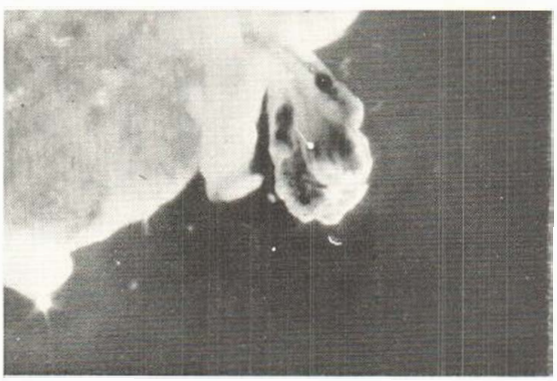

Fig. 8. Shows an isolated sebaceous gland attached to the epidermis.

3. TECHNIQUES FOR INVESTIGATING BACTERIA

Although the numbers of bacteria do not necessarily explain acne nor its severity, bacteria, especially *Propionibacterium acnes*, and to a lesser extent *Staphylococcus epidermidis*, play a role in the aetiology of acne. The aerobic *S. epidermidis* is easy to grow but not many laboratories can grow the anaerobic *P. acnes* easily without experience. Several media, such as reinforced clostridium media and heated blood agar can be used to grow these bacteria. Table 1 summarizes relevant details. Three types of sampling techniques can be used.

Table 1.

	P. Acnes	S. Epidermidis
Media	Reinforced clostridial media (Oxoid)	7% v/v heated horse blood agar (Oxoid)
Time	7 days	2 days
Oxygen requirement	Anaerobic	Aerobic

a) Routine clinical swabbing. An ordinary swab can be used in clinical practice. Occasionally we see patients with acne not responding to treatment. Microbiologically there are two reasons, firstly, they may have developed a gram negative folliculitis (11) and secondly, one occasionally isolates *P. acnes* resistant to the commonly used antibiotics. Thus, taking an ordinary swab soaked in the appropriate culture fluid and sent directly to a microbiologist who is aware of the clinical problem can help to resolve a difficult clinical problem.

b) "Scrub" technique. For most, if not all, assessments of new and established treatments in clinical trials the surface scrubbing technique is valuable (12). In this technique a cylinder of metal is placed on the skin into which is pipetted 1 ml of Triton X100. The lesion is then scrubbed firmly with a metal or plastic hockey-stick-shaped object for one minute (Fig. 9). The fluid is then removed and the procedure repeated with a further 1 ml of fluid. The two 1 ml aliquots are then combined and inoculated onto the appropriate medium. This technique samples both the epidermal surface and the upper pilosebaceous duct.

c) Pilosebaceous duct sampling. It is possible to sample bacteria from the pilosebaceous duct by three techniques. In one technique (13) a cyanoacrylate gel is placed on the skin within the confines of a small Teflon ring. Onto this gel is then placed a 'T'-shaped glass structure which fits accurately into the ring (Fig. 10), the glass sampler is pressed firmly for one minute. The sampler is removed and it removes with it the upper part of the stratum corneum and the upper part of the pilosebaceous duct structure. The sample is then spun with number 9 Ballotini beads at 2.000 revolutions per minute in a Universal bottle containing 10 ml of the appropriate culture fluid; the sample is then plated out. Control studies have shown that there is no surface contamination with this technique but it does not sample from all pilosebaceous units within the given bit of skin. Repeated samples can be taken from the same site but the technique does not sample the depths of all the pilosebaceous ducts.

At this same symposium Leyden has described a further modification of this technique. It is based on the principle that cyanoacrylate gel removes part of the ductal contents including the keratin and bacteria. He micro-dissects the keratin under a microscope and samples the bacteria from individual ducts. However, as in the technique just described, not all of the ductal contents is removed.

The third technique for estimating number of bacteria in the pilosebaceous duct is the sebaceous gland isolation technique as described earlier. It has been shown that calcium chloride has a minimum effect in reducing the number of viable bacteria (14). The ad-

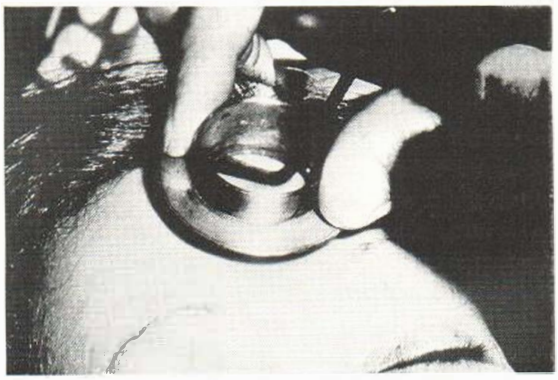

Fig. 9. Shows the surface of the skin being rubbed with the hockey-stick shaped metal.

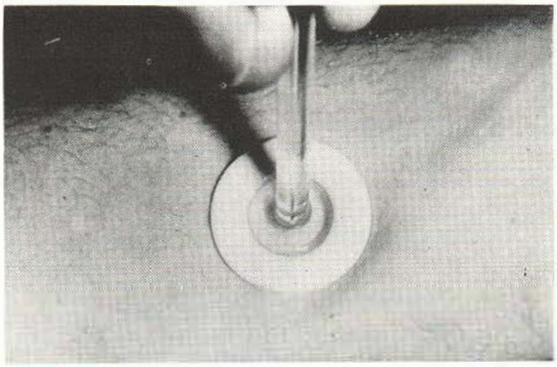

Fig. 10. Shows the follicular sampling technique of Holland & Cunliffe in which the glass sampler is pressed firmly onto the skin within the confines of a Teflon ring.

vantages and disadvantages of these three follicular sampling techniques are summarized in Table 2.

d) In vitro techniques. In vitro techniques can help in the understanding of the role of bacteria in acne and possibly in the development of more active antimicrobial therapy. Such a relevant technique involves the use of a chemostat, which allows the growth of bacteria under a controlled environment. One can control the oxygen, the pH, the carbon dioxide and the supply of nutrients to the bacteria and thus by sampling under controlled environments at different times of the culture one can obtain information on the characteristics of the organisms, their growth rate, enzyme production and antigen production (Fig. 11).

Table 2. *Advantages and disadvantages of follicular sampling techniques*

1	Cyanoacrylate gel technique. (Holland et al.)	Non-invasive	Samples only part of the ducts.
2	Cyanoacrylate gel technique. (Leyden et al.)	Non-invasive	Samples only part of the ducts.
3	Sebaceous gland isolation technique. (Puhvel et al.)	Requires biopsy	Samples all of the duct.

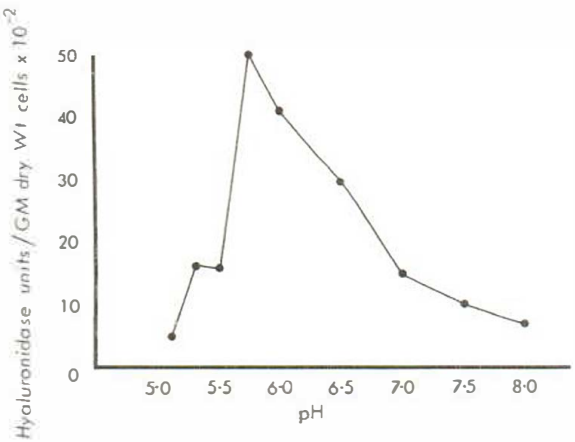

Fig. 11. Shows the relationship between enzyme production (hyaluronidase) with pH of the culture media; the bacteria being grown in a chemostat.

4. ASSESSMENT OF SURFACE FREE FATTY ACIDS (SFFA)

Other authors in this book have discussed the lessening importance of FFA in the aetiology of acne, but measurement of surface FFA is an important laboratory tool in assessing the efficiency of antimicrobial preparations in acne. *P. acnes* produce lipases, which hydrolyze sebaceous triglycerides to FFA. Thus, drugs which reduce or modify the *P. acne* population will reduce SFFA and SFFA are easier to quantitate than are surface bacteria. To measure SFFA the forehead is wiped in a constant way such as firmly three times with a polyurethane sponge soaked in ether. The subject is told not to touch the sampling area. One hour later the area is re-wiped; the sample is filtered through fibre glass, evaporated to dryness and then weight determined. The sample is then reconstituted in 5 ml of hexane and the FFA are extracted with Dole's fluid (15). The FFA are then titrated against N/200 tetra-n-butyl ammonium hydroxide using thymol blue as the indicator. The concentration of FFA is determined by use of an appropriate calibration curve.

5. CLINICAL TRIALS

Clinical trials were not the major part of my presentation, but I consider it necessary to summarize a few aspects of such trials.

In clinical trials the same physician must assess the same patient in a constant way with a good diffuse light, such as a cold fluorescent lamp. Whatever the technique, the physician must show that it is reproducible both between himself on the same day and between the different observers on the same patient on the same day. It is useful to perform two control visits, usually on adjacent days. Thereafter the patient should visit at monthly intervals whilst on treatment because of the known pre-menstrual flare of acne in females. It is also useful to assess the patient at the same time of the day as with men, for example, the state of the beard can markedly affect the picture. We all have our favourite ideas of how to grade the acne. It is a pity that in 1978 — although we know how to measure blood pressure, pulse rate and so forth, but on either side of the Atlantic there is no consistency in the way in which we grade our patients in clinical trials. However, the following variables are worth mentioning. The overall grade. This gives an overall assessment of the patient's severity of acne. It is a technique performed in Leeds and is reproducible. This system is also useful for the practising physician to develop so that he or she can quantify improvement or deterioration of the patients' acne as they come to his clinic. Initially the physician may develop a scor-

ing scale say from 0 (zero) to 5 (five), but with increasing experience we find that many physicians can extend this from 0—10.

The second type of grading involves counting individual lesions such as non-inflamed lesions (whiteheads and blackheads) and inflamed lesions. The latter can be divided into superficial lesions such as papules and superficial pustules and the deeper nodules and pustules. Once a physician gets involved in clinical trials he then realizes the problems. For example, one must not count the prominent follicular lesions on the nose; nor is it worthwhile counting the many small blackheads around the chin as some patients have vast numbers of lesions at this site which makes it an impractical exercise. In addition some non-inflamed lesions are partly blackhead and partly whitehead, but as long as the physician is consistent and counts this type of lesion as either one or the other then this will reduce the error. Attempts have been made recently to quantify the lesions by giving each lesion a score, for example giving a small score to a blackhead or a whitehead, a larger score to a papule and an even larger score to a deep inflamed lesion. The value of this latter technique is still not proven.

The third type of assessment is photographic assessment. Success of this depends entirely on the presence of a professional photographer. The patient's assessment of his treatment is also important. As yet, I do not know the ideal way of a patient assessing his own acne, but at the end of the day this is what the patient is most interested in — his own impression. Currently we have been using a 20 cm visual analogue. Basically this consists of a horizontal 20 cm line divided into two equal parts by a vertical line at the centre. The middle point represents the patient's impression of his acne at the start of treatment. At each treatment visit the patient is asked to draw a vertical line across the horizontal line in an attempt to indicate to the clinician how much better or worse his spots are compared to the initial visit. A line to the right indicates improvement; to the left deterioration. The distance of this line from the middle is measured.

Whatever the clinical techniques, statistical techniques then enter the arena. I only wish to mention three points:

1) If the preparation really works well then statistics are hardly necessary!
2) Secondly, before assessing the data the frequency distribution of each set of data must be plotted as often it is not normally distributed but skewed.
3) We have shown from several studies, both clinical and involving sebum excretion rate studies, that patients with different types of acne and varying levels of sebum excretion rate may behave differently from other sub-groups of the same population. Thus, in any double-blind trial the patients should be placed into matched pairs before starting the treatment so that an equal number of patients with an equal type of condition enters each therapy group; this can save problems at a later date in the trial. Homogenous groups are better than heterogenous groups in statistical analysis and therein lies the problem with acne. It represents an interplay of several heterogenous but inter-related factors but such factors make the problem more interesting for the clinical investigator.

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DISCUSSION

Shuster, Newcastle-upon-Tyne: I want to question Dr. Cunliffe on a more technical matter. I just wonder why you have not improved the technique for collecting lipids you are using and have been using for about five years? You have a method which picks up a ratio of epidermal to sebaceous lipid of about 50%, whereas in the original technique, which is much simplier than the one you are using, we picked up somewheres between 90—95% of sebaceous gland lipids. Do you think there is really any advantage to your procedure?

Cunliffe, Leeds: We are in fact looking at somewhat different things. We are anxious to get one individual sebaceous follicle and one individual sebaceous duct, based on the principle that one pilosebaceousunit may not be quite like its neighbour in terms of what it is doing. So that is why we are using this technique.

Schaefer, Berlin: Dr. Cunliffe, I was interested in your method of isolating sebaceous glands with acetic acid or calcium chloride. But I am concerned that your studies on the metabolic turnover of these isolated sebaceous glands will be influenced by these strong agents. Lipid synthesis depends on an enzyme system which is situated within the cell and depends on the intact undisturbed cell. Did you control the isolation technique with biopsy examination to make sure that your sebaceous walls were intact?

Strauss, Iowa: I think I can answer that question. Dr. Cunliffe is doing the separations after he has run his incubation. So it is just a terminal separation. We have worked with the calcium chloride method and as soon as the tissue comes in contact with calcium chloride, all metabolic activity disappears.

Cunliffe, Leeds: Most of you will have realized over the course of the last 24 hours that free fatty acids have very little to do with the primary etiology of acne. But I think it would be wrong if you went away thinking that to analyze fatty acids is useless in clinical investigations of acne. If you are looking at a medication which is antimicrobial, and you want to assess this effect, you have two opportunities open to you. First, you count the number of bacteria, and second, you can use free fatty acid levels in skin surface lipids as an indirect marker for bacterial activity. The reason that this is worthwhile doing is based on the principle that the microorganisms produce lipases which act on sebaceous triglycerides to form fatty acids. Therefore if the number of bacteria is decreased there are fewer lipases and fewer fatty acids. The advantage of looking at this indirect measurement of fatty acids, is that it technically is much much easier than doing bacterial counts. I think most of you in this room could manage with a little experience and with very little equipment to measure surface lipid fatty acids. In contrast, the cuhuring of *P. acnes* properly acquires much more experience. The technique for collecting the skin lipids that we use is basically a modification of the one used by Dr. Strauss for many years. The skin surface is wiped with a polyurethane sponge soaked in hexane, being careful to avoid the hair. The patient is instructed not to touch the forehead for the next hour and at the end of one hour the forehead is rewiped in the same way with another polyurethane sponge. The sponge is extracted with hexane which is then evaporated down to dryness. The residue is weighed and then redissolved in a known quantity of hexane. Then fatty acids are extracted with Dole's extraction fluid. The fatty acids are titrated with an automatic buret. In summary, I think that we should not throw fatty acids out of court in terms of clinical assessment. If you are using a preparation which has antibacterial activity, the fatty acids can be used to assess whether the compound is active.