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Measurement of enzyme kinetics on the intact skin— a new method to study the biological effects of cosmetics on the epidermis

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Synopsis

A new DIRECT FLUOROMETRIC method allows the MEASUREMENT of ENZYME ACTIVITY on the INTACT SKIN of various body surfaces. This permits normal physiological parameters to operate on the system studied. The pentose phosphate pathway (Entner-Doudoroff), previously shown to operate in the epidermis, provides several enzymes which are used here to assess and compare various dermatological conditions. Glucose-6-phosphate dehydrogenase and lactic dehydrogenase are measured by fluorometric determination of changes in NADPH and NADH. Other enzymes of the Embden Myerhoff and Krebs cycles are measured directly or indirectly by this method. Various COSMETIC base ingredients and compounded formulations were studied to determine their EFFECTS on epidermal metabolism. Enzyme action was recorded as increased, decreased or not affected. This new method is simple and relatively inexpensive, and allows extremely wide applications.

INTRODUCTION

The need for new methods to study the biochemistry of the epidermis *in situ* is becoming more apparent. Methods involving extrapolation from animal models are not always applicable to the human epidermis because of species differences. Excised skin from human volunteers has certain inherent drawbacks including pain, disfigurement and the problem of an isolated specimen removed from its normal milieu. The use of an *in vivo* and *in situ* method would obviate many of these problems. This paper describes such a method.

The skin is the largest body organ, representing approximately one-sixth of the body weight. Far from being a mere barrier to the internal and external environment, the skin is proving to be a dynamic organ with a profound effect on the internal metabolism. A review of the extensive literature on the metabolic functions of the epidermis reveals carbohydrate metabolism to be unique (1–4). For this reason we chose epidermal carbohydrate metabolism as our biochemical system. Previous studies have demonstrated enzyme activity in histochemical sections (5, 6), cell homogenates (7) and epidermal stripping (8). These experiments have been both comprehensive and

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elegant with conclusive demonstration of enzyme activity in the upper layers of the epidermis. Recently Schalla *et al.* (9) demonstrated enzyme activity in the intact epidermis by perfusion of a glass chamber attached to the surface of the skin *in situ*. Their measurements were recorded as a change in absorbance of the perfusate in a flow-through spectrophotometer.

Early studies by Chance *et al.* (10) have shown that fluorescence changes can be detected in the intact organ and that these changes reflect a state of oxidation-reduction within the organ cells. This study and prior studies by Chance *et al.* (11) indicate that tissue irradiated at 366 μ emitted fluorescence characteristic of the reduced pyridine nucleotides. The preponderance of nucleotide fluorescence in the tissues precludes the measurement of other cellular fluorophores with similar fluorescence characteristics. It was further concluded that the assay of reduced pyridine nucleotides is insensitive to the state of oxygenation of hemoglobin and thus this system can be used to follow oxidation-reduction ratios in anoxic states. Based on these previous studies, we designed a system that would measure fluorescence changes of the pyridine nucleotides involved in carbohydrate metabolism in the epidermis.

METHODS AND MATERIALS

THE FLUOROMETER

Our system employs a sensitive fluorometer that measures the change in fluorescence of nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide phosphate (NADP) when they are in the reduced state, NADH or NADPH, respectively. Excitation is at 340 to 365 nm and emission is at 460 nm. In oxidized form (NAD or NAPD) they are not fluorescent. The emission intensity is directly proportional to the amount of reduced nucleotide. Measurements of changes in fluorescence, either increased or decreased, can be utilized with this system. Our instrument employed the electronic components of the Metabolite Fluorometer E704, designed and built at the Johnson Foundation of the University of Pennsylvania. Essentially, this instrument employs a GEF4T4 Germicidal UV lamp with a E062 socket. The ultraviolet light from this source is filtered through a Corning No. 5840 filter or Wratten 18A Kodak filter. The visible emitted light is detected by a photomultiplier tube (PMT) EM19524B (supplied by EMI Gencom Inc., 80 Express Street, Plainview, New York). Ultraviolet light is filtered out by inserting a UV filter 2B (Kodak) or a Corning No. 3389 filter at the face of the PMT. Power supply to the PMT is provided by a Model 6515A DC Power Supply from Hewlett Packard. The recorder is a Honeywell Electronik 19 with 100 mv sensitivity, usually set at a speed of 0.5 in./min. The amplifier and output circuit diagram for the E704 is available from Johnson Foundation, University of Pennsylvania, Philadelphia, Pennsylvania.

For our instrument, we designed the metabolite chamber and detection systems to allow direct measurement on the skin as follows (Figure 1). An aluminum block (D) was machined to allow insertion of the ultraviolet light source (E) and photomultiplier (F) with respective filters (G and H) at an angle of 22.5° from the perpendicular. The bottom of the block was machined to accommodate a closure mechanism (B) and a reaction chamber (A). The slide closure mechanism (C) and the reaction chamber are of stainless steel. By closing the reaction chamber, *in vitro* enzymatic reactions can be

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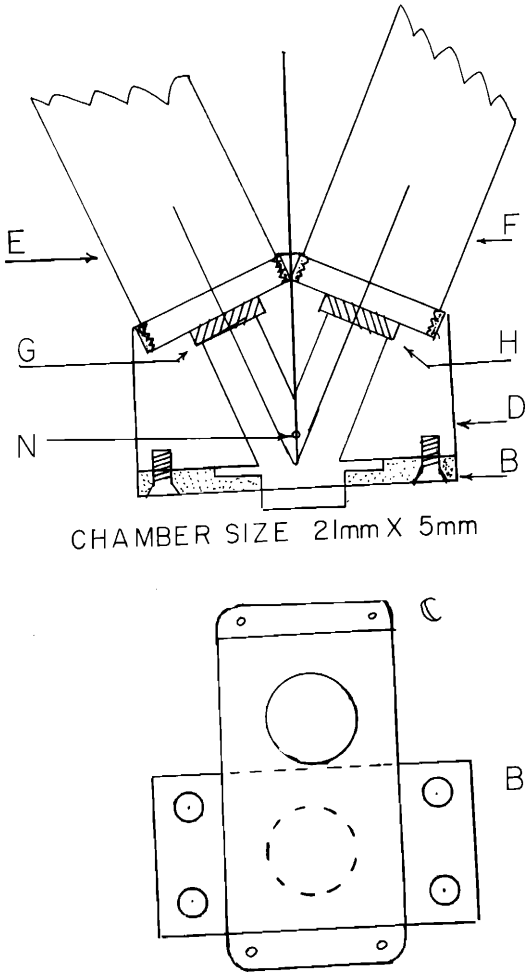

Figure 1. Design of instrument for direct measurement of enzymes on skin

conducted to calibrate the instrument. The closure also protects the PMT when the instrument is being moved or adjusted on the subject's skin. Provision to introduce reactants onto the skin is accomplished by holes (N) drilled at 22.5° through the block to accommodate 22-gauge needles.

All enzyme substrates and control enzymes used in this study were purchased from Sigma Chemical Company. Reagents were analytical grade.

CALIBRATION OF THE INSTRUMENT

The instrument was calibrated to give a full-scale deflection with 1 millimicromole (μM) of NADPH when added in 0.1-ml volume to 2 ml of reaction media. After the instrument is allowed to warm up, 2 ml of distilled water is added to the reaction chamber and the base line is allowed to stabilize. NADPH is then added and adjustments made to allow maximum sensitivity of 1 μM for a full-scale deflection. This is

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Table I
Concentration of Reactants

Enzyme	Reactants
1. Glucose-6-Phosphate Dehydrogenase	5.8×10^{-3}M glucose-6-phosphate in 0.063M, Tris $MgCl_2$ 0.1M pH 7.4, NADP 1 mg/5 ml
2. Isocitric Dehydrogenase	Isocitrate 3×10^{-4}M Manganese Cl_2 0.1MMnCl_2 in NACl 0.15 per cent, Tris buffer 0.063M pH 7.5, NADP 1 mg/ml
3. Lactic Dehydrogenase	Pyruvate Na 2×10^{-5}M, Phosphate buffer 0.05M pH 8.5, $NADH_2$ 0.5 mg/ml
4. Uridine 5-diphospho-glucose dehydrogenase	UDPG 6.28×10^{-5}M Phosphate buffer 0.05M pH 7.0, NAD 1 mg/ml

generally a much greater sensitivity than needed for most measurements. We found many kinetic measurements could be made at a sensitivity of 20 to 60 μM full scale.

After sensitivity is established, the chamber is thoroughly cleansed with distilled water. The instrument is then ready for an *in vitro* assessment of the conditions necessary. In the case of glucose-6-phosphate dehydrogenase, we used the following concentrations and quantities: glucose-6-phosphate, 5.8×10^{-3}M, in tris buffer with $MgCl_2$ at pH 7.4; glucose-6-phosphate dehydrogenase from Bakers Yeast (Sigma #G-6378), 0.05 unit in distilled water; and NADP, 0.04 mg/ml. The coenzyme, NADP, is added incrementally by 0.1-ml aliquots (0.004 mg) until a maximum velocity (Vmax) is obtained. (The absorption of this reaction mixture without added enzyme is 0.01). Measurements of skin reactions are taken as rates rather than Vmax and the yeast enzyme is not used as an internal standard. Measurements are made at 1-min intervals for a total of 5 to 20 min.

The instrument is calibrated each time a new enzyme system is studied. Before the *in vivo* measurements are made, the chamber is thoroughly cleansed and an additional aliquot of substrate and cofactor is added to ensure that no residual enzyme remains

Table II
Summary of Enzyme Activity*

Subject	Sex	Age	G-6-PD	LDH**	ICD	UGDPD
1. L.M.	F	20	2.0	0.5		0.5
2. M.R.	F	20	1.6	8.0		
3. M.H.	F	20	0.9	0.1	2.7	3.0
4. G.G.	F	20	1.0	0.7		
5. J.P.	F	45	2.9	3.5	1.5	2.0
6. T.A.	M	31	1.0	3.2		
7. S.S.	F	41	2.1	7.1		
8. P.P.	M	49	2.4	5.0	0.5	
9. M.S.	M	36	1.9	2.7		
10. S.G.	F	31	1.5	2.5		
11. B.B.	M	22	1.2	3.0		3.4
12. J.C.	F	37	1.9	1.4		

*Calculated in μM/min cm^2.
**Random group of people studied without regard to fasting or nonfasting states. Note marked variations in LDH activity.
Values represent an average of three determinations on the dorsal surface of the forearm. Reproducibility $\pm 0.4\mu$M/min/cm^2.

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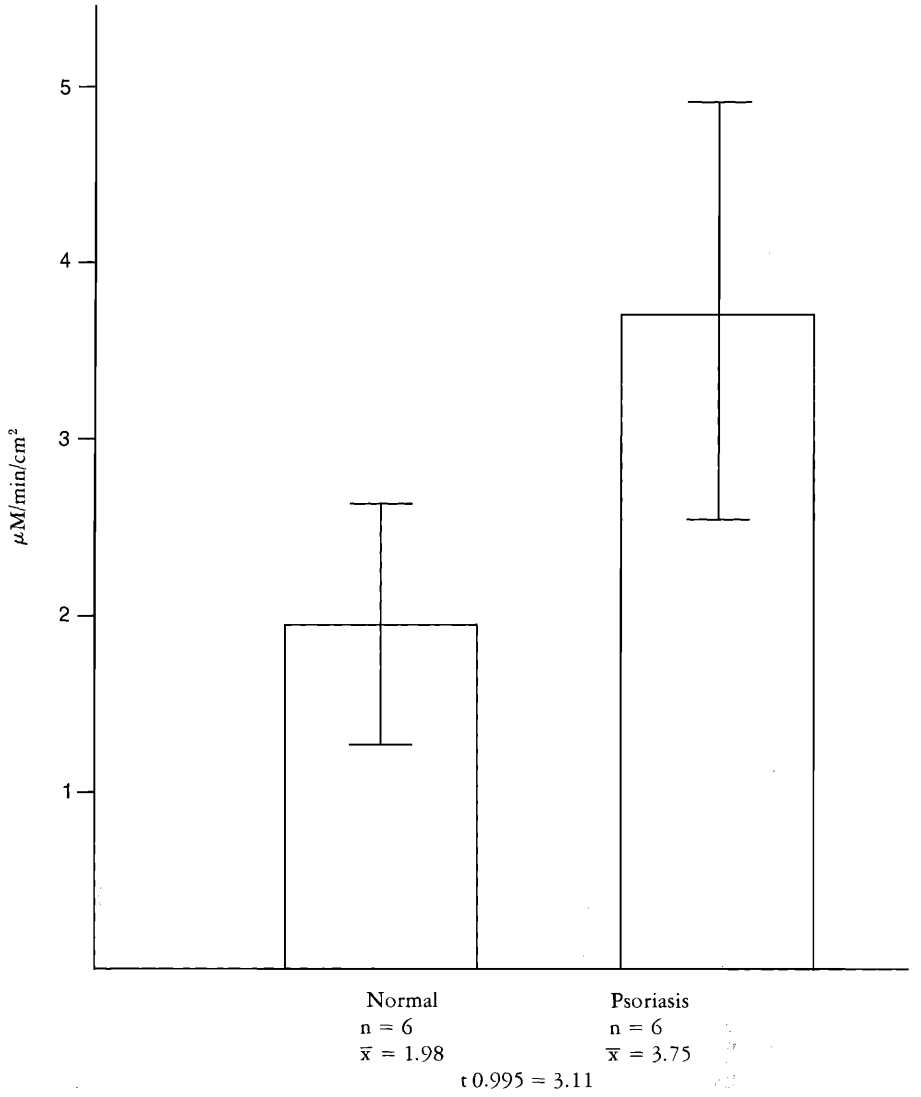

Figure 2. Epidermal glucose-6-phosphate activity in normal subjects and subjects with active psoriasis

within the chamber. The system, after cleansing again with distilled water, is ready for *in vivo* measurements. Enzyme activity was calculated in μM/min/cm$_2$ (millimicromoles per minute per square centimeter of skin surface). This is equivalent to mU/min/cm^2 (miliunits per minute per square centimeter) which is the method used by Schalla *et al.* (9). The concentration of the reactants used in these experiments is given in Table I.

The results of the glucose-6-phosphate dehydrogenase activity measurement on six normal subjects and six subjects with psoriasis are presented in Figure 2. Table II summarizes the activity of the enzymes measured on 12 normal subjects. Activities are recorded for the dorsal surface of the forearm as considerable variation is noted in enzyme activity from different areas of the body (see Table III). We were unable to

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Table III
Comparison of G-6-PD Activity on Various Parts of the Body

Site	G-6-PD Activity*	
	Normal	Psoriasis
Arm: palmar surface	2.2	3.9
Arm: dorsal surface	1.5	3.5
Calf	0.6	1.0
Thigh	—	—
Lower back	—	—

*Averages calculated in μM NAPDH/3min/cm^2.
Six normal subjects.
Six subjects with psoriasis.
Average of 3 measurements per area, per patient. Reproducibility ± 0.6 μM.

get consistent measurements of isocitric dehydrogenase in normal subjects and not at all in individuals with active psoriasis.

STUDIES WITH COSMETIC RAW MATERIALS

Using the values obtained in Table II as a base line, we studied the effects of certain common cosmetic ingredients on the enzyme activity of the epidermis. The dorsal surface of the forearm was used throughout the study. The test material was placed on the test site after a base line enzyme activity was obtained; care was exercised to place the material in a preselected area of 3.8 cm^2. Subject had been instructed not to wash arm for 24 hr prior to the test. Material was placed on the test site with a microliter syringe delivering 10 μl, followed by gentle rubbing into the epidermis. Repeat enzyme determinations were made 4 hr later. Anoxic states were induced by applying a standard 1-in.-wide rubber tourniquet for 3 min above the elbow. Table IV summarizes the results reported as an increase or decrease of enzyme activity over the control rate.

DISCUSSION OF RESULTS

There is an immediate question of what is really being measured in these studies. It is believed by the author that we are actually measuring the oxidation-reduction state of

Table IV
Effect of Various Cosmetic Agents on the Reaction of Two Enzymes in Normal Subjects*

Agent	G-6-PD	G-6-PD Anoxic state	LDH	LDH Anoxic state
Mineral oil (70 visc.)	−80	No effect	−>80	No effect
Lanolin alcohol	−10 to −20	+50	+50	+>80
Isopropyl palmitate	−10 to −20	+50	+50	+>80
**Bath oil	−10 to −20	+50	+50	+>80
Lactic acid/sodium lactate	−90	No effect	+>100	+>100
***Sodium lauryl ether sulfate	−>100	Inhibited	Not studied	Not studied

*Activity expressed as % increase (+) or decrease (−) over control rate.
**Experimental bath oil containing no isopropyl myristate, isopropyl palmitate, or mineral oil.
***The skin was tested 4 hr after application of 10 μl of the agents used, with the exception of the sodium lauryl ether sulfate, which was tested after 0.5 hr.

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the pyridine nucleotides in the first few layers of the epidermis, probably in the upper granular layer. It was shown by Schalla *et al.* (9) in studies carried out with C^{14} labelled NAD that less than 1 per cent of the NAD crossed the cell wall (10^{-11} mol/mg), compared to 10^{-7} mol/mg of hydrogen. It is obvious therefore that the hydrogen ion is the actively moving ion that causes the change in fluorescence.

The question of bacteria activity can be raised also as a source of fluorescence. The studies of Marples (13) on skin flora indicate a figure of 1.05 million bacteria per m^2 on the forearm. Our own values on the 12 subjects used in the study yield an average value of 10.6 colonies over 120 cm^2 area for the unwashed arm and a value of 1.1 colonies per 120 cm^2 for the water-washed arm. Since less than 4 cm^2 were used in the study, the bacteria effect may be discounted. Finally, the question of absorbancy of liquids from the chamber by the epidermis may fairly well be ruled out by the studies of Scheuplein (14) on percutaneous absorption. The changes observed in this study are almost instantaneous and precluded diffusional rates mechanisms.

While the enzyme rates reported in this study are far below those reported for cell-free extracts and whole-cell suspensions, they represent an entirely different class of enzyme kinetics. Possibly they follow the kinetics outlined by Blum and Jender (15) and by O'Sullivan (16) which deal with geometrically constrained enzyme systems and slowly diffusing substrates. Since we were unable to calculate an actual Km value for glucose-6-phosphate dehydrogenase, we calculated an "apparent KM" of 2.9×10^{-4} for the conditions used.

One very interesting observation on several patients with psoriasis was the unexpected *increase* in fluorescence in the presence of partial epidermal anoxia induced by local

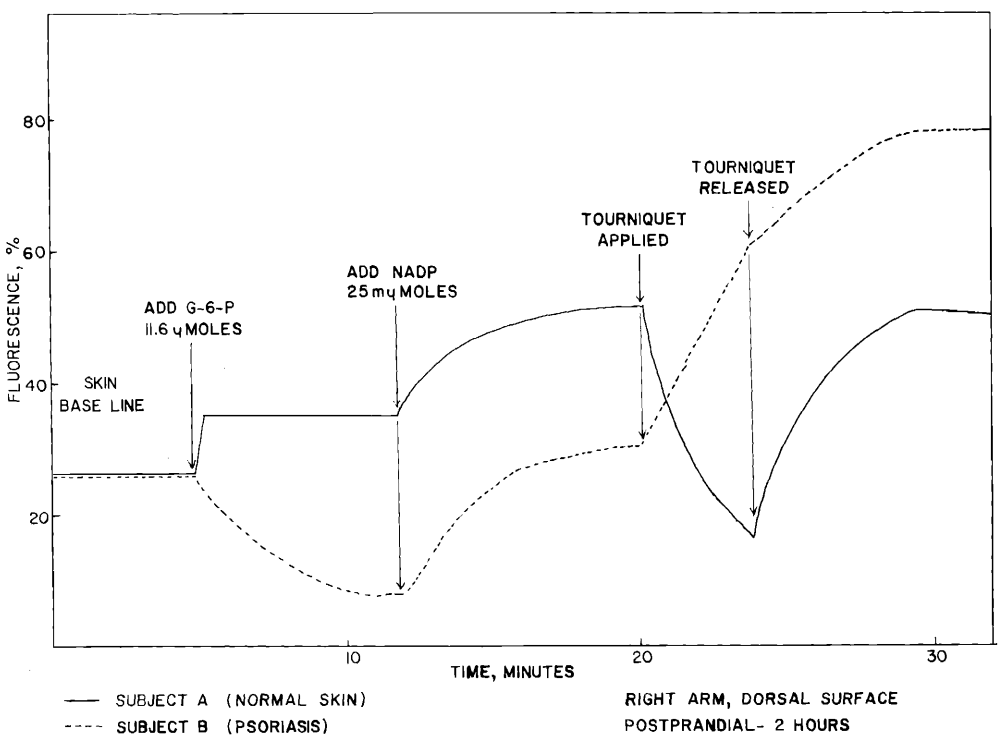

Figure 3. Comparison of effect of partial anoxia in normal individuals and individuals with psoriasis

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venous occlusion. Partial anoxia causes a decrease in fluorescence in the normal individual. This is seen in Figure 3. At present, we have no explanation for this phenomenom. It is found only in patients with active psoriasis and quickly returns to normal when the skin is treated with steroids, or exposed to sunlight for 4 to 6 hr. The effect appears to be related to the severity of the disease, though we have observed it in individuals who have inactive psoriasis and are not under treatment of any type.

A great deal of additional work needs to be done on this system before it will become a routine procedure. The ease of performing the test, the lack of epidermal invasion, and the use of human subjects should make the procedure generally applicable for the study of epidermal effects of applied agents.

CONCLUSIONS

1. A new *in vivo* and *in situ* method for studying epidermal biochemistry has been outlined.
2. The method is reproducible and correlates with similar methods—that is—perfusion and stripping techniques.
3. Classical Michaels-Menten kinetics do not appear to hold for this technique. Precise kinetic studies are needed.
4. Applications appear almost limitless, since the epidermis is accessible and the method adaptable to any reaction in which fluorescence or absorption changes can be measured.

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