QUANTITATIVE ENZYME HISTOCHEMISTRY ON EXPERIMENTAL INJURIES IN THE EPIDERMIS OF THE RHESUS MONKEY (MACACA MULATTA)

by

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TABLE OF CONTENTS

	Page
INTRODUCTION	1
I. Cutaneous carbohydrate metabolism: Review	2
A. Pentose phosphate shunt	4
B. Glycolysis	5
C. Tricarboxylic acid cycle	7
II. Metabolic pattern in the normal epidermis of rhesus monkey	12
A. Enzymatic profile of glucose metabolism	12
B. Concept of metabolic control	13
III. Epidermal enzymatic responses to various trauma: Review	17
A. Responses to carcinogens	17
B. Responses to ionizing radiations	18
C. Responses to wounding	18
IV. Historical survey of the quantitative histochemical method	20
V. Summary	24
MATERIALS AND METHODS	25
I. Animals and their care	25

II. Experimental methods 26

A. Painting with a carcinogen and cocarcinogen . . . 26

B. Ultraviolet light irradiation 27

C. Scraping wound 28

III. Microchemical assays 28

A. Sampling 28

1. Carcinogen experiment 28

2. Ultraviolet light irradiation experiment . . . 29

3. Wound experiment 29

B. Reagents 30

1. Substrates 31

2. Buffers 31

3. Coenzymes 31

4. Cofactors 32

5. Fluorescence reagents 32

C. Assay methods 33

1. The native fluorescence method for NADPH or NADH assay 36

2. The strong alkali method for NAD+ assay . . 36

IV. Statistical analyses 37

RESULTS 39

I. Chemical injury induced by DMBA 39

A. The effect of DMBA on the enzymes of the pentose phosphate shunt 39

B. The effect of DMBA on glycolytic enzymes 40

C. The effect of DMBA on tricarboxylic acid cycle enzymes 40

II. Physical injury induced by ultraviolet light 49

A. The effect of UVL on the enzymes of the pentose phosphate shunt 49

B. The effect of UVL on glycolytic enzymes 50

C. The effect of UVL on tricarboxylic acid cycle enzymes 51

D. Sequential enzymatic changes in the epidermis after ultraviolet irradiation 52

III. Mechanical injury by wound scraping 62

DISCUSSION 72

SUMMARY AND CONCLUSIONS 77

REFERENCES 80

LIST OF TABLES

I. The constituents of reaction mixtures and assay conditions.

II. The constituents of reaction mixtures and assay conditions (cont).

III. Summary of DMBA experiment.

IV. Statistical analysis on the data of G-6-PDH activity of epidermis treated with DMBA.

V. Statistical analysis on the data of 6-PGDH activity of epidermis treated with DMBA.

VI. Statistical analysis on the data of GAPDH activity of epidermis treated with DMBA.

VII. Analyses of variance from the data of LDH, ICDH, and MDH activity in the epidermis treated with DMBA.

VIII. Comparison of enzyme activities among the normal, control and tumor epidermis.

IX. The effects of ultraviolet light on the activity of G-6-PDH.

X. The effects of ultraviolet light on the activity of 6-PGDH.

XI. The effects of ultraviolet light on the activity of GAPDH.

XII. The effects of ultraviolet light on the activity of LDH.

XIII. The effects of ultraviolet light on the activity of ICDH.

XIV. The effects of ultraviolet light on the activity of MDH.

List of Tables (cont.)

XV. Analyses of variance on 90 determinations of each enzyme activity in the control epidermis of ultraviolet light irradiation experiment.

XVI. G-6-PDH activity of wounded and control epidermis.

XVII. 6-PGDH activity of wounded and control epidermis.

XVIII. GAPDH activity of wounded and control epidermis.

XIX. LDH activity of wounded and control epidermis.

XX. ICDH activity of wounded and control epidermis.

XXI. MDH activity of wounded and control epidermis.

LIST OF FIGURES

1. The metabolic map of the pentose phosphate shunt and the enzyme activities in the normal epidermis of the rhesus monkey.

2. The metabolic map of the glycolytic pathway and the enzyme activities in the normal epidermis of the rhesus monkey.

3. The metabolic map of the tricarboxylic acid cycle and the enzyme activities in the normal epidermis of the rhesus monkey.

4. Control sites involved in the glycolytic pathway.

5. The histograms of the enzymatic changes in the epidermis treated with DMBA.

6. The changing curves of enzyme activities in the epidermis after UVL irradiation.

7. The changing curves of the activities of the pentose phosphate shunt enzymes after wounding.

8. The changing curves of the glycolytic enzyme activities after wounding.

9. The changing curves of the TCA cycle enzyme activities after wounding.

INTRODUCTION

Generally speaking, the metabolism of the skin has been studied far less frequently and understood less well than that of other tissues such as the liver and kidney. This appears to be due mainly to the technical difficulties encountered: skin is not a uniform tissue, consisting of many different structures. The use of whole skin, therefore, is unsatisfactory. In an attempt to obtain homogeneous samples of skin, the epidermis has become the material of choice for metabolic studies in the past decade.

Notwithstanding the upsurge of studies on the carbohydrate metabolism of "normal" epidermis in some rodents and in man, there is practically no literature describing the possible alterations in carbohydrate metabolism of "injured" epidermis in any species. This paucity of material also appears to be due to the technical difficulties of obtaining target tissues. For example, regrowing epidermal cells in the wound-healing process are probably limited to those directly adjacent to the wound. An early lesion of papilloma or carcinoma produced by painting with chemical carcinogen may be confined to 10 or 20 cells. To study the possible metabolic changes in these minute lesions, the

application of the quantitative histochemical methods of Lowry are eminently appropriate for assaying certain enzymes in μg amounts of tissue.

In this study, different experimental injuries were produced in the epidermis of rhesus monkeys (Macaca mulatta) and changes in the activities of certain enzymes, participating in carbohydrate metabolism were measured specifically in the "injured" histopathological lesion.

In the following chapters, the characteristic features of carbohydrate metabolism in skin as well as the literature on enzyme changes following epidermal injuries are reviewed and summarized. The pertinence of, and the need for the present study are thus justified.

I. Cutaneous carbohydrate metabolism: Review.

Glucose, synthesized by photosynthesis in plants, plays an important role in the transaction of energy in the biological system of animals. Carbohydrate metabolism in the skin has been reviewed by Rothman in 1954 (1), by Lorincz and Stoughton in 1958 (2) and by Freinkel in 1964 (3). The existence of the metabolic pathway has been demonstrated by enzyme determinations and Weber summarized the broad enzyme spectrum observed histochemically or biochemically up to 1964 (4).

The level of free glucose, β D-glucopyranose, was estimated in the epidermis (5, 6, 7, 8, 9, 10); the amount of glucose present in the epidermis depends on the blood sugar level. It is assumed that the passage of glucose into the epidermal cells is a simple diffusion process so that the utilization of glucose is not limited (7, 10). It was found that glucose was not converted to gluconic acid or to sorbitol in the skin (11). Therefore, upon entering the cell, glucose becomes phosphorylated to glucose-6-phosphate. Hexokinase, the enzyme that catalyzes this reaction, has been demonstrated in the skin (12, 13, 14, 15, 16).

About 15 per cent of this glucose-6-phosphate is hydrolyzed back to free glucose and inorganic phosphate by glucose-6-phosphatase (14). Five per cent of glucose-6-phosphate present in the epidermis is converted to glucose-1-phosphate through the reaction catalyzed by phosphoglucomutase (14). Glucose-1-phosphate is in turn utilized in glycogenesis; biosynthesis and degradation of glycogen by the skin were found and their processes were considered to be rapid and dynamic by Adachi in 1961 (17, 18). Another 5 per cent of glucose-6-phosphate is converted to 6-phosphogluconate by the action of glucose-6-phosphate dehydrogenase, which is the first step of the pentose phosphate shunt; the remaining

75 per cent is converted to fructose-6-phosphate by phosphoglucoisomerase, which is the initial step of glycolysis and the complete oxidation to carbon dioxide and water (14, 19). Less than two per cent of glucose is oxidized in the tricarboxylic acid cycle (19, 20).

Skin tissues require both metabolic energy in the form of adenosine triphosphate (ATP) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) as a reducing agent for biosynthesis. ATP is formed in the glycolysis and tricarboxylic acid cycle, and NADPH is formed by oxidative decarboxylation in the pentose phosphate shunt and tricarboxylic acid cycle.

A. Pentose phosphate shunt

Many reports have been published since Barron et al. in 1948 (13) first suggested the operation of the pentose phosphate shunt in skin. Both C-1 and C-6 of glucose are converted to the methyl group of pyruvate and are, therefore, metabolized equally via the glycolysis and tricarboxylic acid cycle. The loss of C-1 of glucose as CO_2 gives a different metabolism of C-1 and C-6 of glucose in the pentose phosphate shunt. Freinkel (19) found that the ratios of CO_2 derived from C-1 to CO_2 from C-6 glucose in human skin were between 2.8 to 3.1, which indicate the activity

of the pentose phosphate shunt. A similar qualitative study on rat skin was done by Pomerantz and Asbornsen (20). The intermediates such as sedoheptulose-7-phosphate and ribose-5-phosphate of this shunt were determined in the skin of the rat (12) and guinea pig (22).

The activities of glucose-6-phosphate dehydrogenase (14, 23, 24, 25, 26) and 6-phosphogluconate dehydrogenase (26, 27) were estimated quantitatively in human skin. Glucose-6-phosphate dehydrogenase catalyzes the oxidation of glycose-6-phosphate to 6-phosphogluconolactone, which is rapidly hydrolyzed to 6-phosphogluconic acid by the enzyme lactonase. 6-Phosphogluconate dehydrogenase catalyzes the oxidative decarboxylation of 6-phosphogluconic acid to ribulose-5-phosphate (Fig. 1).

The significant roles of this alternative pathway of glucose metabolism in the skin may be to provide pentose phosphate for the synthesis of nucleic acids (ribose nucleic and deoxribose nucleic) and to generate reduced nicotinamide adenine dinucleotide phosphate (NADPH) for reductive processes in lipogenesis and keratinization.

B. <u>Glycolysis</u>

The formation of lactic acid from glucose in human skin was

reported as early as 1926 (28). Intense glycolysis in the presence of oxygen is a specific feature of cutaneous epithelial structure (20, 29, 30, 31, 32, 33); the rate of conversion of glucose to lactic acid under aerobic conditions is approximately 3 µg/mg/hr (31). Lactate dehydrogenase (14, 24, 34), catalyzing the last reaction step and most of other enzymes of glycolysis (14), has been assayed in human skin.

All of the intermediates in the glycolytic sequence are esters of phosphoric acid; only the initial reactant, glucose and the final products, pyruvate and lactate, are not phosphorylated compounds. The excess amount of lactate formed in the epidermis can easily be absorbed by the blood stream from the cell.

The phosphate group of these intermediates is the chemical instrument for generating ATP from ADP. As shown in Figure 2, glycolysis yields a net of 2 ATP molecules per 1 molecule of glucose in anaerobic conditions or 8 ATP molecules in aerobic conditions. Recent isotope studies show that 40-70 per cent of labelled glucose is converted to lactic acid in human and rat skin (19, 20). Thus, for every 100 moles of glucose, 80-140 moles of ATP are produced via glycolysis.

C. Tricarboxylic acid cycle

Since Barron et al. (13) were unable to demonstrate isocitrate dehydrogenase activity and the oxidation of citrate and α-ketoglutarate in the *in vitro* respiration of the skin, they suggested the hypothesis of the succinate-fumarate system as a final step of glucose metabolism. Griesemer and Gould (35) later found that tricarboxylic acid cycle substrates, including α-ketoglutarate but not citrate, stimulated the epidermal respiration; thus, they assumed the presence of the enzymes in the epidermis for the oxidation of those substrates. However, there was still the short-cut glucose metabolism through the succinate-fumarate system of Barron et al. (13) to contend with.

Supplementing those of Barron et al. and Griesemer and Gould, the works of Cruickshunk et al. (36, 37) have provided the findings of transitory stimulation of citrate to the epidermal respiration and of the presence of isocitrate dehydrogenase in the epidermis. Furthermore, Hershey et al. (23, 24, 27) and other investigators (14, 38, 39) have evaluated other tricarboxylic acid cycle enzymes--succinate dehydrogenase, fumarase, and malate dehydrogenase--in human and animal skin. Subsequently, it has become apparent that the tricarboxylic acid cycle can operate in skin as well as in other mammalian tissues.

Since less than two per cent of glucose is oxidized via the tricarboxylic acid cycle in the skin (19, 20), ATP production in this cycle is less than 60 moles for 100 moles of glucose (Fig. 3). NADP-dependent isocitrate dehydrogenase is thought to function as a pathway for the generation of NADPH for biosynthetic purpose.

Figure 1

Metabolic map of the pentose phosphate shunt

Numerical figures are the enzyme activities in the epidermis of the rhesus monkey expressed as moles per hour per kilogram dry weight (40).

Figure I

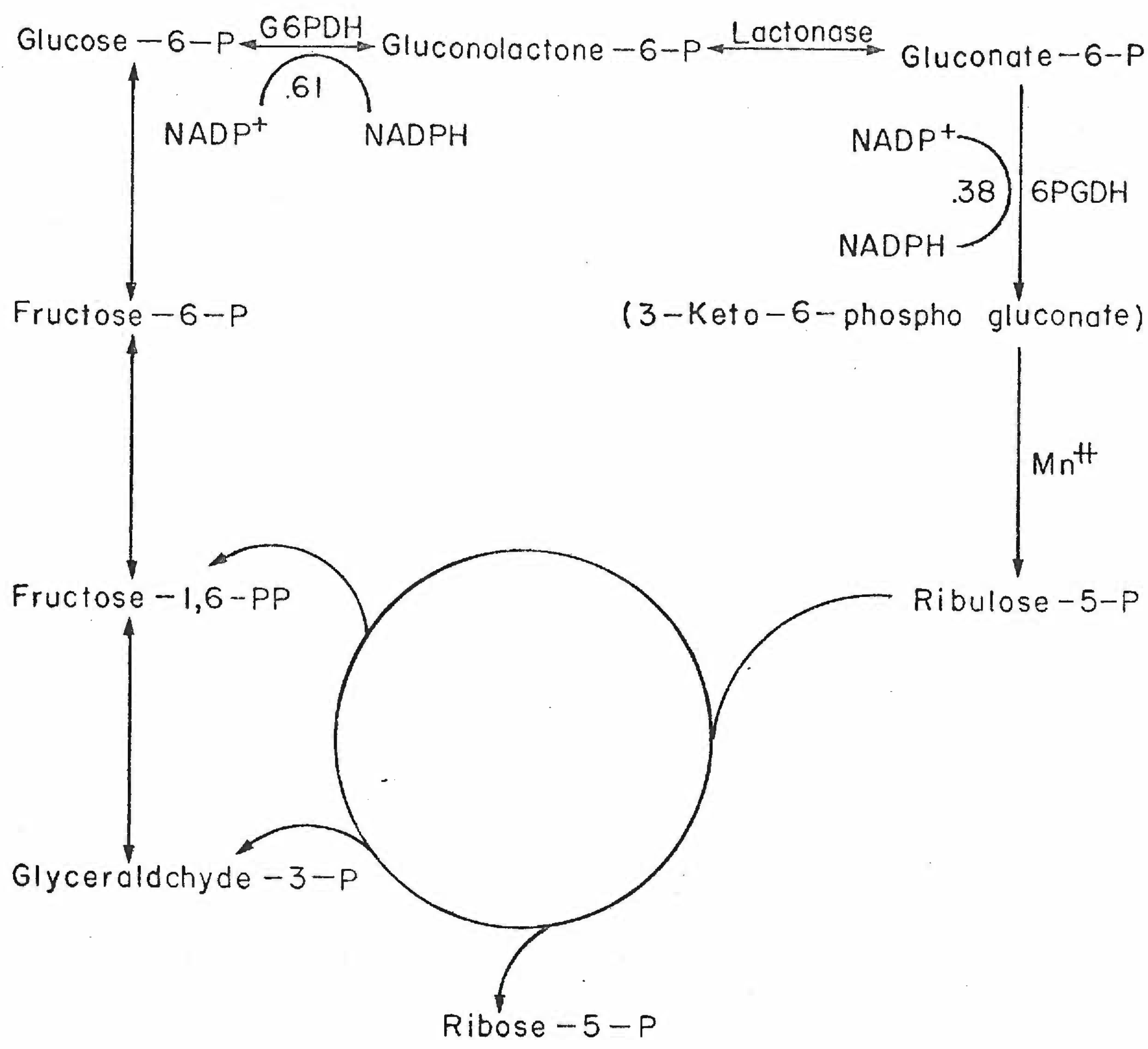


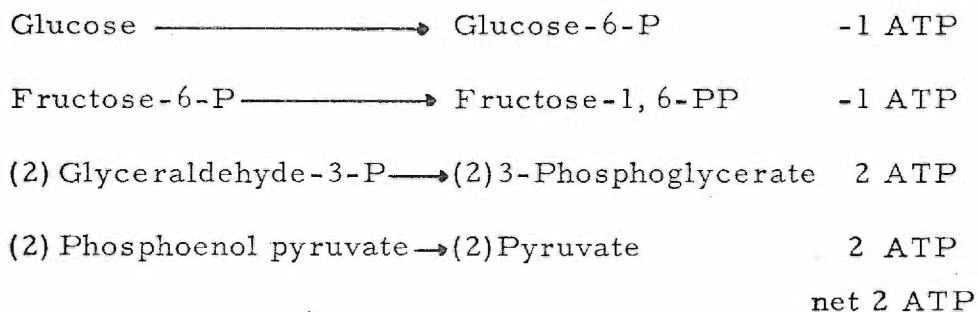
Figure 2

Metabolic map of the glycolysis

Numerical figures are the enzyme activities in the epidermis of the rhesus monkey expressed as moles/hr/kg dry weight (16, 40, 41, 42, 43, 44, 45, 46, 47, 48).

ATP production

In anaerobic condition



In aerobic condition

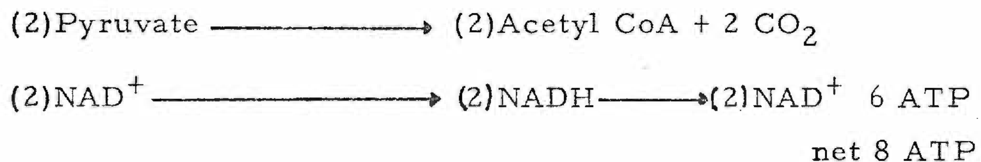


Figure 2

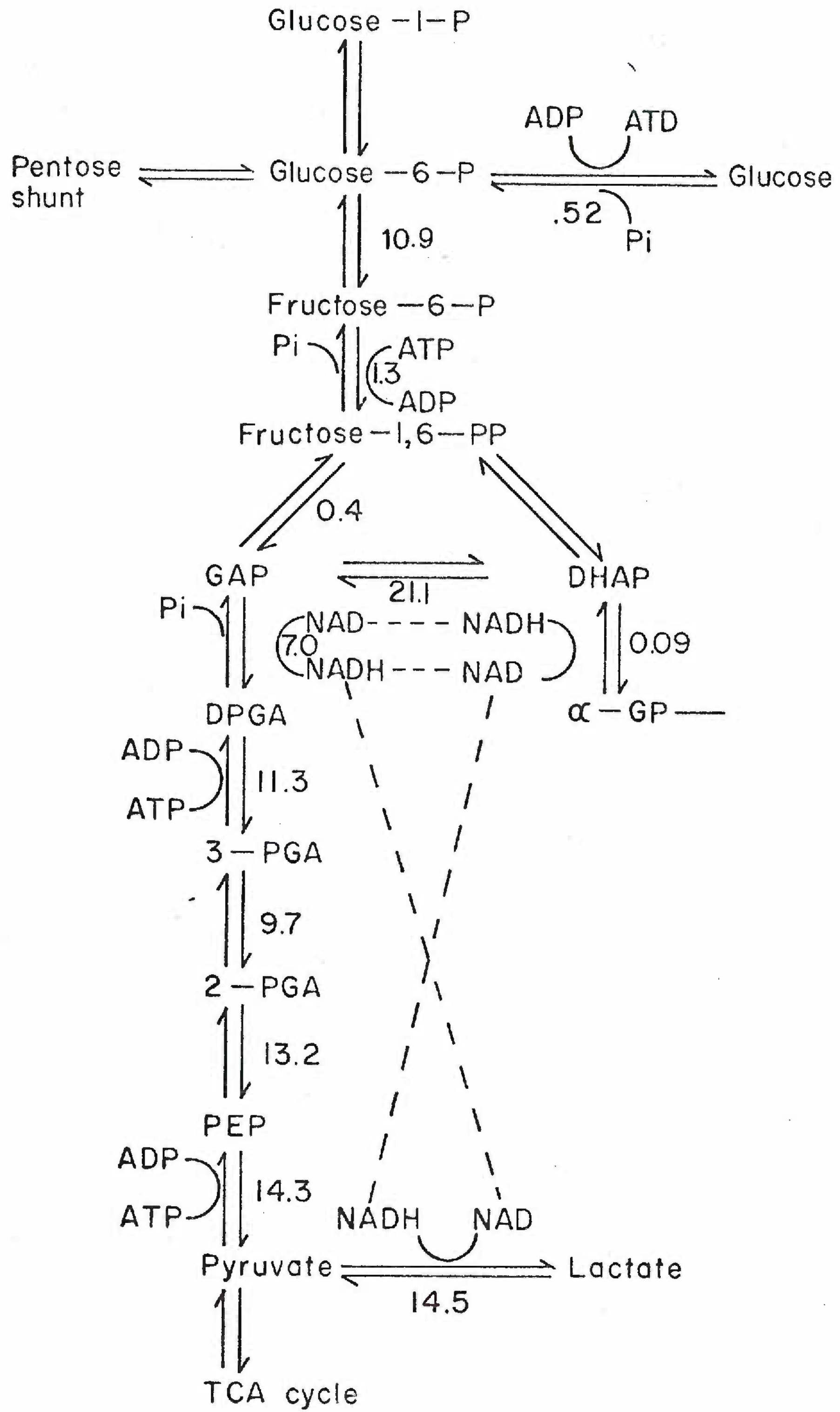
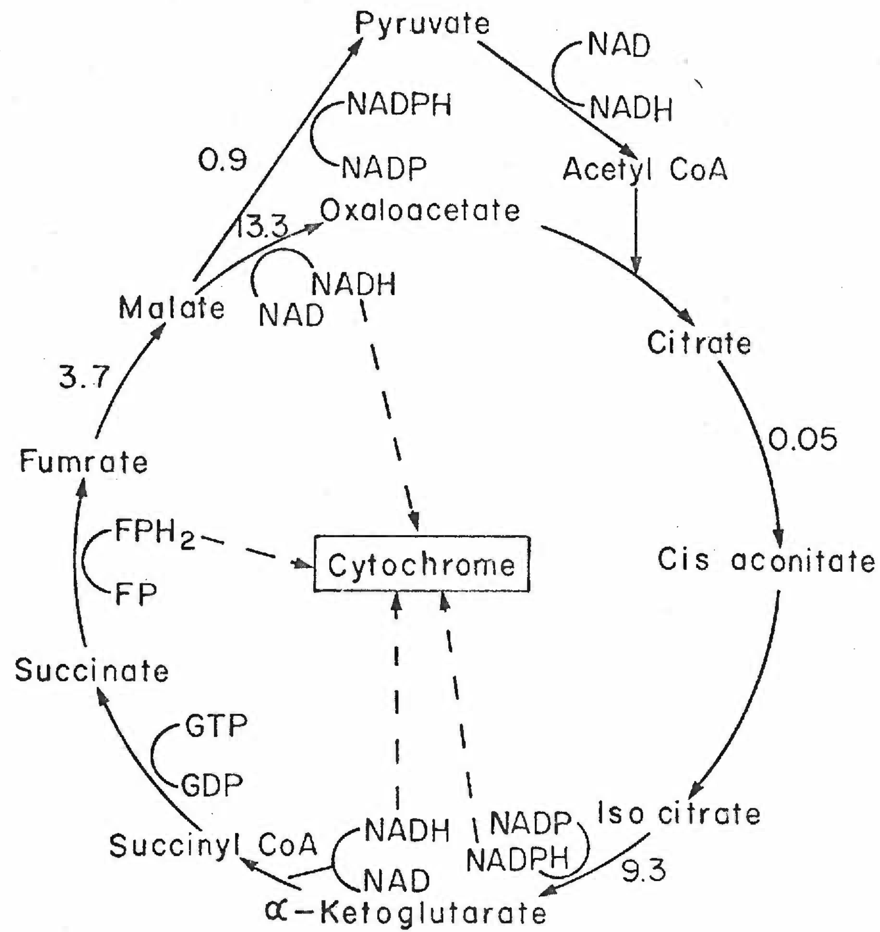


Figure 3

The metabolic map of TCA cycle

Numerical figures are the enzyme activities in the epidermis of the rhesus monkey expressed as moles/hr/kg dry weight (39).

Figure 3



ATP Production

Pyruvate ——→	Acetyl CoA	+ 3 ATP
Isocitrate ——→	α − Ketoglutarate	+ 3 ATP
α − Ketoglutorate ——→	Succinyl CoA	+ 3 ATP
Succinyl CoA ——→	Succinate	+ 1 ATP
Succinate ——→	Fumarate	+ 2 ATP
Malate ——→	Oxaloacetate	+ 3 ATP
or Malate ——→	Pyruvate	
		+15 ATP

Net ATP per hexose unit oxidzed

+30 ATP

II. Metabolic pattern in the normal epidermis of the rhesus monkey.

A. Enzymatic profile of glucose metabolism

Most of the enzymes involved in the pentose phosphate shunt, glycolysis and tricarboxylic acid cycle, have been demonstrated in this laboratory in the skin of rhesus monkeys. The specific activity of each enzyme in the normal epidermis is shown in the metabolic maps represented in Figures 1, 2 and 3. The only pathway of glucose utilization in the skin of this primate is the phosphorylation of glucose to glucose-6-phosphate (11); an active hexokinase (EC 2.7.1.1) system was evaluated in the skin of the rhesus (16).

The demonstration of glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (EC 1.1.1.44) suggested an active participation of the pentose phosphate shunt of glucose metabolism in the epidermis of the rhesus monkey (40) (Fig. 1).

A profile of the other 11 enzymes of the glycolytic pathway established in the epidermis includes glucosephosphate isomerase (EC 5.3.1.9) (41), phosphofructokinase (EC 2.7.1.11) (41), aldolase (EC 4.1.2.b) (42), triosephosphate isomerase (EC 5.3.1.1) (41), glycerophosphate dehydrogenase (EC 1.1.1.8) (43), glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) (44), phosphoglycerate

kinase (EC 2.7.2.3) (45), phosphoglyceromutase (EC 2.7.5.4) (45), enolase (phosphopyruvate hydratase) (EC 4.2.1.11) (46), pyruvate kinase (EC 2.7.1.40) (47), and lactate dehydrogenase (EC 1.1.1.27) (48) (Fig. 2).

The enzymes of the tricarboxylic acid cycle assayed are aconitase (aconitate hydratase) (EC 4.2.1.3), isocitrate dehydrogenase (EC 1.1.1.42), fumarase (fumarate hydratase) (EC 4.2.1.2), malate dehydrogenase (EC 1.1.1.37) and malic enzyme (EC 1.1.1.40) (39) (Fig. 3).

B. Concept of metabolic control

The availability of orthophosphate and phosphate acceptor in tissues has been known to have a basic influence on the rates of oxidation and glycolysis (49, 50, 51). Energy transfer is coupled to the oxidation-reduction reaction in the respiratory chain, and the equilibrium of this reaction is controlled by the phosphate potential, i.e., the ratio of ATP to ADP and P_i. An increased amount of ATP inhibits the respiration (52).

Besides the mitochondrial respiratory control another type of regulating mechanism has been suggested in connection with the sequence of metabolic reactions. In this allosteric control, specific metabolites regulate many enzyme activities.

When these regulatory agents, allosteric effectors, bind an enzyme on its allosteric sites, a reversible conformational alteration occurs in the molecular structure of enzyme protein. This conformational change, known as allosteric transition, modifies the properties of the active site on the enzyme molecule. It is suggested that this allosteric control plays an essential role in the control of metabolic activity and possibly of protein synthesis (53, 54).

Several enzymes participating in energy metabolism have been reported to have allosteric properties and to be controlled by the level of adenosine nucleotides--ATP, ADP, and AMP (53).

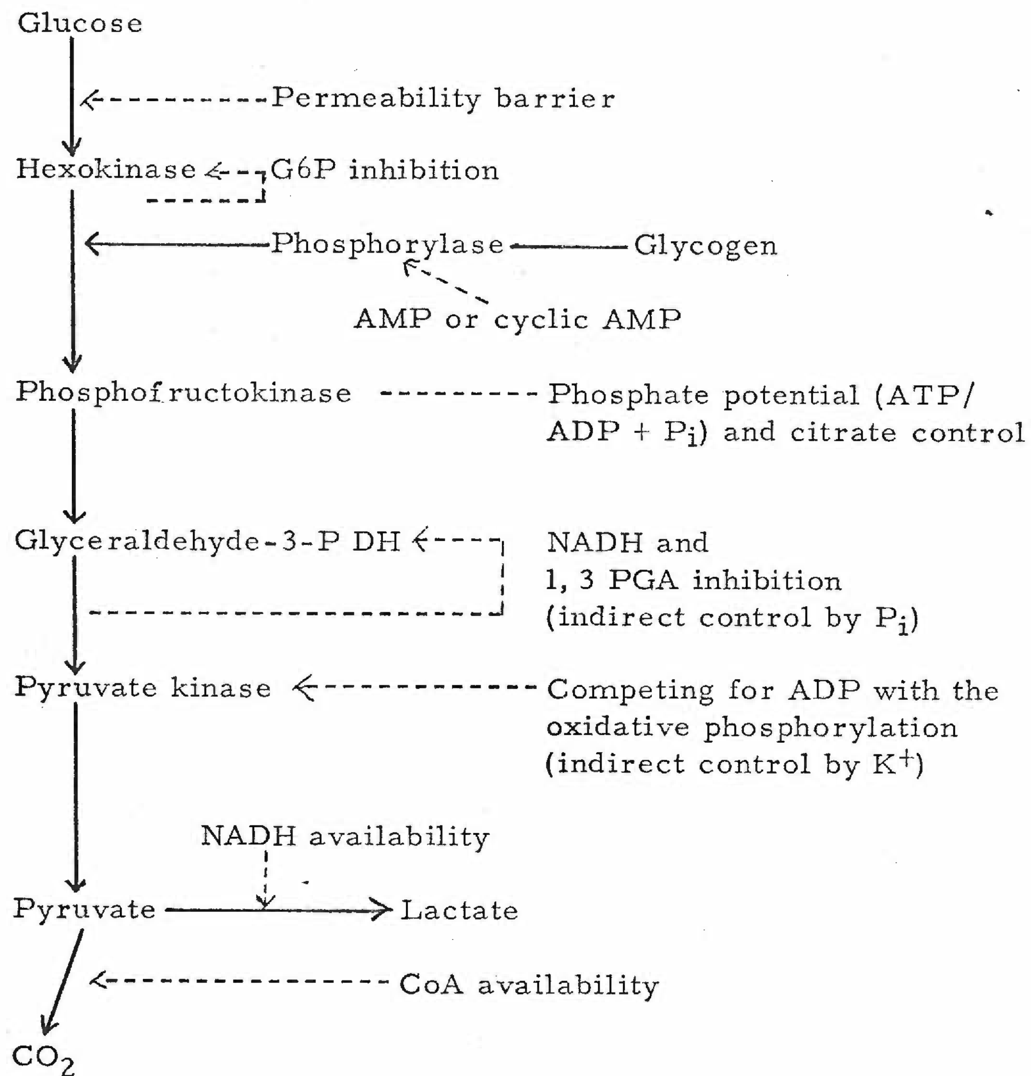
The present concepts of metabolic control in the glycolytic pathway are summarized in Fig. 4. Multi-site control interaction, or multiple feed-back control, are involved in the glycolytic control mechanism (55).

Three control sites in tricarboxylic acid cycle have been found at the points of oxidation of pyruvate, isocitrate and succinate (56).

Although we did not intend to study the role of each enzyme in this laboratory, we found that the rate-limiting enzymes were strongly influenced by various activators and inhibitors. Therefore, it may be possible that in the skin some of the key enzymes shown

in Fig. 4 might work under a control mechanism similar to that of other tissues.

Figure 4



Control sites (indicated by dotted arrows) involved in the glycolytic pathway.

III. Epidermal enzymatic responses to various traumas: Review

The glycogen content of normal mammalian epidermis is known to be small: it was found to be only one-tenth that of the total skin glycogen or 19 mg % in dry weight (17, 57). Histochemically demonstrable glycogen is found only in the cells of the upper stratum spinosum in normal state (58), and biochemical findings show a 50% higher concentration of glycogen in the upper layer than in the basal layer (7). Histochemical studies have indicated that glycogen is stored in the epidermis at the initial stages of various kinds of injuries and disappears during recovery (59, 60).

A. Responses to carcinogens

Experimental skin carcinogenesis has received attention since 1915, when Yamagiwa and Ichikawa (61) induced skin cancer in the ears of rabbits by prolonged applications of a coal tar condensate. Although pathological and morphological studies in skin treated with carcinogen have been made over many decades (62, 63, 64, 65), no study of metabolic changes has been reported. The treatment of skin with carcinogens decreased the deposition of formazan, which may indicate mitochondrial function, and decreased O_2 consumption (63, 64).

B. Responses to ionizing radiations

It has been claimed (66) since 1927 that ultraviolet light and X-ray irradiation cause a depression in glycolysis of the skin. The inhibition of enzymes by X-ray and ultraviolet light have also been reported (67, 68).

One erythema dose of X-ray causes an initial decrease followed by an increase in the glucose incorporation into glycogen in the skin at 24 hours after irradiation (17). Minimum erythema doses (2×10^7 ergs/cm^2) of ultraviolet light produce an accumulation of glycogen in the basal cell layer after 12 hours and morphological changes of the epidermis after 24 hours (69). The accumulation of glycogen by irradiation damage is due to the imbalance in activity between phosphorylase and glycogen synthetase (17).

In a short-range study on ultraviolet irradiation, it was reported that ultraviolet irradiation inhibited or inactivated hexokinase and, in turn, brought about remarkable reductions in glucose oxidation and oxygen consumption at the initial stages, up to two hours after exposure (68). More recently, an *in vitro* study has shown that ultraviolet light inactivates tyrosinase, ATPase, acid phosphatase, succinic dehydrogenase, and oxidative phosphorylation (70).

C. Responses to wounding

Eight hours after stripping the corneum with scotch tape in

man, glycogen is accumulated in the basal cells (71). Histochemically, the activity of several hydrolytic enzymes has been shown to increase during the early phase of wound healing. Alkaline phosphatase appeared in the malpighian layer at the wound margin 24 hours after wounding (72, 73), acid phosphatase was found after 4 hours (73, 74), and leucine aminopeptidase was present 2 hours after injury (74, 75). Before these increases in enzyme activity, a traumatic inhibition of the enzyme or of its synthesis was observed during the first two postoperative hours (75). Oxidative enzymes--monoamine oxidase, succinate dehydrogenase, cytochrome oxidase--have also been shown to increase in the peripheral zone during the early phase (73).

IV. Historical survey of the quantitative histochemical method

The general histochemical procedure was developed by Linderstrøm-Lang et al. in 1935, who found that pepsin was found to be localized in the chief cells of the stomach (76). The results of the microchemical analysis in the original technique was compared with the histological preparation as a control. This quantitative histochemical technique was further developed by Anfinse and Lowry et al. in 1942 (77) and Lowry in 1953 (78, 79, 80), who applied it to the nervous system, retina and brain. Frozen dried sections of brain tissue were prepared and the measurement of the volume and dry weight of fragments of these sections were established as a substitute for the indirect histological control of Linderstrøm-Lang's technique (78). For the preparation of frozen-dried section Lowry carried out the quick freezing and drying method of Hoerr (81), dehydration in vacuum at -30° to -40°, and dissection from the dry sections. For the measurement of the sample, the evaluation of sample size was based on either the protein content, the sample volume, or the dry weight. Protein can be measured on a small tissue with the folin phenol reagent (83). The sample volume is calculated from the thickness of the slice multiplied by the area. The dry weight can be determined with either a quartz torsion balance (84) or a quartz "fish-pole"

balance (85), the latter, with a sensitivity and reproducibility of about 0.01 μg, being much more convenient; the moisture and gases of the air give constant increment in weight under usual laboratory conditions (78).

Since it was difficult to dissect the tissue out larger than a few micrograms in weight, sufficient for histochemical purposes, it was necessary to use rather small-scale chemical methods. General procedures and tools were developed for measuring small amounts, 10^{-10}-10^{-13} mole, of chemicals such as chloride, riboflavin, and phosphorus fractions with as little as 10 μg wet weight of brain tissue (78, 79).

With the development of fluorometry, which has many advantages over spectrophotometry, the procedures for quantitative microchemistry of enzymes, such as adenosine triphosphatase, acid and alkaline phosphatase, cholinesterase, fumarase and aldolase, were also elaborated on small scales with as little as 5 or 10 μg of brain tissue (80). Fluorometry procedures are about a thousand times more sensitive than spectrophotometry and therefore require only a thousandth part of material. This sensitive method came to be particularly suited for studies involving the localization of enzymes and related substrate and coenzymes within the tissues.

Kaplan et al. (86) found that the oxidized pyridine nucleotides are converted to highly fluorescent derivatives when heated in alkali or with carbonyl compound; on the other hand, reduced forms are not influenced by alkali but have a native fluorescence. Lowry et al. (87) modified the Kaplan procedure to stabilize the fluorophor and to measure its fluorescence spectrum. Many of the fluorometric procedures for enzyme assay have been developed, based on the fluorescent properties of pyridine nucleotide, oxidized and reduced. The first enzymes assayed by this principle were lactate dehydrogenase, 6-phosphogluconate dehydrogenase, and glucose-6-phosphate dehydrogenase (87). Furthermore, with the addition of appropriate accessory enzymes, almost any enzyme reaction can be assayed fluorometrically, depending on the oxidation of reduced NAD or reduced NADP or the reduction of NAD^+ or $NADP^+$ (87); and the procedures for many other enzymes have been developed.

The microchemical methods thus devised by Lowry have permitted accurate sampling and assays on various cell types or tissue structures in the nervous system. These micromethods were quickly modified and adapted during the past decade for the study of various tissues in human skin by Hershey and his associates (23, 24, 27). In addition to the procedures modified by Hershey

the quantitative enzyme histochemistry in the skin has been further developed by Adachi in this laboratory, based on the application of the principle of coupling reaction in the various enzyme systems.

V. Summary

The available literature indicates that the "normal" epidermis of man and some rodents utilizes glucose via the glycolytic pathway, the pentose phosphate shunt, and the TCA cycle. The major end-product of glucose catabolism is lactate, and the contribution of the TCA cycle to ATP production is relatively minor. The glucose utilization through the pentose shunt in the epidermis is signficantly active and appears to contribute to nucleic acids and fatty acids metabolism in the epidermis.

The studies of glucose oxidation in the "injured" epidermis, on the other hand, are scanty and fragmentary. The obstacles to these studies are largely due to the histological complexity of the skin which makes it difficult to procure an accurate sampling. Thus, the underlying metabolic alteration in pathological skin has never been understood. The applications of the quantitative micro-enzyme assay method of Lowry to skin appear to be the ideal approach to this problem.

The experiments in this study are designed to approximate a fundamental concept of possible alteration in glucose catabolism in "injured" epidermis by analyzing some of the key enzymes in each metabolic cycle of glucose in 3 types of injury. The enzymes chosen were the following: 1) Glucose-6-phosphate dehydrogenase

(EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (EC 1.1.1.44) analyzed for the pentose phosphate shunt; 2) glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) and lactate dehydrogenase (EC 1.1.1.27) for the glycolytic pathway; 3) isocitrate dehydrogenase (EC 1.1.1.42) and malate dehydrogenase (EC 1.1.1.37) for tricarboxylic acid cycle.

The traumas chosen were: 1) chemical injury (topical carcinogen application); 2) physical injury (ultraviolet light irradiation); 3) mechanical injury (scraping of wound, removal of epidermis).

MATERIALS AND METHODS

I. Animals and their care

Six rhesus monkeys (*Macaca mulatta*) 2 years old at the beginning of this experiment were used for the painting of 9,10-dimethyl-1,2-benzanthracene (DMBA). They were kept in individual cages in the animal care room of the Oregon Regional Primate Research Center.

Three rhesus monkeys, 1-1/2 years old, were used for the wound study. Before the experiment began, they were conditioned for one week to a "sitting chair." After the back of each animal had been wounded by scraping, they continued to sit on the chair

for the next 2 weeks in the intensive care room of the Center to prevent their scratching the wound sites.

Another set of 3 rhesus monkeys, 1-1/2 years old, was used for ultraviolet light irradiation. These animals were given the same training and intensive care as the animals used for wound study during the one-week experiment.

All animals were fed Purina "monkey chow" supplemented by apples and biscuits.

II. Experimental methods

A. Painting with a carcinogen and cocarcinogen

The back of each animal was mapped out into 4 areas and the hairs clipped for the application of carcinogen and cocarcinogen and for the control areas. Two parts of the experimental skin, approximately 5 x 5 cm each, were painted with .8 ml of freshly prepared 1% solution of dimethyl benzanthracene (DMBA)[1] in acetone as a carcinogen and 1 ml of 100% dedecylbenzene[2] as a

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[1]Calbiochem, Los Angeles, California.

[2]California Chemical Company, Oronite Division, San Francisco, California.

cocarcinogen (65). Before these chemicals were applied, one of the two experimental areas was exposed to ultraviolet light from a Westinghouse sun-lamp, FS fluorescent lamp as another accelerator (62). The amount of ultraviolet light energy was 12 x 10^4 ergs/ cm^2, which was calibrated with a Rentschler click meter (Model SM 200) equipped with a WL767 phototube (precalibrated against an FS fluorescent lamp standardized by the United States Bureau of Standards). One of the 2 control parts, about 2.5 x 2.5 cm each, was treated with .5 ml of dodecylbenzene and of acetone after ultraviolet irradiation. Another control was treated with acetone only, after ultraviolet irradiation. The animals were painted 3 times a week for the first 5 months and twice a week for the next 7 months.

B. Ultraviolet light irradiation

Six spots 2.5 cm in diameter on one side of the back of each animal received 10 erythema doses of ultraviolet light. Symmetrical areas on the other side of the back were used as controls.

The source of ultraviolet light was Aero-Kromayer (Model 2221A)[1], the intensity of which for the spectral region 3130A to

[1]Hanovia Lamp Division, Newark, New Jersey.

1850A was 70,000 microwatts/cm^2. Erythema time is one second according to the manufacturer.

C. Scraping wound

The back skin was scraped with a Keratotom[1] to a depth of 0.2 mm, which was thick enough to remove the epidermis.

III. Microchemical assays

A. Sampling

1. DMBA experiment.

Specimens, approximately 1 x 2 cm, of benign tumors (kerato-acanthoma) (65), hyperplasia, and control skin were taken from the three animals one year after the application of DMBA. Tissues were frozen quickly in liquid nitrogen, cut 24 micra in thickness at -20° in a cryostat, and dried at -5° in vacuum of 0.1 mm or less Hg with an acetone-dry ice trap to absorb the moisture for 6-12 hours. Frozen dried tissues were kept in a vacuum at -20° until the enzyme analyses.

Injured and control samples of epidermis were dissected out from the frozen dried sections under the stereomicroscope, weighed out 0.5 to 2.0 µg on a quartz fiber "fishpole" balance

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[1]Storz Instrument Company, St. Louis, Missouri.

(84), and transferred into the small "micro" test tube, with a 2.5 mm inner diameter and 25 mm in length.

2. Ultraviolet light irradiation experiment.

Biopsy specimens, 1 x 1 cm, were taken from the irradiated and the control skin on the 6th, 12th, 24th, 48th, 72nd, and 168th (one week) hour after irradiation. The dissection and measurement of preparations of frozen dried sample proceeded as described above.

3. Wound experiment.

Biopsy specimens, approximately 1 x 2 cm, were obtained from the wound sites and the adjacent portion to it on the 1st, 2nd, 4th, 8th, 14th, and 21st day after wounding. The area adjacent to the wound site (approximately 1 cm), was used as a control for this experiment. The preparation of samples was the same as for those of the DMBA experiment.

Epidermal cells started to migrate inward from the wound margin but did not cover the wound on the first day after wounding. On the 2nd day, a newly-grown epidermal sheath covered the wound; occasionally the advancing epithelial cells moved through the dermis below the leucocytic barrier. Thereafter the inward

migration of the epithelium advanced further. The wounded sample on the first day after wounding, therefore, was taken from the immediate margin of the wound and from the 2nd day on, the newly-formed or migrated epidermal tissues were chosen as wounded epidermis.

B. Reagents

The partial properties and characteristics of six enzymes studied in this thesis were studied previously with an epidermal homogenate from normal rhesus monkeys in this laboratory (39, 40, 44, 48). The optimal assay conditions determined in the previous studies were directly applied to this experiment.

The stability of certain reagents was also checked regularly. For example, nicotinamide adenine dinucleotide (NAD^+) and nicotinamide adenine dinucleotide phosphate ($NADP^+$) were standardized at least monthly as follows: NAD^+ to be standardized was added to 0.1 M tris buffer, pH 8.7, containing ethanol and alcohol dehydrogenase. For $NADP^+$ standardization 0.1 M tris buffer, pH 8.8, containing 1 mM $MgCl_2$, glucose-6-phosphate and glucose-6-phosphate dehydrogenase was used. Consequently NAD^+ and $NADP^+$ were converted to NADH and NADPH, respectively, which were determined spectrophotometrically at 340 mμ. Fifty micromolar NADH in 0.1 M tris buffer, pH 8.7, was used for

spectrophotometrical standardization.

1. Substrates:

Glucose-6-phosphate (Sigma), disodium salt; 100 mM; stored at -20°. 6-Phosphogluconate, trisodium salt (General Biochemicals); 100 mM; stored at -20°.

Fructose-1, 6-diphosphate (Calbiochem); 50 mM; stored at -20°.

Aldolase from rabbit muscle, crystalline suspension in $(NH_4)_2SO_4$, 10 mg/ml, (Sigma); stored at 4°.

Pyruvate, sodium (Sigma); 100 mM; stored at -20°.

Isocitrate, trisodium (Sigma); 100 mM; stored at -20°.

Oxaloacetic acid (Sigma); 100 mM; stored at -20°.

2. Buffers:

Tris (hydroxymethyl) amino methane-hydrochloric acid buffer, 0.5 M; pH 7.5, 8.1, 8.2 and 8.8; stored at -20°.

2-Amino-2-methyl-1, 3-propanediol buffer, 0.5 M; pH 8.6 and 8.8; stored at -20°.

3. Coenzymes:

Nicotinamide adenine dinucleotide, oxidized form, (NAD^+) (Sigma); 100 mM; stored at -20°.

Nicotinamide adenine dinucleotide, reduced form,(NADH)

(Sigma); 50 mM; prepared in a Tris. buffer, pH 8.1-8.6, just prior to use.

Nicotinamide adenine dinucleotide phosphate (NADP+) (Sigma); 20 mM; stored at -20°.

4. Cofactors:

$MgCl_2$; 1 M; stored at 4°.

$MnCl_2$; 1 M; stored at 4°.

Ethyledinitrilotetraacetic acid (EDTA); 100 mM adjusted pH to 7.5; stored at 4°.

Mercaptoethanol solution (Sigma), 1.0 M in 0.02 N KOH; stored at 4°.

Albumin (bovine plasma) (Calbiochem); 5% aqueous solution; kept at 4° or -20°.

5. Fluorescence reagents:

Carbonate buffer, 0.1 M, pH 10.5; stored at room temperature. (The fluorescence of NADPH and NADH is stable in this buffer.)

6.6 N NaOH.

(to develop the fluorescence of NAD+)

1, 2 and 6 N HCl.

(to destroy the native fluorescence of NADPH and NADH)

C. Assay methods

The assay conditions and constituents of the reaction mixture for G-6-PDH, 6-PGDH and GAPDH are given in Table I and those for LDH, ICDH and MDH in Table II.

After an appropriate incubation, the assays of NADP-dependent enzymes, G-6-PDH, 6-PGDH and ICDH, were based on the stoichiometrical reduction of $NADP^+$ to NADPH. The GAPDH activity was measured based on the reduction of NAD^+ to NADH. NADPH or NADH produced by enzyme reaction was measured by the native fluorescence method. The assay system for LDH and MDH was based on the oxidation of NADH to NAD^+. The strong alkali method was used for NAD^+ assay as described below.

Blank and standard tubes were run simultaneously; the reaction mixture without tissue was used as a blank for each enzyme assay and the standard tube contained 0.5×10^{-5} to 1.5×10^{-5} M NAD^+ (NADH) or 0.5×10^{-6} to 2.0×10^{-6} M NADPH. NADPH for $NADP^+$ -dependent enzyme assay was substituted by NADH. In all six enzyme assays, no reaction followed when their respective substrates were omitted from their reaction mixtures. An enzyme blank was negligible and did not increase blank value, when 0.2 to 5 μg of the dissected epidermis were used as enzyme source in 10 to 15 μl of substrate reagent.

TABLE I
The constituents of reaction mixtures and assay conditions

	G-6-PDH	6-PGDH	GAPDH
Substrate	Glucose-6-phosphate, disodium salt, 2 mM	Sodium phosphogluconate, 5 mM	Glyceraldehyde-3-phosphate*
Buffer	2-amino-2-methyl-1,3-propanediol buffer, 0.1 M, pH 8.8	Tris buffer, 0.1 M, pH 8.8	2-amino-2-methyl-1,3-propanediol buffer, 0.1 M, pH 8.6
Coenzyme	$NADP^+$, 0.3 mM	$NADP^+$, 0.3 mM	NAD^+, 3.0 mM
Activator	$MgCl_2$, 2.5 mM	$MgCl_2$, 2.5 mM	Mercaptoethanol, 5 mM
	EDTA,** pH adjusted to 7.5, 0.5 mM	EDTA (pH 7.5), 0.5 mM	EDTA (pH 7.5), 1 mM
Other	Albumin (Bovine plasma), 0.05%	Albumin, 0.05%	Albumin, 0.05%
Final volume	9 µl	9 µl	15 µl
Standard conc.	0.5-2.0 mµ moles/tube	0.5-2.0 mµ moles/tube	8-30 mµ moles/tube
Incubation time	60 min. at 37°	60 min. at 37°	60 min. at 37°

* Glyceraldehyde-3-phosphate is produced in the reagent mixture by the system consisted of 50 mM fructose-1,6-diphosphate and crystalline aldolase from rabbit muscle, 30 µg per ml reagent mixture.

** EDTA (ethyledinitrilotetraacetic acid) may activate the reaction by binding of inhibitory heavy metals, if present, in the assay medium.

TABLE II
The constituents of reaction mixtures and assay conditions (cont.)

	LDH	ICDH	MDH
Substrate	Sodium pyruvate, 1 mM	Isocitrate, trisodium, 4 mM	Oxaloacetic acid, 1 mM
Buffer	Tris buffer, 0.1 M, pH 7.5	Tris buffer, 0.1 M, pH 8.1	Tris buffer, 0.1 M, pH 8.2
Coenzyme	NADH, 2 mM	$NADP^+$, 1 mM	NADH, 2 mM
Activator	---	$MnCl_2$, 0.25 mM	---
Other	Albumin, 0.05%	Albumin, 0.05%	Albumin, 0.05%
Final volume	15 µl	10 µl	15 µl
Standard conc.	8-30 mµ moles/tube	0.5-2.0 mµ moles/tube	8-30 mµ moles/tube
Incubation time	30 min. at 37°	60 min. at 37°	30 min. at 37°

1. The native fluorescence method for NADPH or NADH assay.

The enzyme reaction was stopped by placing the tubes into ice-chilled water. Subsequently the mixture was diluted with 1 ml of carbonate buffer, 0.1 M, pH 10.5. The fluorescence of reduced NAD or NADP is stable in this solution.

Fluorescent light was activated and transmitted in a Farrand fluorometer (Model A2) with a primary filter of Corning glass filter No. 5860 and a secondary filter complex of Corning No. 4308, 5562, and 3387. The activation (excitation) and fluorescence maximum for pyridine nucleotide was 360 mμ and 460 mμ respectively. The primary filter used isolated the mercury line at 365 mμ for activation and the secondary filter combination had maximum transmission at 470 mμ (87).

The intensity of the fluorescence of NADPH or NADH was directly proportional to its concentration and was amplified and measured on a galvanometer.

2. The strong alkali method for NAD^+ assay.

After incubation, the reaction was stopped by the addition of HCl (final concentration of 0.2 to 0.5 N). This procedure also destroyed unreacted NADH. The aliquot treated with acid was then

transferred into 100 μl of strong NaOH to give a concentration of 5.0 to 8.0 N and was allowed to stand for one hour at room temperature or 30 minutes at 37° or 15 minutes at 60°. In this process the NAD^+ produced by enzyme reaction was converted to a stable fluorescent product by the action of strong alkali. The fluorescence of this product was measured as described in the native fluorescence method after 5- to 10-fold dilution with distilled water.

IV. Statistical analyses

The student t-test and analysis of variance were run for the significance of the result. When an analysis of variance on the data was significant, Newman-Keuls test was done to find where the significance lay (88).

Computational formulas

a. The t-test

$$t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{S_{\bar{X}_1} + S_{\bar{X}_2}}}$$

where $\bar{X}_1$, $\bar{X}_2$ = the mean values of two sample

$S_{\bar{X}_1}$, $S_{\bar{X}_2}$ = the standard errors of the two sample means

b. Analysis of variance

(1) G^2/kn

(2) $\sum X_{ij}^2$ — Where $G = X_{ij}$ = total of all raw scores

(3) $\sum T_j^2/n$ — T = the sum of all scores for treatment, i.e., tumor, hyperplasia and normal, etc.

(4) $\sum A_j^2/k$ — A = the sum of all scores for each animal

k = number of groups

n = number of individuals in a group

Source	Sum of square	Degree of freedom	Means of square	F
Between animals	(4)-(1)	n-1		
Within animals	(2)-(4)	n(k-1)	SS/df	$MS_{Bet.}/MS_{Within}$
Treatments	(3)-(1)	k-1		
Residual	(2)-(3)-(4)+(1)	(n-1)(k-1)		
Total	(2)-(1)	nk-1		

RESULTS

I. Chemical injury induced by DMBA

The enzyme activities in the hyperplastic epidermis and benign tumor induced by the combined application of DMBA and dodecylbenzene are summarized in Table III and Figure 5. Statististical analyses, analysis of variance, and t-test, are shown in Tables IV, V, VI, VII and VIII.

A. The effect of DMBA on the enzymes of the pentose phosphate shunt

The most remarkable effect of DMBA was on both glucose-6-phosphate and 6-phosphogluconate dehydrogenase. F-value in the analysis of variance indicated that individual variations among the three animals were insignificant in the assay of each enzyme and that the effect of DMBA treatment was significant on both enzymes (Table IV, V and VIII, Figure 5).

In general, there was no statistically significant difference in enzyme activity between tumor and hyperplastic tissue, whereas significant changes were seen between normal control, and tumor or hyperplastic tissue. As shown in Table VIII, G-6-PDH activity in tumor tissue was 5 times higher than in normal (P 0.05) and 1.5 times higher than in cocarcinogen control (P 0.05).

6-PGDH activity in tumor was about 4 times (P 0.01) and 1.4 times (P 0.05) higher than in normal and in cocarcinogen control tissue, respectively. The control cocarcinogen also enhanced G-6-PDH activity to 3.3 times and 6PGDH activity to 2.4 times higher level than normal (P 0.01). Acetone alone also seemed to affect both enzyme activities.

B. The effect of DMBA on glycolytic enzymes

Glyceraldehyde-3-phosphate dehydrogenase activity had insignificant individual variations and was changed in the DMBA treatment (Table VI), whereas practically no change of lactate dehydrogenase activity was observed. Individual variation in the assay of LDH activity was significant at P 0.05 level (Table VII).

GAPDH activity in the tumor was 3 times higher than in normal and 2 times higher than in control tissue (P 0.01-0.001) (Table VIII). LDH activity of tumor tissue was increased only 30% of normal level, but this increase was statistically insignificant and indicated no difference in LDH activity among the treated, control, and normal epidermis (Table VIII, Figure 5).

C. The effect of DMBA on tricarboxylic acid cycle enzyme

Individual variations among animals for the assay of isocitrate dehydrogenase were insignificant (Table VII). All of the treated

epidermis showed approximately 3 times higher activity of ICDH than normal epidermis (P 0.001) (Table VIII). No significant difference in the ICDH activity was observed among the tumor, hyperplasia, and cocarcinogen control epidermis (Table VIII). Individual variations in malate dehydrogenase were very large (P 0.001), and the activity of this enzyme did not show any statistically significant changes between the normal and experimental epidermis (Table VII and VIII, Figure 5).

TABLE III
SUMMARY OF DMBA EXPERIMENT

Tissue	Animal #	G6PDH	6 PGDH	GAPDH	LDH*	ICDH	MDH
Tumor	1368	1.36 ± .34	.86 ± .17	5.97 ± .76	24.6 ± 8.9	1.32 ± .28	18.2 ± 7.3
	1374	2.30 ± .39	.80 ± .05	6.04 ± 1.87	16.2 ± 6.0	1.08 ± .46	10.8 ± 4.7
	1425	1.89 ± .97	1.03 ± .20	8.66 ± 2.41	8.4 ± 1.8	2.31 ± .42	25.1 ± 12.3
Hyperplasia adjacent to tumor	1368	1.34 ± .41	.59 ± .12	5.12 ± 2.14	10.3 ± 2.6	1.57 ± .42	16.6 ± 7.6
	1374	2.16 ± .34	.76 ± .19	5.39 ± .36	17.0 ± 3.8	1.17 ± .18	14.7 ± 4.9
	1425	1.92 ± 1.03	.84 ± .19	7.65 ± 1.84	13.4 ± 3.3	2.27 ± .32	13.8 ± 4.0
Hyperplasia induced by DMBA & UVL	1368	1.26 ± .22	.63 ± .09	3.76 ± 1.38	13.4 ± 4.3	1.55 ± .06	16.3 ± 2.8
	1374	1.54 ± .38	.57 ± .12	3.64 ± .92	15.0 ± 2.4	.90 ± .08	10.1 ± 2.1
	1425	1.28 ± .20	.59 ± .12	3.41 ± .77	12.5 ± 4.8	1.79 ± .42	21.3 ± 13.2
Hyperplasia induced by DMBA	1368	1.27 ± .18	.67 ± .08	4.13 ± .79	17.5 ± 7.7	1.51 ± .04	13.5 ± 5.8
	1374	1.86 ± .32	.69 ± .22	5.44 ± .90	15.3 ± 5.1	1.19 ± .07	13.4 ± 4.3
	1425	2.23 ± .54	.75 ± .16	3.94 ± .71	10.7 ± 7.1	1.78 ± .34	15.9 ± 6.2
Dodecyl and acetone control	1368	.84 ± .17	.66 ± .14	4.27 ± .65	18.7 ± 6.9	1.21 ± .24	20.7 ± 6.4
	1374	1.66 ± .39	.66 ± .14	5.97 ± .64	13.5 ± 6.7	1.15 ± .08	13.2 ± 3.0
	1425	1.18 ± .38	.57 ± .10	2.90 ± .43	11.6 ± 4.2	1.64 ± .24	17.1 ± 10.2
Acetone control	1368	.71 ± .17	.56 ± .12	3.35 ± .59	19.3 ± 3.0	.95 ± .12	18.0 ± 6.1
	1374	.80 ± .13	.42 ± .09	4.62 ± .52	9.3 ± 5.3	.73 ± .17	10.3 ± 4.4
	1425	.51 ± .10	.49 ± .06	3.69 ± .92	11.6 ± 6.1	1.10 ± .27	19.4 ± 5.3
Normal epidermis	1368	.29 ± .09	.18 ± .02	2.04 ± .51	13.5 ± 6.2	.40 ± .12	13.0 ± 5.9
	1374	.48 ± .16	.45 ± .09	4.30 ± .92	13.5 ± 1.4	.57 ± .14	15.4 ± 3.5
	1425	.31 ± .12	.24 ± .06	1.16 ± .15	7.9 ± 1.7	.63 ± .13	15.2 ± 5.1

Figures are the means ± S.D. of enzyme activities expressed as moles per hour per kilogram dry weight of epidermis. Each enzyme activity was determined with 5 microtest tubes of samples. *LDH activity was assayed with an average of 8 microtest tubes.

TABLE IV

Statistical analysis on the data of glucose-6-phosphate dehydrogenase activity of epidermis treated with DMBA.

Analysis of variance

Source	SS	df	MS	F	
Bet. Animals	1.01	2	0.51	1.3 < 3.5(P=0.05)	Individual variations are insignificant
Within Animal	6.98	18	0.39		
Treatments	6.32	6	1.05	19.0 > 4.8(P=0.01)	Treatments are significant at P=0.01 level
Residual	0.66	12	0.06		
Total	7.99	20			

Newman-Keuls test

	Tumor 5.55†	Hyper. T. 5.42	Hyper.DMBA 5.36	Hyper.DMBA&UVL 4.08	Do+acet cont. 3.68	Acet.cont. 2.02	Normal 1.08
Tumor	----	0.13	0.19	1.47	1.87*	3.53**	4.47**
Hyper. T.		----	0.06	1.34	1.74*	3.40**	4.34**
Hyper. DMBA			----	1.28*	1.68*	3.34**	4.28**
Hyper. DMBA & UVL				----	0.40	2.06*	3.00**
Do+acet. cont.					----	1.66*	2.60**
Acet. control						----	0.94
Normal							----

† Total of enzyme activities of three individuals

* The difference is significant at P=0.05

**The difference is significant at P=0.01

TABLE V

Statistical analysis on the data of 6 phosphogluconate dehydrogenase activity of epidermis treated with DMBA.

Analysis of variance

Source	SS	df	MS	F	
Bet. Animals	0.01	2	0.004	0.1 <3.5(P=0.05)	Individual variations are insignificant
Within animal	0.78	18	0.043		
Treatments	0.67	6	0.112	12.4 >4.8(P=0.01)	Treatments are significant at P=0.01 level
Residual	0.11	12	0.009		
Total	0.79	20			

Newman-Keuls test

	Tumor 2.69†	Hyper.T. 2.19	Hyper.DMBA 2.11	Do+acet cont. 1.89	Hyper. DMBA&UVL 1.79	Acet.cont. 1.47	Normal .87
Tumor	----	.50	.58	.80*	.90*	1.22**	1.82**
Hyper.T.		----	.08	.30	.40	.72	1.32**
Hyper.DMBA			----	.22	.32	.64	1.24**
Do+acet.cont.				----	.10	.42	1.02**
Hyper.DMBA & UVL					----	.32	.92**
Acet. control						----	.60*
Normal							----

† Total of enzyme activities of three individuals

* The difference is significant at P=0.05

**The difference is significant at P=0.01

TABLE VI

Statistical analysis on the data of glyceraldehyde-3-phosphate dehydrogenase activity of epidermis treated with DMBA.

Analysis of variance

Source	SS	df	MS	F	
Bet. Animals	3.3	2	1.65	$0.9 < 3.5 (P=0.05)$	Individual variations are insignificant
Within animal	57.4	18	3.18		
Treatments	39.9	6	6.65	$4.6 > 3.0 (P=0.05)$	Treatments are significant at P=0.05 level
Residual	17.5	12	1.45		
Total	60.7	20			

Newman-Keuls test

	Tumor 20.67+	Hyper.T. 18.16	Hyper.DMBA 13.51	Do+acet cont. 13.14	Acet.cont. 11.66	Hyper. DMBA&UVL 10.81	Normal 7.50
Tumor	-----	2.51	7.16	7.54	9.01	9.86	13.17*
Hyper. T.		-----	4.65	5.03	6.50	7.35	10.66*
Hyper. DMBA			-----	.38	1.85	2.70	6.01
Do+acet cont.				-----	1.47	2.32	5.63
Acet.control					-----	.85	4.16
Hyper. DMBA & UVL						-----	3.31
Normal							-----

+ Total of enzyme activities of three individuals.

* The difference is significant at P=0.05.

TABLE VII

Analyses of variance from the data of LDH, ICDH, and MDH activity in the epidermis treated with DMBA.

LDH

Source	SS	df	MS	F	
Bet. animals	124.59	2	62.30	5.5*	> 3.5 (P 0.05)
Within animal	200.97	18	11.16		
Treatments	38.19	6	6.37	0.5***	< 3.0 (P 0.05)
Residual	162.78	12	13.57		
Total	325.56	20			

ICDH

Source	SS	df	MS	F	
Bet. animals	1.64	2	0.82	2.5**	< 3.5
Within animal	7.19	18	0.40		
Treatments	2.96	6	0.49	1.4***	< 3.0
Residual	4.23	12	0.35		
Total	8.83	20			

MDH

Source	SS	df	MS	F	
Bet. animals	135.0	2	67.5	10.2*	> 6.0 (P 0.01)
Within animal	118.6	18	6.6		
Treatments	33.2	6	5.5	0.7***	< 3.0
Residual	85.4	12	7.1		
Total	253.6	20			

* Individual variations are significant

** Individual variations are insignificant

***Treatments are insignificant at P 0.05 (see t-test in Table VIII)

TABLE VIII

Comparison of enzyme activities among the normal, control and tumor epidermis. Enzyme activities are expressed as moles per hour per kilogram dry weight.

Enzyme	Animal	Tumor (A)	Do+acet. control (B)	Normal (C)	A/C	P**	B/C	P**	A/B	P**
G6PDH	1368	1.36 ± .15*	.84 ± .07	.29 ± .04	4.6	.001	2.8	.001	1.6	.05
	1374	2.30 ± .17	1.66 ± .17	.48 ± .07	4.8	.001	3.4	.001	1.4	.05
	1425	1.89 ± .48	1.18 ± .19	.31 ± .06	6.0	.05	3.8	.001	1.5	--
6PGDH	1368	.86 ± .07	.66 ± .06	.18 ± .01	4.9	.001	3.7	.001	1.3	--
	1374	.80 ± .02	.66 ± .06	.45 ± .04	1.8	.001	1.4	--	1.2	--
	1425	1.03 ± .08	.57 ± .04	.24 ± .02	4.2	.001	2.3	.001	1.7	.001
GAPDH	1368	5.97 ± .34	4.27 ± .32	2.04 ± .22	2.9	.001	2.1	.001	1.4	.01
	1374	6.04 ± .83	5.97 ± .28	4.30 ± .41	1.4	.001	1.4	.01	1.0	--
	1425	8.66 ± 1.08	2.90 ± .43	1.16 ± .06	6.9	.001	2.3	.01	3.0	.001
LDH	1368	24.6 ± 3.9	18.7 ± 2.4	13.5 ± 2.3	1.8	.05	1.4	--	1.3	--
	1374	16.2 ± 2.7	13.5 ± 3.0	13.5 ± .7	1.2	--	1.0	--	1.1	--
	1425	8.4 ± .5	11.6 ± 1.3	7.9 ± .5	1.1	--	1.5	.05	0.7	--
ICDH	1368	1.32 ± .12	1.21 ± .10	.40 ± .05	3.3	.001	3.0	.001	1.0	--
	1374	1.08 ± .20	1.15 ± .03	.57 ± .06	1.9	.05	2.0	.001	0.9	--
	1425	2.31 ± .19	1.64 ± .10	.63 ± .05	3.6	.001	2.6	.001	1.4	.05
MDH	1368	18.2 ± 2.7	20.7 ± 2.2	13.0 ± 2.0	1.4	--	1.5	.05	0.9	--
	1374	10.8 ± 2.1	13.2 ± 1.3	15.4 ± 1.5	0.7	--	0.9	--	0.8	--
	1425	25.1 ± 4.3	17.1 ± 4.5	15.2 ± 2.3	1.6	--	1.1	--	1.4	--

* Values are the mean ± standard error of 5 determinations.

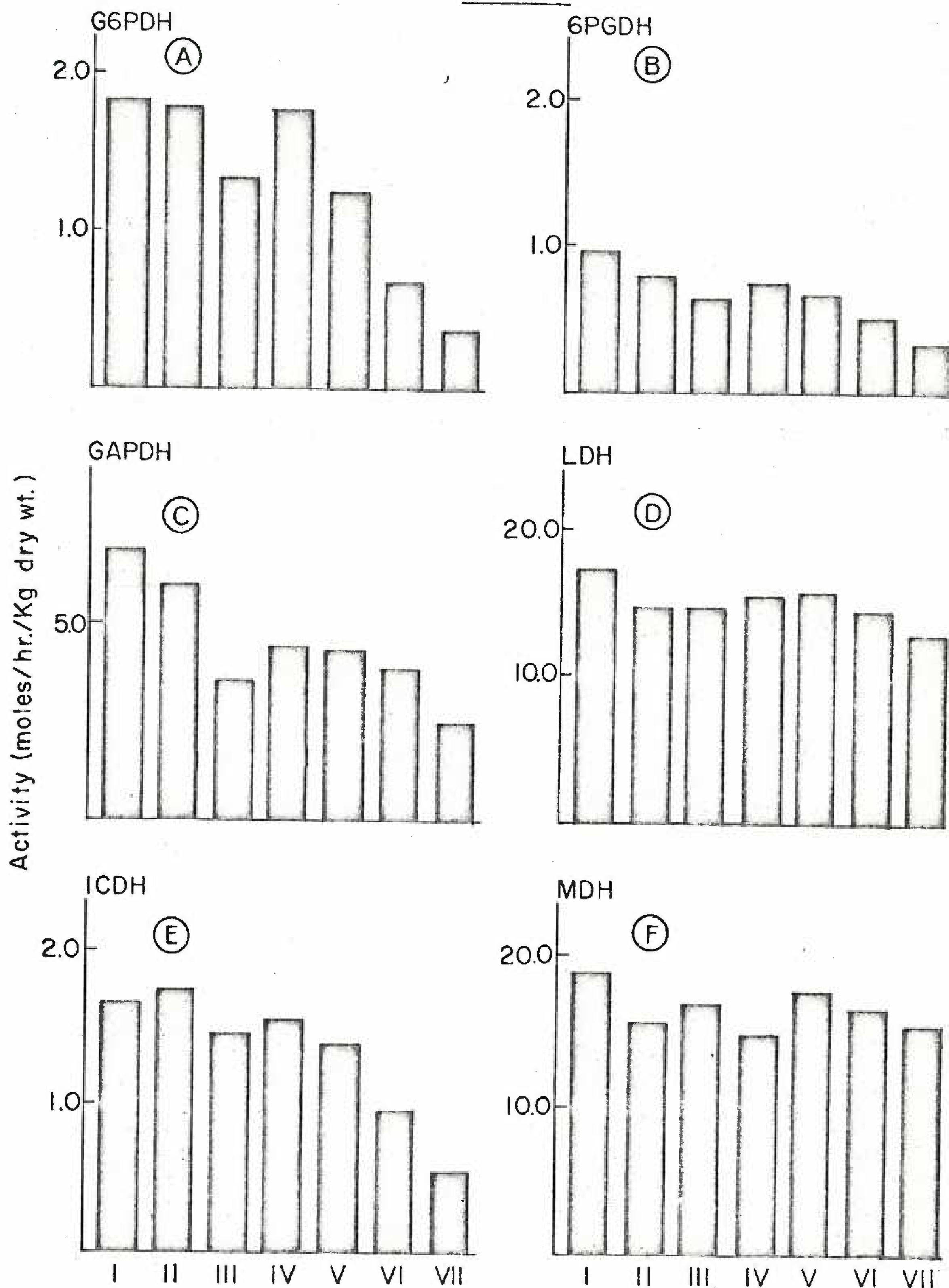
** P values are from t-test and P values bigger than 0.05 are omitted.

Figure 5

Histograms of enzymatic changes in the epidermis treated with DMBA and dodecylbenzene together.

In the ordinate the enzyme activity is expressed as moles per hour per kilogram dry weight tissue. In abscissa I stands for tumor, II for the hyperplasia adjacent to tumor, III for hyperplasia produced by DMBA and UVL, IV for hyperplasia produced by DMBA, V for dodecylbenzene and acetone control, VI for acetone control, and VII for the normal epidermis.

Figure 5



II. Physical injury induced by ultraviolet light

The enzyme activities affected by ultraviolet light are shown in Tables IX, X, XI, XII, XIII, and XIV and the changes during one week of U. V. irradiation are outlined in Figure 6. In general there were initial increases in enzyme activity at 6 hours, then decreases between 12 to 48 hours, and again increases at 72 hours after irradiation.

A. The effect of UVL on the enzymes of the pentose phosphate shunt (Table IX and X, Figure 6)

The mean of 90 determinations of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activity ranged between .50 and .59 and between .51 and .58 mole per hour per kilogram dry weight, respectively, in the control epidermis, depending on the individual animal. An analysis of variance from these data indicated that this extent of individual variation was not significant (Table XV). Initial increases of 30% and 20% in the activity of G-6-PDH and 6-PGDH, respectively, were observed in the irradiated epidermis 6 hours after irradiation. After these increases, the greatest decreases of both enzymes were found during 24 to 48 hours after irradiation: 20% (P 0.05) decrease in G-6-PDH and 40-50% (P 0.001) decrease in 6-PGDH activity in two of three

animals. These depressions of the enzyme activities were followed by maximal increases in both enzymes at 72 hours; thereafter the values of specific activity remained or decreased slightly until 168 hours (1 week) after irradiation: 40-140% (P 0.001) in G-6-PDH and 40-50% (P 0.001) increase in 6-PGDH activity.

B. The effect of UVL on the glycolytic enzymes (Table XI and XII, Figure 6)

The data on glyceraldehyde-3-phosphate dehydrogenase activity in normal epidermis showed statistically significant (P 0.01) individual variation which ranged from 3.4-4.3 moles per hour per kilogram dry weight of epidermis. The normal range of lactate dehydrogenase activity from 16.7-21.8 moles per hour per kilogram dry weight was not statistically significant (Table XV).

An initial increase of an average of 20% normal activity was found in the assays of GAPDH and of LDH in the epidermis irradiated by ultraviolet light. There was a rapid drop in GAPDH activity at 12 hours, and a significant decrease, 30-50% (P 0.05), was found at 48 hours after irradiation. There was about 10% decrease in LDH activity during the period of 12 to 48 hours after irradiation;

animals. These depressions of the enzyme activities were followed by maximal increases in both enzymes at 72 hours; thereafter the values of specific activity remained or decreased slightly until 168 hours (1 week) after irradiation: 40-140% (P 0.001) in G-6-PDH and 40-50% (P 0.001) increase in 6-PGDH activity.

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An initial increase of an average of 20% normal activity was found in the assays of GAPDH and of LDH in the epidermis irradiated by ultraviolet light. There was a rapid drop in GAPDH activity at 12 hours, and a significant decrease, 30-50% (P 0.05), was found at 48 hours after irradiation. There was about 10% decrease in LDH activity during the period of 12 to 48 hours after irradiation;

however, this value of decrease was not statistically significant. The greatest increase, 50% (P 0.001), in GAPDH was reached at 72 hours and was maintained until 1 week after irradiation. A continuous increase in LDH activity was observed after 48 hours and reached in 50% increase (P 0.001) at one week after irradiation.

C. The effect of UVL on tricarboxylic acid cycle enzymes (Table XIII and XIV, Figure 6)

The mean activity of 90 determinations of isocitrate dehydrogenase in the normal epidermis was 0.93 moles per hour per kilogram dry weight. The range of individual variation for this enzyme activity from 0.87-0.99 moles per hour per kilogram dry weight of epidermis was not statistically significant. The mean activity of 90 determinations of malate dehydrogenase in the normal epidermis was 13.3 moles per hour per kilogram dry weight with an insignificant range of 12.0-14.5 moles/hr/kg dry weight.

Ultraviolet radiation caused an initial and conclusive increase in the activities of enzymes participating in the pentose phosphate shunt and in the glycolytic cycle. In contrast to the above enzymes, there were no such increases in the activity of ICDH and MDH of tricarboxylic acid cycle. However, significant depression of ICDH was observed in the period from 12 to 48 hours after irradiation:

the greatest depression being 50% decrease (P 0. 001) of the normal level at 48 hours. A normal level of ICDH activity was recovered at 72 hours and remained until one week after irradiation which was the termination of this experiment. MDH seemed to be the least changeable enzyme by ultraviolet radiation. Practically no MDH response was measurable in the irradiated epidermis, except for only a short period of depression, 30% decrease (P 0. 001) of normal activity at 48 hours after irradiation.

D. Sequential enzymatic changes in the epidermis after ultraviolet irradiation

At 6 hours after UVL irradiation, G-6-PDH and 6-PGDH were enhanced by 20-30% increase in their activities, GAPDH and LDH were activated 20% more than in the normal, and ICDH and MDH showed no changes.

At 12 hours after UVL irradiation, the pentose phosphate shunt enzymes showed normal level of activity, glycolytic enzymes were depressed by 10-20% and TCA cycle enzymes also depressed by 10-30% of normal level of activity.

At 24 hours after irradiation, G-6-PDH activity was gradually increased and 6- PGDH activity was decreased by 10% while glycolytic and TCA cycle enzymes maintained the decreased level.

At 48 hours the pentose phosphate shunt enzyme showed gradual increases and glycolytic and TCA cycle enzymes had their greatest depressions.

At 72 hours the pentose shunt enzymes had 40-80% increased activities, GAPDH had an effect similar to the pentose enzymes, LDH had 10% increased activity, and TCA cycle enzymes recovered to the normal level of activity.

At one week after irradiation the pentose shunt enzyme maintained the increased activities, glycolytic enzymes had 40-50% increased activity, and TCA cycle enzyme maintained the normal level of activity.

TABLE IX

The effects of ultraviolet light on the activity of G-6-PDH.

Hour after irradiation	Animal number	non irradiated epidermis (A)*	Irradiated epidermis (B)*	(B)/(A)	P
6	1663	.58 ± .06	.60 ± .05	1.0	---
	1703	.36 ± .10	.56 ± .09	1.5	.01
	1781	.37 ± .03	.51 ± .10	1.3	.05
	Ave.	.44 ± .12	.56 ± .08	1.3	.001
12	1663	.75 ± .29	.75 ± .20	1.0	---
	1703	.48 ± .16	.40 ± .16	0.8	---
	1781	.47 ± .07	.57 ± .07	1.2	---
	Ave.	.57 ± .15	.57 ± .21	1.0	---
24	1663	.69 ± .07	.56 ± .07	0.8	.05
	1703	.41 ± .06	.61 ± .18	1.5	---
	1781	.56 ± .14	.63 ± .10	1.1	---
	Ave.	.55 ± .15	.60 ± .12	1.1	---
48	1663	.47 ± .14	.41 ± .18	0.9	---
	1703	.77 ± .05	.61 ± .12	0.8	.05
	1781	.58 ± .04	1.33 ± .15	2.3	.001
	Ave.	.61 ± .15	.78 ± .43	1.3	---
72	1663	.41 ± .12	.54 ± .15	1.3	---
	1703	.68 ± .05	1.20 ± .30	1.8	.01
	1781	.51 ± .04	1.24 ± .13	2.4	.001
	Ave.	.54 ± .13	.99 ± .38	1.8	.001
168 (1 wk.)	1663	.68 + .08	.98 ± .19	1.4	.05
	1703	.48 ± .10	.84 ± .13	1.8	.01
	1781	.48 ± .04	.96 ± .14	2.0	.001
	Ave.	.53 ± .13	.92 ± .16	1.7	.001

*Numerical figures express the enzyme activities, moles of substrate converted per kilogram of dry weight tissue per hour (moles/kg dry wt./hr.), and all values are the mean ± S.D. of 5 determinations.

TABLE X

The effects of ultraviolet light on the activity of 6-PGDH.

Hour after irradiation	Animal number	Non-irradiated epidermis (A)*	Irradiated epidermis (B)*	(B)/(A)	P
6	1663	.60 ± .16	.72 ± .18	1.2	---
	1703	.50 ± .14	.63 ± .08	1.2	---
	1781	.40 ± .02	.38 ± .04	1.0	---
	Ave.	.50 ± .14	.58 ± .18	1.2	---
12	1663	.69 ± .06	.46 ± .05	0.7	.001
	1703	.32 ± .04	.55 ± .12	1.7	.01
	1781	.42 ± .08	.38 ± .09	0.9	---
	Ave.	.48 ± .17	.46 ± .11	1.0	---
24	1663	.56 ± .03	.40 ± .07	0.7	.01
	1703	.39 ± .08	.36 ± .06	0.9	---
	1781	.44 ± .09	.43 ± .12	1.0	---
	Ave.	.46 ± .10	.40 ± .09	0.9	.05
48	1663	.40 ± .08	.23 ± .03	0.5	.001
	1703	.67 ± .12	.39 ± .08	0.6	.01
	1781	.71 ± .11	1.09 ± .12	1.5	.001
	Ave.	.62 ± .14	.57 ± .39	0.9	---
72	1663	.50 ± .11	.53 ± .12	1.1	---
	1703	.61 ± .16	.92 ± .14	1.5	.05
	1781	.57 ± .09	.86 ± .17	1.5	.05
	Ave.	.56 ± .12	.77 ± .22	1.4	.001
168 (1 wk.)	1663	.62 ± .06	.84 ± .06	1.4	.001
	1703	.57 ± .07	.68 ± .13	1.2	.01
	1781	.57 ± .04	.59 ± .06	1.0	--
	Ave.	.59 ± .06	.70 ± .13	1.2	.001

* Numerical figures express the enzyme activities, moles of substrate converted per kilogram of dry weight tissue per hour (moles/kg dry wt./hr.), and all values are the mean ± S.D. of 5 determinations.

TABLE XI

The effects of ultraviolet light on the activity of GAPDH.

Hour after irradiation	Animal number	Non-irradiated epidermis (A)*	Irradiated epidermis (B)*	(B)/(A)	P
6	1663	3.64 ± .60	4.41 ± .47	1.2	---
	1703	2.75 ± .85	3.57 ± .40	1.3	---
	1781	2.31 ± .30	2.57 ± .72	1.1	---
	Ave.	2.90 ± .81	3.51 ± .93	1.2	---
12	1663	3.17 ± .52	2.34 ± .56	0.7	.05
	1703	1.57 ± .18	1.65 ± .40	1.0	---
	1781	3.27 ± .65	2.66 ± .71	0.8	---
	Ave.	2.67 ± .93	2.22 ± .68	0.8	---
24	1663	6.88 ± .57	3.88 ± .79	0.6	.001
	1703	4.79 ± 1.70	4.84 ± 1.56	1.0	---
	1781	5.67 ± .49	5.90 ± .92	1.0	---
	Ave.	5.74 ± 1.29	4.87 ± 1.36	0.9	---
48	1663	3.76 ± .26	1.81 ± .23	0.5	.001
	1703	3.46 ± .49	2.53 ± .47	0.7	.05
	1781	3.62 ± .49	4.22 ± .45	1.2	---
	Ave.	3.61 ± .41	2.85 ± 1.10	0.8	.05
72	1663	3.80 ± .67	4.30 ± .93	1.1	---
	1703	3.67 ± .37	7.06 ± .80	1.9	.001
	1781	3.81 ± .31	6.07 ± .44	1.6	.001
	Ave.	3.76 ± .44	5.81 ± 1.37	1.5	.001
168 (1 wk.)	1663	4.27 ± .38	6.42 ± .23	1.5	.001
	1703	4.15 ± .38	5.25 ± 1.52	1.3	---
	1781	3.42 ± .90	5.05 ± .61	1.5	.01
	Ave.	3.95 ± .68	5.57 ± 1.08	1.4	.001

*Numerical figures express the enzyme activities, moles of substrate converted per kilogram of dry weight tissue per hour (moles/kg dry wt./hr.), and all values are the mean ± S.D. of 5 determinations.

TABLE XII

The effects of ultraviolet light on the activity of LDH.

Hour after irradiation	Animal number	Non-irradiated epidermis(A)*	Irradiated epidermis(B)*	(B)/(A)	P
6	1663	15.6 ± 2.7	17.7 ± 4.0	1.1	---
	1703	13.1 ± 2.7	20.4 ± 5.8	1.5	.05
	1781	11.6 ± 2.3	9.4 ± 1.4	0.8	---
	Ave.	13.4 ± 2.9	15.8 ± 6.2	1.2	---
12	1663	24.1 ± 2.1	23.4 ± 8.0	1.0	---
	1703	16.1 ± 5.3	13.7 ± 2.4	0.9	---
	1781	20.2 ± 4.4	16.1 ± 5.3	0.8	---
	Ave.	20.1 ± 5.1	17.8 ± 6.4	0.9	---
24	1663	22.2 ± 6.4	14.8 ± 2.8	0.7	.05
	1703	20.0 ± 9.5	24.7 ± 1.9	1.2	---
	1781	16.2 ± 4.1	19.7 ± 1.7	1.2	---
	Ave.	21.2 ± 6.1	19.4 ± 4.7	0.9	---
48	1663	26.5 ± 4.7	21.0 ± 5.3	0.8	---
	1703	17.5 ± 5.4	12.4 ± 3.2	0.7	---
	1781	19.4 ± 6.0	26.6 ± 7.4	1.4	---
	Ave.	21.1 ± 6.4	20.0 ± 7.9	1.0	---
72	1663	22.4 ± 4.5	16.7 ± 3.2	0.8	.05
	1703	18.5 ± 2.3	32.3 ± 6.1	1.7	.01
	1781	18.4 ± 2.1	19.7 ± 3.8	1.1	---
	Ave.	19.7 ± 3.5	22.2 ± 7.9	1.1	---
168 (1 wk.)	1663	20.1 ± 2.7	32.8 ± 8.4	1.6	.05
	1703	15.4 ± 5.1	22.3 ± 5.8	1.5	---
	1781	14.6 ± 2.7	22.1 ± 5.3	1.5	.05
	Ave.	16.7 ± 4.2	25.7 ± 8.0	1.5	.001

*Numerical figures express the enzyme activities, moles of substrate converted per kilogram of dry weight tissue per hour (moles/kg dry wt./hr.), and all values are the mean ± S.D. of 5 determinations.

TABLE XIII

The effects of ultraviolet light on the activity of ICDH.

Hour after irradiation	Animal number	Non-irradiated epidermis (A)*	Irradiated epidermis (B)*	(B)/(A)	P
6	1663	.84 ± .17	.89 ± .18	1.1	---
	1703	.78 ± .10	.91 ± .06	1.2	---
	1781	.64 ± .08	.60 ± .16	0.9	---
	Ave.	.76 ± .14	.80 ± .19	1.0	---
12	1663	1.08 ± .09	.55 ± .10	0.5	.001
	1703	.54 ± .09	.52 ± .06	0.9	---
	1781	.87 ± .10	.75 ± .07	0.9	---
	Ave.	.83 ± .24	.61 ± .12	0.7	.01
24	1663	.94 ± .12	.56 ± .07	0.6	.001
	1703	.81 ± .16	.64 ± .16	0.8	---
	1781	1.00 ± .09	.80 ± .14	0.8	.05
	Ave.	.91 ± .14	.67 ± .16	0.7	.001
48	1663	1.08 ± .32	.55 ± .14	0.5	.05
	1703	1.14 ± .23	.36 ± .09	0.3	.001
	1781	1.12 ± .11	.88 ± .23	0.8	---
	Ave.	1.11 ± .22	.60 ± .26	0.5	.05
72	1663	1.12 ± .19	.62 ± .06	0.6	.001
	1703	.94 ± .22	1.17 ± .26	1.2	---
	1781	1.01 ± .23	1.20 ± .29	1.2	---
	Ave.	1.02 ± .21	1.00 ± .34	1.0	---
168 (1 wk.)	1663	.96 ± .43	1.24 ± .22	1.3	---
	1703	1.00 ± .30	.92 ± .15	0.9	---
	1781	1.03 ± .11	1.17 ± .22	1.1	---
	Ave.	1.00 ± .18	1.11 ± .23	1.1	---

*Numerical figures express the enzyme activities, moles of substrate converted per kilogram of dry weight tissue per hour (moles/kg dry wt./hr.), and all values are the mean ± S.D. of 5 determinations.

TABLE XIV

The effects of ultraviolet light on the activity of MDH.

Hour after irradiation	Animal number	Non-irradiated epidermis(A)*	Irradiated epidermis(B)*	(B)/(A)	P
6	1663	14.0 ± 2.3	13.3 ± 4.4	1.0	---
	1703	11.7 ± 1.7	12.4 ± 2.4	1.1	---
	1781	11.1 ± 1.7	11.2 ± 1.0	1.0	---
	Ave.	12.3 ± 2.2	12.3 ± 2.9	1.0	---
12	1663	15.1 ± 5.5	16.9 ± 2.5	1.1	---
	1703	12.2 ± 1.0	13.3 ± 1.9	1.1	---
	1781	14.0 ± 4.4	12.0 ± 1.1	0.9	---
	Ave.	13.8 ± 4.0	14.1 ± 2.8	0.9	---
24	1663	14.4 ± 1.2	12.3 ± 1.2	0.9	.05
	1703	9.8 ± 3.9	11.8 ± 3.0	1.2	---
	1781	13.3 ± 2.8	13.9 ± 2.9	1.0	---
	Ave.	12.5 ± 3.3	12.7 ± 2.5	1.0	---
48	1663	14.8 ± 3.9	10.0 ± 2.8	0.7	---
	1703	12.7 ± 2.9	8.6 ± 1.7	0.7	.05
	1781	15.3 ± 1.7	11.4 ± 1.5	0.7	.01
	Ave.	14.3 ± 3.0	10.0 ± 2.3	0.7	.001
72	1663	11.0 ± 2.3	14.1 ± 3.4	1.3	---
	1703	16.1 ± 3.9	14.9 ± 1.8	0.9	---
	1781	12.5 ± 1.8	13.1 ± 1.7	1.0	---
	Ave.	13.2 ± 3.4	14.1 ± 2.4	1.1	---
168 (1 wk.)	1663	18.0 ± 5.0	15.4 ± 2.7	0.9	---
	1703	14.5 ± 3.2	9.7 ± 2.1	0.7	.05
	1781	14.6 ± 2.6	12.5 ± 2.0	0.9	---
	Ave.	15.7 ± 3.8	12.5 ± 3.2	0.8	.05

*Numerical figures express the enzyme activities, moles of substrate converted per kilogram of dry weight tissue per hour (moles/kg dry wt./hr.), and all values are the mean ± S.D. of 5 determinations.

TABLE XV

Analyses of variance on 90 determinations of each enzyme activity in the control epidermis of ultraviolet irradiation experiment.

G-6-PDH

Source	SS	df	MS	F
Between group	0.05	5	0.010	0.52* < 3.11
Within group	0.23	12	0.019	
Total	0.28	17		

6-PGDH

Source	SS	df	MS	F
Between group	0.02	5	0.004	0.04* < 3.11
Within group	0.12	12	0.01	
Total	0.14	17		

GAPDH

Source	SS	df	MS	F
Between group	18.3	5	3.66	6.6** > 5.06 (P 0.01)
Within group	6.6	12	0.55	
Total	24.9	17		

LDH

Source	SS	df	MS	F
Between group	123.6	5	24.72	1.2* < 3.11
Within group	231.6	12	19.30	
Total	355.2	17		

ICDH

Source	SS	df	MS	F
Between group	0.25	5	0.05	2.5* < 3.11
Within group	0.23	12	0.02	
Total	0.48	17		

MDH

Source	SS	df	MS	F
Between group	10.49	5	2.10	0.3* < 3.11
Within group	72.88	12	6.07	
Total	83.37	17		

* Individual variation is insignificant

**Individual variation is significant

Figure 6

Changing curves of enzyme activities in the epidermis after ultraviolet light irradiation.

The dotted lines indicate the means of each enzyme activity in the nonirradiated epidermis which has no significant individual variation (Table XV). In ordinate enzyme activity is expressed as moles per hour per kilogram dry weight epidermis. Abscissa indicates hours after UVL irradiation.

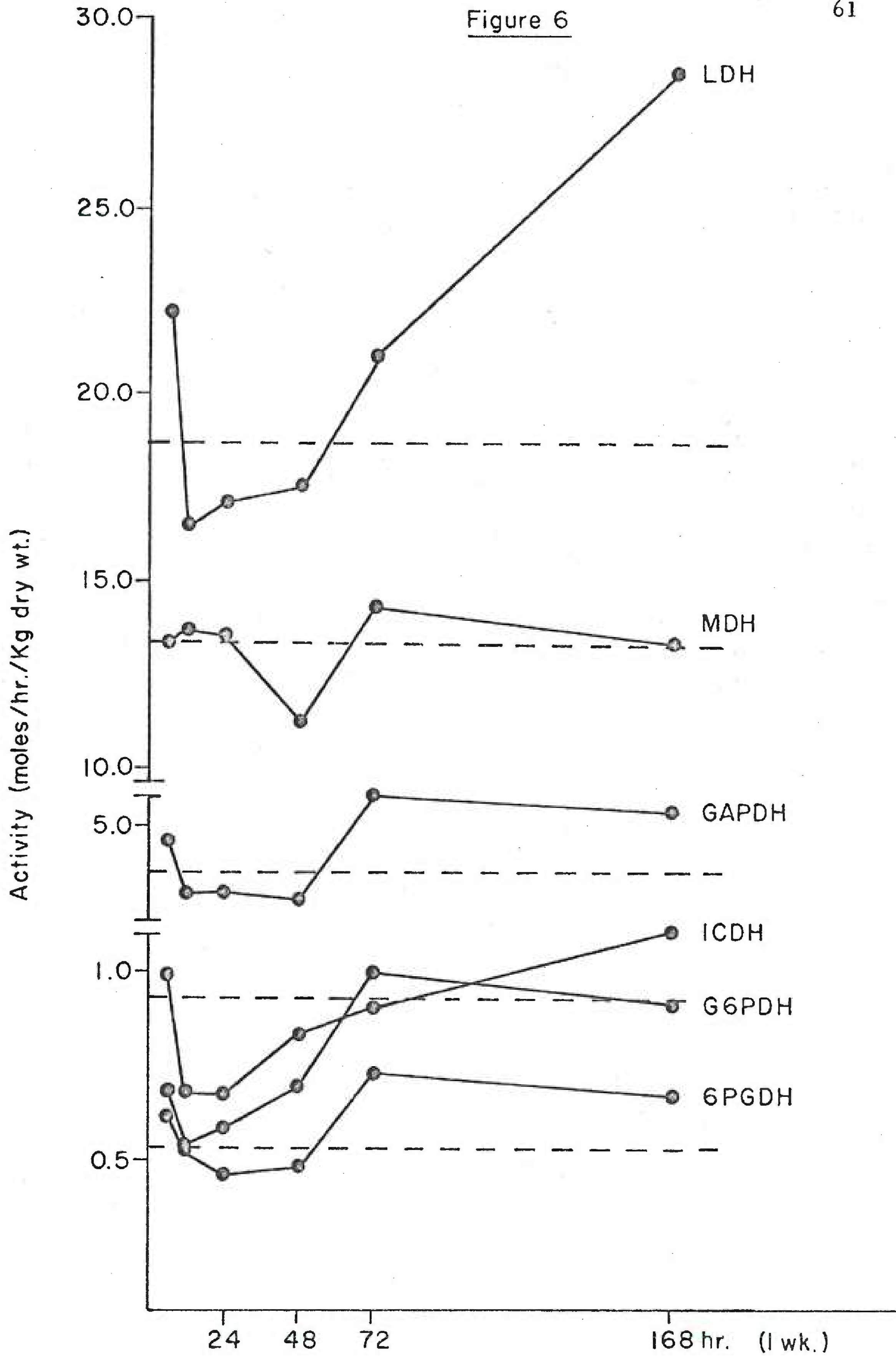


Figure 6
30.0
25.0
20.0
15.0
10.0
5.0
1.0
0.5
Activity (moles/hr./Kg dry wt.)
LDH
MDH
GAPDH
ICDH
G6PDH
6PGDH
24
48
72
168 hr.
(1 wk.)

III. Mechanical injury by scraping

All enzymatic changes during wound healing are listed in Tables XVI, XVII, XVIII, XIX, XX, and XXI and are summarized in Figures 7, 8, and 9. The enzyme activity of the control epidermis, which was taken from the adjacent area approximately 1 cm away from the wound site, showed remarkable variation and fluctuated during the wound healing as it did in the wounded epidermis. Therefore, the control tissue may not have been an appropriate control for the wounded tissue. However, these unusual variations in the control tissue may indicate that the epidermis adjacent to the wound may be involved in the process of wound healing.

A comparison of the data from the wound experiment with those from normal epidermis in the ultraviolet irradiation experiment showed that the enzymatic changes during the wound healing were within the normal ranges with the exception of glucose-6-phosphate dehydrogenase. The G-6-PDH activity of the control epidermis was in about the normal range, and a significant 40%-60% increase in activity was observed from the 2nd day on after wounding (Table XVI, Fig. 7).

TABLE XVI

Glucose-6-phosphate dehydrogenase activity of wounded and control epidermis expressed as moles substrate converted per hour per kilogram dry weight of epidermal tissue.

Day after wound	Animal #	Control epidermis (A)	Wounded epidermis (B)	(B)/(A)	P
1	1770	.26 ± .05*	.27 ± .08	1.0	---
	1799	.32 ± .22	.90 ± .42	2.8	.01
	1801	.21 ± .13	.21 ± .07	1.0	---
	Ave.	.27 ± .16	.57 ± .45	2.1	---
2	1770	.41 ± .04	.89 ± .34	2.2	.05
	1799	.67 ± .21	.63 ± .17	0.9	---
	1801	.54 ± .27	.85 ± .14	1.6	.05
	Ave.	.57 ± .22	.75 ± .24	1.3	.05
4	1770	.29 ± .14	1.01 ± .28	3.5	.01
	1799	.96 ± .20	.84 ± .19	0.9	---
	1801	.61 ± .10	.77 ± .14	1.3	---
	Ave.	.62 ± .31	.87 ± .22	1.4	.05
8	1770	.28 ± .16	.61 ± .14	2.2	.05
	1799	.47 ± .12	.68 ± .30	1.4	---
	1801	.49 ± .15	.74 ± .41	1.5	---
	Ave.	.42 ± .16	.68 ± .29	1.6	.05
14	1770	.45 ± .11	.65 ± .08	1.5	.05
	1799	.64 ± .03	.83 ± .13	1.3	.001
	1801	.60 ± .03	.85 ± .09	1.4	.001
	Ave.	.56 ± .10	.78 ± .13	1.4	.001
21	1770	.37 ± .07	.40 ± .15	1.1	---
	1799	.25 ± .06	.61 ± .23	2.4	.05
	1801	.76 ± .21	1.04 ± .35	1.4	---
	Ave.	.47 ± .27	.63 ± .33	1.3	---

*Figures represent the mean ± standard deviation of 5 determinations.

TABLE XVII

6-Phosphogluconate dehydrogenase activity of wounded and control epidermis expressed as moles substrate converted per hour per kilogram dry weight of epidermal tissue.

Day after wound	Animal #	Control epidermis (A)	Wounded epidermis (B)	(B)/(A)	P
1	1770	.25 ± .07*	.34 ± .06	1.4	---
	1799	.25 ± .04	.34 ± .10	1.4	---
	1801	.26 ± .03	.41 ± .28	1.6	---
	Ave.	.25 ± .05	.36 ± .16	1.4	.05
2	1770	.34 ± .05	.27 ± .05	0.8	---
	1799	.26 ± .09	.35 ± .04	1.3	---
	1801	.42 ± .06	.48 ± .06	1.1	---
	Ave.	.34 ± .09	.36 ± .10	1.1	---
4	1770	.21 ± .01	.47 ± .03	2.2	.001
	1799	.39 ± .07	.46 ± .06	1.2	---
	1801	.43 ± .08	.59 ± .07	1.4	.05
	Ave.	.34 ± .11	.51 ± .08	1.5	.001
8	1770	.20 ± .03	.32 ± .09	1.6	.05
	1799	.26 ± .05	.36 ± .04	1.4	.01
	1801	.23 ± .09	.49 ± .17	2.1	.05
	Ave.	.23 ± .06	.39 ± .13	1.7	.001
14	1770	.22 ± .04	.28 ± .04	1.3	---
	1799	.30 ± .03	.40 ± .08	1.3	---
	1801	.27 ± .05	.36 ± .04	1.3	.05
	Ave.	.26 ± .05	.35 ± .07	1.3	.01
21	1770	.35 ± .16	.28 ± .16	0.8	---
	1799	.24 ± .05	.24 ± .04	1.0	---
	1801	.40 ± .04	.45 ± .07	1.1	---
	Ave.	.33 ± .11	.33 ± .13	1.0	---

*Figures represent the mean ± standard deviation of 5 determinations.

TABLE XVIII

Glyceraldehyde-3-phosphate dehydrogenase activity of wounded and control epidermis expressed as moles substrate converted per hour per kilogram dry weight of epidermal tissue.

Day after wound	Animal #	Control epidermis (A)	Wounded epidermis (B)	(B)/(A)	P
1	1770	1.77 ± .39*	3.25 ± .24	1.8	.001
	1799	2.81 ± .67	3.80 ± 1.14	1.4	---
	1801	2.47 ± .45	3.90 ± .36	1.6	.001
	Ave.	2.35 ± .65	3.65 ± .71	1.6	.001
2	1770	1.60 ± .13	2.64 ± .54	1.7	.01
	1799	2.31 ± .27	3.50 ± .58	1.5	.001
	1801	3.57 ± .31	4.28 ± .70	1.2	---
	Ave.	2.49 ± .87	3.47 ± .89	1.4	.05
4	1770	3.25 ± .32	7.57 ± 1.19	2.3	.001
	1799	2.82 ± 1.25	6.75 ± .95	2.4	.001
	1801	3.94 ± 1.51	4.33 ± .78	1.1	---
	Ave.	3.46 ± 1.27	5.82 ± 1.72	1.7	.001
8	1770	2.14 ± .12	3.20 ± .74	1.5	.05
	1799	2.55 ± .73	5.76 ± 1.91	2.3	.01
	1801	3.11 ± .93	5.31 ± 1.06	1.7	.01
	Ave.	2.60 ± .76	4.87 ± 1.68	1.9	.01
14	1770	1.35 ± .36	1.77 ± .13	1.3	---
	1799	2.10 ± .28	2.60 ± .53	1.2	---
	1801	1.84 ± .50	3.20 ± .46	1.7	.01
	Ave.	1.76 ± .48	2.52 ± .71	1.4	.05
21	1770	1.97 ± .42	1.75 ± .85	0.9	---
	1799	1.32 ± .51	2.01 ± .30	1.5	.05
	1801	3.35 ± .77	2.96 ± .47	0.9	---
	Ave.	2.21 ± 1.03	2.19 ± .75	1.0	---

*Figures represent the mean ± standard deviation of 5 determinations.

TABLE XIX

Lactate dehydrogenase activity of wounded and control epidermis expressed as moles substrate converted per hour per kilogram dry weight of epidermal tissue.

Day after wound	Animal #	Control epidermis (A)	Wounded epidermis (B)	(B)/(A)	P
1	1770	12.2 ± 2.7*	17.3 ± 4.7	1.4	---
	1799	15.1 ± 4.2	13.6 ± 5.1	0.9	---
	1801	17.7 ± 4.5	20.6 ± 2.1	1.2	---
	Ave.	15.0 ± 4.3	17.2 ± 4.9	1.1	---
2	1770	8.7 ± 2.4	12.5 ± 1.5	1.4	.05
	1799	10.9 ± 1.0	10.1 ± 2.7	0.9	---
	1801	11.9 ± 3.5	19.9 ± 1.5	1.7	.01
	Ave.	10.6 ± 2.7	14.2 ± 4.6	1.3	.05
4	1770	8.3 ± .9	8.8 ± 3.4	1.1	---
	1799	10.2 ± 3.7	13.8 ± 1.1	1.3	---
	1801	16.7 ± 4.2	13.6 ± 3.9	0.8	---
	Ave.	12.1 ± 4.9	12.1 ± 3.7	1.0	---
8	1770	13.0 ± 2.7	10.9 ± 3.1	0.8	---
	1799	9.4 ± .9	17.2 ± 5.6	1.8	.05
	1801	8.9 ± 2.0	16.1 ± 2.8	1.8	.01
	Ave.	10.4 ± 2.6	14.7 ± 4.7	1.4	.05
14	1770	11.5 ± 3.9	12.3 ± 4.0	1.1	---
	1799	14.5 ± 3.6	12.0 ± 2.8	0.8	---
	1801	15.9 ± 2.7	16.2 ± 3.3	1.0	---
	Ave.	14.0 ± 3.7	13.5 ± 3.7	1.0	---
21	1770	9.9 ± 3.5	11.0 ± 3.2	1.1	---
	1799	11.7 ± 1.8	9.8 ± 2.4	0.8	---
	1801	19.6 ± 7.4	14.9 ± 1.6	0.8	---
	Ave.	13.5 ± 6.0	11.9 ± 3.2	0.9	---

*Figures represent the mean ± standard deviation of 5 determinations.

TABLE XX

Isocitrate dehydrogenase activity of wounded and control epidermis expressed as moles substrate converted per hour per kilogram dry weight of epidermal tissue.

Day after wound	Animal #	Control epidermis (A)	Wounded epidermis (B)	(B)/(A)	P
1	1770	.74 ± .08*	1.08 ± .36	1.4	---
	1799	1.08 ± .22	.82 ± .28	0.8	---
	1801	.93 ± .20	.95 ± .10	1.0	---
	Ave.	.92 ± .22	.95 ± .27	1.0	---
2	1770	.43 ± .08	.92 ± .10	2.1	.001
	1799	.81 ± .24	.76 ± .27	0.9	---
	1801	1.01 ± .25	1.08 ± .16	0.9	---
	Ave.	.83 ± .30	.89 ± .26	1.1	.05
4	1770	.68 ± .17	.90 ± .32	1.3	---
	1799	.89 ± .31	.93 ± .17	1.0	---
	1801	1.20 ± .25	1.13 ± .21	0.9	---
	Ave.	.93 ± .32	.99 ± .24	1.1	---
8	1770	.54 ± .06	.79 ± .15	1.5	.05
	1799	.77 ± .12	1.32 ± .10	1.7	.001
	1801	.92 ± .18	1.33 ± .28	1.5	.05
	Ave.	.74 ± .20	1.14 ± .31	1.5	.001
14	1770	.96 ± .18	.73 ± .06	0.8	.001
	1799	1.38 ± .25	1.18 ± .25	0.9	---
	1801	.95 ± .10	1.54 ± .17	1.6	.001
	Ave.	1.10 ± .25	1.15 ± .38	1.0	---
21	1770	.75 ± .16	.66 ± .18	0.9	---
	1799	.80 ± .17	.84 ± .19	1.0	---
	1801	1.07 ± .22	1.47 ± .28	1.4	.05
	Ave.	.87 ± .22	.99 ± .41	1.1	---

*Figures represent the mean ± standard deviation of 5 determinations.

TABLE XXI

Malate dehydrogenase activity of wounded and control epidermis expressed as moles substrate converted per hour per kilogram dry weight of epidermal tissue.

Day after wound	Animal #	Control epidermis (A)	Wounded epidermis (B)	(B)/(A)	P
1	1770	12.7 ± .9*	21.1 ± 3.7	1.7	.01
	1799	15.6 ± 1.3	14.8 ± 6.8	1.0	---
	1801	16.3 ± 2.3	15.1 ± 5.9	0.9	---
	Ave.	15.0 ± 2.2	17.0 ± 6.0	1.1	---
2	1770	10.6 ± 2.6	9.1 ± 1.9	0.9	---
	1799	10.5 ± 1.5	14.9 ± 1.0	1.4	.001
	1801	16.2 ± 1.6	20.8 ± 2.9	1.3	.05
	Ave.	12.4 ± 3.3	14.9 ± 5.2	1.2	---
4	1770	6.8 ± 1.2	6.9 ± 2.1	1.0	---
	1799	10.1 ± .8	15.7 ± 2.4	1.6	.01
	1801	13.2 ± 2.3	11.1 ± 1.1	0.8	---
	Ave.	10.0 ± 3.1	11.2 ± 4.1	1.1	---
8	1770	9.3 ± 1.9	10.3 ± 1.2	1.1	---
	1799	11.2 ± 2.0	19.9 ± 2.6	1.8	.001
	1801	13.8 ± 1.6	14.3 ± 1.5	1.0	---
	Ave.	11.4 ± 2.5	14.8 ± 4.4	1.3	.05
14	1770	9.2 ± 1.5	8.7 ± 2.0	0.9	---
	1799	14.5 ± 2.1	14.9 ± 4.1	1.0	---
	1801	15.3 ± 3.3	13.6 ± 2.5	0.9	---
	Ave.	13.0 ± 3.6	12.4 ± 3.9	1.0	---
21	1770	7.5 ± .9	8.1 ± 1.3	1.1	---
	1799	7.7 ± 1.4	6.8 ± 1.1	0.9	---
	1801	12.6 ± 3.7	11.8 ± 3.0	0.9	.01
	Ave.	9.3 ± 3.2	8.9 ± 2.8	1.0	---

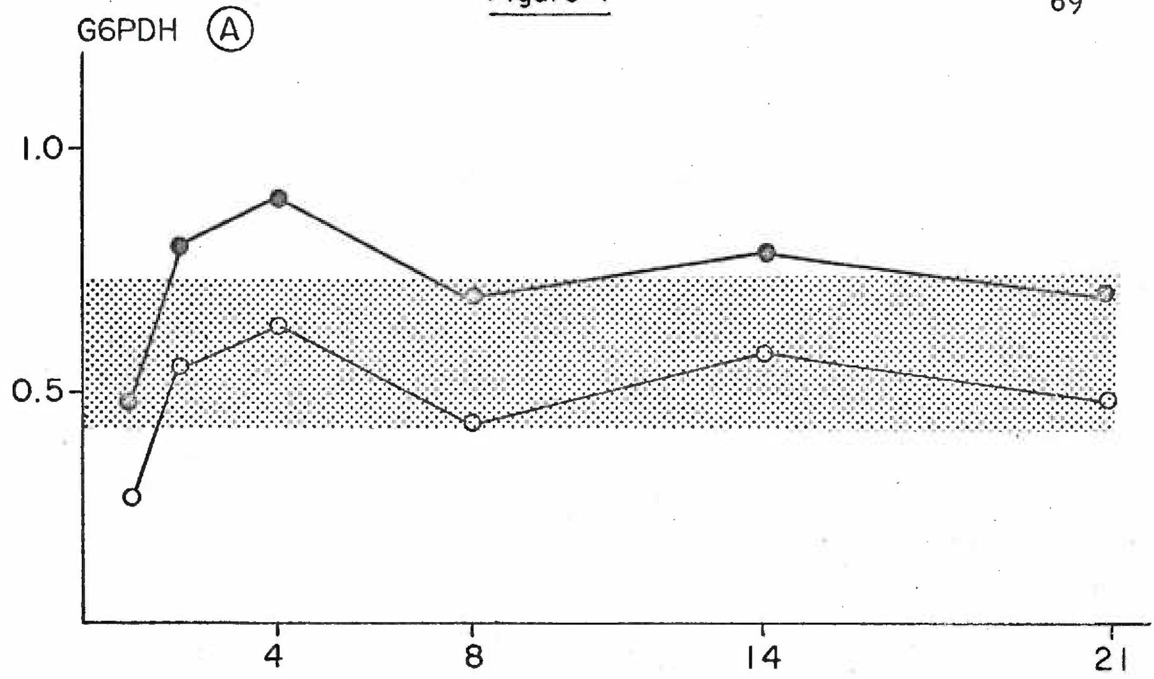
*Figures represent the mean ± standard deviation of 5 determinations.

FIGURE 7

Changing curves of the enzyme activities of the pentose phosphate shunt in the epidermis after wounding.

Changes in enzyme activity in the wounded (solid) and its adjacent epidermis (circle) are compared. The shaded area represents the normal range of each enzyme activity obtained in the UVL experiment. In ordinate the enzyme activity is expressed as moles per hour per kilogram dry weight tissue. Abscissa indicates the days after wounding.

Figure 7



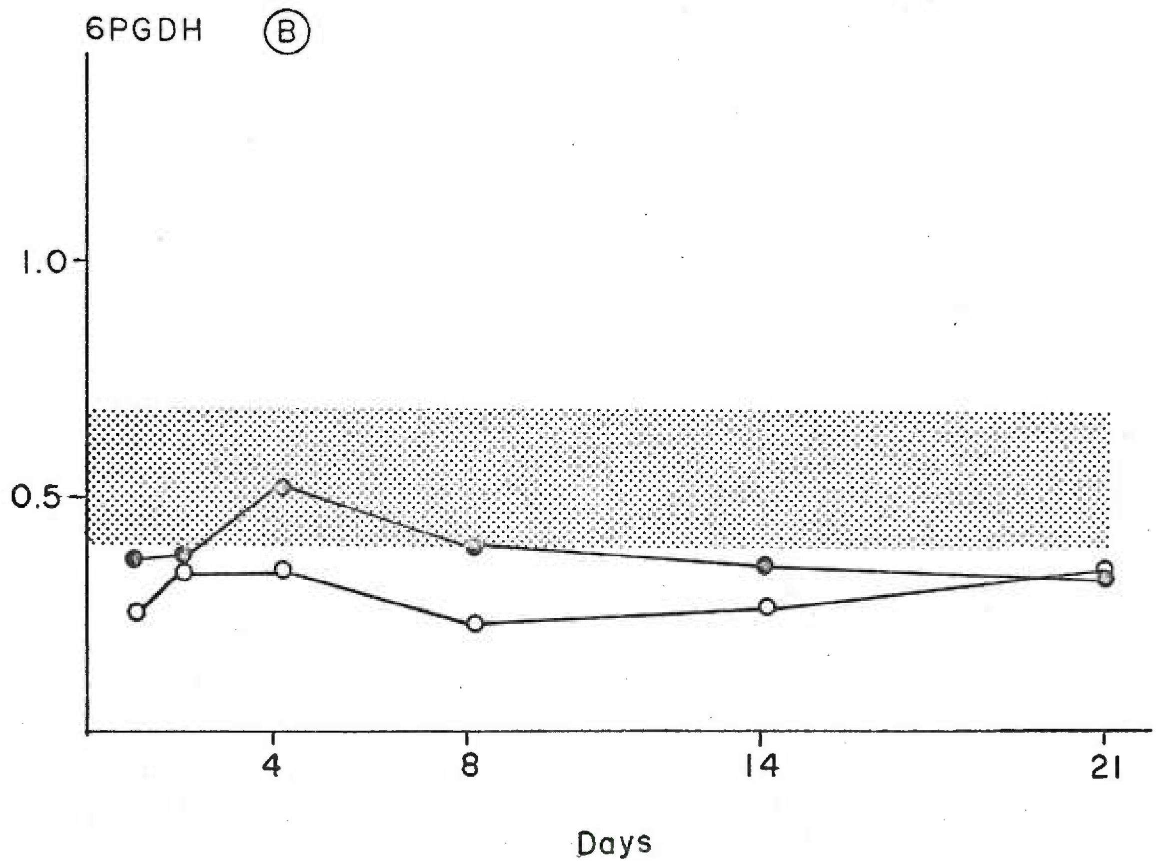
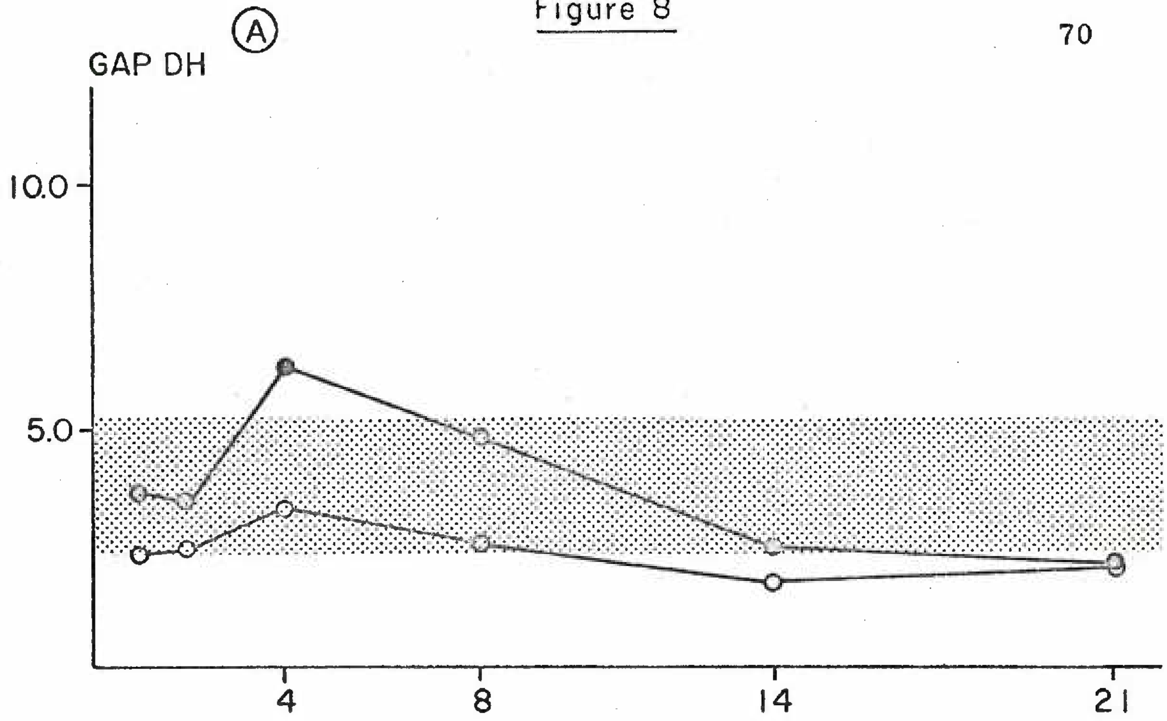


FIGURE 8

Changing curves of the glycolytic enzyme activities in the epidermis after wounding.

Changes in enzyme activity in the wounded (solid) and its adjacent epidermis (circle) are compared. The shaded area represents the normal range of each enzyme activity obtained in the UVL experiment. In ordinate the enzyme activity is expressed as moles per hour per kilogram dry weight tissue. Abscissa indicates the days after wounding.

Figure 8



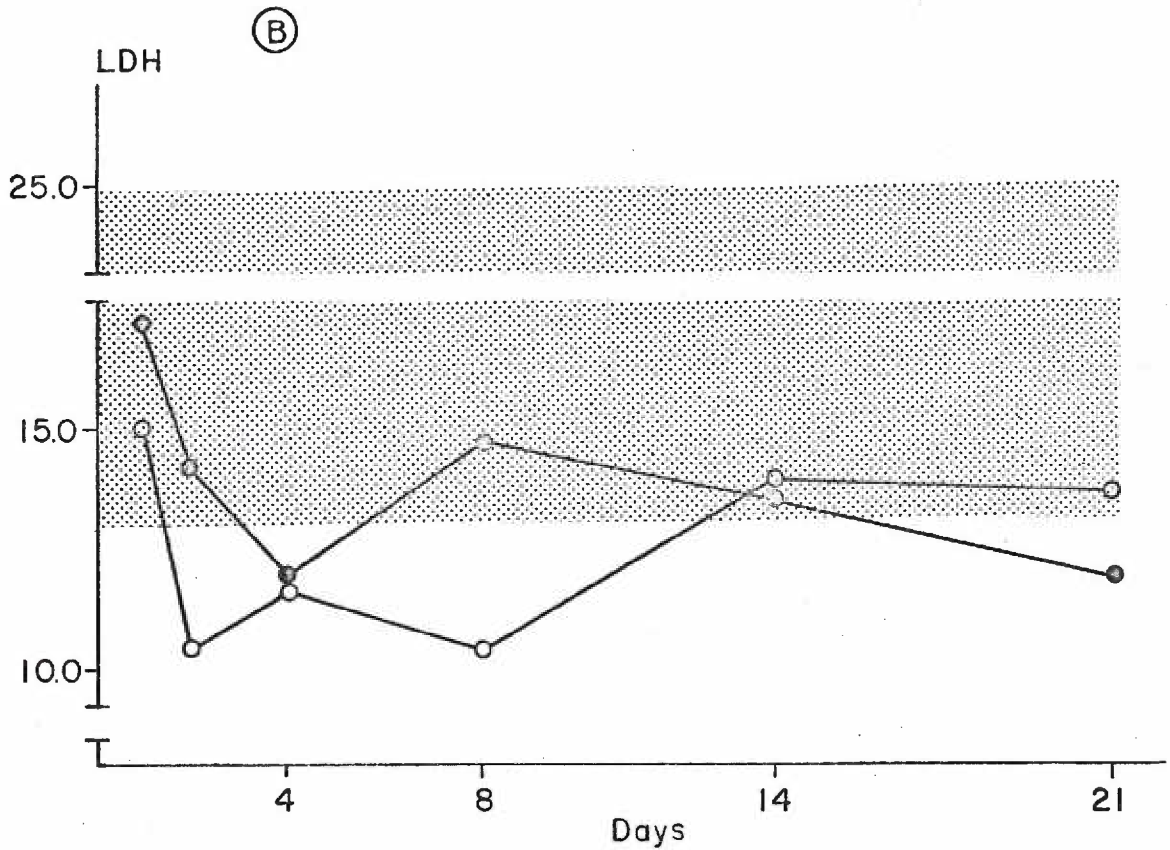
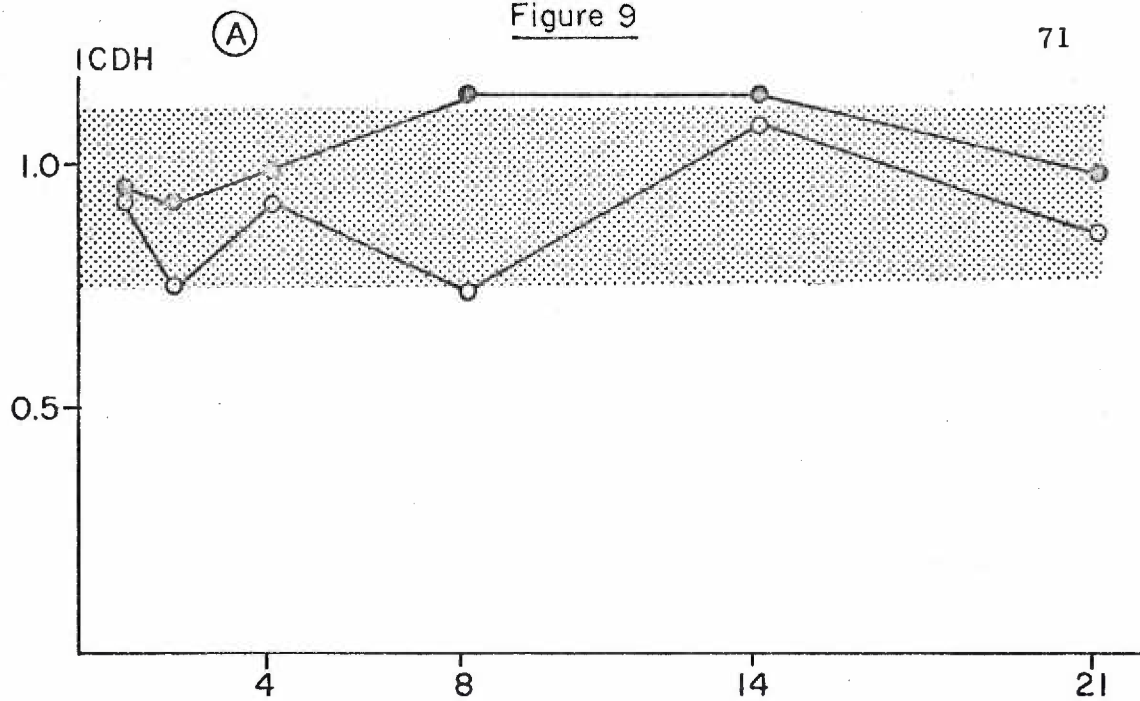


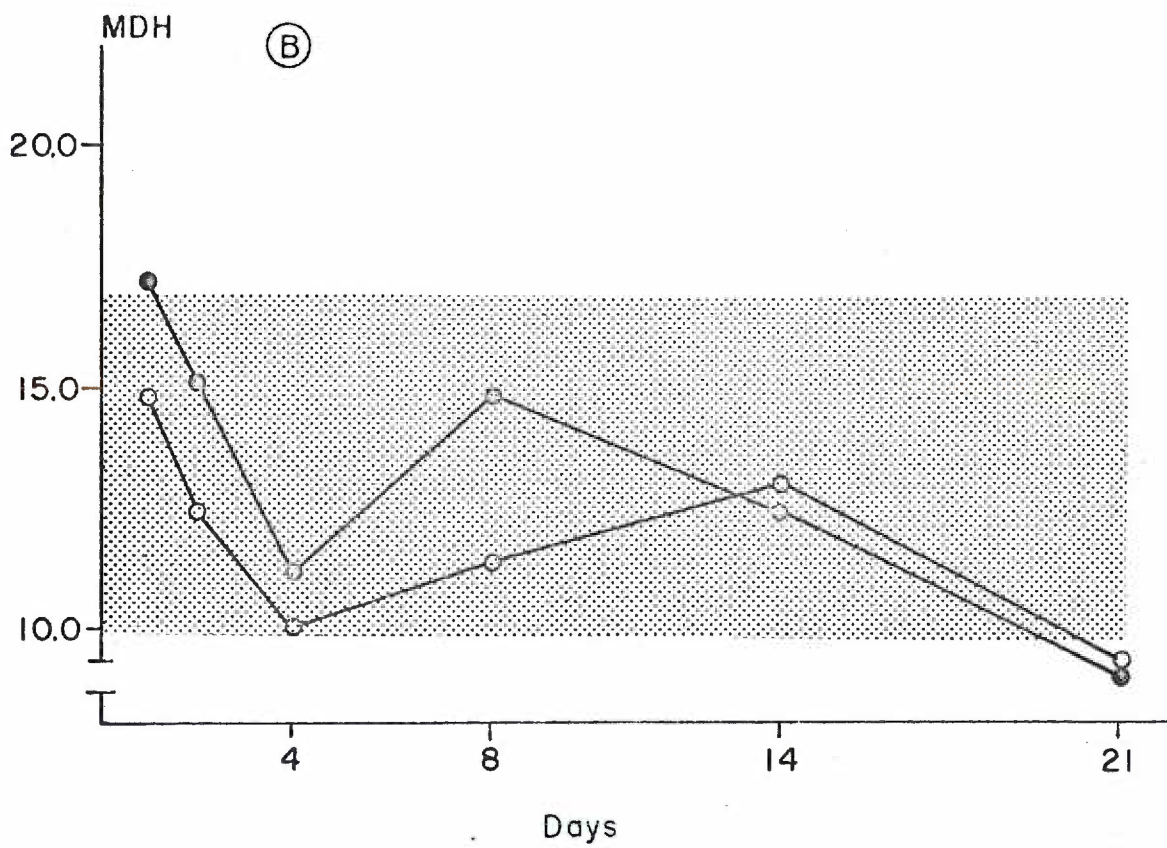
FIGURE 9

Changing curves of the TCA cycle enzyme activities in the epidermis after wounding.

Changes in enzyme activity in the wounded (solid) and its adjacent epidermis (circle) are compared. The shaded area represents the normal range of each enzyme activity obtained in the UVL experiment. In ordinate the enzyme activity is expressed as moles per hour per kilogram dry weight tissue. Abscissa indicates the days after wounding.

Figure 9





DISCUSSION

Glucose metabolism can be studied by investigating either the overall reaction or certain intermediate reactions or both. In this work, we evaluated some of the key enzymes participating in the intermediate reaction. All enzymes studied were analyzed under the optimal assay condition, determined beforehand by means of epidermal homogenates from normal animals as enzyme source. The enzyme activity assayed under the optimal condition (the maximal activity) cannot be used for a functional definition of the metabolic state but may be used for an interpretation of the metabolic potential in the experimental tissue _in vivo_.

The application of DMBA with dodecylbenzene on the skin produced erythema the first week, hyperkeratosis and fissuring with bleeding the first month, papilloma at 2-1/2 months, and keratoacanthoma at the eighth month. Therefore, the tumor and hyperplasia used in this study for enzyme assay can be considered to be severely "injured" tissue, and the degree of response of the epidermis to the damage concurs with present findings: that marked quantitative differences in the enzyme activity were found between experimental and normal tissues. These changes in enzyme activities possibly reflect increased rates of their

respective metabolic pathways, i.e., The Embden-Meyerhof, the pentose, and the TCA cycles. The remarkable changes in glucose-6-phosphate dehydrogenase and 6-phosphogluconate activities suggest that the pentose cycle contributes significantly to the tissue repair process. This provides a reasonable explanation for the glucose path in hyperplastic epidermis, i.e., that glucose is utilized by the active tissue not only to produce energy (ATP) but also to provide certain basic substances for nucleic acids and fatty acids synthesis. In the present experiment, however, no malignancy developed even after 1-1/2 years of treatment with the carcinogen.

The enzyme content and overall metabolism of tumors are known to be qualitatively the same but quantitatively different from those of normal tissues (89). The adaptation of an enzyme to the environment occurs readily in microorganisms, but it has been discovered only recently that mammalian enzymes also are capable of adaptation (90, 91, 92). Enzyme induction in animal tissue may be controlled by 2 mechanisms: 1) increase in the rate of enzyme synthesis; 2) decrease in the rate of enzyme degradation. For example, the accumulation of liver tryptophan pyrrolase (enzyme induction) may be brought about by either hydrocortisone (hormone)

through the first mechanism or by tryptophan (substrate) through the second mechanism (91). Both inducers acted as allosteric effectors to give an allosteric change in the enzyme molecule as described in the introduction. The results of increased enzyme activities observed in this study might indicate an in vivo enzyme induction in the process of epidermal repair; however, future study at the level of an enzyme-producing system is required to understand this process further. The site of protein synthesis at which the enzyme induction occurs appears to be at the transcription level from DNA to RNA (93); a carcinogen stimulated RNA polymerase activity in the nuclei of rat liver (94).

Since tumor tissue is characterized by an increased production of lactate, one may expect some increases in lactate dehydrogenase activity. Yet, lactate dehydrogenase and malate dehydrogenase activity resulted in little change in the tumor, and hyperplasia required a further study on the subunit level of these enzymes. Some changes in the isozyme components have been found in squamous cell carcinoma (95), malignant melanoma (96), thyroid and colon tumor (97, 98); LDH_1 (M, fastest migrating subunit) was increased and LDH_5 (H, slowest migrating subunit) remained unchanged or decreased slightly and thus changed the ratio of M to H subunit.

Changes in the ratio of M to H suggest direct evidence of the differential synthesis of proteins which may be under the differential regulation of genes (97).

It would be of great interest and importance to conduct similar micro-enzyme assays on the actively invading sites of malignant tumors (but not the malignant tumor as a whole). Since an increase in the pentose shunt activity in malignant tumor was reported (99, 100), one might observe greater increases in glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities in the highly malignant tissue than in benign hyperplastic epidermis. If this were the case, the microenzyme assay methods applied here would greatly facilitate the clinical diagnosis of early malignancy.

A biphasic change during recovery from the erythema produced by ultraviolet light was observed in most of the enzyme activities. The initial increases and subsequent decreases in enzyme activity during the first 48 hours in the UVL-irradiated epidermis may be due to a conformational change in the enzyme molecules by a photoactively produced electron which may provide a retention of enzyme catabolism at the initial stage. The increased enzyme activity in the later phase (3rd day - 1st week) may indicate an increased biosynthesis of enzyme or an increased population of the metabolically active basal cells.

The isocitrate dehydrogenase studied is NADP-dependent and is believed to be localized in the cytoplasm (89), and its increased activity in the "injured" epidermis is thought to be related to the generation of NADPH for the biosynthetic purpose.

SUMMARY AND CONCLUSIONS

1. Three types of injuries (chemical, physical, and mechanical) were produced experimentally on the epidermis of adult rhesus monkeys (*Macaca mulatta*). Fluorometric microenzyme assay with 0.5 - 2.0 μg tissue sample provided a sufficient measurement of enzyme activities in the limited area of the "injured" epidermis as well as in the normal.

2. Keratoacanthomas and epidermal hyperplasias were developed on the back skin of the rhesus monkey by a topical application of dimethylbenzanthracene (DMBA). These tissues produced contained the following enzyme activities quantitatively different from those of the normal epidermis:

a. There was no significant difference in enzyme activities between the tumor and the hyperplastic epidermis; this concurs with the nature of the tumor which is benign.

b. In the pentose phosphate shunt, glucose-6-phosphate dehydrogenase activity was 5x higher and 6-phosphogluconate dehydrogenase activity was 4 times higher in the tumor and hyperplastic epidermis than in the normal epidermis.

c. In glycolysis glyceraldehyde-3-phosphate dehydrogenase activity was increased 3-fold whereas lactate dehydrogenase activity

did not show any changes in the tumor and hyperplastic epidermis.

d. In the tricarboxylic acid cycle, a 3-fold increase in isocitrate dehydrogenase activity and little change in malate dehydrogenase activity were observed.

3. The epidermis injured by the 10 erythema doses of ultraviolet light responded with the following biphasic change in enzyme activity:

a. The enzymes of the pentose phosphate shunt in the irradiated epidermis were increased to 20-30% at 6 hours, decreased to 10% at 24 hours, and again increased in their activities up to 80% of the normal at 72 hours post-irradiation.

b. The enzymes of glycolysis were increased to 20% at 6 hours, decreased to 20% during 12-48 hours, and increased in activities to 50% of the normal.

c. The enzymes of tricarboxylic acid cycle showed no initial change at 6 hours but 30-50% decrease in activity during 12-48 hours, followed by 10-20% increase.

4. No dramatic changes in enzyme activity in the newly-formed epidermal tissue and its adjacent tissue were observed during wound healing.

5. In all types of epidermal injury, the response of enzymes participating in the pentose cycle, glucose-6-phosphate dehydrogenase in particular, was remarkable. Therefore, we speculate that in the process of epidermal repair the path of glucose might be shifted significantly via the pentose cycle so that the tissue would also provide basic substances for nucleic acids and fatty acids synthesis in addition to the energy (ATP).

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