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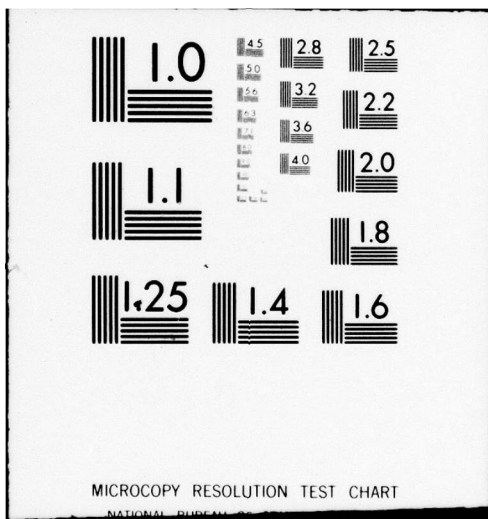
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A PORCINE BIOASSAY METHOD FOR ANALYSIS OF THERMALLY PROTECTIVE FABRICS: A HISTOLOGICAL AND BURN DEPTH GRADING SYSTEM.

Final report

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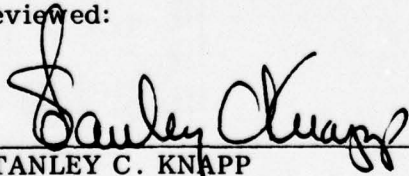
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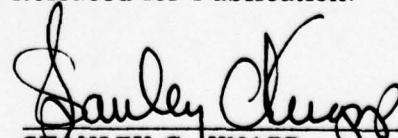


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20. ABSTRACT

A histopathological and burn depth grading system that can be employed in a porcine bioassay of thermal injury is described. Biopsy specimens taken from burn sites including both burned and normal skin were fixed in unbuffered formalin, embedded in Paraplast, sectioned at 6-7 microns, and stained using a Naval Aerospace Medical Research Laboratory modification of an Armed Forces Institute of Pathology hematoxylin and eosin method. Completed slides were graded by a pathologist using a scale of from 0 for no thermal damage, to 10 for thermal damage into the subcutaneous fat. Measurements of normal epidermal thickness (A), normal dermal thickness (B), and burned dermal depth (from the epidermal-dermal border down to the damaged/normal tissue border (D-C), were made using standard optical techniques. In order to account for swelling or shrinkage at the burn site, additional measurements including (D) dermal thickness at burn site, (E) total skin thickness at the burn site, and (C) burn depth as measured from the fat/dermal border up to the junction between normal and damaged skin were subsequently made. A burn depth, corrected for shrinkage, was then calculated as follows:

$$(A + B) - C [(A + B)/E] = \text{corrected burn depth.}$$

This shrinkage correction amounted to 40-50% in burns with histopathological burn grades of 9 or 10.

A set of photomicrographs, one for each histopathological grade, is presented as an aid to others attempting to use this method.

The method provided the quantitative burn depths required for mathematical model development but is somewhat tedious to be used in screening thermally protective fabrics.

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PREFACE

The vivarium of the U. S. Army Aeromedical Research Laboratory (USAARL) is fully accredited by the American Association for Accreditation of Laboratory Animal Care.

The animals used in this study were procured, maintained, and used in accordance with the Animal Welfare Act of 1970 and AR 70-18. In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Resources, National Academy of Sciences-National Research Council. Humane procedures were utilized throughout, and a graduate veterinarian was in constant attendance to perform all surgical procedures and to insure that all animals were fully anesthetized and insensitive to pain.

All authors were research investigators at the USAARL during the conduct of the experiments described herein.

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INTRODUCTION

The bioassay method for evaluation of thermally protective fabrics subjected to conflagration was developed at the U. S. Army Aeromedical Research Laboratory (USAARL) in order to provide medically sound information upon which to base judgments regarding the relative protective capabilities of new candidate fabrics (Knox, Wachtel, McCahan 1978). Other investigators have used a similar approach to study the effect of thermal radiation from simulated atomic explosions (Mixer and Pearse 1953). In both cases, pigs were used as human skin analogs, and burn severity was determined by clinical and histopathological grading systems.

We have described the clinical grading system for a porcine bioassay model and have utilized the grading system for evaluating competitive fabrics (Wachtel, Knox, McCahan 1978). More recently, we have used the porcine bioassay technique to establish a burn data base to be used in calibrating thermal sensors and in developing a computerized burn model which will accurately convert time-heat flux or time-temperature histories into predicted burn depths (Knox, Wachtel, Knapp 1978a).

This report describes the histopathological and burn depth grading system which has evolved during development of the porcine bioassay of thermal damage. No attempt is made herein to describe the correlation between the clinical and histopathological grading systems.

METHODS AND MATERIALS

ANIMALS

One hundred forty-seven white domestic swine weighing 43 ± 8 kg were procured, quarantined, freed of internal and external parasites, and verified to be healthy prior to use in this study. The swine were fasted overnight, premedicated with Atropine (0.04 mg/kg) and fentanyl-droperidol (0.1 ml/kg) or phencyclidine hydrochloride (100 mg) and chlorpromazine hydrochloride (50 mg), intubated, and anesthetized with halothane USP or

methoxyflurane (McCahan and Wachtel 1972) (Ragan and Gillis 1975). All hair was clipped closely with a #40 clipper head (Wachtel and McCahan 1973). When the cutaneous sensation had disappeared (determined by the scratch test), the animal was transported from the vivarium to the test site on a specially constructed transporting device. The experimental animal was maintained in Stage III anesthesia during exposure to the thermal source. The room temperatures were stable during exposure of any one pig, but were not constant during an entire day nor over the several months of data collection. Thus, skin temperatures were an uncontrolled variable.

THERMAL SOURCE AND SHUTTER SYSTEM

The thermal source consisted of a flame gun (modified gun type-conversion oil burner using kerosene fuel) set to deliver 14 ± 0.5 BTU/ft²/sec (Knox and others 1971), or a furnace (modified NASA-Ames T-3 furnace using JP-4 fuel) set to deliver from 0.7 to 3.92 cal cm⁻² sec⁻¹ (Knox, McCahan, Wachtel 1974) (Knox, Wachtel, Knapp 1978b). Each animal was protected from the thermal source by a shutter system and template (Knox and others 1971) (Knox, McCahan, Wachtel 1974). The animal was placed against a laminated wood-transite (or double transite) template with circular, countersunk openings (1½ - 2 inches in diameter) which defined the exposure sites and areas. The time of exposure was controlled by an electrically activated, pneumatically (and gravity and spring also) driven shutter and ranged from 0.5 to 15 seconds (Knox and others 1971) (Knox, McCahan, Wachtel 1974). Each animal received from 12 to 14 burn sites so that a total of over 1,700 sites were evaluated for this study.

DOCUMENTATION

The severity of the resultant cutaneous burn lesions was evaluated immediately and at 24 hours postburn using photographic techniques (still color photographs at constant focal length and light source and 16 mm high-speed motion pictures) and clinical observations. Details of the clinical grading system have been reported (Wachtel, Knox, McCahan 1978).

Excisional biopsies were taken from each burn site including both normal, undamaged skin and damaged skin typical of the lesion. These tissue specimens were fixed in unbuffered 10% formalin and forwarded to

the Veterinary Pathology Department of the Naval Aerospace Medical Research Laboratory, Pensacola, Florida, for processing.

HISTOLOGICAL PROCEDURES

A pathology accession number was assigned to each formalin fixed skin specimen upon delivery to the pathology laboratory and was used to identify each specimen processed in the histopathology laboratory. Each specimen, together with its identification number, was placed in a tissue capsule in a receptacle basket which was attached to the autotechnicon. The tissue was then processed according to the following schedule:

80% alcohol	1 hour
95% alcohol	2 hours
95% alcohol	1 hour
95% alcohol	1 hour
95% alcohol	1 hour
95% alcohol	1 hour
100% alcohol	1 hour
100% alcohol	2 hours
Xylene	2 hours
Xylene	1 hour
Paraplast	1 hour
Paraplast	1 hour

The Paraplast in the autotechnicon was maintained at 60° C.

The autotechnicon was loaded and turned on at the end of the day. The tissues were then processed automatically during the night and were ready for embedding the following morning. The tissues were embedded in Paraplast in square embedding molds, and the identification number was written on the mold. After cooling, the blocks were removed from the molds.

The blocks were then sectioned on a rotary microtome at 6-7 microns, with the sections being floated on a 57° C water bath containing gelatin. The gelatin facilitates adherence of the sections to the glass slides. Satisfactory sections were picked from the water bath and placed on the microscope slides. The case number was recorded on the slide. The slides were then placed on the warming plate for one hour, at which time they were

placed in a metal staining rack and stained using the method developed at the Armed Forces Institute of Pathology (AFIP Manual of Histologic and Special Staining Technique, 2d Ed) as modified by the Naval Aerospace Medical Research Laboratory in the following manner:

Xylene	10 minutes
Xylene	5 minutes
Absolute alcohol	5 minutes
Absolute alcohol	5 minutes
95% alcohol	5 minutes
Distilled water wash	
Hematoxylin Harris	10 minutes
Wash in tap water	
Blue in ammonia water	10 dips
Tap water wash	
Alcoholic eosin	1 minute
95% alcohol	5 minutes
Absolute alcohol	5 minutes
Absolute alcohol	5 minutes
Xylene	5 minutes
Xylene	5 minutes

When the slides were placed in a new solution, they were rinsed up and down five to six times. When taken from the solution, excess was allowed to drain from the rack prior to being placed in the next solution.

After staining was completed, the slides were coverslipped, using Permunt as the mounting medium.

The following procedures were employed in making the staining solutions:

Hematoxylin Harris

Used "as is" from container

Alcoholic Eosin

Eosin Y, water soluble	2 grams
Distilled water	160 ml
Alcohol, 95%	640 ml
Glacial acetic acid	2 ml

Ammonia Water

NH ₄ OH	1 ml
Distilled water	500 ml

CLASSIFICATION OF BURNS

Pathology of Local Hyperthermia

According to Anderson (1966) and Jobb and Kennedy (1970), burns are generally classified according to the depth of injury.

The earliest evidence of hyperthermia is functional, with small blood vessels and capillaries dilating. As the capillary walls become more permeable, the fluid components of the blood leave the vessel and enter the interstitial spaces, with resultant edema. This causes vesiculation if the fluid collects between the epidermis and the dermis.

Cellular damage is first evidenced by a redistribution of the fluid and solid components of the nuclei, followed by nuclear swelling due to imbibition of fluid, rupture of the nuclear membranes, and, finally, pyknosis. The cytoplasm of thermally injured cells becomes at first granular and later homogeneously coagulated. The collagen tends to lose its fibrillar character and to take on the appearance of a dense and more or less homogeneous gel. There is a fall in pH of the injured cells as is indicated by their increased affinity for basic stains (Eosin).

Heat is absorbed such that the epidermis is the first and most severely injured. First degree burns are manifested by erythema and edema with no morphological sign of injury to the epithelial cells. However, after a few days, the surface may desquamate. In second degree burns, the epidermis is destroyed without significant irreversible damage to the dermis. Vascular changes are prominent and vesicles form in and beneath the epidermis. These may contain serum, cellular debris, and leukocytes and may suppurate or rupture quickly. At high heat flux levels, tissue water boils resulting in the typical "steam bleb" upon gross surface examination. The cytoplasm of the epithelial cells is coagulated and the nuclei are shrunken or ruptured.

Damaged epithelium becomes distinctly acidophilic. New epithelium is derived from the margins of the burned area and from the underlying hair follicles.

Third degree burns show sufficient damage to the dermis, with coagulation of part of the connective tissue, blood vessels, and adnexa, as to interface with regeneration. Heat of sufficient intensity or duration to penetrate this deep dessicates and chars the outer dermis. An amorphous agglomeration is produced causing coagulation of the epidermis and dermis. This necrotic tissue sloughs and the defect is filled in by granulation tissue. Permanent scarring with loss of adnexa results. Fourth degree burns are similar in character to third degree but penetrate below the dermis to and beyond the subcutaneous fascia.* The preceding criteria were used to judge the degree of burn to the skin specimens.

Grading

The biopsies were graded microscopically according to the scheme developed by the University of Rochester (Lyon, Davis, Pearse 1955). Whenever the depth of burn was not uniform, the maximum depth of tissue damage was used in the grading. A grade of zero was given to all tissue sections showing no thermal damage. Burned tissue was categorized into epidermal, transepidermal, dermal, and adipose. Epidermal burns were subclassified as to whether cellular change without acidophilism was present (grade 1), whether acidophilism was present involving only partial depth of the epidermis (grade 2), or all the epidermal depth (grade 3). Transepidermal burns (those showing separation between the epidermis and the dermis) were subcategorized as to whether the separation was focal (grade 4) or complete (grade 5). Dermal burns were classified as to whether the damage was superficial, mid, deep, or complete (grades 6, 7, 8, and 9 respectively). Grade 10 was assigned to burns extending beyond the dermis into the adipose tissue. The grades with associated descriptions are summarized in Table 1 (page 11).

*Classification of burns into degrees is being replaced by a classification using "partial" or full thickness where the latter implies that the dermal appendages from which re-epithelialization occurs have been destroyed; thus, necessitating grafting.

TABLE 1
HISTOPATHOLOGICAL AND BURN DEPTH GRADING DEFINITIONS

Grade	Description	Approximate Depth
0	No thermal damage	0
1	Cell damage without acidophilism	1-20 μ
2	Partial epidermal acidophilism	20-50 μ
3	Complete epidermal acidophilism	50-100 μ
4	Partial dermal-epidermal separation	100-150 μ
5	Complete dermal-epidermal separation	150-250 μ
6	Superficial dermal	250-500 μ
7	Mid dermal	500-1000 μ
8	Deep dermal	1000-1500 μ
9	Complete dermal to adipose border	1500-2000 μ
10	Adipose	>2000 μ

In addition to the numerical grades assigned to each section, linear measurements were made on the normal epidermal thickness (A), the normal dermal thickness (B), and the thickness of the burned dermis (D-C), where applicable (see Figure 1, page 13). The measurements were made using an ocular micrometer disc calibrated with a stage micrometer. The measurements involving the epidermis were made from the top of the keratinized layer to the bottom of the Malpighian layer. This was always made above one of the dermal papillae, since this area gave the most consistent readings. Dermal measurements were made from the epidermal-dermal junction to the subcutaneous tissue. The burned dermis was measured from the epidermal-dermal junction to the bottom of the damaged tissue.

In the earliest experiments, it was noticed that tissue shrinkage was a problem; so the depth measurement scheme was restructured to include "burn depth" (C) measured from the damaged/undamaged boundary to the fat/dermal boundary and dermal thickness at the burn (D) and total skin

thickness at the burn (E). A burn depth, corrected for thermal shrinkage was then calculated as follows:

$$(A + B) - C[(A+B)/E] = \text{corrected burn depth}$$

RESULTS

The animals tolerated the small burn sites without systemic or unusual local effects. The experimental design worked well for acquiring excisional biopsy material from the burn sites for histopathological and burn depth determination.

Microscopic examination of the skin specimens revealed damage ranging from none (Grade 0) in control biopsies, to almost fourth degree burn (Grade 10) in unprotected 7.0-15 second exposures.

As an aid to those who might wish to employ this method in the future, the following set of figures is presented representing various increasing levels of burn severity.

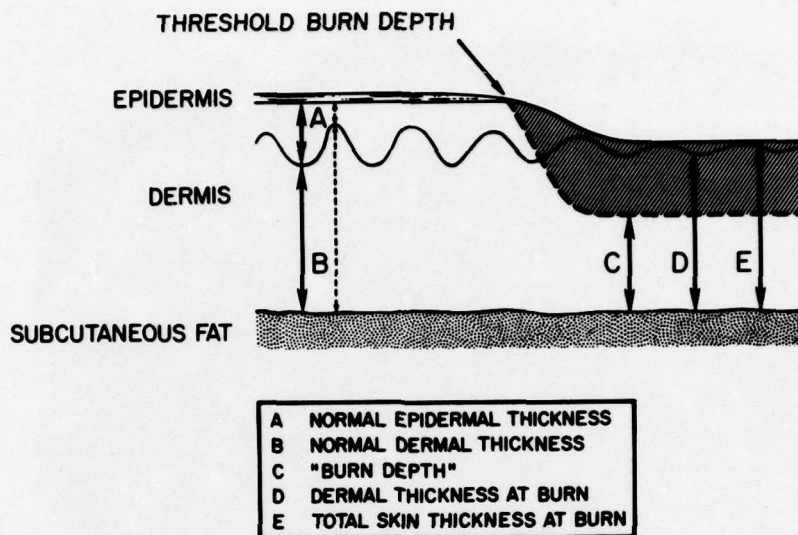


FIGURE 1. A diagrammatic view of a biopsy specimen showing the shaded burn area and the location of the depth measurements. The measurements shown are the latest version which allows corrections for tissue swelling and shrinkage to be made.

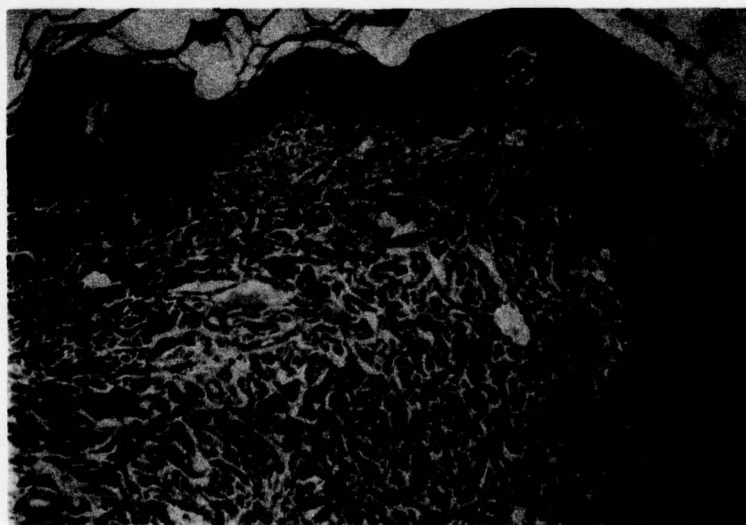


FIGURE 2. Normal porcine epidermis with well defined capillary loops in dermal papillae (arrow) H&E stain 400 x mag.

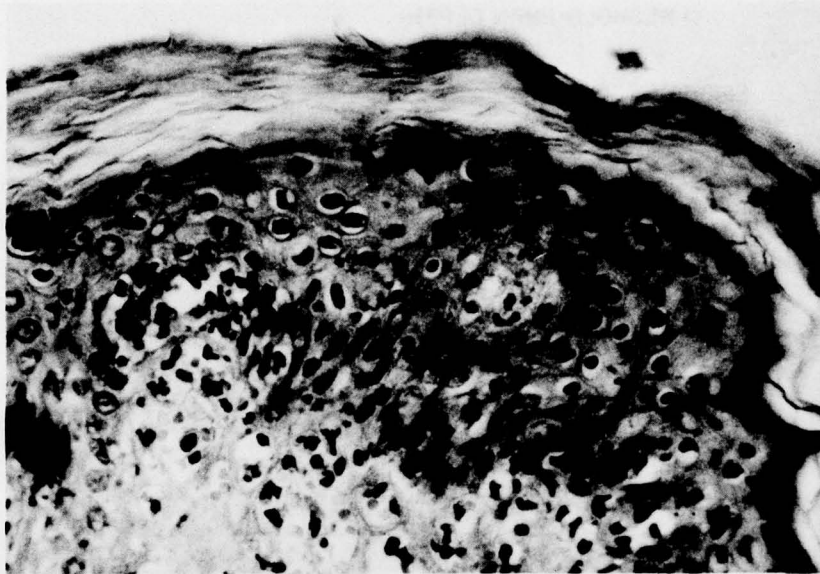


FIGURE 3. Close-up view of epidermis showing swollen nuclei typical of Grade 1 burn. H&E stain approximately 800 x mag.

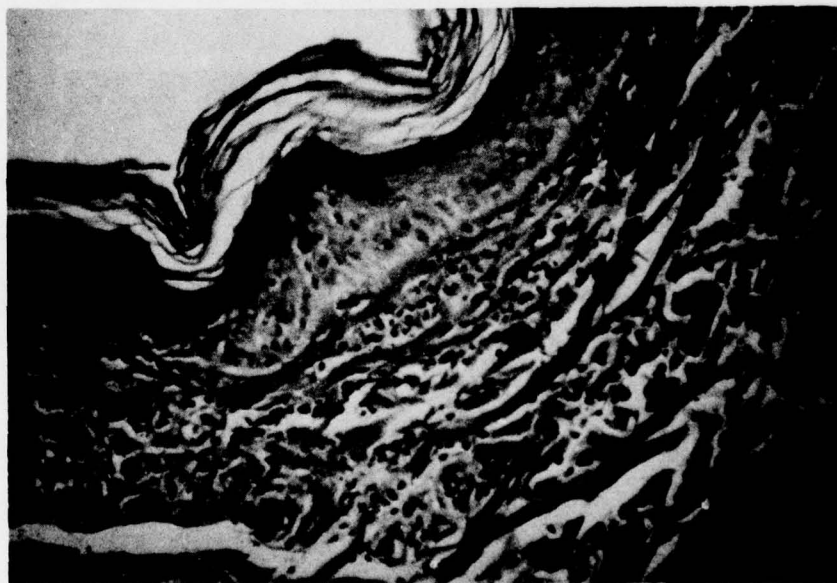


FIGURE 4. Partial epidermal acidophilism (eosinophilism) typical of Grade 2. H&E stain approximately 500 x mag.

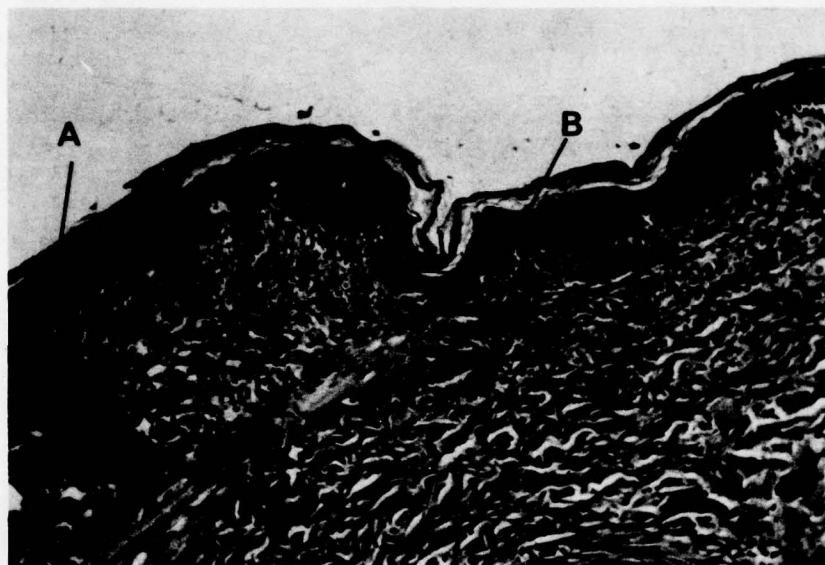


FIGURE 5. Range of burn grades from 0 (normal) at A to 3 at B. H&E stain approximately 400 x mag.

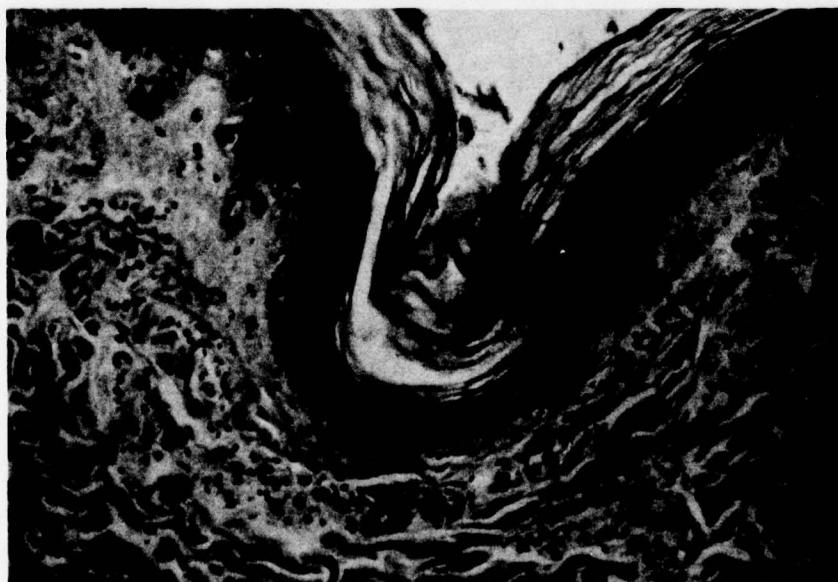


FIGURE 6. Complete epidermal acidophilism (eosinophilism), Grade 3. H&E stain 500 x mag.

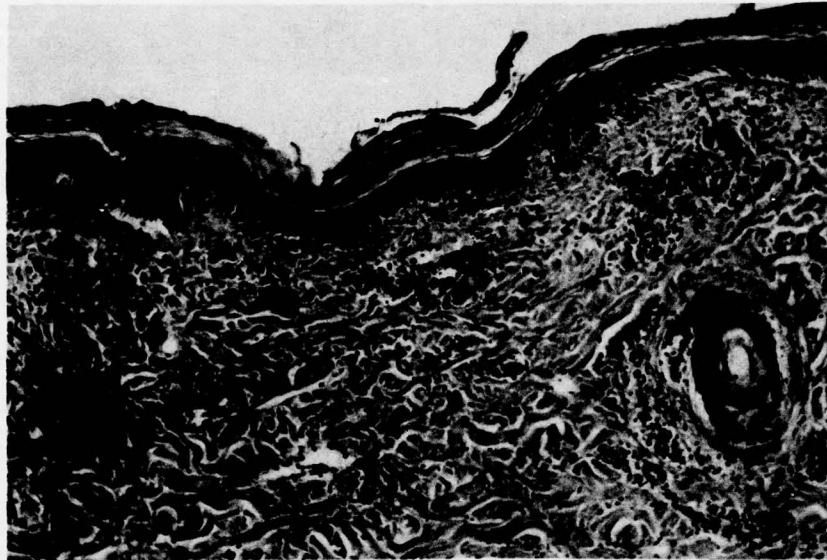


FIGURE 7. Partial epidermal-dermal separation (arrows) Grade 4. Still no dermal changes. H&E stain 400 x mag.

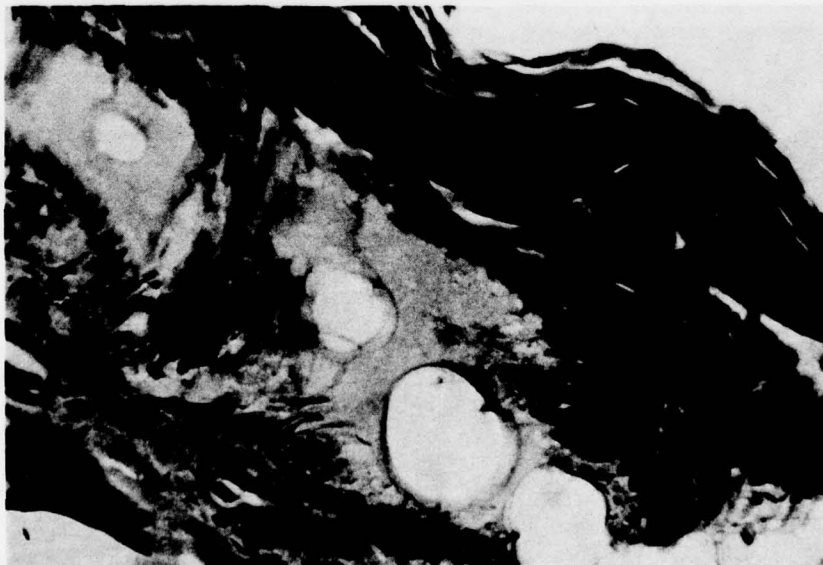


FIGURE 8. Close-up of an area of partial epidermal-dermal separation showing fluid collection, steam vacuole, and cell distortion. H&E stain approximately 800 x mag.



FIGURE 9. Some partial but mostly complete epidermal-dermal separation characteristic of Grade 5. H&E stain 400 x mag.

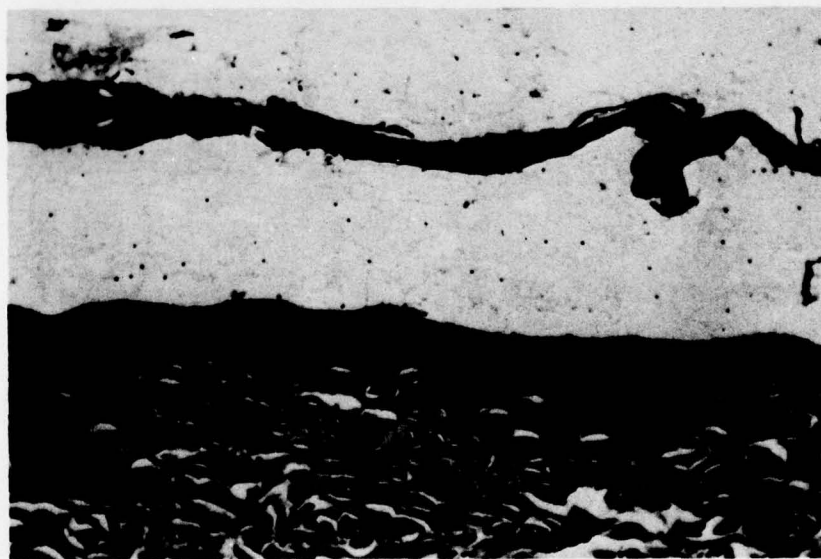


FIGURE 10. Complete separation with superficial dermal damage. Characteristic of a mild Grade 6. H&E stain 400 x mag.

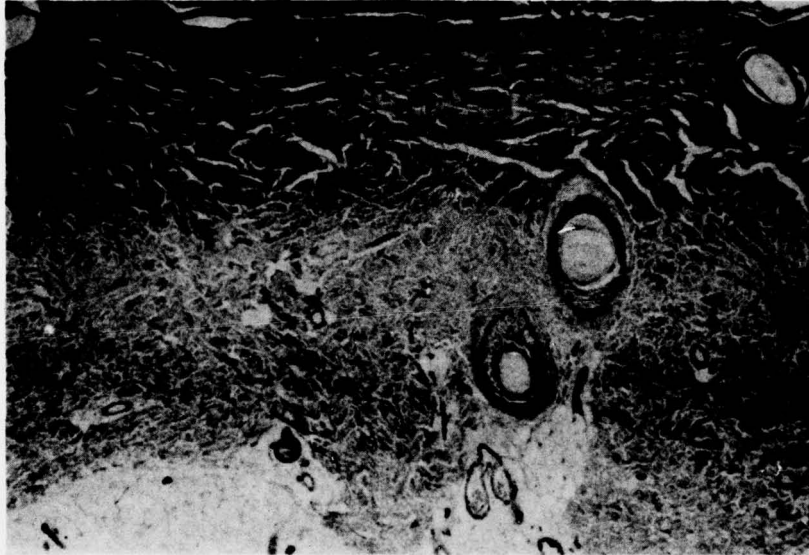


FIGURE 11. Superficial to mid dermal damage characteristic of very deep Grade 6 or Grade 7. H&E stain 100 x mag.

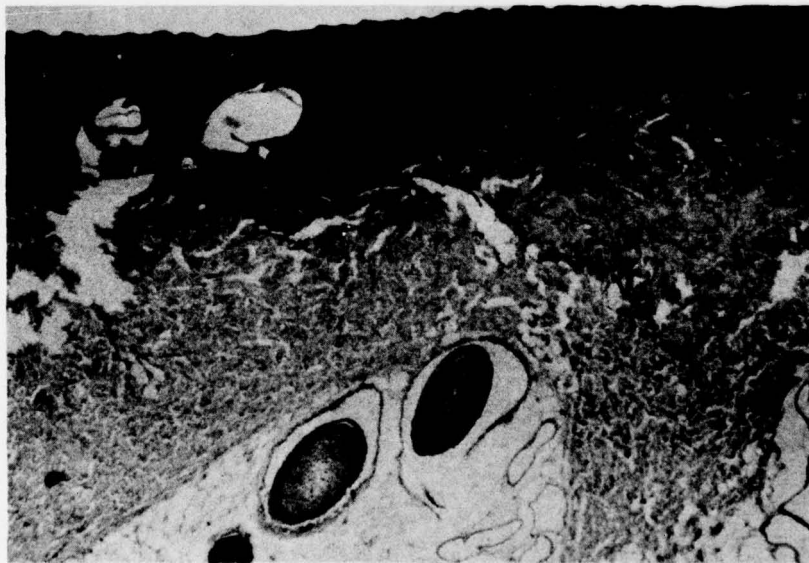


FIGURE 12. Deep dermal burn characteristic of Grade 8. Note fat dermal border is not even but distorted by the presence of hair shafts here cut in cross section. H&E stain 100 x mag.

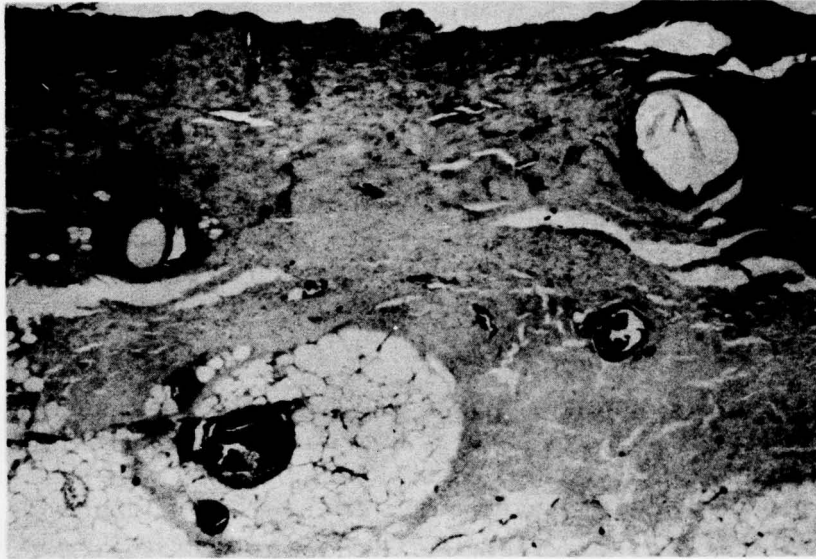


FIGURE 13. Complete dermal to adipose border with no changes in underlying adipose tissue. Grade 9. H&E stain 100 x mag.

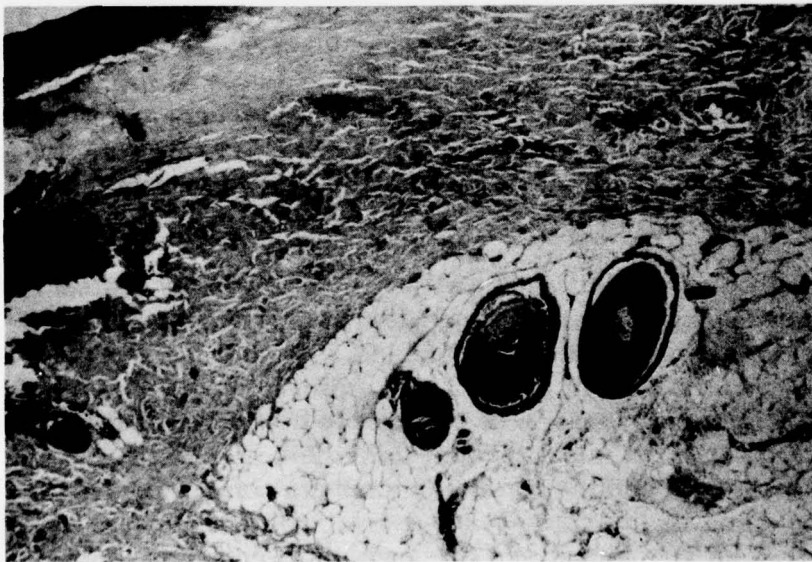


FIGURE 14. Full thickness burn with damage to adipose tissue. Grade 10. Not as clear as the grade indicates since the deep dermis overlying the adipose tissue retains some of its normal fibrillar appearance, but the changes to the hair shafts and adipose tissue push the grade to a 10. H&E stain 100 x mag.

DISCUSSION

The general method of Lyon, et al (1955), has been modified here to include linear depth measurements which allow for correction of thermal shrinkage or swelling. This correction is important in arriving at an agreement between observed burn depths and predicted burn depths from mathematical models (Knox, Wachtel, Knapp 1978b).

Various difficulties and inconsistencies were discovered during the microscopic grading which should be pointed out. In most cases, only one microslide was made and evaluated from each skin biopsy. On some biopsies, where proper evaluation could not be made on the original slide, another section was requested. In a few cases, the numerical grade between the sections differed by two or three grades. Therefore, if the biopsy received is not evenly burned throughout, a single section might not give a fair evaluation. Adding to this problem is one of supplying the pathologist with a good tissue section. As a rule of thumb, the more badly burned a tissue section is, the more difficult it is to cut a good section on the microtome. Should a biopsy not be evenly burned, and the lesser burned area sections more easily, it is probable that the pathologist will receive this type section. The results are thereby immediately biased towards a lower grade. Two skin specimens were selected for serial sectioning to determine how much variation was present. In one specimen of twenty sections, the grade remained constant (grade 6). In the other of forty sections, the grade varied between 8 and 9.

A second problem which arose was within the grading system itself, specifically grades 4 and 5. In both grades, the damage is the same-- separation of the dermal-epidermal interface. The difference could be that the heat applied to the skin in grade 5 was uniform, while it was not in grade 4. In which case, there is no real grade distinction between 4 and 5 as far as severity of burn is concerned. What is being distinguished is the uniformity of heat application. On the other hand, one could postulate that as fluid collects and begins to boil, the dermal-epidermal separation begins at points of weakest attachment and proceeds until complete separation is effected.

There is no direct evidence to support one hypothesis over the other, but intuitively the authors lean toward the latter hypothesis.

Further discussion of error and "pitfalls" in applying this method will be the subject of a future report when the results of applying the linear measurements and shrinkage corrections to some 400 specimens from the University of Rochester studies are available.

CONCLUSIONS

A histopathological burn grading scheme is available.

If applied with care, the grading scheme provides consistent results which are useful for mathematical model development.

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APPENDIX A
LIST OF EQUIPMENT

Veterinary

1. Heidbrink Model 970 - Veterinary Anesthesia Unit
2. CAP-CHUR Equipment (Palmer Chemical and Equipment Company)
3. Drugs
 - a. Sernylan (phyencyclidine hydrochloride - Parke-Davis)
 - b. Thorazine (chlorpromazine - Pitman-Moore)
 - c. Penthrane (methoxyflurane - Abbott)
 - d. Atropine Sulfate
 - e. Innovar-Vet (fentanyl-droperidol)

Laboratory

1. Autotechnicon Model 2A
2. Tissue-Tek Thermoelectric Center
3. Paraffin Dispenser
4. A/O Spencer Microtome Knife Sharpener
5. A/O Spencer "820" Microtome
6. Lipshaw Electric Tissue Flotation, Bath
7. Lipshaw and A/O Spencer Microtome Knives

8. Lab-Line Warming Plate
9. Staining Dishes, Slide Racks, etc.

Experimental Apparatus

1. Flame gun - Conversion oil burner, modified Lennox, Model OB-32 (loaned by the National Aviation Flight Engineering Center, NAFEC, Atlantic City, NJ) that burned kerosene.
2. USAARL T-1 Furnace (NASA-Ames T-3 designed by Richard Fish - modified and built by Lynn Alford - insulating fire brick lined steel box heated by a commercial oil burner (Ray Burner Co., Type RCR, Size 00-1)) that burned JP-4.

Other Materials

1. Black Silk Stove Polish - J. L. Prescott Co., Passaic, NJ
2. Praplast Plus
3. Uni-Tech Hematoxylin Harris
4. Eosin Y
5. 95% Alcohol
6. Absolute Alcohol
7. Course Abrasive
8. Fine Abrasive
9. Hone Glass Compound
10. Xylene
11. Glacial Acetic Acid

12. Ammonium Hydroxide

13. Microscope Slides

14. Cover Slips

15. Permout

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