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Bioassays Used in Development of Topical Dosage Forms

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One main cause of the substantial decline in the introduction of new drugs in the United States in recent years is the prohibitive expense of exhaustive clinical trials required under Federal regulations (the cost of introducing a new drug entity is estimated to range from $2.25 to $15 million with an average of over $10.5 million) (1). Therefore, the pharmaceutical formulator cannot afford to submit to clinical investigation suboptimal dosage forms, which subsequently have to be reformulated and tested again.

To avoid this pitfall, model bioassays have been developed to screen various topical formulations prior to clinical trial. Many articles reviewing these bioassays have appeared in the medical and pharmaceutical literature, and a few review the entire field. Some bioassays used in developing topical corticosteroids (2–4) and for antimicrobials and corticosteroids (5) were reviewed. Similarly, a review of recent progress in the field of topical corticosteroid formulation was published (6).

The present review not only deals with the various bioassays used to test topical corticosteroid and antimicrobial formulations but also covers bioassays for antibacterials, antifungals, antiyeast formulations, antimitotics, antiperspirants, sunscreen agents, antidandruff formulations, and antipruritics. Hopefully, the reader will become informed about the use of these bioassays in the development of optimal formulations and thus avoid prematurely submitting dosage forms for expensive clinical evaluation.

ANTIMICROBIAL BIOASSAYS

Cultivating a model experimental infection that can be consistently reproduced and that persists long enough to evaluate formulations for their relative efficacy is one of the most difficult undertakings in the development of topical antimicrobial drugs. Several

antimicrobial bioassays that satisfy these requirements have been developed recently. For the purpose of this review, they are grouped under three headings: antibacterial formulations, antifungal formulations, and antiyeast formulations.

Antibacterial Formulations—A bioassay was developed which determines the penetration of bacteriostatic agents into the corium of human skin obtained from surgical specimens (7, 8). The antimicrobial agents are used either in their commercial vehicles or in special vehicles as indicated. In this procedure, plastic cylinders are attached to the epidermis with liquid adhesive, and the agent is applied to the skin circumvented by the wall of the plastic cylinder. Liquid vehicles are measured with pipets, and solid vehicles are weighed on a microbalance and then carefully transferred to the skin with a glass rod.

The specimens are incubated for 20–48 hr in a temperature- and humidity-controlled cabinet at 30° dry bulb, 27° wet bulb. After washing with soap and water, the epidermis is removed by exposure to a thermal plate (58–60°) for 2–4 min. Six-millimeter punches of the corium are taken and implanted on the culture medium, with the epidermal side of the corium in contact with the medium. Standard plastic petri dishes are used for all culture media, and bacteria are grown on a standard Mueller–Hinton medium. When inhibition of growth is seen around the implant of corium, the radius of inhibition is measured. This value refers to the distance from the edge of the corium implant to where active growth can be seen.

Penicillin and erythromycin showed definite evidence of penetration when this technique was used (7, 8). The differences in topical antibacterial effectiveness of various erythromycin esters were evaluated; ilotycin was three times as effective as its propionate, valerate, and benzoate esters. Vehicles varied in their ability to effect transport of antimicrobial agents through the epidermis. For example, 2% erythromycin in dimethylacetamide produced an average 5-mm radius of inhibition, while erythromycin in wool alcohols ointment[1] base only produced an average 1-mm radius of inhibition.

The efficacy of antibacterial formulations was studied in experimental human infections with *Staphylococcus aureus* (9–12). Infection sites were prepared by marking three 2 × 2-cm squares on the volar surface of each forearm. After delimiting the squares with adhesive tape, the horny layer was removed with cellophane tape down to the "glistening layer." These stripped sites were then left exposed for 24 hr. The inoculum was prepared by diluting 100-fold a "just hazy" saline suspension of a strain of *S. aureus* sensitive to ordinary antibiotics. Ten microliters of inoculum was applied to each square with a capillary micropipet. Then an occlusive dressing, consisting of a 2 × 2-cm square of plastic film and overlapping strips of cloth-backed adhesive tape, was applied immediately.

Six hours later, the bandages were removed and approximately 30 mg of antibacterial cream was applied in a double-blind fashion to each site (one site received no treatment). This quantity amply covered the surface when spread out under a 2 × 2-cm square of plastic film. The occlusive dressing was then reapplied for another 18 hr. The quantitative effect of the treatment on the density of *S. aureus* was measured by the detergent-scrub technique; clinical severity was assessed on a 0–4 scale, where 4 represents purulent erosion and 0 represents merely the effect of stripping (comparable to the uninoculated site) (9).

By using this technique, steroid–antibacterial formulations containing triamcinolone acetonide, neomycin, gramicidin, and nystatin were evaluated; the antibacterials were more effective in the steroid combination than when used alone.

In another set of experiments, three procedures were developed for appraising the ability of antibacterial chemicals and formulations to suppress the growth of microorganisms on human skin (13, 14). In each of the following tests, the microflora was quantified after the skin had been occluded for 1 day or more, a circumstance that normally leads to an explosive overgrowth of resident organisms.

Occlusion Test—This test is bacteriostatic and estimates the degree to which an antibacterial test agent prevents a relatively small number of microorganisms from increasing in number. In this procedure, two 5 × 5-cm squares are delimited with a marking pencil on the under surface of each forearm. The antibacterial test material is applied to one site, leaving the other site untreated as a control. Unformulated agents are applied in a volatile solvent such as water or alcohol, and rapid evaporation eliminates any antibacterial effect from the solvent. The sites are then covered with plastic film. (With creams and ointments, the materials are spread out over the test site by "massaging" the square of plastic over the site.) The plastic film is tightly sealed by encircling the arm with plastic tape. After 48 hr, the plastic is removed and the sites are immediately sampled by a detergent-scrub procedure and counted.

With this procedure, bacitracin, chloramphenicol, and neomycin were markedly inhibitory at 0.01% concentrations, while methylbenzethonium chloride and sodium pyrithione were effective at 0.1% concentrations. Hexachlorophene and povidone-iodine were effective only at 1% concentrations.

Expanded Flora Test—The expanded flora test was devised to account for circumstances in which huge numbers of organisms are already present and a bactericidal effect is also desirable. For this test, the resident organisms are allowed to reach a high population density by occluding the site for 48 hr before applying the test agent. The entire length of the forearm is wrapped snugly with several layers of plastic[2] wrap, which is sealed to the skin with plastic adhesive tape at the wrist and just below the elbow. The plastic wrapping is removed after 48 hr, and two 5 × 5-cm square test sites are marked out on each forearm. Test materials and plastic covering are then applied as in the occlusion test. After 48 hr, sampling and microbiological assays are conducted.

[1] Eucerin.

[2] Saran.

By this method, chloramphenicol and bacitracin were judged effective at 0.01%, neomycin at 0.1%, and hexachlorophene and sodium pyrithione at 1%. Methylbenzethonium chloride and povidone-iodine were ineffective.

Persistence Test—The persistence test was devised to measure the substantivity of the test antibacterial agent and to determine residual effectiveness several days after application. In this test, the 5 × 5-cm squares are marked out on the forearms as already described; test materials are applied to the sites three times per day for 3 days. The sites are left uncovered without restrictions for 3 days (washing with bland soap is permitted), followed by occlusion for 24 hr. The occluding film is then removed, and the sites are sampled and assayed as described previously. An untreated control site is not included, because there can be a transfer of activity with some very active materials, even to distant sites.

Antibiotic and chemotherapeutic compounds at 1% concentration were tested using this procedure. Of the antibiotics, chloramphenicol, neomycin, penicillin G, and gentamicin were effective.

Antifungal Formulations—Reiss *et al.* (15, 16) were among the earliest investigators to induce experimental *Microsporum lanosum* infections in dogs, cats, and rabbits for the purpose of screening antifungal compounds. The best models were newborn rabbits infected with *M. lanosum,* since they showed the least individual variation during the infection.

A controlled *in vivo* laboratory method was developed for screening antifungal ointments (17). In general, the screening procedure compares the efficacy of antifungal ointments in preventing typical *Trichophyton mentagrophytes* infections in guinea pigs.

A semi *in vivo* procedure was reported for testing antifungal agents for topical use (18). The potential activity of antifungal agents in solution is evaluated by measuring the immersion time necessary to kill *T. mentagrophytes* in infected epidermal scales obtained from guinea pigs. The fungicidal effect is dependent upon the penetrating and fungicidal activity of the test material.

Another *in vivo* model tested various vehicles for clotrimazole in experimentally induced *T. mentagrophytes* and *T. quinckeanum* infections in guinea pigs (19). A cream and a polyethylene glycol solution were identified as optimal formulations, were assessed clinically in open trials, and were highly efficacious (20).

Stoughton (7, 8) used his antibacterial technique to determine the penetration of antifungals into the corium of the human skin. Antifungal agents showed a surprising difference in their activity. For example, 1% tolnaftate[3] solution showed an average 3-mm radius of inhibition, while 1% thiabendazole showed an average 11-mm radius of inhibition.

Another experimental method, which induced inflammatory dermatophytosis on the skin of human volunteers, used purified spores of *T. mentagrophytes* (21, 22). Reproducible infections could be obtained by the application of a quantitated dose of spores to a measured skin area followed by continuous occlusion with a plastic patch for 4 days. Although not presently in use in antifungal bioassays, these infections may prove useful in the development of topical antifungals.

Knight (23–26) developed the most comprehensive human models for *in vivo* and *in vitro* assessments of topical antifungal formulations: induction of experimental human *T. mentagrophytes* infections and an *in vitro* assay using stripped human stratum corneum, respectively.

Experimental Human T. mentagrophytes *Infections*—In this bioassay, the infections are induced by applying 10,000 spores in 0.05 ml of sterile water to the upper arms. Four test sites are marked 5 cm apart on each arm. Each test site is a 4-cm square. A different antifungal compound is carefully applied to each site on one arm; the other arm serves as an untreated control. The test compound is left in place for 15 min, dabbed dry, and then washed and allowed to dry.

Inoculations are made on control and treated sites either immediately after drying or after a delay of 24 or 48 hr of normal activity. The spore solutions are covered with damp gauze, and the entire area is then covered with a square of polyethylene[4] pressed over previously sprayed adhesive to achieve a seal. The dressings are removed after 72 hr, and the sites are examined culturally and microscopically daily for 7 days. A positive infection is either culturally or microscopically positive.

In Vitro *Assay for Antifungal Compounds in Stratum Corneum*—In this bioassay, stratum corneum is stripped off by repeated application of translucent adhesive tape. The tape is then placed, with the stratum corneum surface uppermost, onto a microscope slide and fixed at each end by additional adhesive tape. The slide is placed on a bent glass rod in a petri dish and covered. The dish is sterilized in ethylene oxide gas for 4 hr at 53° at a pressure of 3.64 kg/6.45 cm^2 (8 $lb/in.^2$). The gas is allowed to disperse for 48 hr. Eight milliliters of sterile water is run into the bottom of the dish, and 0.005 ml of a spore suspension of *T. mentagrophytes* is applied to the center of the tape and spread. The lid is replaced, and the dish is stored at 30°.

After 7 days, the inoculated area is covered with a net-like mass of hyphae. If the fungus is growing under less than optimal conditions or if the stratum corneum contains an antifungal compound, the hyphae tend to be shorter. If the stratum corneum contains a high concentration of a fungistatic agent, only small, swollen, germinal tubes are produced (23). All cultures are graded from 0 to 3 on a severity scale.

The results of the evaluation of four antifungal formulations in experimental human *T. mentagrophytes* infections (23) are shown in Table I. All control sites showed positive infection when inoculated immediately after washing, while only one treated site (haloprogin) was infected. Sites treated with 1.0% dipyrithione sodium[5] cream and 5% thiabendazole in dimethylacet-

[3] Tinactin, Schering.

[4] Polythene.

[5] Omadine.

Table I—Results of Human Experimental *T. mentagrophytes* Infections after Prophylactic Use of Topical Antifungal Agents[a]

Hours	Number of Positive Infections				
		Treated Sites			
	Normal Sites	Haloprogin	Tolnaftate	Dipyrithione Sodium[b]	Thiabendazole
0	40/40	1/10	0/10	0/10	0/10
24	38/40	3/10	5/10	0/10	1/10
48	38/40	6/10	7/10	1/10	3/10

[a] Data from Ref. 23. [b] Omadine.

amide were well protected from inoculations 24 and 48 hr following application, while haloprogin and tolnaftate solutions were less effective.

Table II shows results with the same formulations using the *in vitro* stratum corneum bioassay (23). Twelve strippings were used, and all pretreatment scores approached the maximum. (On the 0–3 scale, the maximum degree of growth was 36.) At 0 hr, haloprogin, dipyrithione sodium[5], and thiabendazole completely inhibited hyphal growth, while tolnaftate proved ineffective. Haloprogin's effectiveness declined after 8 hr, and dipyrithione sodium and thiabendazole provided some protection for as long as 72 hr.

These two techniques using human models are relatively simple and produce reasonably consistent results. They indicate that topical antifungal agents are effective for prophylaxis and that one brief application may give protection lasting over 72 hr. With the exception of tolnaftate, the stratum corneum technique results indicate the same degree of prophylaxis with each agent as found in the *in vivo* human models. This finding may be helpful in future assays, because studies with the stratum corneum dermatophyte culture model are considerably easier to perform than those using *in vivo* human experimental infections.

Antiyeast Formulations—Experimental cutaneous *Candida albicans* infections have been attempted in dogs (27), mice (28), guinea pigs (29), rabbits (30), and humans (31–33). But the short duration of experimentally induced cutaneous infections has hampered the evaluation of topical antiyeast formulations. This shortcoming was overcome by using triamcinolone acetonide injections to lower the resistance of rabbits and to prolong the *Candida* infections for 1 month (30). *C. albicans* was introduced by a single scarification on the skin of albino rabbits injected subcutaneously with 10 mg of triamcinolone acetonide 1 week before infection and with 5 mg/week thereafter. The lesions were of the acute inflammatory type for about 1 week, entered a chronic stage with the formation of a thick crust (back) or desquamation of the epidermis (ear), and remained clinically evident and culturally positive for at least 35 days.

Table II—Comparison of the Effectiveness of Topical Antifungal Compounds as Shown by Inhibition of *T. mentagrophytes* Spore Growth on Stratum Corneum *In Vitro*[a]

Hours	Degree of Growth			
	Haloprogin	Tolnaftate	Dipyrithione Sodium[b]	Thiabendazole
Pretreatment	34	32	34	34
0	2	30	0	0
8	14	28	0	0
24	32	30	13	2
48	33	33	13	16
72	32	35	17	23

[a] Data from Ref. 23. [b] Omadine.

This method was used to compare the efficacy of various medications, including some currently used in the treatment of human candidiasis (30). Each rabbit was infected at 10 sites (1.5-cm diameter) on its back. One site in each rabbit was left untreated (infection control), one received a placebo, and the rest received the test medications in a random fashion. The medications were applied once a day for 5 consecutive days, and the cycle was repeated three times. Formulations containing 100,000 units of nystatin/g alone or in combination with other antibiotics and triamcinolone acetonide, 3% amphotericin B, 1% gentian violet, 1% 5-chloro-8-quinolinol, 1% 2-pyridinol 1-oxide, or 1% bis(2-pyridinyl 1-oxide) disulfide–stannous chloride complex were quite active. The latter compound was remarkably active, even at 0.25%.

Attempts to develop a human antiyeast bioassay have shown that a pustular dermatitis can be rapidly induced when a large number of *C. albicans* cells are placed on normal skin and occluded (31–33). The experimental *C. albicans* infection is produced by applying 10 μl of saline containing 10^3, 10^4, and 10^5 yeast cells on the volar surface of the forearm and immediately covering with a 2 × 2-cm square plastic film. This film is firmly sealed to the skin with 2.54-cm (1-in.) cloth-backed tape for 24 hr. Twenty-four hours after removing the dressing, the severity of dermatitis is rated + to +++++. Although this experimental human *C. albicans* infection has never been used to evaluate topical antiyeast formulations, it has a good potential for development of a useful human bioassay.

BIOASSAYS FOR ANTIMITOTIC DRUGS FOR TOPICAL USE

The effectiveness of drugs in diseases where there is an increase in cell proliferation has been generally attributed to their ability to interfere with the mitotic cycle. Since epidermal cells of skin normally have low mitotic rates, vaginal mucosa cells, which have a comparatively high rate of replication, were used (34–37). In this bioassay, test compounds were instilled intravaginally in 5–7-week-old virginal mice in 0.05-ml doses. The first dose was at time 0 and was repeated 2 hr later (concurrent with intraperitoneal injection of 2 mg of podophyllum resin). All mice were killed 6 hr later, and the vaginas were removed *en bloc* and fixed in 10% buffered formalin. Six-micrometer transverse sections were stained with hematoxylin and eosin and examined with an ocular micrometer to measure the number of mitoses per centimeter of mucosa. Only those specimens demonstrating the histological characteristics of the

estrogenic phase of the estrus cycle were included for analysis. A test drug yielding less than 100 mitoses/cm is considered antimitotic.

This bioassay was used to evaluate the efficacy of daunorubicin (daunomycin) and carmustine[6] (34), methotrexate analogs (35), and hydroxyurea and its derivatives (37). Also, it was used to compare bioassay results of 1-methylhydroxyurea and mechlorethamine to clinical efficacy in psoriasis lesions (35).

The vaginal mucosa bioassay was modified to study the effect of antimitotic agents on local DNA, RNA, and protein syntheses, and its usefulness in predicting the efficacy of topical treatments for psoriasis was recommended (38).

A bioassay using UV light was developed to stimulate epidermal hyperplasia (simulating psoriasis) in hairless mice (39–41). Antimitotic activity is measured by the mitotic index and radioactivity of extracted DNA. Among those compounds screened with the bioassay, cycloheximide has proven very effective; preliminary clinical studies with psoriasis confirmed this finding (42).

BIOASSAYS FOR ANTIPERSPIRANT FORMULATIONS

Two human bioassays were developed to determine the antiperspirant activity of topically applied anticholinergics (43, 44). In the first, the forearm antiperspirant test, 4.5-cm^2 circles are marked 1.9 cm (0.75 in.) apart on the inner aspect of the forearm. Test solutions are applied to each circle with a cotton-tipped swab and allowed to dry. This procedure is repeated three times, applying a total of 0.1–0.2 ml of solution. After about 5 hr, the forearm is washed with soap and water to remove any excess.

Sweat inhibition is measured by the starch–iodine technique (45) and is repeated without further treatment at 24 and 48 hr. Sweat inhibition is graded on a scale of 0–4: 0 = no sweat inhibition, control and treated areas are identical; 1 = poor sweat inhibition, treated area is just discernible because of fewer droplets; 2 = fair inhibition, treated area has only about half of its glands functioning; 3 = good inhibition, only a few scattered droplets are present in the treated area; and 4 = excellent inhibition, no sweat droplets are present in the treated area.

The alternative test is the axillary antiperspirant test in which 0.5–1.0 ml of the antiperspirant solution is applied to the entire axillary surface with a cotton-tipped swab or from a commercial roll-on dispenser. After a suitable time interval, the subject is placed in a room at a temperature of 37.8° (100°F) and a relative humidity of about 40–50%. Gravimetric sweat collections are made with tared pads, which are placed snugly in the axillary vault for 10 min. The pads are then placed in tared, tightly covered jars and weighed for comparison with a previously established, normal sweating pattern for each subject.

By using these techniques, various anticholinergic compounds have been found to inhibit sweating when topically applied. Among the compounds investigated, esters that contain one or more elements of atropine and scopolamine structure have the best activity of a long-lasting nature.

BIOASSAYS FOR SUNSCREEN FORMULATIONS

Since 1922, it has been known that sunburn and suntan in humans are caused by the action of only a narrow band within the UV spectrum (290–320 nm) (46). Many methods have been used to prevent actinic injury to the skin, but the most widely used is the topical application of sunscreens which absorb light and screen out harmful radiations. There are two major classifications of bioassays used to evaluate sunscreens: those using artificial light sources that mimic the UV spectrum of sunlight and those using direct sunlight.

The xenon solar simulator is one preferred artificial light source for sunscreen bioassays, because it reproduces spectral features of sunlight in the UV region (47). By means of a template, the skin of untanned adult volunteers is subdivided into small areas; fixed quantities of test sunscreens then are spread over each area. The areas are exposed for a predetermined time and examined at 24, 48, and 72 hr for severity of erythema. This technique was used to evaluate 3% concentrations of the sunscreen dipropylene glycol salicylate (dipsal) in six different vehicles, and the oil-in-water emulsions were found to be superior in promoting tanning (48). The technique also is useful in comparing the efficacy of sunscreen agents alone (47).

The procedure using direct sunlight is quite similar in that the untanned skin of adult volunteers is subdivided by a template prior to application of sunscreens and exposure to sunlight. This technique was used to evaluate selected sunscreens; 5% aminobenzoic acid in 70% ethanol and a cream containing oxybenzone and dioxybenzone were effective sunscreens (49). This procedure also can be used before and after bathing to show the water washability of various sunscreen preparations (49, 50).

Several *in vitro* methods not considered to be bioassays are also used to evaluate sunscreens. They include photographic and spectrophotometric methods (51, 52), thin-film techniques, and the solution–dilution method (53). Spectrophotometric methods also are used to evaluate the effect of vehicle components on the absorption characteristics of sunscreen compounds (54). A method was developed to evaluate the efficacy of sunscreens by analyzing stripped layers of epidermis following sunscreen application (55).

BIOASSAYS FOR ANTIDANDRUFF FORMULATIONS

Dandruff is a chronic noninflammatory scaling of the scalp in which mitosis is accelerated. Antidandruff preparations were evaluated in human volunteers with at least moderately severe dandruff (56–58). Subjects followed the shampoo regimen illustrated in Table III, and efficacy was evaluated by clinical grading or with an objective test (corneocyte count) in which the quantity of horny cells produced was measured with a hemocytometer. Clinical grading was done on a scale of 0–10: 0–1, very little scaling; 2–3, mild scaling; 4–5,

[6] BCNU.

Table III—Protocol for Complete Analysis of a Shampoo[a]

	Pretreatment Days		Treatment Days								Post-treatment Days
	−7	−4	0	3	7	10	14	17	21	24	28
Bland shampoo	x	x									
Test shampoo			x	x	x	x	x	x	x	x	
Grade			x		x		x		x		x
Corneocyte count			x				x				x

[a] Data from Ref. 56.

moderate scaling; 6–7, severe scaling; and 8–10, very strong scaling.

For the corneocyte count, a site on each side of the vertex was clipped so a glass cylinder could be snugly applied to the surface. One milliliter of buffered 0.1% octoxynol[7] was placed in a cup, and the surface was rubbed with a blunt rod[8] for 1 min. The wash fluid was then aspirated, the procedure was repeated, and the two samples were pooled. After appropriate dilution (usually 1:10 but with lesser dilutions after effective treatment), 2 drops of 2% gentian violet were added, the cell suspension was agitated mechanically, and the dispersed cells were counted in a Fuchs–Rosenthal hemocytometer (56). Table IV shows the results of a typical comparison between zinc pyrithione and its detergent base.

A more intensive version of the shampooing schedule was developed in which the test agent was applied once each weekday for 2 weeks (59). The corneocyte counts and clinical gradings were made 4, 8, and 12 days following the last exposure. This shortened version is best suited for screening and evaluating formulation changes.

Other methods measuring the severity of dandruff in humans may be useful tools for evaluating various antidandruff preparations. A method was reported in which a small vacuum cleaner is used to collect dandruff scurf from the scalp for weighing (60, 61). This technique was improved (62) by substituting a scalp brushing technique to help collect dandruff scurf for weighing. A model was described for producing a dandruff-like syndrome in guinea pigs, which requires the presence of lipid mixtures in addition to scalp organism (63). The sloughing reaction observed is probably due to the liberation of free fatty acids and the relative increase in C_{18} monoenoic acid at the irritated site. Although this method has not been used as a bioassay for the evaluation of antidandruff formulations, it could prove to be a useful model.

BIOASSAYS FOR ANESTHETIC–ANALGESIC FORMULATIONS

Both itch and pain are subserved by the same network of cutaneous nerves (64–66). Increasing the intensity of a stimulus converts the itching sensation to pricking or burning; increasing it still more induces pain.

Table IV—Comparison of 2.0% Zinc Pyrithione Shampoo with the Detergent Base Alone[a]

Treatment	Corneocyte Count			Clinical Grades		
	Day 0	Day 14	Day 28	Day 0	Day 14	Day 28
Detergent base	1,256,000	1,148,000	1,174,000	5.4	4.9	4.7
Zinc pyrithione	968,000	678,000	732,000	5.6	3.1	1.9

[a] Data from Ref. 56.

Hardy *et al.* (67) used the Hardy–Wolff–Goodell pain threshold apparatus (68) to evaluate the effect of topical anesthetics–analgesics on pain threshold measurements in human skin. In this bioassay, a 3-sec exposure to the rays from a 500-w incandescent lamp is used to produce a thermal stimulus on the volar surface of the forearm. The test areas are blackened with India ink to ensure complete absorption of the radiation and to prevent effects due to penetration of the rays below the skin surface. The stimulus is thus a purely thermal one. The subject reports on the sensation experienced at the end of the 3-sec exposure, and the stimulus intensity evoking the least perceptible pricking pain is considered to be the pain threshold. The test areas are then measured at 15-min intervals for 1.5 hr after the application of various formulations.

The effects of 3% phenol, 2% menthol, 2% tetracaine[9], and 1% dibucaine[10] in petrolatum were studied, and no significant pain threshold-rising action was found (67). When using the mucous membranes of the lips as target organs, both 5% benzocaine and 1% dibucaine in petrolatum caused marked anesthesia and analgesia.

The ointment base efficiency of local anesthetics was evaluated on the tails of albino rats using a modified Hardy–Wolff–Goodell pain threshold apparatus (69, 70). Although the technique was successful with rat tails, this success is not an indication of its usefulness in evaluating anesthetics on human skin.

Adriani *et al.* (71, 72) evaluated the effectiveness of topical anesthetics in humans. They found that local anesthetics abolished the tingling sensation caused by an electric current applied to the tip of the tongue. In this bioassay, a Burdick muscle stimulator is used to deliver a pulsatile current of 0.1–5.0 v at a frequency of 20 Hz. Although the voltage necessary to cause the tingling sensation varies from subject to subject (0.75–3.0 v), it remains constant for a given subject throughout an experiment and varies little if at all from 1 day to the next. After the application of various anesthetics, the increase in voltage necessary to elicit the tingling response serves as an index of effectiveness and makes possible a comparison of the potency of individual drugs.

The comparative potency and efficacy of more than 40 topical anesthetics were evaluated, and it was found

[7] Triton X-100, Rohm and Haas.
[8] Lined with Teflon (du Pont).
[9] Pontocaine, Winthrop.
[10] Nupercaine, Ciba.

that the latent period was shortened as the concentration was increased (71, 72). There was also a maximum effective concentration for each drug. The most effective drugs, in the order of decreasing activity, were 1% tetracaine, 0.5% dibucaine, 20% cocaine, 1% dyclonine, 4% lidocaine, 5% piperocaine, 2% pramoxine, and 5% hexylcaine.

The efficacy of local anesthetics also was evaluated using normal skin; salts of local anesthetics were ineffective in reducing itching and burning in intact normal skin (73). Only concentrated solutions of bases were efficacious. This result is not surprising since most salts are ionized and do not penetrate the intact skin. This method can be useful in developing topical anesthetic formulations, especially for anesthetics applied to mucous membranes.

BIOASSAYS FOR ANTIPRURITIC FORMULATIONS

Itching is considered a modified form of pain and is carried on afferent pain fibers with subsequent discharges of impulses in the spinothalamic tract of the sensory cortex (74). An experimental method was developed for the measurement of pruritus, and a freshly prepared histamine phosphate solution was the most satisfactory itch stimulus (74, 75). A quantitative technique was devised so that the end-point of subjective reactivity could be measured. This end-point of reactivity, called the "itch threshold," is defined as the greatest dilution of histamine that produces a recognizable pruritus. The itch threshold is then utilized as a tool to determine the effect of various topically applied drugs.

In tests with individual drugs, the itch threshold is first measured, the test drug is then administered, and the itch threshold is measured again after a suitable interval when the effect of the drug is at its peak (76). With this method, five antipruritic preparations were administered topically to 20 subjects with normal skin. The degree of threshold elevation was probably insignificant, since it was very near that of oral placebo medications. Similar results also were reported with 54 topical antipruritic preparations tested in experimental localized pruritus induced by intracutaneous injection of histamine phosphate in three normal adult male subjects (77). Not one of the 54 preparations had a consistent definite effect on histamine pruritus.

A simple method was proposed for recording the degree and duration of pruritus resulting from application of cowhage (*Mucuna pruritum*) to human skin (78). A series of antipruritics and anesthetics was studied using this method, but none of the agents significantly altered the duration of the experimental pruritus. The investigators suggested that the action of most antipruritic preparations on itching skin disease is probably a psychological one.

Although various bioassays for the evaluation of antipruritic formulations exist, none is refined enough to be useful in the development of topical antipruritics. A considerable amount of work is still needed.

BIOASSAY FOR ANTIWART FORMULATIONS

The development of topical antiwart medications is complicated, since most warts spontaneously disappear and warts are difficult to grow under experimental conditions. However, Mendelson and Kligman (79) were successful in isolating wart virus in tissue cultures and subsequently reinoculating humans. Half of 20 subjects inoculated developed banal warts.

Since such a procedure for developing experimental warts in humans has been established, it may be worthwhile to pursue the development of an animal model in which the wart virus in tissue cultures can be inoculated into the experimental animal and the subsequent warts can be treated with antiwart formulations.

BIOASSAYS FOR TOPICAL FORMULATIONS USED IN TREATMENT OF RHUS DERMATITIS

Rhus (poison oak/ivy) dermatitis is one of the most common contact dermatidides seen in the United States. A human bioassay was developed in which the oleoresin of Rhus is applied to four sites on the forearms of human volunteers (80). When dermatitis develops, three sites are treated with three different formulations; the fourth site is left as a control. Interpretation of the results is difficult.

Hypersensitivity was induced in guinea pigs, and topical anti-Rhus formulations were tested (81). This method is a modification of a previously reported method (82) for the sensitization of guinea pigs with the Rhus antigen. On Day 0, female Hartley guinea pigs, ~350 g, are sensitized with an emulsion of poison ivy/oak extract and complete Freund's adjuvant (1:1). The antigen is administered intradermally at four sites over the scapular area and also into each hindpaw, using 0.1 ml/site. On Day 10 and periodically thereafter, the guinea pigs are challenged on a shaved flank to determine sensitivity. Guinea pigs showing a reaction after 24 hr are used for testing. Guinea pigs not sensitized within 20 days are resensitized.

The test challenge consists of applying by inunction 0.01 ml of nondiluted antigen to the external basilar portion of the ear. Beginning 24 hr later, a test formulation is applied to the challenged site twice daily for 2 days. Challenged ears with or without vehicle treatment serve as controls. Nonchallenged, untreated ears are included for comparison.

The guinea pigs are sacrificed 72 hr after challenge, at which time the ears are removed and a biopsy specimen from the challenged ear is punched out with a No. 4 cork borer. The hair is clipped and the biopsy specimens are weighed. Using this bioassay, Kepel *et al.* (81) found that 0.05% fluocinonide gel markedly reduced the weight of challenged ears in comparison with those receiving vehicle alone.

This model may be useful in screening agents for the treatment of human delayed hypersensitivity reactions such as Rhus dermatitis.

BIOASSAY FOR TOPICAL FORMULATIONS USED IN TREATMENT OF PSORIASIS

A model was developed using mouse tail epidermis for the psoriatic keratinization process (83). The mouse tail scale epidermis, unlike the rest of the mouse body

skin, is histologically and biochemically similar to psoriatic skin; there is an absence of a granular layer, which is a prerequisite for orthokeratinization. Upon application of the antipsoriatic medication, formerly parakeratotic scale areas undergo granular layer induction.

Various coal tar fraction ointments were applied to male mouse tails on a daily basis (84–87). At the end of 14–21 days, the animals were sacrificed and the skin was removed and examined histologically for granular layer induction. The tar acids (phenols) with high boiling points (~400°) induced a granular layer in formerly parakeratotic skin. This mouse tail bioassay may be a useful model in the development of topical formulations used in the treatment of psoriasis.

BIOASSAYS FOR TOPICAL CORTICOSTEROIDS

Of all bioassays used in the development of topical formulations, those used for topical corticosteroids are the most sophisticated and refined. The fibroblast assay, the thymus involution bioassay, and the alcoholic vasoconstriction bioassay have been developed for the evaluation of relative potencies of the active ingredient. Other bioassays have been developed to evaluate the relative potencies of finished formulations and to measure both suppression of inflammation and formulation vasoconstriction. One main advantage of the bioassays of topical corticosteroids is their correlation with clinical trials.

Fibroblast Inhibition—It is a well-recognized phenomenon that corticosteroids act on a large number of cells, both *in vivo* and *in vitro*, where anti-inflammatory activity is one important function (88, 89). Connective tissue, which is composed mostly of fibroblasts, responds to inflammatory stimuli with a series of reactions which are inhibited by corticosteroids. At the cellular level, fibroblasts are intimately concerned with the process of inflammation, both as the manufacturer of connective tissue components and as the cells of origin or transformation for many other cell types. Therefore, it is not surprising that fibroblasts react to steroids with morphological changes, such as the disintegration of the cytoplasm and nucleus, and with inhibition of the fibroblast growth. Both of these reactions can be directly correlated with the anti-inflammatory activity of corticosteroids (89, 90).

A sensitive assay was developed based on the reaction of fibroblasts to corticosteroids (89–97). Fibroblasts of clone 929, strain L, are grown in roller tubes. Various concentrations of test corticosteroids dissolved in propylene glycol are incubated, and cells attached to the surface of the glass are counted 5 days after inoculation. Results of the experiments are expressed as dose–response curves; Fig. 1 shows a plot of fibroblast growth under the influence of hydrocortisone and fluocinolone acetonide. The dose–response lines are statistically fitted and present the relative potency of fluocinolone acetonide and hydrocortisone.

Thymus Involution—Systemic assays for assessing thymolytic anti-inflammatory activity of corticosteroids have been used to predict their possible value as a topical corticoid (98–100). The thymolytic activity is

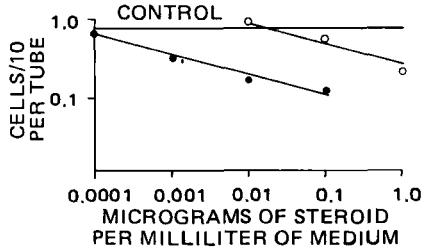

Figure 1—*Log–log plot of fibroblast growth under the influence of hydrocortisone (○) and fluocinolone acetonide (●). The lines are statistically fitted, with dose of steroid on the abscissa and millions of cells on the ordinate. (Adapted, with permission, from Ref. 90.)*

evaluated using 21–23-day-old, bilaterally adrenalectomized rats. At the time of surgery, 50-mg cotton pellets are inserted subcutaneously, one on each side of the animal. The test corticosteroid is administered subcutaneously once daily for 6 days, starting on the day of surgery. Twenty-four hours after the last injection, the thymus is removed, blotted dry, and weighed. The results are plotted as log dose–response curves, where the relative inhibition of the thymus indicates the relative potency of the corticosteroids tested.

Antigranuloma—The antigranuloma bioassay is similar to the thymus involution bioassay in that it is used to determine the relative potency of topical corticosteroids (98–102). The antigranuloma bioassay is evaluated by variants of the Hershberger and Calhoun (101) technique. Male rats, 170–200 g, are adrenalectomized and maintained on a normal diet and saline. Cotton pellets (5–8 mg) are impregnated with the test compound on the day following adrenalectomy and are implanted bilaterally into the loose subcutaneous connective tissue of the pectoral and dorsolateral neck regions. Four pellets are implanted in each animal, two impregnated with the test substance and two controls.

Six days after implantation, the pellets are removed and dried to constant weights. The results are plotted as log dose–log response curves, where the relative inhibition of granuloma formation indicates the relative potency of the corticosteroids tested.

Table V presents the relative potencies of various corticosteroids determined by the fibroblast inhibition assay, thymus involution bioassay, and antigranuloma bioassay.

Inflammation—Since topical corticosteroids are used as anti-inflammatory agents, some important bioassays involve the development of inflammation models which can then be used to evaluate the anti-inflammatory characteristics of various corticosteroid formulations.

Croton Oil—Rooks and Dorfman (98) modified the method of Tonelli *et al.* (104) and studied the relative potencies of anti-inflammatory compounds. The method employs 21-day-old male rats. A 0.05-ml mixture of 20% pyridine, 5% water, 74% ether, and 1% croton oil, with or without the test compound, is applied to the ears. One ear is treated and the other serves as an untreated control. The ears are removed 6 hr after drug administration, and uniform areas of the ear are punched out and weighed.

The anti-inflammatory effect of various corticoste-

Table V—Relative Potency of Corticosteroids Using the Fibroblast Inhibition Assay, Thymus Involution Bioassay, and Antigranuloma Bioassay

	Potency Ratios (Hydrocortisone = 1)		
	Fibroblast Inhibition Assay[a]	Anti-granuloma Bioassay[b]	Thymus Involution Bioassay[c]
Corticosterone	0.48	—	0.17
Hydrocortisone	1.0	1.0	1.0
Prednisolone	1.7	2.7	4.0
			4.1
Dexamethasone	7.5	104.0	47.0
			83.0
Paramethasone	11.3	63.6	45.1
Δ^1,9,11-Dichloro-cortisone 16,-17-acetonide	43.7	—	<1
Triamcinolone acetonide	156.0	48.5	37.7
Fluocinolone acetonide	440.0	446.0	263.0

[a] Data from Ref. 91. [b] Data from Refs. 100 and 103. [c] Data from Refs. 100, 103, and 104.

roids is determined by the relative suppression of the inflammation caused by croton oil. The results are plotted as log dose–response curves, and relative potencies are established. By using this bioassay, fluocinolone acetonide was estimated to be 150 times more potent than hydrocortisone (98).

The inflammation induced in human skin by topically applied croton oil has been suggested as a bioassay for corticosteroid potency, since it approaches the clinical inflammatory situation (105). In croton oil studies, 32 7 × 7-mm squares punched on tape[11] are applied to each forearm. Then 7 μl of 100% croton oil is applied to each square, spread evenly over the entire site, and covered with a plastic film for 3 hr. The sites are carefully marked, and the arms are washed and dried. New tapes with 12 × 12-mm punched-out squares are carefully superimposed over the croton oil-treated sites, and 3 mg of the corticosteroid preparations is applied to the sites. Each forearm is occluded with a plastic film for 18–20 hr. Reading is done at 24 hr on a 0–5 scale based on the degree of erythema, induration, and vesiculation present. The results, after decoding, are subtracted from the score of untreated croton oil sites (106, 107).

The relative potencies of several commercially available corticosteroids were evaluated using this technique. Fluocinonide 0.05% cream was found to be superior to betamethasone valerate, flumethasone pivalate, triamcinolone acetonide, and fluocortolone–fluocortolone caproate creams (107).

The anti-inflammatory activity of various corticosteroids was evaluated by measuring the suppression of croton oil-provoked pustules, and the rank order of anti-inflammatory efficacy correlated reasonably well with clinical judgments of comparative effectiveness (108).

Primary Irritants—A method was developed for evaluating the topical effectiveness of corticosteroids by inducing inflammation with the primary irritants mustard oil and nitric acid (109). Various concentrations of mustard oil were prepared by dilution with liquid petrolatum, but an 80% concentration was used primarily. Concentrations of 4, 10, and 15% nitric acid in aqueous solution were used in a small group for comparison, but the 15% concentration was employed for the main test group. Both primary irritants were kept in contact with the skin site for 24 hr by a cellophane patch and removed with a suitable solvent.

All tests are performed on the normal skin of human volunteers. The test site is a 1 × 1-cm area on the upper part of the back below the level of the dorsal spine of the scapula, and there is no pretreatment of the area. Fifty milligrams of the test ointment is applied and massaged into the skin, and the area is covered with a transparent cellophane disk. Applications are made at time intervals ranging from 24 hr before to 24 hr after the introduction of the inflammatory stimulus, and contact of the corticosteroid ointment with the skin is maintained for 0.5–8 hr. The corticosteroid ointment is removed with water, alcohol, and ether at the desired time.

In all cases, observations are made immediately after application of the inflammatory stimulus and at approximately 2-hr intervals until the maximum development of the inflammatory reaction is achieved. The time of appearance of the reaction, its rate of progress, and the final degree of response are noted. The inflammatory reaction is graded as: erythema, erythema plus obvious edema, additional formation of papules or vesicles, or necrosis.

Five major factors influenced the results obtained in the test situations (109):

1. Relationship between time of corticosteroid application and induction of inflammation. Effective inhibition of inflammation resulted from the application of the corticosteroid 2–8 hr prior to the stimulus. The maximum effect was achieved in about 6 hr. A time interval over 16 hr failed to produce any effect. If applied immediately before or after the stimulus, the corticosteroid produced no alteration in most cases, an exacerbation of inflammation in about one-third of the tests, and inhibition in only a few cases.
2. Duration of contact of corticosteroid with skin. The minimal effective contact time was 1 hr. Prolongation of the contact time over 2 hr gave no increase in effect.
3. Concentration of corticosteroid applied. Optimal and about equal effects were seen with concentrations of 1% hydrocortisone, 0.25% fluorocortisone, and 5% corticotropin.
4. Intensity of inflammatory stimulus applied. There was an inverse relationship between the intensity of the stimulus and the degree of inhibition observed in the inflammatory response.
5. Thickness of epidermis. An inverse relationship was suggested between the thickness of the epidermis in terms of cell layers and the anti-inflammatory effect.

Tetrahydrofurfuryl Alcohol—A laboratory method was developed, utilizing tetrahydrofurfuryl alcohol, which permits the rapid evaluation of topical anti-inflammatory agents (110). This method involves simultaneous application of an irritant and the test agent to human skin. Activity is judged by the ability of the

[11] Blenderm.

agent to inhibit the development of erythema. The test compounds to be evaluated are dissolved in redistilled tetrahydrofurfuryl alcohol. This agent is used because it produces a moderate irritant reaction suitable for test purposes when applied to human skin and is also an excellent solvent for a wide range of steroids and other compounds.

Flannel square patches, measuring 3.8 cm (1.5 in.) and sewn to a thin polyethylene backing, are saturated with the test solution of mixed irritant and anti-inflammatory agent. The patches are applied to the inner surface of the upper arm and held in place from late afternoon to the following morning. This time interval is sufficient to produce a moderate erythema at the control site. When the patches are removed, the sites are marked so they can be located later. Erythema at the test sites is evaluated about 1 hr after removal of the patches.

A seven-point scale is employed, ranging from 0 to +6 (severe) erythema. When the skin sites treated with the experimental agents in tetrahydrofurfuryl alcohol exhibit significantly less erythema than the irritant alone, the agent is rated as having anti-inflammatory activity. When the treated sites exhibit erythema that is equal to or greater than the control, the agent is rated as being without activity.

With this bioassay, fluorometholone was approximately 40 times as potent as hydrocortisone applied topically yet only about equal to hydrocortisone's systemic activity (111). It was suggested that fluorometholone may be a unique compound for anti-inflammatory therapy.

Tetrahydrofurfuryl alcohol was used to study the effects of flurandrenolide as an anti-inflammatory agent (112). The anti-inflammatory effect of flurandrenolide also was compared with that of hydrocortisone acetate, and 0.05% flurandrenolide was more effective than 1% hydrocortisone acetate in inhibition of erythema produced by tetrahydrofurfuryl alcohol.

Stripped Epidermis—Stripping epidermis with adhesive tape was first used by Wolf (113) in epidermal cytology studies. Since then, this method has been used to produce a standardized trauma. Möller and Rorsman (114) studied the vasodilation and exudation after the stripping of the epidermis and noticed an erythema 1–8 hr after the trauma. Wells (115), utilizing this local damage and repair stimulus, suggested that it might lend itself to a bioassay for the study of the local action of hydrocortisone and derivatives.

A flat area of normal skin with uniform surface is chosen (usually the flexor aspect of the forearm), and 1.3-cm (0.5-in.) wide cellophane tape is smoothed onto the skin for a length of about 8 cm. It is then peeled back from one end and sharply stripped off. A fresh piece of cellophane tape is applied to exactly the same area of skin and again is immediately stripped off. This process is repeated until, after about 30 pulls, glistening macules appear on the skin surface. With further stripping, they become confluent. The even, pinkish-red surface is glistening but not moist, and no more scales will peel away. Erythema persists in the strip for several days, and it is neatly confined to the rectangle that has been denuded of scales.

Immediately after stripping, the damaged area is divided into two or more parts; one part is treated with the control of unmedicated vehicle, and the other parts are treated with the hydrocortisone or other local applications. Applications are made three times in 24 hr if the experiment is prolonged. Dressings of soft plastic material are usually applied to cover the stripped area and prevent friction from clothing. Four hours after the first application of hydrocortisone at the start of the experiment, some pallor is apparent at the edges of the strip while the control is uniformly red. By 6 hr, the whole of the hydrocortisone-treated strip is more pale than the control. Twelve hours from the start of the experiment, the difference is maximal, the hydrocortisone-treated area often being as pale as normal skin.

By using the tape method (115), lotions containing 0.01, 0.025, and 0.1% triamcinolone acetonide and 1% hydrocortisone were compared (116). The results showed that 0.025 and 0.1% triamcinolone acetonide had longer and greater activity than the 1% hydrocortisone. Hydrocortisone formulations in various bases were studied using the same method (117).

UV Erythema Suppression—Since most corticosteroid responsive diseases are inflammatory in nature, the UV erythema suppression bioassay may be a useful model in evaluating the relative activity of corticosteroids.

Everall and Fisher (118) first evaluated the effect of topical corticosteroids in preventing the effects of UV rays on human skin, and they found that both cortisone acetate and hydrocortisone acetate ointment were ineffective. There were no alterations of the erythema response of the skin to UV rays if inunctions with hydrocortisone were performed after irradiation (119). When inunctions were carried out immediately prior to irradiation, the erythema response was weaker in the hydrocortisone-treated areas. A method was developed which inhibits Grenz-ray erythema by a single application of a topical corticosteroid (109, 120, 121). To be effective, formulations should be applied prior to irradiation with Grenz rays and provide an adequate concentration of corticosteroids in the tissues at the time of application of the inflammatory stimulus.

A precise UV erythema suppression bioassay was reported which could be correlated with vasoconstrictor bioassays and clinical studies (106, 122). In this bioassay, an air-cooled, hot quartz, high intensity lamp is used as the UV source. The minimum erythema dose (MED) is carefully determined on the inner upper arm of each volunteer the day before the assay is performed. On the following day, 7 × 7-mm squares punched on tape are applied, and each square is irradiated with that volunteer's minimum erythema dose before application of the preparations under study. Three milligrams of each formulation is applied to each square, and the arms are wrapped.

Six hours later, the wrapping and tapes are removed and the arms are washed with soap and water. Two readers independently assess the arms at 8 and 24 hr for both vasoconstriction and erythema. Individual sites are read as follows: 0 = no erythema, 1 = part of a square showing erythema, and 2 = a completely red square. After decoding the study, the results are calculated and

the sum of the readings for each preparation is subtracted from the sum of the readings from "blank" squares. When this bioassay was used, 0.05% fluocinonide ointment was the most active of the topical corticosteroids tested (122). The use of the UV erythema suppression bioassay was recommended along with the vasoconstrictor bioassay in evaluating various formulations before initiating expensive clinical trials.

Kerosene Inflammation Suppression—Kerosene-induced experimental inflammation in human skin has been used to evaluate various commercial corticosteroid formulations (108). In this procedure, the corticosteroids are applied directly to 1.5-cm squares of skin outlined in ink by a rubber stamp. Ointments and creams are applied in excess, spread evenly with a cotton swab, and then covered with 1.5-cm squares of nonwoven cotton. The cotton cloth mainly serves as a reservoir to keep the materials in place, and the patches are fixed to the skin by the occlusive dressing. A control patch receives hydrophilic ointment USP, an oil-in-water emulsion base.

The patches are left in place for 6 hr and then wiped dry. The irritant patches of kerosene are applied 1 hr later, at which time the degree of vasoconstriction is graded. The dressings are removed 20 hr later and the sites are immediately graded on a 5-point scale: 0 = complete absence of pustules or vesicles, 1 = pustules or vesicles not exceeding one-quarter of the area of the square, 2 = pustules or vesicles covering at most one-half of the area, 3 = pustules or vesicles occupying three-quarters of the square, and 4 = bullae. The rank order of anti-inflammatory efficacy of a series of marketed corticosteroids corresponded reasonably well with clinical judgments of comparative effectiveness (108).

By using the same technique, the relative potencies of 0.1% betamethasone valerate, 0.025% fluocinolone acetonide, and 0.1% triamcinolone acetonide creams were evaluated and found to have practically the same potency (123). These results correlated well with double-blind clinical studies. It was, therefore, suggested (108, 123) that the kerosene bioassay be used in evaluating the activity of topical corticosteroids.

6-Chloro-2,4-dinitrobenzene Inflammation Suppression—One problem of preclinical evaluation of new drugs is the simulation of human disease conditions in laboratory animals. To test the ability of topical corticosteroids to suppress eczema, a technique was developed whereby eczematous lesions are induced in guinea pigs sensitized with 6-chloro-2,4-dinitrobenzene (124). In this procedure, 0.02 ml of 50% 6-chloro-2,4-dinitrobenzene in acetone is applied to the shaved necks of young adult albino guinea pigs. Within 24 hr, an acute inflammatory reaction with ulceration develops and lasts 3–5 days before completely healing.

Ten days following this first application, a second external application of 1% 6-chloro-2,4-dinitrobenzene in acetone to any site on the animal's body provokes a local eczematous response in almost 100% of test animals. The reaction lasts 5–7 days before healing is complete, and the hypersensitive state usually lasts up to 3 months. A single application of 1% 6-chloro-2,4-dinitrobenzene fails to provoke the eczematous response in nonsensitized animals.

Scott (124) used this method to evaluate the efficacy of six commercially available corticosteroid ointments: 1% hydrocortisone, 0.25% methylprednisolone, 0.1% prednisolone, 0.1% triamcinolone acetonide, 0.05% flurandrenolide, and 0.025% fluocinolone acetonide. The shaved backs of six sensitized albino guinea pigs were marked into eight sections, and 0.02 ml of 1% 6-chloro-2,4-dinitrobenzene in acetone was applied to each section. Twenty-four hours later (when eczema had developed), one corticosteroid was applied to each of the six sites. The base alone was applied to a seventh site, while no application was made to the remaining site. The effects were observed and recorded at 2 and 4 days after treatment. Those sites treated with hydrocortisone, methylprednisolone, and prednisolone failed to show any difference compared to the control eczema site. The remainder of the compounds hastened and completed healing of the treated sites compared with the control.

This method also was used to determine the minimum concentration of fluocinolone acetonide that could suppress an established eczema (124). This bioassay was suggested for testing different dilutions and bases for a single anti-inflammatory compound in addition to tests for the comparative efficacy of any number of compounds.

Thinning of Epidermis—Two bioassays that utilize the epidermis-thinning potential of topical corticosteroids have been developed: a mouse tail epidermis bioassay and a guinea pig epidermis bioassay.

Mouse Tail Epidermis—In this bioassay, various corticosteroid preparations are applied daily to the tail skin of male mice of the "to" strain for 3 weeks (125). After the mice are killed with chloroform, the dorsal tail skin, about 3 cm from the base of the tail, is removed for histological examination. The tissue is fixed in 70% ethanol, and sagittal sections are prepared and stained with hematoxylin and eosin.

Epidermal thickness from the dermoepidermal junction to the base of the horny layer is measured with an eyepiece graticule. The mouse tail is particularly suitable for this purpose since the dermoepidermal junction is virtually flat and runs parallel to the surface. Ten different measurements are made on each tail scale, and 10 scale regions are examined from each tail.

Of the corticosteroids tested, fluocinolone acetonide and sintisone demonstrated the greatest effect. It was suggested (125) that the thinning effect of the corticosteroid preparations tested closely followed their clinical efficacy in the treatment of proliferative epidermal disorders.

Guinea Pig Epidermis—Topical application of ointment bases causes various degrees of measurable and reproducible epidermal thickening in guinea pigs (126, 127). The addition of steroids in various concentrations to the bases reduces the thickening caused by the ointment base alone. A bioassay was developed on the reduction of the epidermis thickening caused by the ointment base alone (128). Six groups of albino guinea pigs are used. The backs are clipped, and the area is divided into six equal parts. One site is used as a control, the ointment base alone is applied to a second site, and four different concentrations of steroid in the ointment

Table VI—Relative Potency of Commercial Topical Corticosteroids Using the Vasoconstrictor Bioassay[a]

Compound	Commercial Concentration, %	Potency According to Concentration	Relative Vasoconstrictor Activity (Hydrocortisone Acetate = 1)
Hydrocortisone acetate	1	1	1
Prednisolone phosphate	0.5	2	0.1
Prednisolone acetate	0.5	2	1
Prednisolone	0.5	2	0.1
Methylprednisolone acetate	0.25	4	1
9α-Fluorohydrocortisone	0.1	10	1
Dexamethasone phosphate	0.1	10	0.01
Dexamethasone	0.1	10	1
Flurandrenolide	0.05	20	10
Triamcinolone acetonide	0.1	10	100
Fluocinolone acetonide	0.025	40	100

[a] Data from Ref. 136.

base are rubbed into the four remaining sites daily for 4 or 7 days.

The animals are killed on the 5th or 8th day, the hair is removed with barium sulfide, and the treated areas and control sites are fixed in formaldehyde saline and processed for histology. Sections of 5 μm are stained with hematoxylin and eosin, and epidermal thickness is measured at 20 points on each specimen using an eyepiece graticule.

The inhibiting effect of the steroids tested was related to the type of corticosteroid, its concentration, and the base. Fluocinolone acetonide and fluocinonide in fatty alcohol–propylene glycol cream base[12] were most effective, and this bioassay was recommended to select the most suitable vehicle for topical corticosteroids (128).

Inhibition of Hair Growth—Naturally occurring corticosteroids induced inhibition of hair growth in the rat when applied directly to the skin (129, 130). Based on this observation, a bioassay was developed using the rate of hair growth in rats; it shows the comparative potency of hydrocortisone analogs (131, 132). Adult male rats of the Long-Evans strain are distributed among the experimental groups so that littermates are compared under different treatments. The analogs are dissolved in 25% ethanol, and 5.0 μg of each corticosteroid in 0.1 ml of solution is applied daily to a small area caudal to the right ear. An equal volume of 25% ethanol is applied to the same area on the control animals.

A record is made of the extent and pattern of hair growth, and hair is clipped from the dorsum of the neck prior to initiation of treatment and weekly thereafter. This procedure is continued for 7 weeks. Since the pattern of hair growth in the rat is bilaterally symmetrical, inhibition is present if hair fails to appear in the treatment area but grows in the comparable area on the untreated side.

In the scoring system, two points are given for each week in which inhibition is noted. Promptness in response is recognized by adding a figure equal to the number of weeks between the first appearance of inhibition and the end of the 7-week period of study. Two points are subtracted for each observation in which more hair is found in the treated area than in the untreated area. One point is subtracted for each week in which a small amount of hair appears in the treated area that was previously without hair. The maximum score is 21 points.

The scores for all rats treated with one analog are averaged and corrected by subtracting the average score from the alcohol-treated controls. Then, the effectiveness of each analog is compared with that of hydrocortisone in the ratio of the score for an analog/the score for hydrocortisone.

In seven hydrocortisone analogs, unsaturation at C-1 and C-2 induced moderate increases in activity according to this bioassay (131, 132). Methylation at C-2 produced a greater increase. Fluorination at C-9 produced a more highly active compound, while fluorination of 21-deoxyhydrocortisone at C-9 and C-21 produced the most potent compound (132).

Reduction of Mitotic Rate—Fisher and Maibach (133) suggested that the reduction in mitotic rate produced by corticosteroids might be used as a bioassay. A bioassay was developed using the dorsal skin of the hairless mouse which, when "stripped" with adhesive tape, provides a suitable model for the study of the antimitotic effect of corticosteroid formulations (134). Equal quantities of different formulations are applied to at least six mice. Dressings are applied and kept in place for 5–196 hr. Four hours before sacrifice, the mice are injected intraperitoneally with a solution of demecolcine[13]. After sacrifice, the dorsal skin is removed and fixed, and the mitotic index is established. When this bioassay was used, betamethasone valerate had a significant antimitotic effect at a concentration of 10^{-4}, while hydrocortisone was antimitotic at a concentration of 10^{-2} (134). This animal bioassay may be used to assay the relative potency of topical corticosteroids.

Vasoconstrictor—McKenzie and Stoughton (135, 136) noticed that, when using topical corticosteroids under plastic[2] wrap, both the lesion and the surrounding normal skin displayed a pallor. The observed vasoconstriction might be used as an index of the percutaneous absorption of steroids, and a bioassay was developed in which solutions or suspensions of various dilutions of corticosteroids are prepared in 95% alcohol and applied to areas 2.54 cm (1 in.) in diameter on the forearms. One

[12] FAPG.

[13] Colcemid.

arm is left uncovered, and the other is wrapped with plastic[2] wrap. The treated areas are left undisturbed for 16 hr, and the absence or presence of vasoconstriction is recorded.

Plastic[2] wrap caused a 100-fold increase in absorption as determined by vasoconstriction (136). The relative potency of commercially available topical corticosteroids was established (Table VI).

Alcoholic Vasoconstriction—These discoveries opened one of the most active fields of research in the development of topical corticosteroids. The alcoholic vasoconstrictor bioassay was used to evaluate the topical activity of betamethasone esters in humans, and betamethasone 17-valerate was the most active compound (137, 138). It was also utilized in the assessment of four new fluocortolone analogs (139). In these studies, diflucortolone trimethylacetate, which had 10 times the potency of triamcinolone acetonide, was the most potent analog.

The alcoholic vasoconstrictor activity and percutaneous absorption of glucocorticoids was compared (140). Contrary to previous work (137), fluocinolone acetonide was equivalent to betamethasone valerate, and fluocinolone acetonide acetate (fluocinonide) was five times more potent than both betamethasone valerate and fluocinolone acetonide (140).

In alcoholic vasoconstriction tests, flucloronide was more potent than betamethasone valerate, flurandrenolide, beclomethasone propionate, fluocortolone caproate, and flumethasone pivalate (141–143).

The vasoconstrictor bioassay was modified (144) to evaluate precisely the potency of topically applied corticosteroids. In this assay, 10-fold serial dilutions from 10^{-2} (1.0%) to 10^{-7} (0.00001%) of various corticosteroids in ethanol are first prepared. Adult white subjects are utilized, and each subject receives all serial dilutions randomly assigned to one arm. Duplicate applications randomized differently on the opposite arm provide a check on the assay. The number of sites in each study equals the number of preparations to be tested so that every test site receives every preparation.

The skin of the forearm is prepared by washing with soap and water, and the test sites are outlined by a thin film of silicone grease applied with a rubber stamp. There are three or four horizontal and six or eight vertical rows, giving 18 or 32 uniform, 7 × 7-mm squares. The square pattern reduces the problem of recognition of test sites, and the grid pattern is marked with gentian violet to assist in individual site identification. Rapid and accurate application without cross-contamination of glassware is accomplished by using a separate 10-μl pipet for each application. After evaporation of the diluent, the area is covered with plastic[2] wrap and protected by tubular gauze.

After 16–18 hr of occlusion, the dressings are removed; 1–2 hr later, the sites are viewed. Two observers independently read and record the presence or absence of vasoconstriction at each test site. Observations as well as applications are always done under double-blind conditions. For each dose of each compound, the number of responding sites is tabulated, and this value is expressed as a percentage of the number of sites to which that dose–compound combination was applied. Figure 2 shows a typical plot of results in which fluocinolone acetonide was equipotent to betamethasone valerate, while fluocinonide was five times more potent than both fluocinolone acetonide and betamethasone valerate. These results are in agreement with those of Stoughton (140).

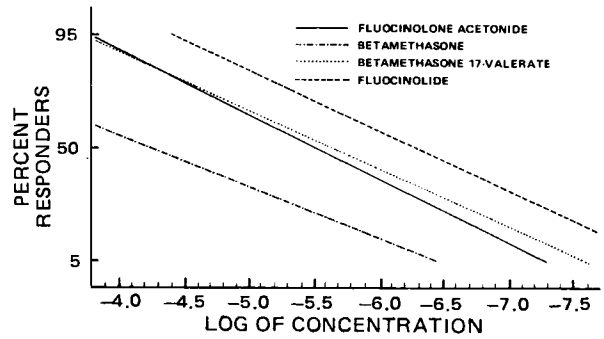

Figure 2—*Dose–response curves of various corticosteroids using the alcoholic vasoconstriction bioassay. (Reprinted, with permission, from Ref. 144.)*

Seven topical corticosteroids were evaluated with a series of vasoconstrictor tests, one of which was the alcoholic vasoconstrictor test (145).

A modified alcoholic vasoconstrictor test was reported using glassine-backed squares of absorbent paper saturated with different concentrations of alcoholic solutions and applied to the skin (146). Four new difluoroprednisolone diesters were more active than betamethasone valerate.

Barry and Brace (147), following the suggestion of Burdick *et al.* (122), adopted the "area under the curve" concept, commonly used in pharmacokinetic studies of systemic drugs, for their alcoholic vasoconstrictor studies. They evaluated vasoconstriction at 17 reading times over 26 hr and determined complete blanching curves for a series of structurally related compounds. These area under the curve determinations are indicative of the intensity and duration of action of the corticosteroids studied. Substitution or removal of the 21-hydroxy group on steroids produced compounds with a wide range of activity. Poor activity correlated with a hemisuccinate salt grouping at position 21 or with the absence of 11β-hydroxy groups.

Formulation Vasoconstriction—After selecting the compound of choice with the alcoholic vasoconstriction test, the next step in the development of topical corticosteroids is the evaluation of the active drug in various vehicles and comparisons with marketed products. The regular Stoughton–McKenzie alcoholic vasoconstriction bioassay was refined (106, 122, 148, 149) in such a way that all preparations are placed in identical tubes and coded. For each study, the forearms of 16–20 volunteers are prepared by gentle washing and drying. Strips of double adhesive-coated tape[11] with 7 × 7-mm prepunched squares are applied to both forearms so that each arm has 32 application sites. Tables of randomization are used to assign each preparation to the individual square test sites.

Three milligrams of each test formulation is applied to the skin in each square and spread evenly. On one arm, a protective cage is placed over the sites for "open"

application. On the other arm, the remaining backing is removed from the strips and the application sites are occluded with plastic[2] wrap. After 6 hr of exposure to the corticosteroid base preparations, all tapes are removed and the arms are washed. At 8, 24, and 32 hr after the original application of the formulation, the presence or absence of vasoconstriction is read independently by two experienced observers under standard lighting conditions.

The results for each application are expressed as the percentage of the total number of sites in which vasoconstriction is observed. Since variations were seen in the slopes of percent sites responding *versus* time curves for the different formulations tested, adding all readings to give a rough area under the curve evaluation was suggested (106). Commenting on the vagaries of the vasoconstriction test, Burdick (150) also suggested the following to eliminate errors inherent in the test: (*a*) preselection of subjects and use of sufficient numbers of subjects and test sites, (*b*) use of two independent readers to reduce interpretative errors, (*c*) randomization of test materials, and (*d*) use of a patterned square site and standard lighting conditions. The method was improved (151, 152) by increasing reading times to determine a complete blanching profile.

A pharmacokinetic–pharmacodynamic relationship was developed for topical corticosteroids used in the vasoconstrictor test (153, 154). Two models were obtained with good results using two-compartment open models. Model 1 makes no assumption about the progress of the blanching before the time, s, at which the source of steroid is effectively removed and thus treats times $t \geq s$ only. It assumes the concentration of steroid in stratum corneum is maximal at s. The response at s is A_s; B is the observed response at any later time. The kinetic model is $A \xrightarrow{k_1} B \xrightarrow{k_2}$, and the equation for the observed responses is solved as:

$$B_{t \geq s} = \frac{k_1 A_s}{k_2 - k_1}\left[e^{-k_1(t-s)} - e^{-k_2(t-s)}\right] + B_s e^{-k_2(t-s)} \quad \text{(Eq. 1)}$$

where k_1 and k_2 are first-order rate constants for blanching. This model gave an excellent fit to data from alcoholic steroid solution bioassays.

Model 2 extends Model 1 by assuming that the steroid enters the stratum corneum from the vehicle at a constant rate and thus permits analysis from initial application, $t = 0$. The kinetic model is source–$P \rightarrow A \xrightarrow{k_1} B \xrightarrow{k_2}$, and the equation for the observed responses is solved as forms of:

$$B_{t \geq s} = \frac{P}{k_2(k_2 - k_1)}\left[k_2 e^{-k_1 t}(e^{k_1 s} - 1) - k_1 e^{-k_2 t}(e^{k_2 s} - 1)\right] \quad \text{(Eq. 2)}$$

where P represents the constant input of pharmacological response from the source. The assumption of a constant rate of input allows A_s and B_s to be expressed in terms of P and k_1, so they do not appear in the equation. This model produced good fits to blanching data from several proprietary creams and ointments but poor fits for the alcoholic data (154).

Considerable work has been done on the evaluation of relative potencies of various topical corticosteroids using the formulation vasoconstriction bioassay. Table VII lists the corticosteroids tested along with dosage forms and the type of vasoconstrictor studies performed with each compound.

Paired Comparison Vasoconstriction—Although regular vasoconstriction studies are useful in evaluating the effect of vehicles and are used in the comparative evaluation of marketed products, they have some disadvantages. Most new corticosteroids produce maximum responses when administered at therapeutic concentrations under occlusion, thus making it difficult to demonstrate potency differences between different corticosteroids or between different concentrations of a specific drug above a certain threshold concentration. The other apparent major difficulty with the vasoconstrictor bioassay is its reliance upon subjective visual measurements. Although the human eye can perform as an effective rating device, its reliability suffers whenever comparisons are separated by distance and time.

To avoid these pitfalls, a technique was developed which involves paired comparison of vasoconstriction at adjacent sites (177). In these studies, only two formulations are tested and the direct comparison of the formulations as pairs is required. For each pair, results are reported as: one site exhibits a greater degree of vasoconstriction, both sites show equal vasoconstriction, or no vasoconstriction is observed at either site. By using this technique, statistically significant differences were found between 0.05% fluocinonide cream and 0.1% betamethasone valerate cream when applied under occlusion.

Xenon-133 Method for Evaluation of Vasoconstriction—Most methods for evaluating vasoconstriction resulting from application of topical corticosteroids are not quantitative. A method was reported which measures corticoid-induced vasoconstriction by xenon-133 clearance (178). Subjects between the ages of 18 and 50, without evidence of dermatological or vascular disease, are selected. Total resting skin blood flow in the forearm is determined by the clearance of epicutaneously applied xenon-133 in 17 control subjects. In an additional seven subjects, a comparison of differences in flow of bilateral symmetrical sites of the forearm is obtained. The experimental group consists of 14 subjects. Polyethylene tape with 4 $\mu g/cm^2$ of flurandrenolide dispersed in the adhesive layer and a similar nonmedicated tape are applied to symmetrical sites of the forearm 18–24 hr prior to the study.

Total skin blood flow is measured by a modification of the atraumatic epicutaneous labeling technique of Sejrsen (179), in which xenon-133 enters the cutaneous tissues through the intact epithelial surface. A 5 × 5-cm vinyl membrane is affixed to the skin with polyethylene tape at its margins; xenon-133 gas, with a specific activity of 5–10 mCi/ml, is introduced under the membrane from a syringe and left in contact with the skin for 2 min. The membrane is then removed, and the excess gas is blown away. This epicutaneous labeling results in diffusion into the cutaneous tissue of a minute fraction of the isotope initially brought into contact with the skin. A counting rate of 1–10 × 10^5 counts/min is obtained.

Counting is performed using a nuclear probe with a 5.1-cm (2-in.) sodium iodide crystal immediately fol-

Table VII—Review of Published Vasoconstrictor Studies with Marketed Corticosteroid Products

Compound	Formulations	Remarks	References
Beclomethasone dipropionate	Cream	Comparison with marketed products	151
	Ointment	Comparison with marketed products	152
Betamethasone benzoate	Cream, lotion, gel	Comparison with marketed products	155
	Ointment	Vehicle evaluation	156
Betamethasone dipropionate	2-Propanol–water solution	Comparison with betamethasone valerate	157
Betamethasone valerate	Cream	Comparison with marketed products	151, 155, 158, 159
		Comparison with fluocinonide	161
		Vehicle evaluation	162
		Comparison with fluclorolone acetonide	163
	Ointment	Comparison with marketed products	122, 152, 155, 159
		Comparison with fluocinonide	161
		Vehicle evaluation	162
		Comparison with flucloronide	163
		Comparison with betamethasone 17-benzoate	156
		Comparison with 21-desoxybetamethasone 17-dipropionate	160
	Lotion	Comparison with marketed products	164
	2-Propanol–water solution	Comparison with betamethasone dipropionate	157
Clobetasol propionate	Cream	Comparison with marketed products	151
	Ointment	Comparison with marketed products	152
Desonide	Cream	Comparison with marketed products	151
21-Desoxybetamethasone 17-propionate	Ointment	Comparison with betamethasone valerate	160
Flucloronide	Cream	Comparison with marketed products	151
		Comparison with betamethasone valerate	163
		Vehicle evaluation	163, 165
	Ointment	Comparison with marketed products	152
		Comparison with betamethasone valerate	163
		Vehicle evaluation	163, 165
	Gel	Vehicle evaluation and comparison with betamethasone valerate	163
Flumethasone pivalate	Cream	Comparison with marketed products	151, 155, 158, 159
	Ointment	Comparison with marketed products	152, 159
	Lotion	Comparison with marketed products	164
Fluocinolone acetonide	Cream	Comparison with marketed products	151, 155, 159
		Comparison with extemporaneous hydrocortisone creams	148
		Comparison with fluorometholone	167
		Vehicle evaluation	162, 166
	Ointment	Comparison with marketed products	152, 155, 159
		Comparison with triamcinolone acetonide	169
		Vehicle evaluation	162, 166, 168
	Gel	Comparison with marketed products	151, 159
	Lotion	Comparison with marketed products	164
		Comparison with triamcinolone acetonide	170
Fluocinonide	Fatty alcohol–propylene glycol[a]	Comparison with marketed products	151, 155, 158
		Comparison with betamethasone valerate	161
		Vehicle evaluation	106, 152, 171, 172
	Ointment	Comparison with marketed products	122
		Vehicle evaluation	152
Fluocortolone plus fluocortolone caproate	Cream, ointment	Comparison with marketed products	159
Fluocortolone pivalate plus fluocortolone hexanoate	Cream	Comparison with marketed products	151
	Ointment	Comparison with marketed products	152
Fluorometholone	Cream, ointment, solution	Vehicle evaluation	167
Fluperolone 21-acetate	Ointment	Comparison with marketed products	164
Flurandrenolide	Cream	Comparison with marketed products	151, 155, 164
	Ointment	Comparison with marketed products	122, 152, 155
Formocortol	Cream	Comparison with marketed products	151
	Ointment	Comparison with marketed products	152
Triamcinolone acetonide	Cream	Comparison with marketed products	151, 155, 158 159
		Comparison with fluocinonide	161
		Comparison with fluocinolone acetonide	148
	Ointment	Comparison with marketed products	122, 152, 155, 159
		Comparison with fluocinonide	161
		Comparison with fluocinolone acetonide	169
	Lotion	Comparison with marketed products	155, 164
		Comparison with fluocinolone acetonide	170
Miscellaneous		Placebo response to white soft paraffin–propylene glycol	173–176

[a] FAPG.

lowing removal of the vinyl membrane. The scintillation detector is coupled to a scaler registering the counting rate at 5-sec intervals and records on a strip chart for at least 30 min (178). After correction for background, the differences in blood flow are determined for symmetrical sites that received flurandrenolide and control tapes.

In one study of flurandrenolide, a mean blood flow of 5.55 ml/100 g/min was determined in the control sites compared to 3.84 ml/100 g/min in sites treated with flurandrenolide (178). Xenon-133 clearance may be better than visual observation to measure vasoconstrictor activity of topical corticosteroids.

Intradermal Injection Vasoconstriction—Studies of topical corticoid vasoconstriction involve both percutaneous penetration and inherent vasoconstrictor activity. To eliminate this penetration aspect, steroids have been studied after intradermal injection. Assays of steroids for possible local activity in human skin (180) found that 9α-fluorohydrocortisone acetate and free alcohol were superior to hydrocortisone acetate and free alcohol (181). The relative potencies of prednisone and prednisolone were studied, and prednisolone was more active (182).

A precise intradermal injection bioassay was reported in which the steroids are serially diluted and 0.1 ml of each dilution is injected intradermally into the backs and forearms of volunteers (183). In the back, five to 11 injections are made in rows, up to six rows per subject (three rows on each side). Injections are about 1.9 cm (0.75 in.) apart; rows are about 2.54 cm (1 in.) apart, always at least 3.8 cm (1.5 in.) from the midline and centered over the muscle belly. The dilutions are injected in descending order of strength from top to bottom, and no randomization is attempted. Three saline control injections are made, one at the top, middle, and bottom of the areas between rows.

In the forearm, injection sites are measured 1.9 cm (0.75 in.) apart on the flexor aspect at the edge of the hairline and not closer than 3.8 cm (1.5 in.) from the cubital fossa and wrist. Only one row of injections is made per arm with not more than seven injections in the row. One saline control is injected medial to the center of the row, about 2.54 cm (1 in.) away. The dilutions are injected in descending order of strength starting near the cubital fossa and working toward the wrist. Different subjects are used in the back and the back *versus* arm studies.

In the back *versus* arm experiment, each subject is injected with one compound on the left back and left arm and vice versa. The injection sites are read at varying intervals from 1.5 to 6 hr. The end-point, which is the most dilute concentration giving definite vasoconstriction, is noted for each subject reacting; an average end-point is figured using all subjects tested, although some fail to react. Vasoconstriction is noted as present or absent. Any questionable blanching is considered negative.

Using this technique, Sutton *et al.* (183) avoided the penetration problems and determined the relative potency of various corticosteroids as determined by skin blanching following intradermal injection. They tested a series of topically used corticosteroids and found hydrocortisone to be least effective and fluocinolone acetonide to be most effective.

Intradermal injections also were used to evaluate both vasoconstriction and atrophy produced by corticosteroids (184). Desonide and triamcinolone acetonide, which differ only by the presence of a fluorine atom, were tested. Both exhibited similar vasoconstrictor potency but only triamcinolone acetonide, the fluorinated steroid, produced atrophy (184).

Mechanism of Action—The vasoconstrictor effect of topical corticosteroids is by now well established, but its mechanism is not clear. Juhlin (185), in his work with vascular reactions in skin treated with fluocinolone acetonide, suggested that the blanching of the skin is due to a vasoconstrictor effect of the steroid *per se*. Thune (186), evaluating the vasoconstrictive effect of steroids by plethysmographic recording of skin pulses, concluded that it is due mainly to decongestion of the capillaries and small veins beneath the epidermis. Solomon *et al.* (187) demonstrated that oral guanethidine, a norepinephrine antagonist, prevents topically applied steroid vasoconstriction in normotensive subjects and suggested that corticosteroids are capable of releasing norepinephrine from cutaneous stores. Others suggested that norepinephrine is not the only factor involved.

Reis (188) showed that steroids have a local and primary effect on normal human bulbar conjunctival vessels as well as potentiating norepinephrine. Steroids suppressed the vasodilation caused by such agents as histamine, alcohol, and bradykinin as well as potentiating the effects of norepinephrine (189). In the modified hamster cheek pouch and in humans, topically applied corticosteroids potentiated catecholamine-induced vasoconstriction. Since this effect was blocked by phentolamine but not by propranolol, this action might be through the increased sensitivity of α-adrenergic receptors (190).

duVivier and Stoughton's (191) demonstration of tachyphylaxis to topically applied steroids might suggest that they act indirectly by releasing endogenous norepinephrine from nerve storage vesicles. They might also influence the metabolism of the neurotransmitter or its recapture by the vesicles after release. Alternatively, the steroid may act by attaching itself to a receptor site, causing the release of the intracellular mediator cyclic adenosine monophosphate or guanosine

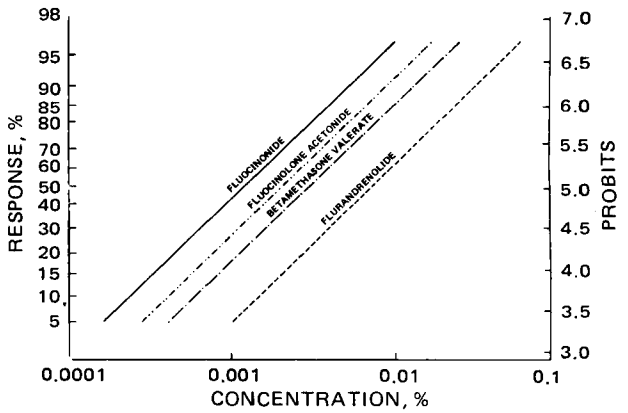

Figure 3—*Dose-response curves of various corticosteroids using the psoriasis bioassay. (Reprinted, with permission, from Ref. 193.)*

Table VIII— Vasoconstrictor and Clinical Response with Solubilized and Suspended 0.025% Fluocinolone Acetonide Ointments[a]

	Degree of Vasoconstriction[b]	Clinical Trials, % of Patients Showing Better Response		
		Eczema	Psoriasis	Total
0.025% fluocinolone acetonide in petrolatum, micronized particles	1.4	12	12	12
0.025% fluocinolone acetonide in petrolatum, solubilized in propylene glycol	1.8	56	36	46

[a] Data from Refs. 194 and 195. [b] Average of 10 subjects.

monophosphate and, thus, causing vasoconstriction (191).

Psoriasis Bioassay—Most bioassays fail to deal with spontaneously occurring skin diseases. The psoriasis bioassay was developed to deal directly with the disease state and, therefore, is the nearest approximation of the clinical trial. Chronic stabilized psoriatic patients are used as test subjects, and the test site is outlined with precut 1.1-cm^2 adhesive squares. One-half minim of medication is applied in a double-blind fashion to each site and covered with a square of plastic film. The medication is spread by light pressure on the film, and the plastic is then sealed to the underlying border. Dressings are applied daily and remain in place 24 hr.

The area is cleaned and read as either unchanged or completely normal. The final reading is made on the 5th day (106, 192, 193). The results can be presented as log dose–percentage cleared (probit) graphs. By using this technique, the relative potencies of various corticosteroids were calculated using fluocinolone acetonide as a standard (Fig. 3). Fluocinonide had 1.85 times the activity of fluocinolone acetonide; betamethasone valerate had 0.69 times the activity of fluocinolone acetonide.

CLINICAL CORRELATION

The ultimate usefulness of any bioassay is displayed when it can be correlated with clinical trials; the vasoconstrictor bioassay is one bioassay that is well correlated with clinical trials. The effect of solubilization of the corticosteroid in the vehicle has been studied (166, 194). Both the vasoconstrictor and clinical response of solubilized *versus* suspended fluocinolone acetonide in ointment form were evaluated. The results, which show the advantage of solubilization of the steroid, are given in Table VIII.

Table IX—Results of Comparison between Formulation Bioassay and Clinical Trial[a]

	Results	
	Formulation Bioassay	Double-Blind Clinical Trial
0.1% betamethasone valerate ointment *versus* 0.025% fluocinolone acetonide ointment	Betamethasone valerate superior; $p < 0.05$	Betamethasone valerate superior; $p < 0.05$
0.1% betamethasone valerate cream *versus* 0.05% fluocinonide cream	Fluocinonide superior; $p < 0.05$	Fluocinonide superior; $p < 0.05$

[a] Data from Ref. 155.

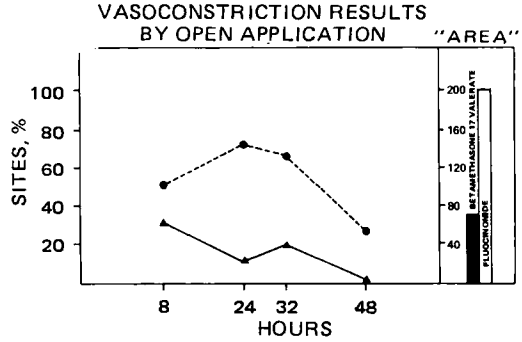

Figure 4—*Results of vasoconstrictor studies by open 16-hr application techniques and comparing 0.05% fluocinonide in fatty alcohol–propylene glycol cream (●) with 0.1% betamethasone 17-valerate cream (▲). (Adapted, with permission, from Ref. 158.)*

Stoughton (155) applied the vasoconstriction *versus* clinical correlations to comparison of marketed products and compared the vasoconstrictor response of 0.1% betamethasone valerate ointment *versus* 0.025% fluocinolone acetonide ointment and 0.05% fluocinonide cream *versus* 0.1% betamethasone valerate cream. In both vasoconstrictor studies and clinical trials, the 0.1% betamethasone valerate ointment and 0.05% fluocinonide cream were statistically superior to 0.025% fluocinolone acetonide ointment and 0.1% betamethasone valerate cream, respectively (Table IX).

The vasoconstrictor and clinical response of 0.05% fluocinonide cream and 0.1% betamethasone valerate cream also were compared (158). The 0.05% fluocinonide cream was superior to 0.1% betamethasone valerate cream as measured with the vasoconstriction bioassay (Fig. 4) and in clinical trials (Fig. 5).

These results clearly indicate that the vasoconstrictor bioassay is an excellent model in evaluating the efficacy of topical corticosteroids prior to their final testing during clinical trials.

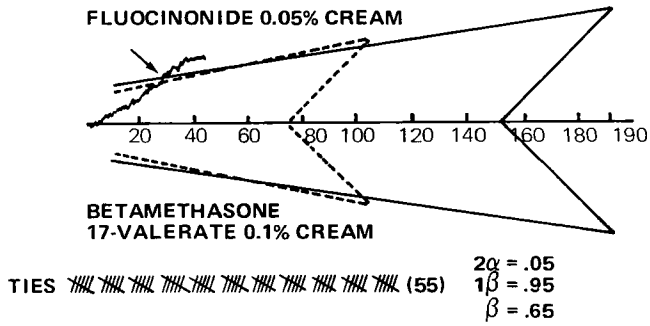

Figure 5—*Fourteen-day results in a sequential trial by paired comparisons of 0.05% fluocinonide in fatty alcohol–propylene glycol cream against 0.1% betamethasone 17-valerate cream. (Reprinted, with permission, from Ref. 158.)*

CONCLUSIONS

The ultimate systems for establishing the therapeutic efficacy of topical formulations are the qualitative and quantitative clinical trials. Since these therapeutic appraisals are full of variables and very costly to perform, they should be reserved only for careful evaluation of preselected candidates using double-blind clinical comparison between new and marketed products.

With the development of bioassays for topical antimicrobials, antimitotics, antiperspirants, sunscreens, antidandruff formulations, anesthetics, antipruritics, antiwart formulations, and corticosteroids, it is now possible to evaluate the inherent potencies of various topically active chemicals. It is also possible to evaluate the drug delivery characteristics of various vehicles.

Some bioassays discussed in this review have already been successfully used in the development of topical dosage forms. The vasoconstrictor bioassay also has been correlated with clinical trials. It is hoped that the newer bioassays which have not been used in the development of topical formulations will be regularly used along with established bioassays in the selection of optimum formulations for clinical trials, thus reducing the exorbitant cost of developing new drugs.

It is this reviewer's firm belief that intelligent use of available bioassays to evaluate test formulations of topical drugs will greatly facilitate the development of more efficacious and safer drugs. Used appropriately, these models can provide added confidence and reliability in those formulations subjected to clinical evaluation.

REFERENCES

(1) A. Burger, *Chem. Eng. News*, **53,** 37(1975).

(2) C. A. Schlagel, *J. Pharm. Sci.*, **54,** 335(1965).

(3) C. A. Schlagel, in "Pharmacology and the Skin," W. Montagna, E. J. Van Scott, and R. B. Stoughton, Eds., Appleton-Century-Crofts, New York, N.Y., 1972, p. 339.

(4) C. A. Schlagel, in "Animal Models in Dermatology," H. Maibach, Ed., Churchill Livingstone, Edinburgh, Scotland, 1975, p. 203.

(5) R. B. Stoughton, in "Pharmacology and the Skin," W. Montagna, E. J. Van Scott, and R. B. Stoughton, Eds., Appleton-Century-Crofts, New York, N.Y., 1972, p. 535.

(6) J. W. Hadgraft, *Br. J. Dermatol.*, **87,** 386(1972).

(7) R. B. Stoughton, *Arch. Dermatol.*, **101,** 160(1970).

(8) R. B. Stoughton, in "Animal Models in Dermatology," H. Maibach, Ed., Churchill Livingstone, Edinburgh, Scotland, 1975, p. 121.

(9) R. R. Marples, A. Rebora, and A. M. Kligman, *Arch. Dermatol.*, **108,** 237(1973).

(10) R. R. Marples and A. M. Kligman, in "Epidermal Wound Healing," H. Maibach and D. Rovee, Eds., Yearbook Medical Publishers, Chicago, Ill., 1972, p. 241.

(11) P. Williamson and A. M. Kligman, *J. Invest. Dermatol.*, **45,** 498(1965).

(12) G. Singh, R. R. Marples, and A. M. Kligman, *ibid.*, **57,** 149(1971).

(13) R. R. Marples and A. M. Kligman, *Antimicrob. Ag. Chemother.*, **5,** 323(1974).

(14) R. R. Marples and A. M. Kligman, *Toilet Goods Assoc. Cosmet. J.*, **1,** 26(1969).

(15) F. Reiss, L. Caroline, and L. Leonard, *J. Invest. Dermatol.*, **24,** 575(1955).

(16) F. Reiss and L. Leonard, *ibid.*, **24,** 589(1955).

(17) S. Molinas, *ibid.*, **25,** 33(1955).

(18) M. M. Dolan, J. S. Ebelhare, A. M. Kligman, and R. C. Bard, *ibid.*, **28,** 359(1957).

(19) M. Plempel and K. Bartmann, *Arzneim.-Forsch.*, **22,** 1280(1972).

(20) H. Weuta, *ibid.*, **22,** 1295(1972).

(21) J. H. Reinhardt, A. M. Allen, D. Gunnison, and W. A. Akers, *J. Invest. Dermatol.*, **63,** 419(1974).

(22) H. E. Jones, J. H. Reinhardt, and M. G. Rinaldi, *Arch. Dermatol.*, **110,** 369(1974).

(23) A. G. Knight, *Br. J. Dermatol.*, **89,** 509(1973).

(24) *Ibid.*, **91,** 49(1974).

(25) A. G. Knight, *J. Invest. Dermatol.*, **59,** 354(1972).

(26) *Ibid.*, **59,** 427(1972).

(27) R. M. Schwartzman, M. J. Deubler, and P. F. Dice, II, *J. Small Anim. Pract.*, **6,** 327(1965).

(28) I. Kubo, M. Hori, H. Niitani, I. Yokoyama, and T. Takemoto, "Studies on the Pathogenesis of Candidiasis," Studies on Candidiasis in Japan, 145, Tokyo-Research Committee of Candidiasis, Education Ministry of Japan.

(29) J. Van Cutsem and D. Thienpont, *Sabouraudia*, **9,** 17(1971).

(30) G. Maestrone and R. Semar, *Naturwissenschaften*, **55,** 87(1968).

(31) H. I. Maibach and A. M. Kligman, *Arch. Dermatol.*, **85,** 233(1962).

(32) M. C. Spencer, *ibid.*, **88,** 925(1963).

(33) A. Rebora, R. R. Marples, and A. M. Kligman, *ibid.*, **108,** 69(1973).

(34) E. J. Van Scott and R. H. Bonder, *J. Invest. Dermatol.*, **56,** 132(1971).

(35) E. J. Van Scott and R. J. Yu, in "Animal Models in Dermatology," H. Maibach, Ed., Churchill Livingstone, Edinburgh, Scotland, 1975, p. 248.

(36) E. J. Van Scott and R. J. Yu, *J. Invest. Dermatol.*, **62,** 378(1974).

(37) R. J. Yu and E. J. Van Scott, *ibid.*, **63,** 279(1974).

(38) J. L. McCullough and G. D. Weinstein, *ibid.*, **65,** 394(1975).

(39) A. duVivier and R. B. Stoughton, *Clin. Res.*, **23,** 166A(1975).

(40) A. duVivier and R. B. Stoughton, *Br. J. Dermatol., Suppl. 11,* **93,** 27(1975).

(41) A. duVivier and R. B. Stoughton, *J. Invest. Dermatol.*, **65,** 235(1975).

(42) A. duVivier, R. Bible, Jr., R. K. Mikuriya, and R. B. Stoughton, *Br. J. Dermatol.*, **94,** 1(1976).

(43) R. B. Stoughton, F. Chiu, W. Fritsch, and D. Nurse, *J. Invest. Dermatol.*, **42,** 151(1964).

(44) F. S. K. MacMillan, H. H. Reller, and F. H. Snyder, *ibid.*, **43,** 363(1964).

(45) M. Wada, *Science*, **111,** 376(1950).

(46) H. F. Blum, "Photodynamic Action and Diseases Caused by Light," American Chemical Society Monograph Series No. 85, Reinhold, New York, N.Y., 1941.

(47) I. Willis and A. M. Kligman, *J. Soc. Cosmet. Chem.*, **20,** 639(1969).

(48) E. R. Kraft, S. G. Hoch, R. A. Quisno, and E. A. Newcomb, *ibid.*, **23,** 383(1972).

(49) S. I. Katz, *Arch. Dermatol.*, **101,** 466(1970).

(50) M. A. Pathak, T. B. Fitzpatrick, and E. Frenk, *N. Engl. J. Med.*, **280,** 1459(1969).

(51) G. Kahn and G. Wilcox, *J. Soc. Cosmet. Chem.*, **20,** 807(1969).

(52) G. Kahn and G. Wilcox, *J. Invest. Dermatol.*, **53,** 200(1969).

(53) K. J. Master, R. A. Sayre, and M. A. Everett, *J. Soc. Cosmet. Chem.*, **17,** 581(1966).

(54) S. Riegelman and R. P. Penna, *ibid.*, **11,** 280(1961).

(55) O. J. Lorenzetti, J. Boltralik, E. Busby, and B. Fortenberry, *ibid.*, **26,** 593(1975).

(56) A. M. Kligman, R. R. Marples, L. R. Lantis, and K. J. McGinley, *ibid.*, **25,** 73(1974).

(57) A. B. Ackerman and A. M. Kligman, *ibid.*, **20,** 81(1969).

(58) G. Plewig and A. M. Kligman, *ibid.*, **20,** 767(1969).

(59) J. J. Leyden, K. J. McGinley, and A. M. Kligman, *ibid.*, **26,** 573(1975).

(60) R. W. Vander Wyk and F. C. Roia, Jr., *ibid.*, **15,** 761 (1964).

(61) R. W. Vander Wyk and K. E. Hechemy, *ibid.*, **18,** 629(1967).
(62) P. Finkelstein and K. Laden, *ibid.*, **19,** 669(1968).
(63) J. A. Troller, *ibid.*, **22,** 187(1971).
(64) M. von Frey, *Arch. Neerl. Physiol.*, **7,** 42(1922).
(65) W. Montagna, "The Structure and Function of Skin," 2nd ed., Academic, New York, N.Y., 1962, p. 10.
(66) E. B. Titchener, "A Textbook of Physiology," Macmillan, New York, N.Y., 1909, p. 56.
(67) J. D. Hardy, C. B. Potelunas, and M. D. Meixner, *J. Invest. Dermatol.*, **16,** 369(1951).
(68) J. D. Hardy, A. G. Wolff, and H. Goodell, *J. Clin. Invest.*, **19,** 649(1940).
(69) W. Lucas and E. P. Guth, *J. Am. Pharm. Assoc., Sci. Ed.*, **42,** 344(1953).
(70) E. W. Brockemeyer and E. P. Guth, *ibid.*, **44,** 706(1955).
(71) J. Adriani, R. Zepernick, J. Arens, and E. Authement, *Clin. Pharmacol. Ther.*, **5,** 49(1964).
(72) J. Adriani and R. Zepernick, *J. Am. Med. Assoc.*, **188,** 711(1964).
(73) H. Dalili and J. Adriani, *Clin. Pharmacol. Ther.*, **12,** 913(1971).
(74) F. E. Corima, *J. Invest. Dermatol.*, **19,** 21(1952).
(75) F. E. Corima and V. Kuykendall, *ibid.*, **20,** 429(1953).
(76) F. E. Corima and V. Kuykendall, *Arch. Dermatol.*, **69,** 206(1954).
(77) F. M. Melton and W. B. Shelley, *J. Invest. Dermatol.*, **15,** 325(1950).
(78) S. G. Macris, I. H. Blank, and H. K. Beecher, *ibid.*, **33,** 15(1959).
(79) C. G. Mendelson and A. M. Kligman, *Arch. Dermatol.*, **83,** 559(1961).
(80) L. Frank, C. Stritzler, and J. Kaufman, *ibid.*, **71,** 117(1955).
(81) E. Kepel, W. H. Rooks, II, R. W. Ferraresi, and L. D. Shott, *J. Invest. Dermatol.*, **62,** 595(1974).
(82) R. T. Bowser and H. Baer, *J. Immunol.*, **91,** 791(1963).
(83) A. Jarrett and R. I. C. Spearman, "Histochemistry of Skin-Psoriasis," English University Press, London, England, 1974.
(84) R. Wrench and A. Z. Britten, *Br. J. Dermatol.*, **92,** 569(1975).
(85) *Ibid.*, **92,** 575(1975).
(86) *Ibid.*, **93,** 67(1975).
(87) *Ibid.*, **93,** 75(1975).
(88) T. F. Dougherty and G. L. Schneebeli, *Ann. N.Y. Acad. Sci.*, **61,** 328(1955).
(89) A. G. Ruhmann and D. L. Berliner, *Endocrinology*, **76,** 916(1965).
(90) D. L. Berliner and A. G. Ruhmann, *J. Invest. Dermatol.*, **49,** 117(1967).
(91) A. G. Ruhmann and D. L. Berliner, *ibid.*, **49,** 123(1967).
(92) D. L. Berliner, *Ann. N.Y. Acad. Sci.*, **116,** 1078(1964).
(93) D. L. Berliner, A. J. Gallegos, and G. L. Schneebeli, *J. Invest. Dermatol.*, **48,** 44(1967).
(94) D. L. Berliner and A. G. Ruhmann, *Endocrinology*, **78,** 373(1966).
(95) D. L. Berliner, A. G. Ruhmann, M. L. Berliner, and T. F. Dougherty, *Excerpta Med.*, **111,** 180(1966).
(96) D. L. Berliner, M. H. Bartley, G. H. Kenner, and W. S. S. Jee, *Br. J. Dermatol., Suppl. 6,* **82,** 53(1970).
(97) D. L. Berliner and C. J. Nabors, Jr., *J. Reticuloendothel. Soc.*, **4,** 284(1967).
(98) R. I. Dorfman, *Br. J. Dermatol., Suppl. 6,* **82,** 45(1970).
(99) I. Ringler, in "Methods in Hormone Research," vol. III, R. I. Dorfman, Ed., Academic, New York, N.Y., 1964, p. 227.
(100) L. J. Lerner, A. Bianchi, A. R. Turkheimer, F. M. Singer, and A. Borman, *Ann. N. Y. Acad. Sci.*, **116,** 1071(1964).
(101) L. G. Hershberger and D. W. Calhoun, *Endocrinology*, **60,** 153(1957).
(102) G. Dipasquale, C. L. Rassaert, and E. McDougall, *J. Pharm. Sci.*, **59,** 267(1970).
(103) L. J. Lerner, A. R. Turkheimer, A. Bianchi, F. M. Singer, and A. Borman, *Proc. Soc. Exp. Biol. Med.*, **116,** 385(1964).
(104) G. Tonelli, L. Thibault, and I. Ringler, *Endocrinology*, **77,** 625(1965).
(105) J. A. Witkowski and A. M. Kligman, *J. Invest. Dermatol.*, **32,** 481(1959).
(106) K. H. Burdick, *Acta Dermatol. Venereol., Suppl. 67,* **52,** 19(1972).
(107) E. Ortega, C. Rodriguez, K. H. Burdick, V. A. Place, and L. Gonzales, *ibid.*, **52,** 95(1972).
(108) K. H. Kaidbey and A. M. Kligman, *J. Invest. Dermatol.*, **63,** 292(1974).
(109) A. Scott and F. Kalz, *ibid.*, **26,** 361(1956).
(110) M. J. Brunner and P. Finkelstein, *Arch. Dermatol.*, **81,** 453(1960).
(111) C. A. Schlagel and J. I. Northam, *Proc. Soc. Exp. Biol. Med.*, **101,** 629(1959).
(112) H. R. Gray, R. L. Wolf, and R. H. Doneff, *Arch. Dermatol.*, **84,** 18(1961).
(113) J. Wolf, *Z. Mikrosk.-Anat. Forsch.*, **46,** 170(1939).
(114) H. Möller and H. Rorsman, *Acta Dermatol. Venereol.*, **40,** 381(1960).
(115) G. C. Wells, *Br. J. Dermatol.*, **69,** 11(1957).
(116) W. W. Heseltine, J. M. McGilchrist, and R. Gartside, *Nature*, **196,** 486(1962).
(117) J. B. Shelmire, *Arch. Dermatol.*, **78,** 191(1958).
(118) J. Everall and L. Fisher, *J. Invest. Dermatol.*, **19,** 97(1952).
(119) N. B. Kanof, *ibid.*, **25,** 329(1955).
(120) A. Scott and F. Kalz, *ibid.*, **26,** 149(1956).
(121) F. Kalz and A. Scott, *ibid.*, **26,** 165(1956).
(122) K. H. Burdick, J. K. Haleblian, B. J. Poulsen, and S. E. Cobner, *Curr. Ther. Res.*, **15,** 233(1973).
(123) S. T. Zaynoun and A. K. Kurban, *Br. J. Dermatol.*, **90,** 85(1974).
(124) A. I. Scott, *ibid.*, **77,** 586(1965).
(125) R. I. C. Spearman and A. Jarrett, *ibid.*, **92,** 581(1975).
(126) I. Sarkany and P. M. Gaylarde, *Trans. St. John's Hosp. Dermatol. Soc.*, **59,** 241(1973).
(127) F. Schaaf and F. Gross, *Dermatologica*, **106,** 357(1953).
(128) H. Barnes, P. M. Gaylarde, A. P. Brock, and I. Sarkany, *Br. J. Dermatol.*, **93,** 459(1975).
(129) W. L. Whitaker and B. L. Baker, *Science*, **108,** 207(1948).
(130) B. L. Baker, *Ann. N.Y. Acad. Sci.*, **53,** 690(1951).
(131) W. L. Whitaker and B. L. Baker, *Univ. Mich. Med. Bull.*, **17,** 384(1951).
(132) K. Fukuyama and B. L. Baker, *J. Invest. Dermatol.*, **31,** 327(1958).
(133) L. B. Fisher and H. I. Maibach, *Arch. Dermatol.*, **103,** 39(1971).
(134) R. Marks, D. Pongsehirun, and T. Saylan, *Br. J. Dermatol.*, **88,** 69(1973).
(135) A. W. McKenzie and R. B. Stoughton, *Arch. Dermatol.*, **86,** 608(1962).
(136) A. W. McKenzie, *ibid.*, **86,** 611(1962).
(137) A. W. McKenzie and R. M. Atkinson, *ibid.*, **89,** 741(1964).
(138) A. W. McKenzie, in "Progress in the Biological Sciences in Relation to Dermatology," A. Rook and R. H. Champion, Eds., Cambridge University Press, Cambridge, England, 1964, pp. 283–290.
(139) H. Baker and H. A. Sattar, *Br. J. Dermatol.*, **80,** 46(1968).
(140) R. B. Stoughton, *Arch. Dermatol.*, **99,** 753(1969).
(141) M. Moore-Robinson and G. A. Christie, *Br. J. Dermatol., Suppl. 6,* **82,** 86(1970).
(142) M. Moore-Robinson, *Clin. Trials J.*, **2,** 45(1971).
(143) J. P. Garnier, *ibid.*, **2,** 55(1971).
(144) V. A. Place, J. Giner-Velazquez, and K. H. Burdick, *Arch. Dermatol.*, **101,** 531(1970).
(145) E. G. Weirich and U. Lutz, *Dermatologica*, **147,** 353 (1973).
(146) G. Falconi and G. L. Rossi, *Arch. Dermatol.*, **105,** 856(1972).
(147) B. W. Barry and A. R. Brace, *J. Invest. Dermatol.*, **64,** 418(1975).
(148) K. H. Burdick, B. Poulsen, and V. Place, *J. Am. Med. Assoc.*, **211,** 462(1970).
(149) J. Ostrenga, J. Haleblian, B. Poulsen, B. Ferrell, N. Mueller, and S. Shastri, *J. Invest. Dermatol.*, **56,** 392(1971).
(150) K. H. Burdick, *Arch. Dermatol.*, **110,** 238(1974).
(151) B. W. Barry and R. Woodford, *Br. J. Dermatol.*, **91,** 323(1974).

(152) *Ibid.*, **93,** 563(1975).
(153) B. W. Barry and A. R. Brace, *J. Pharm. Pharmacol., Suppl.*, **26,** 67P(1974).
(154) B. W. Barry, A. R. Brace, A. C. Norris, and R. Woodford, *ibid.*, **27,** 75P(1975).
(155) R. B. Stoughton, *Arch. Dermatol.*, **106,** 825(1972).
(156) A. F. Pepler, R. Woodford, and J. C. Morrison, *Br. J. Dermatol.*, **85,** 171(1971).
(157) T. Fredriksson, L. Gip, and A. Hamfelt, *Curr. Ther. Res.*, **18,** 321(1975).
(158) K. H. Burdick, *Acta Dermatol. Venereol., Suppl. 67,* **52,** 24(1972).
(159) M. F. Coldman, L. Lockerbie, and E. A. Laws, *Br. J. Dermatol.*, **85,** 381(1971).
(160) M. J. Busse, P. Hunt, K. A. Lees, P. N. D. Maggs, and T. M. McCarthy, *ibid., Suppl. 4,* **81,** 103(1969).
(161) M. F. Coldman, L. Lockerbie, and E. A. Laws, *ibid.*, **85,** 573(1971).
(162) I. Sarkany, J. W. Hadgraft, G. A. Caron, and C. W. Barrett, *ibid.*, **77,** 569(1965).
(163) G. A. Christie and M. Moore-Robinson, *ibid., Suppl. 6,* **82,** 93(1970).
(164) K. J. Child, A. F. English, H. G. Gilbert, A. Hewitt, and E. Woollett, *Arch. Dermatol.*, **97,** 407(1968).
(165) T. Malone, J. K. Haleblian, B. J. Poulsen, and K. H. Burdick, *Br. J. Dermatol.*, **90,** 187(1974).
(166) C. W. Barrett, J. W. Hadgraft, G. A. Caron, and I. Sarkany, *ibid.*, **77,** 576(1965).
(167) J. Tissot and P. E. Osmundsen, *Acta Dermatol. Venereol.*, **46,** 447(1966).
(168) M. F. Coldman and L. Lockerbie, *Br. J. Dermatol.*, **85,** 398(1971).
(169) J. Reid and D. B. Brookes, *ibid.*, **80,** 328(1968).
(170) W. W. Heseltine, J. M. McGilchrist, and R. Gartside, *ibid.*, **76,** 71(1964).
(171) M. Katz and B. J. Poulsen, *J. Soc. Cosmet. Chem.*, **23,** 565(1972).
(172) J. Reinstein, J. Ostrenga, J. Haleblian, S. Shastri, B. Poulsen, and M. Katz, *Acta Dermatol. Venereol., Suppl. 67,* **52,** 13(1972).
(173) B. W. Barry and R. Woodford, presented at British Pharmaceutical Conference, North Staffordshire, Keele, England, Paper 86, 1972.
(174) R. Woodford and B. W. Barry, *Br. J. Dermatol.*, **89,** 53(1973).
(175) *Ibid.*, **90,** 233(1974).
(176) B. W. Barry and R. Woodford, *J. Pharm. Pharmacol., Suppl.*, **24,** 174P(1972).
(177) B. J. Poulsen, K. Burdick, and S. Bessler, *Arch. Dermatol.*, **109,** 367(1974).
(178) T. P. Greeson, N. E. Levan, R. I. Freedman, and W. H. Wong, *J. Invest. Dermatol.*, **61,** 242(1973).
(179) P. Sejrsen, *Scand. J. Clin. Lab. Invest., Suppl.*, **99,** 52(1966).
(180) L. Goldman, R. Flatt, and J. Baskett, *J. Invest. Dermatol.*, **23,** 251(1954).
(181) *Ibid.*, **24,** 81(1955).
(182) *Ibid.*, **25,** 75(1955).
(183) P. M. Sutton, R. J. Feldmann, and H. I. Maibach, *J. Invest. Dermatol.*, **57,** 371(1971).
(184) D. S. Snyder and R. A. Greenberg, *ibid.*, **63,** 461(1974).
(185) L. Juhlin, *Acta Dermatol. Venereol.*, **44,** 322(1964).
(186) P. Thune, *ibid.*, **51,** 261(1971).
(187) L. M. Solomon, E. Wentzel, and M. S. Greenberg, *J. Invest. Dermatol.*, **44,** 129(1965).
(188) D. J. Reis, *J. Clin. Endocrinol.*, **20,** 446(1960).
(189) L. Frank, Y. Rapp, and L. Bird, *Arch. Dermatol.*, **89,** 55(1964).
(190) J. E. Wolf, W. R. Hubler, and N. D. Guzick, through *ibid.*, **111,** 581(1975).
(191) A. duVivier and R. B. Stoughton, *ibid.*, **111,** 581(1975).
(192) J. R. Scholtz and K. J. Dumas, "13th International Congress of Dermatology," vol. 2, Springer-Verlag, New York, N.Y., 1968, p. 179.
(193) K. J. Dumas and J. R. Scholtz, *Acta Dermatol. Venereol.*, **52,** 43(1972).
(194) B. Portnoy, *Br. J. Dermatol.*, **77,** 579(1965).

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