the CONH group of polyamide and the sample and adsorption or partition between the silica gel and the sample. In the mixed layer, polyamide also serves as a strong binder and makes the layer very durable and easy to handle. Also the layer did not crack or peel and could be stored easily. Both sides of the glass are independent of each other and chromatography can be performed simultaneously on both sides. The addition of a small amount of salt (about 0.05% sodium chloride or 0.4% ammonium chloride) in the solvent mixture is essential to break hydrogen bonding between the polyamide and the dyes. Oil-soluble dyes of yellow AB and yellow OB are rather difficult to separate because of the close similarity of their structures (different only in one methyl group). The content of polyamide (13.6%) in this mixed layer was above that of the previous report (12%) (7) in order to obtain a more durable layer.

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Modified Granuloma Pouch Procedure for the Evaluation of Topically Applied Anti-inflammatory Steroids

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Abstract □ A laboratory procedure for evaluating topically applied anti-inflammatory preparations has been described. A modified croton oil-induced granuloma pouch served as the site of drug application and also as a physical barrier to avoid drug ingestion. Increasing concentrations and/or doses of test compounds are correlated with thymolysis, catabolism, and reduced exudate formation. The procedure can differentiate steroidal modifications, percutaneous absorption, and alterations of the vehicle. However, it is difficult to ascertain how much of the action of the test compound is due to its local and how much to its systemic properties. The assay also evaluated test compounds as pharmaceutical preparations. The order of increasing activity of the test substances can be listed as hydrocortisone acetate (1% cream) < methylprednisolone acetate (0.25% cream) < betamethasone 17-valerate (0.025% cream) < triamcinolone 16,17-acetonide (0.025% cream) < betamethasone (0.2% cream) < fluocinolone acetonide (0.025% alcoholic gel or cream).

Keyphrases □ Steroids, anti-inflammatory—topical activity determination □ Granuloma pouch test procedure—topical steroid activity □ Croton oil-air—granuloma pouch formation

Potential anti-inflammatory substances, topical or systemic, have been evaluated in a variety of procedures which apparently represent different modes of actions and/or different stages of the inflammatory process. Some of these procedures include: cotton pellet, granuloma pouch, paw edema, adjuvant-induced polyarthritis, cell cultures, uncoupling of mitochondrial oxidative phosphorylation, plasma protein changes, ear test, lung inflammation, and inhibition of erythema (1–4). It is evident that each test procedure has some merit; however, one cannot avoid nor explain discrepancies which occur between the various animal *in vitro* and clinical procedures. Nevertheless, most clinically efficacious drugs are active in most procedures, although in some assays a divergence may occur between steroidal and nonsteroidal drugs. Avoidance of ingestion of topically applied drugs in animals has presented some perplexing problems. Alternatives appear to introduce other problems; for example the use of occlusive dressings may alter absorption whereas the use of collars and other restraining devices produces stress which elaborates endogenous adrenocorticosteroids. The use of an air pouch which serves as the site of drug application and a physical barrier to avoid ingestion has been tried by the authors. They have also tried to observe whether or not the modified granuloma pouch procedure will distinguish steroidal modifications and/or alterations of the vehicle. It is known that these changes will affect drug absorption, retention, and biological activity (5, 6). It should also be mentioned that most animal (*in vivo* and/or *in vitro*) procedures require a solubilized test compound which requires organic solvents. The present study also evaluated drugs as pharmaceutical preparations.

MATERIALS AND METHODS

The method used was a modification of Selye's procedure (7). Male rats weighing 150–170 g. were individually housed and arranged in groups as indicated in the tables. The dorsal surface of the animal was shaved with animal clippers and the pouches were formed with the subcutaneous injection of 25 ml. of air followed by an injection of 0.5 ml. of a 1% solution of croton oil in sesame oil directly into the pouch. The test compounds included the commercial preparations: fluocinolone acetonide (Synalar cream, Syntex Laboratories Inc.); betamethasone (Celestone cream, Schering Corp.); methylprednisolone acetate (Medrol acetate cream, The Upjohn Co.); triamcinolone 16,17-acetonide (Kenalog cream, E. R. Squibb & Sons, Inc.); and also these laboratory preparations: hydrocortisone acetate in a cream base; betamethasone 17-valerate in a cream base; triamcinolone in a cream base; triamcinolone 16,17-acetonide in a cream base; and fluocinolone acetonide in an alcoholic gel base.

Table I—Effect of Several Topical Anti-inflammatory Substances in a Modified Granuloma Pouch Procedure

Test	Test Preparation, %	Daily Cream Dose, mg./ Animal	No. Animal, I/F	Body Wt. Change, g. ± *SE*	Rel. Organ Weights, mg./100 g. Body Wt. ± *SE* Adrenal	Thymus	Exudate, ml. ± *SE*	Change from Control Cream or Alc. Gel, %
1	Alc. gel control	100	20/20	63 ± 3.6	21.0 ± 0.8	205 ± 8.3	16.7 ± 2.1	—
	Fluocinolone acetonide (cream base) (0.025)	5	12/11	44 ± 4.2[a]	19.2 ± 0.6	144 ± 13.1[a]	14.9 ± 2.7	−11
	Fluocinolone acetonide (cream base) (0.025)	25	12/12	14 ± 4.1[a]	21.8 ± 1.6	54 ± 6.7[a]	5.8 ± 0.9[a]	−65[a]
	Fluocinolone acetonide (cream base) (0.025)	100	12/12	−18 ± 3.6[a]	21.6 ± 0.7	31 ± 1.7[a]	1.6 ± 0.6[a]	−90[a]
	Fluocinolone acetonide (alc. gel base) (0.025)	5	12/12	−14 ± 5.6[ab]	19.9 ± 0.9	67 ± 9.1[ab]	7.4 ± 1.2[ab]	−56[a]
	Fluocinolone acetonide (alc. gel base) (0.025)	25	12/11	− 1 ± 4.0[ab]	20.9 ± 1.1	28 ± 2.2[ab]	4.2 ± 1.0[a]	−75[a]
	Fluocinolone acetonide (alc. gel base) (0.025)	100	12/10	−29 ± 2.9[ab]	22.6 ± 1.3	30 ± 5.0[a]	0.8 ± 0.2[a]	−95[a]
2	Cream control	100	20/20	52 ± 3.9	19.3 ± 0.5	186 ± 10.3	20.0 ± 2.6	—
	Control (untreated)	—	10/10	57 ± 4.6	18.2 ± 0.5	211 ± 16.9	19.6 ± 3.3	—
	Fluocinolone acetonide (cream base) (0.001)	25	10/10	60 ± 4.1	18.6 ± 0.7	211 ± 15.4	19.0 ± 2.1	−2
	Fluocinolone acetonide (cream base) (0.0025)	25	10/10	50 ± 8.1	18.8 ± 1.2	167 ± 18.5	22.4 ± 5.0	+12
	Fluocinolone acetonide (cream base) (0.01)	25	10/9	33 ± 6.2[a]	16.4 ± 0.4[a]	106 ± 10.5[a]	15.0 ± 3.5	−25
	Fluocinolone acetonide (cream base) (0.025)	25	10/10	4 ± 4.7[a]	17.2 ± 0.6[a]	39.5 ± 4.2[a]	7.0 ± 1.8[a]	−65[a]
	Fluocinolone acetonide (cream base) (0.001)	100	10/10	43 ± 5.2	19.7 ± 1.2	186 ± 19.5	18.0 ± 2.8	−10
	Fluocinolone acetonide (cream base) (0.0025)	100	10/10	37 ± 4.4[a]	18.1 ± 0.4	130 ± 19.8	14.9 ± 1.9	−25
	Fluocinolone acetonide (cream base) (0.01)	100	10/10	−13 ± 3.4[a]	17.5 ± 0.8	31 ± 2.1[a]	2.8 ± 0.7[a]	−86[a]
	Fluocinolone acetonide (cream base) (0.025)	100	10/9	−26 ± 3.4[a]	16.3 ± 0.9[a]	30 ± 2.0[a]	0.8 ± 0.2[a]	−96[a]
3	Cream control	125	10/10	55 ± 6.7	17.7 ± 0.7	239 ± 11.9	13.0 ± 1.6	—
	Hydrocortisone acetate (cream base) (1)	125	10/9	34 ± 7.9	16.2 ± 1.1	115 ± 10.3[a]	8.1 ± 1.5[a]	−34[a]
	Hydrocortisone acetate (cream base) (1)	250	10/9	14 ± 4.3[a]	16.4 ± 0.9	72 ± 8.6[a]	4.4 ± 1.4[a]	−66[a]
	Hydrocortisone acetate (cream base) (2)	125	10/10	23 ± 4.9[a]	18.1 ± 1.0	89 ± 13.6[a]	8.0 ± 0.9[a]	−35[a]
	Hydrocortisone acetate (cream base) (2)	250	10/10	9 ± 5.8[a]	16.9 ± 1.3	52 ± 3.4[a]	3.5 ± 1.6[a]	−73[a]
	Betamethasone 17-valerate (cream base) (0.025)	125	10/8	52 ± 5.3	17.0 ± 0.7	182 ± 12.9[a]	14.2 ± 3.1	+9
	Betamethasone 17-valerate (cream base) (0.025)	250	9/9	41 ± 2.4[a]	19.2 ± 0.7	188 ± 11.7[a]	7.6 ± 0.8[a]	−42[a]
4	Cream control	100	15/15	54 ± 5.4	23.2 ± 0.8	200 ± 7.7	21.3 ± 2.5	—
	Triamcinolone (cream base) (0.001)	100	10/10	45 ± 4.9	23.6 ± 0.7	183 ± 10.9	21.0 ± 3.1	−1
	Triamcinolone (cream base) (0.01)	100	10/10	50 ± 7.3	22.0 ± 0.7	187 ± 15.6	17.2 ± 3.1	−19
	Triamcinolone (cream base) (0.025)	25	10/10	47 ± 6.2	22.8 ± 1.2	189 ± 10.6	18.6 ± 3.2	−13
	Triamcinolone (cream base) (0.025)	100	10/10	52 ± 5.4	20.8 ± 0.9	151 ± 10.6[a]	21.6 ± 3.4	+1
	Triamcinolone 16,17-acetonide (cream base) (0.001)	100	10/10	42 ± 5.5	24.0 ± 1.0	192 ± 13.8	15.0 ± 2.5	−30
	Triamcinolone 16,17-acetonide (cream base) (0.01)	100	10/9	29 ± 3.6[a]	20.5 ± 1.0	96 ± 11.1[a]	20.5 ± 1.0	−4
	Triamcinolone 16,17-acetonide (cream base) (0.025)	25	10/10	33 ± 8.1[a]	22.5 ± 1.0	128 ± 16.5[a]	17.8 ± 2.7	−16
	Triamcinolone 16,17-acetonide (cream base) (0.025)	100	10/10	4 ± 4.5[a]	21.9 ± 0.9	46 ± 4.1[a]	9.7 ± 1.6[a]	−55[a]

Table I—(*Continued*)

Test	Test Preparation, %	Daily Cream Dose, mg./ Animal	No. Animal, I/F	Body Wt. Change, g. ± *SE*	Rel. Organ Weights, mg./100 g. Body Wt. ± *SE* Adrenal	Thymus	Exudate, ml. ± *SE*	Change from Control Cream or Alc. Gel, %
	Triamcinolone acetonide[c] (0.025)	100	10/10	5 ± 6.6[a]	20.5 ± 1.6	59 ± 9.6[a]	10.3 ± 1.7[a]	−52[a]
5	Cream control	125	19/19	55 ± 2.9	20.5 ± 0.7	215 ± 10.5	18.2 ± 2.2	—
	Methylprednisolone acetate (cream base) (0.25)	125	10/10	25 ± 3.5[a]	18.3 ± 0.8	117 ± 22.7[a]	4.2 ± 1.0[a]	−53[a]
	Betamethasone (cream base) (0.2)	125	10/10	−25 ± 2.7[a]	19.3 ± 1.7	33 ± 3.0[a]	0.6 ± 0.1[a]	−100[a]

I = Initial number. F = Final number. [a] Significantly different from cream control of alcoholic gel, $p < 0.05$. [b] Significantly different from corresponding cream base preparation (Test 2). [c] Kenalog.

The control creams[1] and the test compounds in either creams or alcoholic gel[2] were applied topically to the surface of the pouch and spread evenly over a controlled area (35 mm. diameter) daily for 8 days starting on Day 2.

On Day 4, all the pouches were reshaved and slightly reinflated to original turgidity and 0.5 ml. of a 3% croton oil solution was injected directly into the pouch. All the animals were killed on Day 10 and the exudate volume was measured. The final body weights and adrenal and thymus weights were also recorded. The groups and their respective treatments are described in the tables. The significance between the control and treated groups was estimated by determining the standard error of the means and application of Student's *t* test.

RESULTS

Topically applied fluocinolone acetonide–alcoholic gel base (0.025%) at 5 mg./animal/day significantly inhibited thymus weights and the normal rate of body weight gain. On the other hand, 25 and 100 mg./animal/day significantly decreased exudate formation, thymus weights, and the normal rates of body weight gain. The comparison of fluocinolone acetonide in an alcoholic gel vehicle (0.025%) with fluocinolone acetonide in a cream vehicle (0.025%) indicates that although the latter also inhibited the same parameters, the former is more active in all the test parameters. Application of various concentrations of fluocinolone acetonide or various amounts of the cream preparations indicates that a dose-related response is obtained with fluocinolone acetonide. The biological activity is directly related to the amount of steroid and not to the quantity of the cream preparation applied (Test 2, Table I). Creams, which contained 1 or 2% hydrocortisone acetate when administered at 250 mg./animal/day significantly reduced exudate formation, thymus weights, and the body weight gain. At 125 mg./animal/day, both concentrations inhibited only the thymus weights and exudate formation whereas the 2% concentration also inhibited the body weight gain. It should be noted that the adrenal weights appeared to be the least sensitive test parameter and no dose-related response was evident.

It is also interesting to note that triamcinolone cream (0.025%) at 100 mg./animal/day significantly inhibited the thymus weight whereas triamcinolone 16,17-acetonide cream (0.025%) at all dose schedules inhibited the thymus weights and the body weight gain. Similarly, triamcinolone 16,17-acetonide (0.025%) at 100 mg./animal/day inhibited exudate formation. At equivalent concentrations the laboratory prepared triamcinolone 16,17-acetonide cream base was comparable to the commercially obtained preparation (Kenalog, 0.025%). Methylprednisolone acetate cream (0.25%) at 125 mg./animal/day had a similar activity whereas betamethasone cream (0.2%) caused a further reduction of the normal rate of body weight gain, thymus weight, and exudate formation. Betamethasone 17-valerate (0.025%) at 250 mg./animal/day significantly inhibited exudate formation, the body weight gain, and the thymus weights. At one-half the dose, only the thymus weights were inhibited.

CONCLUSIONS

The test procedure is capable of evaluating topically applied anti-inflammatory preparations and apparently percutaneous absorption. It should be mentioned that although the air pouch makes the test substances inaccessible to licking and cleaning, one cannot exclude the possibility that the rat may rub the air pouch and the test compound onto the top of the cage and then lick or ingest the steroids. However, this does not appear to be an important factor since the larger cream doses would have been more accessible for ingestion and yet one observes that the biological activity is related to the amount of active ingredient and not to the amount of the cream base applied. As with all other local/topical anti-inflammatory assays, it is difficult to ascertain how much of the action of the test compound is due to its local and how much to its systemic properties since the anti-inflammatory effect is usually associated with thymolysis and a reduced rate of body weight gain.

Katz and Shaikh (8) indicated that the relative percutaneous absorption produced by the molecular modification of corticosteroids is related to changes in solubility and partition coefficient. The physical properties of the vehicle also influence percutaneous absorption and biological activity (9, 10). It should also be kept in mind that the biological activity is also dependent upon many other factors such as plasma protein binding, receptor specificity, rate of metabolic transformation or inaction, and tissue retention subsequent to absorption to produce a more efficient local therapeutic action (8–10). The results demonstrate that an alcoholic gel vehicle for fluocinolone acetonide or the addition of 16,17-acetonide to triamcinolone enhances the biological activity when compared, respectively, to the fluocinolone acetonide cream or the triamcinolone cream. It appears that the alcoholic gel vehicle (fluocinolone acetonide) increases percutaneous absorption as indicated by the greater effect on the body weight and thymus weights. The correlation between biological activity and drug penetration was previously shown clinically where commercially available drugs were reported to be more active when applied to abraded skin or under occlusive dressing (5, 6, 11, 12).

The molecular modification of the test compound such as the formation of the acetonide apparently also leads to an enhanced percutaneous absorption. McKenzie (6) indicated that the addition of a 16,17-acetonide to specific steroids promotes a greater rat skin penetration and that the acetate is also better absorbed than the parent alcohol. The authors also observed that triamcinolone 16,17-acetonide cream base is more active in all the test parameters than triamcinolone, and the laboratory preparation is comparable to commercially obtained triamcinolone 16,17-acetonide (Kenalog). Similarly, commercially obtained methylprednisolone acetate and

[1] The laboratory creams were prepared to be similar to that employed in Synalar cream.

[2] Contains Carbopol (1.75%), diisopropanolamine (0.18%), methylparaben (0.2%), propyleneglycol (5%), alcohol (40%), and water (52.87%).

betamethasone also inhibited exudate formation, body weight gain, and the thymus weights. It is difficult to obtain a relative activity of betamethasone since the concentration used was high and may represent a maximum response.

Unlike clinical observations of topical activity in dermatologic diseases (13–15) in which betamethasone 17-valerate is generally described as comparable to fluocinolone acetonide, the authors' test procedure indicates that fluocinolone acetonide is more active. An attempt to grade the order of activity from the experimental results indicates that fluocinolone acetonide (0.025%) > betamethasone (0.2%) > triamcinolone 16,17-acetonide (0.025%) > betamethasone 17-valerate (0.025%) > methylprednisolone acetate (0.25%) > hydrocortisone acetate (1.0%). This order of decreasing activity is closely related to that described by others (16, 17). Similarly, one may also correlate steroidal modifications such as the addition of Δ^1, 6-methyl, 6-fluoro, 9-fluoro, 6,9-difluoro, 16-methyl, and 16,17-acetonide to the increasing order of biological activity (5, 16, 18, 19).

In conclusion, the modified granuloma pouch procedure not only serves as a site for drug application but is also useful for the evaluation of commercially available corticosteroids. The assay can also distinguish structural modifications and alteration of the vehicle. However, these changes did not always reflect potency differences observed in the clinic.

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Gas-Liquid Chromatography of Salicylate Metabolites

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Abstract □ A gas-liquid chromatographic separation of the methyl ester-methyl ether derivatives of acetylsalicylic acid, salicylic acid, 2,3-dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid, and 2,3,4-trihydroxybenzoic acid is described. Separations were carried out at 155° on a column packed with 5% SE-30 on diatomaceous earth. A hydrogen-flame ionization detector was used.

Keyphrases □ Salicylate metabolites—separation, determination □ Column chromatography—separation □ GLC—analysis

It was decided to develop a gas-liquid chromatographic technique, for possible use by the authors and others, which would effect the separation and identification of acetylsalicylic acid, salicylic acid, 2,3-dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid, and 2,3,4-trihydroxybenzoic acid. Horning *et al.* (1) report retention times for trimethylsilylether (TMS)–methyl ester derivatives of acetylsalicylic acid, salicylic acid, gentisic acid (2,5-dihydroxybenzoic acid), and salicyluric acid, but they do not mention either 2,3-dihydroxybenzoic acid, or 2,3,5-trihydroxybenzoic acid. Williams (2) reports retention times for methyl ester and methyl ester–methyl ether derivatives of several dihydroxybenzoic acids including 2,3- and 2,5-dihydroxybenzoic acids but he presents no data for acetylsalicylic acid, salicylic acid, or 2,3,5-trihydroxybenzoic acid. The following technique will effectively separate acetylsalicylic acid from its hydroxy metabolites.

Table I—Relative Retention Times

Methyl Ester–Methyl Ether Derivative of	Relative	Retention Time
Benzoic acid (internal standard)	—	1.00[a]
Salicylic acid	1.240	2.12
Acetylsalicylic acid	—	2.91
2,5-Dihydroxybenzoic acid	—	3.37
2,3-Dihydroxybenzoic acid	—	3.91
2,3,4-Trihydroxybenzoic acid	—	8.43

[a] Retention time = 1.026 min.

MATERIALS AND METHODS

Identity of the individual compounds used was established by melting point.

An ethereal alcoholic solution of diazomethane was prepared by reacting an ethereal solution of *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide with ethanolic potassium hydroxide in a distilling apparatus (3). The resulting ethereal distillate contained approximately 3 g. of diazomethane.

The methyl ester–methyl ether derivatives of the respective compounds were prepared as follows. Approximately 0.25 g. of each compound was dissolved in a minimum amount of absolute ethanol. Ethereal diazomethane was added dropwise until a yellow color