Quantitative Effect of Topically Applied Anti-Inflammatory Agents on External Ocular Inflammation in Rats

ITALO MAISTRELLO▲, GIUSEPPINA RIGAMONTI, CARLO FROVA, and PIETRO de RUGGIERI

Abstract □ A new quantitative approach to investigate the effect of topically applied anti-inflammatory agents is proposed. An external ocular inflammation was produced in the rat by topical instillation of ocular irritants. The method requires a short time and is based upon quantitatively determining the degree of edema by weighing the eyelid. This end-point can be evaluated objectively, in contrast to the subjective results obtained by many widely used methods. Hydrocortisone, triamcinolone acetonide, triamcinolone, fluocinolone acetonide, and dexamethasone were tested. Dose-response relationships were obtained, and relative potencies were determined by parallel-line assay, using hydrocortisone as a reference standard. Fluocinolone acetonide, the most potent steroid studied, had a topical relative potency of 2745; triamcinolone, the least potent, had a topical relative potency of 4.9. The systemic relative potency was also investigated. The results obtained show that the experimental model of external ocular inflammation appears to be a reliable method for quantitative assay of topical anti-inflammatory agents.

Keyphrases □ Anti-inflammatory steroids, topical—quantitation of relative effects using external ocular inflammation technique in rats □ Ocular inflammation technique—used to quantitate effects of topical anti-inflammatory steroids, rats □ Steroids, anti-inflammatory—quantitation of relative effects using external ocular inflammation technique in rats

Several methods have been reported for provoking ocular inflammation to study the effect of anti-inflammatory substances. The most frequently used procedures are the lamellar graft technique, consisting of the insertion of a pig corneal button intralamellarly into the rabbit cornea (1); the immuno uveitis model (2–4); and methods based upon the topical instillation (5, 6) or injection (7) of ocular irritants. Opacity and vascularization of the cornea, edema, and hyperemia of the conjunctiva and iris and exudation in the anterior chamber are the parameters used for determining the degree of ocular inflammation produced. However, scoring is subjective and arbitrary due to the lack of criteria that can be evaluated objectively. Furthermore, some methods are too time consuming for routine screening.

Therefore, the development of a method to produce temporary ocular inflammation in the rat having an end-point that could be evaluated objectively was desirable. In these experiments, the quantitative determination of the weight of the inflamed eyelid was the end-point. By using this method, dose-response rela-

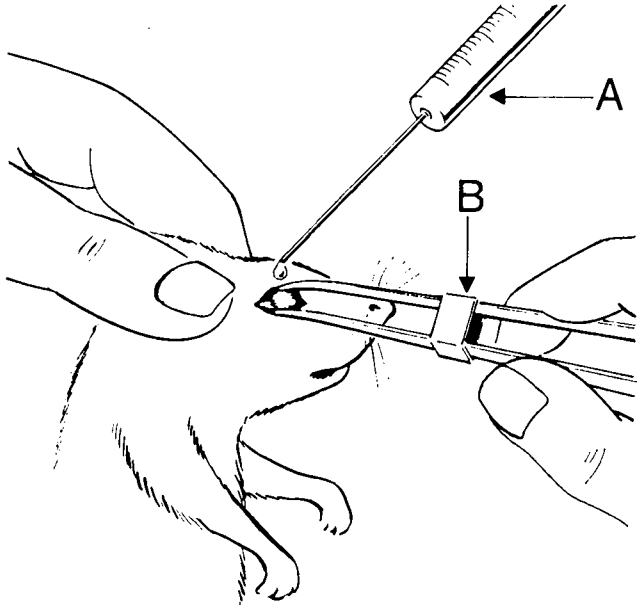

Figure 1—*Technique for instilling substances into the eye of the rat. Key: A, 25-μl. syringe clamped at a 45° angle relative to the horizontal plane; and B, arrest of the curved forceps to allow a maximum spread of 5 mm. at the tip.*

Table I—Effect of Topical Administration of Corticoids on Rat Palpebral Edema

Experiment	Compound	Total Dose, mcg./Animal	Eyelid Weight, mg. (Mean ± *SE*)[a]	Deviation from Control Value, %
A	Control	—	44.61 ± 1.79	
	Hydrocortisone	73.2	38.33 ± 1.88[b]	−14
		131.8	36.65 ± 2.30[c]	−18
		237.2	32.16 ± 1.18[c]	−28
		427	27.44 ± 0.95[c]	−38
	Dexamethasone	0.83	37.83 ± 1.98[b]	−15
		1.5	35.88 ± 2.11[c]	−20
		2.7	29.61 ± 1.18[c]	−34
B	Control	—	49.12 ± 3.99	
	Hydrocortisone	73.2	39.78 ± 1.77[c]	−19
		131.8	34.37 ± 1.13[c]	−30
		237.2	31.66 ± 2.17[c]	−35
		427	24.94 ± 0.68[c]	−49
	Triamcinolone	30	34.26 ± 1.54[c]	−30
		60	27.35 ± 1.39[c]	−44
		120	24.92 ± 1.37[c]	−49
C	Control	—	45.90 ± 2.37	
	Hydrocortisone	73.2	39.11 ± 2.56[b]	−15
		131.8	37.40 ± 1.82[c]	−18
		237.2	33.51 ± 2.14[c]	−27
		427	24.51 ± 0.61[c]	−47
	Fluocinolone acetonide	0.034	34.90 ± 2.28[c]	−24
		0.067	34.15 ± 1.61[c]	−26
		0.135	29.48 ± 1.70[c]	−36
		0.27	26.16 ± 1.16[c]	−43
D	Control	—	44.04 ± 2.73	
	Hydrocortisone	73.2	37.34 ± 2.43[b]	−15
		131.8	33.35 ± 1.39[c]	−24
		237.2	29.31 ± 1.41[c]	−33
		427	25.54 ± 0.99[c]	−42
	Triamcinolone acetonide	0.150	37.15 ± 1.68[b]	−16
		0.312	35.93 ± 2.42[c]	−18
		0.625	34.40 ± 1.29[c]	−22
		1.25	29.52 ± 1.34[c]	−33
		2.5	29.34 ± 1.33[c]	−33

[a] Nine rats per group. [b] $p < 0.05$ when compared with the control. [c] $p < 0.01$ when compared with the control.

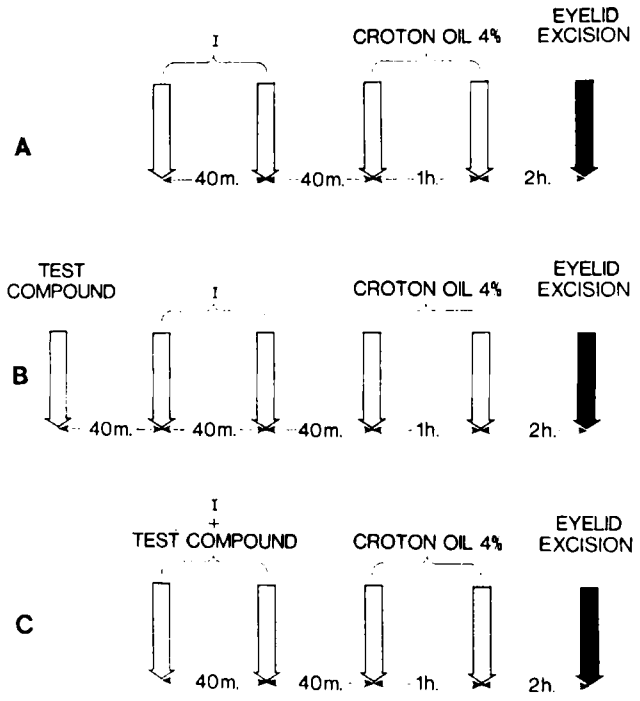

Figure 2—*Treatment schedule for producing ocular inflammation and for testing the effect of anti-inflammatory steroids administered topically or systemically. (A) Production of ocular inflammation. Two doses of 2.5 μl. each of I were instilled into the right eye 40 min. apart, followed 40 min. later by two doses of 4% croton oil in I 60 min. apart. Two hours after the last dose of croton oil, the eyelid was excised. (B) Test of anti-inflammatory potency by systemic administration of the test compound prior to producing inflammation. (C) Test of anti-inflammatory activity by topical instillation of the test compound into the right eye. Note that the test compound is dissolved in I and administered as in A.*

tionships and the relative potency of several anti-inflammatory substances were determined after topical and systemic administration.

MATERIALS AND METHODS

Experimental Animals—Female Sprague Dawley rats, weighing 60–70 g., were randomly divided into groups of nine or 10 animals. The animals were fed standard rat chow and water *ad libitum* up to sacrifice.

Induction of Palpebral Edema—A combination of two irritants, 2-(2-ethoxyethoxy)ethanol[1] (I) and croton oil, was selected to produce a satisfactory external ocular inflammation. Both substances were instilled in the conjunctival sac of the right eye of the rat. 2-(2-Ethoxyethoxy)ethanol was administered as such, while croton oil was administered as a 4% solution in 2-(2-ethoxyethoxy)-ethanol.

Figure 1 illustrates the handling of the animal for drug instillation. Particular care was taken to prevent mechanical damage to the ocular tissue by the needle; a 25-μl. syringe[2] was fixed in a clamp at about 45° to the horizontal plane to prevent spontaneous leakage of the drop of solution. The eyelids were spread apart with curved forceps which were prevented from opening more than 5 mm. at the tips. The rat was then delicately moved toward the syringe until a drop was applied to the center of the cornea, from where it spread onto the entire conjunctival sac. The lids were kept apart for 2–4 sec. to assure adequate diffusion of the substance in the conjunctival sac. The volume of instillation was 2.5 μl.

External ocular inflammation was obtained by instilling two doses of I followed by two doses of croton oil (Fig. 2A). The steroids used for the study of anti-inflammatory activity were administered both systemically and topically. For systemic administration, the drugs were given orally by stomach tube in 1 ml./100 g. body weight of an aqueous vehicle containing 0.9% sodium chloride, 0.5% sodium carboxymethylcellulose, 0.4% polysorbate 80, and 0.9% benzyl alcohol. The treatment schedule is shown in Fig. 2B. For topical administration, the compounds were dissolved in I, which is an optimal solvent for steroidal substances (8), and administered by instillation into the conjunctival sac in two doses of 2.5 μl. each. The treatment schedule is shown in Fig. 2C.

The effect of the irritants and the anti-inflammatory activity of the products tested were evaluated by isolating and weighing the inflamed upper eyelid. Two hours after the last administration (Fig. 2), the animal was killed by cervical fracture and the upper eyelid was isolated, using straight and sharply honed scissors (Fig. 3). After making an incision in the skin of the cranium, the skin was peeled away toward the eyelids (Fig. 3A). When arriving at the palpebra, the skin was cut along the palpebral edge (Fig. 3B). The

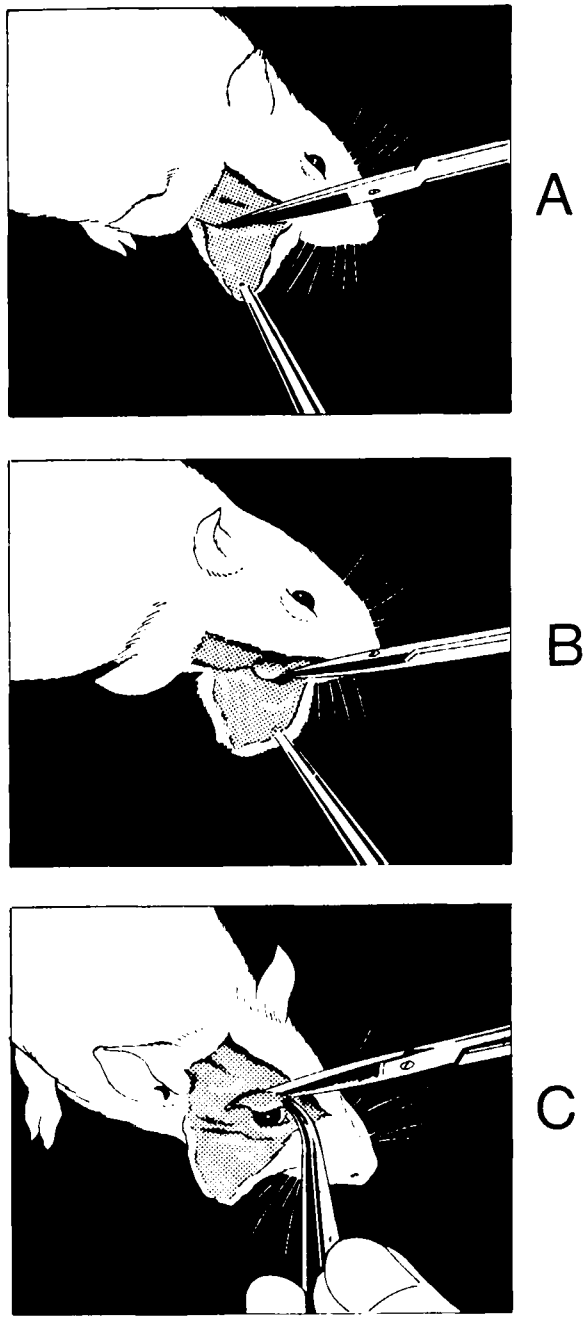

Figure 3—*Procedure for isolation of the rat eyelid. Key: A, peeling off of the cranial skin toward the eyelid; B, cutting along the palpebral edge; and C, removal of the eyelid.*

[1] Carbitol, Union Carbide Chemicals Co., New York, N. Y.
[2] Hamilton.

Table II—Effect of Systemic Administration of Corticoids on Rat Palpebral Edema

Experiment	Compound	Total Dose, mcg./kg.	Eyelid Weight, mg. (Mean ± *SE*)[a]	Deviation from Control Value, %
E	Control	—	42.14 ± 1.41	
	Hydrocortisone	9,250	35.44 ± 1.77[b]	−16
		18,500	31.71 ± 1.03[b]	−25
		37,000	25.71 ± 0.73[b]	−39
	Dexamethasone	45	34.61 ± 0.84[b]	−18
		90	29.58 ± 0.82[b]	−30
		180	25.76 ± 0.76[b]	−39
F	Control	—	44.93 ± 1.96	
	Hydrocortisone	12,650	34.60 ± 1.80[b]	−23
		22,800	32.00 ± 1.22[b]	−29
		41,000	25.57 ± 1.32[b]	−43
	Triamcinolone	1,250	37.20 ± 1.48[c]	−17
		2,500	32.35 ± 1.30[b]	−28
		5,000	26.43 ± 0.88[b]	−41
G	Control	—	46.18 ± 2.17	
	Hydrocortisone	12,650	29.20 ± 1.29[b]	−37
		22,800	26.04 ± 1.20[b]	−44
		41,000	22.66 ± 0.53[b]	−51
	Fluocinolone acetonide	15	39.03 ± 1.21[b]	−15
		30	36.18 ± 1.10[b]	−22
		60	26.93 ± 1.52[b]	−42
		120	23.06 ± 0.58[b]	−50
H	Control	—	47.26 ± 2.00	
	Hydrocortisone	7,030	33.74 ± 1.39[b]	−29
		12,650	33.05 ± 1.79[b]	−30
		22,800	27.82 ± 1.01[b]	−41
		41,000	22.24 ± 0.71[b]	−53
	Triamcinolone acetonide	25	40.82 ± 1.33[b]	−14
		50	35.95 ± 1.69[b]	−24
		100	30.11 ± 1.19[b]	−36
		200	23.13 ± 0.71[b]	−51

[a] Nine rats per group. [b] $p < 0.01$ when compared with the control. [c] $p < 0.05$ when compared with the control.

skin-free palpebra was then removed by cutting along the orbital arc (Fig. 3C). No bleeding was evident during the isolation procedure. The excised eyelid tissue was then immediately weighed on a torsion balance[3] to the nearest 0.2 mg. without blotting. The whole procedure can be carried out in 40–45 sec. after some practice.

Drugs—The anti-inflammatory activity of the following substances was tested: hydrocortisone, triamcinolone (alcohol), triamcinolone acetonide, dexamethasone, and fluocinolone acetonide.

Statistical Analysis—Relative potencies were determined by the parallel-line assay (9). The precision index of the test (λ) was obtained according to Bliss (10).

RESULTS

Time Course of Palpebral Edema Formation—For this experiment, 150 rats were subdivided into 15 groups of 10 animals each. The animals were killed at various times after beginning treatment with the irritants. Increase in eyelid weight began immediately after the first instillation of irritant and reached a peak 2 hr. after the last instillation (Fig. 4). At the peak, the eyelids weighed 50.6 ± 1.5 mg. (mean value ± standard error) compared to the controls weighing 15.5 ± 0.46 mg.—an increase of 226%. The edema remained elevated for 2 hr. more and then began to decline slowly, reaching normal values 10 hr. after the last instillation of irritant.

Anti-Inflammatory Bioassay—The anti-inflammatory effect of various compounds was investigated in eight different experiments. The substances were administered topically in four of these experiments (A, B, C, and D of Table I) and systemically in the other experiments (E, F, G, and H of Table II). In each experiment, the rats were subdivided into various groups of nine animals each,

[3] Sauter.

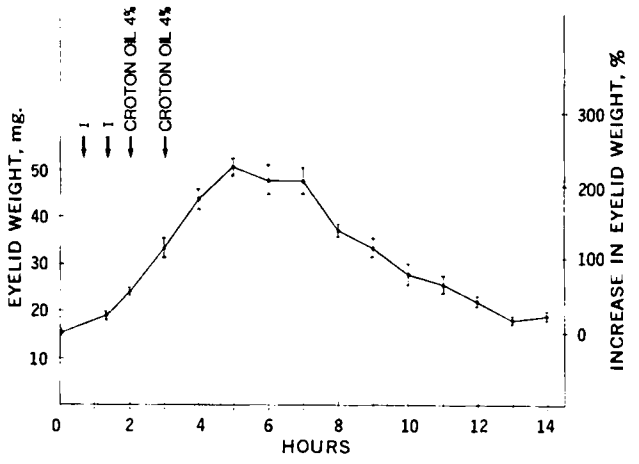

Figure 4—*I–croton oil palpebral edema as a function of time. I and croton oil were administered as described in Fig. 2A. Points and vertical bars indicate the mean and* SE.

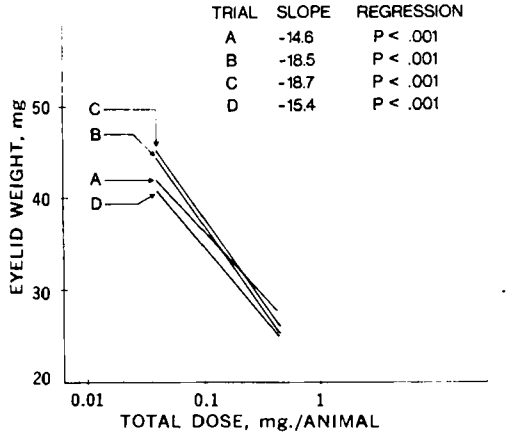

Figure 5—*Regression lines of hydrocortisone, administered topically, obtained in several experiments.*

arranged as indicated in Tables I and II. The animal treatment schedule employed was that shown in Fig. 2; 2 hr. after the last instillation of croton oil in I, the animals were killed and the eyelids were excised and immediately weighed.

Hydrocortisone was used as a reference standard in each test, and at least three doses of the test compounds were used. All compounds significantly reduced palpebral edema, and good linear log dose–response regressions were obtained.

To demonstrate the reproducibility of the method, the regression lines for the topically applied hydrocortisone standard of Table I are shown in Fig. 5. The slopes of the lines were only slightly varied, and the regressions were highly significant by statistical analysis.

The dose–response data for the various assays are plotted in Figs. 6–9. Each figure gives the results of a single steroid applied

Table III—Relative Potencies of Corticoids after Topical and Systemic Administration

Compound	Topical Activity Potency	Topical Activity λ[a]	Systemic Activity Potency	Systemic Activity λ[a]
Hydrocortisone	1		1	
Dexamethasone	103 (67–160)	0.35	238 (185–310)	0.21
Triamcinolone	4.9 (3.6–7.0)	0.25	7.7 (5.6–10.3)	0.23
Fluocinolone acetonide	2745 (1813–4512)	0.38	267 (203–338)	0.19
Triamcinolone acetonide	180[b]	—	157 (122–197)	0.22

[a] Precision index. [b] Graphical estimation (40% inhibition).

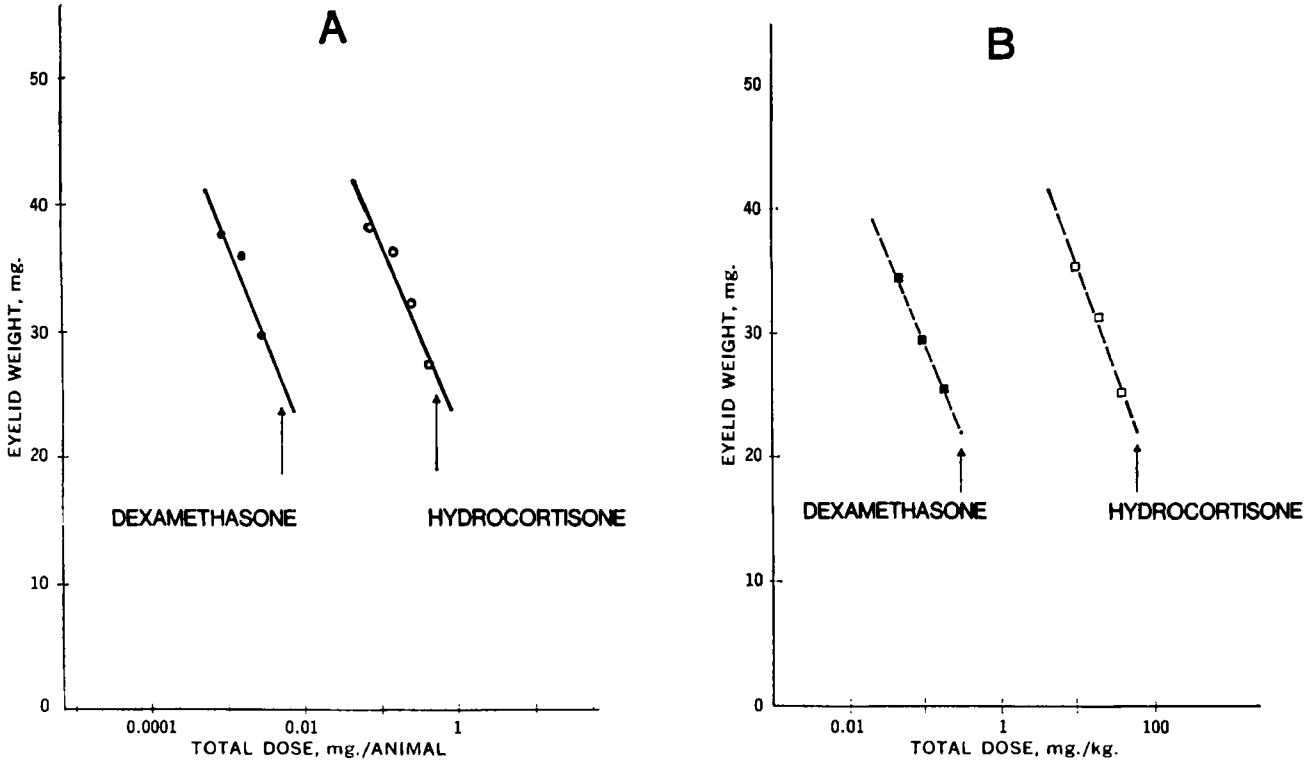

Figure 6—*Anti-inflammatory activity of dexamethasone* versus *hydrocortisone after topical (A) and systemic (B) administration.*

topically and systemically. The regression lines for each test compound, applied either topically or systemically, showed no significant dèparture from linearity. With the exception of triamcinolone acetonide applied topically, the regression line for each compound was parallel to the hydrocortisone standard administered by the same route. Thus, in each case, the relative potency could be determined by the parallel-line assay method. Table III reports the relative potencies of these compounds, with their 95% confidence limits and the precision index (λ).

Triamcinolone acetonide, applied topically, presented a significantly different and less steep regression than hydrocortisone. Thus, a precise relative potency estimate could not be made. However, a graphical estimate was made by comparing the doses producing a 40% inhibition in palpebral edema. Fluocinolone acetonide, applied topically, was the most active compound, already producing a significant reduction in edema at a total dose of 0.034 mcg./animal. The relative potency was 2745. The activity of fluocinolone acetonide was lower after oral administration, having a relative

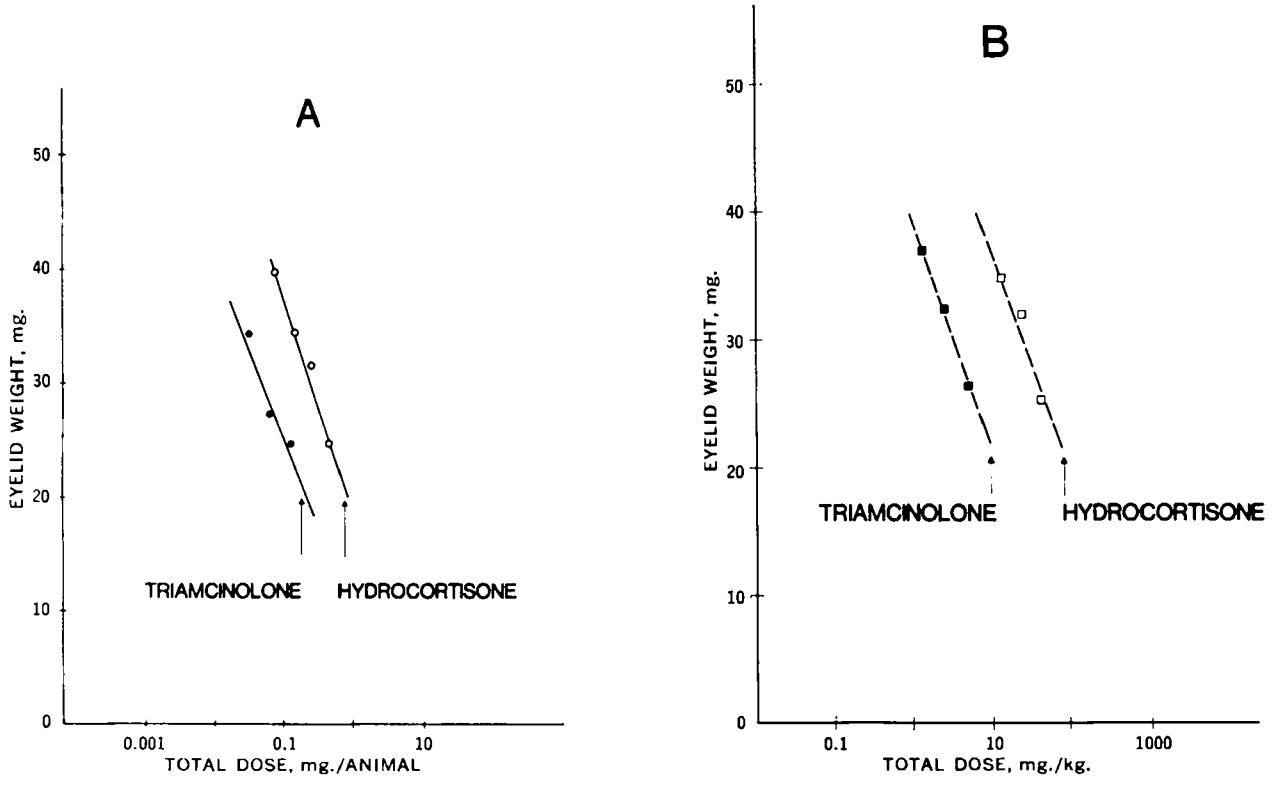

Figure 7—*Anti-inflammatory activity of triamcinolone* versus *hydrocortisone after topical (A) and systemic (B) administration.*

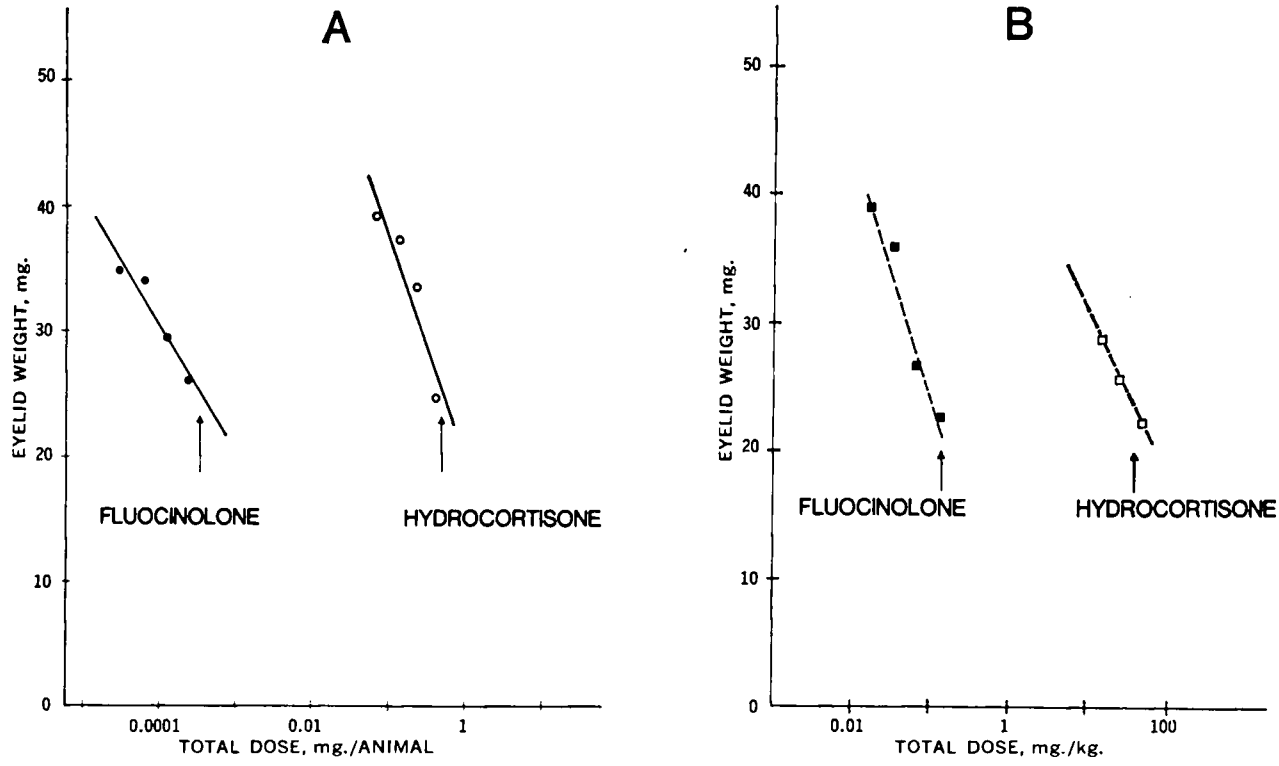

Figure 8—*Anti-inflammatory activity of fluocinolone acetonide* versus *hydrocortisone after topical (A) and systemic (B) administration.*

potency of 267. The least active product was triamcinolone, having relative potencies after topical and systemic administrations of 4.9 and 7.7, respectively. The relative potencies of dexamethasone were 103 (topical) and 238 (systemic).

The graphical estimate of topical relative potency (TRP) for triamcinolone acetonide was 180, while the systemic relative potency (SRP) for this compound was 157. A smaller quantity of the given substance was required to produce an anti-inflammatory effect when applied topically rather than systemically.

DISCUSSION

Preliminary experiments indicated that it is relatively simple to produce inflammation by instilling various types of irritants into

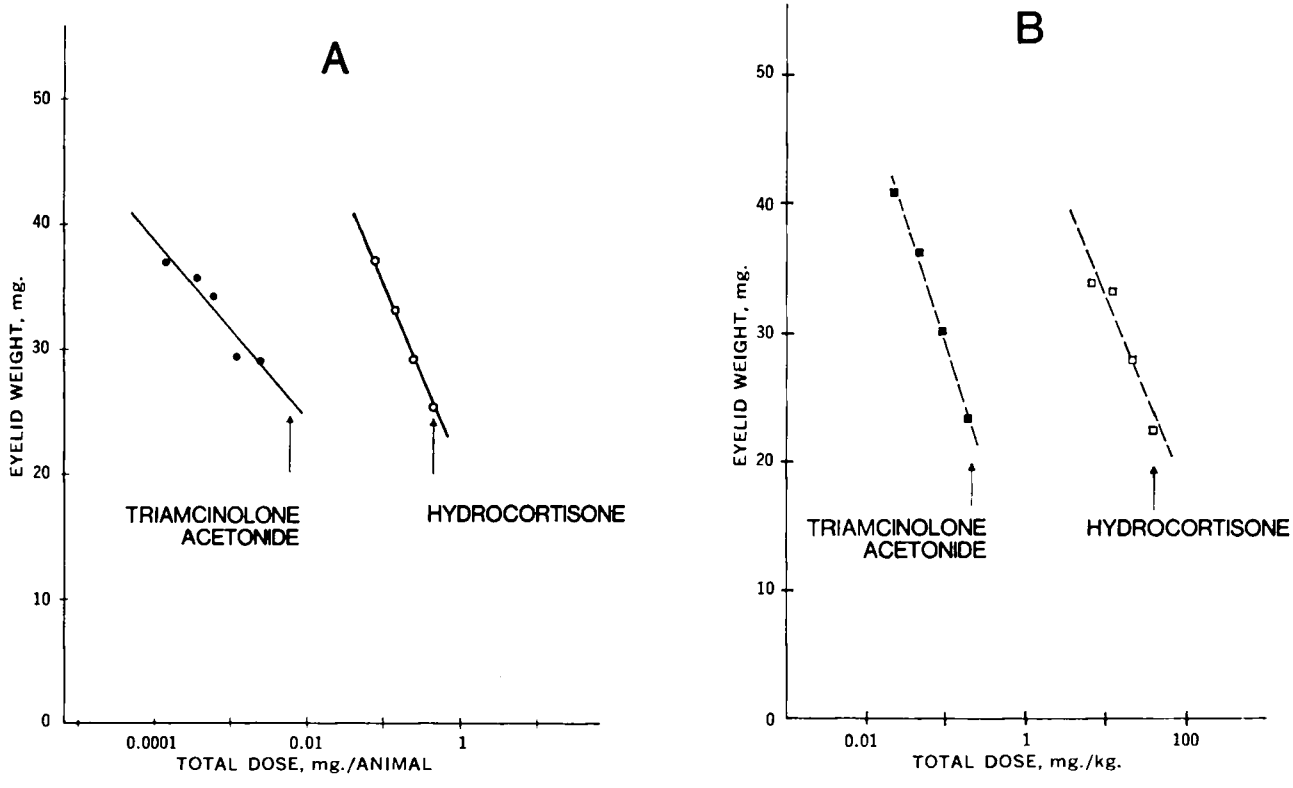

Figure 9—*Anti-inflammatory activity of triamcinolone acetonide* versus *hydrocortisone after topical (A) and systemic (B) administration.*

the conjunctival sac; the inflammation is also reduced significantly by treatment with anti-inflammatory steroids (11). The difficulty is increased considerably, however, when statistically valid and reproducible dose–response relationships are desired. The described scheme for producing irritation was adopted after considerable trial and error and is the only one that permitted quantitative determinations of relative potency within statistically acceptable limits.

Previously described methods on various types of ocular inflammation have permitted only an approximate ranking of the products or have yielded relative potency values with high confidence limits due to great biological variations (4). Moreover, it appears arbitrary to transfer results obtained from topical cutaneous tests or other general tests such as the carrageenin edema assay and the pellet granuloma inhibition assay to the eye.

The differences observed between the topical relative potency and systemic relative potency values demonstrate that it is not possible to ignore the importance of the conjunctiva as a biological membrane on the activity of anti-inflammatory substances. With triamcinolone acetonide, the dose–response relationship of the topically administered compound tended to be flatter than the dose–response curve of the same product given orally.

Regarding the characteristics of inflammation produced in these experiments, it was observed that they pertain to the anterior segment of the eye. The most evident symptoms were exudation, conjunctival hyperemia, and edema. The edemal component of inflammation was used as the sole parameter, since it is an anatomical part easily isolatable and measurable by objective criteria. This model of inflammation, although limited to the anterior segment of the eye, is of interest since the most frequent ocular diseases and the use and abuse of the glucocorticoids involve the lid edge and conjunctiva (12).

The method requires a short time, similar in this respect to methods producing inflammation in the rat paw by injection of irritating substances such as carrageenin and egg white.

The results of the method are highly reproducible. The reproducibility is more notable when compared with the method most frequently used, carrageenin-induced paw edema, which shows great variability from experiment to experiment and from day to day (13, 14).

The method is sensitive, particularly to substances administered topically. Therefore, only small quantities of substances are required for testing.

The use of rats rather than rabbits, which are generally used in ocular inflammation methods, is advantageous in bioassay experiments where large groups of animals are required.

REFERENCES

(1) D. W. C. Lorenzetti and H. E. Kaufman, *Arch. Ophthalmol.*, **76,** 274(1966).

(2) R. M. Wood and M. W. Bick, *ibid.*, **62,** 112(1959).

(3) C. Hanna and H. C. Keatts, *ibid.*, **77,** 554(1967).

(4) J. G. Harter, A. R. Borgmann, and F. E. Leaders, in "Symposium on Ocular Anti-Inflammatory Therapy," 1st ed., H. E. Kaufman, Ed., Charles C Thomas, Springfield, Ill., 1970, chap. 18.

(5) H. Ehlers, *Acta Ophthalmol.*, **5,** 99(1927).

(6) D. Andreani and S. Capalbi, *Gazz. Ital. Oftalmol.*, **9,** 418 (1956).

(7) G. Abbondati, U. Cornelli, M. Preti, and F. Trimarchi, *Ann. Ottal.*, **96,** 161(1970).

(8) J. A. Ostrenga and C. Steinmetz, *J. Pharm. Sci.*, **59,** 414 (1970).

(9) D. J. Finney, in "Statistical Method in Biological Assay," 2nd ed., D. J. Finney, Ed., C. Griffin and Co., London, England, 1964, chap. 4.

(10) C. I. Bliss, "The Statistics of Bioassay," vol. II, Academic, New York, N. Y., 1952, p. 469.

(11) P. de Ruggieri, I. Maistrello, and G. Gavazzi, *Proc. Int. Congr. Horm. Steroids, 3rd, Hamburg,* **1971,** 891.

(12) M. F. Armaly, "Symposium on Ocular Anti-Inflammatory Therapy," 1st ed., H. E. Kaufman, Ed., Charles C Thomas, Springfield, Ill., 1970, chap. 7.

(13) D. T. Walz, M. J. Di Martino, C. L. Griffin, and A. Misher, *Arch. Int. Pharmacodyn. Ther.*, **185,** 337(1970).

(14) A. Y. Green, D. Green, P. A. Murray, and A. B. Wilson, *Brit. J. Pharmacol.*, **41,** 132(1971).

ACKNOWLEDGMENTS AND ADDRESSES

Received September 25, 1972, from the *Pharmacological Research Laboratories, Farmila Società per Azioni, Settimo Milanese, Milan, Italy.*

Accepted for publication May 2, 1973.

Presented to the Fifth International Congress on Pharmacology, San Francisco meeting, July 1972.

The technical assistance of Miss Giovanna Villa and Miss Umberta Baroni is gratefully acknowledged.

▲ To whom inquiries should be directed.