Effects of Cryogenine on Adjuvant-Induced Arthritis and Serum Turbidity in Rats

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Abstract □ Daily oral administration of 100 mg./kg. of cryogenine significantly suppressed the inflammation and the development of disseminated lesions in rats with adjuvant-induced (*Mycobacterium butyricum*) polyarthritis. Significant anti-inflammatory effects continued for 10 days after cessation of therapy (20 days after pedal injection of adjuvant). Cryogenine also partially prevented the decreased growth rate and decreased serum turbidity documented for untreated rats with adjuvant-induced polyarthritis. This profile of activity was similar to that established for 10 mg./kg. daily of hydrocortisone but unlike that for 2 mg./kg. daily of mercaptopurine. Cryogenine appeared equipotent (milligrams per kilogram) to phenylbutazone in inhibiting adjuvant-induced polyarthritis.

Keyphrases □ Cryogenine—effects on adjuvant-induced arthritis and serum turbidity in rats □ Arthritis, adjuvant induced—effect of cryogenine on inflammation and serum turbidity, rats □ Anti-inflammatory activity—effects of cryogenine on adjuvant-induced arthritis and serum turbidity in rats

Adjuvant-induced polyarthritis in rats produced by pedal injection of a killed *Mycobacterium* species suspended in a vehicle of light liquid petrolatum (1) has many similarities to human rheumatoid arthritis (2). While the initial edema and soft tissue thickening in the injected hindfoot may be due partially to irritation, the subsequent swelling in the injected foot and disseminated polyarthritis in the contralateral foot, front limbs, and tail appear to be immunological events (3). In 1967, Kaplan *et al.* (4) reported that chronic oral administration of cryogenine was effective in inhibiting this adjuvant-induced polyarthritis. This study was qualitative rather than quantitative since it was conducted in a very limited number of rats for a limited time and monitored only by caliper measurements of medial foot thickness. Therefore, a quantitative study appeared indicated when an adequate supply of this rare alkaloid became available.

Omaye *et al.* (5) reported that erythrocyte sedimentation rate is significantly increased by adjuvant-induced polyarthritis in a two-step process (significant elevation within 6 days after pedal injection and maximum elevation around Day +15 when secondary lesions develop). This phenomenon was paralleled by increases in total white cell count associated with lymphopenia and neutrophilia. In the same study, orally administered cryogenine and phenylbutazone appeared equipotent (milligrams per kilogram) in moderating these hematological changes.

The interrelationships between lysozyme activity and serum turbidity in rats with adjuvant-induced polyarthritis also were investigated (6). Increased lysozyme levels and decreases in serum turbidity (determined by the stability of serum protein against heat denaturation) were found to be correlated with the severity of the polyarthritic condition. Therapeutically useful anti-inflammatory drugs were shown to reverse both of these parameters. Weissman (7) proposed that degradative enzymes released from lysosomes may denature native connective tissue. The denatured products in turn may then induce an immune response like that seen in adjuvant-induced polyarthritis. Therefore, the present study also attempts to establish the effect of cryogenine and various prototype agents on serum turbidity.

EXPERIMENTAL

Adjuvant-Induced Polyarthritis—Adult female rats of the Sprague–Dawley strain, weighing approximately 200 g., were allowed to acclimatize in this laboratory for 1 week with free access to food pellets[1] and tap water. The animals were randomized into nine treatment groups of 10 rats each, and the test drugs[2] (suspended or dissolved in aqueous 0.25% agar) were orally administered (5 ml./kg.) daily beginning on Day −1 and continuing through Day +20. Both control groups received only the agar dosing vehicle. The oral route was selected to minimize counterirritant effects associated with parenteral injection (8). On Day 0, each rat to receive adjuvant was anesthetized with 30 mg./kg. i.p. of sodium pentobarbital; then 0.05 ml. of a sterile 5-mg./ml. homogenized suspension of heat-killed, desiccated *Mycobacterium butyricum*[3] in light mineral oil was injected into the left hindfoot, using a sterile micrometer syringe and a sterile disposable needle. To prevent external leakage of the injected material and to minimize vascular involvement, the 25-gauge needle was inserted through the radial foot pad and directed medially into the metatarsal region, keeping the needle's path as near to the skin surface as possible. Paw volume was measured up to an india ink line across the lateral malleolus by displacement of mercury similar to the technique described by Van Arman *et al.* (9). These plethysmographic determinations were made every 3 days for both the injected foot and the contralateral foot through Day +30. The inhibitory effects of the various drug treatments were estimated using Eq. 1 (1):

$$\text{percent inhibition} = 100\,\{1 - [(a - x)/(b - y)]\} \quad \text{(Eq. 1)}$$

where y = mean foot volume of the adjuvant control rats (receiving adjuvant injection but no drug treatment) immediately prior to adjuvant administration, b = mean foot volume of adjuvant control rats on a particular day, x = mean foot volume of drug-treated rats immediately prior to adjuvant injection, and a = mean foot volume of drug-treated rats on a particular day.

A subjective scoring system was also used to evaluate the polyarthritis. Every 3rd day the severity of the arthritic lesions in each of the four limbs was rated on a scale of 0–4. Criteria for grading included: severity and extent of edema and erythema of the periarticular tissue plus the degree of enlargement, distortion, and ankylosis of the joints. Therefore, the score for any one animal at any one time could range from 0 to a maximum of 16.

Serum Turbidity—Another 36 rats of the same stock were randomized into nine groups and treated with adjuvant and drugs as already described. However, on Day +20, they were lightly

[1] Purina rat chow.

[2] Sources of test agents were: phenylbutazone, Geigy Pharmaceuticals, Ardsley, N. Y.; indomethacin, Merck Sharp & Dohme Research Laboratories, West Point, Pa.; mefenamic acid, Parke, Davis & Co., Detroit, Mich.; paramethasone, Eli Lilly & Co., Indianapolis, Ind.; and hydrocortisone and mercaptopurine, Atlas Chemical Industries, Inc., Wilmington, Del. The cryogenine alkaloid (mol. wt. 435.53) was isolated from Mexican *Heimia salicifolia* Link and Otto and is not to be confused with the trade name product Cryogénine (phenylsemicarbazide, mol. wt. 151.2) distributed by Laboratoires Sarbach of Châtillon, France.

[3] Number 0640-33, Difco Laboratories.

Table I—Drug Effects on Volume of the Adjuvant-Injected Paw in Adjuvant-Induced Polyarthritis

Treatment (Day −1 to Day +20)	Daily Oral Dose, mg./kg.	Mean Foot Volume, ml. ± *SE* (Percent Inhibition Inflammation) Day +3	Day +9	Day +15	Day +21	Day +24	Day +30
Untreated control	—	1.21 ± 0.02	1.24 ± 0.03	1.26 ± 0.04	1.26 ± 0.04	1.28 ± 0.06	1.27 ± 0.04
Adjuvant control	—	2.07 ± 0.06 (0)	2.44 ± 0.11 (0)	2.51 ± 0.19 (0)	2.65 ± 0.15 (0)	2.85 ± 0.19 (0)	3.03 ± 0.21 (0)
Cryogenine	100	1.84 ± 0.07 (26)	1.89 ± 0.07 (45)	1.80 ± 0.08 (55)	1.85 ± 0.07 (56)	1.84 ± 0.09 (62)	1.94 ± 0.09 (60)
Phenylbutazone	100	1.91 ± 0.04 (21)	1.92 ± 0.04 (45)	1.99 ± 0.06 (42)	1.92 ± 0.06 (53)	1.94 ± 0.05 (57)	1.95 ± 0.05 (61)
Mefenamic acid	25	1.91 ± 0.05 (18)[a]	2.16 ± 0.04 (22)	2.26 ± 0.10 (19)[a]	2.61 ± 0.08 (2)[a]	2.57 ± 0.08 (17)[a]	2.69 ± 0.08 (18)[a]
Indomethacin	1	1.97 ± 0.08 (15)[a]	1.89 ± 0.07 (52)	2.08 ± 0.05 (58)	2.02 ± 0.13 (46)	2.10 ± 0.18 (48)	2.37 ± 0.18 (38)
Paramethasone	0.5	1.81 ± 0.05 (27)	1.97 ± 0.10 (36)	1.86 ± 0.07 (48)	2.08 ± 0.05 (38)	2.02 ± 0.05 (49)	2.06 ± 0.04 (52)
Hydrocortisone	10	1.87 ± 0.05 (21)	1.83 ± 0.03 (49)	1.75 ± 0.05 (58)	1.67 ± 0.06 (68)	1.82 ± 0.07 (62)	1.99 ± 0.09 (57)
Mercaptopurine	2	1.77 ± 0.04 (39)	2.02 ± 0.05 (37)	1.93 ± 0.07 (48)	1.96 ± 0.07 (51)	1.94 ± 0.08 (58)	2.20 ± 0.05 (48)

[a] Mean foot volume is not significantly different from that of the adjuvant control group ($p > 0.05$).

anesthetized with ether and exsanguinated by cardiac puncture. To 0.1 ml. of nonhemolyzed serum, 2.9 ml. of 0.067 *M* Sørensen phosphate buffer[4] in saline (pH 5.2) was added and gently agitated. The mixture was allowed to stabilize at room temperature for 15 min. before being incubated in a constant-temperature water bath at 69° for 30 min. After removal, the sample was cooled in an ice bath and read in a spectrophotometer[5] at a wavelength of 645 nm. (10). Two separate determinations were made per rat.

RESULTS AND DISCUSSION

Adjuvant-Induced Polyarthritis—In the adjuvant control rats, marked inflammatory swelling was noted in the injected paw within 2 hr. after adjuvant injection. Paw volume increased in a linear fashion until a plateau effect was achieved between Days +6 and +12; thereafter volume again increased in a linear fashion through Day +30 (Table I). None of the test agents prevented the acute inflammatory reaction seen immediately following adjuvant injection. If one discounts the Day +3 foot volume readings, which still may reflect trauma and irritation due to the injection, all test drugs inhibited to some degree the subsequent inflammatory processes. With the exception of mefenamic acid, significant inhibition of inflammation in the injected foot was achieved with all test drugs during the dosing period and after cessation of dosage. Significant inhibition of inflammation in the injected foot was documented for mefenamic acid only at Days +6 and +9.

By Day +12, the development of secondary lesions was noted in the adjuvant control animals (increased vascularity of the ears, muzzle, and tail accompanied by swelling of the front and contralateral hindpaws). This secondary involvement increased in severity through Day +30, with the volume of the contralateral foot increasing in a near linear fashion. The initial reaction in the contralateral paw was a diffuse erythematous swelling of the dorsum of the tarsal region and the ankle joint, generally accompanied by a fusiform swelling of the digits. Nodular lesions eventually developed in the ears, muzzle, and tail. Inflammation in the contralateral paw as measured by volume (Table II) was significantly suppressed during the medication period by all drugs except indomethacin (all days) and phenylbutazone (Day +15). While indomethacin also lacked significant effect after cessation of therapy, some beneficial anti-inflammatory effects persisted for the other drugs through Day +30, especially in rats treated with paramethasone and to a lesser extent with phenylbutazone and mercaptopurine. Contralateral feet of the paramethasone-treated rats appeared grossly equivalent to the feet of untreated control animals at the termination of the 30-day observation period. The anti-inflammatory effects in the contralateral feet of animals treated with cryogenine, mefenamic acid, and hydrocortisone were not well sustained after cessation of drug dosage.

The arbitrary scoring system used in Table III should provide an index reflecting the total degree of polyarthritic involvement since both the fore- and hindpaws were rated. Only cryogenine, hydrocortisone, and mercaptopurine appeared to provide significant early protection (Day +12), while all test drugs except indomethacin appeared to protect against the development of secondary lesions (Day +21). All medicaments except indomethacin and mefenamic acid provided some continuing protection after the cessation of therapy (Day +30). The oral doses used were selected to be equiactive based on other anti-inflammatory testing in this laboratory using this rat stock; however, the present results indicate that the test compounds were not equiactive against adjuvant-induced polyarthritis at these doses.

Body Weight Changes—The mean weight of all animals was 218 g. at 0 time. The adjuvant control group achieved only a mean net gain of 5 g. in body weight during the entire observation period. There was a significant loss of mean weight (−11 g.) during the first 3 days following adjuvant injection, after which there was a

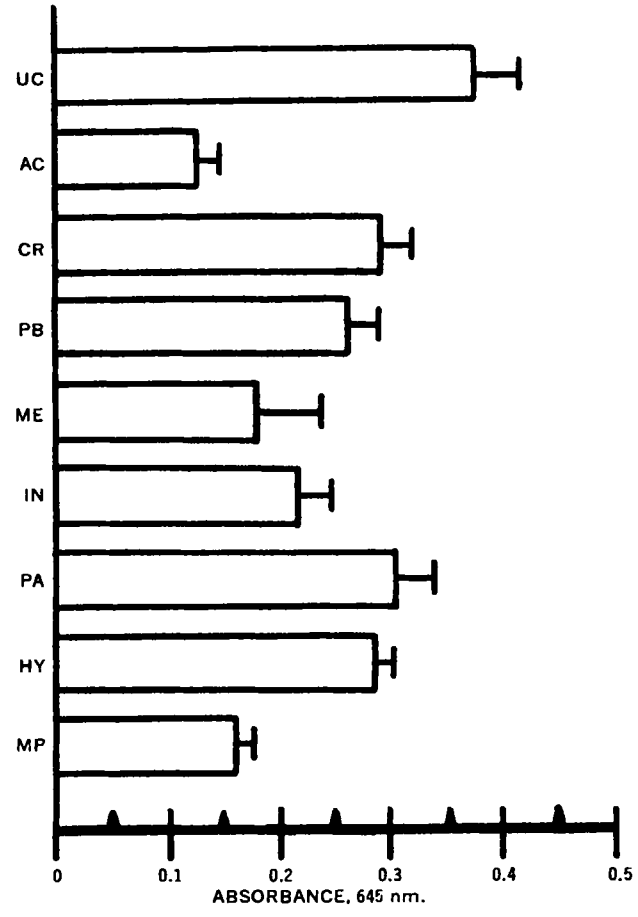

Figure 1—*Serum turbidity (absorbance + 1 SE) on Day +20 of untreated controls (UC) relative to adjuvant controls (AC) plus the effects on serum turbidity of adjuvant-treated rats receiving daily oral administration of cryogenine (CR), phenylbutazone (PB), mefenamic acid (ME), indomethacin (IN), paramethasone (PA), hydrocortisone (HY), and mercaptopurine (MP).*

[4] Composition: 2 ml. 0.067 *M* monopotassium phosphate + 98 ml. 0.067 *M* disodium phosphate.
[5] Spectronic 20, Bausch & Lomb.

Table II—Drug Effects on Volume of the Contralateral Paw in Adjuvant-Induced Polyarthritis

Treatment (Day −1 to Day +20)	Daily Oral Dose, mg./kg.	Mean Foot Volume, ml. ± *SE* (Percent Inhibition Inflammation) Day +15	Day +18	Day +21	Day +24	Day +30
Untreated control	—	1.19 ± 0.02	1.22 ± 0.02	1.24 ± 0.04	1.25 ± 0.05	1.26 ± 0.06
Adjuvant control	—	1.50 ± 0.10 (0)	1.67 ± 0.15 (0)	1.78 ± 0.16 (0)	1.87 ± 0.21 (0)	2.00 ± 0.21 (0)
Cryogenine	100	1.27 ± 0.03 (94)	1.31 ± 0.03 (88)	1.33 ± 0.05 (86)	1.37 ± 0.06 (82)	1.61 ± 0.04 (56)[a]
Phenylbutazone	100	1.30 ± 0.02 (74)[a]	1.31 ± 0.02 (81)	1.32 ± 0.02 (83)	1.35 ± 0.04 (80)	1.48 ± 0.04 (68)
Mefenamic acid	25	1.19 ± 0.02 (113)	1.26 ± 0.01 (94)	1.29 ± 0.01 (90)	1.39 ± 0.02 (75)	1.74 ± 0.04 (37)[a]
Indomethacin	1	1.34 ± 0.05 (71)[a]	1.43 ± 0.05 (62)[a]	1.45 ± 0.07 (66)[a]	1.41 ± 0.09 (75)[a]	1.69 ± 0.10 (46)[a]
Paramethasone	0.5	1.10 ± 0.01 (126)	1.17 ± 0.02 (102)	1.15 ± 0.02 (105)	1.18 ± 0.01 (100)	1.22 ± 0.06 (95)
Hydrocortisone	10	1.26 ± 0.02 (90)	1.29 ± 0.04 (88)	1.31 ± 0.04 (86)	1.44 ± 0.04 (68)[a]	1.56 ± 0.04 (59)[a]
Mercaptopurine	2	1.20 ± 0.03 (103)	1.21 ± 0.02 (100)	1.26 ± 0.03 (92)	1.34 ± 0.05 (80)	1.41 ± 0.03 (75)

[a] Mean foot volume is not significantly different from that of the adjuvant control group ($p > 0.05$).

slow recovery to preinjection weight by Day +12. Body weight then fluctuated within a narrow range during the remaining days of the study. These results are similar to those of Glenn *et al.* (11) and intermediate between the results of Pearson (12), who noted a normal weight gain, and of Newbould (1), who reported severe weight losses.

Considering the three compounds in Table III that provided consistent protection at Days +12, +21, and +30, and considering the patterns of body weight change documented in Table IV, cryogenine closely resembles hydrocortisone. Cryogenine partially prevented the acute loss of body weight noted in the adjuvant controls at Day +3 and thereafter the cryogenine-treated animals consistently gained weight, although at a slower rate than the untreated controls (mean gain of 21 g. by Day +30 as compared to 41 g. for the controls). Rats medicated with mercaptopurine progressively lost body weight through Day +9, and these animals still averaged 10 g. lighter than their starting weight by Day +30. Of the remaining partially protective compounds (Day +21 and/or Day +30) in Table III, the weight change pattern of the animals receiving paramethasone (Table IV) resembles that described for mercaptopurine. Such decreases in total body weight may contribute indirectly to the apparent "anti-inflammatory" effects recorded in Tables I and II but should not distort the arthritic index data in Table III. The weight change effects of mefenamic acid generally resembled the pattern described for cryogenine and hydrocortisone, while the pattern with phenylbutazone differed slightly in that the Day +3 weight loss seen with the adjuvant controls was generally blocked.

Table III—Drug Effects on the Arthritic Index in Rats with Adjuvant-Induced Polyarthritis

Treatment (Day −1 to Day +20)	Daily Oral Dose, mg./kg.	Mean Arthritic Index (Percent Inhibition) Day +12	Day +21	Day +30
Untreated control	—	0	0	0
Adjuvant control	—	3.3 (0)	6.5 (0)	10.2 (0)
Cryogenine	100	2.0 (39)[a]	3.2 (51)[a]	6.2 (39)[a]
Phenylbutazone	100	2.9 (12)	3.6 (45)[a]	6.7 (34)[a]
Mefenamic acid	25	2.4 (27)	4.0 (38)[a]	8.6 (16)
Indomethacin	1	3.1 (6)	4.7 (28)	7.3 (28)
Paramethasone	0.5	2.4 (27)	2.4 (63)[a]	3.6 (65)[a]
Hydrocortisone	10	1.7 (48)[a]	3.3 (49)[a]	3.9 (62)[a]
Mercaptopurine	2	2.3 (30)[a]	2.6 (60)[a]	4.9 (51)[a]

[a] Mean arthritic index is significantly different from that of the adjuvant control group ($p < 0.05$).

The adjuvant control animals resented handling, developed coarse ruffled fur, and began to vocalize and assume defensive fighting postures when paired with a fellow rat. Animals receiving cryogenine appeared quite healthy, displayed normal spontaneous motor activity, and did not show aggressive or defensive behavior patterns when paired with another rat. This tranquil attitude may be due to the weak ataractic activity documented for parenterally administered cryogenine (13) but is more likely due to the anti-inflammatory capacity of cryogenine providing relative freedom from the polyarthritic pain.

Serum Turbidity—As shown in Fig. 1, there was a significant ($p < 0.05$) decrease in serum turbidity in adjuvant control animals as compared to the untreated controls. While the immunosuppressive mercaptopurine was effective in all phases of adjuvant-induced polyarthritis studied (Days +12, +21, and +30, Table III), this effect was accompanied by severe losses in total body weight, and the serum turbidity in mercaptopurine-treated rats at Day +21 was not significantly different from the depressed levels documented for the adjuvant controls. Paramethasone, cryogenine, hydrocortisone, and phenylbutazone had a significant but intermediate degree of effect in preventing the adjuvant-induced decrease in serum turbidity, but the acute loss of body weight produced by paramethasone seems to place this compound in a different pharmacological category than the other three agents.

In studies with intact and adrenalectomized rats using both proliferative (cotton-pellet granuloma) and exudative (carrageenin-induced pedal edema) models of inflammation, DeCato (14) suggested that the anti-inflammatory efficacy of cryogenine may be due, in part, to the release of endogenous corticosteroids. This hypothesis is supported by the limited effectiveness of cryogenine (relative to phenylbutazone) in preventing UV-induced erythema in guinea pigs, a pharmacological model of inflammation that is

Table IV—Drug Effects on Whole Body Weight of Rats with Adjuvant-Induced Polyarthritis

Treatment (Day −1 to Day +20)	Daily Oral Dose, mg./kg.	Mean Body Weight, g. Day 0	Day +3	Day +6	Day +12	Day +18	Day +21	Day +24	Day +30
Untreated control	—	228	232[a]	234[a]	235[a]	253[a]	253[a]	258[a]	269[a]
Adjuvant control	—	215	204	209	217	213	216	212	220
Cryogenine	100	220	215[a]	217	222	229	234[a]	235[a]	241[a]
Phenylbutazone	100	212	210	216	221	218	222	224	231
Mefenamic acid	25	217	212	215	223	227	226	229	236
Indomethacin	1	216	206	205	214	216	219	224	230
Paramethasone	0.5	210	190[a]	186[a]	183[a]	191[a]	190[a]	194	202
Hydrocortisone	10	226	219[a]	218	221	228	230	236[a]	244[a]
Mercaptopurine	2	218	205	198	198[a]	201	199	200	208

[a] Significantly different from values of the adjuvant control group ($p < 0.05$).

unresponsive to corticosteroid-like agents (15). The results of the present study do not conflict with this theory and do establish that the anti-inflammatory activity of cryogenine probably is not due to a mercaptopurine-like immunosuppressive capacity.

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Structure–Side-Effect Sorting of Drugs II: Skin Sensitization

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Abstract □ A computerized sorting program was used to sort out 120 drugs that have been reported to cause skin sensitization from a data bank of 540 clinically useful drugs. Based on an analysis of functional groups, a number of unique common denominators were found for skin sensitization due to covalent bond formation with body proteins. These include potential epoxide-forming groups such as an electron-deficient benzene ring, a vinyl group, hydroxylamine and *N*-oxide precursors, drugs capable of forming a benzylic radical, β-lactam derivatives, and α,β-unsaturated ketones. By using multiple-regression analysis, the quantitative structure–activity relationship for skin-sensitizing properties of 3-alkyl catechols and 2,4-dinitrobenzene derivatives are also reported.

Keyphrases □ Side effects, computer sorting of 540 drugs—skin sensitization related to structure □ Computer sorting of side effects of 540 drugs—skin sensitization related to structure □ Structure–activity relationships—features related to skin sensitization effects, result of computer sorting of 540 drugs □ Skin sensitization effects—related to structural features, result of computer sorting of 540 drugs □ Drug sorting by side effects—structure–activity relationships

As a continuation and extension of an effort to sort and correlate side effects of drugs with their chemical structures (1), attention was focused on the problem of skin sensitization. Numerous drugs are known to cause dermatitis, urticaria, eczema, photosensitization, or other signs of skin sensitization following either systemic or topical applications. The main purpose of this study was to utilize a computerized sorting program to list as many as possible of the drugs known to cause various types of skin sensitization and to find out the common structural features. Attempts were made to categorize these drugs on the basis of functional group analysis and the types of possible chemical reactions with proteins. It is hoped that the findings will not only suggest working hypotheses for skin sensitization by various drugs but will also provide some rational chemical basis of skin sensitization. Quantitative structure–activity analysis was applied to a series of catechols and dinitrobenzene derivatives where quantitative data on series of compounds were available.

METHOD

The computerized sorting program was described previously (1). The following descriptive terms of signs were sorted out from the data bank: skin sensitization, lesions, necrosis, rashes, eruptions, reactions, irritations, opacities, dermatitis, photosensitivity, and urticaria. The results are given in Tables I and II.

The method of least squares was used to derive the equations correlating the physicochemical parameters with the delayed contact skin-sensitizing activity. The biological data and the physicochemical constants used are assembled in Table III.

RESULTS AND DISCUSSION

Skin-Sensitizing Drugs—A total of 120 drugs or drug groups from the data bank of 540 drugs have been reported to cause various types of skin sensitization (2–8). In analyzing the common structural features, many of these drugs were found to contain a benzene ring