Anticonvulsant Activity and Inhibition of Respiration in Rat Brain Homogenates by Substituted Trimethoxybenzamides

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Abstract □ Ten *N*-[4-[4-(arylthiosemicarbazido)carbonyl]phenyl]-3,4,5-trimethoxybenzamides were synthesized and characterized by their sharp melting points, elemental analyses, and IR and NMR spectra. All substituted benzamides were evaluated for their anticonvulsant activity. The degree of protection afforded by these compounds (100 mg/kg ip) against pentylenetetrazol (90 mg/kg sc)-induced convulsions in mice ranged from 10 to 80%. The ability of substituted benzamides to inhibit respiratory activity was observed by inhibition of oxidation of pyruvate, α-ketoglutarate, NADH, and succinate by rat brain homogenates. Inhibition of pyruvic acid oxidation was concentration dependent. The anticonvulsant activity of substituted benzamides was not related to their ability to inhibit cellular respiratory activity.

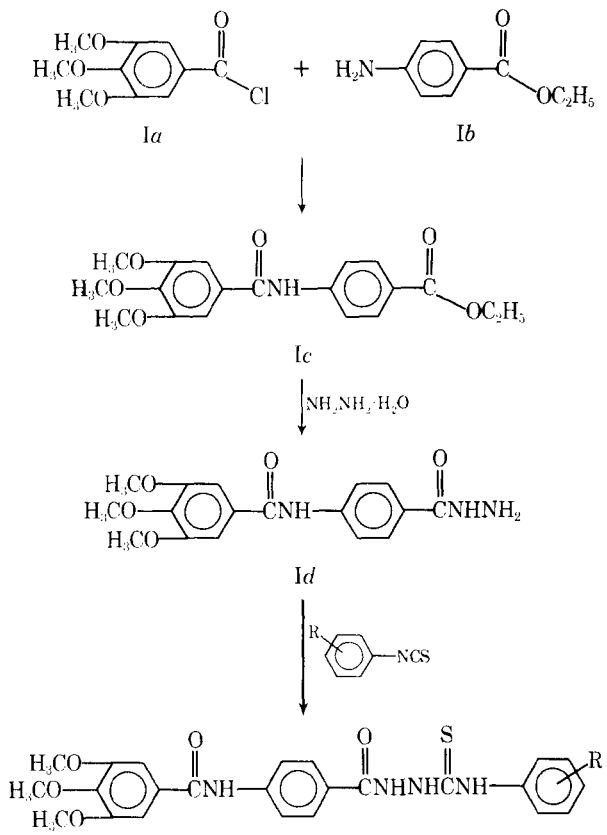
Keyphrases □ Trimethoxybenzamides, various substituted—synthesized, evaluated for anticonvulsant activity in mice and effect on enzyme activity in rat brain homogenates □ Anticonvulsant activity—various substituted trimethoxybenzamides evaluated in mice □ Enzyme activity—NAD dependent and independent oxidations in rat brain homogenates, effect of various substituted trimethoxybenzamides □ Oxidations, NAD dependent and independent—effect of various substituted trimethoxybenzamides in rat brain homogenates □ Structure–activity relationships—various substituted trimethoxybenzamides evaluated for anticonvulsant activity in mice and effect on enzyme activity in rat brain homogenates

Central nervous system (CNS) depressant and hypotensive properties associated with reserpine are due to the presence of a 3,4,5-trimethoxybenzene moiety (1–6). Many drugs affecting CNS activity have amide or thioamide linkage as the common molecular structural units (7, 8). CNS-affecting properties of trimethoxybenzamides were reported (9, 10). Amides also possess greater CNS depressant activity as compared to their corresponding esters (11). The anticonvulsant activity of nitrobenzamides and their ability to inhibit nicotinamide adenine dinucleotide (NAD)-dependent oxidations by rat brain homogenates were reported recently (12, 13).

These observations and the functional role of the 3,4,5-trimethoxyphenyl moiety for CNS activity (14) prompted the synthesis of some *N*-[4-[4-(arylthiosemicarbazido)carbonyl]phenyl]-3,4,5-trimethoxybenzamides. These substituted trimethoxybenzamides were evaluated for their anticonvulsant activity. The ability of these compounds to inhibit cellular respiratory activity of rat brain homogenates also was investigated to elucidate their biochemical mechanism of action. The various trimethoxybenzamides were synthesized by following the methods outlined in Scheme I.

EXPERIMENTAL

Chemistry—The condensation of 3,4,5-trimethoxybenzoyl chloride (Ia, Scheme I) with ethyl *p*-aminobenzoate (Ib) gave ethyl 4-(3,4,5-trimethoxybenzamido)benzoate (Ic) which, on reaction with hydrazine hydrate, yielded 4-(3,4,5-trimethoxybenzamido)benzohydrazide (Id). This trimethoxybenzamide, on treatment with suitable aryl isothiocyanates, gave *N*-[4-[4-(arylthiosemicarbazido)carbonyl]phenyl]-3,4,5-trimethoxybenzamides (II–XI).



Scheme I

Analyses for carbon, hydrogen, and nitrogen were performed; melting points were taken in open capillary tubes and are corrected. IR spectra were taken[1] in mineral oil mulls in the range of 1000 to 4000 cm^{-1}. The NMR spectra were obtained[2] using dimethyl sulfoxide-d_6 as solvent and tetramethylsilane as a reference.

Ethyl 4-(3,4,5-Trimethoxybenzamido)benzoate (*Ic*)—A solution of Ia (0.2 mole) in dry tetrahydrofuran was added slowly, with stirring, to a cooled (below 10°) solution of Ib (0.4 mole) in the same solvent. The mixture was stirred for 1 hr and allowed to stand overnight in a refrigerator. The solvent was removed by distillation under reduced pressure. The solid residue was collected by filtration and washed first with 5% HCl, then with 5% sodium carbonate, and then with water repeatedly.

The compound was recrystallized from ethanol, mp 120° [lit. (14) mp 117°]. The IR spectrum showed peaks at 1690 ($COOC_2H_5$), 1675 (CONH), and 3350 (CO*NH*) cm^{-1}. The NMR spectrum gave signals at δ 1.27 (t, 3H, $COOCH_2CH_3$, J = 7 Hz), 3.75 (s, 3H, 4-OCH_3), 3.87 (s, 6H, 3- and 5-OCH_3), 4.33 (q, 2H, $COOCH_2CH_3$), 7.33 (s, 2H, C_6H_2), 8.00 (s, 4H, C_6H_4), and 10.35 (b, 1H, CONH) ppm.

4-(3,4,5-Trimethoxybenzamido)benzohydrazide (*Id*)—A mixture of

[1] Beckman IR-12 spectrophotometer.
[2] Varian Associates model A-60.

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Table I—Physical Constants and Anticonvulsant Activity of *N*-[4-[4-(Arylthiosemicarbazido)carbonyl]phenyl]-3,4,5-trimethoxybenzamides

Compound	R	Melting Point	Yield, %	Molecular Formula		Analysis, % Calc.	Analysis, % Found	Anticonvulsant Activity, % Protection[a]	Anticonvulsant Activity, % Mortality[b]
II	H	144°	70	$C_{24}H_{24}N_4O_5S$	C	60.00	60.32	20	80
					H	5.00	4.81		
					N	11.66	11.42		
III	2-CH_3	128°	75	$C_{25}H_{26}N_4O_5S$	C	61.13	60.84	60	40
					H	5.26	5.38		
					N	11.33	11.67		
IV	3-CH_3	172°	80	$C_{25}H_{26}N_4O_5S$	C	61.13	61.44	10	90
					H	5.26	5.34		
					N	11.33	11.12		
V	4-CH_3	121°	72	$C_{25}H_{26}N_4O_5S$	C	61.13	61.43	80	20
					H	5.26	5.32		
					N	11.33	11.61		
VI	2-OCH_3	214°	78	$C_{25}H_{26}N_4O_6S$	C	58.82	58.64	10	90
					H	5.09	5.00		
					N	10.90	11.15		
VII	4-OCH_3	113°	70	$C_{25}H_{26}N_4O_6S$	C	58.82	59.11	20	60
					H	5.09	5.27		
					N	10.90	10.65		
VIII	2-OC_2H_5	168°	68	$C_{26}H_{28}N_4O_6S$	C	59.54	59.76	40	60
					H	5.34	5.41		
					N	10.68	10.47		
IX	4-OC_2H_5	128°	65	$C_{26}H_{28}N_4O_6S$	C	59.54	59.29	50	40
					H	5.34	5.23		
					N	10.68	10.48		
X	4-Cl	116°	80	$C_{24}H_{23}ClN_4O_5S$	C	55.97	56.18	10	80
					H	4.47	4.53		
					N	10.88	10.59		
XI	4-Br	125°	82	$C_{24}H_{23}BrN_4O_5S$	C	51.52	51.79	20	40
					H	4.11	4.37		
					N	10.01	10.25		

[a] Anticonvulsant activity is represented as percent protection against pentylenetetrazol-induced seizures. [b] Toxicity is represented as percent deaths of animals treated with pentylenetetrazol during 24 hr.

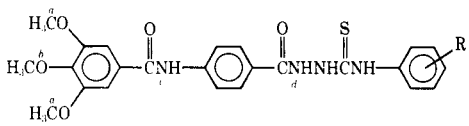

Table II—Chemical Shifts of Some Protons of *N*-[4-[4-(Arylthiosemicarbazido)carbonyl]phenyl]-3,4,5-trimethoxybenzamides

Compound	Chemical Shift[a] OCH_3 (*a*)	OCH_3 (*b*)	CONH (*c*)	CONH (*d*)	R
II	3.90 (s, 6H)	3.78 (s, 3H)	10.50 (b, 1H)	9.80 (b, 1H)	—
III	3.90 (s, 6H)	3.77 (s, 3H)	10.43 (b, 1H)	9.50 (b, 1H)	2.23 (s, 3H)
IV	3.91 (s, 6H)	3.76 (s, 3H)	10.37 (b, 1H)	9.50 (b, 1H)	2.25 (s, 3H)
V	3.88 (s, 6H)	3.77 (s, 3H)	10.28 (b, 1H)	9.60 (b, 1H)	2.28 (s, 3H)
VI	3.90 (s, 6H)	3.78 (s, 3H)	10.38 (b, 1H)	9.30 (b, 1H)	3.78 (s, 3H)
VII	3.88 (s, 6H)	3.75 (s, 3H)	10.30 (b, 1H)	9.60 (b, 1H)	3.75 (s, 3H)
VIII	3.88 (s, 6H)	3.75 (s, 3H)	10.40 (b, 1H)	9.13 (b, 1H)	1.10 (t, 3H, J = 7 Hz) and 4.00 (q, 2H)
IX	3.90 (s, 6H)	3.78 (s, 3H)	10.36 (b, 1H)	9.75 (b, 1H)	1.3 (t, 3H, J = 7 Hz) and 4.03 (q, 2H)
X	3.91 (s, 6H)	3.80 (s, 3H)	10.23 (b, 1H)	9.76 (b, 1H)	—
XI	3.90 (s, 6H)	3.75 (s, 3H)	10.35 (b, 1H)	9.93 (b, 1H)	—

[a] Chemical shifts are expressed in parts per million downfield relative to tetramethylsilane as the internal standard. All spectra were taken in dimethyl sulfoxide-d_6. Multiplicities of the peaks are expressed as singlet (s), triplet (t), and quartet (q). All amidic protons were exchangeable with deuterium oxide.

Ic (0.01 mole) and hydrazine hydrate (0.02 mole, 99–100%) was refluxed on a steam bath in absolute ethanol (200 ml) for 8–10 hr. Excess solvent was distilled under reduced pressure, and the hydrazide that separated out was collected by filtration and recrystallized from ethanol in a 70% yield, mp 202°.

The IR spectrum showed peaks at 1670 (CONH) and 3370 (CONHNH_2) cm^{-1}. The NMR spectrum gave the signals at δ 3.75 (s, 3H, 4-OCH_3), 3.88 (s, 6H, 3- and 5-OCH_3), 4.45 (b, 2H, CONHNH_2), 7.33 (s, 2H, C_6H_2), 7.90 (s, 4H, C_6H_4), 9.91 (b, 1H, CON*H*NH_2), and 10.53 (b, 1H, CON*H*) ppm.

Anal.—Calc. for $C_{17}H_{19}N_3O_5$: C, 59.13; H, 5.50; N, 12.17. Found: C, 59.38; H, 5.41; N, 11.86.

N-[4-[4-(*Arylthiosemicarbazido)carbonyl*]*phenyl*] *-3,4,5- trimethoxybenzamides (II–XI)*—Suitable aryl isothiocyanates (0.01 mole) were added to Id (0.01 mole) in 25 ml of absolute ethanol, and the mixture was refluxed on a steam bath for 6 hr. Then the reaction mixture was concentrated under reduced pressure. On cooling, the solid mass that separated out was filtered and washed with ether. The product thus obtained was dried and recrystallized from ethanol.

The various substituted trimethoxybenzamides (Table I) were characterized by their sharp melting points, elemental analyses, and IR and NMR spectra. The IR spectra showed peaks between 1660 and 1675 cm^{-1} and 3320 and 3360 cm^{-1}, characteristic of *CO*NH and CO*NH* groups, respectively. The chemical shifts in NMR spectra of these compounds are recorded in Table II.

Determination of Anticonvulsant Activity—Anticonvulsant activity against pentylenetetrazol-induced seizures was determined in albino mice of either sex weighing 25–30 g. The mice were divided into groups of 10, and the group weights were kept as near the same as possible. Each test compound was suspended in a 5% aqueous suspension of gum acacia to give a concentration of 1% (w/v). The test compounds (100 mg/kg) were injected intraperitoneally in a group of 10 animals. Pentylenetetrazol (90 mg/kg) was injected subcutaneously 4 hr after drug administration.

This dose of pentylenetetrazol produces not only convulsions in almost all untreated mice but also exhibits 100% mortality over 24 hr. No mortality was observed over 24 hr in animals pretreated with 100-mg/kg doses of test compound. The mice were then observed for seizures for 60 min.

Table III—Effect of *N*-[4-[4-(Arylthiosemicarbazido)carbonyl]phenyl]-3,4,5-trimethoxybenzamides on Rat Brain Respiratory Activity

Compound	Inhibition[a], %					
	Pyruvate			α-Ketoglutarate,	NADH,	Succinate,
	1.0×10^{-3} M[b]	5.0×10^{-4} M[b]	1.0×10^{-4} M[b]	1.0×10^{-3} M[b]	1.0×10^{-3} M[b]	1.0×10^{-3} M[b]
II	65.40 ± 0.52	24.88 ± 0.37	Nil	93.72 ± 0.82	18.92 ± 0.71	Nil
III	95.21 ± 0.81	60.38 ± 0.58	19.74 ± 0.55	38.31 ± 0.50	23.15 ± 0.42	31.78 ± 0.51
IV	88.49 ± 0.77	51.77 ± 0.61	19.30 ± 0.63	98.12 ± 0.78	26.18 ± 0.56	Nil
V	70.38 ± 0.54	52.85 ± 0.91	11.36 ± 0.85	98.41 ± 0.61	Nil	Nil
VI	38.01 ± 0.32	31.08 ± 0.79	Nil	64.01 ± 0.53	Nil	7.35 ± 0.82
VII	36.24 ± 0.79	20.44 ± 0.54	14.39 ± 0.61	60.38 ± 0.49	11.01 ± 0.82	Nil
VIII	54.03 ± 0.82	46.48 ± 0.39	30.26 ± 0.73	46.43 ± 0.37	5.87 ± 0.94	33.17 ± 0.69
IX	66.82 ± 0.92	56.84 ± 0.65	48.89 ± 0.65	88.24 ± 0.12	25.17 ± 0.66	Nil
X	42.86 ± 0.79	27.43 ± 0.87	5.89 ± 0.97	88.81 ± 0.75	53.89 ± 0.71	16.86 ± 0.59
XI	39.02 ± 0.59	15.04 ± 0.73	Nil	56.28 ± 0.34	37.61 ± 0.67	Nil

[a] Each experiment was done in duplicate. All values represent mean values of percent inhibition with the standard error calculated from three separate experiments. Inhibition was determined by the decrease in oxygen uptake per 100 mg wet weight of tissue per hour. [b] Molar concentrations given are the final concentrations of II–XI in the assay system. Assay conditions are as indicated in the text.

An episode of clonic spasm that persisted for a minimum of 5 sec was considered a threshold convulsion. Transient intermittent jerks and tremulousness were not counted.

Animals devoid of threshold convulsions during 60 min were considered protected. The number of animals protected in each group was recorded, and the anticonvulsant activity of these substituted trimethoxybenzamides was represented as percent protection. The mortality was recorded after 24 hr to obtain an idea of the toxicity of these compounds in pentylenetetrazol-treated experimental animals.

Determination of Respiratory Activity of Rat Brain Homogenate[3]—Male albino rats were kept on an *ad libitum* diet. Rat brains, isolated from decapitated animals, were immediately homogenized in ice-cold 0.25 *M* sucrose in a homogenizer[4] in a ratio of 1:9 (w/v). All incubations were carried out at 37°, and the oxygen uptake was measured using air as the gas phase (13). Fresh rat brain homogenate (1 ml), equivalent to 100 mg wet weight, was added to chilled Warburg vessels containing 6.7 m*M* magnesium sulfate, 20 m*M* sodium hydrogen phosphate buffer solution (pH 7.4), 1 m*M* adenosine monophosphate (sodium salt), 33 m*M* potassium chloride, and 500 μg of cytochrome c in a final volume of 3 ml, unless otherwise stated. The central well contained 0.2 ml of 20% KOH.

The final concentration of pyruvate, α-ketoglutarate, and succinate was 1 m*M* while that of NADH was 0.5 m*M*. It was presumed that endogenous NAD, present in rat brain homogenates, was sufficient for these oxidative processes. All substituted trimethoxybenzamides were dissolved in propylene glycol and were used at final concentrations of 1 m*M* during oxidation of α-ketoglutarate, NADH, and succinate and of 0.1, 0.5, and 1 m*M* during oxidation of pyruvate by rat brain homogenates. An equal volume of propylene glycol was added to the control vessels.

RESULTS AND DISCUSSION

The anticonvulsant activity of II–XI is recorded in Table I. All compounds provided protection against pentylenetetrazol-induced seizures at a dose of 100 mg/kg. The degree of protection ranged from 10 to 80%. Most compounds exhibited significantly low anticonvulsant activity. The maximum degree of protection was observed with the compound having a methyl group at position 4 of the phenyl nucleus (V), and minimum protection was observed with IV, VI, and X.

The replacement of the methyl group by a methoxy, ethoxy, or halogen group caused a marked decrease in the anticonvulsant activity. Compounds possessing an ethoxy group on the phenyl moiety possessed higher anticonvulsant activity as compared to compounds having a methoxy group. As is evident from Table I, compounds having low anticonvulsant activity produced less protection against 24-hr mortality in pentylenetetrazol-treated animals. These results failed to provide evidence for any definite structural requirements in the molecular makeup of these substituted trimethoxybenzamides that could be responsible for their anticonvulsant activity.

The inhibition of cellular respiratory activity of rat brain homogenates by II–XI is recorded in Table III. Almost all compounds inhibited oxidation of pyruvate, α-ketoglutarate, NADH, and succinate. The inhibition of pyruvate oxidation was concentration dependent; an increase in the concentration of test compound increased the inhibition of pyruvic acid oxidation by rat brain homogenates. The degree of inhibition by substituted trimethoxybenzamides was of low order during oxidation of NADH and succinate while maximum inhibition by most compounds was observed during oxidation of α-ketoglutarate by rat brain homogenates. However, it can be assumed that the use of higher concentrations of compounds devoid of the inhibitory property could possibly inhibit the respiratory activity of rat brain homogenates.

These results indicated the unspecific inhibition of both the NAD-dependent oxidation of pyruvate and α-ketoglutarate and the NAD-independent oxidation of succinate by rat brain homogenates. The *in vitro* inhibition of the oxidation of NADH was observed, as reported earlier for nitrobenzamides (XII). In general, the nature of different substituents on the phenyl moiety of these compounds did not contribute significantly in their ability to inhibit respiratory activity of rat brain homogenates. These results provided evidence regarding the possible sensitivity of substituted trimethoxybenzamides toward both NADH-COQ (oxido) reductase (Complex I) and succinate-COQ (oxido) reductase (Complex II) of the electron transport chain. Hydro COQ-cytochrome c (oxido) reductase (Complex III) and/or cytochrome c-O_2 (oxido) reductase (Complex IV) also may be susceptible to these substituted trimethoxybenzamides, which might account for their ability to inhibit flavine adenine dinucleotide-dependent oxidation of succinate (NAD-independent) and NAD-dependent oxidation of pyruvate and α-ketoglutarate.

These results did not provide a correlation between the anticonvulsant activity of substituted trimethoxybenzamides and their ability to inhibit the respiratory activity of rat brain homogenates as a biochemical basis of their anticonvulsant activity. Further studies dealing with the synthesis of related structures carrying different substituents on the phenyl moiety and the determination of effectiveness to inhibit purified enzyme preparation may possibly reflect the biochemical basis for the anticonvulsant activity of these compounds.

[3] Sodium pyruvate, sodium α-ketoglutarate, NADH, sodium succinate, adenosine monophosphate (AMP), and cytochrome c were obtained from Sigma Chemical Co., St. Louis, Mo.

[4] Potter-Elvehjem homogenizer.

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Effect of Repeated Skin Application on Percutaneous Absorption of Salicylic Acid

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Abstract □ Various concentrations of salicylic acid in hydrophilic ointment were applied repeatedly at daily or weekly intervals to rats *in vivo*. Salicylic acid absorption through treated skin was monitored by determining the penetration fluxes of salicylic acid through skin excised at various times. A gradual decrease in the salicylic acid penetration flux was observed following weekly applications of either 5 or 10% salicylic acid in hydrophilic ointment. The penetration flux of 1% salicylic acid remained constant. In the daily applications of 5 and 10% salicylic acid, the penetration flux increased after approximately 2 days of treatment and declined thereafter. The penetration flux of salicylic acid from the 1% salicylic acid increased slightly after 3–4 days of treatment.

Keyphrases □ Salicylic acid—percutaneous absorption, effect of repeated application, rats □ Absorption, percutaneous—salicylic acid, effect of repeated application, rats □ Keratolytic agents—salicylic acid, percutaneous absorption, effect of repeated application, rats

The percutaneous absorption of salicylates has been reported extensively (1–5). Barry *et al.* (6) examined blood salicylate levels in rabbits treated with 10% salicylic acid in hydrophilic ointment at weekly intervals. They reported that a progressive and statistically significant decrease in percutaneous absorption of salicylic acid occurred over the treatment period. Skin dehydration and decreased emotional arousal of the animals were suggested as possible explanations for this result.

BACKGROUND

Since salicylic acid is usually applied to the skin in topical therapy with repeated applications, it was decided to evaluate the effect of repeated applications of salicylic acid on its percutaneous absorption. Blood salicylate levels resulting from salicylic acid absorption following repeated topical applications (6) may reflect not only changes in absorption rates due to the dermatological effects of salicylic acid but also other pharmacokinetic alterations in metabolism and excretion due to the prolonged therapy.

Müller *et al.* (7) reported decreased steady-state plasma salicylic acid levels associated with chronic aspirin ingestion. Percutaneous absorption of substances was examined using excised skin (8, 9). Excised skin allows intracutaneous penetration to be measured simply, directly, reproducibly, and precisely, the fundamental assumption being that the stratum corneum is a dead tissue whose impermeability is unaffected by excision. The use of skin excised following repeated applications of salicylic acid to the skin *in vivo* was employed in the present study to examine directly the consequences of the dermatological effects of salicylic acid on its absorption.

The wide range of dermatotherapeutic effects of salicylic acid is also concentration dependent (10). In concentrations of 5% or more, salicylic acid exerts a keratolytic effect on the skin; in concentrations of less than 5%, a keratoplastic effect is apparent (10). Consequently, the objective of this study was to assess: (*a*) the effect of daily or weekly repeated topical applications of salicylic acid on its absorption, and (*b*) the effect of the concentration of salicylic acid in the hydrophilic ointment on its absorption.

EXPERIMENTAL

Animals—Female Wistar rats[1], 250–300 g, were housed in a constant-temperature room and given food[2] and water *ad libitum*.

Ointment Base—Salicylic acid BP was passed through an 80-mesh sieve, and appropriate amounts were incorporated into hydrophilic ointment USP. Accordingly, hydrophilic ointments containing 10, 5, and 1% salicylic acid were prepared.

Test Procedures—Under light ether anesthesia (anesthetic ether BP), the rats were weighed and hair was removed from the flanks with an animal clipper[3]. A sample of ointment was rubbed into the area of shaved skin; this area (3 cm^2) was approximately twice the cross-sectional area for diffusion in the diffusion cells. Additional ointment (total ~2 g) was spread uniformly over the area on the dull side of a sheet of aluminum foil. The foil was held in place by adhesive tape and an elastic bandage.

The animals were kept in restraining cages for the duration of the treatment. After the 7.5-hr test period, the bandages were removed from the animals and the animals were returned to the animal house. Repeated treatments at daily or weekly intervals were then employed. At various stages during the treatment, the animals were asphyxiated by an overdose of ether, the skin immediately was excised, and the appropriate ointment was applied to the epidermis. The treated excised skin was placed in a diffusion cell (9); the receptor compartment contained 0.1% chlorhexidine solution to prevent microbial growth at 37°.

To measure the amount of salicylic acid penetrating the skin, 3-ml aliquots of receptor solution were removed and treated with 1 ml of 13.2% ferric chloride in 0.4 *N* HCl. The resultant color was measured[4] at 540 nm, and the salicylate concentration was computed from a Beer's law plot. The 3-ml aliquot of solution removed from the diffusion cell was replaced with 3 ml of the chlorhexidine solution. A minimum of three animals was used in the estimation of each reported penetration flux. The possible interaction between salicylic acid and the skin on the initial application was examined by comparing the fluxes of various concentrations of salicylic acid in hydrophilic ointment to its flux across an inert membrane

[1] Southern Biological Services, Parkdale, Victoria.
[2] M & V Mouse Cubes, W. M. Charlick, Adelaide, South Australia.
[3] Breville model 900.
[4] Beckman spectrophotometer.

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