this drug was slow and whether it is due to physical or physiological factors has yet to be ascertained. Polyethylene glycol 300 has proved to be a useful solvent for griseofulvin and this and other low molecular weight polyethylene glycols might enable the pharmacokinetics of other insoluble neutral drugs to be evaluated in man.

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Keyphrases

Griseofulvin—absorption
Absorption kinetics—griseofulvin
Distribution, excretion—griseofulvin
Plasma—griseofulvin analysis
Fluorometry—analysis

Analgesic and Anti-Inflammatory Evaluation of Thymotic Acid and Certain Homologs

By FRED J. MAROZZI and MARVIN H. MALONE

Ortho-thymotic acid and nine homologs were submitted for general, analgesic, and antistress evaluations in the rat. While all appeared to be nonspecific central nervous system depressants, only 2-methyl-5-*tert*-butylsalicylic acid appeared to have significant analgesic ability in the rat tail flick test. 2-Hydroxy-4-methyl-5-isopropylbenzoic acid, 2-hydroxy-4-isopropyl-6-methylbenzoic acid, and 2-hydroxy-3-methyl-6-isopropylbenzoic aicd were superior to sodium salicylate and inferior to morphine sulfate in providing significant protection from stress induced by unilateral hind leg tourniquets. The latter two compounds were active in suppressing the acute inflammatory process produced by pedal injection of carrageenin, but were ineffective against chronic inflammation induced by the *Mycobacterium butyricum* adjuvant.

O'BRIEN AND Thoms (1) investigated the antipyretic and analgesic activity of the sodium salts of *o*- and *p*-thymotic acid in 1958. Von Kaulla (2) demonstrated in 1965 that *o*-thymotic acid possessed significant fibrinolytic activity. More recently, Rader and Wulf (3) investigated the fibrinolytic activity of nine homologs of *o*-thymotic acid on the human and cat fibrinolytic systems and were able to show that several compounds possessed significant fibrinolytic activity beyond that of *o*-thymotic acid. The present investigation is concerned with the analgesic and general evaluation of certain of these homologs along with *o*-thymotic acid, itself.

EXPERIMENTAL

The compounds shown in Table I were obtained in limited amounts from the Division of Medicinal Chemistry of the University of Connecticut. This group was selected so as to reveal possible structure–activity relationships. Table I also summarizes the fibrinolytic activity of the various homologs as determined by Rader and Wulf (3, 4).

Qualitative Screening—The hippocratic method of Malone and Robichaud (5) was used as a prelim-

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TABLE I—THYMOTIC ACID DERIVATIVES

Compd.	Chemical Name	Observed M.p., °C.	Fibrinolytic Concn., M[a]
I	2-Methyl-5-ethylsalicylic acid	162–163	>0.04
II	2-Methyl-5-isopropylsalicylic acid[b]	122–128	0.02
III	2-Methyl-5-*sec*-butylsalicylic acid	169	0.005
IV	2-Methyl-5-[2-(3-methyl-butyl)]salicylic acid	166–167	0.03
V	2-Methyl-5-*tert*-butylsalicylic acid	182–183	0.0075
VI	2-Methyl-5-*tert*-amylsalicylic acid	176	0.035
VII	2-Methyl-4-hydroxy-5-isopropylbenzoic acid[c]	156–160	>0.04
VIII	2-Hydroxy-4-isopropyl-6-methylbenzoic acid	115–122	0.02
IX	2-Hydroxy-4-methyl-5-isopropylbenzoic acid	141–144	0.03
X	2-Hydroxy-3-methyl-6-isopropylbenzoic acid[d]	142–145	0.035

[a] Concentration to obtain ≥ 80% dissolution of clot. [b] *Ortho*-thymotic acid. [c] *Para*-thymotic acid. [d] *Ortho*-carvacrotic acid.

inary gross observational screen of all test compounds. Adult albino Holtzman rats (320–500 Gm.) were injected intraperitoneally using at least three logarithmetically spaced dosage levels (at least one of which was lethal), and qualitative and semiquantitative symptomatology was observed and noted on a standardized work sheet (5) at the required times.

Tail Flick Studies—A conduction dolorimeter (Metro Scientific Inc., Long Island, N.Y.) was used to measure the rat tail flick response to a thermal stimulus. The apparatus was modified by the addition of a photo cell in series with a timer in order to provide more accurate recordings of reaction times. Adult albino rats of the Wistar strain (Steinhilber) weighing between 125–150 Gm. were used for the initial analgesic evaluation of each test compound. Each compound was tested on at least five animals per dosage level. All test compounds and the two reference standards (morphine sulfate and sodium salicylate) were injected intraperitoneally in 0.25% agar after determination of control reaction times. Reaction times were again determined at +15, 30, 45, 60, and 120 min. after injection. A reaction time of 20 sec. was considered indicative of 100% analgesia and any animal failing to respond within 20 sec. was manually removed from the dolorimeter. Percent analgesia was calculated for each animal according to the following formula:

$$\% \text{ analgesia} = \left[\frac{R_x - C_0}{20 - C_0}\right] 100$$

where R_x = reaction time in sec. of an individual rat at time x; C_0 = the control reaction time in seconds of the same individual at time 0. Due to the limited amounts of drug available, the compounds were screened for analgesic activity at only one dosage level (100 mg./Kg.). This dosage was considered to be sublethal and without major CNS effects considering the hippocratic screening data.

Hepatic Glutathione Depletion—The methods used were essentially those described by Cooper *et al.* (6). Adult male rats of the Wistar strain (Steinhilber) ranging in weight between 260–375 Gm. were injected intraperitoneally with the test compounds dissolved or suspended in 0.25% aqueous agar. One-half hour after this injection, unilateral right hind limb tourniquets were tied around the thigh muscle proximal to the hip joint using heavy suture thread (Dermalon skin sutures, size 3). Food and water were allowed *ad libitum*. Four hours after the application of the tourniquets the rats were sacrificed by cranio-vertebral dislocation and peripheral portions of the central lobe of the liver (approximately 2 Gm.) were excised immediately and frozen using dry ice. Sacrifice of all animals was arranged so as to occur between the hours of 3:30 and 7:30 p.m. to control for the diurnal variation in liver sulfhydryl content reported by Beck (7). Hepatic glutathione (GSH) levels were determined amperometrically according to the method described by Benesch and Benesch (8, 9). Filtrates of the liver samples were prepared by homogenizing 500 mg. wet weight of liver in 10 ml. of 2.5% sulfosalicylic acid, followed by centrifugation and filtration. Aliquots of each filtrate were then titrated using 0.0005 *N* silver nitrate and a rotating platinum electrode (10).

Acute Antiphlogistic Evaluation—Using methods similar to those described by Winter *et al.* (11), nonfasted male and female Wistar strain rats (Steinhilber) were randomized into 12 cages (2 males and 2 females per cage) to which the test treatments were randomly assigned. Volume changes in the rats' paws were recorded using a modified plethysmographic apparatus similar to that described by Harris and Spencer (12). All drugs were administered orally as aqueous suspensions in 0.25% agar to hydrated animals. One hour after drug administration, 0.05 ml. of a 1.0% suspension of carrageenin in sterile physiological saline was injected into the plantar aponeurosis of the left hind paw. Volume changes in the injected paw were recorded 3 hr. later. The antiphlogistic properties of the three compounds tested were compared statistically to a series of reference agents using Duncan's multiple-range test (13).

Chronic Anti-Inflammatory Activity—Young adult male albino rats (Steinhilber) were used in this study. Foot thickness was determined daily with a vernier caliper for both the injected and contralateral paws. The inflammatory state was induced by the injection of 0.05 ml. of a suspension of desiccated *Mycobacterium butyricum* cells in light liquid petrolatum prepared according to the method of Nuss (14, 15). The test drugs were administered orally daily (100 mg./Kg.) as aqueous suspensions in 0.25% agar for a period of 13 days after which drug treatment was discontinued. Measurements of body weight and foot thickness were continued for another 17 days. Both food and water were freely accessible throughout the test period, and consumption records were maintained. The percent inhibition of the inflammatory response on days 13 and 30 of the test period was calculated according to the following formula (16):

$$\% \text{ inhibition} = 100\left[\frac{1 - (a - x)}{(b - y)}\right]$$

where y = mean injected foot thickness of control rats before injection; b = mean injected foot thickness of control rats on a particular day (13th or 30th); x = mean injected foot thickness of treated rats before injection, and a = mean injected foot thickness of treated rats on a given day (13th or 30th). Thirty animals constituted the control group, and five rats were used for each of the test treatments.

RESULTS AND DISCUSSION

Qualitative Screening—All of the compounds tested were qualitatively similar in their gross pharmacologic effects. Dose-response patterns for decreased motor activity were seen as well as generalized central nervous system depression. The latter appeared to be nonspecific and was characterized by decreased spontaneous motor activity, ataxia, and decreased skeletal muscle tone. Analgesia was evaluated subjectively by observing an animal's response to sharp pressure applied to one of the hind paws by the operator's thumb nail. Analgesia, when noted, was accompanied by ataxia. Compound I differed from all others in that some delayed onset stimulatory activity was noted. *Para*-thymotic acid (VII), in addition to CNS depression, induced personality changes (fearful reactions) at all doses studied (56.2, 100, 177.8, 316.2 mg./Kg.). The following gross estimates of the intraperitoneal lethal dosages were made: compound VI and VII: $\leqslant 100$; compound V, VIII, IX, and X: $\leqslant 237$; compound II and IV: $\leqslant 316$; compound III: $\leqslant 562$; compound I: $\leqslant 1{,}000$ mg./Kg.

Tail Flick Studies—As shown in Table II, the rat tail flick evaluation indicated either a lack of or a very low order of analgesic activity for all compounds tested with the exception of morphine sulfate. This is consistent with the fact that thermal methods have proven generally unsatisfactory for the detection of nonnarcotic analgesics except when these drugs have been administered in very high doses.

Decreased reaction time from preinjection values for the 0.25% agar control animals is indicated by a tendency for "negative analgesia" as shown in Fig. 1.

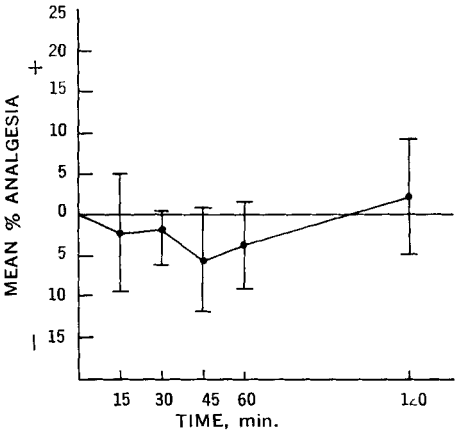

Fig. 1—Analgesic effect of 25 control rats receiving 0.15% aqueous agar solution intraperitoneally, 5 ml./Kg. I, 95% C.L.

This is indicative of a conditioning phenomenon whereby animals prematurely react to sensations of warmth rather than at the actual painful stimulus. Individual variation among control rats (as indicated by 95% C.L. in Fig. 1) was quite small and remained constant at the various times of measurement. In contrast, large variations were observed in responses after the test compounds. These large variances are consistent with similar results which have been reported in the literature (17). The very wide range of values which was seen following 10 mg./Kg. of morphine sulfate, a very potent analgesic, indicated that the extreme values encountered with the test homologs need not be considered aberrant. Of all the test compounds, only compound V may have some analgesic potential since significant analgesia was detected up through the 60-min. readings. In contrast, compound IV definitely appeared to facilitate the pain response, and compound III, likewise.

The study of O'Brien and Thoms (1) concluded that *o*- and *p*-thymotic acid possessed both analgesic and antipyretic activity. Using Davies' modification of the classical D'Amour-Smith technique (17), they reported sodium *o*-thymotate to possess 15 times the analgesic activity of sodium salicylate and

TABLE II—ANALGESIC SCREENING OF TEST COMPOUNDS AND REFERENCE ANALGESICS

Treatment	Mean % Analgesia +15	+30	+45	+60	+120 min.
Control[a]	−2	−2	−6	−4	+2
Morphine sulfate[b]	+68[c]	+86[c]	+88[c]	+80[c]	+87[c]
Sodium salicylate	+3	−4	−4	−5	−4
Sodium phenylbutazone	+1	−2	−2	−2	−1
I	−4	−7[d]	−7[d]	−5	−4
II	+1	−2	−4	−1	+2
III	+3	+11[c]	−6	−13[d]	−15[d]
IV	+8[c]	+7[c]	−15[d]	−20[d]	−25[d]
V	+21[c]	+45[c]	+36[c]	+34[c]	−5
VI	+3	−5	−1	−2	−6[d]
VII	−3	−6	−5	−4	−7[d]
VIII	−1	+5[c]	+1	−3	−2
IX	−4	−7[d]	−5	−5	−6[d]
X	−1	−9[d]	−8	−6	−6[d]

[a] The mean reaction times in seconds (observed range) for the 25 control animal are: preinjection: 4.58 (2.7–10.2); +15 min.: 4.46 (2.9–9.0); +30 min.: 4.41 (2.5–7.6); +45 min.: 3.94 (2.7–6.5); +60 min.: 4.22(2.4–5.7); and +120 min.: 3.81 (2.7–6.0). [b] The intraperitoneal dose of morphine sufate was 10 mg./Kg., while that for all of the other compounds was 100 mg./Kg. [c] Significant analgesia in that the mean percent analgesia is greater than the mean +2 standard errors for controls. [d] Significant facilitation of pain response in that the mean percent analgesia is less than the mean −2 standard errors for controls.

TABLE III—COMPARISON OF TEST TREATMENTS TO TOURNIQUET CONTROLS

Treatment	Dosage, mg./Kg.	N	Mean GSH Level, mg. %	Calcd. F	Significance Level, P
Tourniquet	—	10	145.5	—	—
No tourniquet	—	10	276.0	62.51	<0.001
Morphine sulfate	10	10	269.5	33.96	<0.001
Sodium salicylate	400	10	194.2	5.74	0.025–0.05
I	100	10	142.2	0.05	>0.50
II	100	9	168.1	2.05	0.10–0.20
III	100	10	158.8	0.61	0.25–0.50
IV	100	9	178.9	3.44	0.05–0.10
V	100	9	150.3	0.08	>0.50
VI	100	9	160.3	0.96	>0.50
VII	100	10	193.2	4.30	0.025–0.05
VIII	100	10	212.0	17.02	<0.001
IX	100	10	222.8	15.10	0.001–0.005
X	100	10	221.5	27.02	<0.001

one-tenth the activity of morphine sulfate. In the present study neither *o*- nor *p*-thymotic acid were found to possess significant analgesic activity. The discrepancy between these results and those of O'Brien and Thoms appears to lie in the manner chosen for the evaluation of the tail flick data.

Hepatic Glutathione Depletion—The effectiveness of unilateral hind leg tourniquets in inducing hepatic depletion of glutathione was clearly demonstrated. Statistical comparison of animals receiving 5 ml./Kg. of a 0.25% aqueous agar solution and no tourniquet (no pain) to animals receiving the same dose of agar and unilateral hind leg tourniquets for 4 hr. revealed a highly significant difference between the two means (Table III). In all comparisons, the variances of the control animal populations and the respective test animal populations were not significantly different at $p = 0.05$ (F test).

When the test compound means were compared to tourniquet means, morphine sulfate (10 mg./Kg.), sodium salicylate (400 mg./Kg.), and compounds VII, VIII, IX, and X (100 mg./Kg.) could be considered active ($p < 0.05$). Although tested only at one dosage level, the degree of effectiveness relative to each other can be estimated from the levels of significance presented in Table III. Morphine and compounds VIII, IX, and X all have definite protective capacity, as compared to the moderate activity of sodium salicylate and the slight but detectable effects of compound VII. Considering mg./Kg. dosage levels, compounds VIII, IX, and X are clearly more potent than sodium salicylate but less potent than morphine sulfate. The highly significant protection afforded by morphine indicated the nonspecificity of protection from hepatic GSH depletion as a biochemical metameter for nonnarcotic analgesia. Takesue and Miya (18) have reported protective activity with morphine sulfate (10 mg./Kg.) and phenobarbital (50 mg./Kg.), and concluded that protection from liver GSH depletion is not specific for the nonnarcotic analgesics. The finding that sodium salicylate was able to provide some protection from GSH depletion is significant inasmuch as this compound has been reported (18) as being capable of inducing hepatic GSH depletion (in small doses) in the absence of stress (tourniquets). This observation may be a reflection of the diurnal variation in rat sulfhydryl reported by Beck (7) in 1958.

The exact mechanisms whereby a drug may prevent stress-induced hepatic GSH depletion are at present unknown. It has been postulated that activation of the adrenal medulla under stress with resulting release of epinephrine and increased intermediary metabolism may be responsible for this phenomenon (19). This seems to be supported by Krimsky and Racker's finding that glutathione functions as a prosthetic group in glyceraldehyde-3-phosphate dehydrogenase (20), however, the mechanism (or mechanisms) by which a drug may interfere with the normal hepatic glutathione depletion induced by stress are not immediately accessible from current data and literature reports. Hepatic GSH depletion appears to be more indicative of biochemical alterations induced by stress rather than a true metameter of pain, however, the stress component in the human experience of pain cannot be neglected.

It is interesting to observe that compound V which appeared most active of the homologs in the rat tail flick evaluation (as well as equivocally active compounds III and IV) did not prevent stress-induced GHS depletion. Only compound VIII of the homologs appeared active by both techniques (equivocal: rat tail flick; highly significant: GSH depletion protection).

Acute Antiphlogistic Evaluation—Table IV shows a division of the various treatments employed in this study into subsets according to their relative activity at the administered doses. This Table indicates that while chlorpromazine, cryogenine, papain, compound VIII, and compound X were all significantly different from the controls at a dosage of 100 mg./Kg. orally ($p < 0.05$), indomethacin was clearly the most potent compound tested (5 mg./Kg.).

Chronic Anti-Inflammatory Activity—The results of a chronic anti-inflammatory screen of compounds VIII and X are presented graphically in Figs. 2 and 3. Compound IX was not tested by this procedure due to its lack of activity during the acute anti-inflammatory testing (Table IV) and general scarcity of the compound. Figure 2 indicates that compound VIII was of no benefit in reducing the inflammatory state and, in fact, appeared to aggravate the inflammatory response. Compound X (Fig. 3) did not affect the inflammatory state since the plotted test data closely parallel that of the large control population. Figure 4 depicts the anti-inflammatory response profile of acetylsalicylic acid, an agent of known effectiveness. None of the test agents significantly affected food and water consumption and rate of weight gain. The percentage inhibition of the increase in thickness of the injected foot of the animals treated with compound VIII was −10 and

TABLE IV—MULTIPLE-DRUG COMPARISONS OF ANTI-INFLAMMATORY ACTIVITY IN RATS WITH ACUTE CARRAGEENIN-INDUCED PEDAL EDEMA

Treatment	Oral Dosage, mg./Kg.	Mean Increase in Paw Volume, ml.	Significant Subsets[a]
Control	—	0.45(0.37–0.56)[b]	A
Control	—	0.44(0.39–0.54)	A
IX	100	0.46(0.38–0.64)	A
X	100	0.40(0.34–0.54)	B
VIII	100	0.36(0.31–0.40)	B
Papain	100	0.35(0.29–0.40)	B
Cryogenine[c]	100	0.27(0.19–0.36)	B
Indomethacin	5	0.22(0.18–0.31)	B
Chlorpromazine	100	0.21(0.05–0.33)	B

[a] The treatments effects represented by A and B differ significantly from each other ($p < 0.05$); however, the treatments within these subsets do not differ significantly ($p \geq 0.05$) from one another. [b] The figures within the parentheses represent the observed range of the four experimental values. [c] An alkaloid from *Heima salicifolia* Link and Otto recently shown to have anti-inflammatory potency (15).

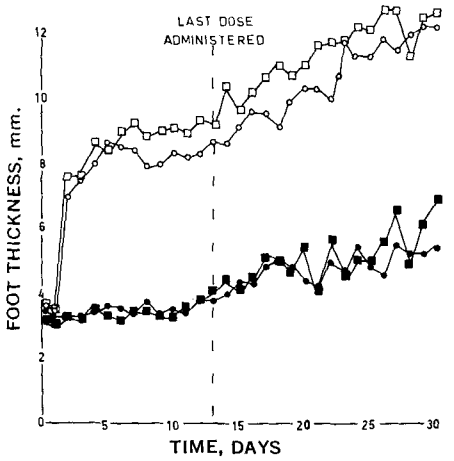

Fig. 2—Effect of 2 weeks of daily oral administration of 100 mg./Kg. of compound VIII on the development of adjuvant-induced polyarthritis. Key: ○, mean width of injected foot (controls); ●, mean width of contralateral foot (controls); □, mean width of injected foot (VIII); ■, mean width of contralateral foot (VIII).

−7% on Days 13 and 30, respectively. The calculated values for compound X were −0.3% on Day 13 and +1% on Day 30. The effectiveness of aspirin in inhibiting the inflammatory response in this study is indicated by a +33% inhibition on Day 13 and a +29% inhibition on Day 30.

SUMMARY

General hippocratic screening indicated that *o*-thymotic acid and nine homologs possessed some CNS depressant activity of a rather nonspecific nature. Rat tail flick data indicated a general lack of morphine-like analgesic activity, although 2-methyl-5-*tert*-butylsalicylic acid appeared to have some potential. Evidence obtained from hepatic glutathione depletion assays indicated that 2-hydroxy-4-isopropyl-6-methylbenzoic acid, 2-hydroxy-4-methyl-5-isopropylbenzoic acid, and 2-hydroxy-3-methyl-6-isopropylbenzoic acid may have some antistress effects being more potent than sodium salicylate and less potent than morphine sulfate in this regard. Only 2-hydroxy-4-isopropyl-6-methylbenzoic acid and 2-hydroxy-3-methyl-6-isopropylbenzoic acid were shown to have some acute anti-inflammatory activity, although their potency was clearly inferior

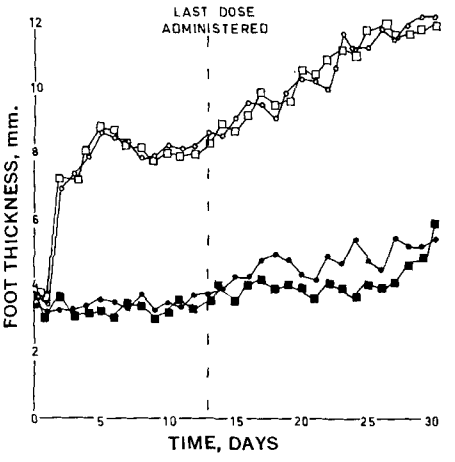

Fig. 3—Effects of 2 weeks of daily oral administration of 100 mg./Kg. of compound X on the development of adjuvant-induced polyarthritis. Key: ○, mean width of injected foot (controls); ●, mean width of contralateral foot (controls); □, mean width of injected foot (X); ■, mean width of contralateral foot (X).

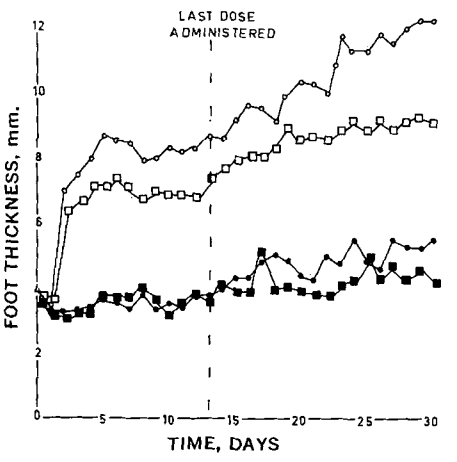

Fig. 4—Effects of 2 weeks of daily oral administration of 100 mg./Kg. of aspirin on the development of adjuvant-induced polyarthritis. Key: ○, mean width of injected foot (controls); ●, mean width of contralateral foot (controls); □, mean width of injected foot (aspirin); ■, mean width of contralateral foot (aspirin).

to that of indomethacin and they were without effect on the chronic inflammatory state induced by the mycobacterium adjuvant. No apparent structure-activity relationships could be deduced from the above studies.

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Keyphrases

Thymotic acid and homologs—activity
Analgesia rat tail flick method
Antiphlogistic activity—pedal carrageenin injection
Anti-inflammatory activity-*Mycobacterium butyricum* cell injection.
Hepatic glutathione depletion—hind leg tourniquet

Lag Time Before Essentially Constant Urinary Excretion Rate Is Attained

By JOHN G. WAGNER and JACK I. NORTHAM

If a substance is continuously infused intravenously at a constant rate, the plasma concentration will increase until it reaches an asymptotic concentration. If the urinary excretion rate of the substance is directly proportional to its plasma concentration, the urinary excretion rate will become essentially constant as the asymptotic plasma concentration is approached. Analogously, if a metabolite is formed at a constant rate, due to saturation of an enzyme system metabolizing the drug, the plasma concentration of the metabolite would be expected to approac hsome asymptotic concentration. If the urinary excretion rate of the metabolite is directly proportional to its plasma concentration, the urinary excretion rate of the metabolite would become essentially constant as the asymptotic plasma concentration is approached. Equations were derived to estimate the lag time between initiation of the maintained constant input rate to the plasma compartment and the time when, for all practical purposes, the asymptotic plasma concentration and the constant urinary excretion rate may be considered to have been reached for both the one- and two-compartment open models. The theoretical expectation is that, if the cumulative excretion curve is nearly linear, then there must be an appreciable negative intercept when the line is extrapolated back to zero time. It is theoretically impossible for a cumulative urinary excretion curve to be linear and the line extrapolate through the origin corresponding to zero excretion at zero time.

IF A SUBSTANCE is continuously infused intravenously at a constant rate the blood (serum or plasma) concentration will increase until it reaches an asymptotic value (1). However, as the curves of Rescigno and Segre (1) and Wagner and Nelson (2) illustrate, it requires an appreciable time to approach the asymptotic concentration. If the urinary excretion rate of the substance is directly proportional to its plasma concentration, then the urinary excretion rate will become essentially constant, and the cumulative urinary excretion curve will become essentially linear, as the asymptotic plasma concentration is approached.

Analogously, if a metabolite is formed at a constant rate, due to saturation of the enzyme system metabolizing the drug, the plasma concentration of the metabolite would be expected to approach some asymptotic value,

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