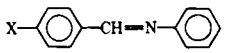
Table III—Activation Parameters for Addition of Diethyl Phosphonate to Substituted Benzylideneanilines



Substituent X	E_a, kcal.	ΔS^{303}, e.u.	ΔS^{313}, e.u.	ΔS^{323}, e.u.
NMe_2	11.29	−23.3	−22.4	−20.4
H	11.30	−18.3	−17.1	−16.1
Cl	11.61	−16.6	−15.9	−14.3

of the line obtained by plotting the pseudo-second-order rate constant *versus* diethyl phosphonate concentration.

At least four runs were made at each diethyl phosphonate concentration and three diethyl phosphonate concentrations were used, making a minimum of 12 runs for each rate constant. The third-order rate constants with the appropriate standard deviations are summarized in Table II.

Determination of Activation Parameters—The activation parameters were calculated by means of two computer programs. The slope of the Arrhenius plot was obtained by means of the normal least-squares computation, and this slope was used to calculate the heat of activation. The entropy of activation was computed by means of a second computer program using the usual equations. The results are shown in Table III.

Determination of Hammett ρ-Constants—The values of the log of the rate constants and standard Hammett σ-constants were supplied to a computer program which calculated the slopes by a normal least-squares method.

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Drug Transport VI: Functional Integrity of the Rat Everted Small Intestine with Respect to Passive Transfer

MILO GIBALDI▲ and BARBARA GRUNDHOFER

Abstract □ Mucosal-to-serosal flux of a number of solutes across the rat everted small intestine was determined as a function of time. Certain compounds including aniline, benzocaine, salicylamide, and antipyrine, which demonstrated initial clearance values of >1 ml./hr., showed little change in permeability over the entire 2-hr. period of study under the experimental conditions. On the other hand, the transfer rates of compounds which were initially cleared at substantially lower rates, such as pralidoxime, riboflavin, methyl orange, eosine blue, and bromthymol blue, increased markedly and continually during the time course of the experiment. Determination of drug transfer rates after the intestine was incubated in buffer revealed that the marked increase in the clearance of these polar compounds with time is due, in part, to a loss of functional integrity of the preparation. The permeability of the everted intestine with respect to riboflavin and methyl orange increased by a factor of about two after only 30 min. of incubation in drug-free buffer solution.

Keyphrases □ Flux, mucosal to serosal—determined for solutes as a function of time, rat everted small intestine □ Drug transfer rates—variability over time, rat everted small intestine □ Drug transport—functional integrity, rat everted small intestine, passive transfer □ Everted small intestine, rat—functional integrity with respect to passive transfer

The everted gut technique for studying intestinal absorption was first introduced in 1954 (1) and since that time has found wide application, particularly in elucidating the transport mechanisms for various nutrients and physiologic substrates (2–4). More recently, the technique has been employed to study the influence of various factors on drug absorption (4). Its use as a potential screen for the absorption characteristics of new drugs has also been considered (5). Despite the widespread and continuing interest in this isolated preparation, there is comparatively little information regarding its functional and structural integrity. Although there are few studies in the area, there is nevertheless strong evidence that the intestinal preparation is "viable" with respect to certain metabolic processes and active transport mechanisms for several hours after removal from an intact blood supply. Bramford (6) found that the rate of oxygen consumption by isolated ileal and jejunal segments of 18-day-old rats was essentially invariant over 3 hr. Similar findings were reported by Jordana and Ponz (7) using intestinal segments from adult rats. These workers also

Table I—Permeability of Rat Everted Intestine to Various Solutes as a Function of Time

	Clearance in Period I[a], ml./hr.		Clearance Ratio, Period IV/Period I		Clearance Ratio, Period IV/Period II	
	Segment 1	Segment 2	Segment 1	Segment 2	Segment 1	Segment 2
Aniline (100)[b]	4.16	3.89	0.97	1.04	1.03	0.95
Benzocaine (100)	3.12	3.39	1.00	0.94	1.14	0.92
Salicylamide (1000)	2.84	2.88	1.20	1.30	1.06	1.15
Antipyrine (500)	1.54	1.43	1.32	1.34	1.07	1.05
Salicylate (2000)	0.70	0.68	1.45	1.59	1.16	1.23
Sulfanilamide (100)	0.47	0.46	1.67	1.56	1.18	1.13
Pralidoxime (400)	0.52	0.40	2.32	2.96	1.30	1.36
Riboflavin (20)	0.33	0.26	2.60	3.22	1.30	1.48
Methyl orange (250)	0.12	0.07	8.21	12.19	1.87	2.23
Eosine blue (500)	0.04	0.04	10.09	10.15	2.57	1.92
Bromthymol blue (1000)	0.003	0.002	7.74	7.88	4.09	4.06

[a] Clearance = 2 (amount transferred in 30 min.)/mucosal concentration. Each value represents the mean of five determinations. [b] Parenthetic values denote mucosal concentrations in micrograms per milliliter.

observed substantial respiratory activity for up to 7 hr. after excision of the intestine. Robinson and Felber (8) showed that the active uptake of L-methionine and L-phenylalanine by rat intestine at 37° was maintained for 2–3 hr. after isolation.

Despite the strong case that may be made for the "viability" of the everted gut preparation, the utility of this technique in drug absorption studies (where passive mechanisms appear to predominate) probably is considerably more dependent on structural integrity than on metabolic integrity. Until recently, no systematic study or evidence of the structural integrity of the rat everted sac preparation under the conditions of its use had been reported. In 1970, Levine *et al.* (9) observed that intestinal sacs of rats are morphologically intact after eversion but progressively lose structural integrity. Changes in the epithelial tissue from animals sacrificed by decapitation were apparent 5 min. after incubation at 37° in oxygenated buffer; at 30 min., 50–75% of the normal epithelium had disappeared; at 1 hr., there was total disruption of the epithelial border. Tissue damage was slower in tissues of animals sacrificed under anesthesia. Even after 60 min. of incubation, only 10–15% destruction and disruption of the epithelial cells was noted.

In light of the rather important implications of the Levine *et al.* (9) investigation, the present study was undertaken to assess the functional integrity of the rat everted gut with respect to the presumed passive transport of various drugs and chemicals.

EXPERIMENTAL

Sprague-Dawley strain rats, weighing approximately 250 g., were fasted 20–24 hr. prior to the experiment. Water was allowed *ad*

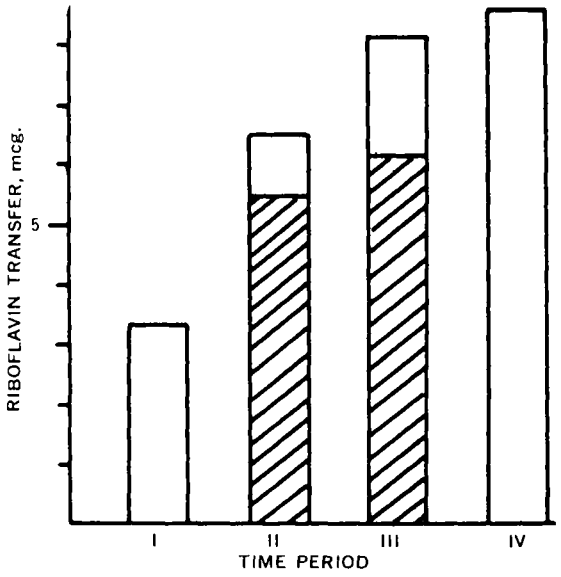

Figure 1—*Riboflavin transfer across the rat everted intestine as a function of time and buffer incubation. The open bars show the amount of riboflavin transferred in each of four consecutive 30-min. periods. The crosshatched bars in Periods II and III show the amount of riboflavin transferred in a 30-min. period after the intestine was incubated in drug-free buffer solution for 30 and 60 min., respectively.*

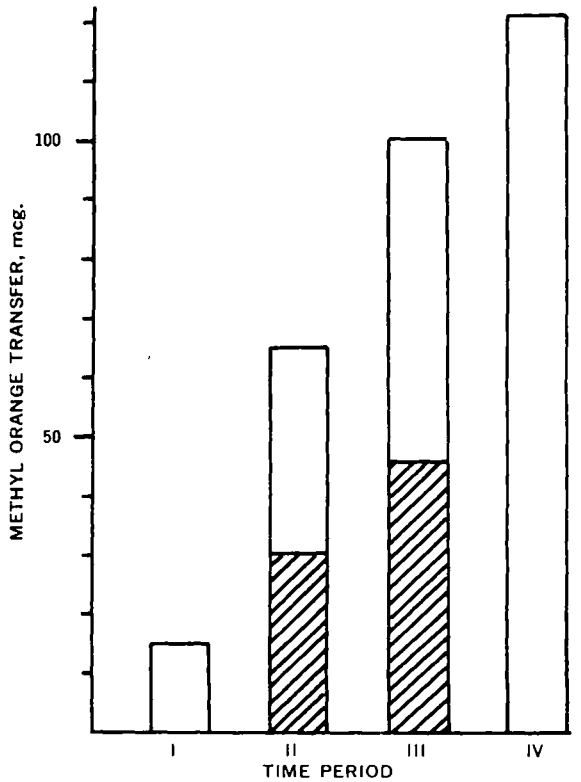

Figure 2—*Methyl orange transfer across the rat everted intestine as a function of time and buffer incubation. The open bars show the amount of methyl orange transferred in each of four consecutive 30-min. periods. The crosshatched bars in Periods II and III show the amount of methyl orange transferred in a 30-min. period after the intestine was incubated in drug-free buffer solution for 30 and 60 min., respectively.*

Table II—Effect of Buffer Incubation on Permeability of Rat Everted Intestine to Riboflavin and Methyl Orange

	Buffer	Segment	Amount Transferred in 30 min. (mcg.) ± SD[a] No Incubation	30 min. Incubation	60 min. Incubation
Riboflavin (20 mcg./ml.)	Na^+	1	3.28 ± 0.46	5.48 ± 0.39	6.13 ± 1.36
		2	2.63 ± 0.36	4.65 ± 0.50	5.47 ± 1.12
Methyl orange (250 mcg./ml.)	Na^+	1	14.8 ± 6.1	30.1 ± 9.5	45.7 ± 9.2
		2	8.7 ± 1.5	24.6 ± 9.4	38.0 ± 7.1
Methyl orange (250 mcg./ml.)	K^+	1	5.4 ± 2.5	12.4 ± 7.8	—
		2	2.7 ± 0.9	5.3 ± 3.6	—

[a] Each value represents the mean ± standard deviation of the mean of five determinations.

libitum. The animals were sacrificed under ether. The experimental method for preparing the everted intestine preparation was detailed in a previous report (10). After severing the intestine at the pyloric junction, the first 15 cm. of intestine was discarded, the gut was everted, and the proximal portion was divided into two 10-cm. segments. The most proximal segment was designated Segment 1, and the distal portion was designated Segment 2. A modified physiologic Krebs bicarbonate buffer[1], pH 7.4 upon gassing, was prepared and is referred to as the *Na^+ buffer*. A *K^+ buffer* was also prepared in which the sodium salts of the Na^+ buffer were quantitatively replaced by the respective potassium salts. Two milliliters of buffer was placed inside the sac (serosal solution); then the entire preparation was placed into approximately 100 ml. of buffer solution containing the drug (mucosal solution), which was continually gassed with O_2–CO_2 (95:5 v/v). In any given experiment, the initial mucosal and serosal solutions were identical except for the presence of drug in the mucosal solution. The serosal compartment was quantitatively sampled every 10 min. during the transfer experiment, and the sample was assayed for drug content as previously described (10, 11). The concentration of a given compound in the mucosal solution remained essentially constant throughout the experiment due to the large volume of mucosal solution.

In some experiments, the everted gut was incubated in drug-free buffer solution for up to 60 min. During incubation, sham samples of serosal solution were taken at 10-min. intervals and discarded. After incubation, the preparation was immediately transferred to the mucosal solution containing the drug, and transfer rates were determined in the usual manner.

RESULTS AND DISCUSSION

An approach to assessing the functional integrity of the everted gut preparation is to determine the constancy of transfer rate as a function of time, as suggested by Benet *et al.* (12). While this method is limited since it does not consider the possibility of drug accumulation in tissue and the finite times that may be required to attain steady-state conditions, it is nevertheless useful and informative. Comparisons of this type are presented for a number of compounds in Table I, which shows the clearance of each compound over the first 30 min. of the study as well as the ratios of the clearance in the fourth 30-min. period (Period IV) to that determined in the first and second 30-min. periods (Periods I and II, respectively).

Inspection of these data reveals two distinct temporal patterns. Certain compounds including aniline, benzocaine, salicylamide, and antipyrine, all of which demonstrated initial clearance values of >1 ml./hr. and are relatively lipid soluble[2], showed little if any change in permeability over the entire 2-hr. period of study. On the other hand, the transfer rates of compounds which were initially cleared at a substantially lower rate, such as pralidoxime, riboflavin, methyl orange, eosine blue, and bromthymol blue, increased markedly and continually during the experiment. The increase in clearance of these compounds in Period IV was 100–900% relative to Period I and 30–300% relative to Period II. The transfer rate–time patterns of two additional compounds, salicylate and sulfanilamide, appeared to be intermediate.

The temporal pattern exhibited by those compounds which were rapidly cleared was previously observed by Benet *et al.* (12). These investigators, using an everted intestine preparation similar to that used in the present study, found no significant change in the transfer rates of salicylate and acetanilide in Krebs bicarbonate buffer over 2 hr. Based on these findings, the authors suggested that the epithelial border may not be the rate-limiting barrier to transport across the *in vitro* rat intestinal segment. The present results with aniline, benzocaine, salicylamide, and antipyrine support this conclusion. However, the transfer rate of a number of other compounds was found to increase with time, and it was necessary to determine whether these changes were due to a time lag in attainment of steady state or loss of integrity before any general conclusions regarding the rate-limiting barrier could be made.

The marked increase in the clearance of pralidoxime, riboflavin, methyl orange, eosine blue, and bromthymol blue as a function of time can be explained by considering either: (*a*) accumulation of drug in the tissue requiring finite times before the attainment of steady state, or (*b*) a loss of functional integrity of the preparation with respect to transport of these compounds, or (*c*) a combination of these mechanisms. Hence, two of the compounds—*viz.*, riboflavin and methyl orange, were selected and buffer incubation studies were conducted to evaluate the mechanism. It was reasoned that if drug accumulation in the tissue was solely responsible for the change in clearance as a function of time, then the initial clearance of a compound should be the same whether the intestine is used immediately upon removal from the blood supply or preincubated in buffer for a given period.

The results of these studies are presented in Figs. 1 and 2 and in Table II. The data clearly show that the permeability of the everted intestine with respect to riboflavin and methyl orange increased by a factor of about two after only 30 min. of incubation in drug-free buffer solution. Further increases in permeability were apparent after a 60-min. incubation. Figure 1 shows that much of the change in transfer rates of riboflavin in Periods II and III compared to that observed in Period I was due to changes in the permeability of the tissue. About 70% of the increase in transfer rate of riboflavin in Period II relative to Period I was directly attributable to the loss in functional integrity over this time period. Although the increase in transfer rate of methyl orange with time was largely due to accumulation in the tissue, loss of functional integrity did contribute significantly to these changes (Fig. 1). Table II also shows that the permeability changes due to incubation were not limited to the Na^+ buffer. Incubation in the K^+ buffer increased the permeability of the tissue to methyl orange to about the same extent as incubation in the Na^+ buffer. The substantial inhibitory effect of K^+ on methyl orange transfer across the everted intestine (11) was evident in both incubated and nonincubated tissue.

In conclusion, the changes in functional integrity of the everted intestine with respect to the mucosal-to-serosal transfer of riboflavin and methyl orange appear to parallel the changes in structural integrity of the mucosal epithelium of isolated intestine as reported by Levine *et al.* (9). The apparent lack of effect of loss of structural integrity on the everted intestine transfer rate of nonpolar, lipid-soluble compounds suggests, in accord with Benet *et al.* (12), that the epithelial border is not the rate-limiting barrier to transfer of these compounds across the rat everted intestine.

[1] KCl, 5 m*M*; KH_2PO_4, 1 m*M*; $NaHCO_3$, 26 m*M*; and NaCl, 122 m*M*.

[2] At the concentrations noted in Table I, these compounds have butanol–water partition coefficients (PC) of >4. All other compounds studied have partition coefficients of <1 except for sulfanilamide (PC = 1.6) and bromthymol blue (PC = 9).

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Distribution and Excretion of ^{3}H-Venoms of *Crotalus adamanteus, Crotalus atrox,* and *Agkistrodon piscivorus* and ^{3}H-Serum of *Lampropeltis getulus* in Rats

C. L. HUANG*▲, G. N. MIR, S. J. LIU, K. L. HEMNANI, and E. T. YAU

Abstract □ ^{3}H-Labeled king snake serum and three pit viper venoms were administered intraperitoneally to rats to study the tissue distribution and rate of excretion. The peak blood level of king snake serum corresponded to that of the three venoms. The liver played a major role in metabolizing the venoms and king snake serum. Fecal excretion was the major route of elimination of these substances.

Keyphrases □ Snake serum and venoms (king and pit viper), radiolabeled - tissue distribution, urinary and fecal excretion, rats □ *Crotalus adamanteus*, radiolabeled—distribution and excretion, rats □ *Crotalus atrox*, radiolabeled—distribution and excretion, rats □ *Agkistrodon piscivorus*, radiolabeled—distribution and excretion, rats □ *Lampropeltis getulus*, radiolabeled—distribution and excretion, rats

Many snake venoms of the pit viper family manifest proteolytic activities (1–3). The most characteristic pathological changes associated with proteolysis is hemorrhage (4, 5). The prominent findings on the activity of the pit viper venoms are the hydrolyses of casein, gelatin, denatured hemoglobin, and hemin. King snake serum has been demonstrated to counteract the proteolytic activity and lethal effect of pit viper venoms (6); however, the mechanism of action is not known. It is of interest to explore the *in vivo* behavior of the king snake serum and the snake venoms that might yield information as to the mechanism of interaction of these counteracting substances. The purpose of this paper is to describe the *in vivo* behavior of the venoms of pit viper, *Crotalus adamanteus*, *Crotalus atrox*, and *Agkistrodon piscivorus*, and king snake serum in rats.

MATERIALS AND METHODS[1]

Preparation of Tritiated Snake Venoms—Tritium labeling of the snake venoms was performed according to Wilzbach's method (7). One gram each of the lyophilized venom of *C. adamanteus*, *C. atrox*, and *A. piscivorus* was dissolved in a small amount of water and thinly spread and dried in a round-bottom flask by using a vacuum evaporator. Tritium gas (3 c.) was introduced into the flask and the container was sealed. The reaction was allowed to proceed at 4° for 2 weeks. After the seal was opened and excess tritium gas was removed, a small amount of water was added to the flask to dissolve the venom and lyophilized to remove labile tritium. The LD_{50} of the ^{3}H-venoms on albino mice was found to be approximately the same as that of the original venom before tritiation (*C. atrox*, 6 mg./kg. i.p.; *C. adamanteus*, 25 mg./kg.i.p.; and *A. piscivorus*, 10 mg./kg. i.p.). The specific activity of each tritiated venom was as follows: *C. adamanteus*, 37 μc./mg.; *A. piscivorus*, 40 μc./mg.; and *C. atrox*, 27 μc./mg. Similarly, 1 g. of king snake serum was tritiated and purified according to the same procedure. The specific activity was 45 μc./mg.

Urinary and Fecal Excretion—A dose equivalent to LD_{25} of each ^{3}H-venom was dissolved in normal saline solution and injected intraperitoneally to three albino rats weighing 200–240 g. The animals were housed in individual metabolic cages and fed with food and water *ad libitum*. Urine and feces specimens were collected every 8 hr. Urine specimens were evaporated to dryness in a vacuum evaporator. Then an aliquot (0.1 g.) was placed in a liquid scintillation bottle containing scintillator fluid[2], and the activity was recorded in a liquid scintillation counter[3]. No hyamine hydroxide was used for the urine specimens. Feces specimens were

[1] Snake venoms were obtained from Miami Serpentarium Laboratories, Miami, Fla. Albino rats were purchased from Southern Animal Farms, Prattville, Ala. King snakes (*Lampropeltis getulus*) were obtained from Charles Chase Co., Fla., and Miami Serpentarium Laboratories.

[2] 10 g. 2,5-diphenyloxazole, 0.25 g. 1,4-bis[2-(5-phenyloxazolyl)]-benzene, 200 g. naphthalene, and dioxane *q.s.* to 1 l.

[3] Tracerlab, Corumatic 100 a.