Use of Cannulated Everted Intestinal Sac for Serial Sampling as a Drug Absorbability (Permeability) Screen

STANLEY A. KAPLAN[▲] and STANLEY COTLER

Abstract ☐ The *in vitro* everted sac technique was evaluated for screening the permeability parameter of drug substances associated with their *in vivo* absorbability. The time of onset of permeability across the sac, lag time in minutes, and the cumulative amount of drug transferred per unit concentration of drug in mucosal solution, clearance in milliliters per minute, were shown to provide an indication of the permeability of a drug substance. The everted sac permeability screen, when coupled with a dissolution screen, allows for the systematic evaluation of the potential absorbability of a drug in its initial stages of development. This overall program indicates the nature of an absorbability problem, either dissolution or permeability, and therefore is a guide to overcoming such problems.

Keyphrases ☐ Cannulated everted intestinal sac—absorbability screening, drugs in solution ☐ Bioavailability—*in vitro* transfer across everted intestinal sac correlated with *in vivo* absorbability ☐ Permeability parameters across everted intestinal sac—correlated with *in vivo* absorbability ☐ Absorbability, drugs—correlated with *in vitro* transfer across everted intestinal sac

The oral administration of a drug is the route of choice in most cases. With relatively insoluble drugs, the rate of solution is usually the rate-determining step in the overall absorption process following oral administration. When considering relatively soluble drugs, permeability may become the rate-determining step.

The extent and rate of absorption depend upon a number of physicochemical factors such as solubility, dissolution rate, particle size, stability in aqueous media, pKa, partition coefficient, and permeability of the drug. Evaluation of these parameters can provide information vital to the ultimate development of the compound for clinical application.

Emphasis has been placed on screening for dissolution properties, but little work has been done in screening for permeability characteristics. Obviously this type of procedure should be available in the initial stages of drug development and used in conjunction with a dissolution test to choose the compound form that shows the greatest potential for absorption. Therefore, one would be able to determine which parameter associated with the absorption process is the rate-limiting step, as well as be provided with an indication of how the absorbability problem might be overcome. An absorption or permeability screen can reduce the time and cost of developing a new drug if performed prior to the formulation and clinical stages of development.

The present study concerns the utility of the everted intestinal sac technique (1, 2) as a screening procedure for the intrinsic absorbability (permeability) of new drug substances. Since the drug under study is in solution, the
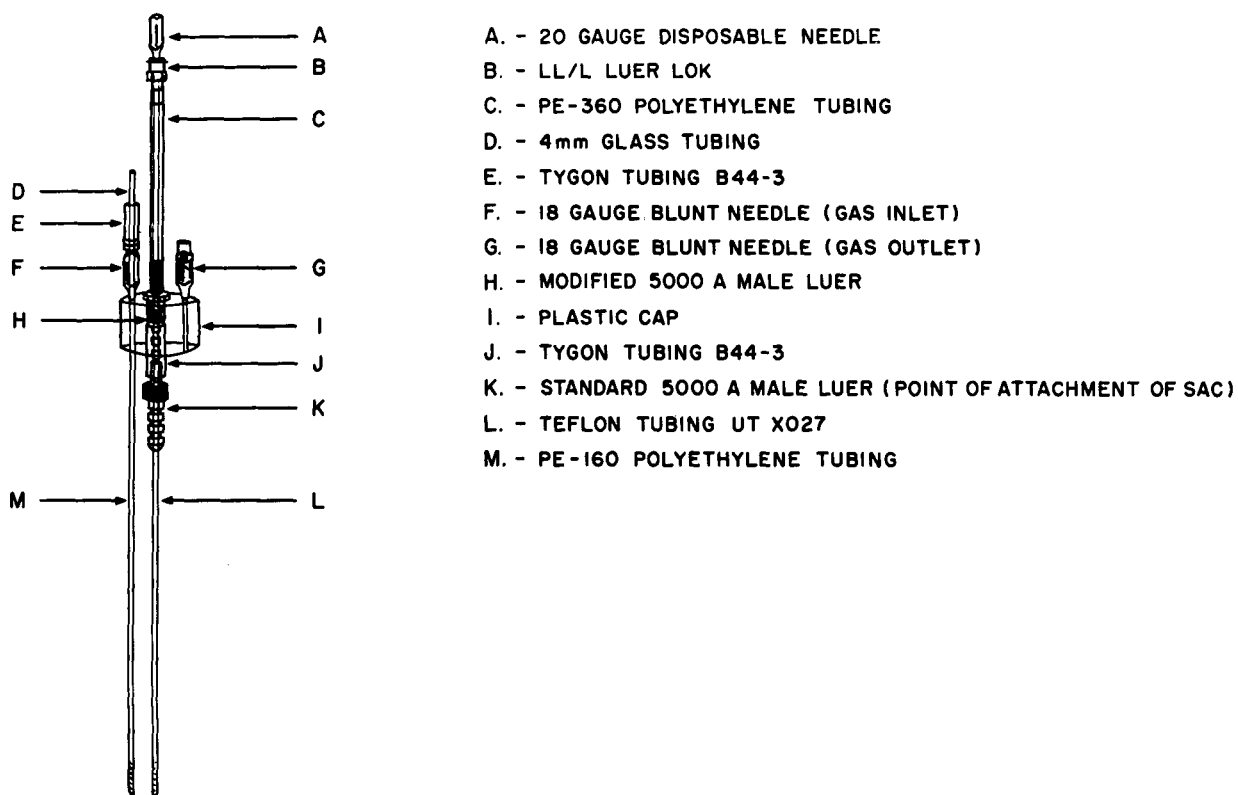

A. - 20 GAUGE DISPOSABLE NEEDLE
B. - LL/L LUER LOK
C. - PE-360 POLYETHYLENE TUBING
D. - 4mm GLASS TUBING
E. - TYGON TUBING B44-3
F. - 18 GAUGE BLUNT NEEDLE (GAS INLET)
G. - 18 GAUGE BLUNT NEEDLE (GAS OUTLET)
H. - MODIFIED 5000 A MALE LUER
I. - PLASTIC CAP
J. - TYGON TUBING B44-3
K. - STANDARD 5000 A MALE LUER (POINT OF ATTACHMENT OF SAC)
L. - TEFLON TUBING UT X027
M. - PE-160 POLYETHYLENE TUBING

Figure 1—*Diagram of incubating apparatus.*

Table I—Compounds Used in the Everted Intestinal Sac Study

Compound Number	pKa	Chemical or Generic Name
1	Neutral	N'-(6-Methoxy-4-pyrimidinyl)-N'-methyl-sulfanilamide
2	3.4	Diazepam
3	9.5	5-(3-Indolyl)-2,3-dihydro-1H-1,4-benzodi-azepine
4	4.8	Chlordiazepoxide hydrochloride
5	10.7	7-Chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one 4-oxide
6	4.9,5.8	Investigational compound
7	8.7	8-Chloro-1-(3-dimethylaminopropyl)-3,5-dihydro-4,1-benzodiazepin-2(1H)-one hydrochloride
8	7.2	Trimethoprim
9	1.5,10.5	5-(2-Chlorophenyl)-7-nitro-3H-1,4-benzo-diazepin-2(1H)-one
10	8.6	N,N'-(Iminoditrimethylene-di-p-toluene-sulfonamide) hydrochloride
11	6.2	Sulfadimethoxine
12	4.9	N⁴-Ethoxyacetyl-N'-(5-methyl-3-isoxa-zolyl)sulfanilamide
13	4.9	Sulfisoxazole
14	5.6	Sulfamethoxazole
15	6.1	Sulphormethoxine
16	5.7	N'-(4,5-Dimethyl-3-isoxazolyl)sulfanila-mide

Table II—Cumulative Micrograms of Compound 13 Transferred per Unit Concentration of Drug as a Function of Increasing Mucosal Concentration of Compound 13

Mucosal Concentration, mcg./ml.	Number of Runs	20	30	40	60	90
				Minutes		
10	3	0.13	0.23	0.32	0.54	0.91
25	4	0.10	0.18	0.29	0.50	0.88
50	4	0.13	0.21	0.29	0.45	0.70
100	12	0.18	0.27	0.38	0.61	0.88
500	10	0.16	0.28	0.41	0.70	1.23
1000	8	0.18	0.32	0.46	0.74	1.13
Mean ± SE		0.15 ± 0.02	0.25 ± 0.03	0.36 ± 0.04	0.59 ± 0.06	0.94 ± 0.08

permeability of the drug across the intestinal mucosa is the absorbability parameter being screened. A method for the use of everted isolated intestinal segments for *in vitro* absorption studies was described by Wilson and Wiseman (1). In their method the serosal fluid could be sampled only at the conclusion of the experimental procedure. Crane and Wilson (2) later devised an everted sac system which permitted serial sampling of the fluid on the serosal side of the intestine. The system described herein follows the Crane and Wilson design with some modifications.

EXPERIMENTAL

Design of Incubating Apparatus—The incubating apparatus (Fig. 1) requires the following materials: Kimax screwtop test tube, size G (25 × 150 mm.), with plastic cap; model LL/L female luer lok with male luer[1]; model 5000 A male luer to hose[1]; hypodermic needle, 20 gauge × 3.81 cm. (1.5 in.); hypodermic needle, 18 gauge × 3.81 cm. (1.5 in.); polyethylene 360 and polyethylene 160 tubing[2]; Teflon medical grade tubing, UTX027[1]; and Tygon tubing B44-3. It was assembled as follows.

A modified 5000 A male luer was passed through the center of a plastic test tube cap, and 55 mm. of polyethylene 360 tubing was attached to the upper end of the luer. A small piece of Tygon tubing was attached to the lower end of the 5000 A luer to which a standard 5000 A male luer was securely attached.

The everted sac was attached to the lower end of the hose. Two 18-gauge × 3.81-cm. (1.5-in.) hypodermic needles were passed through the plastic cap, one on either side of the polyethylene tubing, and sufficient polyethylene 160 tubing was attached to one needle so that it reached the bottom of the incubating flask (gas inlet). The other needle acts as a gas outlet. A Teflon catheter UTX027 was then attached to a 20-gauge needle and passed through the interior of the polyethylene 360 tubing to permit the serial sampling of the serosal fluid.

Preparation of Everted Sac—Charles River male rats, weighing 175–250 g., were fasted for 24 hr. with access to drinking water. The rats were sacrificed and the small intestine was exposed *via* a

midline incision of the abdomen, removed, and cut at the pyloric sphincter and ileocecal junction. The entire small intestine was rinsed with normal saline solution (20–30 ml.) and then sleeved onto a stainless steel rod and everted.

Two 10-cm. lengths of intestine from the upper ileum were cut, ligated at the distal end, and attached at the proximal end of the hose adapter so that the sac was 8 cm. from the tip of the hose to the
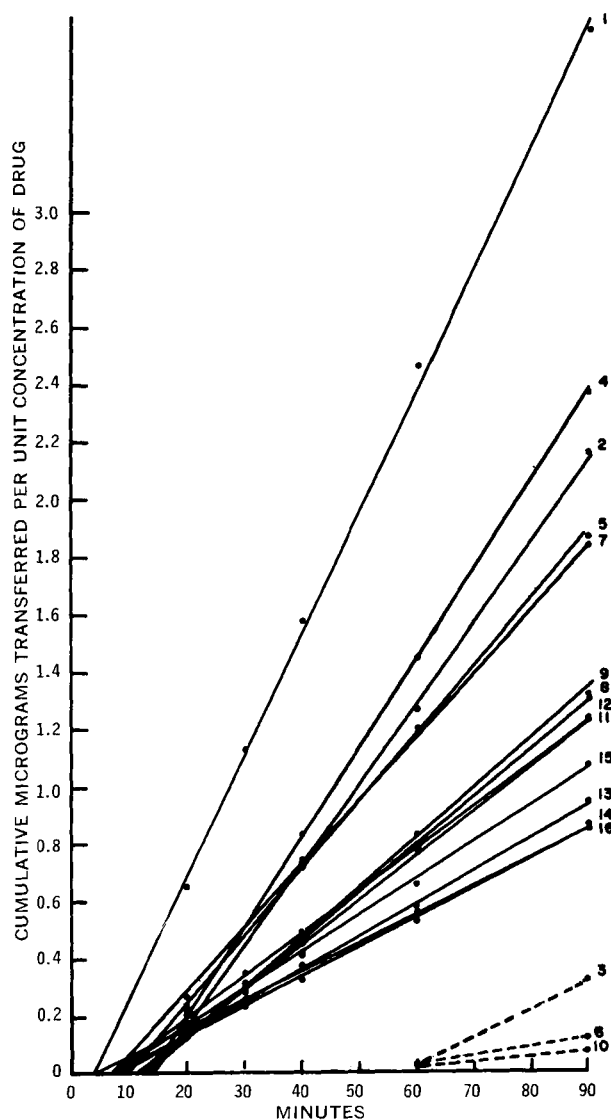
Figure 2—*Cumulative amount of drug transferred per unit concentration of drug in the mucosal solution as a function of time. Compounds 1–16 are identified in Table I.*

Table III—Cumulative Micrograms of Drug Transferred per Unit Concentration of Drug in Mucosal Solution

Compound Number	Number of Runs	Minutes					Lag Time ± SE, min.	Clearance[a] ± SE, ml./min.
		20	30	40	60	90		
1	6	0.66	1.14	1.58	2.48	3.66	3 ± 1	0.041 ± 0.002
2	8	0.21	0.45	0.72	1.28	2.18	18 ± 1	0.021 ± 0.002
3	4	N.M.[b]	N.M.	N.M.	0.04	0.33	>60	N.M.
4	11	0.23	0.50	0.84	1.46	2.39	13 ± 2	0.025 ± 0.001
5	8	0.24	0.47	0.74	1.28	1.88	12 ± 1	0.021 ± 0.002
6	7	N.M.	0.02	0.04	0.08	0.13	49 ± 2	0.001 ± 0.008
7	8	0.28	0.56	0.75	1.20	—	8 ± 1	0.023 ± 0.002
8	5	0.16	0.30	0.47	0.78	1.33	18 ± 1	0.013 ± 0.0007
9	3	0.14	0.29	0.48	0.84	—	13 ± 1	0.014 ± 0.0008
10	7	N.M.	N.M.	N.M.	0.03[c]	0.09	>60	N.M.
11	4	0.21	0.36	0.50	0.81	1.23	6 ± 2	0.014 ± 0.001
12	3	0.17	0.29	0.43	0.79	—	12 ± 3	0.013 ± 0.001
13	41	0.15	0.25	0.36	0.59	0.94	11 ± 1	0.010 ± 0.0004
14	4	0.14	0.24	0.33	0.54	0.88	7 ± 2	0.009 ± 0.002
15	4	0.18	0.29	0.42	0.67	1.08	6 ± 2	0.011 ± 0.001
16	3	0.15	0.26	0.37	0.57	0.86	3 ± 1	0.009 ± 0.0001

[a] Based on the 60-min. data point. [b] Nonmeasurable. [c] Measurable in two out of seven runs.

ligature. A 10-g. weight was attached to the ligated end of the sac to maintain it in a vertical position during the experiment.

The entire apparatus was placed in the incubating flask containing the drug dissolved in 35 ml. of Krebs–Ringer bicarbonate buffer (3) (defined as mucosal fluid) and incubated at 37° in a Dubnoff metabolic shaker (76 excursions/min.) under an atmosphere of carbon dioxide–oxygen (5:95) saturated with water vapor.

Two milliliters of Krebs–Ringer bicarbonate buffer (defined as serosal fluid) was introduced into the sac via the cannula, with the fluid level always visible in the polyethylene 360 tubing above the plastic cap. At 10-min. intervals, the serosal solution was withdrawn by means of a hypodermic syringe attached to the Teflon catheter and the sac was rinsed with 1 ml. of fresh buffer. Two milliliters of buffer was again introduced into the sac for the next 10-min. incubation and the process was repeated. The serosal solution and rinse

were combined and filtered through a "type HA" Millipore filter. Aliquots were then analyzed for drug content.

Preparation of Drug Solutions—The 16 compounds evaluated in the study are listed in Table I. All drug solutions were prepared in pH 7.4 Krebs–Ringer bicarbonate buffer. Solutions of Compound 13 were prepared to contain 10, 25, 50, 100, 500, and 1000 mcg./ml. All other solutions were prepared to contain 100 mcg./ml., except
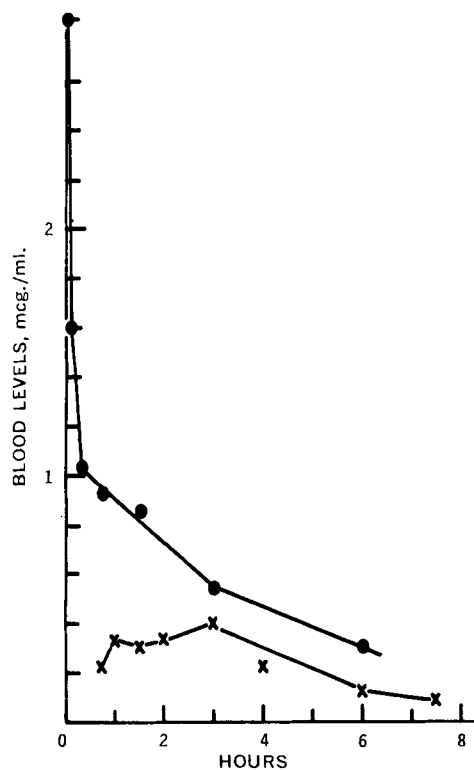

Figure 3—*Blood level curves in a dog following the intravenous (●) and oral capsule (×) administrations of a 5-mg./kg. dose of Compound 3.*
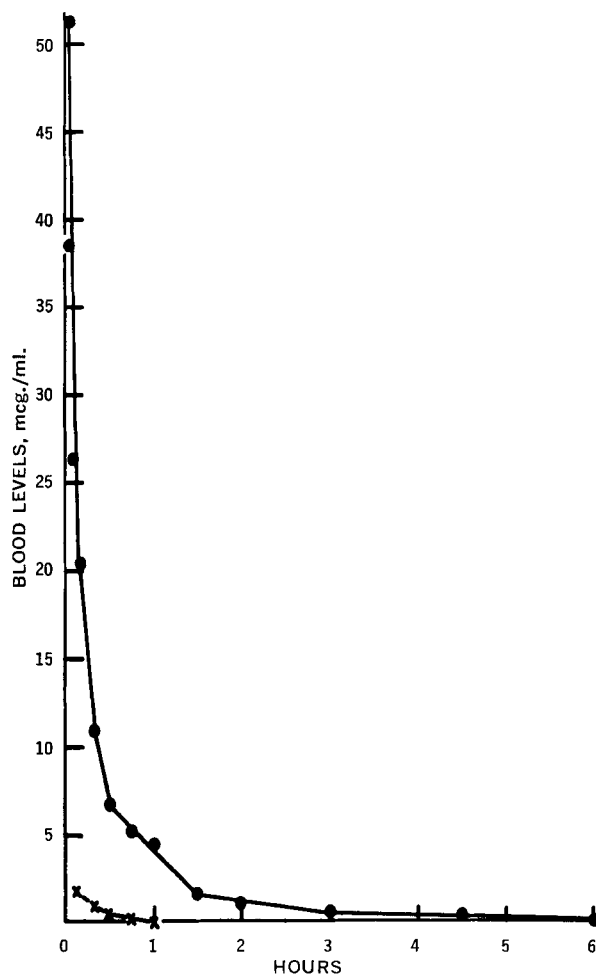

Figure 4—*Blood level curves in a dog following the intravenous (●) and oral solution (×) administrations of a 25-mg./kg. dose of Compound 6.*
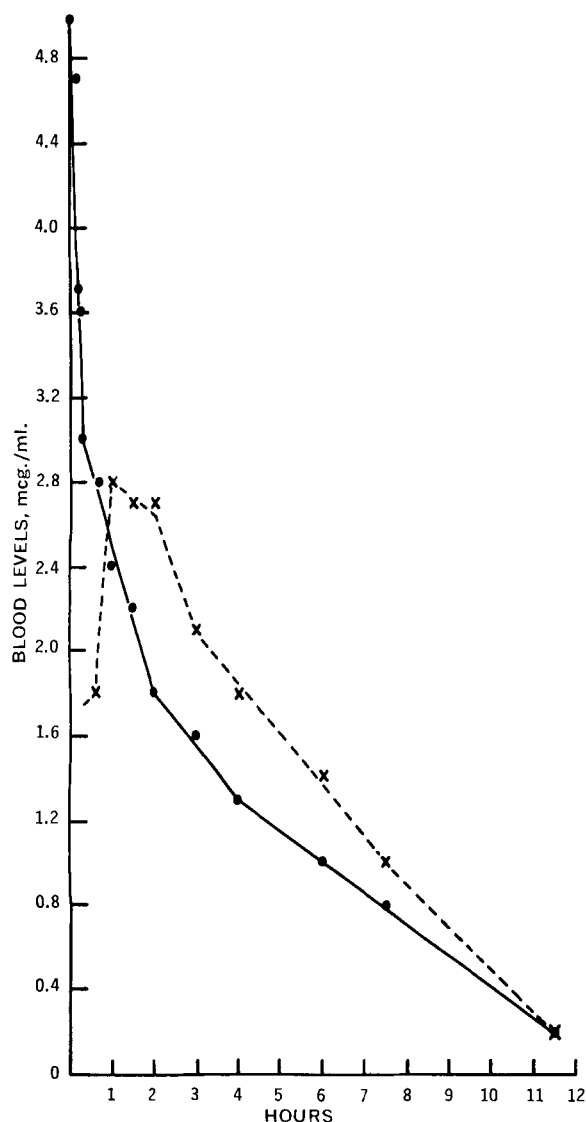
Figure 5—*Blood level curves in a dog following the intravenous (●) and oral (×) administrations of a 5.7-mg./kg. dose of Compound 8.*

for those containing Compound 3 (40 mcg./ml.) and Compound 1 (21 mcg./ml.).

Analytical Methods—The sulfonamides, Compounds 1 and 11–16, were assayed by the standard Bratton–Marshall procedure (4).

Compounds 4 and 9 were hydrolyzed with hydrochloric acid and then assayed by the standard Bratton–Marshall procedure (5). Compound 2 was extracted at pH 9, back-extracted into 2 N HCl, and measured spectrophotometrically at 240 nm. (6). Compound 5 was made alkaline and exposed to UV light for 1 hr. to form a fluorophore, whose fluorescence was measured with activation at 380 nm. and emission at 480 nm. (7). Compound 3 was measured spectrophotometrically at 371 nm. (8). Compound 10 was extracted at pH 9 and reacted with dinitrofluorobenzene. The derivative was then isolated by TLC and determined spectrophotometrically at 380 nm. (8). Compounds 7 and 8 were analyzed by formation of a bromcresol complex and determined spectrophotometrically (8). Compound 6 was made alkaline and exposed to light in the presence of methylene blue to form a fluorophore, whose fluorescence was measured with activation at 335 nm. and emission at 375 nm. (8).

RESULTS AND DISCUSSION

The cumulative amount of Compound 13 transferred per unit concentration of drug at various times as a function of increasing mucosal concentrations of Compound 13 is presented in Table II.

The values presented at each time are independent of the mucosal concentration of drug, suggesting a passive transfer process. The constant transfer observed was due to an essentially constant concentration gradient across the intestinal barrier, which was maintained in the *in vitro* system by replacing the serosal fluid at 10-min. intervals and by using a large volume of mucosal solution.

The permeability characteristics of each drug across the everted sac preparations were determined by tabulating the cumulative amounts of drug transferred with time per unit concentration of drug in the mucosal solution (Table III) and plotting *versus* time on rectilinear paper (Fig. 2).

The *in vitro* permeability parameter was determined for each drug in terms of: (*a*) its lag time, the extrapolation of the linear segment to the abscissa in minutes, and (*b*) its clearance, the cumulative amount of drug transferred in 60 min./unit concentration of drug in mucosal solution reduced to units of milliliters per minute.

The transfer data in Table III for the 16 compounds studied indicate that two of the compounds, 3 and 10, exhibited a lag time greater than 60 min. and a nonmeasurable clearance. Compound 6 exhibited a lag time of 49 min. and a clearance of 0.001 ml./min. The other compounds exhibited a lag time ranging from 3 to 18 min. and a clearance ranging from 0.009 to 0.041 ml./min. The magnitude of the clearance or *in vitro* rate of transfer was found to relate directly to the degree of undissociation of the compound in the pH 7.4 *in vitro* system. *In vivo* intravenous and oral studies were run in a minimum of two or three fasted unanesthetized dogs with each compound. The results presented for single dogs are illustrative of all the animals studied. The *in vivo* data indicate that Compounds 3, 6, and 10, which exhibited permeability characteristics that were not consistent with good absorbability (late lag time and nonmeasurable or very low clearance), were in fact poorly absorbed.

Compound 3 exhibited solubility characteristics consistent with good absorption. Following intravenous and oral administration of
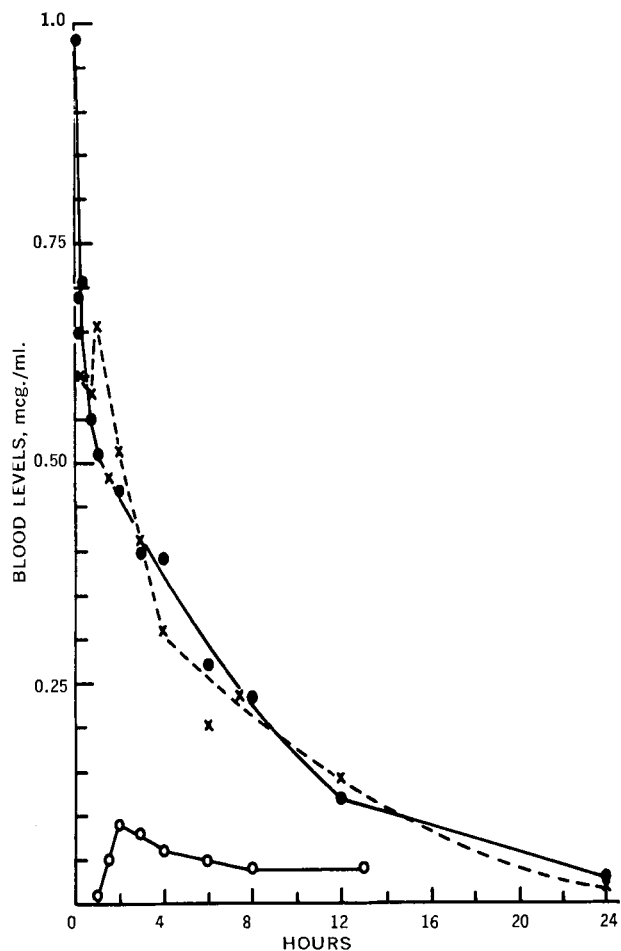

Figure 6—*Blood level curves in a dog following the intravenous (●) and oral (O, capsule; ×, solution) administrations of a 2.3-mg./kg. dose of Compound 9.*

a 5-mg./kg. dose to a dog, the blood level data (Fig. 3) indicate that the orally administered drug was absorbed in an erratic, slow, and incomplete fashion. Compound 6 exhibited both dissolution and permeability characteristics consistent with poor absorption. Following intravenous and oral solution administration of a 25-mg./kg. dose to a dog, the blood level data (Fig. 4) indicate negligible oral absorption. Compound 10 exhibited solubility characteristics capable of providing the drug rapidly for absorption. A 20-mg./kg. dose was administered intravenously and orally to a dog. Following the oral dose, the drug could be measured only at one point, 4 hr. post-administration. The poor absorption of Compound 10 was further reflected in the intraperitoneal/oral LD_{50} ratio of 27/1 in the mouse (8).

In contrast with those compounds where absorption was hindered due to poor permeability, Compound 8 exhibited solubility and permeability characteristics consistent with good absorbability and resulted in the blood level curves presented in Fig. 5 following intravenous and oral administration of a 5.7-mg./kg. dose to a dog (9). Compound 9, however, exhibited poor dissolution characteristics but excellent permeability characteristics. Following intravenous, oral solid, and oral solution administration of a 2.3-mg./kg. dose to a dog, the blood levels (Fig. 6) indicated complete absorption when the drug was administered orally in solution.

The results indicate that the everted intestinal sac technique can provide useful information concerning the permeability characteristics of a drug. Based on the *in vitro* and *in vivo* data obtained with compounds exhibiting either poor or good permeability across the everted sac, the evidence indicates that poor *in vitro* permeability is associated with poor absorbability of the compound. The findings relative to the three compounds, 3, 6, and 10, which were poorly permeable and poorly absorbed, suggest a permeability limitation in their absorption. However, the *in vivo* data *per se* do not allow one to pinpoint the precise nature of the limited absorption of the drug. For example, GI and/or first-pass metabolism in the liver might contribute to the limited availability of these compounds. Although some questions remain relative to the role of permeability in the limited absorption of these compounds, experience to date with the everted sac is sufficiently encouraging to warrant its continued use in the absorbability screening of new compounds.

Aside from its utility as an absorption screen, the everted sac technique may prove of value in studying the rate of disappearance of a drug from the intestinal lumen, the accumulation of a drug in the intestinal wall, absorbability of a drug from different regions of the intestinal tract, effects on transfer of other added substances, and possible biotransformation of the drug by the intestine. These studies should provide future avenues of exploration in obtaining a better understanding of the absorption process.

SUMMARY

1. The cannulated everted intestinal sac adapted for serial sampling was studied as a screening technique to determine the absorbability (permeability) of new drug substances.

2. The experimental conditions were described. In this technique, a 10-cm. segment of rat ileum is everted, cannulated, and suspended in a solution of drug in pH 7.4 Krebs–Ringer bicarbonate buffer. The amount of drug passing across the intestinal mucosa is then determined as a function of time.

3. Graphs of cumulative amount transferred with time were evaluated in terms of its lag time (extrapolation of linear segment to the abscissa) and clearance, the cumulative amount transferred in 60 min./unit concentration of drug. A late lag time and a nonmeasurable or low clearance determined *in vitro* were found to be associated with permeability-limited absorption problems *in vivo*.

4. The data indicate that the cannulated everted intestinal sac is a useful tool in screening the absorbability of new drug substances in solution.

REFERENCES

(1) T. H. Wilson and G. Wiseman, *J. Physiol.*, **123**, 116(1954).

(2) R. K. Crane and T. H. Wilson, *J. Appl. Physiol.*, **12**, 145 (1958).

(3) W. W. Umbreit, R. H. Burris, and J. F. Stauffer, "Manometric Techniques," 3rd ed., Burgess, Minneapolis, Minn., 1957, p. 149.

(4) A. C. Bratton and E. K. Marshall, *J. Biol. Chem.*, **128**, 537 (1939).

(5) L. O. Randall, *Dis. Nerv. Syst.*, *Suppl.*, **22**, 1(1961).

(6) J. A. F. de Silva, B. A. Koechlin, and G. Bader, *J. Pharm. Sci.*, **55**, 692(1966).

(7) B. A. Koechlin and L. D'Arconte, *Anal. Biochem.*, **5**, 195 (1963).

(8) Unpublished data on file, Hoffmann-La Roche Inc., Nutley, NJ 07110

(9) S. A. Kaplan, R. E. Weinfeld, S. Cotler, C. W. Abruzzo, and K. Alexander, *J. Pharm. Sci.*, **59**, 358(1970).