(24) I. Lalezari, P. Nasseri, and R. Asgharian, *J. Pharm. Sci.*, **63**, 1331 (1974).
(25) H. M. N. H. Irving and J. J. Markham, *Anal. Chim. Acta*, **39**, 7 (1967).
(26) P. Job, *Ann. Chim.*, **9**, 113 (1928).
(27) *Ibid.*, **16**, 97 (1936).
(28) J. H. Yoe and A. L. Jones, *Ind. Eng. Chem., Anal. Ed.*, **16**, 111 (1944).
(29) M. E. Auerbach, *ibid.*, **15**, 492 (1943).
(30) P. B. Marshall and E. W. Rogers, *Biochem. J.*, **39**, 258 (1945).

ACKNOWLEDGMENTS

The authors are grateful to Ali Shafiee for providing research facilities.

Inhibition by Paromomycin of R-Factor Transfer of Tetracycline Resistance between *Escherichia coli* and *Salmonella pullorum*

ALDO BUOGO ˣ and PIERANGELO CATTANEO

Received October 12, 1976, from the *Farmitalia Research Laboratories, 20014 Nerviano, Milan, Italy.* Accepted for publication February 2, 1977.

Abstract □ *In vitro* assays showed that low concentrations (5–10 and 20 μg/ml) of the antimicrobial paromomycin sulfate are able to block or diminish significantly the transfer of the tetracycline resistance R-factor between *Escherichia coli* and *Salmonella pullorum*. This observation is important because it offers the possibility of preventing the formation of tetracycline-resistant pathogens, a limiting factor of tetracycline use in both human and veterinary medicine.

Keyphrases □ Paromomycin—effect on R-factor transfer of tetracycline resistance from *Escherichia coli* to *Salmonella pullorum* □ Tetracycline resistance—R-factor transfer from *Escherichia coli* to *Salmonella pullorum*, effect of paromomycin □ Bacterial resistance—to tetracycline, R-factor transfer from *Escherichia coli* to *Salmonella pullorum*, effect of paromomycin □ Antibacterials—effect of paromomycin on R-factor transfer of tetracycline resistance from *Escherichia coli* to *Salmonella pullorum* □ Antimicrobials—effect of paromomycin on R-factor transfer of tetracycline resistance from *Escherichia coli* to *Salmonella pullorum*

Previously (1), it was demonstrated that *in vitro* pretreatment of *Escherichia coli* with low doses of tetracycline inhibits the production of mutants resistant to paromomycin sulfate (I), an oligosaccharide antimicrobial with a broad antibacterial spectrum including Gram-positive, Gram-negative, and acid-fast bacteria (2–4). Compound I also inhibits some protozoa (5, 6).

The effect of I on the development of tetracycline-resistant strains was studied by evaluating its action on the R-factor transfer capacity between a strain of *E. coli* (donor) and a strain of *Salmonella pullorum* (recipient) (7).

Salmonellosis is an increasing health hazard (8–11). The spread of drug-resistant pathogenic *Salmonella* has encouraged investigation of methods to reduce this phenomenon, which affects both human and animal bacterial flora.

EXPERIMENTAL

Bacterial Strains—*E. coli* (FI 3632), tetracycline resistant and rifamide sensitive, and *S. pullorum* (FI 741), tetracycline sensitive and rifamide resistant, were used. The *Salmonella* strain was used because only mutants with acquired resistance to tetracycline develop in subcultures on a solid medium containing 30 μg of tetracycline/ml plus 40 μg of rifamide/ml.

In these transfer assays, the two strains can be immediately distinguished on a solid medium containing lactose and a pH indicator by the formation of acidifying (*E. coli*) or nonacidifying (*S. pullorum*) colonies. The usual cultural, biochemical, and serological tests were employed to identify the parent and revertant strains.

Assay Media—Medium 1 was liquid tryptose broth[1]. Medium 2 (solid) contained tryptose agar[1], 1% lactose, and 0.01% bromthymol blue (Wurtz medium) with and without tetracycline[2] (30 μg/ml), rifamide[3] (40 μg/ml), paromomycin[4] (30 μg/ml), or tetracycline (30 μg/ml) plus rifamide (40 μg/ml). Medium 3 was TSI medium[1], and Medium 4 was SIM medium[1].

Medium 1 was used for the conjugation assays and the determination of the minimum inhibitory concentrations (MIC) of the three antimicrobials. Medium 2 served as a control for the conjugation experiments and for the sensitivity tests following the Kirby–Bauer method. The medicated media were employed for checking the drug resistance of the parent and revertant strains. Media 3 and 4 were employed for an initial differentiation between *E. coli* and *S. pullorum* colonies.

Reagents—Sensitivity disks were prepared according to the "Code of Federal Regulations" (12) and contained 30 μg of tetracycline, 40 μg of rifamide, or 30 μg of I. The somatic serodiagnostic antiserum of *E. coli* was obtained by rabbit immunization as described by Kauffmann (13), and the somatic serodiagnostic antiserum of *S. pullorum* was purchased[1].

Antibiotic Sensitivity Test—This test was performed in tryptose broth by determining the MIC values, in solid Medium 2 according to the Kirby–Bauer method, and by evaluating the bacterial growth in Medium 2 containing 30 μg of the single antimicrobial/ml.

Conjugation Assays in Presence of I—*Single Strains with I and 2–4 hr of Contact Time*—Compound I (final concentrations of 5, 10, and 20 μg/ml) was added to tryptose broth cultures of the single strains after 2 hr of incubation at 37°. After further incubation (2 and 4 hr), the cultures were washed (three times by centrifugation) to remove I and the pellets were resuspended and brought to volume with sterile tryptose broth.

Under these conditions, the drug concentration remaining in the cultures was lower than the minimum assayable microbiologically (0.05 μg/ml) by the agar plate method (12). Broth cultures of the two test strains grown in the absence of I were treated identically. The final bacterial suspensions thus obtained were mixed in a chessboard scheme according to the different concentrations of I with which the bacteria had previously been in contact.

[1] Difco Laboratories, Detroit, Mich.
[2] Lot 241/5, Farmitalia Research Laboratories, Milan, Italy.
[3] Lot 011, Gruppo Lepetit, spa, Milan, Italy.
[4] Aminosidine, lot 501.2, Farmitalia Research Laboratories, Milan, Italy.

Table I—Antibiotic Sensitivity of the Test Strains[a]

Strain	Tetracycline MIC, μg/ml	Tetracycline ST, mm	Tetracycline NC, %	Rifamide MIC, μg/ml	Rifamide ST, mm	Rifamide NC, %	I MIC, μg/ml	I ST, mm	I NC, %
E. coli	200	0	100	12.5	16.7	0	33	12.1	0
S. pullorum	1.5	26.2	0	150	0	100	25	13.5	0

[a] MIC = minimum inhibitory concentration in tryptose broth after 24 hr of incubation; ST = inhibition zone diameter in millimeters (average of four zones); and NC = number of colonies developed in medium containing 30–40 μg/ml of the antimicrobials, reported as percentage of the control tests (number of colonies grown in medium without antibiotic).

After static incubation at 37° for 3, 20, and 40 hr, known quantities of the microbial suspensions were seeded on plates of Medium 2 containing rifamide plus tetracycline. Following incubation at 37° for 18–24 hr, the acidifying and nonacidifying colonies were counted. Two to 10 colonies per plate were checked for identification purposes in Medium 3 and 4 tubes and by slide agglutination.

Mixed Strains with I and 3–20 hr of Contact Time—A few tests were carried out by placing the two strains in contact with I for the whole conjugation time. The subcultures were carried out after 3, 20, and 40 hr.

Tests for Bacteriophages—Experiments were carried out on *E. coli* to exclude bacteriophage carriers and the transduction phenomena. The experiments were performed both by seeding the filtrates of *E. coli* broth cultures on plain Medium 2 inoculated with the recipient strain and by the method of phage liberation after UV irradiation (14).

RESULTS

S. pullorum is resistant to rifamide and sensitive to tetracycline and I; *E. coli* is only resistant to tetracycline (Table I).

Table II—Mean Number (Three Replicates), Reported in Factorial Design, of *S. pullorum* Colonies that Developed on Wurtz Medicated Medium (30 μg of Tetracycline/ml and 40 μg of Rifamide/ml) after Conjugation

Conjugation Time = 20 hr[a]

	E. coli I, μg/ml	0 (A)	10 (B)	20 (C)	Assay
S. pullorum	20 (X)	3,203	2,940	787	a
		4,056	2,201	832	b
		10,446	0	5	c
		5,503	3,616	0	d
	10 (Y)	3,221	2,850	2743	a
		3,922	3,353	2790	b
		12,375	5,019	0	c
		6,070	2,955	2	d
	0 (Z)	3,287	3,102	1807	a
		8,960	5,438	4064	b
		17,220	14,392	1	c
		12,523	12,680	7869	d

Conjugation Time = 40 hr[b]

	E. coli I, μg/ml	0 (A)	10 (B)	20 (C)	Assay
S. pullorum	20 (X)	7,529	8,420	671	a
		94,667	9,366	337	b
		44,098	0	0	c
		21,647	23,298	0	d
	10 (Y)	11,764	7,702	1,522	a
		415,664	56,331	1,450	b
		11,836	28,130	0	c
		30,478	16,504	370	d
	0 (Z)	22,365	8,142	7,431	a
		318,665	115,668	54,667	b
		35,900	2,864	0	c
		44,235	43,574	18,073	d

[a] Out of 72 possible combinations of the data of the same assay, only 14 were statistically nonsignificant ($p > 0.5$): BYa *versus* CYa, AZa *versus* BZa, AZc *versus* BZc, AZd *versus* BZd, CYc *versus* CZc, BXa *versus* BYa, BXd *versus* BYd, BXa *versus* BZa, AXa *versus* AYa, AXb *versus* AYb, AXc *versus* AYc, AXd *versus* AYd, AXa *versus* AZa, and AYa *versus* AZa. [b] Out of 72 possible combinations of the data of the same assay, only eight were statistically nonsignificant ($p > 0.5$): BXc *versus* CXc, AXd *versus* BXd, AZd *versus* BZd, CXc *versus* CYc, CXc *versus* CZc, CYc *versus* CZc, BYa *versus* BZa, and AXc *versus* AZc.

Table II gives the data obtained from four conjugation tests (a, b, c, and d), and Table III gives the results of two tests (a and b). These tables report the average results (three replicates for each determination) of the *S. pullorum* colonies that had become tetracycline resistant after 2 hr of precontact with various concentrations of I and successive conjugation with the donor strain for 20 and 40 hr at 37°. The statistical significance (p) of all possible combinations between *E. coli* and *S. pullorum* treated with I was calculated, and the few nonsignificant confrontations are reported in the tables. On the average, 87% of the combinations showed statistically significant differences, that is, positive R-factor transfer with p values ranging from <0.001 to <0.05 (analysis of variance).

The data from the conjugation tests calculated as percentages of revertants and the range limits reported in Figs. 1 and 2 allow an immediate evaluation of the transfer decrease associated with the I treatment.

The lowest value was reached when both strains were exposed to the highest concentration of I (20 μg/ml). When only the donor strain (*E. coli*) and, to a lesser extent, when only the recipient strain were treated with I, a significant reduction in transfer also was noted. In both cases, the lowest value was again associated with the pretreatment with the highest concentration of I. Conjugation periods of 3 hr or less did not modify the transfer, in agreement with Anderson (15).

Separate analogous tests demonstrated that the presence of 3 μg/ml or less of I did not interfere with the acquisition of tetracycline resistance.

The experiments performed to show the presence of bacteriophages in the donor strain were negative.

DISCUSSION

These assays show that contact with subinhibitory concentrations of I impedes the acquisition and donation of tetracycline resistance in *S.*

Table III—Mean Number (Three Replicates), Reported in Factorial Design, of *S. pullorum* Colonies that Developed on Wurtz Medicated Medium (30 μg of Tetracycline/ml and 40 μg of Rifamide/ml) after Conjugation

Conjugation Time = 20 hr[a]

	E. coli I, μg/ml	0 (A)	5 (B)	10 (C)	Assay
S. pullorum	10 (X)	24,668	31,132	37,332	a
		25,768	35,465	28,536	b
	5 (Y)	28,534	27,466	31,170	a
		43,499	35,600	29,531	b
	0 (Z)	140,334	35,302	27,266	a
		285,666	32,466	31,102	b

Conjugation Time = 40 hr[b]

	E. coli I, μg/ml	0 (A)	5 (B)	10 (C)	Assay
S. pullorum	10 (X)	185,334	302,152	85,766	a
		125,332	95,666	37,800	b
	5 (Y)	165,667	62,168	95,667	a
		105,332	44,798	5,366	b
	0 (Z)	314,666	275,334	163,666	a
		310,332	40,766	121,000	b

[a] Out of 36 possible combinations of the data of the same assay, only five were statistically nonsignificant ($p > 0.5$): AYa *versus* BYa, BZb *versus* CZb, CXb *versus* CYb, BXb *versus* BYb, and CXb *versus* CYb. [b] Out of 36 possible combinations of the data of the same assay, none was statistically nonsignificant.

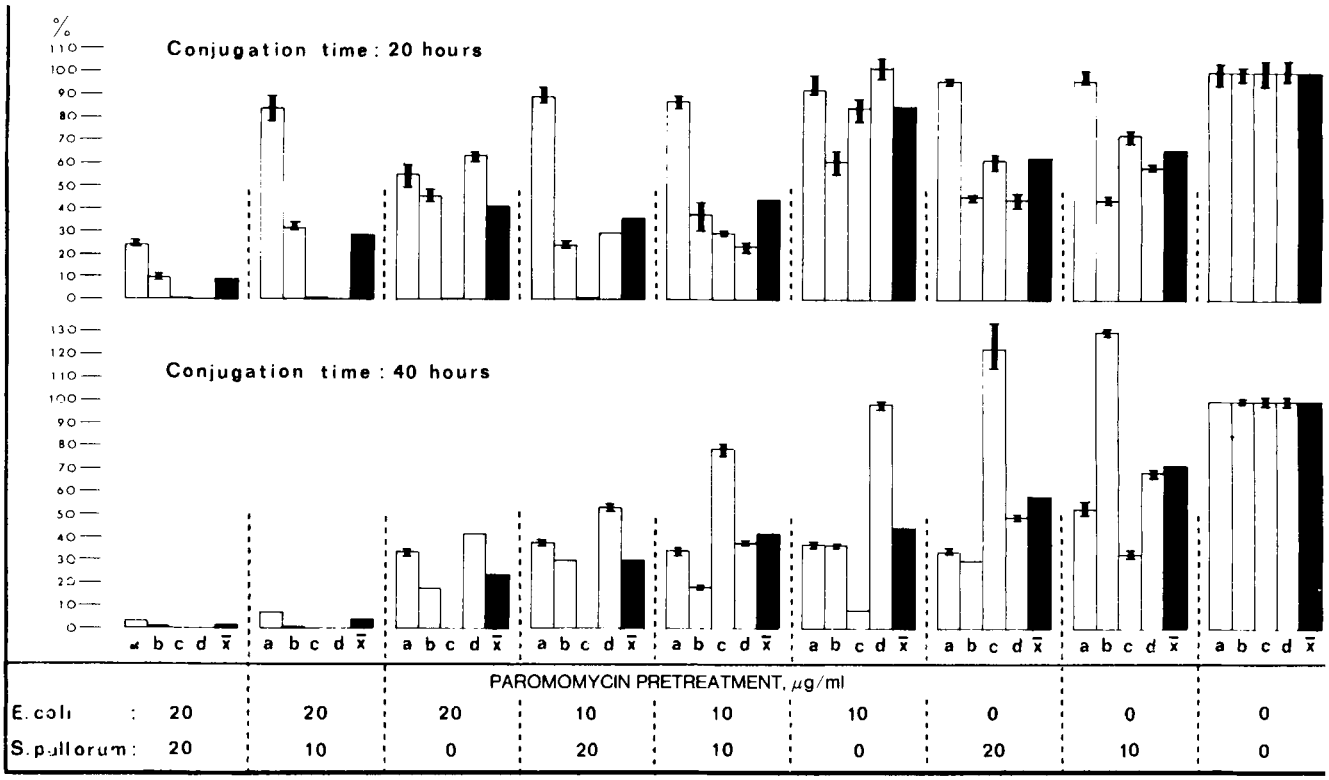

Figure 1—*Percent of* S. pullorum *colonies (with respect to the controls) that became tetracycline resistant after pretreatment of* E. coli *and/or* S. pullorum *with 20 and 10 μg of I/ml during 2 hr at 37°. Data are from four assays (a, b, c, and d) and their averages (x̄). The* I *represents the range limits for three determinations.*

pullorum and *E. coli*. Analogous effects were described for the acridine derivatives, mitomycin and sodium lauryl sulfate (16).

The effects of I may be attributed either to the induced synthesis of anomalous proteins, according to the mechanism of action common to the oligosaccharide antibiotics, or to a I-induced change of the superficial electrical charge of the bacterial cells, in a way analogous to that which occurs in *E. coli* in the presence of streptomycin, another oligosaccharide antibacterial (17).

The experimental evidence suggests that the emergence of tetracycline-resistant bacteria through conjugation resistance transfer may be prevented by the presence of I in low concentrations.

This acquisition seemed to be relevant even *in vivo*, because orally administered I is not absorbed through the GI tract and can reach intestinal concentrations high enough to inhibit the transfer of resistance factors between the ubiquitous, usually harmless, *E. coli* and the pathogenic *Salmonella*.

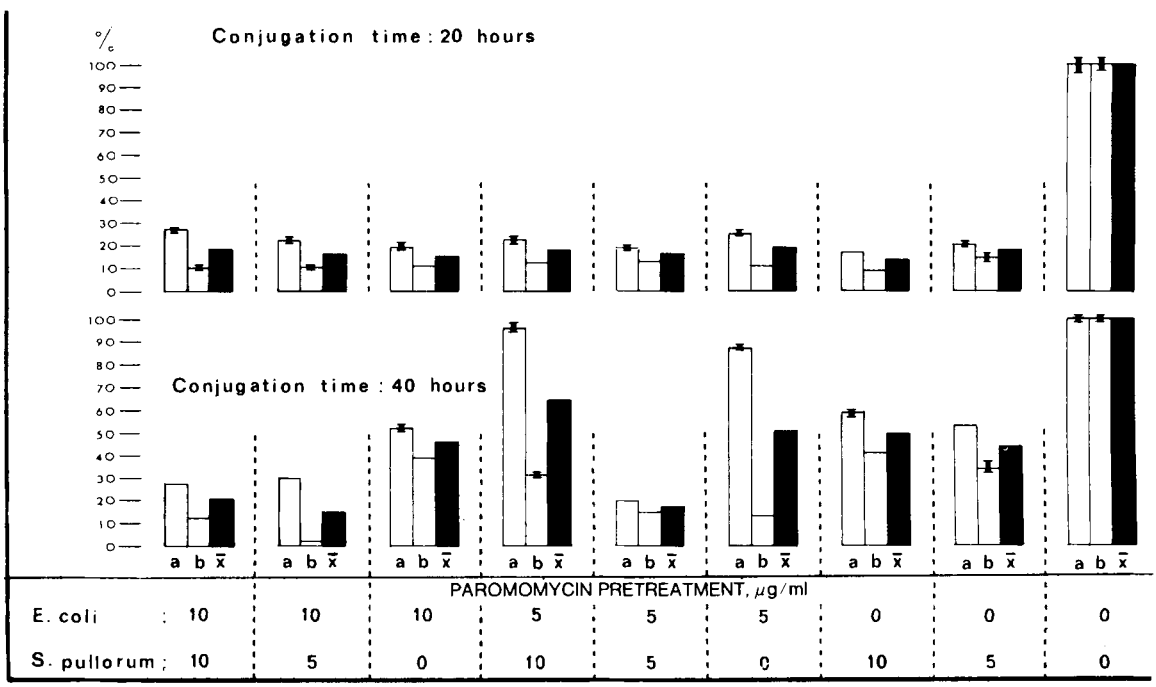

Figure 2—*Percent of* S. pullorum *colonies (with respect to the controls) that became tetracycline resistant after pretreatment of* E. coli *and/or* S. pullorum *with 10 and 5 μg of I/ml during 2 hr at 37°. Data are from two assays (a and b) and their averages (x̄). The* I *represents the range limits for three determinations.*

REFERENCES

(1) E. R. Garrett and J. K. Lewis, *J. Pharm. Sci.*, **64**, 1936 (1975).
(2) M. Ghione, A. Buogo, and A. Sanfilippo, *G. Microbiol.*, **8**, 9 (1960).
(3) F. Arcamone, C. Bertazzoli, A. Buogo, G. Cassinelli, and A. Vigevani, *Panminerva Med.*, **16**, 9 (1974).
(4) A. Buogo and P. Cattaneo, *Gazz. Med. Ital.*, **133**, 1 (1974).
(5) W. Almeida, *Hospital*, **59**, 947 (1961).
(6) G. K. Daikos, P. Kontomichalou, E. Petassis, and D. Bilasis, *Antimicrob. Agents Chemother.*, **4**, 765 (1963).
(7) A. Buogo and P. Cattaneo, *Arch. Vet. Ital.*, **26**, 111 (1975).
(8) P. J. Christopher, P. D. Claxton, D. C. Dorman, B. F. O'Connor, R. W. Proudford, and R. G. A. Sutton, *Med. J. Aust.*, **1**, 337 (1974).
(9) N. Datta, *J. Hyg. (London)*, **60**, 301 (1962).
(10) D. W. Pocurull, S. A. Gaines, and H. D. Mercer, *Appl. Microbiol.*, **21**, 258 (1971).
(11) M. Finland, C. Garner, C. Wilcox, and L. D. Sabath, *J. Am. Med. Assoc.*, **229**, 1309 (1974).
(12) Code of Federal Regulations, 21 Food and Drugs, Parts 300 to 199, Apr. 1, 1975.
(13) F. Kauffmann, "The Bacteriology of Enterobacteriaceae," Munksgaard, Copenhagen, Denmark, 1966, pp. 90, 91.
(14) P. Frédéricq and A. Gratia, *C. R. Soc. Biol.*, **143**, 560 (1949).
(15) E. S. Anderson, *Ann. Rev. Microbiol.*, **22**, 131 (1968).
(16) Y. Masanosuke and M. G. Sevag, *J. Bacteriol.*, **93**, 245 (1967).
(17) A. Buogo, *G. Microbiol.*, **6**, 57 (1958).

Pharmacokinetic Model for Salicylate in Cerebrospinal Fluid, Blood, Organs, and Tissues

C. N. CHEN, D. L. COLEMAN, J. D. ANDRADE ˣ, and A. R. TEMPLE

Received May 10, 1976, from the *College of Engineering and College of Pharmacy, University of Utah, Salt Lake City, UT 84112.*
Accepted for publication April 6, 1977.

Abstract □ The developed pharmacokinetic model, an extension of the Bischoff–Dedrick model, simultaneously predicts the kinetic behavior of salicylate in cerebrospinal fluid, blood, organs, and tissues. The model, which is entirely different from conventional compartment models, is derived from basic considerations of drug distribution with biochemical and physiological meaning. The dog was studied at three different dosages of salicylate: therapeutic, moderate intoxication, and severe intoxication. The predicted kinetics of salicylate in cerebrospinal fluid, blood, plasma, liver, muscle, and adipose tissue by the model agreed well with the experimental data. The effectiveness of hemoperfusion treatment for the severely intoxicated dog by albumin-coated activated carbon and its effect on the kinetic behavior of salicylate in cerebrospinal fluid, blood, organs, and tissues were studied. The model was also applied to predict the kinetic changes of salicylate in the body during and after the extracorporeal treatment. The predicted results also agreed with the experimental data.

Keyphrases □ Models, pharmacokinetic—developed to predict behavior of salicylate in cerebrospinal fluid, blood, organs, and tissues, compared to experimental data with dogs □ Pharmacokinetic models—developed to predict behavior of salicylate in cerebrospinal fluid, blood, organs, and tissues, compared to experimental data with dogs □ Salicylate—pharmacokinetic model developed to predict behavior in cerebrospinal fluid, blood, organs, and tissues, compared to experimental data with dogs

Blood or plasma drug levels have been used as an index of dose scheduling for therapeutics under the assumption that the drug level in blood or plasma corresponds to the pharmacological effect of the drug. Conventional pharmacokinetic models have been widely applied to simulate the kinetic behavior of drug levels in blood or plasma. However, the knowledge of drug levels in blood or plasma with time may not provide sufficient information for adequate therapy. The kinetic information of drug levels in brain, cerebrospinal fluid, blood, organs, and tissues of pharmacological interest may be necessary for the development of more appropriate dosage regimens.

The model developed and used in this study is an extension of the Bischoff–Dedrick model (1, 2). This model is derived from basic considerations of drug distribution with biochemical and physiological meaning. The model has been applied successfully to predict the pharmacokinetics of thiopental (2, 3), methotrexate (1, 4, 5), and cytarabine (6).

Previously, an extended version of the Bischoff–Dedrick model was used to predict thiopental kinetics in the dog (2). In this paper, the model previously presented (2) is modified and applied to salicylate in the dog. The model also is modified to consider the effects of activated carbon hemoperfusion on the pharmacokinetics of salicylate in the dog. Since drug–protein binding plays an important role in pharmacological effect and pharmacokinetics, the model also is applied to predict the pharmacokinetics of free (unbound) salicylate levels in plasma water, which is more related to the pharmacological effect of the drug.

THEORETICAL

A diagram of blood circulation through various body regions is shown in Scheme I. The blood pool is the blood volume excluding the blood contained in the capillary beds of organs and tissues in the body.

The transient mass balance for any organ or body region can be expressed as (2):

$$\begin{bmatrix}\text{drug accumulation rate}\\ \text{in both capillary bed and}\\ \text{tissue portion}\end{bmatrix} = \begin{bmatrix}\text{drug inflow rate from}\\ \text{blood pool and/or}\\ \text{other body regions}\end{bmatrix} + [\text{drug ingestion rate, if any}] - \begin{bmatrix}\text{drug outflow rate}\\ \text{from body region}\end{bmatrix} - \begin{bmatrix}\text{drug metabolism rate and/or}\\ \text{excretion rate, if any}\end{bmatrix} \quad \text{(Eq. 1)}$$

The mathematical equation of the transient mass balance for any body region (Yz) is:

$$\frac{d(V_{YzB}C_{T,YzB})}{dt} + \frac{d(V_{YzT}C_{T,YzT})}{dt} = Q_{Yz}(C_{T,B} - C_{T,YzB}) \quad \text{(Eq. 2)}$$

where the subscripts mean the following: B, blood; T, total or tissue; and Yz, a body region such as Ad (adipose), Br (brain), GI (gastrointestinal), Li (liver), Mu (muscle), and Vi (viscera); and where $C_{T,B}$ is the total