Intestinal Drug Absorption and Metabolism III: Glycine Conjugation and Accumulation of Benzoic Acid in Rat Intestinal Tissue

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Abstract □ Glycine conjugation of ^{14}C-benzoic acid to ^{14}C-hippuric acid was demonstrated in *in vitro* rat intestinal slices and everted intestinal preparations. Hippuric acid was detected and quantified by two independent analytical techniques, a quantitative TLC method, and a reverse isotope dilution method. Comparison of hippuric acid concentration after 2 hr. in mucosal and serosal fluids when hippuric acid was formed endogenously from benzoic acid, and with mucosal and serosal concentrations when hippuric acid was added exogenously to the mucosal fluid, indicated that hippuric acid is formed in the tissue compartment rather than by bacterial or intestinal enzymes in the mucosal fluid. Hippuric acid is apparently formed enzymatically in the mucosal cell according to a previously proposed cell compartment model and subsequently diffuses to both mucosal and serosal fluids. The net amount of hippuric acid formed under the conditions of the study was small (about 1%) and was found to be due to limited synthesis rather than to extensive hydrolysis. Both exogenously added benzoic acid and hippuric acid accumulated in the intestinal tissue compartment (11.2 and 6.4%, respectively) during transfer from the mucosal fluid.

Keyphrases □ Benzoic acid (carboxyl-^{14}C) absorption, metabolism—intestinal □ Metabolism, transfer, intestinal—everted sac, tissue incubation methods □ Hippuric acid formation—intestinal metabolism, benzoic acid □ Scintillometry—analysis □ Reverse isotope dilution—analysis □ TLC—analysis

The intestinal mucosa has enzyme systems capable of metabolizing many drugs by a variety of metabolic pathways, including sulfate and glucuronide conjugation (1–4). These processes can markedly affect the rate and extent of drug absorption. For example, intestinal glucuronide conjugation was found to affect greatly the rate and extent of transport of free salicylamide in both *in vivo* and *in vitro* preparations (4, 5).

The capability of intestinal tissue to conjugate aryl acids with glycine has not been established. The following studies using benzoic acid as a model substrate were therefore undertaken to determine: (*a*) if glycine conjugation does occur in the intestinal mucosa, and (*b*) if the degree of conjugation of benzoic acid to hippuric acid is sufficient to alter benzoic acid transport and, therefore, require consideration in kinetic studies on mechanisms of absorption. Some interesting observations on *in vitro* accumulation and transport of both benzoic acid (BA) and hippuric acid (HA) are also discussed.

EXPERIMENTAL

Materials—Benzoic acid[1] (carboxyl-^{14}C) had a specific activity of 56.0 mc./mmole and was 99% pure as determined by TLC. Hippuric acid[2] (carboxyl-^{14}C) had a specific activity of 6.66 mc./mmole and was 98% pure as determined by TLC. Periodic analysis of aqueous solutions of the two radioactive chemicals confirmed that degradation in solution was negligible during the period of the experiments. Hippuric acid[3] was recrystallized twice from double-distilled water. All other chemicals and reagents were purchased in the highest grades available and used as such.

Intestinal Transfer and Metabolism Measurements—Transfer and metabolism studies were performed *in vitro* using both an everted sac method, as described by Wilson and Wiseman (6), and a tissue incubation procedure. All experiments were carried out at 37° for a 2-hr. period. In the everted sac method, male Sprague-Dawley[4] rats (175–250 g.) were allowed food and water *ad libitum*. They were anesthetized with ether; the abdomen was opened and the entire small intestine was removed. After discarding the first 5 cm. on both ends, the mucosa was washed well with isotonic saline to remove particulate matter, and the intestine was carefully everted with a glass rod. The everted intestine was then cut into equal segments weighing approximately 4 g., filled with 4.0 ml. of Krebs–Ringer bicarbonate buffer (KRB) at pH 7.4, and tied at both ends. The filled sac was quickly transferred to a 50-ml. conical flask containing 25.0 ml. of a 10^{-2} m*M* solution of either ^{14}C-BA or ^{14}C-HA in KRB. Glycine (1 m*M*), adenosine triphosphate (ATP) (8×10^{-1} m*M*), and cysteine (5×10^{-1} m*M*) were also added, and the mucosal solutions were continuously bubbled with a mixture of 95% oxygen and 5% carbon dioxide. Controls were prepared in a similar manner except for the addition of tissue.

For experiments involving HA transfer (exogenous) from the mucosal side to the serosal side, aliquots of mucosal, serosal, and control sample fluids were assayed after 2 hr. for HA. For experiments involving BA transfer from the mucosal side to the serosal side and HA formation (endogenous) by the everted intestine, aliquots of mucosal, serosal, and control sample fluids were assayed after 2 hr. for both BA and HA. In addition, sections of the everted intestine were assayed for total drug at the end of the 2-hr. incubation period.

In a limited number of trials, the tissue incubation procedure was also used. Sections of everted intestine, 1–2 cm. in length, were randomly selected and placed into 4.0 ml. of KRB containing a 2.5 m*M* solution of ^{14}C-BA. Glycine (10 m*M*), ATP (10 m*M*), and cysteine (5 m*M*) were also added, and the incubation fluid was continually bubbled with the oxygen–carbon dioxide mixture. After incubation, the entire tissue incubation fluid was assayed for HA.

Metabolism by Intestinal Lumen Contents—To determine the amount metabolized by enzymes in lumen fluid, the entire lumen washings (about 5 ml.) containing fluid and fecal debris were collected and filtered through a Whatman No. 1 filter paper; 4 ml. of filtered fluid was incubated with 6 ml. of KRB containing ^{14}C-BA (10^{-2} m*M*). Glycine (1 m*M*), ATP (8×10^{-1} m*M*), and cysteine (5×10^{-1} m*M*) were also added, and the incubation fluid was flushed with the oxygen–carbon dioxide mixture. After incubation, an aliquot of the incubation fluid was assayed for HA.

Assay Procedures—Quantitative assays for ^{14}C were performed by liquid scintillation spectrometry[5] using Snyder's dioxane scintillation solution.[6] External standardization was used for quench corrections.

In transfer studies of BA and HA across the everted intestine, total radioactivity in mucosal and serosal samples was related through the specific activity of the compounds used to the amount or concentration of BA or HA present. Tissue sections were analyzed

[1] Amersham/Searle Corp., Des Plaines, Ill.
[2] New England Nuclear, Boston, Mass.
[3] Eastman Organic Chemicals, Rochester, N. Y.
[4] Blue Spruce Farms, Altamont, N. Y.
[5] Packard Tri-Carb model 3320, Packard Instrument Co., Inc., Downers Grove, Ill.
[6] Snyder's dioxane scintillation solution consists of 0.3 g. POPOP, 7.0 g. PPO, and 100 g. naphthalene in 1 l. of dioxane. To this, 200 ml. distilled water is added.

Table I—Percent HA Formed in Three *In Vitro* Preparations as Determined by Two Analytical Procedures

Preparation[a]	Percent HA Formed/Gram Dry Tissue[b] TLC Assay	Reverse Isotope Dilution Assay
Everted sac		
Mucosal solution (10^{-2})	0.630 ± 0.145 (9)	0.576 (0.655, 0.496)
Serosal solution (0)	0.760 ± 0.134 (9)	—
Tissue incubation (2.5)	1.22 ± 0.385 (3)	1.14 ± 0.367 (3)
Lumen contents (10^{-2}) incubation[c]	0.0443 ± 0.0039 (3)	—

[a] The initial BA concentration (m*M*) is given in parenthesis. [b] Calculated as the amount of HA formed in 2 hr./final total amount of BA and HA and normalized to unit gram dry weight. The number of experiments is given in parenthesis following the standard error of the mean. [c] Lumen contents were obtained from sections of an intestine having a dry weight of 1 g.

for total radioactivity after first dissolving aliquots of tissue into soluene-100.[7]

In studies involving HA formation *in vitro* from BA, two assay procedures were used to measure the amount of ^{14}C-HA formed. The first of these, the quantitative TLC method, involved a slight modification of the extraction procedure developed by Chantrenne (7). After extraction of HA at low pH, the organic solvent [chloroform–*n*-butanol (5:1)] was evaporated to dryness, the residue taken up in hot ethanol, and the ethanol evaporated to approximately 0.5 ml. Then 200 μl. of this solution was spotted on 20 × 20-cm. chromatography plates coated with 250 μ of silica gel HF_{254}.[8] The plates were developed in an ascending technique using the system ether (water saturated)–formic acid (90%)–methanol (98:1:1). This system (8) was chosen because BA (R_f 0.87) and HA (R_f 0.34) were widely separated, while residual amounts of protein remained near the origin. Hippuric acid, easily identified as a dark spot on the fluorescent background when the developed plate was exposed to short UV light, was scraped quantitatively into 20 ml. of the counting solution, and the vial was agitated prior to counting. Controls were treated in an identical manner, and the net ^{14}C-HA formed *in vitro* was expressed as the difference between the experimental and control values. The total recovery of HA by this procedure was 81.3 ± 4.0%.

The second assay procedure, used to confirm results obtained from the TLC procedure, was a reverse isotope dilution technique (9). A known quantity (approximately 200 mg.) of unlabeled HA was dissolved with heat in 10.0 ml. of incubation fluid containing both ^{14}C-BA and ^{14}C-HA. After determining the total amount of radioactive materials in the solution, the solution was then cooled to produce crystals of HA. The crystals were recovered by suction filtration, air dried, and weighed, and an aliquot was counted. Recrystallization was repeated four or five times, or until a constant specific activity of the purified hippuric acid was observed. From the data available, the amount of ^{14}C-HA in the incubation fluid was calculated.

Data Analysis—The significance of differences was determined by Student's *t* distribution. Any value of $p < 0.05$ was considered to be significant.

RESULTS AND DISCUSSION

Glycine Conjugation by Intestinal Preparations—Evidence that BA is conjugated with glycine to form HA was clearly shown in three different *in vitro* preparations. The percentage of HA formed following 2 hr. of incubation of BA in each of the intestinal preparations is shown in Table I. In all preparations, the percentage of HA formed was quite low. Because the net amounts of HA formed were so small, some difficulties were encountered in using the TLC assay, which required special considerations. To minimize loss of the small amount of ^{14}C-HA during preparation of the sample, it is necessary to add cold carrier HA to each sample. However, as shown in Table II, radioactivity at the HA band from control samples containing ^{14}C-BA and cold carrier HA but no ^{14}C-HA are much greater than the background radioactivity at the HA band obtained when only ^{14}C-BA without cold carrier HA is spotted. The reason for this anomalous effect is thought to be due to complexation of ^{14}C-BA with the cold carrier HA used in the assay. These higher counts from control samples, if not properly accounted for, would indicate the presence of ^{14}C-HA when none is actually present. HA radioactivity from the tissue experiments was always significantly greater than the values obtained from the control samples.

It was necessary, therefore, to run a control sample on each chromatogram and use the differences between the experimental and control samples as a quantitative measure of net ^{14}C-HA formed by the tissue.

To compare the values obtained from the TLC assay with another independent assay method, the more tedious but quite specific reverse isotope dilution technique was used. A representative example of the recovery of ^{14}C-HA utilizing this method is illustrated in Fig. 1. Excellent correlation between the two analytical methods (Table I) validates the values obtained by the TLC method and confirms the presence of ^{14}C-HA following incubation with ^{14}C-BA.

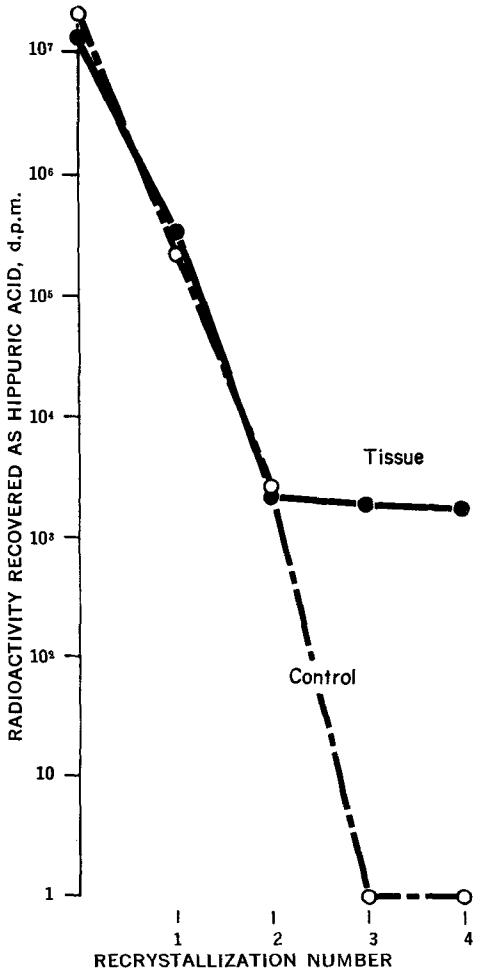


Figure 1—*Demonstration of ^{14}C-HA by isotope dilution technique. The amount of radioactivity after successive recrystallizations of HA initially dissolved in either incubation or control solutions. Key: ●, incubation fluid; and ○, control solution.*

[7] Packard Instrument Co., Inc., Downers Grove, Ill.
[8] E. Merck AG., distributed by Brinkmann Instruments Inc., Westbury, N. Y.

Table II—Recovery of Radioactive Materials at R_f 0.34[a] from the Thin-Layer Plate

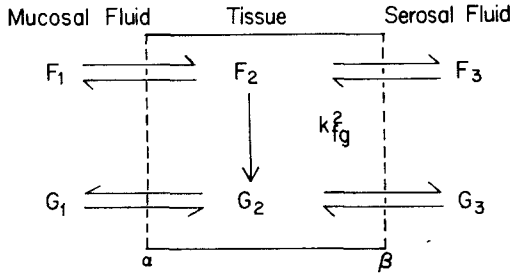
Starting Material	Disintegrations per Minute Recovered × $10^{-3} \pm SE$
^{14}C-BA[b]	0.082 ± 0.0060 (4)[c]
^{14}C-BA + cold carrier HA (before incubation)	0.62 ± 0.090 (6)
^{14}C-BA + cold carrier HA (after incubation	5.72 ± 0.84 (16)

[a] Corresponding to the region where HA is found. [b] Spotted directly as supplied by manufacturer. [c] Number of experiments shown in parenthesis.

Although the tissue incubation procedure appeared to give slightly higher amounts of metabolite than the everted sac preparation, the higher initial concentration of BA used in the tissue incubation procedure precludes direct comparison of the metabolic capacity of these two preparations. The lumen content incubation produced less than one tenth the amount of HA that was produced by the everted sac preparation when both procedures were run under identical incubation conditions. The significance of this finding relative to the site of metabolism is discussed later.

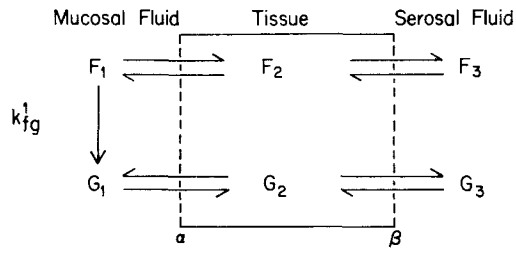
Evidence for Glycine Conjugation by Mucosal Tissue—The presence of ^{14}C-HA following incubation of ^{14}C-BA provided unequivocal evidence for glycine conjugation. Since it is well known that bacterial enzymes can also metabolize drugs (10), two types of experiments were carried out to differentiate between HA formation by the intestinal mucosal cell and by bacterial flora of the intestinal lumen.

These two possibilities can be considered from the previously proposed (4) compartment model which assumes that free drug (F) is conjugated to metabolite (G) in the intestinal mucosal cell (Model I),



Model I

or the alternate case where free drug is converted to metabolite in the lumen by bacterial or other enzymes in the mucosal fluid (Model II), where F_1, F_2, and F_3 are the amounts of free drug



Model II

(BA) in the mucosal fluid, tissue, and serosal fluid, respectively; and G_1, G_2, and G_3 are the amounts of metabolite (HA) in the mucosal fluid, tissue, and serosal fluid, respectively. The rate constants for metabolism in the tissue and in the lumen fluid are represented by $k^2_{f\alpha}$ and $k^1_{f\alpha}$, respectively. The symbols α and β represent the apical (mucosal) and basal (serosal) rate-limiting barriers, as previously defined (4).

The possibility of conjugation in the mucosal fluid (Model II) was evaluated by incubation of the lumen contents with ^{14}C-BA. The entire lumen contents of the rat intestine were collected and filtered with filter paper of sufficient porosity to allow passage of bacteria but exclusion of most cellular and fecal debris. The contents were incubated with ^{14}C-BA under conditions identical to those used for the everted sac studies. The very small amount of HA formed by lumen fluid (0.044%) would, at most, account for only one-tenth the amount of HA formed when tissue is present (Table I).

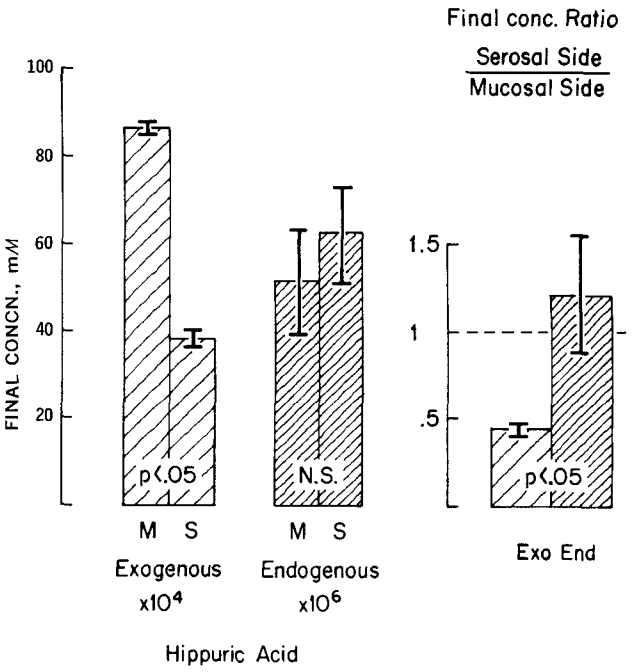


Figure 2—*Comparison of mean final (2 hr.) mucosal and serosal concentrations of HA formed endogenously from BA (8 experiments) or added exogenously to the mucosal fluid (5 experiments) and the corresponding mean of the individual serosal-to-mucosal concentration ratios. Bars indicate standard errors of the mean. Key: M, mucosal solution; and S, serosal solution.*

This study would not rule out HA formation in the lumen compartment by microorganisms that are known to adhere to the villi and microvilli (11). A second experiment was therefore performed to examine this possibility. Exogenous ^{14}C-HA was introduced directly into the mucosal fluid. After 2 hr., the concentrations of ^{14}C-HA in mucosal fluid (C_1), tissue (C_2), and serosal fluid (C_3) were compared to the values obtained when ^{14}C-HA was formed endogenously. If Model II was correct, the observed final concentration gradients of HA should be the same when HA is formed endogenously in the mucosal fluid after incubation with BA as when exogenous HA is added directly to the mucosal fluid—*viz.*, $C_1 \geqslant C_2 \geqslant C_3$.

Figure 2 gives a comparison of the final mucosal[9] and serosal concentrations and serosal-to-mucosal concentration ratios of HA when formed endogenously from BA and when added exogenously to the mucosal fluid. When HA is formed endogenously, the final mucosal and serosal concentrations are not significantly different after 2 hr. incubation. However, when HA (exogenous) is placed initially in the mucosal fluid and allowed to diffuse across the intestine, the final serosal concentration is significantly less than the final mucosal concentration after 2 hr. incubation. The final concentration ratio of HA in the serosal fluid to HA in the mucosal fluid after 2 hr. showed a value of 0.442 for exogenously added HA, which was statistically less than the value of 1.22 for HA formed endogenously after 2 hr. The ratio of 1.22 is not significantly different from unity. It is clear that HA, formed endogenously, shows a different diffusion pattern from HA added exogenously to the mucosal fluid. The observed results are consistent with Model I in which HA is formed in the mucosal cell and diffuses bidirectionally to the mucosal and the serosal fluids, as has been also observed with the glucuronide (5) and sulfate (12) metabolites of salicylamide in *in vitro* rabbit everted intestinal sacs.

Evidence for Limited Glycine Conjugate Hydrolysis—The low net amount of HA formed appears to be due to a low

[9] The word *final* refers to the last sample taken at 2 hr., beyond which the integrity of the tissue becomes questionable. It does not imply that distribution equilibrium within the system has necessarily been achieved.

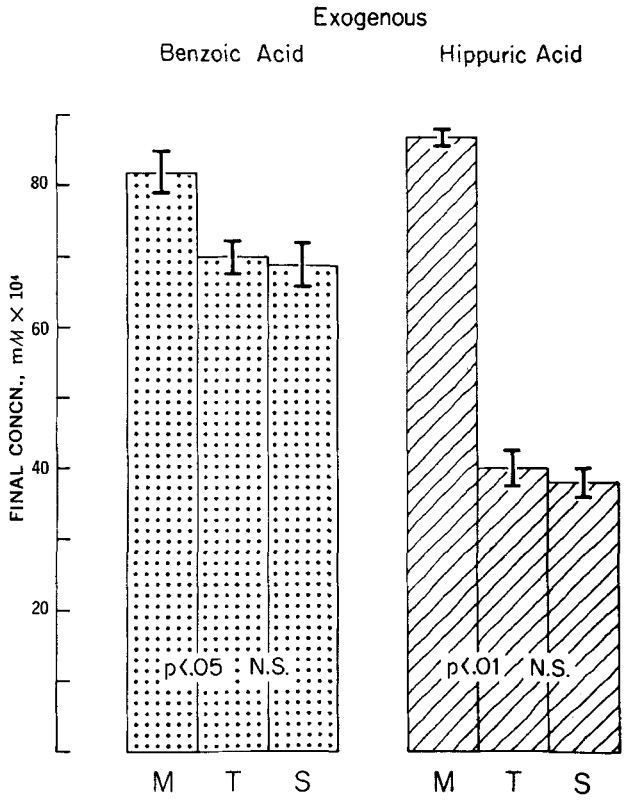


Figure 3—*Comparison of final (2 hr.) concentrations of BA and HA in mucosal fluid (M), tissue (T), and serosal fluid (S) after exogenous drug is placed in mucosal fluid at an initial concentration of 10^{-2} mM.*

synthesis rate rather than extensive hydrolysis after formation, since an insignificant rate of hippuric acid hydrolysis occurs. This was confirmed by comparing total radioactivity in the mucosal fluid, tissue, and the serosal fluid, following addition of exogenous ^{14}C-HA, with the radioactivity determined after organic extraction of an acidified aqueous sample of each compartment. Since exogenous ^{14}C-HA is labeled on the glycine carboxyl moiety, hydrolysis would yield ^{14}C-glycine which, in an acidified solution, would not be extracted into the organic solvent. Appreciable hydrolysis of ^{14}C-HA would, therefore, result in a low fraction of organic extractable ^{14}C. The recovery determined by total radioactivity and organic extractable radioactivity were the same, indicating negligible hydrolysis of HA.

Intestinal Transfer and Accumulation of Exogenous BA and HA—Formation of HA in the intestinal tissue is of biochemical significance in establishing the presence of the glycine conjugating system in the intestinal mucosal tissue; the small amount formed, however, is not significant from a kinetic viewpoint. It will not alter or contribute to the material balance of the system and may be neglected in the kinetics of *in vitro* transfer of BA.

Since neither BA nor HA was appreciably metabolized, it was possible to compare directly the transfer characteristics of ^{14}C-BA and ^{14}C-HA when both were added directly to the mucosal fluid. Under these conditions, both weak acids follow Model I, where k_{fs}^{2} is very small for the conjugation of BA and negligible for the hydrolysis of HA. The amounts of both ^{14}C-BA and ^{14}C-HA in mucosal fluid, tissue, and serosal fluid, 2 hr. after being added exogenously, are shown in Table III.

Accumulation of both BA and HA in the intestinal tissue was quite significant, accounting for 11.2 and 6.40%, respectively, of the initial amount added. Under the conditions of this study, accumulation of these compounds in the tissue must be accounted for if mass balance is to be achieved. The intestinal tissue must be considered as a compartment if either metabolism or accumulation of drug occurs within the tissue at a measurable rate (4, 13). Although metabolism within the tissue is negligible, significant accumulation of drug in the intestinal tissue requires consideration of the tissue as a compartment and a three-compartment model is appropriate.

The data from Table III, based on the compartment volumes indicated, were transformed to concentrations so that the final concentration gradients could be compared as shown in Fig. 3. Comparison of the higher mucosal concentrations to the almost equal concentrations of tissue and serosal fluid demonstates ($p < 0.05$ for both acids) that a gradient between mucosal fluid and tissue, but not between tissue and serosal fluid, exists after 2 hr. The significance of these findings with respect to the rate-limiting steps in aryl acid transfer across the intestine is presently under investigation.

Table III—Comparison of the Amount of Exogenous BA and Exogenous HA in Mucosal, Tissue, and Serosal Compartments

Compartment	Volume, ml.	Percent of Initial Amount ± *SE* BA	HA
Mucosal	25	82.0 ± 2.71 (9)[a]	86.6 ± 0.85 (5)
Tissue	4[b]	11.2 ± 0.22 (9)	6.40 ± 0.36 (5)
Serosal	4	11.0 ± 0.53 (9)	6.12 ± 0.30 (5)
Totals		104.1	99.1

[a] Number of experiments given in parenthesis. [b] Tissue volume assumes a tissue density of 1.0.

SUMMARY AND CONCLUSION

The present study unequivocally established that glycine conjugation can occur during the transfer of BA across the rat everted intestine. The extent of biotransformation was quite small, requiring sensitive tracer techniques for detection. The extent of conjugation may, of course, be greater under other experimental conditions or in other species. The low net amount of conjugate formed is a result of a low synthesis rate rather than to subsequent hydrolysis of the conjugate.

Kinetic evidence indicates that conjugation occurs predominantly in the intestinal tissue, presumably by a cellular enzymatic process. Exit from the mucosal cell is bidirectional, as has also been previously observed with glucuronide and sulfate conjugates.

Although the extent of metabolism is not sufficient to require the intestinal tissue to be considered as a separate compartment, there is significant accumulation of both BA and exogenous HA in the tissue to require the consideration of the intestine as a separate compartment.

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ACKNOWLEDGMENTS AND ADDRESSES

Received April 27, 1970, from the *Departments of Pharmaceutics** † *and Pediatrics*,† *State University of New York at Buffalo, Buffalo, NY 14214*

Accepted for publication September 1, 1970.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Washington, D.C. meeting, April 1970.

Supported by funds from United Health Foundation of Western New York, Grant No. CR-68-UB-7, and National Institutes of Health, Grant No. 1-RO1-GM-17068-01.