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ACKNOWLEDGMENTS

Supported in part by the Belgian "Fonds National de la Recherche Scientifique" and by the "Fonds de Developpement Scientifique" of the University of Louvain, Belgium. P. Couvreur is "Chargé de Recherches" to the Belgian "Fonds National de la Recherche Scientifique."

The authors thank Dr. Dunn, Loctite Co., Ireland, for a generous gift of the monomer samples and Dr. Kramp, Merck Sharp & Dohme, Brussels, Belgium, for a generous gift of dactinomycin.

Effect of Carnitine Analogs on Carnitine Acetyltransferase

MARVIN R. BOOTS *x, MARY LYNN WOLFE ‡, SHARON G. BOOTS *§, and JESSE L. BOBBITT ‡

Received June 1, 1979, from the **Department of Pharmaceutical Chemistry, Health Sciences Division, Medical College of Virginia, Virginia Commonwealth University, Richmond, VA 23298*, and the ‡*Biochemical Research Department, Lilly Research Laboratories, Division of Eli Lilly and Company, Indianapolis, IN 46240.* Accepted for publication September 20, 1979. §Present address: Department of Chemistry, Stanford University, Stanford, CA 94305.

Abstract □ Carnitine analogs with various substituents on the nitrogen were tested for their effect on carnitine acetyltransferase from rat sperm and pigeon breast. A radiometric assay was used to measure the formation of acetylcarnitine in the presence of other enzymes that competed for acetyl coenzyme A in the sperm preparation. The apparent enzyme inhibition caused by the analogs was explained by the analogs serving as alternative substrates with higher K_m and lower V_{max} values. The analogs had no effect on whole sperm.

Keyphrases □ Carnitine analogs—effect on carnitine acetyltransferase, spectrophotometric and radiometric measurements, kinetic parameters, *in vitro* □ Kinetics—analysis of carnitine analogs on carnitine acetyltransferase activity, spectrophotometric and radiometric measurements □ Spectrophotometry—analysis of carnitine analogs on carnitine acetyltransferase activity, kinetics

The primary importance of carnitine acetyltransferase (EC 2.3.1.7) in mitochondria is not known. Since the enzyme is found in high levels in cells that derive much of their energy requirements from lipid metabolism, it was thought that its major function was in lipid metabolism (1). For example, pigeon breast and heart muscle are common sources of the enzyme. The enzyme also is abundant in sperm (2), where the acetylation of carnitine may serve as an important regulator of both lipid and carbohydrate metabolism (3, 4).

Carnitine analogs that inhibit the enzyme may alter the path of energy metabolism (5). Rat and mouse sperm offer a convenient means of testing the biological activity of carnitine analogs. Carnitine appears to be important for sperm metabolism. Not only are carnitine acetyltransferase levels high in sperm, but carnitine is also in high concentration in epididymal fluid (6). The sperm can be isolated easily with little contamination by other cells, and they can be checked easily for viability by examining their motility under a microscope.

Unfortunately, the most commonly used spectrophotometric assay for carnitine acetyltransferase cannot be used with crude sperm enzyme; this assay measures the disappearance of the thiol ester bond of acetyl coenzyme A, and competing reactions using acetyl coenzyme A interfere with this measurement unless the enzyme is purified. However, crude enzyme can be assayed by measuring the transfer of labeled acetate to acetylcarnitine, which can be precipitated as the periodide (7). Since carnitine acetyltransferase probably is essential for sperm function, any inhibitor also should be detected with whole sperm by changes in the fertilization rate.

In this study, carnitine analogs were examined for their effect on the enzyme in both crude sperm preparations and crystalline pigeon breast enzyme. In addition, selected analogs were examined in various test systems to determine the cellular effects of the analogs.

EXPERIMENTAL

Carnitine Analogs—The *dl*-carnitine analogs (I–VII) were prepared as described previously (5).

Spectrophotometric Assay—The spectrophotometric assay of carnitine acetyltransferase activity measured the decrease in absorbance[1] of the thiol ester bond of acetyl coenzyme A at 232 nm (8). The reaction mixture in each cell contained 1.0 m*M* L-carnitine[2], 0.1 m*M* acetyl coenzyme A[2], and the carnitine analog of the desired concentration. The reaction was carried out at 25° in 100 m*M* tromethamine buffer, pH 8.0,

[1] Gilford spectrophotometer.
[2] Acetyl coenzyme A, PL Biochemicals.

0022-3549/80/0200-0202$01.00/0

Table I—Radiometric Precipitation Assay of Carnitine Acetyltransferase Inhibition

Compound	Inhibitors, mg/ml					
	1	2	4	1	2	4
	Inhibition of Sperm Enzyme, %			Inhibition of Pigeon Breast Enzyme, %		
I	15	24	50	12	25	45
II	25	38	45	30	45	50
III	28	38	42	30	45	45
IV	12	20	35	10	18	25
V	0	3	10	10	18	—
VI	5	12	38	10	20	30
VII	0	5	12	5	12	25

after addition of ~20 microunits (80 units/mg of protein) of pigeon breast enzyme[3].

Radiometric Assay—The assay using precipitation of labeled acetylcarnitine was a modification of the semimicro method described by McCaman *et al.* (7). The 0.25-ml reaction mixture contained 24 m*M* DL-carnitine (4.8 mg/ml), 0.5 m*M* ^{3}H-acetyl coenzyme A[4] tritium-labeled to ~1 mCi/mmole, the carnitine analog at various concentrations, and 0.1 *M* phosphate buffer, pH 7.5, with 0.5 m*M* ethylenediaminetetraacetate. The exact amount of sperm or pigeon breast enzyme was adjusted to stay within the linear portion of the standard curve.

After 30 min at 37°, the reaction was stopped, and the solution was deproteinized with 10 μl of cold 50% (w/v) trichloroacetic acid. After centrifugation, 0.20 ml of the supernate was transferred to another small centrifuge tube, and 0.30 ml of potassium iodide–iodine solution was added to precipitate the carnitine, labeled acetylcarnitine, and carnitine analogs as the periodides. The precipitate usually was allowed to stand overnight at 4° to ensure precipitation when the analogs were present. The precipitate was washed with 0.6 ml of cold 2 *N* H_2SO_4, dissolved in 10 ml of scintillator solution[5], and decolorized with 0.3 ml of an organic base[6].

Enzymes—Crystalline pigeon breast carnitine acetyltransferase was purchased[3]. The sperm enzyme was obtained from the mitochondria of rat or mouse epididymal sperm using a differential centrifugation procedure similar to that described previously (2). Fat-free epididymal tissue was homogenized in 0.25 *M* sucrose (5 ml/rat) and centrifuged for 10 min at 1000×*g*. The pellet was rehomogenized, and the combined supernate was centrifuged for 15 min at 10,000×*g* to sediment the mitochondria.

The mitochondria were homogenized twice at 20-min intervals with pH 8.0 phosphate buffer–ethylenediaminetetraacetate solution containing 0.1% deoxycholate (2), using 2 ml of buffer/rat. The homogenate was centrifuged at 10,000×*g* for 20 min. The enzyme was in the supernate, and the protein concentration was ~10 mg/ml. The preparation was stable in the frozen state.

Kinetic Measurements—Kinetic parameters for the carnitine analogs were determined spectrophotometrically using substrate concentrations less than or equal to 1 m*M*. Both K_m and V_{max} values were determined graphically from Lineweaver–Burk plots.

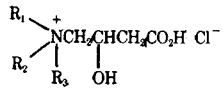
Whole Sperm Effects—Epididymal sperm from mice were expressed and washed twice in Tyrode's solution containing 0.1% glucose and 0.1% bovine serum albumin. The sperm were resuspended in the modified Tyrode's solution. To this suspension was added, in the desired concentration, the analog adjusted to neutral pH.

After 15 min at 37°, the sperm were washed again, and the enzyme was extracted for assay as already described. Comparison was made with control sperm without analog.

***In Vitro* Fertilization and Egg Culture**—Some analogs were tested in an assay designed to detect substances that interfered with sperm function, egg fertilization, or early embryo development. The system contained a measured number of mouse sperm and mouse eggs in an appropriate medium cultured under normal conditions (9). In the absence of test substances, the eggs were fertilized and developed to the morula stage at a known rate. Compounds that interfered with any part of the process, *i.e.*, from sperm viability to early egg metabolism, could be detected by an analysis of the cell types present at the end of the culture period. At 1 μg of the test substance/ml, ~14% of 1000 randomly selected organic compounds showed some activity. At 10 mg/ml, ~40% of the tested compounds interfered with some aspect of egg development.

[3] Sigma Chemical Co.
[4] New England Nuclear.
[5] The liquid scintillation fluid consisted of 2-ethoxyethanol–toluene (1:1) with 0.1 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene and 5 g of 2,5-diphenyloxazole/liter.
[6] Soluene, Packard Instruments.

Table II—Kinetic Parameters for Carnitine Analogs as Substrates for Carnitine Acetyltransferase[a]



Compound	R_1	R_2	R_3	K_m, m*M*	V_{max}, nmoles/min/mg
Carnitine	CH_3	CH_3	CH_3	0.3	11
I	CH_3CH_2	CH_3	CH_3	0.4	8.6
II	CH_3CH_2	CH_3CH_2	CH_3	0.6	6.0
III	$CH_3(CH_2)_3$	CH_3	CH_3	0.6	6.2
IV	CH_3CH_2	CH_3CH_2	H	1.0	3.2
V	$CH_2CH_2OCH_2CH_2$		H	1.0	<1
VI	$CH_2CH_2CH_2CH_2CH_2$		H	1.0	<1
VII	$CH_2CH_2CH_2CH_2$		H	2.0	<1

[a] Data was obtained *via* the spectrophotometric assay using crystalline pigeon breast enzyme.

RESULTS

The radiometric assay for carnitine acetyltransferase, based on precipitation of acetylcarnitine, was linear with the amount of sperm enzyme until ~40% of the labeled acetyl coenzyme A was consumed. It also was linear with time until the same amount of acetyl coenzyme A was consumed. Acetyl coenzyme A was limiting under the assay conditions since excess carnitine was needed to get complete precipitation. The amount of enzyme used for assaying the inhibitory properties of the analogs was adjusted to give 25–30% conversion of acetyl coenzyme A (corresponding to approximately 3 milliunits of the commercial pigeon breast enzyme). Addition of the analogs to the reaction mixture appeared to inhibit the sperm enzyme (Table I). The same inhibition patterns were seen with purified pigeon breast enzyme using the precipitation assay (Table I): I–III were better inhibitors than V–VII.

When the analogs were tested as substrates for the enzyme in the absence of carnitine, they served as substrates to varying degrees. The precipitation of the acetyl analog as the periodide was more variable, however, and K_m and V_{max} values for each analog were determined spectrophotometrically using concentrations up to 1 m*M* (Table II).

Whole Sperm Enzyme Effects—Analogs IV and V were tested for their effect on carnitine acetyltransferase levels in whole sperm. Enzyme levels were unchanged in sperm incubated for 15 min with the analogs and then washed. Compound V was tested at 1 μ*M* and IV was tested at concentrations as high as 100 m*M* without effect on the enzyme extracted from treated sperm.

Effect on *In Vitro* Sperm and Egg Culture—Compounds III–V and VII were tested at 0.1, 1.0, and 10 μg/ml of media. The fertilization rate in the presence of the analogs was indistinguishable from controls.

DISCUSSION

The radiometric assay permitted the measurement of small amounts of sperm carnitine acetyltransferase without extensive purification. Enzyme levels in the epididymal sperm of one mouse could be measured, and numerous assays could be made without large numbers of animals.

Under the usual assay procedures, the analogs first appeared to be typical competitive inhibitors except that inhibition leveled off at high levels. When examined as substrates, the apparent inhibition occurred because they were poorer substrates than carnitine. The 4-mg/ml analog concentration was about twice the concentration of L-carnitine in the radiometric assay. Exact kinetics of the radiometric assay were obscured by the presence of D-carnitine (also an inhibitor) and the variables introduced by the precipitation of the acetyl analog periodides. The spectrophotometric system was used to measure K_m and V_{max} of the analogs with the purified pigeon breast enzyme. The similarity of the enzymes toward the analog effects in the radiometric assay suggested that the data obtained spectrophotometrically with the crystalline enzyme were applicable to the crude sperm enzyme.

The K_m value generally increased and the V_{max} value decreased with increasing bulk and rigidity of the nitrogen substituents. Analogs I–IV accepted the transfer of the acetyl group to the β-hydroxyl if the posi-

tively charged nitrogen atom of the analog fit the binding site on the enzyme. Attempts to isolate the labeled, acetylated analog from the reaction mixture were unsuccessful. The label trailed badly in all separation systems tried.

Although the analogs acted as enzyme inhibitors, they had no effect on the sperm. Whether the analogs were excluded from the cells or entered the cell and were ineffective in altering the enzyme activity cannot be discerned from the available data. These studies confirmed previous work that showed that the analogs had no biological activity (5).

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ACKNOWLEDGMENTS

The authors thank Mr. James F. Hutchins, Lilly Research Laboratories, for testing the analogs in the *in vitro* fertilization system.

Pharmacokinetics of Doxycycline Reabsorption

PETER VENG PEDERSEN [*x] **and RAYMOND MILLER** [‡]

Received August 20, 1979, from the [*]*Department of Pharmacy, School of Pharmacy, and the* [‡]*Division of Clinical Pharmacology, Department of Medicine, University of California, San Francisco, CA 94143.* Accepted for publication September 28, 1979.

Abstract □ Two cyclic linear compartment models are proposed to investigate the reabsorption mechanism of doxycycline. In one model, reabsorption is considered to be continuous; in the other model, it is discontinuous. The continuous model, when fitted, leads to one real and two complex conjugate eigenvalues, corresponding to a regression equation consisting of a regular exponential term and an exponentially damped trigonometric expression. In spite of the apparent oscillatory nature of this regression equation, the fitted curves show no secondary peaks or humps apparent in the data. Simulation studies indicate that it may not be possible to get response profiles showing secondary peaks or humps that are experimentally detectable with linear compartment systems with cyclic pathways and continuous transfer. The model with discontinuous cyclic transfer was more flexible in describing the discrepancies in the data and appeared to be preferable to the continuous cyclic transfer model judged by the Akaike information criterion.

Keyphrases □ Doxycycline—pharmacokinetics of reabsorption, two cyclic linear compartment models proposed □ Pharmacokinetics—doxycycline reabsorption, two cyclic linear compartment models proposed □ Models—pharmacokinetics of doxycycline reabsorption

There does not appear to be general agreement about the pharmacokinetic behavior of doxycycline in humans. Gibaldi (1) claimed that the persistence of serum doxycycline levels is due to a relatively slow absorption compounded by enterohepatic cycling, while other authors proposed that it is due to an intrinsically slow rate of elimination (2, 3). The pharmacokinetic parameters for doxycycline were calculated from the data of Fabre *et al.* (3), assuming a single-compartment model (1). The elimination rate was calculated using the data points from 35 to 72 hr after administration; these values were assumed to be true postabsorptive data (1). The mean half-life for doxycycline of 9.8 hr obtained in this way does not differ greatly from the half-life of other tetracyclines. An apparent absorption half-life of 5.3 hr was calculated by the method of residuals. It was suggested (1) that the persistence of appreciable serum concentrations of doxycycline relative to other tetracyclines is a function of absorption kinetics rather than elimination kinetics.

Using the same data, a subsequent investigator (2) calculated a substantially different absorption half-life of 50 min and a quite different elimination half-life of 22–24 hr and suggested that the persistent serum levels are primarily due to slow elimination. Schach von Witteman (2) pointed out that the low values found 72 hr after dosing are not very accurate and that the emphasis placed on this limited segment of the data is not justified. However, this investigator ignored the last two data points entirely in calculating the elimination rate constant. Inclusion of these two data points results in a considerably shorter half-life.

Although it is difficult to resolve properly the absorption and elimination kinetics from oral data alone, the data (3) show signs of secondary peaks, which support the hypothesis of reabsorption. This hypothesis is further supported by a high affinity to the bile reported for doxycycline and other tetracyclines (4). If reabsorption occurs, then the calculation of intrinsic absorption and elimination half-lives using classical pharmacokinetic approaches as discussed may not apply. It is not valid to assume a postabsorptive phase in a pharmacokinetic system with significant reabsorption.

Therefore, it is of interest to investigate the kinetic behavior of doxycycline using kinetic models that do consider the drug's reabsorption. It is also valuable to investigate whether the reabsorption occurs through a continuous process as suggested previously (4) or whether it is of a discontinuous nature, possibly related to biliary intestinal secretion that occurs primarily in the form of squirts of bile into the intestine (5). This work proposes two simple pharmacokinetic models to investigate these matters using the data of Fabre *et al.* (3).

THEORY

The two linear pharmacokinetic models proposed represent simple forms of drug reabsorption. Both models contain a cyclic disposition and do not belong to the categories of models (mammillary, catenary, and other noncyclic systems) proposed most frequently in pharmacokinetics.

Model 1—This linear model proposes first-order absorption, central

0022-3549/80/0200-0204$01.00/0