activity studies in mice and rats suggested that the lipid and water solubilities of the compounds, rather than their enzyme-catalyzed hydrolysis rates, probably control the availability of acetaminophen following oral administration. These experiments provide additional evidence that the carbonate linkage may be an importantly useful one for the creation of variant physical and chemical properties in an entity with a singular pharmacologic action (11). This might then offer the opportunity to the formulator to choose among a variety of compounds with a singular pharmacologic action for the one which is: (*a*) amenable to physical formulation in a given dosage form; (*b*) stable in a given dosage form where all forms of the drug may not be equally stable; (*c*) satisfactory from the standpoint of taste or consumer acceptability; and (*d*) appropriate therapeutically from the standpoint of its time-action profile.

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Keyphrases

Acetaminophen prodrugs
Carbonate esters, acetaminophen–synthesis
Carboxylic acid esters, acetaminophen–synthesis
Hydrolysis rates, *in vitro*–acetaminophen prodrugs
Toxicity, oral–acetaminophen prodrugs
Analgesic activity–acetaminophen prodrugs

Acetaminophen Prodrugs II

Effect of Structure and Enzyme Source on Enzymatic and Nonenzymatic Hydrolysis of Carbonate Esters

By L. W. DITTERT*, G. M. IRWIN, C. W. CHONG, and J. V. SWINTOSKY*

Hydrolysis rates are reported for acetaminophen prodrugs with the structure CH_3CONH-ϕ-OCOOR at pH 7.4 in phosphate buffer alone or containing 1% human plasma or serum from several animal species. The hydrolysis rates in buffer decreased as the electrophilic character of the R group decreased. Dilute plasma or serum accelerated the hydrolysis; and the number of carbon atoms, the degree of chain branching, aromaticity, and chlorine substitution in the R group variously affected the degree of acceleration. In general, the sera of small rodents (mouse, guinea pig, and rat) were more potent catalysts of the hydrolyses of all types of acetaminophen carbonates than that of other animals (cat, dog, sheep, and rabbit) or human plasma.

THE METHODS of preparation and the physical properties of a series of carbonate esters of acetaminophen have been previously reported (1). These compounds were found to hydrolyze at various rates in dilute (2% v/v) human plasma solutions, and some of them had analgesic activity on the order of acetaminophen in rats. It was postulated that the analgesic activity was due to free acetaminophen released in the blood streams of the rats following oral administration of the prodrugs.

This report discusses the influence of the structure of acetaminophen carbonate prodrugs on the nonenzymatic hydrolysis of the compounds at pH 7.4 and on the enzymatic hydrolysis of the compounds catalyzed by blood plasma from

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humans and blood sera from various animal species.

EXPERIMENTAL

Frozen citrated human blood plasma (Type O^+) was obtained in approximately 100-ml. quantities from single donors through the Philadelphia Serum Exchange. Frozen pooled animal sera were obtained from Colorado Serum Company, Denver, Colorado.

Half-lives for the hydrolysis of the acetaminophen carbonates at 37° in pH 7.4 phosphate buffer (0.1 *M*), with and without human plasma or animal serum, were determined by direct UV analysis in the thermostatted cell compartment of a Cary model 15 spectrophotometer. Fifty milliliters of the plasma or serum solution was warmed to 37° in a 125-ml. glass-stoppered conical flask and a portion placed in the spectrophotometer reference cell. One-half milliliter of 95% ethanol containing an appropriate amount of the acetaminophen carbonate was injected by means of a hypodermic syringe, and the flask was swirled gently until mixing was complete. A portion of this mixture was transferred to the sample cell of the spectrophotometer and the absorbance followed until no further change was observable. In cases in which the reactions were very slow, the final absorbance values were calculated. The half-lives were determined from plots of log Δ absorbance *versus* time (see Fig. 1). In most cases, the spectrophotometer was set at 240 mμ (the absorbance maximum of the esters), where a decrease in absorbance reflected the disappearance of the ester. In several experiments, the spectrophotometer was set at 300 mμ (the absorbance maximum of acetaminophen), where an increase in absorbance reflected the appearance of acetaminophen. For a given ester in a given enzyme system, the same half-lives were obtained at the two wavelengths.

RESULTS AND DISCUSSION

Half-lives for the hydrolysis of 11 carbonate esters of acetaminophen in pH 7.4 phosphate buffer (0.1 *M*), with and without 1% (v/v) human plasma, are shown in Table I. The rates of nonenzymatic cleavage of acetaminophen carbonate esters of straight-chain alcohols decreased with increasing chain length to four carbons; thereafter, they remained essentially constant at a minimum value.

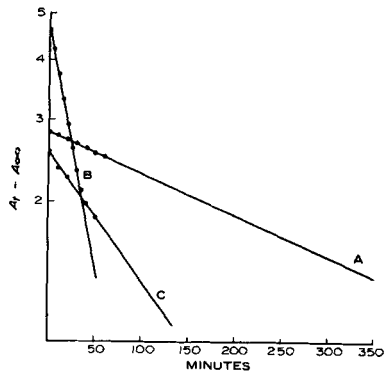
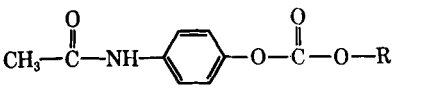


Fig. 1—Representative plots showing the first-order nature of the hydrolysis of carbonate esters of acetaminophen in pH 7.4 phosphate buffer (0.1 M*) containing 1% (v/v) human plasma at 37°. Absorbances were determined at 240 mμ, the absorbance maximum of the esters.* (A_t = *absorbance at time* t; A_∞ = *absorbance when the reaction is apparently complete.*) The *half-lives are as follows*—A, *methylcarbonate*, $t_{1/2}$ = *345 min.;* B, *butylcarbonate*, $t_{1/2}$ = *30 min.;* C, *isopropylcarbonate*, $t_{1/2}$ = *111 min.*

TABLE I—HALF-LIVES FOR HYDROLYSIS OF CARBONATE ESTERS OF ACETAMINOPHEN IN pH 7.4 PHOSPHATE BUFFER (0.1 *M*)[a]



R	Half-lives, min. 1% Human Plasma	Half-lives, min. Buffer	$t_{1/2}$ buffer/ $t_{1/2}$ plasma
$-CH_3$	345	9,000	26
$-C_2H_5$	90	12,000	133
$-C_4H_9$	30	18,000	600
$-C_6H_{13}$	22	22,800	1036
$-C_8H_{17}$	28	19,800	707
$-CH(CH_3)_2$	111	18,000	162
$-CH_2-CH(CH_3)_2$	37	21,000	568
$-C_6H_5$	52	240	4.6
$-C_6H_4-NH-C(=O)-CH_3$	230	420	1.8
$-CH_2-CH_2-Cl$	50	1,320	26
$-CH_2-C-Cl_3$	52	240	4.6

[a] With and without 1% v/v human plasma (37°).

These results are in agreement with the findings of Gordon *et al.* (2), who studied the effect of the carbon chain length of aliphatic alcohols on the ammonolysis of their acetate esters. They found that the rate of ammonolysis decreased to a constant minimum value at alcohol carbon chain lengths of about five. These results suggest that the hydrolysis of the acetaminophen carbonates is subject to essentially the same electronic effects as the hydrolysis of carboxylic acid esters and of other carbonate esters (3); that is, the alkyl groups tend to act as "electron pumps" depolarizing the carbonyl group and making it less susceptible to nucleophilic attack. As with carboxylic acid esters, this inductive effect levels off at alkyl chain lengths of about four or five carbons.

The rates of enzymatic cleavage of the acetaminophen carbonates of straight-chain alcohols in 1% human plasma increased with increasing chain length up to about six carbons. The figures shown in Table I in the column headed $t_{1/2}$ buffer/$t_{1/2}$ enzyme, which indicate the degree of enzyme catalysis relative to the nonenzymatic rate, follow the same pattern. Adams and Whittaker (4), in studies on human plasma cholinesterase, reported that the optimum chain length for either the acyl group or the alkyl group in a homologous series of carboxylic acid esters is four carbons. Hofstee (5) found that esters of fatty acids with seven carbons in the acyl chain are more susceptible to chymotrypsin catalyzed hy-

TABLE II—HALF-LIVES FOR HYDROLYSIS OF CARBONATE ESTERS OF ACETAMINOPHEN IN pH 7.4 PHOSPHATE BUFFER (0.1 *M*)[a]

R	Buffer	Half-lives, min. ($t_{1/2}$ buffer/$t_{1/2}$ enzyme)[b] Mouse	Guinea Pig	Rat	Cat	Human Plasma	Rabbit	Dog	Sheep
$-CH_3$	9000	30 (700)	51 (176)	95 (95)	294 (31)	345 (26)	79 (114)	40 (225)	335 (27)
$-C_2H_5$	12000	15 (800)	35 (343)	101 (119)	86 (140)	90 (133)	115 (104)	156 (77)	557 (22)
$-C_4H_9$	18000	6.9 (2610)	3.7 (4865)	6.7 (2687)	18 (1000)	30 (600)	50 (360)	27 (667)	520 (35)
$-CH(CH_3)_2$	18000	40 (450)	20 (900)	108 (167)	118 (153)	111 (162)	238 (76)	...	...
$-CH_2-CH(CH_3)_2$	21000	7.5 (2800)	5.6 (3750)	7.9 (2658)	12 (1750)	37 (568)	143 (147)	55 (382)	318 (66)
$-C_6H_5$	240	0.17 (1412)	0.25 (960)	1.3 (185)	10 (24)	52 (4.6)	...	100 (2.4)	165 (1.5)
$-C_6H_4-NHCOCH_3$	420	1.5 (280)	1.5 (280)	3.6 (117)	11 (38)	130 (3.2)	71 (5.9)	111 (3.8)	63 (6.7)
$-CH_2-CCl_3$	240	0.21 (1143)	0.93 (258)	1.1 (218)	10 (24)	52 (4.6)	18 (13)	100 (2.4)	50 (4.8)

[a] Containing 1% serum from animals or 1% human plasma (37°). [b] The numbers in parentheses are $t_{1/2}$ buffer/$t_{1/2}$ enzyme ratios which represent the degree of enzymatic catalysis.

drolysis than esters of other fatty acids. The enzymes in human plasma responsible for the hydrolysis of acetaminophen carbonate esters appear to behave in a similar way. However, since human plasma contains a mixture of esterolytic enzymes, at least part of the dependence of the enzymatic rates on structure may be attributed to variations in the contributions made by the several plasma enzymes to the overall hydrolysis rates of the esters.

Half-lives for the hydrolyses of two acetaminophen carbonate esters of branched chain aliphatic alcohols are also shown in Table I. Branching slows both the nonenzymatic and the enzymatic hydrolysis reactions slightly, but the effect is less pronounced in the isobutyl than in the isopropyl case. The slowing effect of branching is probably due to inductive electronic effects similar to those observed by Gordon *et al.* (2) in their studies of the ammonolysis of acetate esters of ethanol, butanol, isopropanol, and isobutanol. Their results show a direct parallel with the nonenzymatic cleavage data for the corresponding acetaminophen carbonate esters shown in Table I.

Chain branching apparently has comparatively little effect on the degree of enzymatic catalysis of the acetaminophen carbonates by human plasma as shown by the closeness of the $t_{1/2}$ buffer/$t_{1/2}$ enzyme values for the ethyl and isopropyl derivatives and for the butyl and isobutyl derivatives. Thus, it would appear that branching in the aliphatic alcohol chain has much less of an effect on the human plasma catalyzed hydrolysis reaction than does the total number of carbons in the chain. This is an important finding with regard to the selection of derivatives for the preparation of carbonate ester prodrugs. Derivatives of branched alcohols generally have lower melting points and higher aqueous and nonaqueous solubilities than those of straight-chain alcohols (1). Since branching seems to have little effect on the hydrolysis of the carbonates, branched derivatives with their greater aqueous solubilities might be more rapidly available for oral absorption but equally susceptible to enzymatic hydrolysis as the straight-chain derivatives.

Table I shows hydrolysis data for the phenyl- and the *p*-acetaminophenylcarbonates of acetaminophen. The results suggest that aromatic groups apparently affected both the electronic state and the affinity of human plasma enzymes for these molecules. The phenolic carbonates were much more rapidly hydrolyzed in pH 7.4 buffer than the hexylcarbonate, probably because of the electrophilic character of the aromatic rings which polarize the carbonyl group and make it more susceptible to nucleophilic attack. The phenolic carbonates, however, were much less susceptible to enzymatic cleavage than is the hexylcarbonate, possibly because the hexyl group has a greater affinity for the catalytic sites on the esterolytic enzymes of human plasma.

The influence of chlorine substitution on the enzymatic and nonenzymatic hydrolysis of the ethylcarbonate of acetaminophen is illustrated in Table I. These data show that increasing chlorine substitution beta to the carbonate linkage progressively increased the nonenzymatic hydrolysis rate. The effect was quite pronounced in going from the ethyl to the monochloroethyl derivative and was apparently due to the capacity of chlorine to act as an "electron sink" causing increased polarization of the carbonyl

group. On the other hand, progressive chlorine substitution appears to decrease the susceptibility of the ethylcarbonate ester to enzymatic hydrolysis. Again this effect might be due either to deteriorating enzyme substrate "fit," or to changes in the contributions made by the various enzymes to the overall hydrolysis rate as chlorine substitution is increased.

The results of the studies on the phenolic carbonates and the chlorine substituted ethylcarbonates of acetaminophen suggest that prodrug carbonates of phenols and of alcohols containing electrophilic substituents are relatively more susceptible to nonenzymatic cleavage but relatively less susceptible to enzymatic cleavage than prodrug carbonates of aliphatic alcohols. Thus, phenols and electrophilic alcohols may form carbonate ester prodrugs which are less stable pharmaceutically and less labile to enzymatic cleavage *in vivo* than those formed by aliphatic alcohols.

Table II shows half-lives for the hydrolysis of eight carbonate esters of acetaminophen in pH 7.4 phosphate buffer (0.1 *M*) containing 1% blood serum from seven animals or 1% human plasma. Values for $t_{1/2}$ buffer/$t_{1/2}$ enzyme for each of the enzyme systems are shown in parentheses. The results show that serum from all the animals contains esterases capable of catalyzing the hydrolysis of the acetaminophen carbonate esters, although in several instances the catalysis is very weak.

For almost every species, the aliphatic carbonate esters can be ranked in the following order of susceptibility to enzymatic attack: butyl $\cong$ isobutyl > isopropyl $\cong$ ethyl > methyl. In serum from small rodents (mouse, guinea pig, and rat) the phenyl- and 2,2,2-trichloroethylcarbonates were about equally susceptible to enzymatic attack and the *p*-acetaminophenylcarbonate was somewhat less susceptible. In serum from the other animals and in human plasma, the *p*-acetaminophenylcarbonate was somewhat more susceptible to enzymatic attack than the phenyl- and 2,2,2-trichloroethylcarbonates.

For almost every compound, the sera of the animals can be ranked in the following approximate order of catalytic potency: mouse > guinea pig > rat > cat > human > rabbit > dog > sheep, although in several cases guinea pig and mouse could be interchanged in this ranking.

Augustinsson (6) reported wide species differences in both the concentrations and the specificities of the esterases normally found in the sera of animals and man. Since serum contains several esterolytic enzymes, each with its own specificities for the substrates, at least part of the dependence of the enzymatic rates on structure in a given sample of serum may be attributed to variations in the contributions made by the plasma enzymes to the overall hydrolysis rates. The specificities and the concentrations of the enzymes in the serum vary from species to species, and are probably largely responsible for the various hydrolysis rates observed for a given substrate in the sera of various animals.

Thus, it is not surprising that it is difficult to make broad generalizations based on the data of Table II. However, the following conclusions can be drawn: (*a*) if an acetaminophen carbonate ester has a comparatively large $t_{1/2}$ buffer/$t_{1/2}$ enzyme ratio in the serum of one species, it will probably also have comparatively large $t_{1/2}$ buffer/$t_{1/2}$ enzyme ratios in the sera of all species, and (*b*) the sera of small rodents seem to possess either higher concentrations of esterases, or esterases which have a greater affinity for acetaminophen carbonate esters than the sera of the other animals or human plasma.

All the compounds hydrolyzed in 1% human plasma. It would be expected that all would release free acetaminophen in the blood of humans fairly rapidly if they indeed reached the blood intact following oral administration. In almost every case, the compounds were hydrolyzed more rapidly in the serum of common laboratory test animals (except rabbit and dog) than in human plasma, and it would be expected that free acetaminophen would be rapidly released following oral absorption in these animals. Thus, human plasma is a convenient test system for studying the hydrolysis of carbonate ester prodrugs because hydrolysis in this system suggests that hydrolysis would also occur in the blood of humans and laboratory test animals *in vivo*. Lack of hydrolysis in dilute human plasma, however, would not necessarily mean that hydrolysis in the tissues of laboratory test animals and humans is not possible.

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Keyphrases

Acetaminophen prodrugs
Carbonate ester, acetaminophen—hydrolysis
Hydrolysis—enzymatic, nonenzymatic
UV spectrophotometry—analysis