Fisher, H. F., Subramanian, S., Stickel, D. C., & Colen, A. H. (1980) *J. Biol. Chem. 255*, 2509–2513.
Johnson, R. E., Andree, P. J., & Fisher, H. F. (1981a) *J. Biol. Chem. 256*, 3817–3821.
Johnson, R. E., Andree, P. J., & Fisher, H. F. (1981b) *J. Biol. Chem. 256*, 6381–6384.
Siegel, J. M., Montgomery, G. A., & Bock, R. M. (1959) *Arch. Biochem. Biophys. 82*, 288–299.

Substrate Specificity of Bacterial Glycerophospholipid:Cholesterol Acyltransferase†

J. Thomas Buckley

ABSTRACT: The substrate specificity of a bacterial analogue of the plasma enzyme lecithin:cholesterol acyltransferase (LCAT) has been examined with small unilamellar liposomes and Triton mixed micelles. In contrast to LCAT, the microbial enzyme is capable of using all of the naturally occurring phospholipids as acyl donors. In general reaction rate depends more on the length or degree of unsaturation of the acyl chains than on the nature of the phospholipid head group. Among a series of disaturated phosphatidylcholines in liposomes, dilauroylphosphatidylcholine is the preferred acyl donor. Like LCAT, the enzyme will catalyze acyl transfer by using other alcohols in addition to cholesterol. Of saturated straight chain primary alcohols 1-decanol is the preferred acyl acceptor. Cholesterol, however, is a far better acceptor than any nonsterol alcohol tested. Other steroids with equatorial hydroxyls at position C-3 and trans-fused A:B rings will also act as acceptors whereas those steroids with axial hydroxyls at C-3 or cis-fused rings are inhibitors of acyl transfer. The ability of steroids to act as acyl acceptors may be due to the nature of their interaction with the phospholipid acyl donor.

The glycerophospholipid:cholesterol acyltransferase (GCAT)[1] released by members of the *Vibrio* family (MacIntyre & Buckley, 1978; MacIntyre et al., 1979) and purified in this laboratory (Buckley et al., 1982) has a number of interesting features. In reaction mechanism it appears similar to the mammalian enzyme lecithin:cholesterol acyltransferase (LCAT). Like LCAT, the enzyme can act as a 2 position specific phospholipase or as an acyltransferase, depending upon the presence or absence of cholesterol. Furthermore, as with LCAT, the enzyme has no divalent cation requirement and its activity is stimulated by apolipoprotein A-1 (Buckley et al., 1982). Since GCAT is far more stable than LCAT and since it is available in large quantity, it should prove valuable in obtaining information in vitro to aid in further studies of the mechanism of LCAT action. In addition, because the enzyme (unlike LCAT) can carry out hydrolysis or acyl transfer using most if not all glycerophospholipids, because it has no divalent cation requirement, and because it apparently acts asymmetrically on plasma membranes (Buckley et al., 1982), it may be used to corroborate and expand information on phospholipid interactions in plasma membranes obtained in studies using eucaryotic phospholipases.

The lack of a divalent cation requirement and the 2 position specificity as well as the ability to acylate cholesterol distinguish the *Vibrio* enzyme from all other microbial phospholipases. It seems quite possible if not likely, however, that the transfer of a fatty acid from a phospholipid such as phosphatidylcholine to cholesterol is not the reaction carried out by the organism in vivo. Thus although it is conceivable that the bacteria use the enzyme to degrade eucaryotic cells, it may normally be used with quite different substrates, for example, in cell wall metabolism.

In this paper the specificity of the bacterial enzyme toward both acyl donors and acyl acceptors is examined in order to extend the comparison with LCAT and to shed more light on the probable function of the enzyme in vivo.

Materials and Methods

Materials. GCAT was purified from cell-free culture supernatants of *Aeromonas salmonicida* as previously described (Buckley et al., 1982). Bovine serum albumin (essentially fatty acid free), cholesterol, and aliphatic primary alcohols were purchased from Sigma Chemical Co. [4-^{14}C]Cholesterol (specific activity 58 mCi/mmol) and [1-^{14}C]oleic acid (57 mCi/mmol) were from Amersham Corp. 2-[1-^{14}C]Oleoylphosphatidylcholine was prepared according to the procedure of Pugh & Kates (1975). Individual phospholipids, and phosphatidylcholine molecular species, were obtained from Sigma, Serdary Research, or Supelco Inc. Steroids were supplied by Sigma, Supelco, or Research Plus Steroids. Individual lipids were examined by thin-layer chromatography prior to use and discarded if less than approximately 99% pure.

Preparation of Substrates. Small unilamellar vesicles of the compositions described in the text were prepared by sonication followed by ultracentrifugation at 110000*g* for 1 h to remove undispersed lipid (Barenholtz et al., 1977). The actual amounts of individual lipids in each preparation were quantitatively determined, and the liposomes were used immediately. Unless otherwise specified the chemical compositions of the liposomes were within 10% of the predicted amounts. Mixed micelles containing Triton X-100, phospholipids, and steroids or primary alcohols were prepared as described by Dennis (1973a,b). The ratio of detergent to total

† From the Department of Biochemistry and Microbiology, University of Victoria, Victoria, British Columbia V8W 2Y2, Canada. *Received June 30, 1982.* This study was supported by a grant from the Natural Sciences and Engineering Research Council of Canada.

[1] Abbreviations: CE, cholesteryl ester; chol, cholesterol; PC, phosphatidylcholine; LCAT, lecithin:cholesterol acyltransferase; GCAT, glycerophospholipid:cholesterol acyltransferase; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/82/0421-6699$01.25/0

Table I: Comparison of Phospholipids as Acyl Donors in Sphingomyelin/Cholesterol Liposomes

phospholipid[a]	cholesteryl ester (nmol)	rel[b] act.
PC (egg)	9.0	1.0
PE (heart)	12.6	1.4
PS (brain)	13.5	1.5
PI (brain)	6.8	0.75
CDL (heart)	53.1	5.9
PC (dipalmitoyl)	3.3	0.37
PE (dipalmitoyl)	6.9	0.77
PG (dipalmitoyl)	4.6	0.51

[a] Small unilamellar liposomes were prepared as described in the text from mixtures containing [^{14}C]cholesterol, sphingomyelin, and the appropriate phospholipid (5:4:1 mol/mol/mol). [b] Ratio of cholesteryl ester produced with each phospholipid to cholesteryl ester produced with egg PC.

lipid was adjusted to 2/1 (mol/mol). In both the unilamellar vesicles and the mixed vesicles the ratio of phospholipid to acyl acceptor was normally 1/1 (mol/mol).

Incubation Conditions. In a typical assay, substrates (0.5 μmol each of acceptor and donor lipids in micelles or liposomes) were incubated with the enzyme for 5 min at 40 °C in 0.5 mL of 0.16 M KCl and 0.05 M Tris, pH 7.4. Reaction mixtures for the liposome assays also contained 1.4% (w/v) albumin. Reactions were terminated by the addition of chloroform/methanol (2:1 v/v), and the lipids were extracted as previously described (Buckley et al., 1982).

Lipid Determinations. Neutral lipids were separated by thin-layer chromatography in petroleum ether/ether/acetic acid (70:30:1), or in chloroform/methanol (20:1). Phospholipids were separated by chromatography in chloroform/methanol/acetic acid/water (65:25:4:1). In general, plastic-backed plates (Polygram Sil G, Brinkmann) were used with radioactive lipids and glass-backed Silica Gel H (Merk) plates for subsequent chemical determinations. Spots were located by brief exposure to iodine and either scraped from glass plates and eluted (Arvidson, 1968) or cut from plastic plates and counted in Scintiverse II (Fisher Scientific).

Other Procedures. Phospholipid phosphorus was measured as described by Bartlett (1959) following sample digestion with perchloric acid. Protein was quantitated by the procedure of Peterson (1977).

Results

Two approaches were taken to minimize changes in the physical properties of the substrates while examining the action of GCAT on individual phospholipids. In the first, each of the glycerophospholipids was incorporated into small unilamellar liposomes in which 80% of the total phospholipid was sphingomyelin, which is not a substrate for this enzyme. It was anticipated that, when a matrix of cholesterol and a large amount of sphingomyelin were maintained, structural variation due to the presence of small amounts of the other phospholipids would be small. The action of GCAT on these liposomes is presented in Table I. It may be seen that all of the common glycerophospholipids can be used for acyl transfer to cholesterol and that the more unsaturated phospholipids from natural sources are better substrates than the synthetic dipalmitoyl species which were tested. Cardiolipin consistently was a much better substrate than any other lipid under these conditions.

Dennis (1973b) has used Triton X-100 mixed lipid micelles in order to reduce changes in substrate physical properties which are due to changes in their composition. Results obtained by using similar micelles containing the detergent, cholesterol, and individual phospholipids are shown in Table II. As in the liposomes, all phospholipid classes could act as acyl donors in the transfer reaction. Phosphatidylinositol was also the least effective donor in this system; however, cardiolipin was no more effective than the other lipids. Saturated PC and PE were poor acyl donors, as was observed in the liposomes, but dipalmitoyl-PG, which was a poor substrate in the liposome system, was as effective an acyl donor in micelles as the naturally occurring unsaturated lipids.

Table II: Comparison of Phospholipids as Acyl Donors in Triton Micelles

phospholipid[a]	cholesteryl ester (nmol)	rel[b] act.
PC (egg)	93.0	1.0
PE (heart)	70.8	0.8
PS (brain)	83.7	0.9
PI (brain)	33.4	0.35
CDL (heart)	85.6	0.9
PC (dipalmitoyl)	8.2	0.09
PE (dipalmitoyl)1	11.2	0.12
PG (dipalmitoyl)	93.4	1.0

[a] Each assay contained 0.5 μmol of phospholipid and 0.5 μmol of [^{14}C]cholesterol in 0.5 mL containing 2.0 μmol of Triton X-100 and the enzyme. [b] Ratio of cholesteryl ester produced with each phospholipid to cholesteryl ester produced with egg PC.

Table III: Molecular Species of Phosphatidylcholine as Acyl Donors in PC/Cholesterol Liposomes

phosphatidylcholine[a]	cholesteryl ester (nmol)	rel[b] act.
didecanoyl (C10)	47	0.8
dilauroyl (C12)	103	1.8
dimyristoyl (C14)	85	1.5
dipalmitoyl (C16)	58	1.0
distearoyl (C18)	41	0.7
dioleoyl (C18:1)	173	3.0

[a] Small unilamellar liposomes were prepared by sonication of individual PC molecular species with equimolar cholesterol followed by ultracentrifugation. The measured ratio of each PL to cholesterol was within 10% of 1/1 except for DPPC/cholesterol liposomes in which the ratio was 1.11/1. Incubation time was 10 min at 40 °C. [b] Relative to cholesteryl ester produced using DPPC/cholesterol liposomes.

Table IV: Molecular Species of Phosphatidylcholine as Acyl Donors in Triton Micelles

phosphatidylcholine[a]	cholesteryl ester (nmol)	rel[b] act.
didecanoyl (C10)	321	4.3
dilauroyl (C12)	152	2.1
dimyristoyl (C14)	117	1.6
dipalmitoyl (C16)	74	1.0
distearoyl (C18)	54	0.7
dioleoyl (C18:1)	250	3.4

[a] Each assay contained 0.5 μmol of the individual PC's, 2.0 μmol of Triton X-100, and 0.5 μmol of [^{14}C]cholesterol in 0.5 mL of 0.25% Triton X-100, pH 7.4. Incubation time was 10 min at 40 °C. [b] Relative to cholesteryl ester produced using DPPC/cholesterol.

The results above indicated that the nature of the fatty acyl chains is a major determinant of the effectiveness of phospholipids as acyl donors. Therefore the influence of fatty acid composition on reaction rate was studied with PC–cholesterol liposomes and the micelle system described above. The data in Table III indicate that among saturated phosphatidylcholines in liposomes, dilauroyl-PC is the preferred substrate but that an unsaturated species such a dioleoyl-PC is considerably more effective than any of the saturated PC's tested.

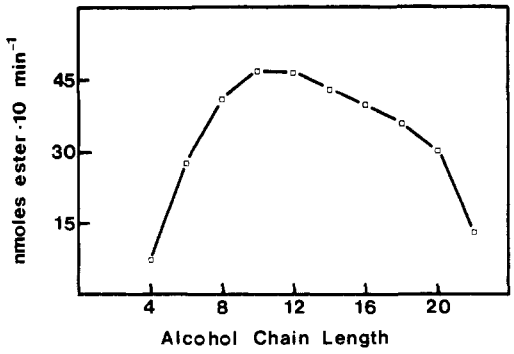

FIGURE 1: Straight-chain primary aliphatic alcohols as acyl acceptors in Triton/phosphatidylcholine mixed micelles. Each of the alcohols indicated (0.5 μmol) was incubated with 2 μmol of Triton and 0.5 μmol of egg PC containing 2-[^{14}C]oleoyl-PC in 0.5 mL, pH 7.4, for 10 min.

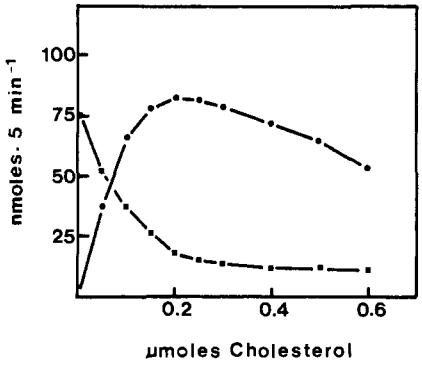

FIGURE 2: Acyl transfer and hydrolysis at the 2 position of phosphatidylcholine as a function of cholesterol concentration. The specified amounts of cholesterol were added to 0.5 μmol of PC and 2 μmol of Triton in 0.5 mL. Production of ^{14}C-labeled fatty acid (■) and [^{14}C]cholesteryl ester (●) was measured after 5-min incubation.

The results obtained using detergent–lipid micelles (Table IV) also pointed to the enzyme's preference for short-chain or unsaturated phosphatidylcholine acyl donors. However, in the micelles the enzyme's activity did not peak with disaturated C12 PC but was highest with didecanoyl-PC, the smallest substrate tested.

Mammalian lecithin:cholesterol acyltransferase is known to carry out acyl transfer to many primary alcohols in addition to cholesterol (Kitabatake et al., 1979), although the enzyme appears to have an absolute requirement for phosphatidylcholine as the acyl donor (Glomset & Norum, 1973). The results in Figure 1 show that the microbial enzyme is also capable of using aliphatic alcohols as acyl acceptors in the micelle system and that among straight chain primary alcohols, *n*-decanol is preferred. In fact virtually all relatively hydrophobic alcohols tested were enzyme substrates although none were as effective as acyl acceptors as cholesterol (data not shown). The action of GCAT in Triton micelles containing PC and increasing amounts of cholesterol is shown in Figure 2. It may be seen that the amount of CE produced increases as the cholesterol concentration increases and that there is a parallel decrease in the appearance of fatty acid (due to simple enzymatic hydrolysis at the 2 position). Hence it would appear that the total loss of fatty acid from this position due to both acyl transfer and hydrolysis is constant and that the proportion of fatty acid appearing as CE depends on the amount of cholesterol relative to PC. A similar observation was made with the aliphatic alcohols as acceptors. Thus relatively less free fatty acid was generated in micelles containing a good acceptor such as *n*-decanol than in micelles containing a poor acceptor such as *n*-butanol (data not shown).

Table V: Comparison of 3-OH Steroids as Acyl Acceptors

steroid[a]	steroid ester (nmol)	free fatty acid (nmol)	total from 2 position of PC (nmol)
cholesterol	83.3	12.9	96.2
epicholesterol	0.4	46.9	47.3
coprostanol	0.3	59.6	59.9
androsterone	0.0	41.6	41.6
epiandrosterone	76.4	25.4	101.8
pregnenolone	72.0	32.4	104.4
5α-androstan-3β-ol	68.3	18.7	87.0
5-androstan-3β-ol-17β-carboxylic acid	37.7	59.6	97.3
5α-androstan-3β-ol-11,17-dione	76.7	29.8	106.5
none		80.5	80.5

[a] 0.5 μmol of each steroid was incubated with 0.5 μmol of egg phosphatidylcholine containing 2-[^{14}C]oleoyl-PC, 2 μmol of Triton X-100, and the enzyme in a total volume of 0.5 mL. Incubation time was 5 min at 40 °C.

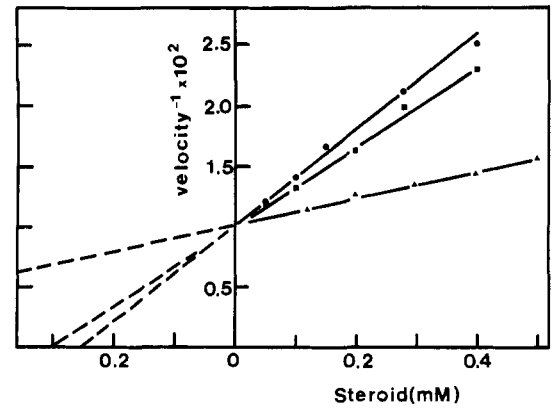

FIGURE 3: Inhibition of acyl transfer to cholesterol by other steroids. Each steroid was incubated with enzyme for 5 min in the Triton mixed micelle system as described in Table V. (●) Androsterone, (■) epicholesterol, and (▲) coprostanol.

Because cholesterol was such a good acyl acceptor, it seemed conceivable that the enzyme specifically recognized a portion of the steroid ring structure. In Table V results obtained by using several other steroids are presented. It may be seen that epicholesterol, in which the hydroxyl at C-3 is below the ring plane (α), and coprostanol, which contains a cis A:B ring fusion, are both virtually inactive as enzyme substrates. Similarily, androsterone, which like epicholesterol contains a 3α-OH, would not act as an acyl acceptor. Other steroids which shared the 3β-OH and the trans A:B rings with cholesterol were also effective enzyme substrates. Clearly an unsubstituted C ring and a side chain at C-17 were not requirements as 5α-androsten-3β-ol-11,17-dione and 5α-androstan-3β-ol were both enzyme substrates. The introduction of a negative charge at the position of the side chain significantly lowered activity; however, 5-androsten-3β-ol-17β-carboxylic acid was nevertheless a far better acyl donor than epicholesterol. The results in Table V also indicate that the total rate of removal of fatty acid from the 2 position of PC was significantly lower in the presence of each of the three steroids which were not enzyme substrates, suggesting that they were inhibiting the reaction. This was confirmed by measuring the rate of cholesterol esterification in the presence of increasing concentrations of the inhibitors. The results are presented as Dixon plots in Figure 3. It can be seen that each of the steroids was an inhibitor of cholesterol esterification and that the apparent inhibitor constants were much lower for the

two 3β-OH steroids than for coprostanol.

Discussion

The bacterial acyltransferase appears to be less specific than cobra venom phospholipase A_2 (Roberts et al., 1978) and much less specific than mammalian LCAT (Glomset & Norum, 1973) in its reaction with phospholipids. Thus all of the major classes of glycerophospholipids are acyl donors, and although phosphatidylinositol seems less suitable than the others, in general the range of activity between classes is relatively narrow. Cardiolipin is a considerably better substrate than the other naturally occurring phospholipids in the liposome system but no better in mixed micelles. The reason for this is not apparent, nor is it clear why dipalmitoylphosphatidylglycerol is a more effective substrate than the other saturated lipids in the micelle system but not in liposomes.

The results in these tables and the results obtained by using different molecular species of phosphatidylcholine (Tables III and IV) clearly indicate that the enzyme has a strong preference for phospholipids carrying short-chain or unsaturated fatty acids. Interestingly, reactivity appears to peak with dilauroyl-PC in the liposome system but continues to increase up to didecanoyl-PC in the micelles. Assman et al. (1978) have shown that the activity of human LCAT toward saturated PC's in 10/1 PC/cholesterol liposomes is maximal using dimyristoyl-PC but that unsaturated PC's containing fatty acids with one or two double bonds are better acyl donors than their saturated homologues. In addition, Roberts et al. (1978) have demonstrated that the enzymatic activity of phospholipase A_2 and phospholipase C increases as the fatty acid chain length decreases in Triton mixed micelles of phosphatidylcholine. These authors argued that the effect of chain length was a reflection of optimization of the interfacial properties of the substrate. It seems quite possible that if this is so, a different chain length might be required to optimize liposome substrates and mixed micelles.

As with both mammalian LCAT (Norby & Norum, 1975; Kitabatake et al., 1979) and rat liver phospholipase A_1 (Waite & Sisson, 1974), a variety of alcohols were able to act as acyl acceptors. Among a series of straight-chain aliphatic alcohols, *n*-decanol was the best acceptor, although it and all the other alcohols tested were much poorer substrates than cholesterol. Kitabatake et al. (1979) have shown using a somewhat different liposome system that human LCAT prefers *n*-hexadecanol among saturated alcohols, and these authors also found cholesterol to be the preferred substrate. The consistent observation that acyl transfer rates were highest with mixtures of a phospholipid and cholesterol implies that the function of the enzyme in vivo is not in cellular metabolism as the bacteria contain no cholesterol. For this reason and because the enzyme is a secretory product of the organism, it seems more likely that it is used in the degradation of eucaryotic plasma membranes.

Perhaps the most surprising aspect of this work is the remarkable specificity the microbial enzyme shows toward steroid acyl acceptors in the Triton mixed micelle system. The results in Table IV show that for acyl transfer from PC to a steroid there is a very strong preference for a planar ring system and a β-OH at the 3 position of the acceptor. One obvious question is whether this requirement is due to specific binding of the steroid to the enzyme or due to an equally specific interaction between the steroid and PC in the micelle. The enzyme may have separate binding sites for acceptor and donor as is the case with phospholipase A_1 (Waite & Sisson, 1974); however, the acceptor site must be flexible enough to allow the binding of a variety of unrelated alcohols yet specific enough to discriminate against steroids such as coprostanol and epicholesterol. In addition if two sites are involved, then the orientation of acyl donor and acceptor must be highly selective as I have shown that acyl transfer is 2 position specific and that simple hydrolysis occurs at the 1 position of egg PC. Furthermore 1-acyllysophosphatidylcholine is not an acyl donor, yet it is hydrolyzed at the same rate as PC (Buckley et al., 1982). Alternatively, the specificity of the steroid interaction could be directed by prior binding of the phospholipid. This seems unlikely, however, as there would be nothing to prevent the rapid hydrolysis which is observed in the absence of the steroid.

It has been known for some time that a planar (trans-fused) ring system and an unblocked equatorial (β) hydroxyl group at position C-3 are required for optimal interaction of steroids and phospholipids in artificial systems (Brockerhoff, 1974; Jain, 1975; Demel & DeKruyff, 1976; Brainard & Cordes, 1981). The molecular basis for these requirements nevertheless remains controversial. One popular view suggests that the steroid hydroxyl forms a linear hydrogen bond with one of the carbonyl oxygens of the phospholipid (Brockerhoff, 1974; Huang, 1976, 1977a,b). Such an interaction may account for the specificity of the transacylation observed here. Thus binding of the enzyme to Triton micelles containing PC/cholesterol complexes would result in acyl transfer of the fatty acid at the two position, due to the very close proximity of the cholesterol hydroxyl in the complex and perhaps due to steroid–carbonyl hydrogen bonding at this position as suggested by Brockerhoff (1974). The ester bond at the 1 position of PC would simply be hydrolyzed as there is no interaction of it with cholesterol and the effective concentration of water would therefore be far greater than the steroid concentration. Similarily, binding to LPC/steroid complexes also would result in simple hydrolysis as the steroid would be improperly positioned for acyl transfer and therefore unable to compete with water. Steroids which can interact with PC to form complexes in which the hydroxyl group is not properly positioned to permit transacylation would lower the reaction rate by competing with cholesterol for PC, not by competing for a specific binding site on the enzyme.

Clearly additional evidence is required to establish the nature of the active substrate complex; however, the steroid specificity of the reaction demonstrated here adds another facet to an already interesting new enzyme. Both Norby & Norum (1975) and Kitabatake et al. (1979) have examined mammalian LCAT in a liposome system using several plant steroids as cholesterol substitutes, but there has been no detailed study of the activity of LCAT toward steroids such as those used here. It will be interesting to see if the mammalian enzyme has similar requirements and especially to examine its activity toward some of the naturally occurring steroid hormones and their derivatives.

Acknowledgments

I thank U. Rink for her technical assistance and Dr. S. MacIntyre for her early involvement in this work.

References

Arvidson, G. A. E. (1968) *Anal. Biochem. 38*, 423.

Assman, G., Schmitz, G., Donath, N., & Lekim, D. (1978) *Scand. J. Clin. Lab. Invest. 35*, 677.

Barenholtz, Y., Gibbes, D., Litman, B. J., Goll, J., Thompson, T. E., & Carlson, F. D. (1977) *Biochemistry 16*, 2806.

Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 466.

Brainard, J. R., & Cordes, E. H. (1981) *Biochemistry 20*, 4607.

Brockerhoff, H. (1974) *Lipids 9*, 645.
Buckley, J. T., Halasa, N., & MacIntyre, S. (1982) *J. Biol. Chem. 257*, 3320.
Demel, R. A., & deKruyff, B. (1976) *Biochim. Biophys. Acta 457*, 109.
Dennis, E. A. (1973a) *Arch. Biochem. Biophys. 158*, 485.
Dennis, E. A. (1973b) *J. Lipid Res. 14*, 152.
Glomset, J. A., & Norum, J. R. (1973) *Adv. Lipid Res. 11*, 1.
Huang, C. H. (1976) *Nature (London) 259*, 242.
Huang, C. H. (1977a) *Lipids 12*, 348.
Huang, C. H. (1977b) *Chem. Phys. Lipids 19*, 150.
Jain, M. K. (1975) *Curr. Top. Membr. Transp. 6*, 1.
Kitabatake, K., Piran, U., Kamio, Y., Doi, Y., & Nishida, T. (1979) *Biochim. Biophys. Acta 573*, 145.
MacIntyre, S., & Buckley, J. T. (1978) *J. Bacteriol. 135*, 402.
MacIntyre, S., Trust, T. J., & Buckley, J. T. (1979) *J. Bacteriol. 139*, 132.
Norby, G., & Norum, K. R. (1975) *Scand. J. Lab. Clin. Invest. 35*, 677.
Peterson, G. L. (1977) *Anal. Biochem. 83*, 346.
Pugh, E. L., & Kates, M. (1975) *Biochim. Biophys. Acta 380*, 442.
Roberts, M. F., Otnaess, A.-B., Kensil, C. A., & Dennis, E. A. (1978) *J. Biol. Chem. 253*, 1252.
Waite, M., & Sisson, P. (1974) *J. Biol. Chem. 249*, 6401.

Secondary α-Hydrogen Isotope Effects on the Interaction of 5-Fluoro-2′-deoxyuridylate and 5,10-Methylenetetrahydrofolate with Thymidylate Synthetase†

Thomas W. Bruice and Daniel V. Santi*

ABSTRACT: Secondary α-hydrogen isotope effects have been used to study the covalent interactions of FdUMP and (6*R*)-L-5,10-methylene-5,6,7,8-tetrahydrofolate (CH_2-H_4folate) with dTMP synthetase. Dissociation of 6-tritiated FdUMP from the FdUMP–CH_2-H_4folate–dTMP synthetase ternary complex proceeds with a large α-hydrogen secondary kinetic isotope effect (k_H/k_T = 1.23). In contrast there is no isotope effect upon initial formation of the complex, but isotopic equilibration slowly occurs with $k = 5.5 \times 10^{-2}$ h^{-1}. The equilibrated complex is enriched in tritium, and the equilibrium isotope effect determined by two independent experimental methods was K_H/K_T = 1.24. This is in excellent agreement with values calculated from simple models and provides the first experimental verification of a fractionation factor for -CR_2CHTSR. Together with other data, these results lead us to conclude that the covalent bond between the cysteine residue of the enzyme and the 6 position of FdUMP is formed after the rate-determining step in the formation of the complex and, by microscopic reversibility, is cleaved before the slow step in dissociation of the complex. Dissociation of [^{3}H]-CH_2-H_4folate from the ternary complex occurs with a normal secondary kinetic isotope effect of k_H/k_T = 1.03 which is some 8% higher than the calculated equilibrium isotope effect between bound and free cofactor. We explain this by proposing that the normal isotope effect which accompanies formation of the putative 5-iminium ion of the cofactor from the complex during dissociation is not completely canceled by the inverse isotope effect which accompanies its subsequent conversion to CH_2-H_4folate in the rate-determining step of the reaction. The rate-determining step may also involve a conformational change of the enzyme, driven by reversible interactions of the cofactor and the protein, which may play a role in the interconversion of CH_2-H_4folate and the 5-iminium ion in the normal enzymic reaction as well as in formation of the FdUMP–CH_2-H_4folate–dTMP synthetase complex.

Thymidylate (dTMP) synthetase (EC 2.1.1.45) catalyzes the conversion of dUMP and CH_2-H_4folate[1] to dTMP and H_2-folate. Much of what is currently known of the mechanism of this enzyme has been derived from studies of its interaction with the mechanism-based inhibitor FdUMP (cf. Pogolotti & Santi, 1977; Danenberg, 1977). This inhibitor participates in two or more steps of the normal enzymic reaction and results in the formation of a stable complex in which the 6 position of FdUMP is covalently bound to the nucleophilic catalyst of the enzyme and the 5 position is linked to the one-carbon unit of the cofactor. Since the ternary complex is structurally analogous to a steady-state intermediate of the normal enzymic reaction, studies of the mechanism of its formation provide an accessible model for understanding features of the partial catalytic reaction it mimics. In this paper we describe secondary α-hydrogen isotope effects at C-6 of FdUMP and the one-carbon unit of CH_2-H_4folate which occur during formation/dissociation of the complex. The results of these studies

† From the Department of Biochemistry and Biophysics and Department of Pharmaceutical Chemistry, University of California, San Francisco, California 94143. *Received July 20, 1982.* This work was supported by U.S. Public Health Service Grant CA 14394 from the National Cancer Institute. T.W.B. was the recipient of a National Institutes of Health Predoctoral Training Grant Fellowship T32 CA09270. This work is described in the Ph.D. dissertation submitted by T.W.B. to the University of California.

* Address correspondence to this author at the Department of Biochemistry and Biophysics, University of California.

[1] Abbreviations: CH_2-H_4folate, (6*R*)-L-5,10-methylene-5,6,7,8-tetrahydrofolate; CH_2-H_4PteGlu$_5$, (6*R*)-L-5,10-methylene-5,6,7,8-tetrahydropteroylpentaglutamate; H_2folate, 7,8-dihydrofolate; FdUMP, 5-fluoro-2′-deoxyuridylate; Tris, tris(hydroxymethyl)aminomethane; NMM, *N*-methylmorpholine; EDTA, ethylenediaminetetraacetic acid; KIE, kinetic isotope effect; FF, fractionation factor; HPLC, high-performance liquid chromatography. All other abbreviations are as suggested by IUPAC.

0006-2960/82/0421-6703$01.25/0