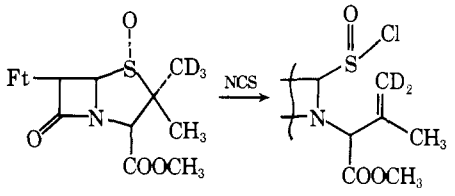
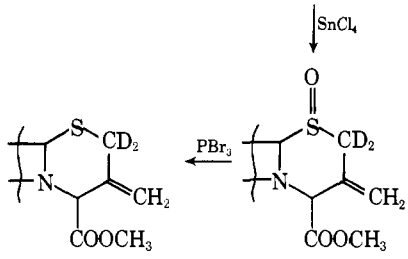
mp 198-201° (CH_2Cl_2/cyclohexane); NMR ($CDCl_3$) δ 3.80 (s, 3, CO_2CH_3), 5.32 (m, 3, exomethylene and C_4-H), 5.46 (d, 1, J = 4.5 Hz, C_6-H), 5.67 (d, 1, J = 4.5 Hz, C_7-H), and 7.03 (m, 4, ArH). The NMR spectrum exhibited only a very small (less than 5%) signal for protons at the C_2 position while the signal for the 3-exomethylene group was of normal intensity, indicating selective incorporation of the CD_2 group into the 2-position.[14]

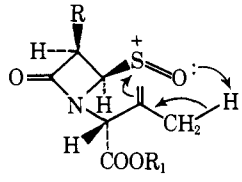


IV V



VII VI

A sulfinium cation VIII is a probable intermediate in the ring closure of sulfinyl chlorides II with Lewis acids, and the mechanism can be visualized as an intramolecular ene reaction.[15]



VIII

It seemed that if the mechanism does involve the intermediacy of a sulfinium cation VIII that any other derivative of the sulfinic acid capable of forming such an intermediate might also be used as a starting material for the synthesis of 3-methylene cephams. In fact, using Bronsted acids (e.g., H_2SO_4, H_3PO_4, CH_3SO_3H), we have been able to cyclize the sulfinic acid (II, X = OH), and some of its derivatives such as esters (II, X = OCH_3), thiol esters (II, X = S-*i*-Pr), amides (II, X = NHPh), imides (II, X = succinimido), and hydrazides (II, X = N(COOR)NH(COOR)). These reactions will be described at a later date in a full paper [16]

Future publications from these laboratories will demonstrate the utilization of 3-methylenecephams in the preparation of various 3-substituted cephalosporins.

Acknowledgment. We are grateful to Drs. M. Gorman and W. A. Spitzer for many helpful discussion.

References and Notes

(1) Azetidinone antibiotics. 17. Paper 16. S. Kukolja, M. R. Gleissner, A. I. Ellis, D. E. Dorman, and J. W. Paschal, *J. Org. Chem.*, **41**, 2276 (1976).
(2) R. B. Morin, B. G. Jackson, R. A. Mueller, E. R. Lavagnino, W. B. Scanlon, and S. L. Andrews, *J. Am. Chem. Soc.*, **85**, 1896 (1963); **91**, 1401 (1969).
(3) C. W. Ryan, R. L. Simon, and E. M. Van Heyningen, *J. Med. Chem.*, **12**, 310 (1969).
(4) R. R. Chauvette and P. A. Pennington, *J. Am. Chem. Soc.*, **96**, 4986 (1974); R. Scartazzini and H. Bickel, *Helv. Chim. Acta*, **57**, 1919 (1974); **58**, 2437 (1975); and ref 12 in this paper.
(5) S. Kukolja and S. R. Lammert, *Angew. Chem., Int. Ed. Engl.*, **12**, 67 (1973). In addition to NCS, other halogenating agents have been also used successfully, e.g., *N*-bromosuccinimide, *N*-chlorophthalimide, *N*-chloro-*N*-methyl-*p*-toluenesulfonamide, *N,N*-dichlorourethane, *tert*-butyl hypochlorite.
(6) When 6-amidopenicillin sulfoxides are employed in this rearrangement the best yields are obtained when the reaction is carried out in the presence of acid scavengers.[16]
(7) The other Lewis acids, such as $TiCl_4$, $AlCl_3$, $ZnCl_2$, $ZnBr_2$, $SbCl_5$, $HgCl_2$, $FeCl_3$, and $ZrCl_4$, were also successfully used for a ring closure of II.
(8) A rearrangement of I to III via the sulfinyl chloride (II) can be performed in two separate steps. However, it is preferable to carry out these two steps as a one pot reaction. For example, (a) a mixture of 18.8 g of methyl 6-phthalimidopenicillanate 1-oxide and 6.7 g of NCS in 1 l. of CCl_4 was refluxed for 70 min, cooled to ca. 25°, and then 6 ml of $SnCl_4$ was added and the reaction mixture was stirred for 45 min. After workup, 18.4 g of a mixture of *R* and *S* sulfoxides of the corresponding 3-methylenecephams was obtained. (b) p-Nitrobenzyl 6-phenoxyacetamidopenicillanate 1-oxide (6.0 g), 1.8 g of NCS, and 500 ml of toluene were refluxed for 90 min, cooled to ca. 50°, and then 1.8 g of $SnCl_4$ was added and the reaction mixture stirred for 90 min. After workup, 2.16 g of *p*-nitrobenzyl 7-phenoxyacetamido-3-methylenecepham-4-carboxylate 1-oxide was obtained.
(9) All new compounds gave good mass spectral or elemental analyses.
(10) G. V. Kaiser, R. D. G. Cooper, R. E. Koehler, C. F. Murphy, J. A. Webber, I. G. Wright, and E. M. Van Heyningen, *J. Org. Chem.*, **35**, 2430 (1970).
(11) Compounds with the other side chains and acid protective groups were also cyclized under a similar condition. The details will be published in a full paper.[16]
(12) R. R. Chauvette and P. A. Pennington, *J. Org. Chem.*, **38**, 2994 (1973).
(13) R. D. G. Cooper, *J. Am. Chem. Soc.*, **92**, 5010 (1970).
(14) Mass spectral analysis gave the following deuterium distribution in the C_2 position: d_0, 2.2%; d_1, 25.5% d_2, 72.3% ± 2%.
(15) H. M. R. Hoffmann, *Angew. Chem., Int. Ed. Engl.*, **8**, 556 (1969); J. A. Marshall, N. H. Andersen, and P. C. Johnson, *J. Org. Chem.*, **35**, 189 (1970); K. B. Sharpless and T. Hori, *ibid.*, **41**, 176 (1976); P. E. Peterson, R. Brockington, and M. Dunham, *J. Am. Chem. Soc.*, **97**, 3517 (1975).
(16) S. Kukolja, S. R. Lammert, M. R. Gleissner, A. I. Ellis, J. L. Pfeil, T. S. Chou, J. R. Burgtorf, W. A. Spitzer, T. Goodson, I. G. Wright, M. A. Schmoll, R. R. Chauvette, and P. A. Pennington, unpublished results.

Stjepan Kukolja,* Steven R. Lammert
Michael R. B. Gleissner, Alvin I. Ellis
The Lilly Research Laboratories, Eli Lilly and Company
Indianapolis, Indiana 46206
Received April 16, 1976

5-Iodoacetamidomethyl-2′-deoxyuridine 5′-Phosphate. A Selective Inhibitor of Mammalian Thymidylate Synthetases

Sir:

In 1959 Baker proposed a unique approach to the control of cancer by drugs that are designed to detect differences between cancerous and normal cells.[1] The theory is based on the fact that, although an enzyme derived from different sources catalyzes the same transformation, the primary structure of the enzyme may vary with the source.[2] This variation in primary structure, in all probability, will not alter the structure of the active site; however, it is reasonable to assume tertiary structural differences will be detectable in other portions of the various isozymes.

We wish to report evidence for the first example of selective irreversible inhibition of thymidylate synthetase (EC 2.2.2.6).[3] 5-Iodoacetamidomethyl-2′-deoxyuridine 5′-phosphate (I), a competitive inhibitor of thymidylate synthetases obtained from calf thymus and Ehrlich ascites tumor, is an irreversible inhibitor of the tumor, but, not the thymus enzyme. It is proposed that the tertiary structural differences existing near the active site of the two enzymes are such that the alkylating portion of I is excluded from the reactive nucleophile in the thymus enzyme. 5-Fluoro-2′-deoxyuridine 5′-phosphate is an irreversible inhibitor of thymidylate synthetase; however, there is no evidence that it is isoenzyme specific.[3]

5-Iodoacetamidomethyl-2′-deoxyuridine 5′-phosphate (I) was prepared from the corresponding nucleoside[4] by selective chemical phosphorylation using phosphorus oxychloride according to the procedure of Yoshikawa and co-workers.[5] Iodoacetamide (II) used was a recrystallized sample of a preparation obtained from Aldrich Chemical Co.

Thymidylate synthetase was extracted from fresh calf thymus according to the method of Jenny and Greenberg[6] through the ammonium sulfate fractionation step. Ehrlich ascites tumor

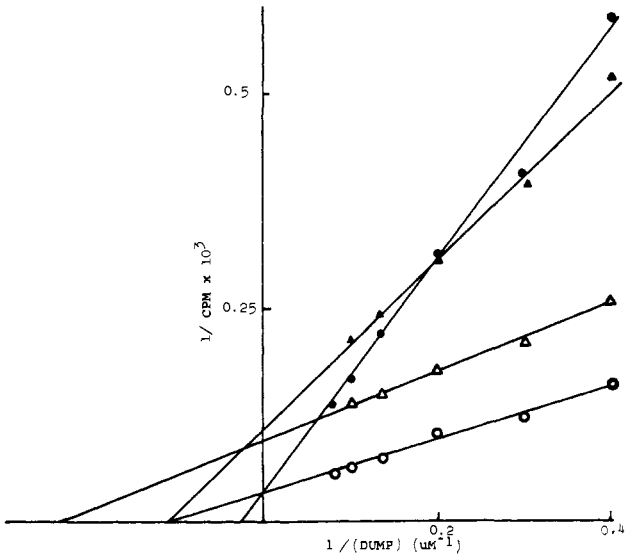


Figure 1. Reciprocal plot of inhibition of calf thymus and Ehrlich ascites tumor thymidylate synthetase by 5-iodoacetamidomethyl-2′-deoxyuridine 5′-phosphate (I): calf thymus, no inhibitor (○); calf thymus, [I] = 1.12 × 10^{-4} M (●); ascites tumor, no inhibitor (△); ascites tumor [I] = 6.2 × 10^{-5} M (▲).

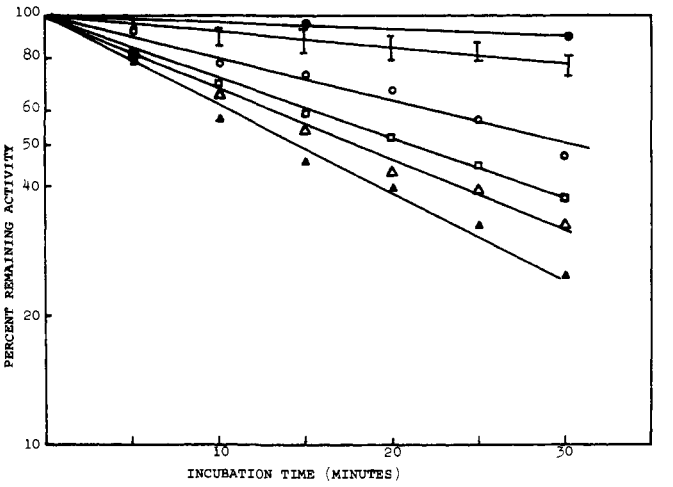


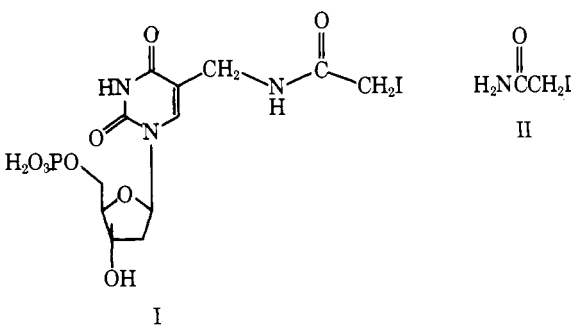
Figure 2. Plot of percent remaining activity of calf thymus and Ehrlich ascites tumor thymidylate synthetase after preincubation with 5-iodoacetamidomethyl-2′-deoxyuridine 5′-phosphate (I): no inhibitor, both enzymes (●); calf thymus enzyme plus I at 15, 46, 155, and 465 μM (I, bars indicate range of remaining activity); ascites tumor enzyme plus 46 μM of I (○); ascites tumor enzyme plus 87 μM of I (□); ascites tumor enzyme plus 155 μM of I (△), ascites tumor enzyme plus 465 μM of I (▲).

cells harvested 8 days after innoculation of male BDF-1 mice were used in the preparation of a cell-free extract of thymidylate synthetase according to the procedure of Fridland and co-workers.[7]

The assay reported by Roberts[8] was modified. For assay of the thymus enzyme the reaction mixture contained 0.02 M formaldehyde, 0.02 M magnesium chloride, 0.05 M Tris HCl pH 7.4, 0.05 M 2-mercaptoethanol, 0.0005 M tetrahydrofolic acid (Sigma Chemical Corp), 5-[^{3}H]dUMP (2.5 to 10 × 10^{-6} M, 400 μCi/μM) (Schwartz-Mann), enzyme, and inhibitor when required. For assay of the ascites tumor enzyme the reaction mixture contained 0.0033 M formaldehyde, 0.033 M mercaptoethanol, 0.1 M phosphate buffer pH 6.7, 0.0002 M tetrahydrofolic acid, substrate, and inhibitor. Controls lacked the cofactor, tetrahydrofolic acid. Assays were run in a total volume of 0.1 ml at 37 °C for 7 min using the ascites tumor enzyme and for 10 min using the thymus enzyme. The reaction was stopped by the addition of 50 μl of 20% trichloroacetic acid and the unreacted substrate was removed by adding 0.25 ml of a 20% suspension of activated charcoal. After standing 15 min the mixture was filtered through a glass wool plugged Pasteur pipette and 0.1 ml of the filtrate was counted in a scintillation fluid containing 0.5% 2,5-diphenyloxazole and 10% Beckman BBS-3 solubilizer in toluene.

The K_m for the thymus preparation measured against the substrate 2′-deoxyuridine 5′-phosphate was 7.8 μM (Figure 1); the reported K_m for the purified thymus enzyme is 9 μM.[9] Ascites tumor enzyme showed a K_m of 4.4 μM (Figure 1), comparable to the reported value of 6.3 μM.[7]

Preincubation of the enzyme with the inhibitors I and II was done in the absence of the cofactor, tetrahydrofolate, and mercaptoethanol since these are both strong nucleophiles and lead to inactivation of the inhibitors. The preincubation mixture for both enzymes contained 0.0033 M formaldehyde and 0.1 M phosphate buffer pH 6.7. At the specified times samples were assayed by additions to give in 0.1 ml 0.033 M formaldehyde, 0.1 M phosphate buffer pH 6.7, 0.0033 M 2-mercaptoethanol, 0.0002 M tetrahydrofolic acid, and 1.25 × 10^{-5} M [5^3H]-2′-deoxyuridine 5′-phosphate (400 μCi/μM). The assays for remaining activity were run as described for the K_m and K_I studies.



I

II

Analysis of the inhibition, without preincubation, by reciprocal plotting (Figure 1) shows that both enzymes were inhibited by I and that the inhibition is competitive with the substrate 2′-deoxyuridine 5′-phosphate. The K_I calculated from Figure 1 for the thymus enzyme is 27 μM and that for the ascites enzyme is 68 μM.

Iodoacetamide has been shown to inactivate ascites tumor thymidylate synthetase and this inactivation was prevented by incubation with the substrate.[10] In our studies 460 μM iodoacetamide (II) showed a half life for inactivation of the thymus enzyme of 30 min. Compound I was less effective against the thymus enzyme; 25% inactivation was observed after 30 min incubation using 465 μM of I.

Analysis of the ascites tumor enzyme showed a half life of 17 min using 460 μM iodoacetamide (II) and 14 min using 460 μM I. Comparing the lower concentration of inhibitors, 46 μM iodoacetamide (II) showed about 15% inactivation at 30 min whereas 46 μM of I showed 50% inactivation (Figure 2).

It is clear from Figure 1 that compound I is an active site inhibitor (competitive with substrate) of both the thymus and ascites enzymes. However, on incubation, compound I shows little inactivation of the thymus enzyme and the inactivation is not dependent on the concentration of I (Figure 2).[11] In contrast compound I is a potent irreversible inhibitor of the ascites tumor enzyme (Figure 2). These results coupled with the fact that I is more potent than iodoacetamide (II) suggest that I is a species isozyme specific active-site-directed irreversible inhibitor of the ascites tumor enzyme. Although thymidylate synthetase isozymes have not been shown to exist in these systems they have been demonstrated in host and phage infected host.[12] Reported molecular weights for thymidylate synthetases are 77 500 for the thymus enzyme[9] and 67 000 to 71 000 for the ascites tumor enzyme.[7]

Evidence is presented that inactivation of the ascites tumor

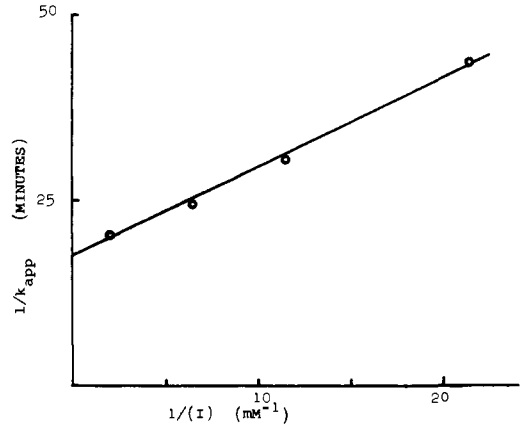


Figure 3. Irreversible inhibition of Ehrlich ascites tumor thymidylate synthetase by 5-iodoacetamidomethyl-2′-deoxyuridine 5′-phosphate (I). Double reciprocal plot of the pseudo-first-order rate constant of inactivation (k_{app}) vs. inhibitor concentration. The values k_3 and K_I were determined from the intercept and slope.

enzyme proceeds through the dissociable complex EI in the equation:

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [EI] \xrightarrow{k_3} [EI^*]$$

The model[13] for this type of inhibition has been described for cholinesterase,[14] trypsin,[15] and carboxypeptidase.[16]

A plot of the reciprocal of the pseudo-first-order rate constants ($1/k_{app}$, obtained from the slope of the lines in Figure 2) vs. the reciprocal of the concentration of I (Figure 3) according to the equation

$$1/k_{app} = \frac{K_I}{k_3(I)} + 1/k_3$$

gives a k_3 for the rate of inactivation of 0.057 min^{-1} and K_I of 70 μM which is in reasonable agreement with a K_I of 68 μM for I calculated from the reciprocal plot shown in Figure 1.

References and Notes

(1) B. R. Baker, *Cancer Chemother. Rep.*, **4**, 1 (1959).
(2) Isozymes are defined as structurally different enzymes catalyzing the same transformation. For an extensive discussion of the control of cancer through isozyme specificity refer to B. R. Baker, "Design of Active-Site-Directed Irreversible Enzyme Inhibitors", Wiley, New York, N.Y., 1967.
(3) M. Friedkin, *Adv. Enzymol.*, **38**, 235 (1973); P. Reyes and C. Heidelberger, *Mol. Pharmacology*, **1**, 14 (1965).
(4) A. Kampf, C. J. Pillar, W. J. Woodford, and M. P. Mertes, *J. Med. Chem.*, **19**, 909 (1976).
(5) M. Yoshikawa, T. Kato, and T. Takenishi, *Bull. Chem. Soc. Jpn.*, **42**, 3505 (1969).
(6) E. Jenny and D. M. Greenberg, *J. Biol. Chem.*, **238**, 3378 (1963).
(7) A. Fridland, R. J. Langenbach, and C. Heidelberger, *J. Biol. Chem.*, **246**, 7110 (1971).
(8) D. Roberts, *Biochemistry*, **5**, 3546 (1966).
(9) H. Horinishi and D. M. Greenberg, *Biochim. Biophys. Acta*, **258**, 741 (1972).
(10) (a) R. J. Langenbach, P. V. Danenberg, A. Fridland, W. W. Cleland, and C. Heidelberger, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, **31**, 419 (1971); (b) P. V. Danenberg, P. J. Langenbach, and C. Heidelberger, *Biochemistry*, **13**, 926 (1974).
(11) The rate of inactivation of the thymus enzyme by compound I (Figure 2) was relatively unchanged over the concentration range of 15–465 μM. This low activity was not a result of decomposition of I in the preincubation media since additions of I at various times during incubation did not alter the rate of inactivation. Furthermore, after incubation of I at 37 °C in the buffer for 6 h, no decomposition could be detected. In contrast after 6 h at 37 °C in 0.5 M KOH, 70% of I remained unchanged.
(12) S. W. Krauss, B. D. Stollar, and M. Friedkin, *J. Virol.*, **11**, 783 (1973).
(13) K. F. Tipton, *Biochem. Pharmacol.*, **22**, 2933 (1973).
(14) R. Kitz and I. B. Wilson, *J. Biol. Chem.*, **237**, 3245 (1962).
(15) E. Shaw and G. Glover, *Arch. Biochim. Biophys.*, **139**, 298 (1970).
(16) G. M. Hass and H. Neurath, *Biochemistry*, **10**, 3535 (1971).

Roxane L. Barfknecht, Rocio A. Huet-Rose
Arieh Kampf, Mathias P. Mertes*
Department of Medicinal Chemistry
The University of Kansas
Lawrence, Kansas 66045

Received March 29, 1976

^{13}C Nuclear Magnetic Resonance Probe of the Active Site of Carbonic Anhydrase

Sir:

We wish to report the successful introduction of a ^{13}C NMR probe into the active site of carbonic anhydrase (carbonate hydrolyase E.C. 4.2.1.1) and its initial use to elucidate important ionization features of the active site. The catalytic activity of this zinc metalloenzyme[1] is known to depend on the ionization of an active site group with p$K_a \sim 7$ whose identity remains in considerable dispute.[1-4] Human carbonic anhydrase B (HCAB) was reacted with 90% [1-^{13}C]bromoacetic acid, an active site directed reagent, under conditions[5] known to lead to the highly specific N$^{\tau}$-monocarboxymethylation of histidine 200 in the amino acid sequence.[6] This active site residue has been found in the crystal structure to lie within a few Angstroms of the essential zinc metal.[7] The carboxymethylated enzyme (CmHCAB) is known to possess its own intrinsic CO_2 hydration[8] and esterase[9] activities that vary between 2.5 and 20% depending on pH and substrate used.

A ^{13}C NMR spectrum of the enzyme modified with enriched bromoacetic acid shows only one peak that is not present in either HCAB or unenriched CmHCAB.[10] This relatively sharp (about 6 Hz line width) resonance is about three times larger than the underlying broad envelope due to the natural abundance signals from the 314 other carbonyl carbons of this 28 900 mol wt enzyme. It is thus due to the *single carbon* of the enriched carboxylate of CmHCAB. In order to test the ability of this potential NMR "probe" to monitor active site events, a titration of the enzyme was carried out while simultaneously measuring the chemical shift of the enriched carboxyl. Figure 1 demonstrates that between pH 5 and 10.5 the probe senses multiple ionizations that must originate in or affect the active site. The smooth curve in Figure 1 is a theoretical fit using a model of two independent ionizing groups with pK_a of 6.0 and 9.2 affecting the resonance.

Reasonable assignments of these pK_a values to active site ionizing groups can be made. The pH 6.0 inflection is almost certainly due to the ionization of the imidazole side chain of

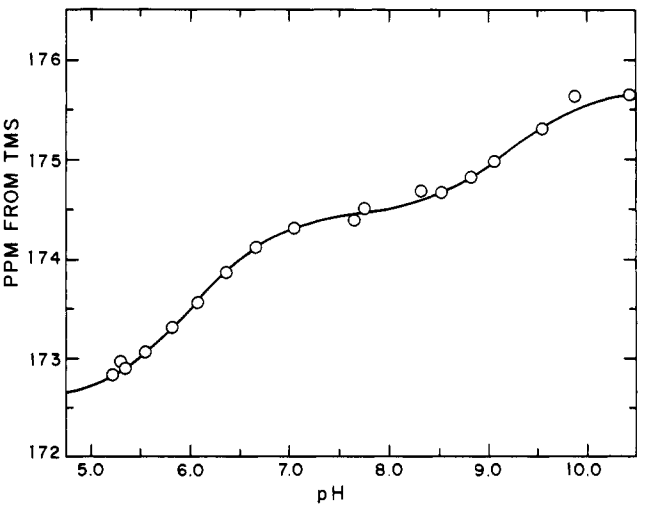


Figure 1. Variation of the chemical shift (Me_4Si scale) of the enriched carboxylate ^{13}C NMR resonance of CmHCAB with pH at 25 °C and 0.2 ionic strength (adjusted with Na_2SO_4 or K_2SO_4). Spectra were obtained at 25.1 MHz on a Jeol PFT-100P/EC 100 FT NMR system using 10-mm sample tubes. Enzyme concentration varied between 1 and 6 mM. All samples contained 10% D_2O for locking and 2–5 μl of dioxane as an internal shift standard (dioxane assumed at 67.40 ppm downfield of Me_4Si). The pH was adjusted by dialysis against buffers and the pH was measured immediately before and immediately after spectra were obtained, rarely changing by more than 0.02 pH units. Smooth curve represents a theoretical fit (see text).