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Studies of the inhibition of lipolytic enzymes—III. The inhibition of a Tween hydrolase from rat adipose tissue *in vitro* and *in vivo* by *n*-butyl carbamic acid methyl ester, U-14641

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n-Butyl carbamic acid methyl ester (BCME) has been demonstrated to be a potent species-specific inhibitor of canine liver and kidney lipase.[1] The isolation and extensive purification of a Tween hydrolase from rat adipose tissue has also been reported.[2] The experiments reported herein demonstrate that BCME is an inhibitor, both *in vitro* and *in vivo*, of Tween hydrolase from rat adipose tissue.

METHODS

For the kinetic experiments, Tween hydrolase was highly purified by the procedure hitherto reported.[2] Enzyme activity *in vivo* was determined in male Sprague-Dawley rats weighing 250–300 g, and fed Purina lab chow *ad libitum*. BCME was made up in water at a concentration of 0·1 M and was administered i.v. into the tail veins. The rats were killed by decapitation, and the epididymal and perirenal fat pads were quickly excised and chilled in cold water. The water was then decanted and the pads, blotted dry and weighed, were homogenized in a chilled Waring Blendor for 1 min at top speed in 2 volumes (v/w) of cold water. The resulting homogenate was centrifuged at 5000 *g* for 10 min at 0°. The supernatant fluid was filtered through cheesecloth to remove particles of fat, and the Tween hydrolase activity was assayed as follows. To 15-ml conical centrifuge tubes was added 500 μmoles ammonium chloride buffer, pH 8·35; 0·25 ml adipose tissue extract prepared as described; 25 μmoles Tween 20 (Atlas Chemical Industries), and water to a final volume of 2 ml. Incubations were for 10 min at 38°.

In the kinetic experiments, because small volumes of highly purified enzyme (30 units*/tube) were needed, the final volume of the incubation mixture was reduced to 1 ml and 250 μmoles of buffer was used, along with varying amounts of substrate. All components were added except the substrate, and a 3-min preincubation period was employed to ensure establishment of steady-state conditions between enzyme and inhibitor. The tubes were then removed from the water bath, chilled, and the Tween substrate added, following which the tubes were again incubated for 10 min at 38°. At this time the tubes were again removed from the bath, chilled in ice slush, and the reaction stopped by addition of 7·5 ml of a solvent mixture of 4:1:0·1 isopropanol:heptane:1 N H_2SO_4 per ml incubation medium. Fatty acids were extracted and titrated by the method of Ko and Royer.† Appropriate enzyme and substrate blanks were carried in every experiment to correct for endogenous fatty acid.

RESULTS AND DISCUSSION

Under the described conditions for kinetic experiments, with 10 μmoles Tween substrate, BCME was found to inhibit the enzyme 93 per cent at a concentration of 5×10^{-3} M, 85 per cent at 5×10^{-4} M, and 56 per cent at 5×10^{-5} M.

* According to international usage, 1 unit of enzyme is that amount of enzyme required to liberate 1 μmole fatty acid/hr under specified conditions of assay.

† Unpublished.

In pilot experiments, BCME killed all rats at i.v. doses of 50 μmoles/100 g body weight. At 40 per cent of this dose, no serious symptoms of toxicity were encountered, and the maximal attainable inhibition was observed to last for at least 5 hr after injection.

In Fig. 1 a dose–response curve is shown in which the animals were injected with indicated doses of BCME and sacrificed 2·5 hr later for enzyme assay. Per cent inhibition was calculated in comparison with Tween hydrolase isolated under identical conditions from saline-injected controls. It is

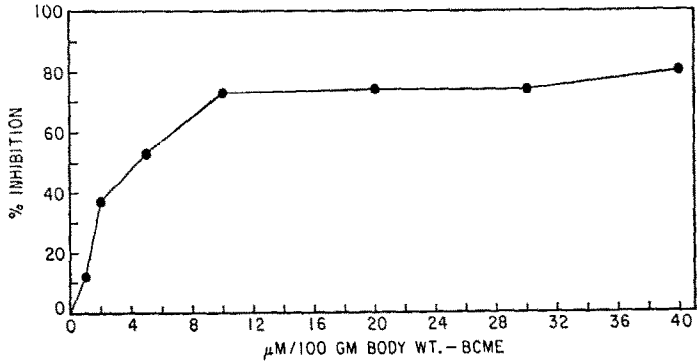

FIG. 1. Dose–response relationship of increasing doses of BCME to per cent inhibition of Tween hydrolase, 2·5 hr after administration in rats.

apparent, as previously reported for canine liver lipase,[1] that the dose range between minimal and maximal response is rather narrow, occurring between 1 and 10 μmoles/100 g body weight. Above this dose there is no appreciable increment of inhibition.

If it is assumed that BCME is equally distributed throughout the organism, and that no substantial degradation takes place during the experimental period, doses in the range of 10–40 μmoles/100 g body weight are equivalent to molar concentrations between 1 and 4×10^{-4}. In this dosage range, as shown in Fig. 1, approximately 80 per cent inhibition of the enzyme was induced. At a somewhat higher concentration (5×10^{-4} M) *in vitro*, 85 per cent inhibition was observed, a figure in reasonable agreement with results *in vivo*. It could also be predicted from results *in vitro*, that at a lethal tenfold higher dose, complete inhibition would still be unattainable.

Failure to obtain 100 per cent inhibition could also be ascribed to the presence of more than one enzyme capable of hydrolyzing Tween in crude adipose tissue extracts. This possibility was checked, by means of the known heat stability of Tween hydrolase previously reported;[2] no inactivation is seen when this enzyme is subjected to 60° for 5 min. When adipose tissue extracts from animals treated with maximal doses were heated as indicated, no further increase in per cent inhibition was observed. If another hydrolase is present, it is as stable to heat as the Tween hydrolase under discussion.

A detailed kinetic analysis of inhibition of Tween hydrolase by BCME was also conducted under described conditions. Figure 2 shows a Lineweaver-Burk[3] plot of the results. Constants were calculated from these curves and also directly from the data in an appropriately programmed IBM 1620 computer.* A diagnostic plot according to Reiner[4] is also presented in Fig. 3. The notation used is the same as previously prescribed for canine kidney lipase.[1] The mechanism of inhibition was found to be identical with that reported for canine kidney lipase,[1] namely, "partial inhibition," in which both enzyme and enzyme–substrate complex are inhibited. Also, the enzyme–inhibitor complex may, to a small extent, dissociate to free and active enzyme, or may exchange an inhibitor molecule for a substrate molecule to form an active enzyme–substrate complex. The Haldane constant (K_s) for the uninhibited Tween hydrolase was $1{\cdot}03 \times 10^{-2}$ M, a figure in close agreement with the published

* The author wishes to thank Mr. J. I. Northam of these laboratories for performing these calculations, and Mr. N. J. Crittenden for technical assistance.

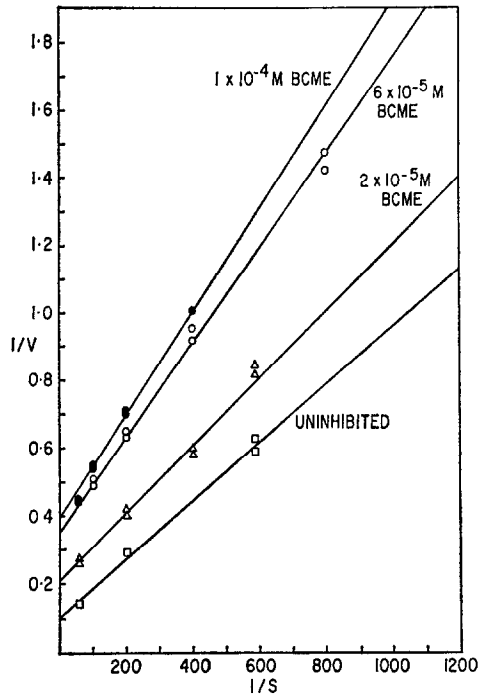

FIG. 2. Inhibition of highly purified Tween hydrolase from rat adipose tissue.

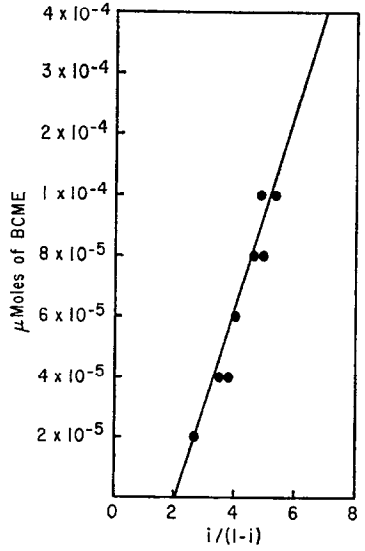

FIG. 3. A plot of I (concentration of BCME) vs. i/(1 − i):(1 − i = r) is the (velocity with inhibition)/(velocity without inhibition). The negative I intercept (positive i/(1 − i)) intercept is consistent with the mechanism of "partial inhibition,"[4] as generalized.[1]

value[1] of $1{\cdot}3 \times 10^{-2}$ M. The value for V_{max} was 11·1. K_3, the dissociation constant of the enzyme–inhibitor complex was $1{\cdot}33 \times 10^{-4}$ M. K_4, the dissociation constant of the enzyme–substrate–inhibitor complex was $2{\cdot}54 \times 10^{-5}$ M.

From the data presented, it is concluded that BCME is an inhibitor, albeit an incomplete one, for Tween hydrolase in rat adipose tissue. It appears that the enzyme is inhibited *in vivo*, that a dose–response relationship exists, and that the concentration of inhibitor required to inhibit the enzyme *in vitro* is comparable to that required to induce comparable degrees of inhibition *in vivo*.

Research Laboratories,
The Upjohn Company,
Kalamazoo, Mich., U.S.A.

DONALD P. WALLACH

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Comparative growth-inhibitory activivity of homofolic acid against cell lines sensitive and resistant to amethopterin

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CERTAIN sublines of neoplastic cells selected for resistance to amethopterin *in vitro*[1, 2] or *in vivo*[3] contain increased amounts of folate reductase (dihydrofolate reductase, tetrahydrofolate dehydrogenase). On the basis of this evidence, attention has been directed to the possible exploitation of this metabolic difference for the selective eradication of such amethopterin-resistant cells.[3] Antimetabolites which are substrates of folate reductase, such as N^{10}-methylfolic acid[4] and homofolic acid,[5] are converted to the corresponding analogs of tetrahydrofolate. Thymidylate synthetase is inhibited by tetrahydrohomofolate[5] and by N^{10}-methyltetrahydrofolate.[6] The increase in folate reductase content of amethopterin-resistant sublines of sarcoma-180 cells *in vitro*[1] or in murine leukemia L-1210 cells *in vivo*[7] was not accompanied by any significant change in the kinetic characteristics of this enzyme. Thus, more rapid formation of the tetrahydrofolate analogs would be expected to occur in cells containing larger amounts of folate reductase. The purpose of this study was to compare the growth-inhibitory activity of homofolic acid in cultures of S-180 cells which differed by 200-fold in their content of folate reductase.

EXPERIMENTAL

The development and characteristics of the sublines of sarcoma-180 cells sensitive (AH/S) and resistant to amethopterin (AT/174 and AT/3000) have been described.[1, 8] Flasks (T-15) were inoculated with 200,000 cells in 2 ml of the corresponding maintenance medium.[8] After incubation overnight at 36°, the medium in each flask was replaced by the appropriate experimental medium in which the growth of the cells depends upon folic acid (no thymidine or purines supplied), and which was supplemented with varied concentrations of homofolate. The media were changed thereafter three times so that the total period of growth in the presence of the analog was 7 days. The total protein content was determined according to the method described by Oyama and Eagle.[9] The control cultures grew 12- to 15-fold in 7 days as measured by increase in protein content over the inoculum.

RESULTS AND DISCUSSION

Homofolate[10] differs from folic acid by the presence of a methylene group between C^9 and N^{10} of pteroylglutamic acid. This structural modification does not prevent the enzymatic reduction of the pteridine ring, but does increase the distance between the pteridine ring and the nitrogen atom