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The enzymic oxidation of certain folic acid antagonists

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WE HAVE previously demonstrated[1] that dichloromethotrexate* (DCM), a folic acid antagonist, undergoes oxidative deamination in animals and humans. Based on chemical, physicochemical and synthetic[2] evidence, the structure of 4,7-dihydroxy-DCM* is proposed for this metabolite. We wish to report that the oxidative deamination is effected *in vitro* by an enzyme system which occurs in the soluble fraction of the liver of several animal species.

Apart from simple pteridines,[3, 4] the enzymic oxidation of folic acid derivatives has not hitherto been described. Attempted oxidation of DCM by the ascorbate-ferrous system[5] and by milk† or calf liver‡ xanthine oxidase was unsuccessful in our hands. This failure, together with the differences in distribution between xanthine oxidase and our enzyme system in various species and organs, suggests they are dissimilar. When 2 μmoles of DCM were aerobically incubated for 3–4 hr with rat liver homogenate equivalent to 1 g of tissue at 37°, with shaking, oxidative deamination was virtually complete. Homogenates were prepared in 3 parts of isotonic KCl, using a Potter homogenizer. For the incubation a simple technique was employed: a measured amount of various liver (or other organ) fractions was placed inside a cellulose (Visking Corp.) sac in a flask containing substrate dissolved in 0·1 M Tris buffer [tris-(hydroxymethyl)-aminomethane] of pH 8·2. A blank containing no substrate was likewise prepared. At the end of incubation, the outside solutions were examined by differential spectrophotometry. Oxidation of DCM was indicated by disappearance of the substrate, as well as by appearance of the metabolite. If oxidation was complete, then the spectrum observed was that of 4,7-dihydroxy-DCM alone. The product of the enzymic oxidation was identical with a specimen isolated from the bile of a DCM-treated rabbit,[6] not only spectrophotometrically and chromatographically, but also with respect to mobility in high-voltage electrophoresis.[7] Also, monochloromethotrexate, monobromomethotrexate, chlorobromomethotrexate, dichloroaminopterin, difluoromethotrexate, monochloroaminopterin, and monofluoromethotrexate all appeared to be similarly oxidized. On the other hand, although gradual disappearance was noticed, folic acid, aminopterin, and methotrexate (amethopterin) afforded no well-defined product. In contrast with both DCM and dichloroaminopterin, neither N^{10}-methyl-dichlorofolic acid nor dichlorofolic acid appeared to be affected by the enzyme system, indicating, perhaps, that the first step in the oxidation is hydroxylation of the 7-position, followed by oxidative deamination of the 4-amino group; however, conclusive evidence must await further investigation.

No oxidation occurred if incubation was carried out with liver homogenate previously heated to about 80° for 3 min, thus implicating an enzymic reaction. Additional supporting evidence was supplied by studies on intracellular localization and the optimal pH-value for the reaction; although

* Generic name for N-{3,5-dichloro-4-[(2,4-diamino-6-pteridinylmethyl)-methylamino]benzoyl}glutamic acid, the corresponding chemical name for 4,7-dihydroxy-DCM is therefore N-{3,5-dichloro-4[2-amino-4,7-dihydroxy-6-pteridinylmethyl)-methylamino] benzoyl}-glutamic acid.

† Worthington Biochemical Corporation, Freehold, N.J.

‡ Courtesy of Dr. Ruth K. Kielley of the National Cancer Institute.

the latter was between 8 and 8·5, this enzyme system differed from a microsomal aromatic hydroxylation enzyme system[8] in that it was found primarily in the soluble (72,000 g-supernatant) fraction. After continuous dialysis for 7 hr, the soluble fraction remained active, thus yielding no information as to cofactor requirements. The oxidation rate was greatly diminished when the reaction was conducted anaerobically. The enzyme system was also inhibited by β-diethylamino-ethyl diphenylpropylacetate (SKF 525-A) at a final concentration of 10^{-3}M.

The presence of the enzyme system has been demonstrated in the liver of the rhesus monkey, rat, mouse, rabbit and guinea pig. Its absence in the liver of the dog is consistent with the finding *in vivo* that no metabolite of DCM was excreted in the bile or urine by this species.[6] Of all the animals thus far studied, the rabbit is the only one that showed enzyme activity in the small intestine. Further work is in progress to characterize the enzyme system, and to elucidate its cofactor requirements and substrate specificity.

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Enzymic conversion of valine⁵-angiotensin-I into an oxytoxic principle by impure preparations of alpha-amylase

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RECENTLY, Bumpus *et al.*[1] have shown that isoleucine^5-angiotensin-II and the heptapeptide, arginyl-valyl-tyrosyl-isoleucyl-histidyl-prolyl-phenylalanine, are the only peptides which could arise from isoleucine^5-angiotensin-I that show significant oxytocic activity. The measure of oxytocic activity, then, is a direct index of the conversion. Previous work by Huggins and Walaszek[2, 3, 4] on a vasopressor polypeptide with oxytocic activity, formed by incubating fraction IV-4 of human plasma protein with a crude preparation of alpha-amylase, suggested that such preparations may contain enzymes which convert angiotensin-I to an oxytocic principle.

Alpha-amylase was obtained from Nutritional Biochemicals Corporation and was labelled batch #7942. Synthetic valine^5-angiotensin I and II were generous gifts of Professor Rolfe Meier, CIBA, Ltd., Basle, Switzerland. Samples of human saliva were obtained from our laboratory associates, separated into two groups, smokers and non-smokers. All assays were carried out on the isolated rat uterus, as described by Carlini, Picarelli and Prado.[5] Uteri were taken from virgin rats weighing approximately 200 g, injected on the previous day with 10 μg of diethylstilbesterol per 100 g of body weight. The uteri were suspended in 10 ml of de Jalon's solution at 30 °C and activity was recorded with a frontal lever on a smoked drum. The contact time of the agonist with the tissue was 1–½ min and 5-min intervals elapsed between applications. Incubations of enzymes with substrates were carried out at 30 °C; for studies of heat denaturation, the enzymes were placed in a boiling water-
min.