Inhibition of Oxidizing Enzymes by Metronidazole

Metronidazole, 1-(2'-hydroxyethyl)-2-methyl-5-nitroimidazole, a drug proposed for the control of chronic alcoholism[1], has been shown to inhibit in vitro several enzymes which include alcohol dehydrogenase (horse liver) and xanthine oxidase (cream and rat liver)[2-4] as well as uricase (hog liver)[4]. Non-competitive inhibition of alcohol dehydrogenase from human liver has also been reported recently[5].

The mechanism of this inhibition is not yet understood. In this connection, one finding from our laboratories may be significant, and may be related to the inhibition of NAD-linked dehydrogenases. When metronidazole and NAD-H are mixed at 25 °C, in absence of enzymes or other substrates, metronidazole causes disappearance of the typical absorption peak at 340 nm, and NAD-H can be titrated by metronidazole (Table).

The high absorbance of metronidazole at 340 nm has been previously mentioned[4,5]. Metronidazole has a sharp maximum at 325 nm; measurements of absorbance at this wave-length can be used for quantitative assays of metronidazole. Linear curves are obtained over a wide range of concentrations, which was previously reported[6] and confirmed by the present author[4]. This intense absorbance in the near UV makes spectrophotometric assays of NAD-H difficult. Success was achieved only when the Beckman DU spectrophotometer was used in conjunction with the photomultiplier attachment.

The mechanism of interaction between metronidazole and NAD-H is not yet understood. Disappearance of the absorption peak at 340 nm may be caused by the formation of a charge-transfer complex with consequent change in absorbtion spectrum[7,8]. In this connection, it is interesting to recall that imidazoles combine with oxidized NAD; however, this reaction occurs only when the 1-position of the imidazole is free[9]. This position is occupied by the (2'-hydroxyethyl)-side chain in metronidazole.

In any case, the interaction of metronidazole and NAD-linked enzymes such as alcohol dehydrogenase, must be re-examined. In both published reports, inhibition of alcohol dehydrogenase is based on measurements of the rate of NAD-H formation. It is possible that a steady-state of NAD-H is formed, which would be interpreted as an inhibition of the enzymatic reaction. In reality, enzyme activity itself may be unimpaired and reduced NAD immediately reoxidized or bound by metronidazole or its reaction products. It is therefore necessary to assay alcohol dehydrogenase activity by other methods which do not depend on NAD-H formation, such as measurement of ethanol or acetaldehyde. These studies are presently being carried out in the author's laboratory. Whether metronidazole is truly an inhibitor of alcohol dehydrogenase and the mechanism of this inhibition – if confirmed by other methods – remains to be established.

Metronidazole, which contains a hydroxyethyl-('ethanol') sidechain was tested as substrate for alcohol dehydrogenase in absence of ethanol. It had been reported[4] not to be a substrate, even during prolonged incubation with high concentration of enzyme. In the light of the non-enzymatic interaction between metronidazole and NAD-H, enzymatic attack on this compound might be masked. Whether metronidazole is a substrate for alcohol dehydrogenase must be re-examined by other assays which do not depend on the measure of the rate of NAD-H formation; this work is being carried out presently.

After the non-enzymatic reaction of metronidazole and NAD-H was found, it became of interest to assay the effect of antabuse (disulfiram) on NAD-H, since disulfiram is also used as an antialcoholic drug. Work with this compound also presents great technical difficulties, due to its high absorbance in the near UV and its insolubility in water. Absorbance could be overcome by the use of the photomultiplier attachment of the Beckmann DU spectrophotometer. For alcohol dehydrogenase assay, disulfiram was dissolved in ethanol, and appropriate corrections were made in the amount of ethanol added to the reaction system as substrate. For other assays, disulfiram was dissolved in ethanol (0.01 *M* solutions were used most frequently). To avoid precipitation when relatively high concentrations of this reagent were mixed with aqueous systems, it was added to solutions of 0.1 *M* phosphate buffer, pH 7.8, containing 2% of 'Unisol' detergent (Schuco Industries, New York). A clear solution was obtained with high concentrations of disulfiram, without any precipitation. The detergent itself did not interfere with the assays here reported. When disulfiram and NAD-H were mixed, no change in absorbance at 340 nm was observed with concentrations of disulfiram up to 0.003 *M*.

It is interesting to note that metronidazole inhibits oxidases belonging to 3 different classes: NAD-linked enzymes (alcohol dehydrogenase), FAD-linked enzymes (xanthine oxidase), and uricase, for which cofactor requirements are not known. In the first 2 cases, it could be shown[3,4] that no inhibition occurs when tetrazolium salts are used as electron acceptors. EDWARDS and PRICE[5] found metronidazole to be a non-competitive inhibitor in relation to ethanol. The findings discussed above

Interaction between metronidazole and NAD-H

Metronidazole (μmoles)	A_{340}		
	NAD-H (μg)		
	100	200	300
0	0.25	0.51	0.74
1	0.19	0.43	0.67
2	0.16	0.29	0.39
3	0.02	0.04	0.06
4	0.00	0.00	0.00

Graded levels of metronidazole were mixed at 25 °C with graded levels of NAD-H (200 μg/ml = 0.254 μmoles/ml) and the volume was completed to 3.0 ml with 0.1 *M* phosphate, pH 7.4. Absorbance was read in a Beckmann DU spectrophotometer with photomultiplier using equivalent amounts of metronidazole for blanks. 1.0 cm light-path.

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require further study of the mechanism of action of metronidazole in presence of reduced NAD-enzymes. It is possible that metronidazole acts as a relatively non-specific electron trap, which can be counteracted by tetrazolium salts. Metronidazole did not act as inhibitor of monoamine-oxidase and diamine oxidase, in reaction systems which were inhibited by other known inhibitors of these 2 enzymes (R. E. RANNEY, Chicago, unpublished)[10].

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Zusammenfassung. Eine unerwartete Möglichkeit, wie Metronidazol zu einer nur scheinbaren Hemmung der Alkoholdehydrogenase führen könnte, wird aufgedeckt.

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Antagonism by Deoxyribosides of the Inhibitory Action of Certain Hydroxamic Acids on Deoxyribonucleic Acid Synthesis

ADAMSON[1] evaluated the activities of a number of congeners of hydroxyurea (HU) against advanced leukemia L1210 in mice and concluded that the essential structural requirement for antitumor activity is the hydroxamic acid group (-CONHOH). From a more recent study of the effects of certain HU analogs on DNA synthesis in HeLa cells in vitro, YOUNG et al.[2] found that a carbonyl group is not a prerequisite in an inhibitory compound in the system under consideration. However, the -NOH group is required for inhibitory activity, and the proton on the hydroxyl group must be unsubstituted.

Reports from this laboratory have revealed additional hydroxamic acids with selectivity of action against DNA synthesis in ascites tumor cell and/or microbial test systems. Salicyl hydroxamic acid (SHA) confers a prompt inhibition of the former system at a 50% inhibitory concentration (IC_{50}) of about $4 \times 10^{-4} M$; inhibition is readily reversible upon removal of the compound[3]. Oxamyl hydroxamic acid (OHA) has an IC_{50} of about $9 \times 10^{-4} M$ in the same system, and its action is also reversible. In addition, this latter compound inhibits growth of, and DNA synthesis by, certain gram-negative bacteria, and induces unbalanced growth characterized by marked elongation of individual cells[4]. Acetoxyoxamide (AOA) shares most of the pharmacological properties of OHA, except that onset of its action is preceded by a latent period which presumably corresponds to the time required for hydrolysis of the O-acetyl group to yield an *N*-hydroxyl group with an unsubstituted proton[5]. 2,3-Dihydroxybenzoyl hydroxamic acid (DHB) has an IC_{50} of about $5 \times 10^{-5} M$ in the ascites tumor system following 1 h exposure of the cells to the compound, and its action is only slowly reversible; i.e. a lag period is evident between removal of the compound from the cells by washing and resumption of the rate of DNA synthesis to near-control values (unpublished data).

It now appears sufficiently documented that a major metabolic defect conferred by HU is an inhibition of the enzymatic conversion of ribonucleotides to deoxyribonucleotides[6–10]. Partial antagonism by a mixture of deoxyadenosine, deoxyguanosine, and deoxycytidine of the action of HU on HeLa cells has been observed[11]; these 3 deoxyribosides plus thymidine virtually completely antagonize the action of HU on mouse fibroblast (L) cells[12]. The observation that HU reverses the orotic aciduria induced in patients by the administration of 6-azauridine, an inhibitor of orotidylic decarboxylase, suggests, in addition, some inhibition by HU of de novo pyrimidine biosynthesis[13]. The following work was consequently initiated to determine if the actions of SHA, OHA, AOA, and DHB on DNA synthesis are likewise antagonized by deoxyribosides. If so, it may be inferred that the mode of action of these agents is similar to that of HU.

Experimental. Determination of the rate of DNA synthesis by Ehrlich ascites tumor cells was substantially as described earlier[3–5]. Eagle's minimum essential medium with Hank's balanced salt solution (MEM) and NCTC-109 medium were from Microbiological Associates. Thymidine-^{3}H was from New England Nuclear Corporation. Deoxyribosides were from Nutritional Biochemicals Corporation or Schwartz Laboratories. The previously reported activity of AOA against DNA synthesis by cells suspended in NCTC-109 medium[5] was found to be reproducible only when this medium was used; no inhibitory action was found when this compound was assessed against the system using MEM. Consequently, all experiments employing AOA were done with NCTC-109 medium, while all others were done with MEM. The MEM reaction mixture consisted of 5.0 ml of a washed 1% cell suspen-

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