3. C. A. ASHFORD and K. C. DIXON, *Biochem. J.* **29**, 157 (1935).
4. F. DICKENS and G. C. GREVILLE, *Biochem. J.* **29**, 1468 (1935).
5. H. MCILWAIN, *Biochem. J.* **49**, 382 (1951).
6. S. S. PARMAR, *Biochem. biophys. Res. Commun.* **4**, 33 (1961).
7. S. S. PARMAR, Ph.D. Thesis, McGill University, Montreal, Canada (1957).
8. E. C. WEINBACK, H. J. LOEW, W. R. FRISELL and L. HELLERMAN, *J. biol. Chem.* **189**, 779 (1951).
9. M. JOWETT and J. H. QUASTEL, *Biochem. J.* **29**, 2143 (1935).
10. J. H. QUASTEL and A. H. WHEATLEY, *Biochem. J.* **27**, 1753 (1933).
11. J. R. KLEIN and H. KAMIN, *J. biol. Chem.* **138**, 507 (1941).
12. J. H. QUASTEL, *Proc. Int. Symposium on Enzyme Chemistry, Tokyo and Kyoto*, p. 510 (1957).

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The effect of Flagyl on xanthine oxidase and alcohol dehydrogenase*

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FLAGYL (metronidazole†), a drug used to combat trichomonal infection of the vagina, has recently been shown to produce aversion to ethanol. Because of the latter effect, the drug is being tested as a repessant of compulsive consumption of alcohol.[1] The mechanism of this antagonism between ethanol and metronidazole is not yet known.

A similar ethanol antagonism is produced by disulfiram (Antabuse). Disulfiram has been shown to inhibit several enzymes related to alcohol metabolism,[2] but the principal effect is attributed to the blocking of acetaldehyde oxidation. One of the enzymes inhibited by disulfiram, which are involved in the oxidation of acetaldehyde, is xanthine oxidase.[3] As part of a survey of the mechanism of action of Flagyl, its effect on purified milk and liver xanthine oxidase (1.2.3.2) and on liver alcohol dehydrogenase (1.1.1.1) was tested.

METHODS

Cream xanthine oxidase and horse liver alcohol dehydrogenase were obtained from Worthington (Freehold, N.J.) and were used without further purification. Liver xanthine oxidase was prepared from the supernatant fraction of rat liver by a modification of a method previously described for beef liver[4] and was purified by ammonium sulfate fractionation; the fraction precipitated between 20 and 50 per cent saturation was used as enzyme source. Flagyl was dissolved in 0·1 M phosphate, pH 7·8, at the concentration of 0·01 M. Xanthine oxidase activity was assayed spectrophotometrically by uric acid formation, at 298 mμ,[5] and xanthine dehydrogenase activity colorimetrically by reduction of nitro-BT tetrazolium salt, in the presence of phenazine-methosulfate and gelatin at 540 mμ.[6] Fine chemicals were obtained from Sigma (St. Louis). The reaction mixture, without xanthine, was pre-incubated for 5 min at 25°, and the reaction was started by the addition of xanthine. The reaction rate

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† Flagyl: 1-(2′-hydroxyethyl)-2-methyl-5-nitroimidazole.

was measured in a Beckman DU spectrophotometer with photomultiplier attachment, at 25° and pH 7·8.

Alcohol dehydrogenase was assayed spectrophotometrically at 340 mμ,[7] in a total volume of 8·0 ml, at pH 8·8 and 37°. Test tubes were incubated in a Dubnoff shaker, and absorbance was read at regular intervals in a Gilford 300 spectrophotometer. NAD was obtained from Sigma. The reagents were mixed in ice, and the reaction was started by the addition of 2·0 ml ethanol 2·0 M.

RESULTS AND DISCUSSION

When liver xanthine oxidase was incubated with Flagyl, the *oxidase* activity was inhibited; complete inhibition was observed with 0·001 M final concentration (Fig. 1). The range of inhibition is quite narrow: 0·0003 M of the drug caused only 5 per cent inhibition. When the same enzyme fraction was tested for *xanthine dehydrogenase* activity, in the presence of phenazine methosulfate and tetrazolium salt, no inhibition was observed even at higher concentrations of Flagyl than those that caused complete inhibition in the oxidase assay. The inhibition of oxidase activity was not affected by the addition of up to 0·1 mg phenazine methosulfate or EDTA, 0·001 M. No change in optical

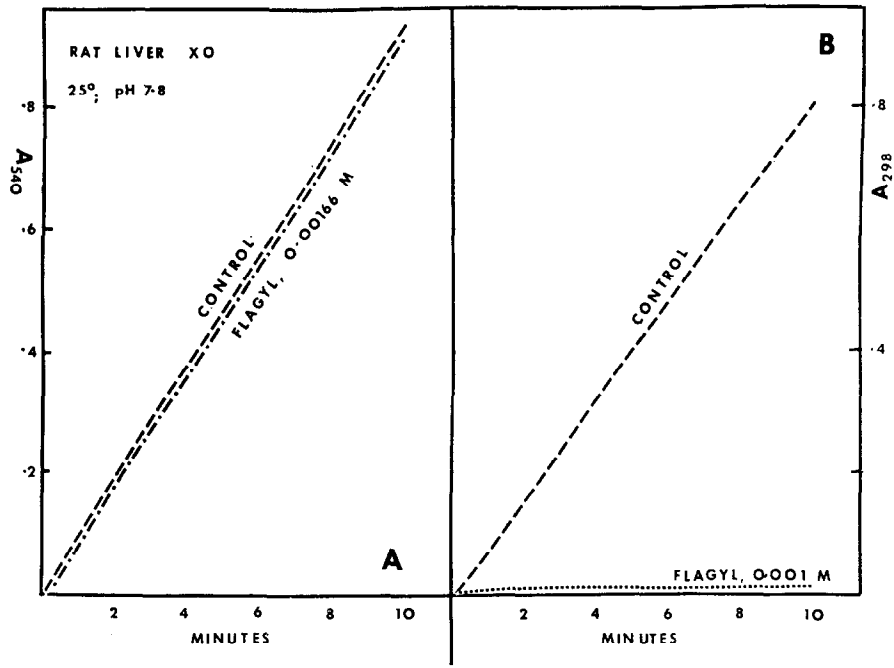

FIG. 1. Effect of Flagyl on liver xanthine oxidase.
A. Dehydrogenase activity; 540 mμ: enzyme, 0·08 mg; 0·5 ml xanthine, 0·001 M; 0·5 ml gelatin, 0·1%; 0·5 ml nitro-BT tetrazolium salt, 4 mg/ml; 0·2 ml phenazine methosulfate, 0·2 mg/ml.
B. Oxidase activity, 298 mμ: 0·08 mg enzyme; 0·5 ml xanthine, 0·001 M; phosphate, pH 7·8, 0·1 M, to complete 3·0 ml. The reaction was started by addition of xanthine.

density was observed either in the oxidase assay at 298 mμ or the dehydrogenase assay at 540 mμ when Flagyl was incubated in the complete system with enzyme or xanthine omitted.

Similar results were observed when cream xanthine oxidase was tested. Flagyl caused 100 per cent inhibition of the oxidase activity at 0·001 M concentration, whereas 0·002 M Flagyl caused no

inhibition of dehydrogenase activity. In order to verify whether Flagyl would inactivate xanthine oxidase irreversibly, xanthine oxidase and Flagyl were preincubated at 37° in the absence of substrate (Table 1). When xanthine was added after 15 min, complete inhibition was observed, as reported above. However, when the mixture of xanthine oxidase and Flagyl was dialyzed after preincubation,

TABLE 1. EFFECT OF DIALYSIS ON INHIBITION OF XANTHINE OXIDASE

Preincubation (min; °)	Flagyl	Dialysis	Activity (%)
15; 37°	−	+	100
15; 37°	+	+	100
none	+	−	0
30; 37°	+	−	0

Two ml cream xanthine oxidase, diluted 1:100 from stock with 0·1 M phosphate, pH 7·8, was incubated in a Dubnoff shaker with 8·0 ml metronidazole, 0·01 M. The mixture was dialyzed for 2 hr against four changes of 50 vol. of buffer, at +2°. For assay, 1·0 ml of the mixture was incubated with 0·5 μmole xanthine in 3·0 ml buffer, pH 7·8, at 25°. Activity was determined as change in absorbance at 298 mμ, during 10 min.

no inhibition was observed, and activity was completely restored. This experiment demonstrates that inactivation of xanthine oxidase by Flagyl is not irreversible.

It is interesting to note that both Flagyl and Antabuse[3] affect only the oxidase activity *in vitro*, while the dehydrogenase activity is not affected. Although the structure of the two inhibitors, disulfiram and metronidazole, is completely different, both of them have the same action on one of the enzymes that may be involved in alcohol detoxication. The antialcoholic effect of Antabuse is ascribed to the blocking of the catabolism of acetaldehyde, which is the oxidation product of ethanol, and the toxic effects are attributed to the accumulation of acetaldehyde.[8] It should be noted that six enzymes are potentially involved in the catabolism of acetaldehyde, several of which are inhibited by Antabuse; xanthine oxidase is one of these, but it is much less sensitive to Antabuse than the other enzymes. Furthermore, Antabuse also inhibits other enzymes, besides those involved in ethanol metabolism; their inhibition may contribute to the overall "alcohol–Antabuse" symptoms.[2, 3, 9]

The inhibition of one of the enzymes involved in acetaldehyde oxidation by metronidazole, i.e. xanthine oxidase, would reinforce the proposed mechanism of action of the "antialcoholic compounds", through inhibition of acetaldehyde oxidation. Other enzymes related to acetaldehyde metabolism, especially aldehyde oxidase (EC 1.2.3.1) should also be tested *in vitro*, in the presence of metronidazole. It would also be interesting to verify whether administration of metronidazole to alcoholics would raise the level of acetaldehyde in blood and organs.

The interaction of metronidazole and xanthine oxidase is similar to that reported for xanthine oxidase and nitro-furan derivatives,* which are chemically similar to metronidazole. These compounds are inhibitors of xanthine oxidase at high concentrations *in vitro*, whereas xanthine oxidase seems to be involved in detoxication of the nitrofurans.[10] Xanthine oxidase may also play a role in the metabolism of Flagyl.†

One should not expect that Flagyl inhibits only one enzyme. Indeed, alcohol dehydrogenase (1.1.1.1) itself is inhibited by low concentrations of metronidazole *in vitro*, 50 per cent inhibition being observed by about 3×10^{-4}M metronidazole (Fig. 2). Addition of $ZnSO_4$ to the reaction mixture, tested up to the level of 8×10^{-4}M final concentration, had no influence on the reaction rate, in presence or

* Furacin: 5-nitro-2-furaldehyde semicarbazone.

† R. Fried, unpublished.

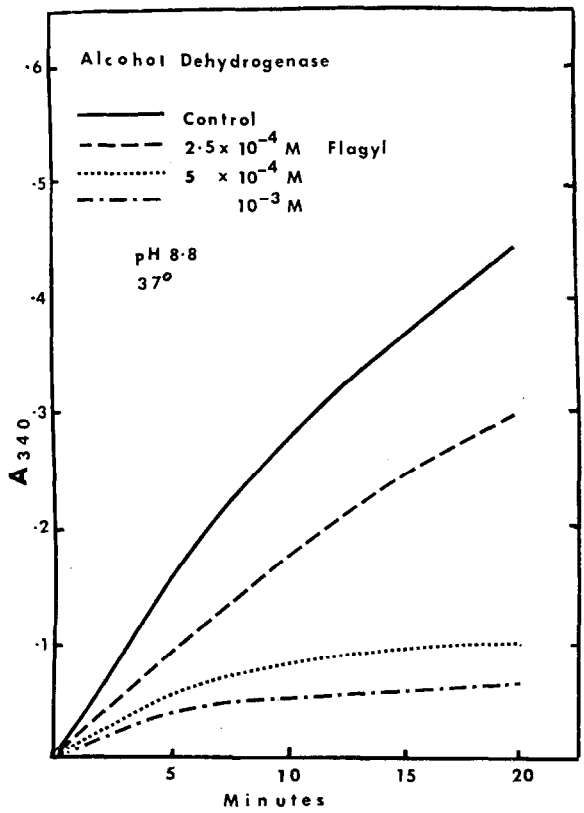

FIG. 2. Inhibition of liver alcohol dehydrogenase by Flagyl; 0·10 mg enzyme; 2 mg NAD; completed to 8·0 ml with sodium pyrophosphate, 0·032 M, pH 8·8. The reaction was started by addition of 2·0 ml ethanol, 2·0 M.

absence of metronidazole. Taylor[1] reports unpublished findings by R. W. Manthei, which also indicate inhibition of alcohol dehydrogenase by metronidazole.

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REFERENCES

1. J. A. T. TAYLOR, *Bull. Los Angeles neurol. Soc.* **29**, 158 (1964).
2. F. E. HUNTER, JR. and O. H. LOWRY, *Pharmac. Rev.* **8**, 89 (1956).
3. D. A. RICHERT, R. VANDERLINDE and W. W. WESTERFELD, *J. biol. Chem.* **186**, 261 (1950).
4. R. FRIED and L. W. FREEMAN, *Abstracts, 142nd Meeting Am. chem. Soc.* p. 31C (1962).
5. H. R. KALCKAR, *J. biol. Chem.* **167**, 429 (1947).
6. R. FRIED, Abstracts, International Neurochemistry Congress, Oxford, p. 30 (1965). *Analyt. Biochem.* (in press).

7. B. L. VALLEE and F. L. HOCH, *Proc. natn. Acad. Sci., U.S.A.* **41**, 327 (1955).
8. E. JACOBSEN, *Pharmac. Rev.* **4**, 107 (1952).
9. W. W. WESTERFELD, *Tex. Rep. Biol. Med.* **13**, 559 (1955).
10. J. D. TAYLOR, H. E. PAUL and M. F. PAUL, *J. biol. Chem.* **191**, 223 (1951).

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Inhibitory effect of barbiturate on brain and serum xanthine oxidase

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BARBITURATES have been shown to inhibit the respiration of brain *in vitro*.[1] Cytochrome *c* reductase was claimed to be the sensitive site of the inhibition by barbiturates in the oxidative system of the cell.[2] Aldridge[3] suggested that the inhibition must occur in the site of the energy transport chain

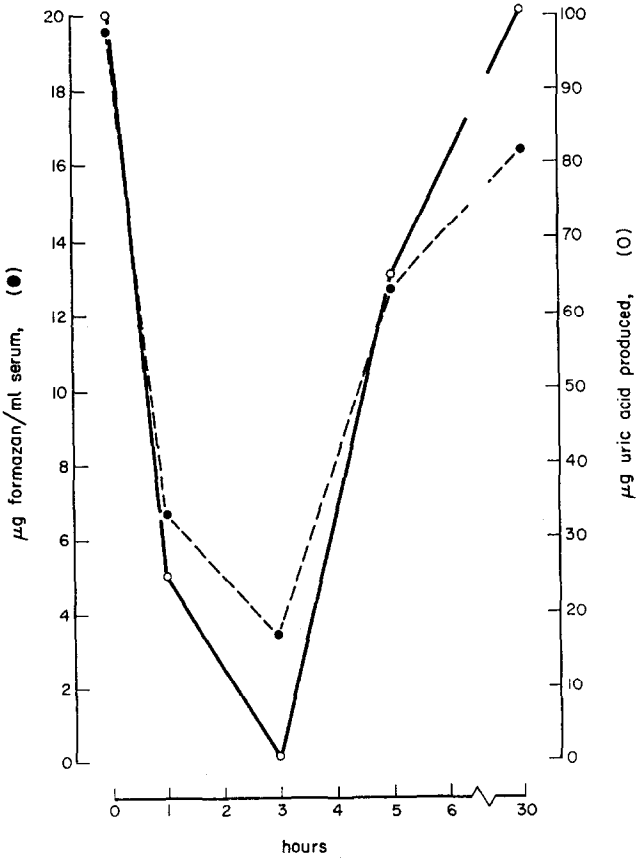

FIG. 1. Effect of the barbiturate on brain and blood xanthine oxidase activity. Time in abcissa is expressed in hours from the beginning of the narcosis. Each point represent average values for three determinations.