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accepted method for treating methanol poisoning (214)

### BIOCHEMISTRY OF METHANOL POISONING—III THE ENZYMIC PATHWAY FOR THE CONVERSION OF METHANOL TO FORMALDEHYDE

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Methanol  
WOW  
Eye  
Metabolism  
P450

**Abstract**—An enzyme system capable of oxidizing both methanol and ethanol in the presence of DPN was purified almost ninety-fold from the liver of the rhesus monkey. Data for the  $K_m$  values for the two alcohols and DPN and for the  $K_i$  values in the presence of some inhibitors are presented. The enzyme appears to be identical to liver alcohol dehydrogenase (ADH).

Ethanol competitively inhibited the oxidation of methanol by this enzyme.

The purified enzyme had appreciable activity when, in place of DPN, TPN was used as the hydrogen-acceptor.

Contrary to the observations of earlier authors, crystalline ADH, purified from horse liver, had an appreciable activity with methanol as substrate.

The data on the elimination of methanol in the blood of monkeys administered methanol by stomach tube agrees with the rates of its oxidation, as observed *in vitro*. It is concluded that the dehydrogenase mechanism, and not the catalase-peroxide system, is responsible for the physiological oxidation of methanol.

IN CONTINUING studies in this laboratory on the possible biochemical lesion in blindness due to methanol poisoning,<sup>1-4</sup> it became of interest to elucidate the nature of the enzyme system which catalyzes the oxidation of methanol to formaldehyde, since the latter compound is implicated as the toxic agent in the ocular damage resulting from methanol intoxication.

The enzymic pathway involved in the physiological oxidation of methanol has been a subject of considerable controversy. Lutwak-Mann<sup>5</sup> demonstrated that a partially purified preparation of horse liver alcohol dehydrogenase could oxidize methanol, as well as ethanol, to the corresponding aldehyde, but found that the reaction proceeded at a much slower rate in the former case. Using a similar preparation, Zatman<sup>6</sup> reported in a brief note that methanol is oxidized at a rate about one-ninth that of ethanol and that ethanol exerts a powerful competitive inhibitory effect on methanol oxidation. However, crystalline horse liver ADH<sup>†</sup>, the properties of which have been thoroughly studied by Theorell and co-workers,<sup>7, 8</sup> did not react with methanol and DPN to any measureable extent, although the enzyme catalysed the reverse reaction, viz. the reduction of formaldehyde by reduced DPN. As a

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‡Abbreviations: DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; ADH, alcohol dehydrogenase.

possible explanation of the discrepancy between their results and those of Zatman<sup>4</sup> and of Roe,<sup>9</sup> who found that ethanol acts as an antidote in clinical cases of methanol poisoning. Theorell and Bonnichsen<sup>7</sup> suggested that "methanol oxidation *in vivo* requires some catalyst besides ADH + DPN."

Keilin and Hartree<sup>10</sup> reported in 1945 that catalase can oxidize methanol or ethanol in an *in vitro*-system in the presence of a hydrogen peroxide-generating enzyme system. These observations were extended by Chance<sup>11</sup> using sensitive spectrophotometric techniques. Utilizing the data of Agner and Belfrage<sup>12</sup> on the disappearance of methanol in the blood of rabbits, Chance observed that sufficient catalase was present in the liver to account for the data of Agner and Belfrage and suggested that the peroxidative removal of methanol by catalase is probably the principal physiological pathway. From work with rats, a similar conclusion was reached by Strittmatter<sup>13</sup> who found that the rates, as calculated for the catalase-peroxidative reaction, closely parallel the maximum calculated rates for methanol oxidation *in vivo*.

Mannerling and Parks<sup>14</sup> have studied the effect of 3-amino-1:2:4-triazole, a potent inhibitor of catalase, both *in vivo* and *in vitro*, on the metabolism of methanol in rats. They found that although methanol oxidation was markedly inhibited in an *in vitro*-system containing catalase from rats treated with aminotriazole, this agent had no effect of the oxidation of methanol *in vivo*.

All these conflicting observations prompted us to investigate the physiologically important pathway involved in the conversion of methanol to formaldehyde. This problem was approached by the isolation and subsequent purification of an enzyme system from liver which catalyzes the oxidation of methanol to formaldehyde.

#### MATERIALS AND METHODS

The alcohols used in these studies were of reagent grade. DPN and TPN were purchased from the Sigma Chemical Co. Preparations of crystalline horse liver ADH were obtained from C. F. Boehringer & Sohne and from Worthington Biochemical Corp.

##### *Enzyme assay method*

Preliminary studies indicated that, in the presence of methanol and semicarbazide, a dialysed, 25,000 g -supernatant fraction derived from an homogenate of monkey liver reduced 2:6-dichlorophenol-indophenol, and that this reduction was increased markedly upon the addition of DPN. The reaction could also be followed, in the absence of the indophenol, by the increase in absorption at 340 m $\mu$ .

The activity of the enzyme responsible for the oxidation of methanol was estimated by following the course of pyridine nucleotide reduction at 340 m $\mu$  in a Beckman model DU spectrophotometer. Thus, in a Beckman cuvette with a 1-cm light path, were mixed 1.5 ml of 0.2 M glycine-NaOH buffer, pH 9.6, 0.1 ml of DPN (or TPN) containing 1 mg of the nucleotide, enzyme solution and water to give a final volume of 2.9 ml. After recording the initial optical density at 340 m $\mu$  the reaction was initiated by the addition of 0.1 ml of 50% methanol or ethanol. The increase in extinction at 340 m $\mu$  was read after 3 min. A unit of enzyme activity is defined as a change of optical density of 0.045 in 3 min; the specific activity is units of activity per mg of protein. The protein content of solutions was assayed by the method of Warburg and Christian.<sup>15</sup>

*Enzyme purification*

All procedures were carried out at 3 °C unless specified otherwise. The liver of freshly killed rhesus monkeys (*Macaca mulatta*) was the tissue of choice, since the earlier studies of Gilger and Potts<sup>16</sup> had indicated that, of all the laboratory animals tested, only the rhesus monkey exhibited signs of toxicity which were completely parallel with those observed in human cases of methanol poisoning.

One kilogram of monkey liver mince (obtained from fourteen monkeys) was stirred overnight with 2 l of ice-cold distilled water and centrifuged at 8000 g. The turbid buff-colored supernatant fraction was heated to 55 °C and maintained at this temperature, with continuous stirring, for 30 min. The solution was rapidly cooled in an ice-bath and recentrifuged at 8000 g; this yielded a clear red supernatant solution which, after separation, was treated slowly, with continuous stirring, with 277 g of ammonium sulfate per liter of fluid; appropriate amounts of ammonia were added to maintain the pH around 7. After 1 hr, the solution was centrifuged at 8000 g for 30 min and the residue was discarded. To each liter of supernatant solution, 195 g of ammonium sulphate was added, in order to raise the saturation from 45 to 70 per cent. After standing for 1 hr the solution was centrifuged and the residue was suspended in 0.01 M K-phosphate buffer, pH 7.0, and dialysed against distilled water to remove the ammonium sulfate. Diethylaminoethanol cellulose (Eastman), purified according to Peterson and Sober,<sup>17</sup> and equilibrated with distilled water, was packed in a column 5 × 40 cm. Chromatography was carried out in the cold room, at a flow rate of approximately 60 ml/hr. The enzyme solution was poured on the column, which then was washed with 400 ml of distilled water to elute the enzyme, the red hematin compounds being completely retained. This effluent was lyophilized, the residue suspended in 0.02 M K-phosphate buffer and dialysed against the same buffer for 18 hr. The denatured protein was removed by centrifugation to give a white opalescent liquid.

It was observed that during the fractionation of the enzyme, ADH was simultaneously purified. All attempts to separate these two enzymic activities, using conventional techniques, have failed; the methods used have included adsorption on alumina C<sub>γ</sub> gel or calcium phosphate gel, anion and cation exchange cellulose columns, alcohol fractionation at various pH values and ionic strengths. Hence, this solution was used as the source of stock enzyme and dilutions were freshly made from this preparation for every set of experiments.

## RESULTS

*Kinetic studies with monkey liver enzyme*

Fig. 1 depicts the results of some experiments on the reaction rates of methanol at pH 7.4 and pH 9.6, as plotted by the method of Lineweaver and Burk<sup>18</sup> to obtain the  $K_m$ . Fig. 2 gives the  $K_m$  for DPN under the conditions given in the legend.

Theorell and Bonnichsen<sup>7</sup> have stated that horse liver ADH is powerfully inhibited by *p*-chloromercuribenzoate (PCMB), while it is unaffected by iodoacetate (IA), and Valee and Hoch<sup>19</sup> have found that *o*-phenantroline (OP), as a result of its combination with the zinc present in ADH, also causes inhibition of the enzyme. Thus, it was of interest to test the effect of these substances on the enzyme system obtained from monkey liver. Both PCMB and OP inhibited the oxidation of methanol, while IA had no effect. All the kinetic data obtained with

52 °C for 15 min  
 used by Topley

ADH

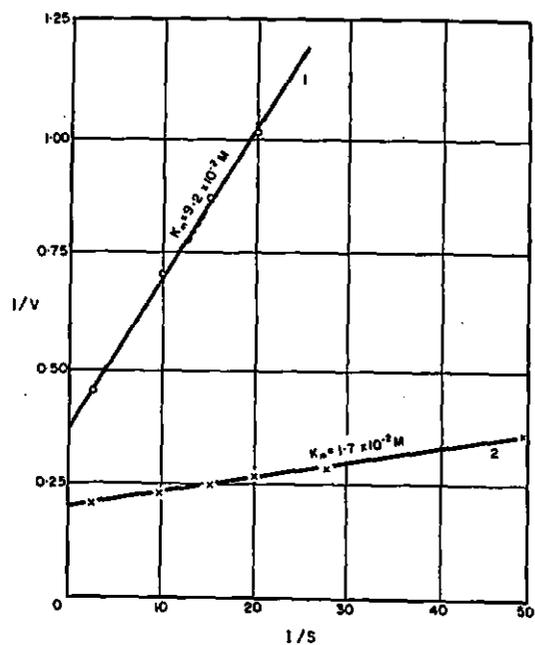


FIG. 1. Michaelis constant,  $K_m$ , for methanol at different pH values: (1), phosphate buffer, pH 7.4; (2), glycine-NaOH buffer, pH 9.6.  $V$  = units of enzyme activity as defined in text.  $S$  = (methanol) in moles/l.

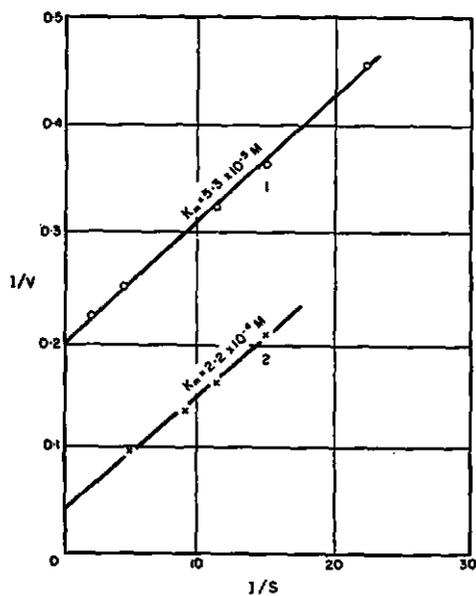


FIG. 2. Michaelis constant,  $K_m$ , for DPN with excess alcohol. Glycine-NaOH buffer, pH 9.6 (1) with methanol; (2) with ethanol.  $V$  = units of enzyme activity as defined in text.  $S$  = (DPN) in moles/l.  $\times 10^{-3}$ .

either methanol or ethanol as substrates are summarized in Table 2, which indicates that the enzyme systems responsible for the oxidation of ethanol and methanol display a marked similarity to each other in their properties, such as susceptibility to inhibition by PCMB and OP and lack of effect in the presence of IA, the only salient differences being in the relative  $K_m$ -values for the substrates and the  $K_i$ -values for the inhibitors.

TABLE 1. PURIFICATION OF "METHANOL DEHYDROGENASE" FROM MONKEY LIVER

Fraction	"Methanol dehydrogenase"			ADH		
	Specific activity	Total activity $\times 10^3$	Purification	Specific activity	Total activity $\times 10^3$	Purification
(1) Supernatant fraction of aqueous extract of 1 kg of liver mince	0.25	18.1	1	0.71	48.4	1
(2) Supernatant fraction after heating to 55 °C	0.99	17.4	4	2.8	43.9	3.9
(3) 45-70% ammonium sulphate fraction	3.7	13.2	14.8	9.9	35.1	13.9
(4) Chromatography on DEAE-cellulose	26.0	4.8	104	63.0	16.3	89

TABLE 2. SUMMARY OF SOME OF THE KINETIC CONSTANTS OF METHANOL AND ALCOHOL DEHYDROGENASE FROM MONKEY LIVER

Kinetic constant studied	Substrate used	
	Methanol	Ethanol
(1) $K_m$ -values of the alcohols in presence of excess DPN at pH 9.6	$1.7 \times 10^{-3}M$	$2.7 \times 10^{-3}M$
(2) $K_m$ -values of DPN in presence of excess alcohol.	$5.3 \times 10^{-5}M$	$2.2 \times 10^{-4}M$
(3) $K_i$ -values using <i>p</i> -chloromercuribenzoate.	$1.8 \times 10^{-5}M$	$0.8 \times 10^{-5}M$
(4) $K_i$ -values using <i>o</i> -phenanthroline.	$8.3 \times 10^{-5}M$	$2.6 \times 10^{-4}M$

In studies of the effect of different amounts of ethanol on the oxidation of methanol, absorbance at 340  $m\mu$  could not be used; accordingly, the formaldehyde formed during the reaction was measured colorimetrically by the method of Tanenbaum and Bricker.<sup>20</sup> The results, plotted according to Lineweaver and Burk,<sup>18</sup> clearly indicate that ethanol is a competitive inhibitor of methanol oxidation (Fig. 3), as has been observed by Zatman,<sup>6</sup> using a horse liver preparation of ADH.

#### Studies with horse and human liver enzyme

The presence and activity of "methanol dehydrogenase" was examined in normal human liver, obtained *post mortem*, to see whether this enzymic activity was present to account for the production of the toxic agent, formaldehyde. The results, summarized in Table 3, indicate that human liver oxidizes methanol at a rate comparable to that observed in monkey liver.

The conflicting reports of Lutwak-mann<sup>6</sup> on the slow oxidation of methanol by a partially purified horse liver ADH preparation, and that of Theorell and Bonnichsen,<sup>7</sup> and more recently, of Merritt and Tomkins<sup>21</sup> and Winer,<sup>22</sup> on the inability of methanol to react with crystalline horse liver ADH, necessitated a detailed examination of the purification steps involved in the isolation of horse liver ADH to see whether the

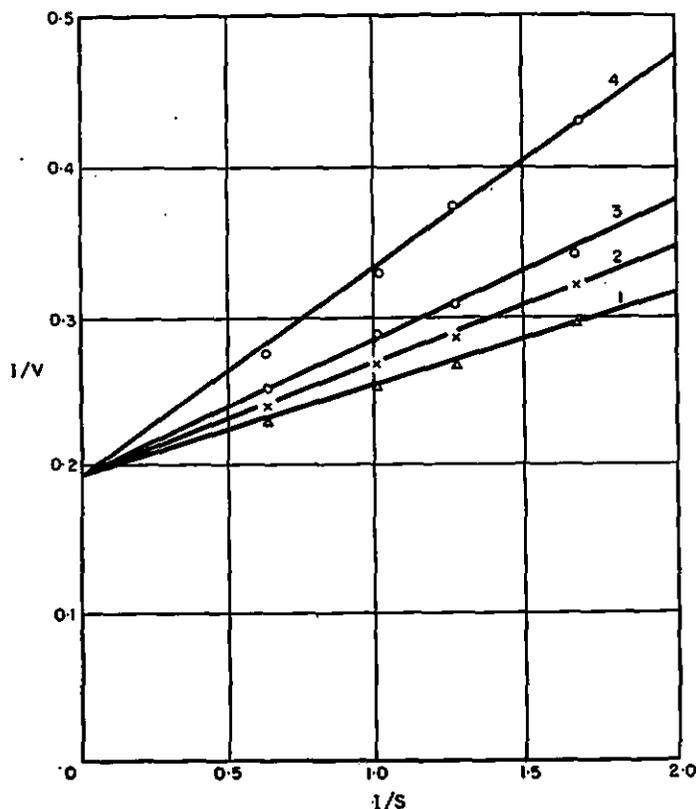


FIG. 3. Competitive inhibition of methanol oxidation by ethanol. Components of the medium were: Na-pyrophosphate buffer, pH 8.8, 0.025 M; DPN, 0.003 M; enzyme, 320  $\mu$ g of protein; substrate (methanol and inhibitor (ethanol) as indicated). Final volume, 1 ml. Temperature of incubation, 34 °C. Time, 10 min. Reaction was stopped by addition of 0.2 ml of 30% trichloroacetic acid and formaldehyde, estimated according to Tanenbaum and Bricker.<sup>20</sup> (1) Control; (2) 10mM ethanol; (3) 25 mM ethanol; (4) 50 mM ethanol.  $V$  =  $\mu$ g formaldehyde formed.  $S$  = (methanol) in moles/l.

methanol-oxidizing activity was lost in any of these steps. Table 3 presents data concerning the activities of the various fractions obtained by the purification procedure of Brink and Bonnichsen<sup>23</sup> for ADH. It is seen that the ratios of the ethanol-to-methanol-oxidizing ability of the different fractions vary within a relatively narrow range; the activity for methanol persisted in the crystals obtained upon ethanol fractionation, even after using the modified recrystallization method of Dalziel.<sup>14</sup> Furthermore, ultracentrifugal studies showed that this horse liver enzyme preparation migrated as a single homogeneous band, under the conditions described in the legend to Fig. 4. It may be mentioned that the twice-recrystallized horse liver ADH supplied

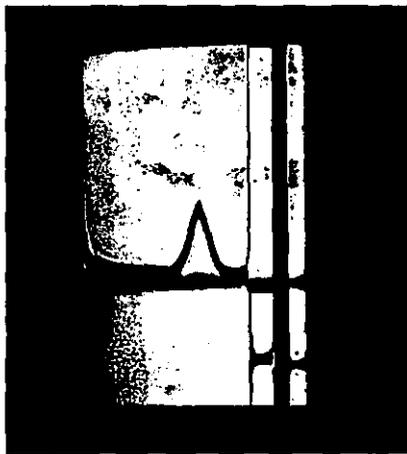


FIG. 4. Ultracentrifugal study of ADH crystallized from horse liver. Sedimentation was carried out at 23 °C at a speed of 59,780 rev/min in 0.01 M phosphate buffer, pH 7.0.

by C. F. Boehringer, had an ethanol/methanol ratio corresponding approximately to our figures and also behaved as a single protein in the ultracentrifuge.

*Activity with TPN as hydrogen acceptor*

During the purification of the dehydrogenase from *monkey* liver, studies were simultaneously made on its specificity towards the pyridine nucleotides. It was observed that both methanol and ethanol were also oxidized in the presence of TPN. The magnitude of the alcohol-TPN activities are given in Table 4. It may be added

TABLE 3. PURIFICATION OF HUMAN AND HORSE LIVER ALCOHOL DEHYDROGENASE

Fraction	Human*			Horse		
	Specific activity for ADH (A)	Specific activity for MDH (B)	Ratio A:B	Specific activity for ADH (A)	Specific activity for MDH (B)	Ratio A:B
(1) Water extract	0.65	0.12	5.4	2.9	0.42	6.8
(2) Supernatant fraction after heat denaturation	1.01	0.18	5.6	5.1	0.85	6.0
(3) 45-70% ammonium sulphate fraction dialysis	2.79	0.48	5.8	15.9	1.93	8.2
(4) Supernatant fraction after ethanol-chloroform precipitation, concentration, and dialysis	—	—	—	46.2	6.2	7.5
(5) Ethanol crystals	—	—	—	199	33	6.1

\* Specimen, 3 hr *post-mortem*, from a 72 year-old white man, who died of congestive heart failure secondary to chronic bronchitis.

TABLE 4. PYRIDINE NUCLEOTIDE SPECIFICITY

Substrate	Activity with DPN (units)	Activity with TPN (units)	Ratio, DPN/TPN
Ethanol	54.5	34.0	1.6
Methanol	19.7	4.1	4.8

Experimental conditions, as listed in "Methods", using monkey liver enzyme having a specific activity of 26 units/mg of protein, with methanol as substrate.

that during the purification of *horse* liver ADH, both methanol-TPN and ethanol-TPN activities were found, but the former was invariably destroyed or lost during the ethanol-fractionation step, while the latter persisted even after recrystallization. Pullman *et al.*<sup>25</sup> found that crystalline horse liver ADH exhibited a slight activity towards TPN; this amounted to about 1 per cent of that observed with DPN. Using the twice-recrystallized commercially prepared ADH, the ratio of activity of DPN to TPN, with ethanol as substrate, varied from 28:1 to 35:1.

#### DISCUSSION

Experiments have been described which suggest that ADH is also capable of oxidizing methanol. We used the rhesus monkey for our studies, since, as Roe<sup>9</sup> has

stressed, there is a fundamental difference between the toxic effects of methanol in man and those seen in lower animals. In addition, Gilger and Potts<sup>16</sup> have shown that the signs observed in the rhesus monkey parallel those observed in the human being.\*

An evaluation was made of the dehydrogenase mechanism in the physiological oxidation of methanol, using lines of reasoning similar to those employed by Theorell and Bonnichsen.<sup>7</sup> The rates of the disappearance of methanol in the blood stream of monkeys were measured by the method of Agner and Belfrage.<sup>13</sup> With an administered oral dose of 6 g/kg of body weight, a mean value of 0.0013 per cent per min was obtained for the disappearance of methanol from the blood over a period of 22 hr.<sup>26</sup> Since our monkeys had a body weight of approximately 3 kg and a presumed blood volume of about 235 ml, it can be calculated that the monkey oxidized a maximum of 10.45  $\mu$ moles methanol per min, assuming that significant pulmonary and urinary losses did not occur. Our best preparations had a specific activity of 26 units/mg of protein and, assuming that sufficient DPN is available to saturate the enzyme, it can be estimated that the observed elimination rate of 10.45  $\mu$ moles per min should require 166 mg of enzyme. By using a similar argument, and the data in Table 1, it can be calculated that the aqueous extract of 70 g of liver (the usual weight of a monkey liver) contains 48 mg of the enzyme. Thus, there is a somewhat reasonable correspondence between the calculated amount of enzyme from experiments *in vivo* and the amount of enzyme obtained from data *in vitro*, especially when one considers other tissue sites of oxidation of methanol, the fact that the kinetic data were obtained at 23 °C, and the probable elimination of some unchanged methanol by both the lung and the kidneys.

Further support for the contention that the dehydrogenase mechanism is the principal pathway in the oxidation of methanol comes from the observation of the competitive inhibitory effect of ethanol. The accepted method of treatment of clinical cases of methanol poisoning is to administer both bicarbonate to combat acidosis and a large dose of ethanol, the latter exerting its action by delaying the oxidation of methanol and thereby facilitating its renal and pulmonary excretion.<sup>9</sup> Such an effect has been demonstrated in experimental cases in man,<sup>27</sup> in monkeys,<sup>28</sup> and in rabbits.<sup>12</sup> In Bartlett's experiments<sup>29</sup> with rats, using <sup>14</sup>C-labelled alcohols, methanol was combusted to <sup>14</sup>CO<sub>2</sub> at about one-seventh the rate of ethanol. If the catalase mechanism of methanol oxidation were operating, the difference in the rates of oxidation of methanol and ethanol would be difficult to explain in view of the fact that both alcohols are oxidized at the same rate by catalase in the presence of peroxide. The observation of Mannering and Parks<sup>14</sup> that the oxidation of methanol was unaffected in aminotriazole-treated rats, in which liver catalase was depressed by over 90 per cent also favors the view that a dehydrogenase mechanism, and not a catalase mechanism, is operating *in vivo*.

Although it is reasonable to assume that the liver is the principal site of elimination of methanol, it is difficult to accept the premise that formaldehyde, the presumed toxic agent in methanol poisoning, is formed in the liver and transported to the eye where it exerts its effect, since formaldehyde has not been successfully demonstrated in the blood in cases of methanol poisoning.<sup>30</sup> Rather, it is more reasonable to support the view that methanol is oxidized *in situ* in the retina by the retinene reductase present

\* Recent work in this laboratory<sup>3</sup> has failed to confirm the retinal changes and metabolic acidosis found by Gilger and Potts<sup>16</sup> in methanol-poisoned rhesus monkeys.

in the rods and cones; this enzyme Wald<sup>31</sup> believes to be identical with ADH. Indeed, crystalline horse liver ADH oxidizes vitamin A to retinene.<sup>32</sup> The relative rates of oxidation of methanol and/or formaldehyde by the retina of different species may hold the clue to the species difference seen in the ocular damage in cases of methanol poisoning. To date, we have not been able to test this hypothesis because of the unavailability of suitable human material.

Our finding that ADH from horse liver, purified and recrystallized to the stage of a homogeneous protein in the ultracentrifuge, still oxidized methanol was surprising since various authors<sup>7, 21, 22</sup> have presented data that this enzyme exhibits no activity towards methanol.

In the work of Merritt and Tomkins<sup>21</sup> and of Winer<sup>22</sup> the explanation for their inability to observe methanol oxidation with crystalline ADH would appear to lie in the low methanol concentration (0.001M) used in their studies. In the work of Theorell and Bonnichsen,<sup>7</sup> however, no immediate explanation is apparent. We can only speculate that repeated ethanol crystallizations perhaps caused some structural modification of the protein which resulted in a loss of methanol-oxidizing ability.

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#### REFERENCES

1. J. R. COOPER and V. MARCHESI, *Biochem. Pharmacol.* **2**, 313 (1959).
2. M. M. KINI and J. R. COOPER, *Biochim. et Biophys. Acta.* **44**, 599 (1960).
3. J. R. COOPER and P. FELIG, *Toxicol. Appl. Pharmacol.* **3**, 202 (1961).
4. M. M. KINI and J. R. COOPER *Biochem. J.* (In press)
5. C. LUTWAK-MANN, *Biochem. J.* **32**, 1364 (1938).
6. L. J. ZATMAN, *Biochem. J.* **40**, 67P (1946).
7. H. THEORELL and R. BONNICHSEN, *Acta Chem. Scand.* **5**, 1105 (1951).
8. H. THEORELL, *Advanc. Enzymol.* **20**, 31 (1958).
9. O. ROE, *Pharm. Rev.* **7**, 399 (1955).
10. D. KEILIN and E. F. HARTREE, *Biochem. J.* **39**, 293 (1945).
11. B. CHANCE, *Acta Chem. Scand.* **1**, 236 (1947).
12. K. AGNER and K. E. BELFRAGE, *Acta Physiol. Scand.* **13**, 87 (1947).
13. P. A. J. STRITTMATTER, Ph.D. Thesis, Harvard University, Division of Medical Sciences (1953).
14. G. J. MANNERING and R. E. PARKS, JR., *Science* **126**, 1241 (1957).
15. O. WARBURG and W. CHRISTIAN, *Biochem. Z.* **310**, 384 (1941).
16. A. P. GILGER and A. M. POTTS, *Amer. J. Ophthal.* **39**, 63 (1955).
17. E. A. PETERSON and H. A. SOBER, *J. Amer. Chem. Soc.* **78**, 751 (1956).
18. H. LINEWEAVER and D. BURK, *J. Amer. Chem. Soc.* **56**, 658 (1934).
19. B. L. VALLEE and F. L. HOCH, *J. Biol. Chem.* **225**, 185 (1957).
20. M. TANENBAUM and C. E. BRICKER, *Analyt. Chem.* **23**, 354 (1951).
21. A. D. MERRITT and G. M. TOMKINS, *J. Biol. Chem.* **234**, 2778 (1959).
22. A. D. WINER, *Acta Chem. Scand.* **12**, 1695 (1958).
23. N. G. BRINK and R. H. BONNICHSEN, *Biochemical Preparations* Vol. 4, p. 29. John Wiley, New York (1955).
24. K. DALZIEL, *Acta Chem. Scand.* **12**, 459 (1958).
25. M. E. PULLMAN, S. P. COLOWICK and N. O. KAPLAN, *J. Biol. Chem.* **194**, 593 (1952).
26. M. M. KINI. Unpublished observations.
27. G. LEAF and L. J. ZATMAN, *Brit. J. Industr. Med.* **9**, 19 (1952).
28. A. P. GILGER, L. S. FARKAS and A. M. POTTS, *Amer. J. Ophthal.* **48**, 153 (1959).
29. G. R. BARTLETT, *Amer. J. Physiol.* **163**, 614 (1950).
30. A. O. GETTLER and A. V. ST. GEORGE, *J. Amer. Med. Ass.* **70**, 145 (1958).
31. G. WALD, *Handbook of Physiology* Vol. 1, Section 1, p. 671. American Physiological Society, Washington (1959).
32. A. F. BLISS, *Arch. Biochem. Biophys.* **31**, 197 (1951).