Reports

Inhibition of Methanol Metabolism with 3-Amino-1,2,4-Triazole

For a number of years a lively controversy has existed concerning the first oxidative step in the *in vivo* metabolism of methanol (*1*). The observation of Bonnichsen (*2*) that crystalline horse liver alcohol dehydrogenase is incapable of converting methanol to formaldehyde revived the view that a peroxidative reaction involving the enzyme catalase is the major mechanism in this oxidation (*3*). A means of testing this hypothesis is now offered by the recent finding that intraperitoneal injection of 3-amino-1,2,4-triazole (AT) causes as much as a 90-percent reduction in liver catalase activity (*4*). Thus, if a marked reduction in liver catalase can be effected by AT, and if catalase operates in the oxidation of methanol, then AT should cause a reduction in methanol metabolism. This rather obvious line of reasoning prompted us to test the effects of AT on the *in vitro* and *in vivo* metabolism of methanol.

Female albino rats weighing between 200 and 250 g were injected intraperitoneally with a 10-percent aqueous solution of AT (*5*) at a dose level of 1 g/kg. Three hours later their livers were removed and prepared as 10-percent homogenates in isotonic KCl. The homogenates were assayed for catalase activity by the method of Feinstein (*6*). The capacity of liver to metabolize methanol was studied by incubating 5 ml of the homogenate in a mixture (total volume, 10 ml; 0.1*M* phosphate buffer at *p*H 7.4) containing the following materials (in micromoles): methanol, 100; semicarbazide, 150; nicotinamide, 80; and $MgCl_2$, 40. The mixture was incubated in a Dubnoff metabolic shaker at 37°C in closed bottles containing air. An equilibration time of 20 minutes was allowed before methanol was added. Aliquots of the mixture were removed for analysis at 0 and 40 minutes. The 40-minute incubation period was chosen in order to remain within the limits of linearity on a previously determined metabolism-time curve. The measure of methanol metabolism is based on the observation of Strittmatter (*7*), which we were able to confirm, that while the oxidation of methanol will proceed to CO_2 when liver slices are employed, homogenates are incapable of carrying the oxidation beyond the formaldehyde stage. The aliquots were distilled from a 30-percent trichloroacetic acid solution, and the distillates thus obtained were analyzed for their formaldehyde content by the chromotropic acid method of MacFadyen (*8*). All values were corrected for a predetermined distillation loss of 10-percent.

Fifteen injected and 15 control animals were employed in this study. Livers from AT-treated rats were found to possess only 4 to 8 percent of the average catalase activity of livers from control animals. The livers from control and triazole-treated rats metabolized methanol at average rates of 6.3 (4.2 to 8.6) and 1.9 (1.4 to 2.4) μmole/g of liver per hour, respectively. Thus, AT administration effected an average 70-percent inhibition of methanol metabolism.

Since it is conceivable that AT could have inhibited enzymes other than catalase which may be responsible for methanol metabolism, an experiment was designed to determine whether or not catalase alone would restore to normal the reduced rate of methanol metabolism resulting from AT administration. Liver homogenates from both AT-treated and control rats, prepared and incubated as described previously, were supplemented with graded volumes of a solution of crystalline beef liver catalase (*9*) which were calculated to supply quantities of catalase equal to, less than, and greater than that lost through AT inhibition. The results are diagramed in Fig. 1.

It may be seen that addition of catalase restored the methanol-metabolizing capacity of AT-treated livers to normal or above normal, while it was without pronounced effect on the methanol metabolism of control livers. Moreover, it was not until 0.25 ml of the catalase solution had been added that the methanol-oxidizing capacity of liver homogenates from AT-treated animals equaled that of unsupplemented homogenates from control rats. The catalase activity of 0.25 ml of the solution used, about 1000 K units (*10*), was approximately equal to that found in the control homogenates. The rate of methanol metabolism reflects the interdependence of catalase and peroxide-generating systems. In normal liver homogenates catalase is not rate-limiting, as was shown by the failure of added catalase to cause an appreciable increase in methanol metabolism. However, we were able to show that, when a known peroxide-generating system [glucose oxidase (*11*) plus glucose] was added to normal liver homogenates, the rate of methanol oxidation could be increased as much as threefold. That catalase and not the peroxide-generating systems is rate-limiting in homogenates of AT-treated livers was shown by the failure of glucose oxidase and glucose to effect an increase in methanol metabolism.

Having established the role of catalase in an *in vitro* system, we attempted to determine whether this peroxidative mechanism actually functions in the *in vivo* metabolism of methanol. Eight adult female rats were given 1 g/kg of AT intraperitoneally. Two hours later methanol (20 percent by weight in saline solution) was injected by way of the tail vein at a dose level of 2 g/kg, and the disappearance of blood methanol was followed at 6-hour intervals for 36 hours. Blood methanol was determined by the method of Agner and Belfrage (*12*). Eight similar rats, which received methanol but no AT, served as controls. A third group of four rats received 1 g/kg doses of AT 2

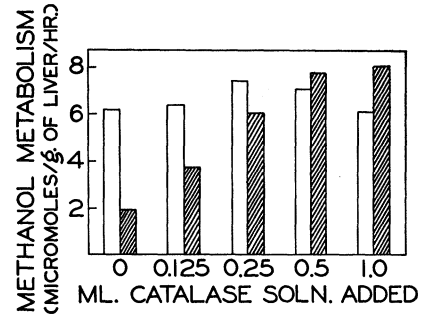

Fig. 1. Effect of added crystalline catalase on the metabolism of methanol by homogenates from livers of AT-treated and control rats. The solid bars represent the AT-treated animals, the open bars, the control animals. The catalase solution possessed about 4000 K units (*10*) of catalase activity per milliliter. Six AT-treated and six control rats were employed at each level of catalase addition except at the 0.125- and 1.0-ml levels, where four animals were used.

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hours before and 1 and 10 hours after methanol injection. In a second study employing four control and four experimental rats, the methanol dosage was raised from 2 to 3 g/kg. In none of these experiments was there sufficient difference between the methanol disappearance from the bloods of treated and nontreated rats to indicate that AT significantly inhibits methanol metabolism *in vivo*.

In view of the disagreement between results obtained *in vitro* and *in vivo*, one is obliged to consider the following possibilities: (i) AT is not an inhibitor of liver catalase *in vivo*; (ii) liver catalase is much more effective in its role as a catalyst of methanol oxidation *in situ* than it is *in vitro*, in which case the small amount of uninhibited catalase remaining after AT treatment is still sufficient to mediate adequate methanol metabolism; and (iii) catalase does not participate in the *in vivo* metabolism of methanol in the rat.

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References and Notes

1. For detailed discussions of both sides of the question the reader is referred to the comments of the following authors: R. Lemberg and J. W. Legge, *Hematin Compounds and Bile Pigments* (Interscience, New York, 1949), pp. 415–419; B. Chance, *Advances in Enzymology* (Interscience, New York, 1951), pp. 185–188; G. R. Bartlett, *Quart. J. Studies Alc.* 13, 583 (1952); E. Jacobsen, *Pharmacol. Revs.* 4, 107 (1952); O. Röe, *ibid.* 7, 399 (1955).
2. R. K. Bonnichsen, *Acta Chem. Scand.* 4, 715 (1950).
3. D. Keilin and E. F. Hartree, *Proc. Roy. Soc. (London)* B119, 141 (1936); *Biochem. J.* 39, 293 (1945).
4. W. G. Heim, D. Appleman, H. T. Pyfrom, *Science* 122, 693 (1955).
5. The 3-amino-1,2,4-triazole was generously supplied by the American Cyanamid Co., Agricultural Chemical Division. It was purified twice according to the directions given by that company, specifically, by placing the compound in the thimble of a Soxhlet extractor and extracting with a mixture of methanol and benzene in the proportions of their azeotrope (39.6 percent by weight methanol and 60.4 percent by weight benzene). The product crystallizes in the boiler of the Soxhlet apparatus.
6. R. N. Feinstein, *J. Biol. Chem.* 180, 1197 (1949).
7. P. A. J. Strittmatter, "The biological oxidation of methanol and formaldehyde, thesis submitted to Harvard University, 1 Dec. 1953.
8. D. A. MacFadyen, *J. Biol. Chem.* 158, 107 (1945).
9. The crystalline beef liver catalase was purchased from Worthington Biochemical Corp., Freehold, N.J. A 1/5 solution of this suspension was made using isotonic KCl containing a trace amount of NH_4OH.
10. One K unit represents the monomolecular velocity constant extrapolated to zero time as described by J. B. Sumner and A. L. Dounce [*Methods in Enzymology* (Academic Press, New York, 1955) vol. 2, p. 779].
11. The glucose oxidase was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio.
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12 August 1957

Response of Weaver Finch to Chorionic Gonadotrophin and Hypophysial Luteinizing Hormone

The specificity of the weaver-finch (*1*) feather reaction for luteinizing hormone has been described by Witschi (*2*), and the work with this assay has been reviewed by him recently (*3*). Preparations of luteinizing hormone (LH) from beef or sheep hypophyses give positive responses at dose levels far below the minimal effective doses of conventional rat assays. The weaver-finch unit for a standard LH preparation (Armour lot No. 227-80), for example, is 5 μg. In rat assays based on augmentation of follicle-stimulating hormone (FSH), the dose required to elicit a positive response with the same material is 1 mg (*4*).

In addition to its high degree of sensitivity, the weaver-finch test possesses the important advantage of being unaffected by the presence of nonluteinizing gonadotrophins. Doses of prolactin (LTH) as high as 5.0 mg fail to give a positive reaction. When this quantity of prolactin is added to the LH standard, the activity of the combined preparation proves to be identical with that of the LH component (Table 1). This same principle applies when combinations of hypophysial FSH and LH are prepared and tested in comparison with the LH factor alone. The FSH used in the experiment reported in Table 1 is inactive at a dose of 0.25 mg. Adding this amount of FSH to graded doses of LH has no measurable effect on the threshold for pigment deposition in the feathers. When the FSH preparation itself is assayed, a dose of 1.0 mg proves to have positive weaver-finch activity. This would seem to result from a small degree of LH contamination in the FSH sample. At this high total dose of FSH (1.0 mg) a 0.5 percent contamination with LH (0.005 mg) would account for the positive reaction. On this basis, 0.75 mg of FSH (inactive, when given alone) would contain LH contamination equivalent to the activity of about 0.003 mg of the LH standard. This factor, together with one-half the minimal effective dose of the LH standard (0.0025 mg), should exceed the threshold for weaver-finch activity. When these two subeffective doses of FSH and LH, respectively, are given simultaneously, this result is obtained (Table 1). The latter finding does not violate the principle that LH activity in the weaver-finch test is not affected by extraneous FSH present. In the combination employed, the FSH preparation simply acts as a carrier for the small amount of LH necessary to bring the total active material up to the required minimal effective dose.

The fact that gonadotrophins lacking LH activity do not synergize or augment in the weaver-finch test for LH is of particular importance in the testing of clinical material as well as in procedures designed to ascertain the purity of hypophysial extracts. When the hypophysectomized male rat is used in assays for LH, it is conceivable that the presence of prolactin in the sample would influence the size of the ventral prostate. Studies reported by several different laboratories indicate that LTH can enhance the responses of the ventral prostate and other sex accessories to LH in the hypophysectomized male rat (*5*). In the case of the immature or hypophysectomized female rat, the ovarian response to FSH in the test material is considerable and probably influences the interpretation of specific LH effects. It has never been established whether or

Table 1. Weaver-finch assay of hypophysial extracts and human chorionic gonadotrophin.

Material and dose	Weaver-finch reaction
Hypophysial extracts	
LH* (0.0025 mg)	−
LH* (0.005 mg)	+
LTH† (5.0 mg)	−
LH* (< 0.005 mg) and LTH† (5.0 mg)	−
LH* (0.005 mg) and LTH† (5.0 mg)	+
FSH‡ (0.25 mg)	−
FSH‡ (0.75 mg)	−
FSH‡ (1.0 mg)	+
LH* (< 0.005 mg) and FSH‡ (0.25 mg)	−
LH* (0.005 mg) and FSH‡ (0.25 mg)	+
LH* (0.0025 mg) and FSH‡ (0.75 mg)	+
Chorionic gonadotrophin (CGH)	
Pregnancy urine, 2nd mo of gestation (0.25 ml)	+
Pregnancy serum, 2nd mo of gestation (0.25 ml)	+
Normal male urine ultrafiltrate (6-hr concentrate)	−
Normal male urine ultrafiltrate (> 6-hr concentrate)	toxic
Male urine from patient with seminoma (0.25 ml)	+
Above male, after orchiectomy (2-hr concentrate)	−
CGH, Ayerst No. 29075 (30 IU)	−
CGH, Ayerst No. 29075 (60 IU)	+
CGH, Scheifflin No. 990331 (30 IU)	−
CGH, Scheifflin No. 990331 (60 IU)	+
CGH, Roche Organon No. 412018 (30 IU)	+
CGH, Roche Organon No. 412018 (60 IU)	+

* Luteinizing hormone, Armour lot No. 227-80. † Prolactin, Squibb lot No. 4B74978. ‡ Follicle-stimulating-hormone, Armour lot No. 377-200.