Manometric Studies on Guinea Pig Choline Oxidase.* (22892)

H. M. MAZZONE AND J. N. WILLIAMS, JR.
Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison.

It has been reported(1) that choline oxidase is absent from guinea pig liver. It was also reported later that the enzyme system is not found at all in the guinea pig and that the formation of methionine from choline is not demonstrable *in vitro* with guinea pig liver or kidney homogenates(2), using standard technics. However, Berg(3) has recently demonstrated, using C^{14} methyl-labeled choline, that both guinea pig liver slices and homogenates are able to methylate homocysteine to form methionine. This reaction was reported to be blocked under anaerobic conditions. From the latter observation, it appeared that choline oxidase must be present in the guinea pig, although its activity might be too low to be easily detected. We have reinvestigated this problem and wish to report our findings in the present paper.

Methods. Oxygen uptake studies using the Warburg manometric apparatus were undertaken to observe whether choline oxidase could be demonstrated in the guinea pig. The experimental animals were young adult guinea pig males fed a normal stock diet. After sacrificing, the liver, kidneys, and other organs were quickly removed and placed in ice. Homogenates (16.7%) were quickly prepared in 0.25 M sucrose using a Potter-Elvehjem homogenizer. The following concentration of buffers, at a pH of 7.3 unless stated otherwise, was used throughout the study: Krebs-Ringer phosphate (less calcium), 1.0 ml; and 0.039 M sodium potassium phosphate (equimolar in disodium phosphate and monopotassium phosphate), 0.5 ml. The filling of the Warburg vessels has been described elsewhere(4). The flasks were preincubated at 37°C for 1 hour before the addition of the substrate, choline chloride, was added from the side arm. The total fluid volume in each flask was brought to 3.7 ml with water. A control flask without substrate, but with water in its place, was used in each assay. Oxygen uptake was recorded for 180 minutes, after adding the substrate or water, at 10-minute intervals for the first 30 minutes and then at 30-minute intervals thereafter. Except for the studies concerning the effect of enzyme concentration on activity, the oxygen uptake was expressed as μl O_2 consumed due to added choline per hour per g tissue. The activity was calculated in every case from that portion of the "difference" (+ choline -- choline) curve which most nearly gave a straight line with maximum activity at least for 1 hour.

Results. The demonstration of the presence of choline oxidase and the optimum conditions for measuring the activity of the enzyme in guinea pig liver and kidney homogenates are presented in this section. Spleen, muscle, and intestine were also studied, but were found to contain no choline oxidase activity as measured in our system.

Preincubation effect. It was observed that endogenous oxidation in guinea pig homogenates was so high that choline oxidase activity is masked unless most of the endogenous oxygen uptake is first removed. For the first 60-90 minutes of the assay, manometer readings of the controls, representing endogenous oxidation, approached and sometimes exceeded manometer readings for the flasks containing added substrate. The difference in readings therefore would tend to lead one to believe that no activity was being measured. However, by preincubating the reaction mixtures for one hour before the addition of substrate, much of the endogenous respiration is removed. Lower manometer values are then observed for the control flasks and the difference values obtained represent a significant amount of oxidation taking place.

Effect of substrate concentration. As shown in Fig. 1, choline oxidase activity increased as the substrate concentration was

* Published with approval of Director of Wisc. Agric. Exper. Sta. Supported in part by grant from Nutrition Foundation, New York City and grant (A-753 (R)) from the National Institute of Arthritis and Metabolic Diseases, of N.I.H., P.H.S.

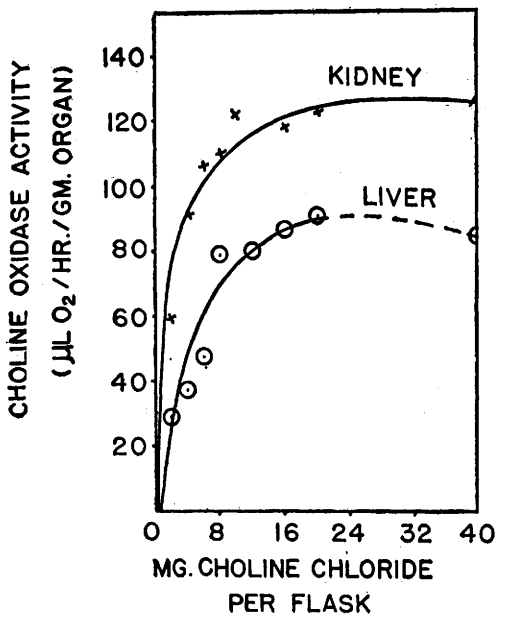

FIG. 1. Effect of substrate concentration on choline oxidase activities of guinea pig liver and kidney homogenates.

raised until about a 20 mg level of choline chloride per flask was reached. At first sight this might appear to be a rather high concentration of substrate but this feature is not unusual in enzyme systems. Possibly the inhibiting materials competing with the site of enzymatic activity in this system tend to cause a higher concentration of substrate to be required. Because of the low enzyme activity observed it cannot be stated with certainty whether a higher (40 mg/flask) level of choline chloride significantly inhibited the enzyme system, especially in the case of the liver homogenates. With rat liver a definite inhibition of the activity was observed for high substrate concentration(4).

Effect of pH. The buffer systems mentioned above were adjusted to various pH values and added to separate duplicate Warburg flasks (Set I). One ml of the appropriate homogenate was added to each flask. A separate set of flasks (Set II), similar to Set I was also prepared. The pH values were obtained by withdrawing the mixtures from the Warburg vessels of Set II after the initial 1-hour preincubation period plus 30 minutes after the addition of 20 mg of the substrate. The enzyme activity of Set I was recorded and plotted against the corresponding pH value. It was observed that a suitable pH optimum for both liver and kidney is 7.3.

Relation of enzyme concentration to activity. Enzyme activity was measured using volumes of 0.3, 0.5, 0.8, and 1.0 ml of 16.7% liver and kidney homogenates. Isotonic sucrose was added to equalize the enzyme volumes in the flasks. The substrate concentration was 20 mg of choline chloride and the pH of the buffers was 7.3. The enzyme activity was observed to be linear over the range of homogenate levels studied.

Location of choline oxidase in the cells of guinea pig liver and kidney. For these experiments 33% homogenates were used, since some activity would be expected to be lost during centrifugal fractionation. An aliquot of each homogenate was centrifuged at 25,000 g for 30 minutes in a refrigerated centrifuge. After separating the supernatant an equal quantity of isotonic sucrose was added to the insoluble residue and the mixture homogenized to resuspend the residue. The original homogenate, the resuspended residue, and the supernatant were then assayed for choline oxidase activity. The results indicate that the enzyme system, for both liver and kidney, is present in the insoluble fraction of the homogenate.

Identification of oxidation product in the kidney. A homogenate of 58 g of kidney in 290 ml of isotonic sucrose was preincubated with the two buffer systems in a 2-liter flask for 1 hour at 37°C. The volume ratio of the essential components of the reaction mixture in the Warburg vessels was maintained here. At the end of the preincubation period 174 ml of choline chloride equal to 6.96 g, stored at 37°C was added to the flask and the reaction mixture allowed to proceed under mechanical stirring for 6 hours. A control without added choline was run in the same manner. The reaction was terminated by precipitating the protein with trichloroacetic acid (T.C.A.). After removal of the T.C.A. with ether, the aqueous solution was concentrated under reduced pressure to a volume of

about 50 ml, and then treated with absolute alcohol to remove inorganic salts. After evaporating the alcohol at low temperature, the resulting aqueous solution was passed through a Dowex 50 column to remove the unused choline(5). Elution of the column with 1N HCl does not remove choline but does wash free other substances including betaine, a likely oxidation product of choline by the action of the enzyme. The hydrochloride elutant was concentrated to a small volume and then heated with charcoal to give a clear filtrate. A final concentrate of 5 ml was used in the identification of betaine by paper chromatography.

The literature reports various solvent systems† and detecting sprays for the paper chromatographic separation and detection of quaternary ammonium bases(6-11). Whatman No. 1 paper was used and the solvent allowed to ascend until a distance of 37 cm had been traversed. A control spot of betaine hydrochloride was applied on each strip. After air drying for 1 hour the detecting spray was applied. Betaine was identified only in the case where choline chloride had been added to the reaction mixture. Betaine aldehyde was not identified by an identical chromatographic process.

Discussion. In many cases, especially with liver homogenates, it was observed that choline oxidase activity was not measurable until a preincubation period of 1 hour and then did not commence until 60-90 minutes after the addition of substrate. This effect might be explained in several ways: 1. the lack of an adequate hydrogen-transport system to react simultaneously with choline dehydrogenase as well as with the endogenous-substrate oxidizing systems; 2. inhibition of certain endogenous oxidizing systems by choline chloride, which would give the impression that no choline oxidase was present until the substrates for those particular systems were used up; or 3. a lag in choline oxidation similar to that encountered with xanthine oxidase.

Although little or no activity was observed in the supernatant fluid of kidney and liver homogenates, it is possible that the enzyme system while insoluble, might require a cofactor. A likely site for this cofactor would be the supernatant fluid.

The identification of betaine as an oxidation product of choline should not rule out altogether the possibility of betaine aldehyde being present. The chromatographic methods employed for the identification of oxidation products of choline might not be sensitive enough to demonstrate the presence of betaine aldehyde.

Summary. 1. The enzyme system, choline oxidase, is present in the liver and kidneys of the guinea pig. Activity is low but measurable, as shown using standard manometric technics. 2. Optimum substrate concentration, pH, and assay conditions are presented. 3. The enzyme response *versus* enzyme concentration was found to be linear over the range tested. 4. The enzyme system in both liver and kidney of the guinea pig is insoluble as shown by centrifugal separation of the soluble and insoluble components of the homogenates in isotonic sucrose. 5. In kidney betaine was found to be an oxidation product by the action of the enzyme on choline.

† n-Butanol : Pyridine : Water(6;7,8)
3 : 1 : 1.5
n-Butanol : Ethanol : Acetic Acid : Water (9;7,10)
8 : 2 : 1 : 3
n-Butanol saturated with water(11;10)

1. Bernheim, F., and Bernheim, M. L. C., *Am. J. Physiol.*, 1933, v104, 438.
2. Dubnoff, J. W., *Arch. Biochem.*, 1949, v24, 251.
3. Berg, P., *J. Biol. Chem.*, 1951, v190, 31.
4. Williams, J. N., Jr., Litwack, G., and Elvehjem, C A., *ibid.*, 1951, v192, 73.
5. Friedman, S., *et al., Arch. Biochem. and Biophys.*, No. 2, 1955, v59, 484.
6. Chargaff, E., *et al., J. Biol. Chem.*, 1948, v67, 175.
7. Brown, F., *Biochem. J.*, 1950, v47, 598.
8. Isherwood, F., *ibid.*, 1953, v48, 515.
9. Augustinsson, K., *Acta Chem. Scand.*, 1953, v7, 906.
10. Brante, G., *Nature*, 1949, v163, 651.
11. Huennekens, F., *J. Biol. Chem.*, 1954, v206, 443.

Received November 14, 1955. P.S.E.B.M., 1957, v94.