Colorimetric Method for Rapid Determination of Arginase Activity.* (22319)

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Existing methods for determining arginase activity generally involve measuring one of the products of arginine hydrolysis. For example, ornithine has been determined by titration(1). The most usual product measured, however, is urea, which can be estimated by the cumbersome procedure of decomposition with urease followed by the manometric measurement of carbon dioxide(2,3) or the colorimetric(4) and titrimetric(5) estimation of the ammonia produced. Urea can be directly estimated colorimetrically, but arginine has been reported to interfere with the color produced(6). In addition, most of these methods require more arginase activity than many studies allow. We recently had need of a rapid method for arginase determination in which only small amounts of tissues were required. A note by Iyer and Krishna Murti (7) appeared to offer a promising method, in which arginine remaining after hydrolysis was estimated colorimetrically by the Sakaguchi reaction as modified by MacPherson (8). However, the method of Iyer and Krishna Murti(7) could not be reproduced since the details of carrying out the method as well as the results presented could not be adequately interpreted. It was also found that the suggested glycine buffer gave marked inhibition of color development.

In the present paper, we wish to present a method which allows the accurate determination of arginase activity in samples of liver as small as 20 γ (fresh weight).

Methods. Reagents. In addition to those reagents listed by Gilboe and Williams for determining free arginine(9), the following reagents are required for estimating arginase activity: 0.4% trichloroacetic acid (TCA); 0.1 M sodium pyrophosphate adjusted to pH 9.5; and a solution containing 50 γ of arginine per ml, prepared as needed from a stock solution containing 605 γ arginine hydrochloride per ml kept at 5°. The last solution is stable for about one month. *Procedure.* A liver homogenate containing 0.1 mg of liver per ml is prepared by homogenizing a weighed portion of rat liver, obtained from, a freshly sacrificed rat, in ice-cold distilled water. The homogenate should be kept in an ice bath and used within 30 minutes after preparation. In a test tube, large enough for mixing of added solutions by swirling, are placed 3 ml of the diluted arginine solution and 1 ml of the pyrophosphate buffer. The mixture is placed in a constant temperature bath maintained at 37°. After a 5-minute temperature equilibration period, 1 ml of the liver homogenate is added; the contents are mixed quickly, and the mixture is incubated for exactly 10 minutes. At the end of the 10-minute period, 1 ml of the incubation mixture is withdrawn and pipetted into a tube containing 4 ml of 0.4% TCA. The protein precipitated from this amount of tissue is so low that centrifugation is not necessary. However, if larger amounts of tissue are used, for example, tissues containing exceptionally low arginase activity, centrifuging would be necessary to obtain a clear extract. The procedure for estimating the unhydrolyzed arginine has been outlined by Gilboe and Williams(9). A blank is run using the same procedure as above except that the arginine is omitted. Since each tube except the blank contains 30 γ of arginine per ml before incubation, the amount of arginine hydrolyzed is determined by subtracting the γ of arginine per ml remaining in the tube from 30 γ. By decreasing all volumes to one-fifth those given above and adding 4 ml of TCA directly into the incubation mixture, only 20 γ of liver are needed.

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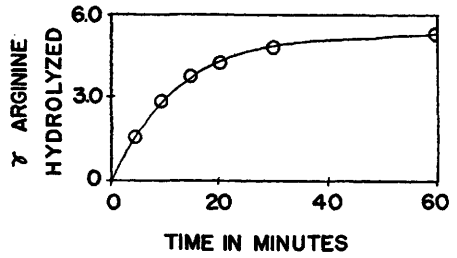

FIG. 1. Rate of arginine hydrolysis in a typical experiment.

A *standard curve* should be established by mixing 0, 1, 2, and 3 ml of the arginine solution (50 γ per ml) in 4 separate tubes, adding 1 ml of 0.1 M sodium pyrophosphate to each tube, diluting to 5 ml with water, and following the procedure outlined above for taking the sample and developing the color. This method of obtaining a standard curve gives both a standard curve and a check on the procedure.

Results. To determine the proper incubation time, a mixture of 6 ml of 50 γ of arginine per ml, 3 ml of the pyrophosphate buffer, 3 ml of distilled water, and 3 ml of the liver homogenate was incubated at 37°. One ml aliquots were taken from the incubation mixture at 5, 10, 15, 30 and 60 minutes. The amount of arginine hydrolyzed was determined as previously described. Fig. 1, which is a plot of the arginine hydrolyzed *vs.* time, indicates there is no longer a straight line relationship between enzyme activity and time after 10 minutes. This observation agrees with the results of Van Slyke and Archibald(6).

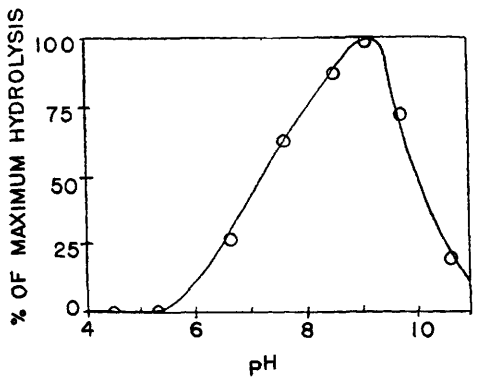

FIG. 2. Effect of pH on arginase activity (avg of 2 experiments).

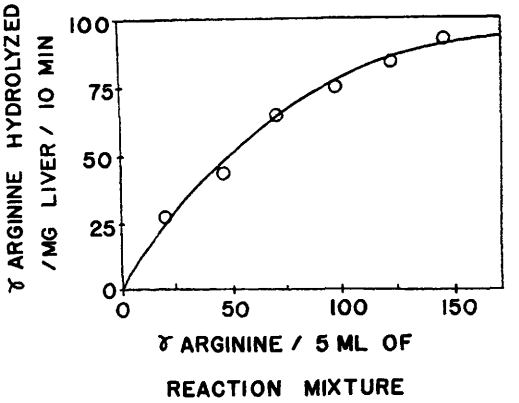

FIG. 3. Arginase activity *vs.* arginine concentration (avg of 7 experiments).

The optimum pH of the enzyme was determined by carrying out the reaction in a compound buffer (0.05 M in each anion) prepared from the following substances: sodium acetate, acetic acid, sodium dihydrogen phosphate, disodium phosphate, sodium pyrophosphate, sodium carbonate, and sodium bicarbonate. Portions of this compound buffer were adjusted to pH 4, 5, 6, 7, 8, 9, 10 and 11 respectively. From Fig. 2 it can be seen that the pH optimum lies between 9.0 and 9.5, which is in agreement with the data of previous workers for arginase(6,10).

The substrate concentration curve for 0.1 mg rat liver per 5 ml total reaction volume is shown in Fig. 3. It can be seen that the curve levels off near 150 γ of arginine. The linearity of the enzyme activity *vs.* enzyme concentration over a 5-fold range using 150 γ arginine is shown in Fig. 4. Thus it may be concluded that the enzyme in 0.1-0.5 mg of liver per 5 ml total reaction volume is saturated when the amount of substrate is 150 γ.

Further experiments have indicated that additional manganese is not needed in the system reported in this paper. This indicates that sufficient manganese is present in the whole liver homogenate to activate the enzyme maximally. Using purified arginase preparations, manganese would undoubtedly need to be added. This is left to the discretion of the operator when preparations other than whole liver homogenates are used. From data in Fig. 3 the Michaelis constant for this

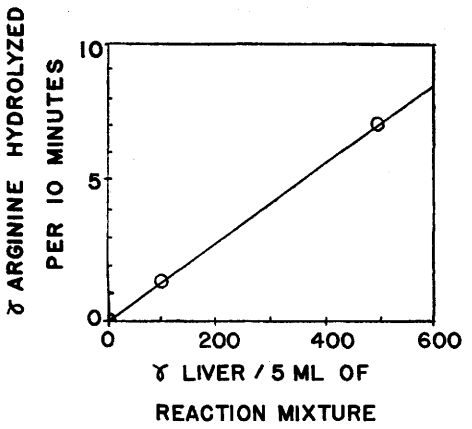

FIG. 4. Linear response of arginase activity *vs.* liver concentration (avg of 2 experiments).

system was calculated to be 1.53 x 10^{-4} M which agrees with a calculation of the Michaelis constant from data presented by Van Slyke and Archibald(6).

Summary. A method has been presented for the rapid determination of arginase with a minimum of operations and equipment. The activity of as low as 20 γ of whole liver can be determined. The arginine remaining after enzymatic hydrolysis is estimated by a quantitative modification(9) of the Sakaguchi reaction. Additional studies are presented indicating the necessary substrate concentration, the pH optimum, and linearity of the enzyme concentration curve for these experimental conditions.

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Effect of Cholinergic Drugs on Development of Chick Embryo.* (22320)

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There is sufficient evidence to indicate the presence of cholinesterase in embryos. Zacks (10) demonstrated the presence of cholinesterase in the embryonic chick from 0-96 hour stages. Wenger(8) followed the development of cholinesterase in spinal cords of chick embryos of 5½ to 11 days of incubation. Nachmansohn(4,5) determined cholinesterase activity in chick nerve and muscle at 6 or 9 days of age. He followed the increasing levels of activity of this enzyme to about the 15th day and showed a higher rate of increase until hatching. Similar observations have been made on early and later stages of amphibian embryos (Youngstrom(9), Sawyer (6,7), Boell and Shen(1,2)).

Since cholinesterase is present during early embryonic stages and increases steadily as the neuromuscular apparatus appears, the administration of enzyme inhibitors and the accumulation of acetylcholine could be attended by developmental abnormalities. To investigate this possibility, 4-day chick embryos were treated with various doses of cholinesterase inhibitors (prostigmine bromide; physostigmine salicylate), the enzyme

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