Although this evidence is consistent with the involvement of calmodulin in the increase in enzyme activity induced by dibutyryl cyclic AMP and the methylxanthines, it is also possible that other, as yet undefined, interactions may contribute to the action of haloperidol in the brain stem slice preparation. What does emerge from these experiments is strong evidence against the involvement of cyclic AMP or any other endogenous cyclic nucleotide in the regulation of tryptophan hydroxylase in the slice preparation.

In summary, pretreatment of rat brain stem slices with dibutyryl cyclic AMP, caffeine, theophylline and 3-isobutyl-1-methylxanthine increased the activity of tryptophan hydroxylase in supernatant preparations of enzyme made from the slices. This effect does not appear to be mediated by a cyclic AMP sensitive mechanism since it was not reproduced by exposure of the slices to 8-bromo cyclic AMP, to papaverine, a nonxanthine phosphodiesterase inhibitor, or to other treatments known to raise tissue cyclic AMP levels. The ability of haloperidol to block this increase in enzyme activity is consistent with a role for calmodulin and calcium as mediators of the enzyme activation, particularly when this observation is considered in conjunction with the evidence that supernatant preparations of this enzyme are activated under phosphorylating conditions by a calcium–calmodulin dependent process [5]. Nevertheless, in view of the high concentrations of haloperidol employed in the present experiments, the possibility that this drug may produce its effects in the brain stem slices through some other action, unrelated to its ability to bind to calmodulin, should be kept in mind.

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Increased sensitivity of the fluorometric method of Snyder and Hendley for oxidase assays

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Snyder and Hendley [1] and Guilbault *et al.* [2] developed a fluorometric assay for amine oxidases, in which hydrogen peroxide formed in the oxidase reaction is measured fluorometrically by converting homovanillic acid to a highly fluorescent compound in the presence of peroxidase. This method is widely used because of its simplicity and high sensitivity, and it is applicable to any hydrogen peroxide-generating system. We have found that much higher sensitivity of this method can be achieved by changing the final pH of reaction mixtures, the details of which are reported in this communication.

As a monoamine oxidase preparation, crude mitochondrial fractions were isolated from the pooled livers of six male Sprague–Dawley rats as described previously [3]. Purified hog kidney diamine oxidase was purchased from the Sigma Chemical Co., St. Louis, MO.

The incubation mixtures contained 0.10 ml of 0.25 M sodium phosphate buffer (pH 7.4), 0.10 ml of peroxidase solution (0.5 mg/ml), 0.10 ml of homovanillic acid solution (1.0 mg/ml), 0.10 ml of enzyme solution (rat liver monoamine oxidase, 0.0159 mg protein/ml; hod kidney diamine oxidase, 0.20 mg/ml; or water for the hydrogen peroxide assay), 0.10 ml of substrate solution (benzylamine for monoamine oxidase, final concentration 1.0 mM; putrescine for diamine oxidase, final concentration 1.0 mM; or hydrogen peroxide, 22 nmoles/ml) and 0.10 ml of water.

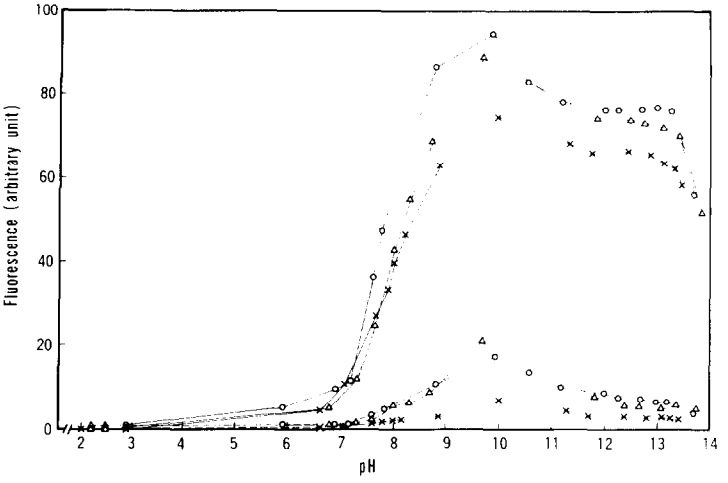

Fig. 1. Effect of the final pH values of reaction mixtures on the fluorescence produced by the reaction of rat liver mitochondrial monoamine oxidase (×——×), hog kidney diamine oxidase (△——△) or hydrogen peroxide (○——○). The upper curves (solid lines) show the fluorescence of test samples and the lower curves (dotted lines) that of their respective blank tests. Enzymatic reactions were carried out at pH 7.4 and the media were brought to various pH values. The amounts of monoamine oxidase, diamine oxidase and hydrogen peroxide were 1.6 and 20 μg of protein and 2.2 nmoles per tube respectively. For details of the assays, see text. Each point is the mean of triplicate determinations with the S.D. not more than 2.0% of the mean.

After incubation at 37° for 60 min, the oxidase activities were stopped by adding 0.10 ml of specific inhibitors (pargyline for monoamine oxidase, final concentration 1.0 mM; semicarbazide for diamine oxidase, final concentration 1.0 mM; or water for the hydrogen peroxide assay). After cooling the tubes in tap water, 2.0 ml of various concentrations of NaOH or HCl solution was added to the mixtures to obtain various final pH values of the mixtures. The fluorescence intensity was measured with excitation at 323 nm and emission at 426 nm (uncorrected). As blank tests, assay mixtures without substrates were incubated; the substrates were mixed after inactivation of the enzymes with the inhibitors. Under these conditions, the enzyme assays were linear with respect to incubation time and also to enzyme concentration. Finally, the pH of each mixture was measured with a Hitachi–Horiba type F-7 pH meter.

Protein was measured by the method of Lowry *et al.* [4].

Figure 1 shows fluorescence intensities as a function of the final pH values of the reaction media in the assays of monoamine oxidase, diamine oxidase and hydrogen peroxide itself, where enzymatic reactions have been carried out at pH 7.4. The fluorescence of the test samples was equally increased rapidly from pH 7.0 to pH 10, and they remained at high values up to pH 13.5, although a slight decline was observed. The fluorescence of the blank tests was also increased up to pH 10.0 and then decreased. Since sensitivity of fluorometric assays is related to the ratio of fluorescence of a test sample to that of a blank test, the most desirable pH range of the mixture was found to be from pH 11.5 to 13.0. The shift of pH of the mixtures to this range resulted in a 3- to 4-fold increase of sensitivity as compared with that measured with the mixture at pH 7.4. The alkalinization contributed also to stopping the enzyme reaction and to clarification of the assay mixtures containing turbid enzyme preparations such as crude mitochondria.

The fluorescence in the alkaline mixture (pH 11.5–13.0) was stable for several hours.

The calibration curve with different amounts of hydrogen peroxide, after alkalinization of the reaction mixture, showed its linearity from 0.1 to at least 10 nmoles per tube and passed through the origin. The detection limit was 0.1 nmole, which gave about 150% of the blank fluorescence.

In the original method of Snyder and Hendley [1], oxidase reaction was stopped by chilling the mixture (pH 7.8), which was then subjected to fluorescence measurements with excitation at 315 nm and emission at 425 nm (uncorrected). In our modified assay, measurements with excitation at 323 nm and emission at 426 nm (uncorrected) were adopted, since the fluorescence maxima were found to shift to these wavelengths under alkaline conditions.

On the basis of the above data, we recommend the following procedure as a modified assay for an amine oxidase or hydrogen peroxide itself. The incubation mixtures (0.6 ml) containing sodium phosphate buffer (pH 7.4), peroxidase, homovanillic acid, an amine oxidase and a substrate (their composition may be the same as described before) are incubated at 37° for an appropriate period (15–60 min), after which 2.0 ml of 0.1 N NaOH is added to the mixture to bring its final pH to 11.5–13.0; then the fluorometric measurements are made as described before. As blank tests, assay mixtures without substrates are incubated; the substrates are mixed after adding the NaOH solution. Internal standards should be taken by adding appropriate amounts of hydrogen peroxide to the mixtures prior to incubation, especially when some quenching due to enzyme preparations is suspected.

In summary, we have modified the method of Snyder and Hendley for oxidase assays by adding NaOH solution after enzyme reaction. This addition increased the sensitivity more than 3-fold and contributed also to stopping the enzyme reaction and to clarification of assay mixtures containing turbid enzyme preparations.

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