The Use of Sodium Bicarbonate-C^{14} to Determine Cholinesterase Activity[1]

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Simple and inexpensive methods of determining cholinesterase activity are useful in many phases of research dealing with organophosphorus and carbamate insecticides. Such a method, based on the same principle utilized in manometric techniques, has been used with success in our laboratory. Sodium bicarbonate-C^{14} is used to react with acetic acid liberated from the enzymatic hydrolysis of acetylcholine. The resulting $C^{14}O_2$ is trapped and quantitatively measured by liquid scintillation counting. The method, described below, requires no special purchase of instruments for laboratories already equipped with an incubator-shaker apparatus and a liquid scintillation counter.

Reagents

(1) Saline solution. - 0.5M NaCl in distilled water.

(2) Substrate. - 0.28M acetylcholine bromide in saline solution.

(3) Carbon dioxide trap solution. - 1:2 mixture of 2-aminoethanol and 2-methoxyethanol.

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(4) Sodium Bicarbonate-C^{14}. - One millicurie of $NaHC^{14}O_3$ (Sp. Act. 19.5 mc/mM; New England Nuclear Corp., Boston, Mass.) + 3 g. of $NaHCO_3$ in 100 ml. of distilled water. One microliter of this solution contained 14,000 cpm as determined by liquid scintillation counting (Packard Tri-Carb Model 3365).

(5) Acetylcholinesterase. - 20,000 units of acetylcholinesterase (Nutritional Biochemical Corp., Cleveland, Ohio) in 100 ml. of sterile water.

Reaction Apparatus

A single reaction vessel used for assaying cholinesterase activity is shown in Figure 1. Twelve of these vessels are routinely used for a single run. Each consists of a 25 ml. large mouth Erlenmeyer flask (F) fitted with a No. 4 Neoprene stopper (E) in which two 18G 1" needles and one 18G 2" needle (G) have been inserted. The longer needle is used as an air inlet and one of the short needles serves as an outlet through which the air is directed to the carbon dioxide trap (K). A ¼ cc syringe (B) is attached to the second short needle for substrate injection into the flask. The plunger of the syringe is held in place with a clip (C).

Clear plastic tubes, (A) and (H), are connected to the needles used for the air inlet and outlet with tips of ¼ cc syringes. With this arrangement the apparatus can be dismantled easily for cleaning. The tubing connected to the long needle (A) is attached to an air source. The air outlet hose (H) connects to a gas

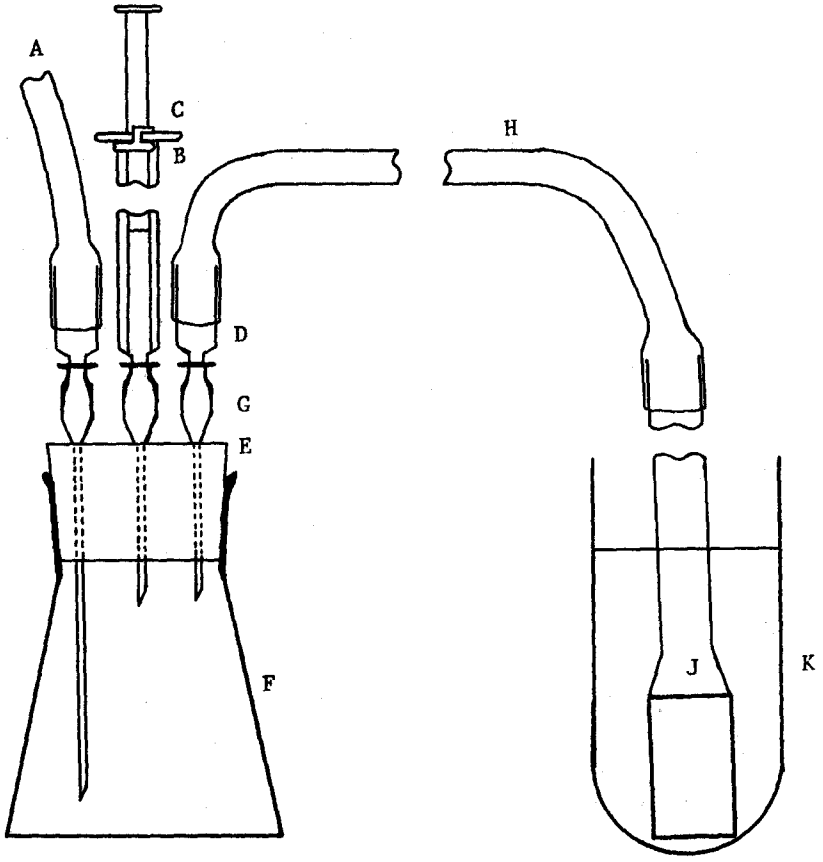

Fig. 1. Reaction vessel for determining cholinesterase activity using sodium bicarbonate-C^{14}: A - air inlet hose, B - ¼ cc syringe for substrate injection, C - clip for holding syringe plunger stationary, D - tips of ¼ cc syringes for connecting tubing to needles, E - No. 4 Neoprene stopper, F - 25 ml. Erlenmeyer flask, G - 18G needles, H - air outlet hose, J - gas dispersion tube, K - carbon dioxide trap consisting of a 40 ml. centrifuge tube containing 20 ml. of trap solution.

dispersion tube (J) which is submerged in 20 ml. of the carbon dioxide trap solution. A 40 ml. round-bottom centrifuge tube serves as a container for the solution. After the apparatus has been completely assembled, the air flow through the system is adjusted to approximately 330 ml. per minute with the aid of a manometer.

Procedure

Place the 25 ml. Erlenmeyer flask in a 37° C water bath (Dubnoff Metabolic Shaking Incubator equipped with holders for 25 ml. flasks) and connect the air flow tubes to the appropriate needles in the Neoprene stopper. Do not fit the stopper to the flask at this time. Draw 0.2 ml. of the substrate solution into a ¼ cc syringe and attach the syringe to the needle used for substrate injection.

Pipette 1.5 ml. of saline solution, 0.1 ml. of radiolabeled sodium bicarbonate solution (approximately 1.4×10^6 cpm) and 0.2 ml. of acetylcholinesterase into the 25 ml. flask. Immediately connect the stopper to the flask and allow the mixture to equilibrate, with shaking, for 5 minutes. During this time the gas dispersion tube should be in trap solution to prevent laboratory contamination by any $C^{14}O_2$ which might escape before addition of the substrate. At the end of the 5-minute equilibration period, transfer the gas dispersion tube to a fresh carbon dioxide trap solution and quickly inject the substrate into the flask. Continue incubation for exactly 30 minutes. One-half ml. aliquots are then removed from the trap for radioassay by liquid scintillation counting.

To determine the amount of $C^{14}O_2$ given off as a result of non-enzymatic hydrolysis of acetylcholine, a second flask is carried through the same procedure as described above except 1.7 ml. of saline solution is added and the 0.2 ml. of enzyme solution omitted. The two runs are made simultaneously.

Cholinesterase Inhibition Determination

Depression and recovery of cholinesterase in houseflies and rats following treatment with organophosphorus and carbamate insecticides have been followed using the sodium bicarbonate-C^{14} method. Once the enzyme concentration necessary to liberate approximately 70% of the radioactivity is determined, the cholinesterase activity in the treated animals is easily and rapidly measured.

The anticholinergic properties of compounds also may be determined in vitro. One such study was conducted where Ciodrin ^{R} (2-methylbenzyl 3-hydroxycrotonate dimethyl phosphate) was used as the inhibitor. In this experiment 10 flasks were carried through the procedure at the same time; one flask was used for both the control (no enzyme) and normal enzyme (no inhibitor) and two for each of the four different levels of inhibitor. The Ciodrin was added to the flasks in an acetone solution and the solvent evaporated. Additions of enzyme to each flask and the subsequent additions of substrate and removal of aliquots from the trap solutions were separated by a one minute time interval to prevent any variation in the time of incubation of each flask. After the introduction of the enzyme, the flasks were incubated for 15 minutes.

A total incubation time of 30 minutes was allowed following the injection of the substrate although aliquots for radioassay were removed from each trap at 10-minute intervals.

The results of this experiment are shown in Figure 2.

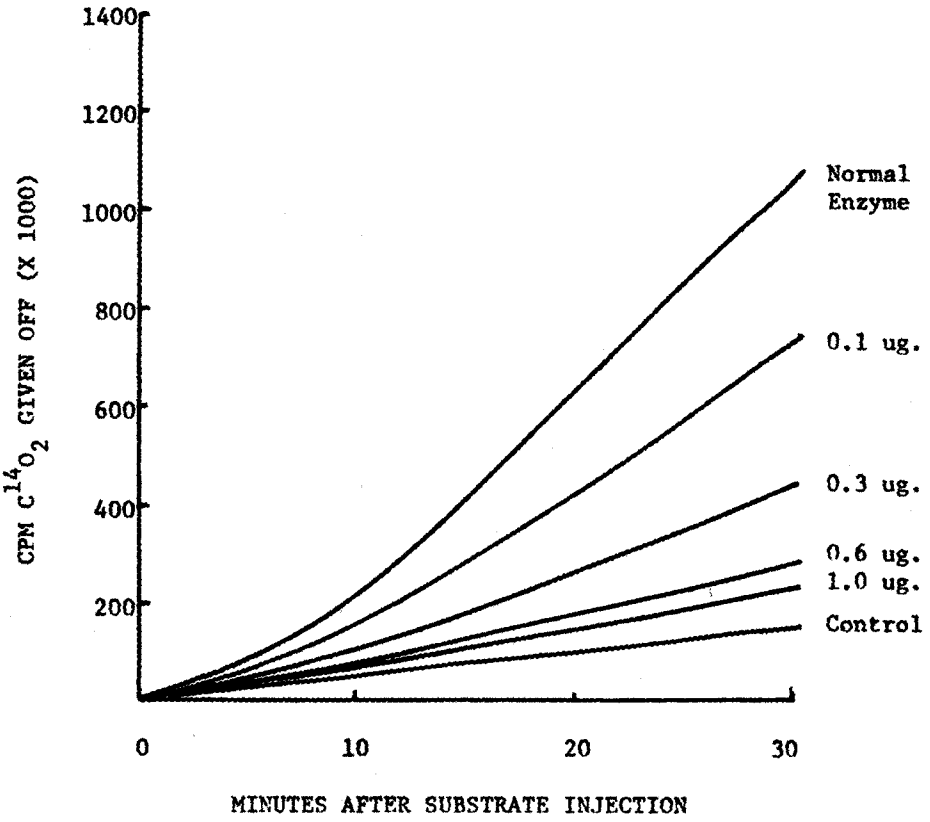

Fig. 2. Ciodrin inhibition of acetylcholinesterase indicated by decreasing quantities of C^{14}-carbon dioxide as inhibitor concentration increases. The figures expressed in microgram amounts represent the level of Ciodrin per flask.

Approximately 75% of the total radioactivity was given off as $C^{14}O_2$ in flasks containing enzyme but no inhibitor (Normal Enzyme). Without enzyme (Control), approximately 10% of the counts were detected in the carbon dioxide trap at the same time interval. It was obvious, then, that the uninhibited enzyme was responsible for the liberation of almost 1×10^6 cpm. The increasing concentration of Ciodrin was clearly evident by the decreasing amount of $C^{14}O_2$ detected in the traps.

The procedure presently described offers a rapid and inexpensive method of estimating cholinesterase activity. Its use thus far has been limited to rather gross determinations but additional refinement of the procedure could result in a useful tool for more precise studies.