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Note

Use of radio gas chromatography for monitoring the *in vivo* labelling of postmortem [^{3}H]choline production

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Many methods exist for the quantitation of endogenous acetylcholine and choline. A few examples include bioassay[1], fluorometry[2], chemiluminescence[3], paper or thin-layer chromatography, gas chromatography[4,5], high-performance liquid chromatography (HPLC)[6], and column chromatography–radioenzymology[7]. However the simultaneous measurement of choline, acetylcholine and tracer isotopes of these compounds is more complex and is basically limited to chromatographic procedures. Radio gas chromatography[8] has been used to separate ^{14}C isotopes of choline and acetylcholine from non-radioactive analogues. However the procedure was limited by low sensitivity and long retention times ($\approx$20 min). Gas chromatography–mass spectrometry[9] (GC–MS) has been used for the separation of deuterated isotopes but requires a major equipment expenditure. More recently Potter *et al.*[10] used HPLC for the separation of tritiated isotopes of choline and acetylcholine from non-radiolabelled analogues. The procedure was sensitive but required several enzymes, post column mixing and reactions.

Using a bioassay procedure, it was established that more choline left the brain than was supplied by the arterial blood[11]. In addition, a marked production of choline occurred in the brain after death[11,12]. The identity of the precursors responsible for the postmortem production of choline at present is incomplete but probably results from the sequential hydrolysis of acetylcholine, glycerophosphorylcholine, and phospholipids[13]. It has also been observed that the specific activity of deuterated choline decreased with postmortem incubation[13], indicating significant dilution with unlabelled choline. However, the postmortem production of deuterated choline was not evident.

It was the purpose of the present study to inexpensively modify an existing GC method of analysis to simultaneously measure choline, acetylcholine, and their tritiated isotopes. The modification was then used to demonstrate *in vivo* labelling of the postmortem production of choline.

MATERIALS AND METHODS

Animals, chemicals and radioisotopes

Male Sprague Dawley albino rats weighing 200–250 g were used for determining incorporation and release of [^{3}H]choline. Tritiated choline and acetylcholine were

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obtained from New England Nuclear. Paraterphenyl crystals used for isotope trapping were obtained from Aldrich.

Synthesis of [^{3}H]hexanoylcholine

[^{3}H]hexanoylcholine was synthesized for use as an internal standard by adding 0.3 ml hexanoyl chloride (Aldrich) to 200 μCi of [^{3}H]choline dissolved in 1.0 ml of 5 m*M* silver *p*-toluene sulfonate in acetonitrile. The mixture was incubated at 80°C for 45 min, centrifuged and the supernatant transferred and evaporated to dryness. After vacuum dessication, the residue was then reconstituted in 1.0 ml sodium acetate buffer (pH 4.0, 0.05 *M*). Radiochemical purity (98%) was determined with cellulose thin-layer chromatography using *n*-butanol–water–ethanol–acetic acid (100:33:70:17) as the eluent.

Simultaneous detection of choline, acetylcholine and tritiated variants

The simultaneous assay of non-radioactive and tritiated choline and acetylcholine was accomplished using a modification of the nitrogen–phosphorus GC procedure of Kosh and Freeman[14]. Brain tissue was homogenized in 4 ml 1 *M* formic acid–tetrahydrofuran (15:85) together with 5 nmol of propionylcholine and $1.3 \cdot 10^6$ dpm of [^{3}H]hexanoylcholine as internal standards. Butyrylchloride was used to convert choline and [^{3}H]choline to the respective butyryl esters.

Several GC modifications were required for the quantitation of the tritiated choline esters. The column packing material used was Chromosorb 750 coated with 10% OV-17 and 10% Triton X-100. During a chromatographic run, oven temperature was 130°C during the initial 3 min, and was then increased to 150°C for the remainder of the run. The gas flow-rates for helium (carrier), hydrogen and air were 40, 3.0 and 50 ml/min, respectively. The column effluent was split using SGE micro valves so that 80% of the flow was diverted to the mass (nitrogen–phosphorus) detector and 20% to a heated side port containing trapping cartridges (see Fig. 1A). The cartridges were held in place by O-rings inserted into a Swagelok (6.25 mm to 1.56 mm) adapter assembly (Fig. 1B). The trapping cartridges were prepared similar to the method of Karmen *et al.*[15] using glass tubing (4.8 cm × 6.5 mm O.D. × 2 mm I.D.) with a glass wool plug at one end, packed with 0.15 g of *p*-terphenyl crystals coated with 10% OV-17 and 10% Triton X-100. Cartridges were changed at 3, 6, and 9 min during the chromatographic run, which corresponded to the acetyl-, propionyl-, and hexanoylcholine radioactivity areas. After trapping, the cartridges were placed in scintillation vials containing 10 ml of scintillation fluid, shaken until the *p*-terphenyl was suspended in the fluid, and counted for radioactivity. Radioactivity was converted to dpm using a computer program which utilized matrix calculations to correct for radioactivity bleed between peaks, similar to the method utilized for correction of deuterated isotope spill-over[9]. Standard curves were prepared using $1.3 \cdot 10^6$ of [^{3}H]hexanoylcholine as the internal standard and known quantities of [^{3}H]choline or [^{3}H]acetylcholine. The ratios of the corrected dpm for [^{3}H]choline or [^{3}H]acetylcholine to [^{3}H]hexanoylcholine were then plotted *versus* the amount of tritiated compound (pmol) used. A suction trapping system was mounted above the detector to prevent the exhausting of radioactivity into the atmosphere. The system relied on a vacuum source to pull air and detector exhaust sequentially through a drying tube containing molecular sieve and then through a solution of mineral oil and water.

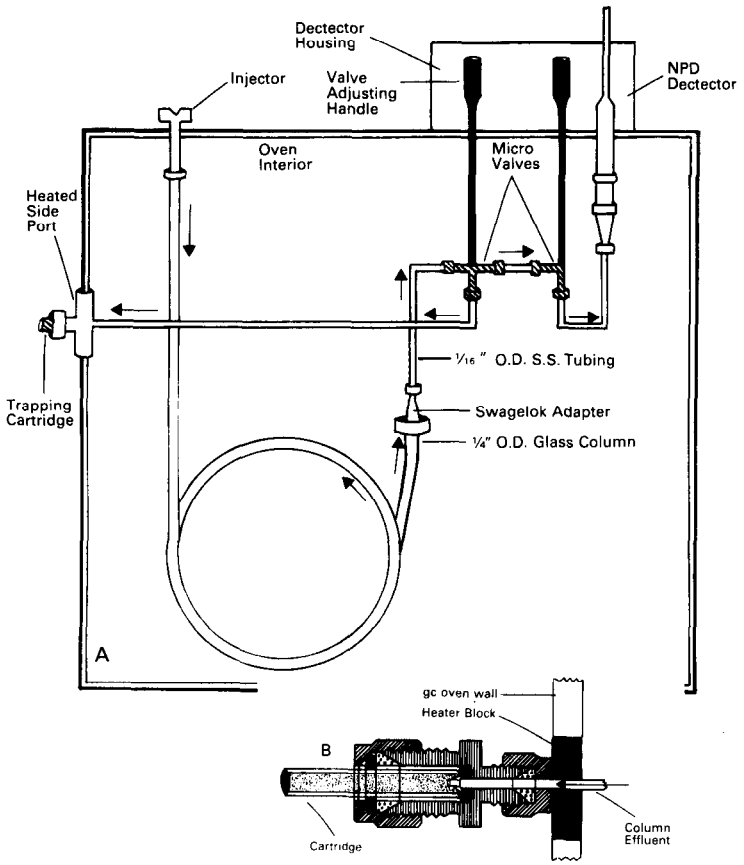

Fig. 1. (A) Plumbing schematic of gas chromatograph modified for split stream operation. Twenty percent of the column effluent was diverted to the heated side port for collection of tritiated compounds in the trapping cartridge. (B) Enlargement of cartridge–Swagelok assembly showing column effluent and O-ring seals.

In vivo incorporation and release of [^{3}H]choline

Male rats were surgically implanted with polyethylene canulas into the lateral ventricle according to the method of DeBalbian–Verster *et al.*[16]. After 24–36 h recovery, the conscious animals were injected through the cannula with 2 μCi [^{3}H]choline (80 Ci/mmol, 1 μCi/μl) at 10 A.M., 2, 6, and 10 P.M. each day for a total of six days. Thirty gauge wire was used to seal the cannula after each injection. Animals were sacrificed by decapitation 12 h after the last injection, the brain removed, and split in half longitudinally. One half was then homogenized 2 min following decapitation and the second half homogenized after 17 min incubation in a water bath at 37°C. The homogenates were then analyzed for tritiated and non-tritiated choline and acetylcholine content as described above.

RESULTS

Quantitation of choline and acetylcholine

The GC assay changes made in the present method did not affect the resolution nor the quantitation of endogenous acetylcholine and choline obtained with our previously published method[14]. One normal formic acid–tetrahydrofuran (15:85) rather than 1 *M* formic acid–acetonitrile (15:85) was used for tissue homogenization to reduce losses of quaternary compounds. Recovery of tritiated acetylcholine, choline and hexanoylcholine through the homogenization procedure averaged 98–99%. When formic acid–acetonitrile was used, recovery ranged from a low of 45–50% for hexanoylcholine to a high of 85–90% for choline. An interesting characteristic observed by homogenizing in tetrahydrofuran was complete dissolution of the brain tissue.

Various solid supports and phase coatings were examined as packing material for the glass cartridges used to trap the tritiated compounds. Maximum trapping efficiency was obtained with a 1 7/8 inch cartridge packed with *p*-terphenyl crystals coated with 10% OV-17 and 10% Triton X-100. Comparison of dpm obtained following direct injection of the tritiated compounds onto the cartridge with total dpm obtained after elution from the GC produced recoveries ranging from 90–95% for all compounds.

The collection time "windows" for acetylcholine (0–3 min), choline (as butyrylcholine, 3–6 min) and the internal standard hexanoylcholine (6–9 min) allowed adequate separation with a minimum amount of radioactive bleed between fractions. Since radioactive bleed was proportionally constant, matrix corrections were utilized to obtain the corrected dpm. A plot of the ratio of tritiated choline or acetylcholine to tritiated hexanoylcholine *versus* molar quantity is shown in Fig. 2. The minimum detectable quantity taken through the assay was 150 (± 83) fmol choline ($2.6 \cdot 10^4$

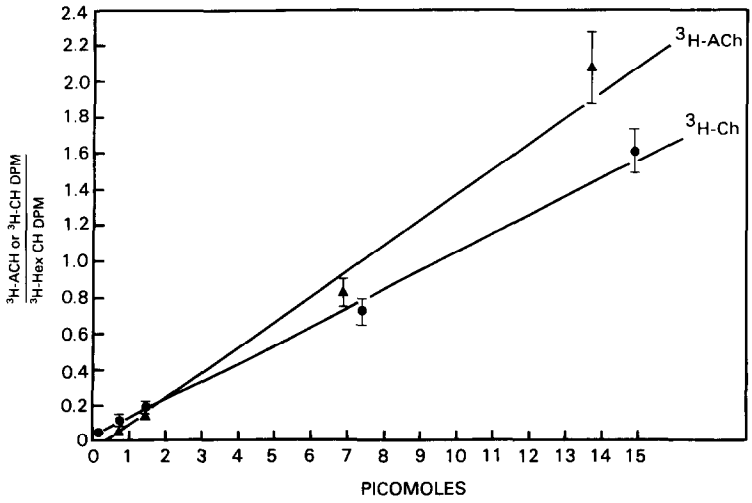

Fig. 2. Standard curve showing linearity of dpm ratio *versus* molar quantity for [^{3}H]choline and [^{3}H]acetylcholine. [^{3}H]Hexanoylcholine (0.6 μCi) was used as internal standard. Samples were analyzed for [^{3}H]choline and [^{3}H]acetylcholine as described in Materials and methods.

TABLE I

POSTMORTEM PRODUCTION OF [^{3}H]CHOLINE AND CHOLINE BY RAT BRAIN TISSUE

*Treatment**	*[^{3}H]Choline (dpm/g)*	*[^{3}H]Choline (fmol/g)*	*Choline (nmol/g)*	*Sp. act.*** *(dpm/nmol)*	*Acetylcholine (nmol/g)*
Incubated 2 min	120 806 ± 58 211	636.4 ± 330	96.7 ± 3.0	1249	14.1 ± 1.0
Incubated 17 min	774 800 ± 191 522***	4402.3 ± 1088***	448 ± 28.3***	1729	9.6 ± 0.8§

* Rats were injected intraventricularly at 10 A.M., 2, 6, and 10 P.M. for six days with [^{3}H]choline (2 μCi/injection, 80 Ci/mmol). Brain tissue was homogenized in formic acid–tetrahydrofuran after incubating at 37°C for 2 or 17 min and then assayed for tritiated and endogenous choline and acetylcholine content.

** Sp. act. = specific activity, defined as (dpm[^{3}H]choline)/(nmol choline).

*** $p \leqq 0.001$, compared to 2-min incubated tissue.

§ $p \leqq 0.005$, compared to 2-min incubated tissue.

dpm) and 730 (± 102) fmol acetylcholine ($1.3 \cdot 10^5$ dpm). Linearity was observed up to 15 pmoles ($2.6 \cdot 10^6$ dpm) and was not examined further. Recovery of tritiated choline and acetylcholine through the complete procedure (chemical assay and GC separation) ranged between 50 and 60%. The value obtained for recovery was good, considering that 33% of the radioactivity is lost during the chemical demethylation step.

Incorporation and postmortem production of [^{3}H]choline

The purpose of this experiment was to utilize the radio gas chromatographic procedure developed above to determine if the postmortem production of choline in brain tissue could be labelled following chronic administration of labelled choline. Tritiated choline (intraventricular) was administered four times a day for six days to rats and the brain tissue examined for endogenous and tritiated choline and acetylcholine content 12 h after the last injection. Brain tissue analyzed at 2 min produced $\approx$121 000 dpm/g of [^{3}H]choline, whereas 17-min incubation produced $\approx$775 000 dpm/g of [^{3}H]choline (Table I). As expected non-labelled choline increased 4–5 fold and acetylcholine decreased approximately 24%. Tritiated acetylcholine could not be detected at either time. The specific activities for choline at 2 and 17 min postmortem were 1249 and 1729, respectively.

DISCUSSION

The GC method of Kosh and Freeman[14] was modified in the present study for the purpose of simultaneously quantitating choline, acetylcholine, and tritiated choline and acetylcholine. The modification was accomplished for less than $500 and enabled rapid GC processing (< 10 min /sample) for both non-labelled and tritiated compounds. The combined assay required the use of propionylcholine and [^{3}H]hexanoylcholine as internal standards for the non-labelled and labelled analogues, respectively. It was found necessary to change the intial homogenizing medium to formic acid–tetrahydrofuran to provide recoveries of 98–99% for all of the quaternary compounds during homogenization.

Residual isotope bleed from the column between sample injections was mini-

mal for the tissue samples. However, in preparing the standard curve, the amount of bleed was found to be proportional to the amount of tritiated choline and acetylcholine used. Even with the highest amounts of radioactivity used, one hour of continued column elution decreased the background to baseline values. The reduced background bleed of radioactivity is an advantage since other methods have required oxidation reduction combustion trains[15] or disposable columns[17] to minimize background bleed from tritium or ^{14}C isotopes respectively.

A high sensitivity for both choline and acetylcholine (150 and 730 fmol, respectively) was obtained in the present assay. The method compares favorably with other assays for labelled choline and acetylcholine in which the limit of sensitivity has been in the pmol range[7,18]. The present assay utilized only a 20% column effluent split for radioactivity trapping. Sensitivity could be further increased if a greater column split ratio were utilized and carrier make-up gas added.

After six consecutive days of intraventricular [^{3}H]choline administration, postmortem production of both non-labelled and [^{3}H]choline was observed in rat brain tissue. While the time course of labelling of phospholipids can vary from minutes[19], to hours[20], to days[13], the present data demonstrates that much longer pretreatment times are required to label the choline precursor(s) involved in postmortem production of choline, suggesting incorporation of choline into phospholipids with long turnover times. In the present study (Table I), labelling of the postmortem production of choline was not observed with less than six days of chronic [^{3}H]choline administration. The specific activity of the [^{3}H]choline produced after 17 min incubation was greater than after 2 min incubation. This may reflect the inability of six days of pretreatment to uniformly label all phosphatidylcholine pools, resulting in the rapidly turning over pool contributing a greater proportion of labelled choline to endogenous choline produced during postmortem incubation.

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