Biochemical Pharmacology, Vol. 20, pp. 2355–2366. Pergamon Press, 1971. Printed in Great Britain

FACTORS INFLUENCING CHOLINE MOVEMENTS IN RAT BRAIN SLICES*

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(*Received* 28 *August* 1970; *accepted* 20 *November* 1970)

Abstract—The kinetics of the accumulation of acid-soluble ^{3}H-material from [^{3}H]-choline by rat brain slices were consistent with the concurrent existence of both passive diffusion and carrier-mediated transport; hemicholinium-3 inhibited accumulation. Addition to or subtraction from the standard incubation medium of 50 mM NaCl did not affect accumulation, whereas the addition of 50 mM LiCl inhibited accumulation of ^{3}H-material. After a 15-min incubation 30 per cent of the accumulated acid-soluble ^{3}H-material chromatographed with phosphorylcholine, 5 per cent with acetylcholine, and 60 per cent with choline. Although the slice : medium ratio of ^{3}H-material was 1·7, that of [^{3}H]choline was only 1·1. Adding azide or 50 mM KCl to the medium decreased the accumulation of ^{3}H-material; however, these agents also decreased the fraction metabolized and the slice : medium ratio for [^{3}H]choline remained near 1·0. Most of the accumulated ^{3}H-material was localized, after subcellular fractionation, in the postmitochondrial supernatant material; the concentration of ^{3}H-material in the nerve-ending fraction, in terms of the synaptosomal volume, was 10 times that in the medium.

SINCE neural tissue depends on exogenous choline for the synthesis of acetylcholine and choline-containing phospholipids,[1] mechanisms for the efficient uptake of extracellular choline are essential. Early studies on the accumulation‡ of [^{3}H]choline by mouse brain slices[2,3] were interpreted in terms of an energy-dependent system, sensitive to ouabain and the concentration of extracellular sodium, in parallel with passive diffusion. More recently, experiments with synaptosomes (isolated nerve-ending particles from brain) demonstrated a carrier-mediated transport system that was not energy-dependent and did not require sodium.[4] A possible explanation for the discrepancy might lie in the metabolism of [^{3}H]choline after accumulation, since the energy-dependent transformation of labeled choline would create a sink, influencing the transmembrane distribution of ^{3}H-materials. It thus seemed of interest to re-examine [^{3}H]choline influx and efflux with brain slices under various experimental conditions, to determine the subcellular localization of the accumulated material, and

* This investigation was supported by U.S. Public Health Service grants NS-05430, GM-00293 and GR-11-8614; W. J. Cooke was a predoctoral fellow of the U.S. Public Health Service. Portions of this work were included in a dissertation submitted by W. J. Cooke in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the State University of New York, Upstate Medical Center.

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‡ The term "accumulation" is used to describe the acquisition of ^{3}H-material by the slice irrespective of the mechanism; it thus refers to accumulation through passive diffusion and carrier-mediated transport.

to estimate the extent of metabolism. These experiments indicate that choline was accumulated by a carrier-mediated transport system in parallel with passive diffusion, that a significant fraction was metabolized, and that certain pharmacological agents could decrease the accumulation of ^{3}H-materials either by inhibiting the carrier-mediated transport system or by interfering with the subsequent metabolism of [^{3}H]choline.

METHODS

Cerebral cortical slices from male Sprague–Dawley rats (150–200 g, Holtzman Farms) were prepared as described by McIlwain and Rodnight.[5] Slices, approximately 0·35 mm thick and weighing 30–40 mg were obtained from each hemisphere with the aid of a Plexiglass guide milled to 0·3 mm; the outer slice was discarded. The slice was transferred to a beaker of the preincubation medium (see below) at 0°, blotted, and weighed on a torsion balance. The slice was then equilibrated for 30 min in 4 ml of the preincubation medium at 37°, with gentle shaking, under an atmosphere of 95% O_2–5% CO_2 maintained by a manifold equipped with a pressure gauge. After equilibration the slice was transferred to 5·0 ml of experimental medium and incubated for 15 min at 37° under an atmosphere of 95% O_2–5% CO_2, unless otherwise specified.

The preincubation medium contained: 124 mM NaCl, 26 mM $NaHCO_3$, 5 mM KCl, 1·2 mM KH_2PO_4, 1·3 mM $MgSO_4$, 0·75 mM $CaCl_2$, and 10 mM glucose, and was equilibrated with 95% O_2–5% CO_2 before use. The standard incubation medium contained, in addition, 33 μM [^{3}H]choline chloride; various experimental media contained the additions or deletions to the standard medium as specified, expressed as final concentrations.

To measure the accumulation of radioisotope during incubation, the slice was removed from the medium and washed twice at 0° for a total of 30 sec in 3 ml of the preincubation medium. The slice was homogenized in 1 ml of 5% TCA* in a TenBroeck homogenizer and extracted, with intermittant mixing, for at least 30 min at room temperature before centrifugation at 17,000 *g* for 30 min. An aliquot of the supernatant material was used for scintillation counting (acid-soluble ^{3}H-material). (Less than 0·5 per cent of the acid-soluble ^{3}H-material remained in the precipitate as determined by re-extracting the residue with TCA.) The incorporation of ^{3}H from [^{3}H]choline into acid-insoluble material was measured in certain experiments by dissolving the TCA precipitate in 0·3 ml of 90% formic acid, and using an aliquot for scintillation counting.

The subcellular distribution of accumulated acid-soluble ^{3}H-material was studied in individual brain slices by differential and density gradient centrifugation.[6,7] Following the incubation and wash the slice was homogenized in 3 ml of 0·32 M sucrose. The homogenate was then centrifuged at 1000 *g* for 10 min, the pellet (P: "nuclei and debris") was collected, and the supernatant material was centrifuged at 15,000 *g* for 15 min. The crude mitochondrial pellet was separated from the postmitochondrial supernatant material (*S*), resuspended in 2 ml of 0·32 M sucrose, and layered on a discontinuous sucrose gradient composed of 1·8 ml each of 0·8 and 1·2 M sucrose in a 5 ml cellulose tube. The completed gradient was centrifuged in a Beckman 50·1

* Abbreviations used: DNP, 2,4-dinitrophenol; GABA, γ-aminobutyric acid; HC-3, hemicholinium; pCMB, p-Chloromercuribenzoic acid; TCA, trichloroacetic acid; TTX, tetrodotoxin.

swinging-bucket rotor at 90,000 *g* for 90 min. The visually distinguishable fractions were designated as follows: A, top of gradient to the beginning of the first band; B, the first band ("myelin fraction"); C, the second band ("synaptosomal fraction"); D, the relatively clear material under the synaptosomal fraction; and E, the "mitochondrial pellet". The fractions were collected with Pasteur pipettes, volumes measured, and the fractions homogenized prior to removal of samples for subsequent measurement of protein and radioactivity.

Efflux of accumulated ^3H was measured after prior incubation of the slices in the standard incubation medium as described above. The slice was washed twice at 37° for a total of 30 sec in 3 ml of standard medium, and then placed in a vessel containing 2·5 ml of the unlabeled standard or experimental efflux media. (The standard efflux medium was identical to the preincubation medium and contained no choline.) At 5-min intervals the efflux medium was withdrawn from the chamber through a stopcock in the bottom of the vessel and 2·5 ml of fresh medium, equilibrated with 95% O_2–5% CO_2 (except where noted) was introduced at the top. At 1-min intervals the slice was gently agitated by lifting a thin stainless-steel mesh on which the slice rested. Temperature was maintained at 37° by a circulating water bath. At the end of the efflux incubation period the slice was removed from the chamber, weighed and the content of acid-soluble ^3H-material analyzed as described above. Results are presented in terms of the amount of radioactivity remaining in the slice at the end of each collection period, and expressed as a percentage of the total amount present at the beginning of the efflux incubation. Rate constants were derived by the use of a computerized version of the "peeling" method suggested by Riggs[8] for solving polycomponent exponential equations.

Protein content was measured by the method of Lowry *et al.*[9] using crystalline bovine serum albumin as the standard. Cytochrome oxidase activity was measured by a modification of the method of Cooperstein and Lazarow[10] and acetylthiocholinesterase activity was determined by an adaptation of the method of Ellman *et al.*[11] The dry weight of brain slices was determined by drying incubated slices in a vacuum oven at 55° for 24 hr. The inulin space was determined by incubating preincubated slices in the medium containing ^{14}C-inulin (0·1 μc/ml) for 15 min. The slices were removed, blotted, and extracted with 5% TCA for scintillation counting. The inulin space was calculated from the inulin content of the slices as a fraction of the inulin concentration in the medium, applying the assumptions delineated by Franck *et al.*[12]

Metabolic products of [^3H]choline were separated by paper chromatography. Brain slices were incubated in the usual fashion with [^3H]choline, rinsed, and homogenized in 0·1 N HCl to avoid the interference attributable to TCA.[13] To the homogenate 100 μg each of phosphorylcholine, choline and acetylcholine (as the chlorides) were added as carrier. The supernatant material, obtained after centrifugation, was lyophilized, dissolved in 50 μl of water, and then spotted on Whatman No. 3 chromatography paper. Standards, prepared in the same manner, were chromatographed simultaneously. The chromatogram was developed for 18 hr in a descending system in butanol–acetic acid–ethanol–water (8:2:1:3, v/v).[14] Standards were located by spraying the dried chromatogram with Dragendorff's reagent or dipicrylamine.[15] Radioactivity was localized by scintillation counting after cutting the chromatogram into 1-cm sections and eluting in the counting vials with water prior to the addition of scintillation fluid. Greater than 94 per cent of the applied material was recovered.

Radioactivity was measured in Bray's solution[16] with a liquid scintillation spectrometer equipped with an external standard for the correction of quenching. In most cases observed activities were converted to disintegrations per minute.

Electron micrographs were obtained as described previously.[7]

Where appropriate, the data are presented as averages of individual observations together with the S.E.M. of *n* observations. Statistical significance was calculated using the Student's *t*-test for paired observations.

All solutions and reagents were made in glass-distilled water. Choline methyl-[^{3}H]chloride (62·7 mc/mM) and carboxyl-[^{14}C]inulin (2·5 mc/g) were purchased from the New England Nuclear Corp. TTX was purchased from Calbiochem. The [^{3}H]-choline was specified as 99 per cent pure; this was confirmed by chromatography, which showed all radioactivity migrating with authentic choline.

RESULTS

Accumulation of ^{3}H-*material from* [^{3}H]*choline.* Brain slices accumulated ^{3}H-material during brief incubations in media containing [^{3}H]choline. The accumulated material could be separated into two fractions: that which could be extracted by 5% TCA ("acid-soluble ^{3}H") and that which could not ("acid-insoluble ^{3}H"), the latter presumably representing phospholipid choline; data are expressed in molar equivalents in terms of the specific activity of [^{3}H]choline (dis./min/μmole) in the medium. The slices accumulated choline for at least 30 min (Fig. 1), and by 15 min had attained a tissue to medium concentration ratio of acid-soluble ^{3}H of 1·75; accumulation of acid-insoluble

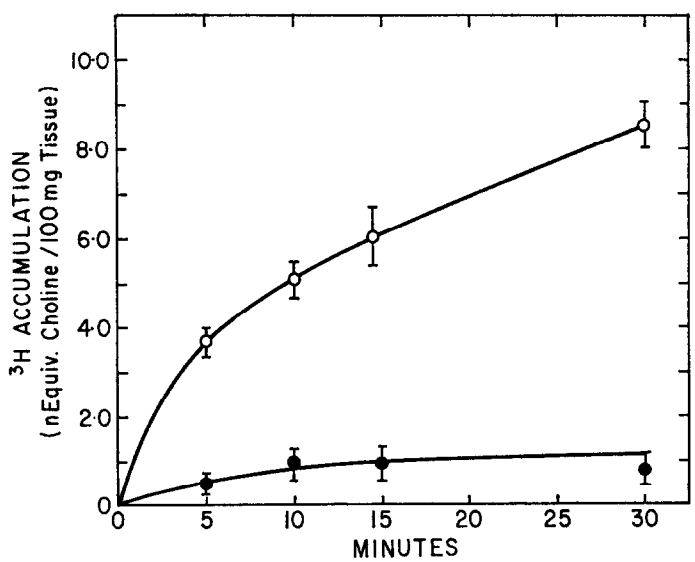

FIG. 1. Time course of accumulation of acid-soluble ^{3}H from [^{3}H]choline by brain slices. Slices were preincubated for 30 min at 37° prior to incubation in the standard medium containing 33 μM [^{3}H]-choline (see Methods). Incubation was for the indicated times at 37° (○——○) or 0° (●——●) and the accumulated acid-soluble ^{3}H extracted and analyzed as described in Methods. Accumulation is expressed as n-equiv. ^{3}H from [^{3}H]choline/100 mg initial wet weight of tissue. Symbols represent the mean ± S.E.M. of eight determinations.

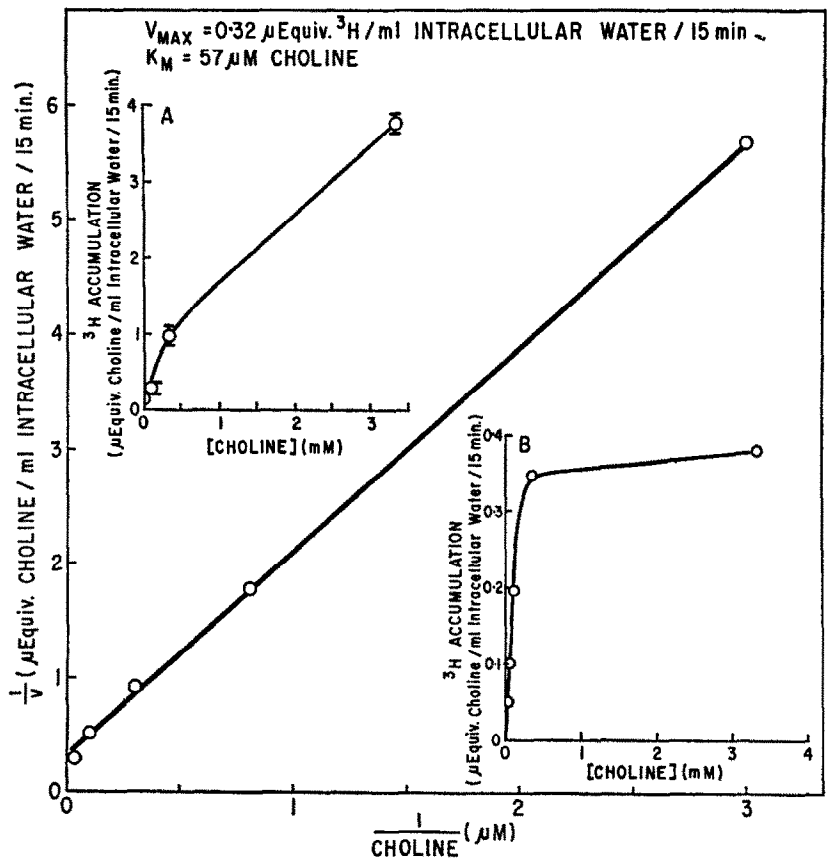

FIG. 2. Kinetics of accumulation of acid-soluble ^{3}H. Slices were prepared as in Fig. 1, then incubated for 15 min at 37° in experimental media containing the indicated concentrations of [^{3}H]choline. The accumulation of ^{3}H is expressed in terms of μ-equiv. choline accumulated per ml of intracellular water per 15 min vs. the concentration of choline in the medium (panel A). Accumulation attributable to diffusion was subtracted from the original data to obtain a measure of the accumulation attributable to a carrier-mediated transport process (panel B). These data were then replotted in the double-reciprocal form with a straight line fitted by the method of least squares to obtain the kinetic parameters K_m and V_{max}. Symbols represent the mean of at least five determinations; S.E.M. are indicated for the original data.

^{3}H was only one-tenth as great at this time. Accumulation was markedly slower at 0° (Fig. 1).

The accumulation of acid-soluble ^{3}H-material by slices increased with the concentration of [^{3}H]choline in the medium (Fig. 2, panel A) in a fashion suggesting both carrier-mediated and diffusional components. That component of accumulation attributable to a carrier-mediated transport process may be calculated by defining the contribution of passive diffusion to the total accumulation, which can be estimated from the accumulation at the higher external choline concentrations where the contribution of the carrier-mediated system is minimal. Subtracting the calculated diffusional component from the total accumulation at each of the lower concentrations then gave an approximation of the carrier-mediated system (Fig. 2, panel B), from

which the K_m and V_{max} for the system were calculated (Fig. 2). Analogous studies on the accumulation of acid-insoluble ^{3}H-material gave similar values: $K_m = 49$ μM; $V_{max} = 2{\cdot}3$ n-equiv./100 mg initial wet weight per 15 min.

Effects of experimental conditions on the accumulation of ^{3}H*-material.* None of the agents studied increased the accumulation of acid-soluble or acid-insoluble ^{3}H-material from [^{3}H]choline (Table 1). Certain metabolic inhibitors (azide, iodoacetate and DNP) significantly decreased the accumulation, as did most of the other agents studied (Table 1). However, TTX antagonized the inhibition induced by ouabain, glutamate, azide and 50 mM KCl. HC-3, which has been shown to be a competitive inhibitor of choline transport in other systems,[17] decreased the accumulation of acid-soluble ^{3}H about 50 per cent at a concentration of 0·1 mM.

To investigate possible ionic requirements for choline accumulation the effects of various solutes were compared. Increasing or decreasing the Na^+ concentration in the incubation medium by 50 m-equiv./l. had no effect on accumulation of ^{3}H-material, although the complete removal of Na^+ (replaced iso-osmotically with sucrose) decreased accumulation (Table 2). Substituting Li^+ for Na^+ or adding Li^+ to the

TABLE 1. EFFECTS OF VARIOUS AGENTS ON THE ACCUMULATION OF ^{3}H-MATERIAL*

Agent	Concentration (mM)	n	^{3}H-material accumulation (Per cent of control) Acid-soluble	Acid-insoluble
None			100	100
TTX	$1{\cdot}5 \times 10^{-4}$	10	100 ± 3	103 ± 3
Ouabain	0·1	10	83 ± 7†	70 ± 3†
+TTX	$1{\cdot}5 \times 10^{-4}$	10	100 ± 4‡	101 ± 4‡
Azide	3·0	12	78 ± 4†	13 ± 2†
+TTX	$1{\cdot}5 \times 10^{-4}$	8	92 ± 3‡	
+KCl	50·0	8	59 ± 2†‡	
KCl	50·0	10	70 ± 1†	26 ± 3†
+TTX	$1{\cdot}5 \times 10^{-4}$	8	80 ± 3†‡	36 ± 3†
Glutamate	5·0	8	91 ± 4	29 ± 2†
+TTX	$1{\cdot}5 \times 10^{-4}$	8	99 ± 3	38 ± 3†
GABA	5·0	6	101 ± 5	66 ± 4†
Glutamine	5·0	6	94 ± 6	80 ± 9†
HC-3	0·1	8	55 ± 3	
Procaine	3·0	8	47 ± 7†	
pCMB	0·1	6	96 ± 4	
DNP	0·1	8	78 ± 4†	
	1·0	9	67 ± 3†	
Iodoacetate	1·0	6	87 ± 5†	21 ± 2†
$MgCl_2$	6·5	6	109 ± 5	105 ± 4
$CaCl_2$	2·5	8	84 ± 6†	
	5·0	8	78 ± 3†	
95% N_2–5% CO_2		8	91 ± 2†	39 ± 6†

* Brain slices were prepared and equilibrated for 30 min at 37°, then incubated for 15 min in the experimental media containing 33 μM [^{3}H]choline and the additions or deletions indicated. After incubation the slices were rinsed and the accumulated acid-soluble and acid-insoluble ^{3}H measured as described in Methods. The ^{3}H accumulation is presented as a percentage of that in the control incubations in the standard medium, ±S.E.M., of n determinations.

† $P < 0{\cdot}05$ with respect to control.

‡ $P < 0{\cdot}05$ with respect to primary agent alone.

TABLE 2. EFFECTS OF Li^+ AND Na^+ ON ACID-SOLUBLE ^3H ACCUMULATION*

Composition of medium				
Na^+ (m-equiv/l.)	Li^+	Sucrose (mM)	n	^3H-accumulation (per cent of control)
150				100
150	50		6	78 ± 3†
150		100	6	102 ± 4
200			8	102 ± 7
100		100	6	82 ± 5†
‡	75	150	6	60 ± 4†
‡		300	6	72 ± 4†

* Experiments were performed as indicated in Table 1. Additions or deletions in the experimental incubation media were made as indicated. Results are presented as the mean ± S.E.M. of n determinations.

† $P < 0.05$ with respect to control.

‡ 26 mM tris HCl used to maintain pH at 7·4.

standard medium decreased accumulation, whereas adding an osmotically equivalent amount of sucrose to the standard medium did not (Table 2). As noted above (Table 1) 50 mM KCl also diminished accumulation.

Identification of ^3H-material accumulated by slices. After a 15-min incubation in the standard medium a significant percentage of the accumulated acid-soluble ^3H-material migrated with phosphorylcholine (32%) and the bulk of the material with free choline (63%) (Fig. 3; Table 3). Incubation with either 50 mM KCl or azide significantly

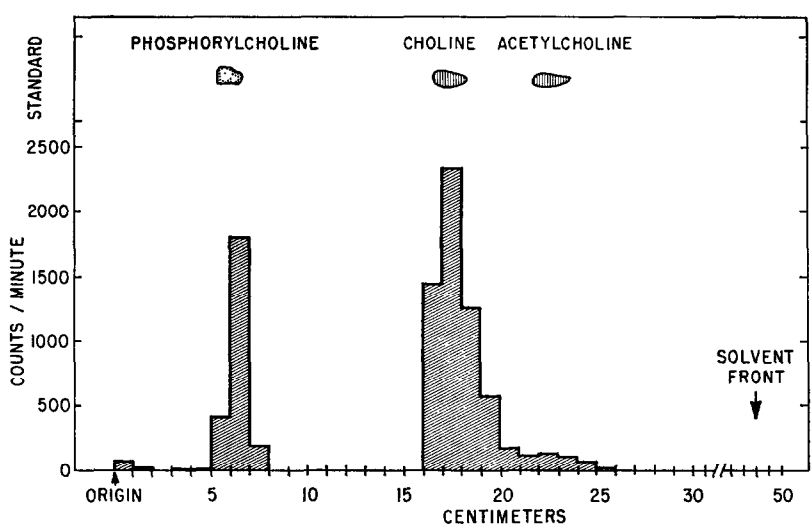

FIG. 3. Paper chromatographic separation of accumulated ^3H-materials. Experiments were performed as in Fig. 1, except that 100 μg each of phosphorylcholine, choline and acetylcholine was added to the slice immediately before the extraction of acid-soluble materials with 0·1 HCl. Chromatography was performed as described under Methods.

TABLE 3. EFFECT OF K^+ AND AZIDE ON ACCUMULATED ACID-SOLUBLE ^3H FROM [^3H]CHOLINE*

Agent	Concentration (mM)	n	Per cent of total recovered activity		
			Phosphorylcholine	Choline	Acetylcholine
Control		8	32 ± 4	63 ± 3	5 ± 1
KCl	50·0	6	15 ± 3†	78 ± 2†	7 ± 1
Azide	3·0	5	12 ± 3†	78 ± 5†	10 ± 3

* Experiments were performed as in Fig. 3. The radioactivity corresponding to the location of the standards is expressed as a percentage of the total amount of activity recovered from the chromatogram. Results are presented as the mean ± S.E.M. of n determinations.
† $P < 0·05$ with respect to control.

decreased the radioactivity in the material migrating as phosphorylcholine, with a consequent increase in that fraction migrating as choline (Table 3). In all cases the amount of labeled material migrating as acetylcholine was small; it should be noted that no cholinesterase inhibitors were used.

Subcellular distribution of acid-soluble ^3H-material. The distribution of acid-soluble ^3H-material was determined after separation of the subcellular fractions by differential and density gradient centrifugation. The effectiveness of the separations was assayed by both enzymatic analysis of the individual fractions and by electron microscopy. The specific activity of cytochrome oxidase, a mitochondrial marker, was 3·7 times greater in the "mitochondrial" fraction (Fraction E) than in the "synaptosomal" fraction (Fraction C), whereas the specific activity of acetylthiocholinesterase, a synaptosomal marker, was five times greater in Fraction C than in E. Electron micrographs of the fractions supported these identifications: there was a preponderance of synaptosomes in Fraction C, and a high concentration of free mitochondria in Fraction E, with a few synaptosomal particles present.

TABLE 4. RELATIVE DISTRIBUTION AND SPECIFIC ACTIVITY OF ACID-SOLUBLE ^3H-MATERIAL IN SUBCELLULAR FRACTIONS FROM BRAIN SLICES*

Fraction	Acid-soluble ^3H-material	
	Relative distribution (per cent of total)	Specific activity (n-equiv. ^3H/mg protein)
Homogenate	100	0·82 ± 0·05
P	16·5 ± 1·9	0·19 ± 0·02†
A	1·7 ± 0·4	
B	2·9 ± 0·6	
C	12·5 ± 0·7	0·91 ± 0·05
D	1·3 ± 0·4	0·51 ± 0·05†
E	1·7 ± 0·2	0·33 ± 0·03†
S	63·4 ± 5·6	3·86 ± 0·14†

* Experiments were performed as in Table 1, and the slices then fractionated as described under Methods. Data are presented ± S.E.M. of 11 determinations.
† $P < 0·05$ with respect to homogenate.

Most of the acid-soluble ^{3}H-material accumulated during the 15-min incubation period was in the postmitochondrial supernatant fraction of the homogenate (Fraction S, in Table 4); Fraction S also attained the highest specific activity (μmoles/mg protein) of acid-soluble ^{3}H-material. The specific activity of acid-soluble ^{3}H-material in Fraction C ("synaptosomal" fraction) was approximately three times greater than that in Fraction E ("mitochondrial" fraction) (Table 4).

HC-3 significantly decreased the specific activity of acid-soluble ^{3}H-material in both the "mitochondrial" and "synaptosomal" fractions (Table 5), in agreement with the decreased accumulation by the whole slice. Similar decreases in the relative specific activities were caused by procaine and by azide plus 50 mM KCl.

TABLE 5. EFFECTS OF VARIOUS AGENTS ON THE RELATIVE SPECIFIC ACTIVITIES OF ACID-SOLUBLE ^{3}H-MATERIAL IN SUBCELLULAR FRACTIONS OF BRAIN SLICES*

Agent	Concentration (mM)	n	Relative specific activity† of acid-soluble ^{3}H-material (per cent of control) Synaptosomes (Fraction C)	Mitochondria (Fraction E)
None		18	100	100
Ouabain	0·1	6	91 ± 11	109 ± 11
Procaine	3·0	6	57 ± 3‡	52 ± 7‡
KCl	50·0	6	100 ± 2	84 ± 8
Azide	3·0	5	91 ± 8	88 ± 7
+KCl	50·0	5	74 ± 5‡	58 ± 11‡
Glutamate	5·0	4	99 ± 5	86 ± 7
HC-3	0·1	6	67 ± 3‡	63 ± 5‡

* Experiments were performed as in Table 4. The specific activity of acid-soluble ^{3}H-material in each fraction was calculated and expressed (relative specific activity) as a percentage of the specific activity of acid-soluble ^{3}H-material in that fraction from control slices concurrently determined. Data are presented ± S.E.M. of n determinations.

† ^{3}H, dis./min/mg protein.

‡ $P < 0·05$ with respect to control.

Efflux of acid-soluble ^{3}H-material. The efflux of ^{3}H-material from the slices, accumulated during the 15-min incubation in the standard medium, could be described by a multi-component exponential function; estimates of rate constants for at least two components of the control efflux curve indicated a slow compartment with $k_3 = 0·0277$, and a second component with $k_2 = 0·148$. A third and still faster component was indicated, but not enough data points were available from which to estimate a rate constant.

Efflux at 0° was markedly slowed (Fig. 4), and the ratio of the rate at 0° to that at 37° was the same as the ratio of the rates of accumulation at those temperatures. Both azide and 50 mM KCl increased the rate of efflux slightly, whereas ouabain (data not presented) and decreased extracellular Na^+ (to 50 m-equiv./l.) had no demonstrable effect. Efflux under an atmosphere of 95% N_2–5% CO_2 was decreased. Because of the small amounts of material available, attempts to identify the ^{3}H-material in the efflux media were unsuccessful.

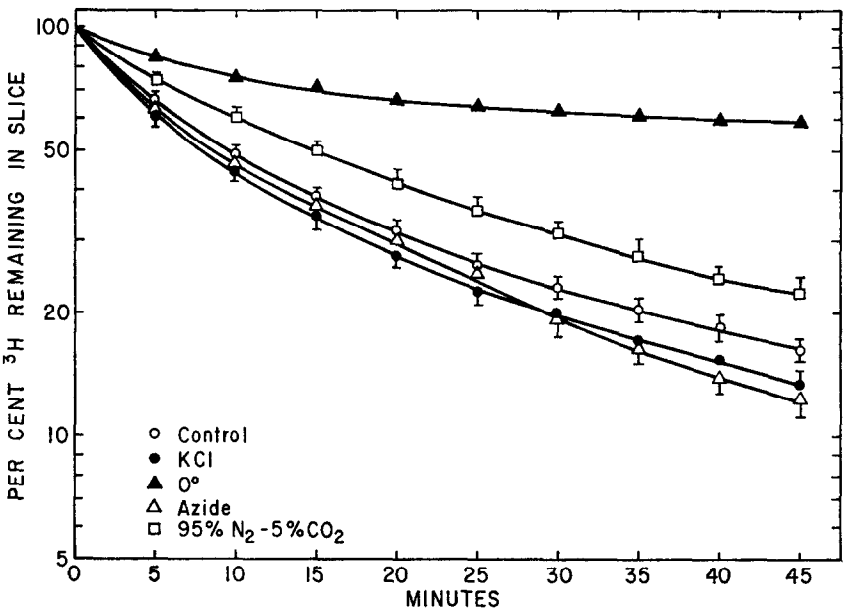

FIG. 4. Effects of various agents on the efflux of accumulated ^{3}H-material. Slices were prepared and efflux curves were constructed as described in Methods. Concentrations: KCl, 50 mM; azide, 3 mM. Symbols represent the mean ± S.E.M. of at least six determinations.

DISCUSSION

The concentration-dependent accumulation of ^{3}H from [^{3}H]choline (Fig. 2) is consistent with the concurrent existence of both passive diffusion and a saturable carrier-mediated transport process. The K_m for the saturable component of acid-soluble ^{3}H accumulation was 57 μM (Fig. 2), and essentially the same K_m was obtained for the accumulation of acid-insoluble ^{3}H-material. These values are in reasonable agreement with that reported for guinea pig synaptosomes[4] where the K_m = 83 μM, although somewhat lower than that reported for mouse brain slices.[2] In addition, HC-3, which is a competitive-inhibitor of choline transport in other systems,[2,4,17,18] inhibited the accumulation of acid-soluble ^{3}H from [^{3}H]choline (Table 1).

Although the tissue to medium concentration ratio of ^{3}H was greater than unity, the data need not indicate accumulation of choline against a gradient, for metabolism of [^{3}H]choline could account for such a distribution in the absence of active transport. In fact, only 63 per cent of the accumulated acid-soluble ^{3}H-material migrated as free choline (Fig. 3; Table 3). Thus, under control conditions, the accumulated acid-soluble ^{3}H-material was equivalent to a tissue to medium ratio of 1·09, whereas the tissue to medium ratio of total acid-soluble material was 1·75. In the presence of 50 mM KCl the accumulation of acid-soluble ^{3}H-material by slices was inhibited (Table 3), and then 78 per cent of the accumulated material migrated with choline; under these conditions the tissue to medium ratio with respect to choline was 0·97. A similar calculation can be made concerning the effects of azide (tissue to medium ratio

of 1·07). Hence the inhibition induced by these two agents may reflect alterations in the metabolism of the accumulated material, rather than a direct inhibition of the carrier-mediated transport system.

Sodium plays a significant role in various carrier-mediated transport systems,[19,20] and a similar dependency has been suggested for the transport of choline in mouse brain slices.[3] In the present experiments an increase or decrease of 50 m-equiv./l. of Na^+ in the incubation medium had no effect on the accumulation of acid-soluble ^{3}H-material (Table 2), although the total deletion of Na^+ (substituting sucrose iso-osmotically) decreased the accumulation; in this latter instance, however, the diminished accumulation may reflect the deleterious effects on brain slices of the total absence of Na^+.[21] It is apparent that Li^+ could not substitute for Na^+ with respect to choline accumulation (Table 2). Diamond and Kennedy[4] also demonstrated an inhibitory effect of Li^+ on the accumulation of choline by synaptosomes; furthermore, they found that choline accumulation was inhibited by Na^+ and K^+, with Na^+ being the least inhibitory of the three. It is interesting that TTX, an agent that specifically inhibits the Na^+-current across excitable membranes,[22] reversed the inhibited accumulation of acid-soluble ^{3}H-material induced by ouabain, azide and glutamate, agents known to alter sodium movements across neural membranes.[23–25] These observations suggest that the inhibition of accumulation may not be due to direct effects on the transport system, but rather to an alteration in the intracellular Na^+ concentration which in turn leads to alterations in metabolic processes sensitive to the ionic environment.[26]

Most of the acid-soluble ^{3}H was in the postmitochondrial supernatant fraction (S) (Table 4), indicating that a large portion of the accumulated material was in a free, unbound form. Although the specific activity of acid-soluble ^{3}H (dis./min/mg protein) in the synaptosomal fraction (C) was the same as that in the original homogenate, if the accumulation of ^{3}H-material is considered in terms of the synaptosomal volume, measured to be 2·6 μl/mg protein,[7] then the concentration of ^{3}H-material within the synaptosomes would be 10 times higher than that in the medium. Because of insufficient material, it was not possible to identify the ^{3}H-material in the synaptosomal fraction; however, it is an attractive possibility that a concentration of ^{3}H-material in the nerve endings might reflect a prior accumulation of ACh at that site.

Studies on the efflux of accumulated ^{3}H-material indicated the existence of at least three compartments. The observed rate constants for two of these compartments are comparable to those seen in rat diaphragm muscle preparations rich in nerve endings.[18] That the identifiable compartments seen in the present study do not reflect efflux from the extracellular space is indicated by studies on the efflux of a presumably extracellular marker, [^{3}H]mannitol;* under identical experimental conditions the efflux of mannitol from brain slices could be described by a single exponential function with $k = 0{\cdot}236$, clearly larger than k_2 found in the present studies (Fig. 4). The increased rate of efflux of ^{3}H-material in the presence of 50 mM KCl and azide may be attributable to the demonstrated metabolic effects of these agents (Table 3); a decrease in the conversion of choline to phosphorylcholine would lead to a greater availability of choline, which would be expected to traverse the neural membrane more readily than phosphorylcholine.

* W. J. Cooke and J. D. Robinson, unpublished observations.

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