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ACETYLCHOLINE, CHOLINE ACETYLTRANSFERASE AND CHOLINESTERASES IN MOTOR AND SENSORY NERVES OF THE BULL FROG*

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Abstract—Analytical procedures developed during the past few years were applied to reinvestigate the distribution of the acetylcholine system in peripheral nerve. Acetylcholine was determined by pyrolysis–gas chromatography, choline acetyltransferase by a radiometric assay using [^{14}C]acetylcoenzyme A, and cholinesterases by a colorimetric technique. The results obtained with the improved methods have unequivocally demonstrated the presence of choline acetyltransferase, acetylcholine, and acetylcholinesterase in motor as well as in sensory nerve fibers. Ventral roots were found to contain much higher concentrations of acetylcholine and choline acetyltransferase than the dorsal root ganglia and the dorsal root fibers. Cholinesterase activity was highest in the ganglion; there was no significant difference in cholinesterase activity of ventral and dorsal root fibers.

SEVERAL studies have been done to determine the presence of the acetylcholine system—cholinesterases, ChE (acetylcholine acetylhydrolase, EC 3.1.1.7, AChE; acylcholine acylhydrolase, EC 3.1.1.8, BuChE); acetylcholine, ACh; choline acetyltransferase (choline *O*-acetyltransferase, EC 2.3.1.6, ChAc)—in the peripheral sensory nervous system. The information obtained has been somewhat contradictory with respect to ACh and especially ChAc. Either no ChAc activity was measured,[1,2] or the values obtained were doubtful and on the borderline of detectability (see review by Hebb[3]). Similar uncertainty applies to the quantitative analysis of ACh in these structures.[1,3,4] Many of the discrepancies are probably due to inadequacies in the methods employed. In the previous studies, ACh was always determined by means of bioassay procedures which limited the specificity and sensitivity for endogenous ACh and for the ester formed *in vitro* by ChAc.

During the past few years, improved methods have been introduced for the determination of ACh and ChAc. There is available a highly sensitive and specific chemical assay for ACh based on gas–liquid chromatography.[5–7] The minimum amount required for the specific detection of ACh is 2 ng.[8]

The development of a radiometric assay for ChAc,[9] as modified for anion-exchange column chromatography,[10] has improved the sensitivity and specificity of previous

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methods. Furthermore, specific inhibitors for ChAc have been described.[11–13] More recently, histochemical procedures to localize ChAc have been developed.[14–17]

These advances in the available methodology led us to reinvestigate the distribution of the cholinergic system in sensory nerve. This work is relevant in view of Nachmansohn's hypothesis[18,19] of a universal and crucial function of the ACh system in the permeability cycle of all excitable membranes, including those of axons. The failure to determine ACh content and ChAc activity in dorsal root ganglia and fibers,[1,2] previously was used as an argument against the role of the ACh system in conduction of the nerve impulse. The demonstration of AChE or BuChE activity alone in these structures is in this respect not critical, since this enzyme is also found in nonconducting tissue.[20,21]

In the present study, we have reinvestigated the presence and concentrations of ACh, ChAc, AChE and ChE in the sensory nervous system of the bull frog. The activities found in dorsal root ganglia and dorsal root fibers as examples of the pure sensory components are compared with the activities found in pure motor fibers, as represented by the ventral roots and by the mixed fiber population of the sciatic nerve.

MATERIALS AND METHODS

Animals and preparation of tissues

Bull frogs were obtained from Southern Biological Supply Company, McKenzie, Tenn., and kept in plastic containers at room temperature (22–25°). Dorsal root ganglia (DRG), dorsal root fibers (DRF), ventral root fibers (VRF) and mixed fibers of the sciatic nerve (SCI) were prepared and collected in frog Ringer's solution (composition (mM): NaCl, 115; KCl, 2·5; $CaCl_2$, 1·8; $NaHCO_3$, 2·4; dextrose, 5) containing 1×10^{-4} M paraoxon when ACh was to be determined. In most of the experiments, each preparation was pooled from three frogs, blotted on filter paper, weighed and homogenized in small glass homogenizers (350 μl) 1:3 (w/v) for DRG and DRF or 1:6 (w/v) for VRF and SCI. All fibers contained sufficient AChE, ChE and ChAc to allow determinations from a single animal. DRG and DRF had to be pooled from three frogs to permit a quantitative analysis of ACh.

Choline acetyltransferase

Biochemical determination. ChAc was determined by means of a radiometric procedure[9] with the modification for column chromatographic separation of the labeled ACh.[10] Analytical grade anion-exchange resin AG 1–X2, 200–400 mesh, in the chloride form (Bio Rad Laboratories, Richmond, Calif.) was used to separate ^{14}C-labeled ACh from [^{14}C]acetyl coenzyme A. The incubation medium had the following composition (final concentration, mM): NaCl, 300; $MgSO_4$, 20; physostigmine sulfate, 0·5; choline iodide, 10; acetyl[1-^{14}C]coenzyme A, 0·57; and phosphate buffer, pH 7·4, 75. The total incubation volume was 110 μl (100 μl medium and 10 μl homogenate). Acetyl-1-[^{14}C]coenzyme A (specific activity, ~50 mc/m-mole) was purchased from New England Nuclear in Boston, Mass., and the specific activity reduced with acetyl coenzyme A (Mann Research Laboratories) to approximately 1·5 mc/m-mole. The incubation time was 15 or 30 min in a 37° shaking water bath. The [^{14}C]acetylcholine was eluted four times with 0·5 ml of distilled water from the

ion-exchange column; the eluate was collected in counting vials and radioactivity determined in a Nuclear Chicago Mark I liquid scintillation counter using 15 ml of Bray's scintillation mixture.[22] Efficiency as determined with an external standard and the channel ratio method was found to be about 72 per cent. Column blanks, which consisted of the complete incubation medium without the enzyme, were run under similar conditions, and the value obtained was deducted from each experimental value.

Cholinesterases

ChE and AChE activity was determined by means of the colorimetric procedure of Hestrin.[23] The substrates used were acetylcholine iodide (ACh), 5×10^{-3} M, butyrylcholine iodide (BuCh), 1×10^{-2} M, and acetyl-DL-β-methylcholine (McCh), 1×10^{-2} M (all from Sigma Chemical Company, St. Louis, Mo.). The substrates were dissolved in frog Ringer's buffered with 0·1 M Tris and HCl to pH 7·5. The reaction was started by the addition of homogenate and the rate of hydrolysis was followed at room temperature (22–25°) in a shaking incubator.

ACh extraction and determination

The tissue was homogenized in 2 ml acetonitrile containing 2% trichloroacetic acid to which propionyl and butyryl choline had been added as internal standards. After centrifugation, the supernatant was diluted by the addition of an equal volume of water and extracted twice with 2 vol. diethyl ether. The ether was discarded and residual ether was removed from the aqueous layer with a stream of nitrogen. The aqueous layer was divided into duplicate samples with the exception of the dorsal root ganglia and dorsal root fibers, in which only a single analysis was possible due to limitation of material. The quaternary compounds were precipitated by potassium iodide–iodine solution with tetramethyl ammonium iodide as coprecipitant, and the precipitate was analyzed for choline esters by pyrolysis–gas chromatography. For quantitative studies, butyrylcholine and propionylcholine as iodide salts were added before homogenization as internal standards. The absolute recovery of ACh varied between 75 and 85 per cent. However, with the use of internal standards, the exact, absolute recovery becomes unimportant. Propionylcholine and butyrylcholine do not appear in the chromatogram of tissue to which no standards have been added. They are not present in significant amounts in the tissue tested. Any dimethylaminoethyl acetate present in the tissue would not be determined by this method. As a tertiary amine, it is insensitive to the precipitation technique used and, in the unlikely event of partial precipitation, it would be lost due to its volatility (b.p. 138°) when samples were placed in the pyrolyzer and purged at 150° prior to pyrolysis.

ACh was analyzed on a Fisher Victoreen model 4400 gas chromatograph equipped with flame ionization detector and a Barber–Coleman pyrolyzer.

RESULTS

Distribution of ChAc

A striking difference was observed in the amount of ACh synthesized in sensory and motor nervous tissue (Table 1). The DRF synthesized 80 nmoles ACh/g of fresh weight/hr, whereas the VRF synthesized 8000 nmoles ACh/g/hr. The DRG synthesized twice as much as the DRF. The activity in the sciatic nerve (motor and sensory

TABLE 1. SYNTHESIS OF ACh IN SELECTED TISSUES FROM THE PERIPHERAL NERVOUS SYSTEM OF BULL FROGS*

Tissue	ACh formed (nmoles/g fresh wt. × hr^{-1})
DRG	173 ± 17 (10)
DRF	80 ± 6 (6)
VRF	8020 ± 350 (11)
SCI	3230 ± 260 (10)

* Values represent mean ± S.E.M. The number of independent experiments is indicated in parentheses. In each individual assay, tissue was pooled from three frogs.

fibers) was 3200 nmoles/g × hr^{-1}. To ensure that the radioactivity eluted with the ACh fraction was really due to enzymatic acetylation of choline by ChAc, the three controls listed below were run.

Boiling of the homogenate. This procedure completely abolished radioactivity in the aqueous eluate. The values obtained were equal to the column blank background and ruled out nonspecific binding of ^{14}C-labeled material to some cation.

Incubation in the presence of an inhibitor of ChAc. Chloroacetylcholine (Cl-ACh), $6{\cdot}25 \times 10^{-5}$ M, has been shown[12] to inhibit partially purified placental ChAc by more than 90 per cent. With Cl-ACh present in the ChAc incubation medium, the amount of ACh formed was reduced, as shown in Table 2. The inhibition of ChAc was

TABLE 2. EFFECT OF CHLOROACETYLCHOLINE (Cl-ACh) ON THE ACTIVITY OF ChAc FROM PERIPHERAL NERVES OF THE BULL FROG

Concn. of Cl-ACh (M)	Source of ChAc activity*			
	DRG	DRF	VRF	SCI
Control	100·0	100·0	100·0	100·0
5×10^{-5}	80·0	55·0	64·0	60·0
1×10^{-4}		37·0	23·0	18·0
5×10^{-4}	53·0	23·0	13·0	

* Data are expressed as per cent of activity remaining in the presence of the inhibitor concentration specified in the left column when compared to control values.

obvious in all tissues, but it was more pronounced at the lower concentration in VRF and SCI, which exhibit high ChAc activity.

Nonspecific acetylation of cationic compounds other than choline. Incubation under the standard conditions in the absence of the substrate choline was then used to establish whether there was any transfer of labeled acetyl groups occurring other than the one due to ChAc activity. It was found that there was some nonspecific transfer of radioactivity to that fraction which normally contains the ^{14}C-ACh. The amount was negligible (< 3 per cent of control values with choline) in VRF and

SCI and was estimated to be about 5–10 per cent of the control values for DRG and DRF. It is possible that this higher percentage of nonspecific acetyl transfer explains, to a certain extent, the lower degree of ChAc inhibition by ClACh (Table 2) in DRG and DRF preparations.

Distribution of cholinesterases (BuChE and AChE)

The highest enzyme activity was found in the DRG, which hydrolyzed 500 μmoles ACh/g $\times$ hr^{-1} (Table 3). Using MeCh, as specific substrate for AChE, the DRG hydrolyzed this ester at 60 per cent of the rate at which it split ACh. However, BuCh, a specific substrate for ChE, was split at only less than 5 per cent of the rate of ACh.

TABLE 3. HYDROLYSIS OF VARIOUS CHOLINE ESTERS BY CHOLINESTERASES FROM PERIPHERAL NERVES OF THE BULL FROG

Substrate	Tissue analyzed*			
	DRG	DRF	VRF	SCI
ACh (5×10^{-3} M)	509·0 ± 43·0 (11)	47·3 ± 4·3 (8)	50·3 ± 6·4 (14)	61·1 ± 9·1 (12)
MeCh (1×10^{-2} M)	298·0 ± 38·0 (3)	10·7 ± 3·5 (3)	31·8 ± 3·0 (3)	30·3 ± 3·7 (4)
BuCh (1×10^{-2} M)	18·7 ± 3·2 (3)	13·6 ± 1·8 (3)	4·7 ± 0·7 (3)	23·3 ± 3·7 (3)

* Values are given in micromoles of substrate hydrolyzed per gram fresh weight per hour, using the substrate indicated at the concentration specified. Enzyme activity was determined with the procedure of Hestrin[23] at room temperature (22–25°). Data represent mean ± S.D. and the number of determinations is indicated in parentheses.

The DRF, which have their cell bodies in neurons located in DRG, had a strikingly different composition of choline ester-hydrolyzing enzymes. In this tissue, BuChE made a significant contribution to the overall hydrolysis of ACh. BuCh was hydrolyzed faster by DRF than was MeCh. The mixed SCI was similar to DRF in its hydrolysis for the different choline esters, while the VRF had more AChE than ChE.

Determination of ACh by pyrolysis–gas chromatography

ACh was estimated not only in the tissue extracts but in many cases also in the bathing fluid in which it had been stored during the dissection procedure ($\sim$3 hr). This was done in an attempt to assess the amount which leaked out during preparation. The levels of ACh in the various tissues are shown in Table 4.

The amount of ACh is smallest in DRG, which also had low ChAc activity (Table 1) but was outstandingly high in AChE (Table 3). A chromatographic record is shown in Fig. 1. Although the amount of ACh in the DRG was small, there was an easily distinguishable peak at the time when authentic ACh eluted from the column. The amount of ACh in this particular case, under the conditions specified in the legend to Fig. 1, corresponds to 3–4 ng. Slightly more of the ester was present in DRF, while the motor VRF and mixed SCI fibers contained 10–20 times as much as the pure sensory tissue. A significant amount of ACh leaked out from the tissue

TABLE 4. LEVELS OF ACh IN PERIPHERAL NERVOUS TISSUE OF THE BULL FROG AS DETERMINED BY PYROLYSIS–GAS CHROMATOGRAPHY*

Source of ACh	DRG	DRF	VRF	SCI
Tissue	1·36 (1·03–1·65) N = 3	2·39 (1·63–3·08) N = 3	29·03 (22·0–39·1) N = 9	18·52 (14·3–22·9) N = 15
Bathing fluid	1·47 (0·85–2·1) N = 2	not determined	16·50 (10·2–23·3) N = 5	3·95 (0·96–7·0) N = 7
Tissue†	13·50 N = 1	8·22 (6·22–10·22) N = 2	67·4 (49·8–90·3) N = 5	18·34 (15·1–21·2) N = 6
Bathing fluid†	2·05 N = 1	5·56 (3·88–7·55) N = 2	13·54 (10·0–19·4) N = 6	4·83 (2·38–7·06) N = 4

* Values are given in nanomoles of ACh per gram fresh weight. Data represent mean and, in parentheses, the range for the number (N) of independent assays indicated. DRG and DRF were pooled from three frogs per assay.

† Determinations were made in February and March 1971. The material in the upper half of the table was analyzed in December 1970, and January 1971.

during the collecting period (line 2, Table 4). The amounts are variable in terms of per cent of the ACh content from tissue to tissue and are only intended to show that leakage has been considered rather than to represent a quantitative evaluation.

An increase in the levels of ACh was found in bull frogs sacrificed in February and March, 1971, as compared to samples taken earlier. These values are given in the lower part of Table 4. The changes occurred in all tissues but SCI, in which the values were identical with those measured earlier and listed in the upper part of Table 4.

DISCUSSION

The application of a radiometric procedure measuring the transfer of ^{14}C-labeled acetyl groups to choline resulting in ^{14}C-ACh formation has established beyond doubt the presence of ChAc in all peripheral nervous tissues of the bull frog. The amount of ACh formed in DRG and DRF is small. This is in contrast to the findings of earlier investigations in which no ChAc activity was found.[1–3] Evidence for the specificity of the reaction measured is provided by the following experiments: (1) the inhibitory effect of ClACh on ^{14}C-ACh formation (Table 2) supports the presence of ChAc, since it is a specific inhibitor of ChAc activity;[12] (2) nonspecific binding of label to cationic compounds does not occur, as shown in incubations where boiled tissue was used; and (3) nonspecific acetyl transfer other than by ChAc activity cannot explain all the ^{14}C label found in the ACh fraction. However, there was some nonspecific contribution as measured in the absence of exogenous choline in the incubation medium.

The presence of ChAc in DRF could be the result of axoplasmic flow from the ChAc-containing neurons in DRG, as was described by Weiss and Hiscoe[24,25] and was demonstrated for ChAc in the sciatic nerve of the goat.[26] The rate of synthesis of

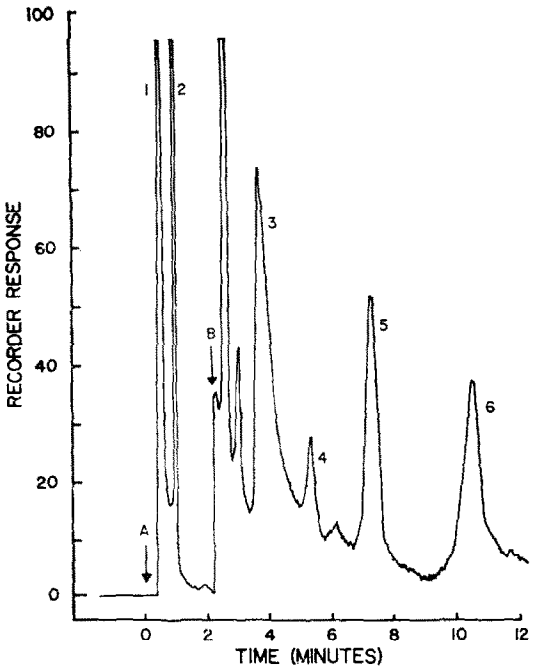

FIG. 1. A chromatogram obtained from pooled dorsal root ganglia from three bull frogs. Point A represents the instant of pyrolysis with an electrometer sensitivity of 2×10^{-9} amps. At point B, sensitivity is increased to 2×10^{-10} amps. Peaks are as follows: 1, trimethylamine; 2, methyl iodide; 3, dimethyl amino ethanol; 4, dimethyl amino ethyl acetate; 5, dimethyl amino ethyl propionate; 6, dimethyl amino ethyl butyrate. Peaks 5 and 6 represent the tertiary amine esters of propionyl and butyrylcholine, respectively, which are used as internal standards. These peaks do not appear in tissue to which none of these standards has been added. Other peaks are unidentified. Chromatographic conditions: 8 ft × ¼ in. aluminum column packed with 20% Carbowax 6000 ion 80/100 mesh Chromosorb W (HMDS). Column temp., 125°; detector temp., 200°; injector and pyrolyzer block temp., 160°. Nitrogen flow, 75 ml/min; oxygen flow, 1·5 l./min; hydrogen flow was adjusted to optimum detector sensitivity.

ACh in DRF of bull frogs is approximately in the same order of magnitude as that of DRF from cat, dog and ox.[18,27,28]

The rate of hydrolysis of ACh in DRF and VRF showed little quantitative difference from the findings in DRF and VRF of ox.[2,18] Exceptions are the frog, *Rana pipiens*,[29] and the dog.[30] The composition of the choline ester-hydrolyzing enzymes in the various tissues investigated shows striking differences. The high ratio (15·9) of MeCh:BuCh hydrolysis indicates that the DRG enzyme is mainly AChE with only a small contribution of ChE. This corresponds very well to the characterization obtained with a microgasometric procedure in isolated neurons from DRG of *Rana pipiens*.[31] The ratio (6·8) for VRF also indicates a large amount of AChE, while SCI and DRF (ratios of 1·3 and 0·8 respectively) have a significant contribution of BuChE in the total hydrolysis of ACh. This finding raises an interesting question concerning the DRF. The large differences in the composition of these enzymes in DRG and DRF of bull frogs could mean that the neurons in DRG are not the only

source for the axonal DRF cholinesterases, since one could expect a closer similarity in their substrate specificities. In view of the concept of axoplasmic flow of cholinesterases,[32] one would have expected a pattern in the fibers similar to the one which was seen in the neurons, unless one assumes different flow patterns for ChE and AChE. Further, one has to consider the possibility that certain proteins could have different turnover times in DRG and DRF. This would result in a different composition of proteins in general and of enzyme proteins in particular. Another possibility is that not all of the enzymes in DRF have their origin in the perikaryon of the dorsal root neurons. There may be a contribution from Schwann cells or local synthesis, or from both. Local synthesis of cholinesterases has been shown to occur in the cat after irreversible inhibition by diisopropylfluorophosphate.[33,34] BuChE could also derive from the Schwann cell.[21] The possibility of a transfer of macromolecules from the Schwann cell to the axon seems to exist.[35,36]

Pyrolysis–gas chromatography has unequivocally established the presence of ACh in tissue extracts from DRG and DRF. The method is highly specific in the detection of various choline esters[6,8] and is superior to bioassay procedures. Tissue extracts from DRG and DRF, though low in ACh content, always showed a peak on the gas chromatogram where authentic ACh would elute from the column (Fig. 1).

There is no satisfactory interpretation as yet to explain the differences in the levels of ACh in some of the tissues (Table 4). It does not seem to be due to the extraction procedure, since SCI, the tissue that is most difficult to homogenize, gave very reproducible results.

It is possible that the ACh-system in the bull frog undergoes "seasonal variations". The analyses were done over a period of several months. The levels of ACh were higher in frogs sacrificed in February and March than in those for November and December, except for SCI. The increase, however, could not be related to a measurable increase in ChAc activity. A systematic collection of data year round may provide a better understanding of the changes observed. Seasonal variations in the ACh-system have been described in the lobster peripheral nervous system.[37]

The release of ACh into the bathing medium during dissection can be explained in part by the technique. The fibers are cut open at both ends of the bundle, thus providing a relatively large surface area for diffusion of ACh into the medium. The ganglion also is stripped of its connective tissue sheath. Spontaneous release of ACh has been studied in dog sciatic nerve,[38] in frog and cat sciatic nerve,[39] and in more detail in lobster walking leg nerves,[40] but to our knowledge has never been assessed in previous studies of ACh content in DRF and VRF.[1–4]

We cannot explain the functional significance of the large differences in ChAc and ACh concentration measured in DRG and DRF on the one hand, and in VRF, on the other. If the ACh were intimately connected with the permeability cycle of all excitable membranes, including those of the axons as postulated,[18,19] one would expect less variable concentrations in motor and sensory fibers. There are suggestions that the ACh-system is involved in regulating tissue permeability and transport functions in non-nervous tissue like placenta.[41] Other results also indicate functions of ACh in the synthesis of membrane phospholipids[42–44] and amino acid uptake and protein synthesis in white blood cells.[45] If one assumes a function for the ACh-system in metabolic reactions in more general terms, then the concentration differences in the two types of fibers are still unexpected, unless their metabolic requirements are

completely different. Some differences in the electrophysiological characteristics of sensory and motor fibers have been described.[46–48]

By use of electron microscopic procedures for histochemistry combined with microgasometry of single neurons of dorsal root ganglia, a high ChE activity, associated with the inner surface of the endoplasmic reticulum membranes and the nuclear envelope, was demonstrated.[31] This intracellular localization of the hydrolyzing enzyme may explain, in part, the high activity in the cells when compared to the activities of ChAc, ACh and ChE in the fiber preparations. Only turnover studies of these enzymes will give a more conclusive explanation of these particular findings. Attempts to establish a direct relationship between the concentrations of ChE and ChAc in a number of tissues have been inconclusive.

The radiometric assay for ChAc and the gas chromatographic ACh determination have established that low concentrations of both components are present in sensory peripheral nerve. This indicates that there are cholinergic fibers originating in the DRG. Giacobini[49] has concluded from histochemical observations that 10–15 per cent of the neurons in DRG contain relatively large amounts of AChE, which suggests the origin of cholinergic fibers. This assumption is supported by the histochemical localization of ChAc activity in some neurons of the DRG in the bull frog (A. M. Burt, in preparation). The presence in DRF of all components of the ACh-system in low concentrations can then be explained by axoplasmic flow toward some cholinergic synapses in the spinal grey where these sensory fibers project. The role of the cholinergic fibers originating in the DRG is still unknown; experiments are planned to study their function.

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