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THE ACETYLATION OF HEMICHOLINIUM-3 BY CHOLINE ACETYLTRANSFERASE

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Abstract—The acetylation of hemicholinium-3 (HC-3) by choline acetyltransferase (ChAc) from a rat brain homogenate has been investigated using several different methods to recover the acetylated product. These methods are based on precipitation by ammonium reineckate, binding to an ion-exchange column (Amberlite CG-50), extraction with sodium tetraphenylboron in butyl ethyl ketone, extraction with mercuric potassium iodide in octanone and paper electrophoresis. Identification of the incubation product by electrophoresis showed that HC-3 was acetylated although it was not established whether the product was the mono- or the bi-acetylated form. The observed acetylation of HC-3 (1 mM) compared with choline (1 mM) varied between 0 and 27 per cent depending on the procedure used for isolation of the acetylated product. It is suggested that this variation arises out of differences in the relative percentage recoveries of acetyl HC-3 and acetylcholine by the different methods; the ion-exchange procedure was shown to recover ACh efficiently but did not recover any acetyl HC-3. The possibility that HC-3 is acetylated *in vivo* has been suggested and the significance of this possibility discussed.

Hemicholinium-3 (HC-3), a bis-quaternary ammonium compound containing two choline-like moieties and first investigated by Schueler [1] has been shown to inhibit the formation of acetylcholine (ACh) in membrane-enclosed systems of cholinergic nerves [2, 3]. An effect on the uptake of choline into the presynaptic nerve terminal has been proposed as the main action of HC-3 responsible for this inhibition [4–7]. However interference with other aspects of ACh metabolism has also been suggested; these suggestions include effects on the conversion of choline to phosphorylcholine, phosphatidylcholine and betaine [5, 8, 9, 10]; inhibition of the transfer of ACh from the cytoplasm into synaptic vesicles [11]; inhibition of brain choline kinase [12]; and acetylation by choline acetyltransferase (ChAc, acetyl-CoA: choline-*O*-acetyltransferase, EC 2.3.1.6) the enzyme responsible for the synthesis of ACh from choline and acetylcoenzyme A [13, 14].

The acetylation of HC-3 has been investigated by several workers and has produced conflicting results. It has been proposed by Rodriguez de Lores Arnaiz *et al.* [13] and Hemsworth [14] that for HC-3 as for triethylcholine (TEC), a compound with pharmacological actions similar to HC-3 [15], that acetylation by ChAc may take place followed by release of the acetylated product as a false transmitter. These workers have reported that HC-3 is acetylated *in vitro* by ChAc but that the acetylation is only about 25 per cent as efficient as the acetylation of choline.

This suggestion has been disputed, however, by Diamond and Milfay [16] who could find no evidence for the acetylation of HC-3 by soluble rat brain ChAc *in vitro*. In addition Collier [11] showed a lack of release of accumulated [^{14}C]HC-3 upon nerve stimulation of a cat superior cervical ganglion preparation.

The present investigation seeks to resolve the controversy surrounding the acetylation of HC-3, by using several established methods to detect any acetylated product that might be formed.

MATERIALS AND METHODS

ChAc enzyme preparation. Fresh rat brains were homogenised on ice in 0.1% Triton X-100 containing 200 mM KCl (10 ml/g wet wt) using a glass homogeniser with a Telfon pestle. The homogenate was centrifuged at 20,000 *g* for 20 min and the supernatant containing the enzyme was used immediately or stored at −20°.

When ChAc was required free from endogenous choline, it was first passed through a Sephadex G-50 column equilibrated with 200 mM KCl.

Incubation system. The rates of acetylation of choline and HC-3 were estimated by incubation with acetyl-1-[^{14}C]coenzyme A and ChAc and by quantitative determination of the amount of labelled product formed.

The incubation mixture was placed in a plastic microfuge tube (Beckman) and contained 5 μl ChAc, 10 μl of distilled water, choline (2.5 mM) or HC-3 (2.5 mM) and 10 μl of a buffer solution containing acetyl-1-[^{14}C]coenzyme A (58 mCi/m-mole), 5×10^{-5} M; $MgSO_4$, 10^{-2} M; NaCl, 6×10^{-2} M; physostigmine sulphate, 4×10^{-4} M; EDTA, 2×10^{-4} M; albumin, 0.1 mg/ml; and potassium phosphate (pH 7.7) 3×10^{-2} M. The total volume of the incubation mixture was 25 μl. The mixture was incubated at 37° for 15 min over which time the reaction followed a linear time course as determined from a graph of cpm of acetylated product against time.

To ascertain that endogenous choline had been removed by passage of the ChAc through Sephadex G-50, incubations were carried out in which no exogenous substrate was added. Blank values were determined in which the enzyme was denatured by precipitation with trichloroacetic acid before incubation.

Isolation and identification of the acetylated product.

(a) *Reineckate precipitation.* The method used was a modification of the procedure described by McCaman and Hunt [17]. After incubation, 5 μl of 50 per cent trichloroacetic acid (TCA) containing

2×10^{-1} M choline chloride as carrier was added to precipitate the enzyme. The tubes were centrifuged in a microfuge (Beckman) and 20 μl of the supernatant was transferred to 50 μl of a saturated ammonium reineckate solution in 0.5 N HCl. The tubes were again centrifuged and after removal of the supernatant the precipitate was washed with 50 μl of 0.2 N HCl. After recentrifugation and removal of the supernatant the precipitate was dissolved in 100 μl of acetone and transferred to Whatman glass-fibre filter discs. The radioactivity was then determined by adding 5 ml of a toluene-based scintillation fluor containing PPO (5 g/litre) and POPOP (0.3 g/litre) and counting in a liquid scintillation counter (Beckman LS-230).

(b) *Extraction with sodium tetraphenylboron in butyl ethyl ketone.* After incubation, 20 μl of the incubation was transferred to 7 ml of 10 mM sodium phosphate buffer (pH 7.4) containing 0.25 mg of acetylcholine chloride according to the method of Fonnum [18]. One ml of butyl ethyl ketone containing 15 mg of sodium tetraphenylboron was then added and after thorough mixing the tubes were centrifuged to separate the layers. One-half ml of the upper ketone layer was then transferred directly to 10 ml of toluene-based scintillation fluor and the radioactivity determined.

(c) *Extraction with mercuric potassium iodide in octanone.* The method used in the present experiments was a modification of that described previously by Glover and Green [19]. As in the reineckate method, the reaction was stopped by the addition of 5 μl of 50 per cent TCA containing 2×10^{-1} M choline chloride. One hundred μl of a solution containing 0.1 M mercuric potassium iodide (K_2HgI_4) in octanone was added and mixed thoroughly. The tubes were then centrifuged in a microfuge to separate the two layers. The modification used in the present experiments consisted of removing 50 μl of the upper octanone layer which was transferred to a Whatman glass-fibre filter disc. The filter disc was dried and was then counted in 10 ml of toluene-based scintillation fluor. In the method used by Glover and Green [19] an aliquot of the octanone layer was added directly to scintillation fluor and counted for radioactivity.

(d) *Electrophoresis.* Electrophoresis was used both to isolate and to identify the acetylated products after incubation. The reaction was stopped by adding 5 μl of acetic acid (1.5 M)/formic acid (0.75 M) buffer to the incubation medium. TCA was not used as it was found to interfere with the subsequent electrophoresis. After centrifugation, 20 μl of the reaction mixture was applied to the mid-line of an electrophoresis paper strip. Four applications of 5 μl were used and the paper was allowed to dry in between the applications in order to obtain a narrow band of sample on the paper. The strips were then subjected to electrophoresis at 500 V for 1 hr in a V-type Durrum cell (Beckman) using 1.5 M acetic acid–0.75 M formic acid buffer as described by Potter and Murphy [20]. After electrophoresis, the strips were stained in iodine vapour whilst barely damp and the position of any stained band was marked in pencil.

Radioactive strips were passed through a Tracerlab 4π strip scanner to determine the position of the radioactivity. For a quantitative assessment of the radioactivity the strips were cut into 1-cm portions and counted in 5 ml of toluene-based scintillation fluor.

The position of the radioactive bands obtained after electrophoresis of incubation mixtures were compared with the stained bands of authentic ACh and acetyl HC-3 similarly subjected to electrophoresis. The authentic compounds were visualised by staining in iodine vapour or by spraying with Dragendorff's reagent.

(e) *Ion-exchange chromatography on Amberlite CG-50 (100–200 mesh).* A modification of the method of Diamond and Kennedy [21] was used to isolate the acetylated products by ion-exchange chromatography.

After incubation 100 μl of 50 per cent ethanol containing 0.6 mg/ml acetylcholine chloride was added to the tubes (Eppendorf 3810) which were then centrifuged for 1 min in an Eppendorf 3200 centrifuge. One hundred μl of the supernatant was then transferred to a 2.5-cm column of Amberlite CG-50 (100–200 mesh) prepared in distilled water and contained in a Pasteur pipette. The sample was allowed to run into the column which was then eluted five times with 0.5 ml sodium acetate (1 mM) to remove unreacted acetyl-[^{14}C]coenzyme A. This eluate was discarded. The column was then eluted with HCl (1 N) until a volume of 2 ml had been collected. The radioactivity in this 2 ml of HCl eluate was then determined using 10 ml of a modified Bray's [22] 1,4-dioxan-based scintillation fluor containing naphthalene (100 g/litre), methanol (100 ml/litre), PPO (4 g/litre) and POPOP (0.2 g/l).

When necessary, the observed recovery of radioactive products was corrected for the fact that only part of the incubation medium was used for the extraction procedure.

Detection of Acetyl HC-3 and ACh in eluates from an Amberlite CG-50 column

One hundred μl of a solution of acetyl HC-3 (10^{-2} M) or ACh (2.5×10^{-2} M) was applied to the top of an Amberlite CG-50 column prepared in a Pasteur pipette. Elution was then carried out using sodium acetate (1 mM) and HCl (1 N) as described above. This procedure was followed by elution with 2 ml NaOH (1 N).

To detect the presence of acetyl HC-3 in the eluates, two methods were used as described below.

(1) Column eluates were passed directly through a Uvicord II spectrophotometer LKB 8303A type and their absorption at 280 nm measured. Under these circumstances, larger volumes of eluants were used than described above.

(2) Sodium acetate and HCl eluates were collected to a volume of 2 ml. The NaOH eluate was collected in 1 ml fractions. One hundred μl from each eluate fraction was then added to 100 μl of a saturated ammonium reineckate solution in 0.5 N HCl. The presence of acetyl HC-3 in the eluate was indicated by the formation of a pink precipitate.

The reineckate precipitation method was also used for the detection of ACh.

Materials. Hemicholinium-3, (HC-3); 2,2'-(4,4'-biphenylene)-bis-(2-hydroxy-4,4-dimethylmorpholin-

ium bromide), was obtained from Aldrich Chemical Co. London.

Acetyl hemicholinium-3, (AcHC-3); 4,4'-biphenylene-bis-(2-oxoethylene)-bis-(2-acetoxyethyl-dimethylammonium bromide), was obtained from Eastman Organic Chemicals, Rochester, New York. Acetyl-1-[^{14}C]coenzyme A was purchased from the Radiochemical Centre, Amersham, England.

RESULTS

Five different methods were used for estimating the rate of acetylation of choline and HC-3 by ChAc by using various methods for isolating the radioactive acetylated products of incubation. These different methods gave very different estimates of HC-3 acetylation and the results are shown in Table 1.

(a) *Reineckate precipitation.* When either HC-3 or choline was used with acetyl-[^{14}C]coenzyme A as a substrate for ChAc, a radioactive substance was precipitated during the isolation procedure by ammonium reineckate. ChAc enzyme preparations from brain homogenates generally contain endogenous choline partially from plasma and possibly from breakdown of phospholipids. The absence of endogenous choline substrate in the ChAc enzyme preparation after passing through the Sephadex G-50 column was evident from incubations in which no exogenous substrate was added: such an incubation gave values for acetylated product which were similar to blank values.

HC-3 was acetylated at a rate of 27.9 per cent compared with the acetylation of choline at the same concentration (Table 1). In the presence of endogenous choline with HC-3 as substrate the recovered radioactivity was greater than when no HC-3 substrate was added also indicating an acetylation of HC-3.

(b) *Sodium tetraphenylboron-butyl ethyl ketone extraction.* Acetylation of both HC-3 and choline was also evident with this method of extraction. The percentage acetylation of HC-3 compared with choline was shown to be 11.0 per cent (Table 1). When HC-3 was used as substrate in the presence of endogenous choline, i.e. when the ChAc enzyme had not been passed through the Sephadex G-50 column; the amount of radioactive product recovered after extraction with sodium tetraphenylboron was greater than when no substrate was added. This recovery of radioactivity indicates that acetyl HC-3 was synthesised by ChAc in the presence of endogenous choline.

(c) *Mercuric potassium iodide–octanone extraction.* Using the present modification in the method of Glover and Green [19] HC-3 was found to be acetylated at a rate of 22.7 per cent compared to the acetylation of choline at the same concentration (Table 1).

(d) *Electrophoresis.* Electrophoresis of an incubation mixture without added substrate and using ChAc which had not been passed through Sephadex G-50, revealed two radioactive peaks (Fig. 1A). These peaks were identified as ACh and acetylcarnitine by comparison with the electrophoresis of authentic compounds located by iodine staining. These peaks were absent when the ChAc preparation has been passed through Sephadex G-50 (Fig. 1B). These results show that choline and carnitine are present as endogenous substrates in brain fractions. Fig. 1C and 1D show that with HC-3 and choline as substrates respectively the acetylated products were obtained, identified as above using authentic biacetyl HC-3 and ACh. It is not known whether the acetylated product of the HC-3 incubation was entirely the biacetylated derivative.

A quantitative analysis of the acetylation of HC-3 and choline was made by counting the radioactivity in 1-cm strips of the electrophoresis paper in the liquid scintillation counter. This method showed that HC-3 was acetylated 16.3 per cent compared with the acetylation of choline (Table 1).

Table 1. Acetylation of HC-3 and choline by ChAc

Extraction procedure	Acetylation of HC-3 ($\times 10^4$ cpm/mg protein/min)	Acetylation of choline ($\times 10^4$ cpm/mg protein/min)	Percentage acetylation of HC-3 compared with choline
Reineckate precipitation (n = 7)	1.50	5.93	27.9 ± 2.7
$NaPh_4B$–butyl ethyl ketone (n = 7)	0.89	8.35	11.0 ± 0.6
K_2HgI_4–octanone (n = 7)	1.32	5.74	22.7 ± 1.2
Electrophoresis (n = 2)	0.98	6.04	16.3 ± 0.2
Ion-exchange Amberlite CG-50 (n = 3)	0	4.63	0

Conditions for the determinations were as described in Materials and Methods. Endogenous choline had been removed from the ChAc by passage through Sephadex G-50. HC-3 and choline substrates were added to the incubation system to obtain a final concentration of 1 mM.

The results shown in the first two columns represent typical results for one determination for each extraction procedure. Those in the third column represent the mean ±S.E.M. from a number of determinations.

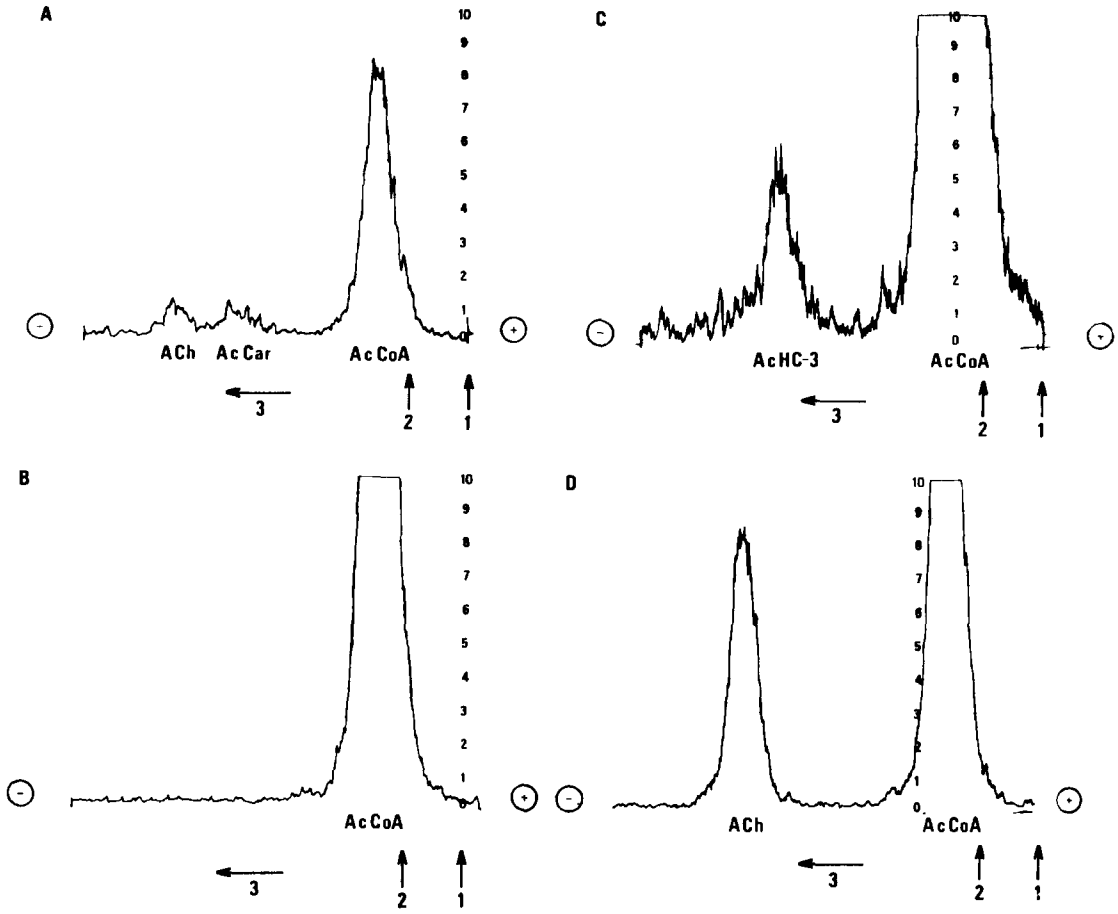

Fig. 1. Determination of radioactive peaks on paper electrophoresis strip passed through Tracerlab strip scanner. Paper electrophoresis was undertaken as described in Materials and Methods. The arrows represent (1) the origin of scanning, (2) the origin of electrophoresis on the paper strip (3) the direction of the applied voltage from anode to cathode. In A; an incubation mixture without added substrate was electrophoresed. Peaks AcCar and ACh represent acetylcarnitine and acetylcholine respectively. In B; ChAc which had been passed through Sephadex G-50 was used in the incubation mixture without added substrate. In C; the incubation was with HC-3 as substrate. In D; the incubation was with choline as substrate. In all records peak AcCoA represents unreacted acetyl[^{14}C] coenzyme A. In A, B and C full scale deflection on the abscissa, 0–10, corresponds to 1000 cpm; in C, 0–10 corresponds to 250 cpm.

(e) *Ion-exchange on Amberlite CG-50.* When choline and HC-3 were used as substrates in the presence of endogenous choline i.e. when the ChAc enzyme had not been passed through a Sephadex G-50 column, no acetylation of HC-3 was observed. The total cpm of radioactive product, when HC-3 was present in the incubation was 1577 cpm which was similar to the total cpm when no HC-3 was added. When 1 mM choline was added to the incubation system, in place of HC-3, [^{14}C]ACh was recovered in the HCl eluate from the Amberlite CG-50 ion-exchange column and the total amount of radioactive acetylated product obtained was 24534 cpm. These results are similar to those obtained by Diamond and Milfay [16].

When a ChAc enzyme was used which contained no endogenous choline substrate the rates of acetylation in terms of cpm/mg protein/min were as shown in Table 1.

Since the other extraction methods used in our experiments indicated that HC-3 was acetylated by ChAc two possible reasons for this discrepancy were considered. Firstly any acetylated HC-3 formed during the incubation might not bind to the ion-exchange column. Alternatively any acetyl HC-3 might be bound to the column but might not be eluted from the ion-exchange column by solutions which elute ACh. These two possibilities were therefore investigated.

Acetyl HC-3 was shown to bind to the Amberlite CG-50 ion-exchange resin but the compound was not eluted by 1 N HCl. Larger volumes of 1 N HCl in excess of the 2 ml normally applied for elution purposes also had no effect on the binding of acetyl HC-3 to the column. Acetyl HC-3 was however shown to be detected in the eluate when 1 N NaOH was used for elution of the ion-exchange column. Detection of the acetyl HC-3 in the solutions applied to and eluted from the column was made by u.v. absorption at 280 nm and by the use of ammonium reineckate (See Materials and Methods).

ACh was found to be completely removed from

solution by passage through the Amberlite CG 50 ion-exchange column. Elution of the ACh from the ion-exchange column was made with 1 N HCl.

These results show therefore that both acetyl HC-3 and acetylcholine bind to the ion-exchange column but that unlike ACh, acetyl HC-3 is not eluted by 1 N HCl.

DISCUSSION

The present results indicate that when HC-3 is used as a substrate for ChAc, together with acetyl CoA, a product is formed which can be recovered by a number of procedures which have been used previously for the isolation of the radioactive products of acetylation by ChAc. Identification of this material by electrophoresis shows that the product is the acetylated form of HC-3. It is suggested that the variation in percentage acetylation of HC-3 compared with choline is caused by the different recoveries of acetyl HC-3 and ACh with respect to each other and with respect to the different methods. The different estimates of HC-3 acetylation go a long way to explain the discrepancies in the literature.

Different methods for determination of ChAc activity have been developed over the past decade since the introduction of radiochemical methods by Schuberth [23]. Modifications of this method were made by McCaman and Hunt [17] who used ammonium reineckate for precipitation of ACh. This method has the disadvantage of producing high blank values partly as a result of the colour of the reineckate precipitate. In addition the method has been criticised for its lack of specificity because all quaternary salts including carnitine and acetylcarnitine are precipitated by this reagent. Fonnum's [18] method has the advantage of being less laborious than the reineckate method and of producing lower blank values. The method of Glover and Green [19] has been shown to suffer from the fact that any hydrolysis of acetyl CoA during the incubation to liberate acetate will yield false high values (Bradshaw and Hemsworth; unpublished observations). This disadvantage has been overcome in the present experiments by drying the organic phase on filter paper.

The method of paper electrophoresis first used by Potter and Murphy [20] has the advantage that the products of acetylation may be identified. It is suggested that because of this the electrophoresis method is the best procedure for investigating the acetylation of substrates for ChAc.

The present results using the ion-exchange resin do show some agreement with the results reported by Diamond and Milfay [16] in that no acetylated radioactive product of incubation is observed when HC-3 is used as substrate. However our conclusions regarding the acetylation of HC-3 differ completely from those of Diamond and Milfay [16]. The reason for this difference in the conclusions is that the present results show that acetyl HC-3 is bound to the Amberlite CG-50 resin but is not eluted by HCl whereas ACh is bound to the resin and is eluted by 1 N HCl. Diamond and Milfay [16] do not give an indication of the recoveries of ACh and acetyl HC-3 obtained with their ion-exchange resin but seem to have assumed that acetyl HC-3 and ACh will react with and elute from the ion-exchange resin in a similar manner. The reason for the resistant binding of acetyl HC-3 to the column is not known but 1 N NaOH was shown to remove the quaternary compound. It would be expected that acetyl HC-3 would bind to the column in a similar manner to ACh, however this does not occur and it may be that the acetyl HC-3 forms some covalent bonding with the matrix of the ion exchange resin. The ion-exchange procedure therefore appears to be less suitable than other methods for studying the acetylation of choline analogues.

A variety of choline analogues have been previously shown to be acetylated by ChAc *in vitro* [24–28] and it appears that HC-3 can be included among these. It also seems possible that the acetylation of HC-3 might take place *in vivo* since it has already been shown that HC-3 is taken up into cholinergic nerve terminals [29] and the present results show that the acetylation occurs even in the presence of endogenous choline. The importance of this possibility has yet to be determined but Slater and Stonier [30] suggest that a competition between choline and HC-3 at an intracellular site may be a more important factor in the inhibition of ACh synthesis by HC-3 than is the impairment of choline uptake and it is possible that such a competition occurs for acetylation by ChAc.

Collier [11] however showed that the cat superior cervical ganglion accumulated [^{14}C]HC-3 but the amount of HC-3 taken up was not changed by nerve stimulation. In addition the accumulated HC-3 was not released by subsequent nerve stimulation. Collier [11] concluded that HC-3 is not transported by the choline transport system and does not form a false cholinergic transmitter. However, Collier's [11] conclusions may be the result of tissue and species variation because Guyenet *et al.* [7] showed that in rat striatal synaptosomes HC-3 had an afffinity for the carrier mechanisms which was one hundred times greater than the affinity of choline itself. Also Haga and Noda [31] and Snyder *et al.* [32] showed that HC-3 had a very low K_i for inhibition of the high affinity component of the total uptake of choline.

It is apparent that further studies are required into the acetylation of HC-3 both *in vitro* and *in vivo* in a number of different tissues and species. Malthe-Sorenssen and Fonnum [33] have demonstrated the heterogeneity of ChAc within the same species and in different species and it may be that these multiple forms of ChAc have different activities towards different substrates.

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