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EFFECTS OF CHOLINERGIC DRUGS ON UPTAKE OF [^{14}C]-α-AMINOISOBUTYRIC ACID INTO FRAGMENTS OF HUMAN TERM PLACENTA

IMPLICATIONS FOR CHOLINERGIC RECOGNITION SITES AND OBSERVATIONS ON THE BINDING OF RADIOACTIVE CHOLINERGIC LIGANDS

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Abstract—Fragments of human term placenta took up [^{14}C]-α-aminoisobutyric acid ([^{14}C]AIB, 100 μM) against a concentration gradient. Intracellular radioactivity rose to levels 6- to 22-fold over that in the surrounding medium depending on whether the tissue was preincubated at 37° or not. Among the cholinergic drugs whose effect on [^{14}C]AIB accumulation was examined, atropine (1 mM), but not *d*-tubocurarine, reduced the concentration ratios significantly. Although this concentration was high by comparison to innervated tissues, it raised the possibility that muscarinic-type cholinergic recognition sites might be present in the non-innervated placenta. Attempts to biochemically label such sites by equilibrium dialysis, a filtration or a centrifugal assay with cholinergic agonists ([^{3}H]acetylcholine, [^{3}H]nicotine, [^{3}H]muscarone), a muscarinic antagonist ([^{3}H]quinuclidinyl benzilate) or a nicotinic antagonist ([^{125}I]-α-bungarotoxin) in sub-nM to μM concentrations failed. None of the ligands showed the high-affinity, saturable and reversible binding characteristics described for cholinergic receptors in a variety of innervated structures. The results do not rule out the existence of cholinergic recognition sites in the placenta, but make it questionable that such macromolecules have the characteristics of muscarinic and nicotinic sites as defined in nervous tissue.

The presence of large concentrations of a constituent with acetylcholine (ACh)-like activity in the human placenta was first described more than forty years ago [1, 2]. Recent gas chromatographic analysis has confirmed the presence of ACh, and the tissue levels in term placentae were found to be more than 100 nmoles/g of fresh tissue [3]. The remarkable feature about this abundance of ACh was that all attempts to establish nervous tissue elements have failed [4], suggesting that placental ACh has functions unrelated to neurotransmission, for which this choline ester is best known.

Speculations about the physiological significance of placental ACh have generally focused on control of permeability and transport across the membranes which separate the fetal and maternal blood stream [5–7]. More recently it has also been postulated that ACh was involved in the release of placental proteohormones [8].

Among substrates whose transport in the placenta has been well characterized were amino acids. Studies *in vivo* revealed higher concentrations in the fetal blood than present in the maternal blood, indicating concentrative uptake mediated by the placenta [9, 10]. Similarly, slices or fragments of human term placenta accumulated the nonmetabolizable amino acid analogue [^{14}C]-α-aminoisobutyric acid (AIB) against a concentration gradient [9, 11–15]. Studies to explore whether ACh or drugs acting on cholinergic structures have any effects on this active uptake process were incomplete [7, 8].

Although an investigation of the distribution of AIB between placental tissue and incubation medium *in vitro* can be considered only indicative of transport, we felt that the preparation would be suitable to examine the effects of a variety of cholinergic drugs on the tissue accumulation of AIB. Furthermore, it was of interest to study the human placenta for its content of tissue components that might react with ligands, which in recent years have been applied successfully to isolate and characterize cholinergic recognition sites in the central and peripheral nervous system. The existence of such a macromolecule in the placenta has been postulated and it was speculated to be nicotinic [7] although more recently incomplete experimental evidence suggested a muscarinic-type cholinergic site [16, 17].

MATERIALS AND METHODS

Tissue sources and preparations

Human placentae from either uncomplicated vaginal deliveries or elective Caesarean sections were placed in an ice chest and transported to the laboratory. Within 0.5 hr of delivery they were placed on a tray immersed in crushed ice with the maternal surface up. A transverse cut approximately one-third of the thickness of the placental disk was made to expose the fetal villous tissue. Small fragments (4–6 mm) were dissected free-hand and collected in ice-cold Krebs–Henseleit buffer (KHB) of pH 7.4 which was equilibrated with 5% CO_2 in oxygen and had the following composition (mM): $NaHCO_3$, 27.2; NaCl, 118.0; KCl, 4.8; KH_2PO_4, 1.0, $MgSO_2$, 1.2; $CaCl_2$, 2.5; and glucose, 11.1. After thorough washing, the fragments were freed from gross contami-

nation with connective tissue, major blood vessels and anchoring villi. These fragments were used directly for the amino acid uptake experiments, while a homogenate was prepared for the cholinergic ligand-binding studies. In the filtration assay a 1:10 (w/v) homogenate was made in 0.32 M sucrose. In the equilibrium dialysis and centrifugal experiments the tissue was homogenized 1:5 (w/v) in KHB containing no cholinesterase inhibitor except where noted. In all cases the homogenization of the washed fragments was achieved with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) at a setting of 10 for two 30-sec periods during which the homogenizing container was submerged into an ice bath. Fractions obtained by differential centrifugation (pellet 1 [P_1] 1,000 *g*, 10 min and supernatant [SN_1]; pellet 2 [P_2] 10,000 *g*, 45 min and supernatant 2 [SN_2]) were used in the [^{3}H]muscarone experiments, while the SN_1 was used in all other cholinergic ligand-binding studies. This fraction was used without further treatment to remove endogenous ACh except when [^{3}H]ACh binding was studied (see below). Upon differential centrifugation of a human placenta homogenate, ACh was recovered primarily in the soluble cell fraction; however, about 60 per cent of the ester was lost in the absence of cholinesterase inhibitor [17]. Protein determinations were performed with the biuret reagent using bovine serum albumin as the standard [18].

Amino acid uptake studies

Three solutions were made for these experiments. Solution 1 was KHB; solution 2 was made from solution 1 and contained inulin (methoxy [^{3}H]) 100 nCi/ml to label the extracellular water space (ECW). All drugs were dissolved in solution 2. Solution 3 contained 125 nCi/ml [^{14}C]AIB and unlabeled AIB (500 μM) dissolved in solution 2. This was diluted 1:5 in the final incubation mixture, resulting in 100 nCi/ml [^{3}H]inulin and 25 nCi/ml [^{14}C]AIB (100 μM). Six placenta fragments weighing approximately 100 mg were transferred into 25-ml Erlenmeyer flasks containing 3.0 ml of solution 2. One ml of solution 2 with or without drug was added. The room atmosphere was displaced with 5% CO_2 in oxygen, the flasks were sealed and placed in a water bath at 37° for a preincubation period lasting up to 120 min. Drugs were present during the last 30 min. After the preincubation period, 1.0 ml of solution 3 containing [^{14}C]AIB was added. The room atmosphere was again displaced and the sealed flasks were incubated at 37°. The incubation with [^{14}C]AIB lasted from 30 to 180 min. At the end of the uptake period, the fragments were subjected to a pressure-blotting procedure to selectively decrease the extracellular water content [11]. The tissue wet weight was then determined. After lyophilization the dry weight was determined in order to calculate the total tissue water (TW) content. The dried placental samples were homogenized in 1.0 ml of ice-cold 10% trichloroacetic acid (TCA) using a motor-driven Teflon pestle. The pestle and homogenizing containers were rinsed twice with 0.5 ml of ice-cold 10% TCA. The homogenate was centrifuged for 15 min in a model GLC-1 centrifuge (Sorvall Instruments) housed in a cold room at 1500 *g*. A 0.5-ml aliquot of the acid supernatant was removed for determination of radioactivity in 10.0 ml of a dioxane-based scintillation [19] fluid or xylene–Triton X-100 [20]. The samples were counted in a model 3380 liquid scintillation spectrometer equipped with a model 544 absolute activity analyzer for automatic external standardization (Packard Instruments, Downers Grove, IL). Percentages of TW (75–86 per cent), ECW as measured by [^{3}H]inulin space (40–60 per cent), and the concentrations of [^{14}C]AIB in the intracellular water space (ICW) in relation to [^{14}C]AIB in the incubation medium (ratio $C_i:C_0$) were calculated using formulas reported elsewhere and a computer program developed for those calculations [11, 21].

Binding studies with radioactive ligands

Equilibrium dialysis studies. The procedure employed was essentially that described by O'Brien *et al.* [22–26]. One ml of the lyophilized SN_1 reconstituted in KRB ($\simeq$ 35 mg protein) was placed into 0.5-in. dialysis tubing prepared according to McPhie [27]. The samples were dialyzed for 24 hr in a cold room against 100 ml KHB containing the ligand(s) under investigation. Quadruplicate 0.1-ml samples were removed from the bag and the bath. The bag contents were solubilized with Soluene-100 (Packard Instruments) prior to the addition of 10.0 ml of toluene scintillation fluid (0.5 g 2,5-diphenyloxazole (PPO)/liter, 0.2 g dimethyl 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]benzene (POPOP)/liter in toluene). The vials were then allowed to sit at 4° in darkness until chemiluminescence had subsided and stable counts could be obtained in the model 3380 liquid scintillation counter. When the binding of [^{3}H]ACh was studied, the homogenate was pretreated with 10 μM diisopropylfluorophosphate (DFP, Sigma Chemicals, Saint Louis, MO) to inhibit acetylcholinesterase (AChE). The inhibitor was present throughout tissue collection and homogenization. Excess DFP was removed from the homogenate by dialysis for 4 hr against two changes of KHB prior to the beginning of the equilibrium dialysis. This step was also expected to remove most of the ACh associated with the soluble fraction[17].

Filtration assay. This procedure was essentially that reported by Yamamura and Snyder [28]. The incubation mixture consisted of 0.05 M sodium phosphate buffer, pH 7.4, 0.1 ml of the SN_1 obtained from a 1:10 homogenate (1.4 to 1.9 mg protein) and drug dissolved in the buffer. The final volume of 2.0 ml was incubated for 60 min at 25° at which time the mixture was diluted with 3.0 ml of ice-cold buffer. The contents were immediately poured over a vacuum pump suction assembly holding Whatman GF/B filters. The trapped material was washed three times with 3.0-ml portions of cold phosphate buffer and the filters were then transferred to scintillation vials containing 10.0 ml of toluene–Triton X-100 scintillation fluid [toluene–Triton X-100, 2:1 (v/v), 4.0 g PPO/liter, 0.1 g dimethyl POPOP/liter]. The samples were gently shaken on a reciprocal shaker for 10 min and subsequently stored at room temperature overnight. The vials were then counted in a model LS-100 liquid scintillation spectrometer (Beckman Instruments, Fullerton, CA) and the results recorded as counts per min.

Centrifugal assay. This procedure was adapted from that reported by Salvaterra and Moore [29]. The 1,000 *g* supernatant was spun at 15,000 *g* for 45 min at 2°. The sediment was resuspended in KHB (1:4, w/v), and 0.1 ml (≃ 5 mg protein) was added to a final volume of 2.0 ml KHB. The samples were preincubated with the unlabeled ligand for 30 min at 25°. The labeled ligand was added and the incubation continued for 30–60 min at 25°. Immediately thereafter, the samples were centrifuged at 15,000 *g* for 45 min at 25°. The pellet surface was washed three times with 1.0 ml of cold KHB and dissolved in 0.5 to 0.75 ml of 88% formic acid. After solubilization, 10.0 ml of toluene–Triton X-100 scintillation fluid was added. The samples were allowed to stand overnight at room temperature prior to counting. When [^{125}I]-α-BT was the ligand, the final pellet was transferred into a polycarbonate-counting tube with four washes of 0.5 ml KHB. All solutions containing labeled or unlabeled α-BT also contained 1.5 mg/ml of bovine serum albumin. ^{125}I-labeled samples were counted in the presence of 0.5 to 2.0 ml buffer or water in a model 3002 gamma scintillation counter (Packard Instruments).

In parallel assays the binding of [^{126}I]-α-BT to nicotinic cholinergic recognition sites was examined with rat hemidiaphragm muscles similar to the descriptions of Colquhoun *et al.* [30]. While one hemidiaphragm was preincubated for 2 or 3 hr in 2.0 ml KHB containing 14 μM unlabeled α-BT, the contralateral muscle was kept as control. This period was followed by a 2- or 3-hr incubation in 125 nM [^{125}I]-α-BT. The tissues were then washed with KHB for 20–24 hr in a cold room with numerous bath changes. The diaphragms were lightly blotted and radioactivity was determined.

Radiochemicals. The radiochemicals employed were obtained from the following sources: Nicotine *d*-bitartrate [G-^{3}H], 250 mCi/m-mole, from Amersham, Arlington Heights, Il., α-aminoisobutyric acid [1-^{14}C], 12.25 mCi/m-mole, inulin-methoxy, [methoxy-^{3}H], 107.5 mCi/g and acetylcholine [acetyl-^{3}H]iodide, 49.5 mCi/m-mole were from New England Nuclear, Boston, MA, [^{125}I]-α-bungarotoxin and carrier were a gift from Dr. R. N. Brady, Vanderbilt University, Nashville, TN. The specific activity at the time of synthesis was 21.8 Ci/m-mole. [^{3}H]quinuclidinyl benzilate (QNB, sp. act. about 2.7 Ci/m-mole) and carrier were a gift from Dr. J. L. Bennett, Michigan State University. Nor-muscarone was a gift from Dr. P. Waser, Zurich, and was labeled with tritiated CH_3I according to the descriptions of O'Brien and Gilmour [31] and further advice obtained from Mr. B. D. Hilton, Cornell University, Ithaca, NY. The specific activity of [^{3}H]muscarone was about 83 mCi/m-mole.

RESULTS

When fragments of human term placenta were incubated with 100 μM [^{14}C]AIB, radioactivity accumulated in the intracellular compartment space against a concentration gradient in a time-dependent manner (Fig. 1). The intracellular radioactivity exceeded that in the incubation medium within 1 hr and rose to levels six times higher over a period of 3 hr. Preincubation of the placental tissue at 37° in KHB increased the concentrating ability several-fold,

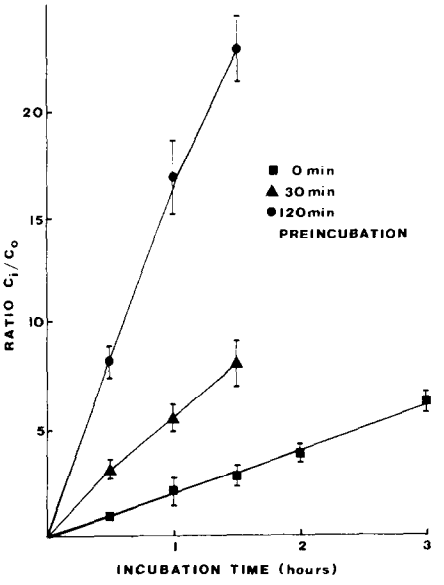

Fig. 1. Accumulation of [^{14}C]-α-aminoisobutyric acid by human term placenta fragments. Fragments (≃ 100 mg wet weight) were incubated in 100 μM [^{14}C]AIB without (■) or with 30-min (▲) and 120-min (●) preincubation at 37° for the time periods indicated. Intracellular radioactivity was calculated and expressed in relation to that of the incubation medium (ratio C_i/C_0). Each point represents the mean ± S.D. of four determinations from one placenta.

depending on the duration of the preincubation as reported by other investigators [11–15] (Fig. 1). We then proceeded to study the effects of drugs which act on muscarinic and/or nicotinic cholinergic receptors as defined in innervated tissues. The compounds were examined with unpreincubated tissue and with fragments which had been preincubated for up to 2 hr. In some experiments the drug and [^{14}C]AIB were added simultaneously, while in others the drug was allowed to act on the tissue for 30 min prior to the addition of the labeled substrate. The most consistent effect on AIB accumulation was observed when atropine sulfate (1 mM) was present. This led to a significant reduction of the concentration ratios regardless of whether the tissue had been preincubated or not (Fig. 2). Although the drug concentration was high using criteria of neuropharmacology, the inhibitory effect was peculiar for atropine because *d*-tubocurarine (Fig. 2) and decamethonium in equimolar concentrations did not affect [^{14}C]AIB accumulation. The quaternary methyl bromide derivative of atropine caused significant reductions of the amino acid uptake only in some of the specimens investigated (two out of six). It was also examined whether the SO_4^{2-} ion could have been responsible for the lower concentration ratios, but Na_2SO_4 (1 mM) was without effect. Considering the concentrations of ACh in the human placenta—which are unusually high compared to the mammalian central nervous system—and supported by the observations concerning the effects of atropine on ACh release from placenta fragments [17], it appeared conceivable that the effect of this drug was indicative of a muscarinic-type recognition site which upon reaction with the antagonist modulated amino acid uptake. Among other drugs

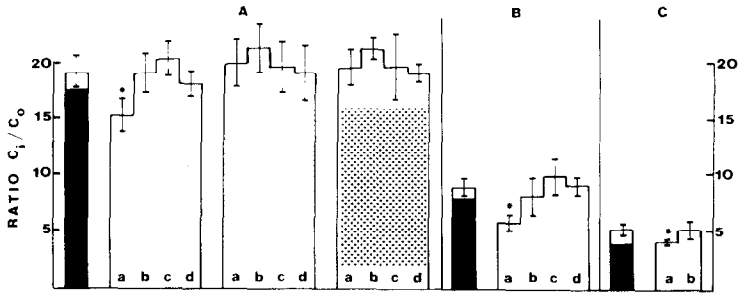

Fig. 2. Effects of cholinergic blocking drugs on the accumulation of [^{14}C]-α-aminoisobutyric acid by human term placenta fragments. Fragments (≃ 100 mg wet weight) were preincubated for 120 min (90 min + 30 min with drug, panel A), 30 min with drug (panel B) or used immediately (panel C) with 100 μM [^{14}C]AIB. Intracellular radioactivity was calculated and expressed in relation to that in the incubation medium (ratio C_i/C_0). Drug concentration of atropine sulfate (open bars), atropine methyl bromide (hatched bars) and *d*-tubocurarine chloride (dotted bars) were: a = 1 × 10^{-3} M, b = 1 × 10^{-4} M, c = 1 × 10^{-5} M and d = 1 × 10^{-6} M. Each point represents the mean ± S.D. of four determinations from one placenta. Values marked with an asterisk differ significantly ($P < 0.05$) from control values (solid bars).

the direct-acting cholinomimetics (nicotine, muscarine and ACh, concentration range 1 μM–1 mM) had no significant effects, while among the primarily indirect-acting drugs (through inhibition of AChE) the carbamate inhibitor physostigmine exerted no action while the organophosphate paraoxon reduced [^{14}C]AIB accumulation. The inhibition by paraoxon required 10 μM and was significant ($P < 0.05$) only after longer incubation times of 2 and 3 hr.

Few attempts have been made to biochemically characterize cholinergic recognition sites in non-innervated tissues. This was accomplished by means of labeled ligands [16, 32, 33] which have been successfully applied to innervated structures for the identification of cholinergic receptor macromolecules. With respect to the human placenta, there were equivocal indications from ACh release experiments [17] and the amino acid uptake results of the present study which did not allow definitive conclusions. It was unclear whether any comparable sites might exist and if present whether they would fit the criteria of muscarinic or nicotinic sites established in nervous tissue. Therefore, ligand-binding studies were initiated with [^{3}H]muscarone, a cholinergic agonist which has affinity for and intrinsic activity on both pharmacologically defined types of cholinergic sites [34–36]. Muscarone is not hydrolyzed by AChE and was successfully used to label cholinergic receptors in various innervated tissues [22–24, 31]. Equilibrium dialysis was chosen, in spite of the fact that it is time consuming, to rule out the possibility of rapid ligand dissociation from binding sites. When equilibrium dialysis against 10 nM [^{3}H]muscarone (about 2000 dis./min/ml) was performed with various fractions of placenta homogenates obtained by differential centrifugation, some indication for binding was obtained as evidenced by differences in the amount of radioactivity in the dialysis bag and in the surrounding bath (Table 1). One of the critical questions was to examine the tissue specificity of this binding. Three different sources of protein were studied: a P_2 pellet from rat brain prepared according to the protocol Farrow and O'Brien [23], a comparable fraction from rat liver and a solution of bovine serum albumin. While the amount of [^{3}H]muscarone bound to the rat brain synaptosome fraction was quite similar to the published values [23], our results differed with respect to the liver preparation because binding comparable to the binding observed with nervous tissue occurred at a bath concentration of 10 nM [^{3}H]muscarone (Table 1). Bovine serum albumin on the other hand did not bind the ligand at all. An equally important consideration was the reversibility of the binding of [^{3}H]muscarone. To evaluate this question, portions of the SN_1 fraction were first dialyzed for 24 hr against 10 nM [^{3}H]muscarone. The dialysis bags were then removed and transferred to fresh bathing media containing again 10 nM [^{3}H]muscarone, plain KHB or KHB containing various concentrations of unlabeled muscarone up to 1 μM. The binding was not reversible after dialysis for 24 hr against KHB, but in the presence of 1 μM muscarone much of the radioactivity was displaced (Table 2). The most disturbing observation, however, was the binding which occurred to the liver preparation where no cholinergic recognition sites were anticipated [23]. This result raised immediate doubts about the meaning of the [^{3}H]muscarone binding to the placental material. Therefore, the ligand-binding studies were continued with the most widely used muscarinic and nicotinic cholinergic antagonists, [^{3}H]QNB and [^{125}I]-α-BT respectively. Both drugs were available at higher specific activities than [^{3}H]muscarone, thus permitting their use in the low or below nM concentration range.

When [^{3}H]QNB was incubated with a rat brain synaptosomal fraction under conditions comparable to the ones described by Yamamura and Snyder [28], specific binding of the ligand was observed which was quite similar except that the binding was maximal at 2 nM instead of 4 nM and thus closer to the value of 1 nM described by Heilbronn [37]. In this filtration binding assay, [^{3}H]QNB (0.1 nM to 3 μM) was bound by the placenta material. However, parallel incubations in the presence of competing unlabeled QNB or atropine in a 100- to 3000-fold molar excess or other muscarinic agonists (oxotremorine, carbachol) revealed that the binding of [^{3}H]QNB remained the same regardless of the presence of other drugs known to compete with the labeled ligand in innervated tissues. Comparable results were obtained in

Table 1. Equilibrium dialysis of fractions from rat brain, rat liver, bovine serum albumin and human placenta against 10 nM [^{3}H]muscarone*

Fraction	Expt.	Radioactivity (dis./min/0.1 ml) Bag	Bath	[^{3}H]muscarone (pmoles bound/ g protein)
Rat liver P_2	1	432 ± 35	174 ± 26	387.7
	2	415 ± 32	184 ± 22	347.1
Rat brain P_2	1	391 ± 35	193 ± 42	303.9
	2	424 ± 13	170 ± 18	389.9
Bovine serum albumin 35 mg	1	162 ± 32	152 ± 11	No binding
	2	158 ± 18	150 ± 14	No binding
Placenta P_1	1	282 ± 25	245 ± 8	
	2	237 ± 26	229 ± 19	
SN_1	1	337 ± 17	235 ± 12	†
	2	301 ± 11	228 ± 9	†
P_2	1	242 ± 12	226 ± 7	
	2	282 ± 29	216 ± 8	†
SN_2	1	250 ± 23	242 ± 10	

* A crude synaptosomal fraction obtained at 10,000 *g* (P_2) from rat brain and a comparable fraction from rat liver were dialyzed (1 ml; 35 mg protein) for 24 hr at 2–4° against [^{3}H]muscarone. Fractions of human placenta were obtained by differential centrifugation as described in Methods. Numbers show mean ± S.D. of quadruplicate counting from samples dialyzed in duplicate sets and represent radioactivity (expressed in dis./min) in 0.1-ml samples from the bag and the bath.

† Experiments marked with a dagger were indicative of binding to placenta material.

equilibrium dialysis assays (Table 3) where the amount of radioactivity bound was directly related to the [^{3}H]QNB concentration, but independent of the presence of large excesses of unlabeled ligand or other cholinergic drugs (muscarinic agonists: carbachol and oxotemorine; muscarinic antagonists: QNB, atropine and scopolamine) which prevented binding of [^{3}H]QNB to synaptosomes obtained from rat brain. These observations suggested nonspecific adsorption of [^{3}H]QNB to placental constituents with extremely high capacity for binding of the ligands and low affinity which appeared to be unrelated to the high affinity expected for cholinergic recognition sites characteristic of tissues with cholinergic innervation.

Quite similar results were obtained with [^{125}I]-α-BT which was examined in the concentration range of 1–15 nM under control conditions and in the presence of a 100-fold M excess of unlabeled toxin or 1×10^{-4} M *d*-tubocurarine with the centrifugal assay. While [^{125}I]-α-BT binding to intact rat hemidiaphragms, a classical nicotinic cholinergic preparation, was blocked by 65 per cent when the tissue was preincubated with 12 μM α-BT, comparable pretreatment of protein derived from the human placenta failed to alter the amount of [^{125}I]-α-BT bound. This unspecific [^{125}I]-α-BT binding was related to the toxin concentration, and, when applied in a fixed concentration with variable amounts of placental protein, showed no signs of saturation of the nonspecific adsorbtion sites.

In order to rule out the possibility that the low temperature of the environment (2–4°) during equilibrium dialysis against [^{3}H]QNB and [^{125}I]-α-BT might have affected ligand binding, some studies were performed under room temperature conditions with

Table 2. Equilibrium dialysis of fraction SN_1 from human term placenta*

Treatment	Expt.	Radioactivity (dis./min/0.1 ml) Bag	Bath
[^{3}H]Muscarone 10 nM	1	309 ± 36	182 ± 16
	2	468 ± 54	217 ± 22
KHB	1	367 ± 46	0
	2	456 ± 32	0
Muscarone 1 μM	1	93 ± 12	0
	2	133 ± 11	0

* Dialysis was first performed against 10 nM [^{3}H]muscarone for 24 hr. Dialysis bags were then transferred to fresh solutions and equilibrium dialysis was continued for 24 hr against 100 vol. each of either [^{3}H]muscarone, Krebs-Henseleit buffer (KHB) or unlabeled muscarone in 100-fold M excess.

Table 3. Equilibrium dialysis of 1000 *g* supernatant from human placenta against [^{3}H]QNB*

Ligand	Competing ligand	Radioactivity (dis./min/0.1 ml) Bag	Bath
[^{3}H]QNB (0.4 nM)		473 ± 33	284 ± 8
	Carbachol (1 mM)	479 ± 28	288 ± 13
[^{3}H]QNB (20 nM)		20,466 ± 1,388	13,045 ± 302
	QNB (2 μM)	20,663 ± 1,222	13,175 ± 265

* Duplicate samples of supernatant 1 (see Materials and Methods for details; 1 ml; 35 mg protein) were dialyzed for 24 hr at 4° (against 100 vol. of the ligand specified). Parallel assays were run in the presence of the competing ligands in the concentrations indicated. Values show the mean ± S.D. of six 0.1-ml samples removed from bags and baths and duplicate assays and are expressed in dis./min.

a centrifugal assay. Again, all the binding which occurred to the protein pelleted after centrifugation and repeated washing was found to be uninfluenced by competing drugs. Additional experiments were done with agonists, although experience from many receptor-labeling studies has shown that only a few agonists were suitable to label their own receptors because their binding affinities were generally much lower than those of the antagonists, and in the concentrations required for binding, unspecific attachment became a major problem [38]. When used [^{3}H]ACh and [^{3}H]nicotine for the ACh-binding experiments, the tissue required pretreatment with a cholinesterase inhibitor because it is almost impossible to remove all erythrocyte-associated AChE with thorough washing or by extensive perfusion [39]. Placenta homogenate was treated with DFP as described in Materials and Methods before exposure to [^{3}H]ACh (20 nM–1 μM) in the equilibrium dialysis and centrifugation assay. No specific binding could be established with either ligand. There were no significant differences in the amounts of radioactivity bound in the presence of competing unlabeled ligand in a 1000-fold M excess with ACh, and nicotine did not bind at all (Table 4).

Table 4. Equilibrium dialysis of 1000 *g* supernatant from human placenta against cholinergic agonists*

Ligand	Competing ligand	Radioactivity (dis./min/0.1 ml) Bag	Bath
[^{3}H]ACh (20 nM)		252 ± 12	164 ± 13
	ACh (20 μM)	245 ± 13	169 ± 8
[^{3}H]ACh (200 nM)		2,430 ± 48	2,154 ± 13
	ACh (0.2 mM)	2,494 ± 63	2,191 ± 54
[^{3}H]ACh (1 μM)		12,763 ± 124	10,718 ± 368
	ACh (2 mM)	12,548 ± 261	10,529 ± 278
[^{3}H]nicotine (4 nM)		177 ± 12	163 ± 13
	Nicotine (5 μM)	165 ± 44	172 ± 21
	Carbachol (0.1 mM)	181 ± 10	158 ± 18
[^{3}H]nicotine (40 nM)		1,734 ± 21	1,808 ± 60
	Nicotine (50 μM)	1,747 ± 18	1,817 ± 41
	Carbachol (0.1 mM)	1,730 ± 45	1,836 ± 30

* Duplicate samples of supernatant 1 (see Materials and Methods for details; 1 ml; 10–15 mg protein) were dialyzed for 24 hr at 4° against 100 vol. of the ligand specified. Parallel assays were run in the presence of the competing ligands in the concentrations indicated. Values show mean ± S.D. of six 0.1-ml samples removed from bags and baths of duplicate assays and are expressed in dis./min.

DISCUSSION

Our experiments on the uptake of [^{14}C]AIB have shown that this non-metabolizable amino acid was taken up against a concentration gradient. The linearity of this process with respect to time, the concentration ratios achieved and the stimulating effect of tissue preincubation were in general agreement with the results of other investigators [9, 11–15]. The results obtained with the tissue fragments indicated that manipulation of the functional state of the cholinergic system by drugs could alter the tissue accumulation of [^{14}C]AIB. However, no unequivocal indication could be derived from the results as to whether ACh stimulated or depressed amino acid accumulation. A proposed working model considered both agonistic and antagonistic actions of ACh with respect to trophoblast permeability, depending on the amount of ACh released [7]. Based on the effects of muscarinic blocking drugs, which reduced the concentration ratios, one would be inclined to conclude that the action of ACh would enhance [^{14}C]AIB movement.

On the other hand, paraoxon, generally thought to act indirectly by inhibition of AChE (at least in the concentration applied), also reduced the concentrating ability of the tissue even though only after 2–3 hr of exposure. A similar effect has been briefly reported and was attributed to ACh accumulation as a consequence of inhibition of the hydrolytic enzyme [8]. The present results did not support that interpretation because physostigmine at 1×10^{-4} M could be expected to cause as complete an inhibition of AChE as 10 μM paraoxon did; yet physostigmine had no effects on [^{14}C]AIB uptake. Although the AChE activity of homogenates prepared from thoroughly perfused human placenta was found to be negligible [39], one cannot rule out *a priori* that the observed effects of paraoxon could not have any relationship to AChE inhibition. Homogenates prepared from tissue that had only been washed lost 60 per cent of their initial ACh content during differential centrifugation [17], which was probably due to hydrolysis caused by blood contamination.

It has been reported that organophosphorus cholinesterase inhibitors, and paraoxon in particular, have a variety of biochemical effects which appeared to be unrelated to the blockade of ACh catabolism. Among those were direct effects on cholinergic receptors [40], alterations of amino acid incorporation into newly synthesized nerve proteins [41] and inhibition of Ca^{2+} uptake by sarcoplasmic reticulum [42]. Ca^{2+} had pronounced effects on the release of ACh from placental tissue [17]. Thus, it appears conceivable that the effects of paraoxon were not due to ACh but should be considered as an action whose underlying mechanism is not clearly established.

The reduction of [^{14}C]AIB accumulation resulting from atropine, but not from *d*-tubocurarine, was caused only by concentrations which by criteria of drug action on innervated tissues must be considered excessive. The effects were present in some experiments at 0.5 mM, but no longer at 0.2 mM. Nevertheless, it would be premature to reject the idea of a muscarinic-like site in the placenta. The investigation of Olubadewo [17] have shown that atropine at 76.9 μM depressed the release of ACh from floating villous tissue, an effect which suggested a muscarinic-type recognition site. In retrospect it appears that choosing amino acid uptake as a physiological function possibly subject to modulation by the placental cholinergic system was not a suitable indicator because [^{14}C]AIB movement was little affected by drugs which in substantially lower concentration affected ACh release. However, in view of the speculations involving the cholinergic system in placental transport regulation [5–7], we felt when the experiments were designed that amino acid uptake *in vitro* might be an indicative process.

The results of the attempts to attach ligands which are of proven value in tagging cholinergic recognition sites of either the muscarinic or nicotinic type as differentiated in nervous tissue [22–26, 28–31] were altogether inconclusive. Among the agonists studied, [^{3}H]ACh and [^{3}H]muscarone bound by what appeared to be unspecific adsorption to placental proteins, while nicotine did not bind at all (see Tables 1, 2 and 4). Yet nicotine (57.9 μM) markedly stimulated the release of ACh from isolated villous tissue, while in higher concentrations (0.766 mM), it depressed ACh release [17]. Both circumstances suggest the presence of a nicotine recognition site. However, in our experiments the use of [^{3}H]nicotine concentrations as high as 10 μM still gave no indication of any binding whatsoever. The observations concerning the attachment of the classical blocking ligands for muscarinic sites ([^{3}H]QNB) and nicotinic sites ([^{125}I]-α-BT) were equally questionable. With respect to the latter ligand, our results and conclusions agreed with those of Kau *et al.* [16]. [^{125}I]-α-BT bound to placental proteins, but the binding could not be altered by the presence of an excess of unlabeled toxin or *d*-tubocurarine, making it unlikely that the binding was related to those cholinergic sites which have been described in brain tissue and in peripheral innervated structures [24, 28, 43]. Although our results on the modulation of [^{14}C]AIB uptake by atropine and more so the effects of that drug on ACh release [17] seemed to indicate a muscarinic-type cholinergic recognition site, the binding of [^{3}H]QNB must also be considered non-specific. No ligand which could reasonably be expected to interfere with [^{3}H]QNB binding [28, 43] had any effects (see Table 3). We were aware of only a few other reports which dealt with the presence of cholinergic recognition sites in tissues without innervation. One preparation was the chick amnion, a non-innervated smooth muscle, to which an irreversible labeled muscarinic antagonist bound with the high affinity and specificity expected from a muscarinic-type cholinergic receptor [32]. The other system was sea urchin sperm cells where the experimental observations suggested a nicotinic-type recognition site which was very sensitive to stimulation by nicotine and blockade by α-BT [33]. The advantage of both models cited was that there existed a clearly defined physiological function as a reference basis, smooth muscle contraction or sperm motility respectively. There is another non-innervated membrane, the rabbit allantois, which responded to cholinergic drugs by changing its permeability as evidenced by electrical potentials [44]. However, no attempts have yet been made to identify the cholinergic recognition sites of that tissue bioche-

mically with the use of the classical ligands. Recently, we became aware of an unpublished study in which the saturable, reversible, high-affinity binding of cholinergic ligands to human placenta fractions was explored. Several of the drugs employed were identical with those used in the present study. The conclusions drawn with respect to the presence of cholinergic receptors resembling the sites defined in innervated tissues were comparable to our observations [45]. A major shortcoming is that in the human placenta there is no functional characteristic known yet that is clearly measurable and intimately associated with the functional state of the ACh system. Therefore, it is extremely difficult to decide what constitutes relevant pharmacological concentrations of drugs.

The results of this study allow a variety of interpretations. One might want to conclude that there were no cholinergic recognition sites in the human placenta, but the pharmacological evidence cited above did not warrant that decision. The classical labeled antagonists which have proven their value in neurochemistry could be unsuitable for this nonnervous tissue because their chemical configuration did not conform to the structural prerequisites of the placental cholinergic recognition sites. Previous experiments in our laboratory have revealed that the placental cholinergic system is fundamentally different from that in innervated tissues [46–48]. Another interpretation could be that there were very few cholinergic sites in the placenta and that their affinity for the ligands was extremely high. Under such conditions, which appeared unlikely because of the high drug concentrations required to change functional characteristics such as amino acid uptake and ACh release, the nM levels of ligands could be too high and lead to substantial nonspecific adsorption, thus masking the small amounts specifically bound. It was impractical to use lower ligand concentrations because the available specific radioactivity was limiting. Alternatively, the postulated placental cholinergic sites could have low affinity for the ligands. Then the situation discussed by Snyder [38] for receptor-labeling studies with agonists could apply where the concentrations required also led to significant labeling of unspecific binding sites, thus making it impossible to distinguish specific binding.

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REFERENCES

1. H. C. Chang and J. H. Gaddum, *J. Physiol., Lond.* **79**, 255 (1933).
2. P. Hauptstein, *Arch. Gynaek.* **151**, 262 (1932).
3. B. V. Rama Sastry, J. Olubadewo, R. D. Harbison and D. E. Schmidt, *Biochem. Pharmac.* **25**, 425 (1976).
4. T. Fujiyama, R. Yamaguchi and K. Noda, *Tohoku J. exp. Med.* **105**, 111 (1971).
5. G. Bull, C. O. Hebb and D. Ratkovic, *Nature, Lond.*, **190**, 1202 (1961).
6. G. B. Koelle, *Neurosci. Res. Prog. Bull.* **5**, 44 (1967).
7. R. D. Harbison, J. Olubadewo, C. Dwivedi and B. V. Rama Sastry, in *Basic and Therapeutic Aspects of Perinatal Pharmacology* (Eds. P. L. Morselli, S. Garattini and F. Sereni), p. 107. Raven Press, New York (1975).
8. R. D. Harbison, M. W. Stevens, C. Dwivedi and M. Fant, *Fedn. Proc.* **35**, 611A (1976).
9. J. Dancis, W. L. Money, D. Springer and M. Levitz, *Am. J. Obstet. Gynec.* **101**, 820 (1968).
10. M. L. Reynolds and M. Young, *J. Physiol., Lond.* **214**, 583 (1971).
11. C. H. Smith, E. W. Addock, F. Teasdale, G. Meschia and F. C. Battaglia, *Am. J. Physiol.* **224**, 558 (1973).
12. C. H. Smith and R. Depper, *Pediat. Res.* **8**, 697 (1974).
13. R. K. Miller and W. O. Berndt, *Am. J. Physiol.* **227**, 1236 (1974).
14. D. J. Gusseck, P. Yuen and L. D. Longo, *Biochim. biophys. Acta* **401**, 278 (1975).
15. R. H. Enders, R. M. Judd, T. M. Donohue and C. H. Smith, *Am. J. Physiol.* **230**, 706 (1976).
16. S. T. Kau, J. Olubadewo and B. V. Rama Sastry, *Fedn. Proc.* **35**, 800A (1976).
17. J. Olubadewo, Ph.D. Thesis, Univ. Microfilms, Ann Arbor, MI, No. 76-22359 (1976).
18. A. G. Gornall, C. S. Barwin and M. M. David, *J. biol. Chem.* **177**, 751 (1949).
19. G. Bray, *Analyt. Biochem.* **1**, 279 (1960).
20. U. Fricke, *Analyt. Biochem.* **63**, 555 (1975).
21. F. Welsch, *Biochem. Pharmac.* **25**, 81 (1976).
22. R. D. O'Brien, L. P. Gilmour and M. E. Eldefrawi, *Proc. natn. Acad. Sci. U.S.A.* **65**, 438 (1970).
23. J. F. Farrow and R. D. O'Brien, *Molec. Pharmac.* **9**, 33 (1973).
24. R. D. O'Brien, M. E. Eldefrawi and A. T. Eldefrawi, *An. Rev. Pharmac.* **12**, 19 (1972).
25. J. L. Denburg, M. E. Eldefrawi and R. D. O'Brien, *Proc. natn. Acad. Sci. U.S.A.* **69**, 177 (1972).
26. L. S. Scheifer and M. E. Eldefrawi, *Neuropharmacology* **13**, 53 (1974).
27. P. McPhie, *Meth. Enzym.* **22**, 23 (1971).
28. H. Yamamura and S. H. Snyder, *Proc. natn. Acad. Sci. U.S.A.* **71**, 1725 (1974).
29. P. M. Salvaterra and W. J. Moore, *Biochem. biophys. Res. Commun.* **55**, 1311 (1973).
30. D. Colquhoun, H. P. Rang and J. M. Ritchie, *J. Physiol., Lond.* **240**, 199 (1974).
31. R. D. O'Brien and L. P. Gilmour, *Proc. natn. Acad. Sci. U.S.A.* **63**, 496 (1969).
32. A. W. Cuthbert and J. M. Young, *Br. J. Pharmac.* **49**, 498 (1973).
33. L. Nelson, *Expl Cell Res.* **101**, 221 (1976).
34. P. Waser, *Experientia* **14**, 356 (1958).
35. P. Waser, *J. Pharm. Pharmac.* **12**, 577 (1960).
36. P. Waser, *Experientia* **17**, 300 (1961).
37. E. Heilbronn, *Croat. chem. Acta* **47**, 395 (1975).
38. S. H. Snyder, *Biochem. Pharmac.* **24**, 1371 (1976).
39. G. A. Ruch, R. Davis and G. B. Koelle, *J. Neurochem.* **26**, 1189 (1976).
40. E. Bartels and D. Nachmansohn, *Archs. Biochem. Biophys.* **133**, 1 (1969).
41. F. Welsch and W-D. Dettbarn, *Comp. Biochem. Physiol.* **39B**, 393 (1971).
42. N. Binder, E. J. Landon, L. Wecker and W-D. Dettbarn, *Biochem. Pharmac.* **25**, 835 (1976).
43. H. Yamamura and S. H. Snyder, *Molec. Pharmac.* **10**, 861 (1974).
44. M. Burt, M. Brzin and J. Davies, *Anat. Rec.* **168**, 453 (1970).
45. M. K. Lewis, M. S. Thesis, Cornell Univ. Ithaca, New York (1975).
46. F. Welsch, *Biochem. Pharmac.* **25**, 1021 (1976).
47. P. Wennerberg and F. Welsch, *Res. Commun. Chem. Path. Pharmac.* **13**, 665 (1976).
48. F. Welsch, *Res. Commun. Chem. Path. Pharmac.* **15**, 457 (1976).