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UPTAKE OF ACETYLCHOLINE BY HUMAN PLACENTA FRAGMENTS AND SLICES FROM GUINEA PIG AND RAT PLACENTA

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Abstract—Fragments of human term placenta took up [^{3}H]acetylcholine (ACh) into the intracellular water fraction against a concentration gradient when incubated in a medium which contained 10 μM paraoxon as an inhibitor of cholinesterases. Intracellular concentrations rose to levels 3- to 4-fold over the concentration in the medium in a time- and temperature-dependent manner. The ACh uptake was saturable and followed the Michaelis–Menten equation. It required metabolic energy and was markedly reduced by drugs and conditions which decreased cell levels of ATP. Concentrative uptake was inhibited by alterations in the ionic environment as introduced by high K^+, Li^+, Rb^+ and Cs^+. Slices from guinea pig placenta reached intracellular concentrations which were only equal to the concentration in the incubation medium, suggesting equilibration by diffusion, while rat placenta slices concentrated ACh more slowly than human placenta and reached ratios of 2·0. These species variations further confirm known differences of the cholinergic system in placenta and stress the need for caution in extrapolating findings from the placenta of one species to another.

Human placenta has been known for a long time to contain high concentrations of an acetylcholine (ACh)-like material [1,2], although there was no indication for any innervation. The tissue levels have been reported to be as high as 459 μg ACh chloride (equivalent to 2500 nmoles)/g of fresh human placenta [1], compared with levels of about 25 nmoles/g of rat brain [3].More recently, gas-chromatographic analysis has confirmed the presence of ACh, and the amount was stated to be 73 μg/g tissue [4] (based on ACh iodide equivalent to 265 nmoles/g). The concentrations of ACh were quite variable depending on the length of pregnancy and were much higher in immature placenta [1,5,6]. Most of the ACh appeared to be in a "bound" form [4,7], although no specific subcellular site has been identified to date which was associated with ACh. Electron micrographs show a vast number of vesicles in the syncytio- and cytotrophoblast [8], and by analogy to nervous tissue it seems conceivable that some of these structures could be storage sites for "bound" ACh.

It has also been shown that human placenta was a rich source of choline acetyltransferase (choline-*O*-acetyltransferase, EC 2.3.1.6, ChAc), the enzyme catalyzing the biosynthesis of ACh [9–11], which had kinetic properties identical to the brain ChAc [12]. The tissue also contained acetylcholinesterase (acetylcholine acetylhydrolase, EC 3.1.1.7, AChE) but the activity was low [13,14] compared to the strikingly high concentrations of ACh and ChAc and was not detectable with Koelle's histochemical method using acetylthiocholine [15]. It is noteworthy that high concentrations of ChAc and ACh were unique for human placenta and that placentas of common domestic and laboratory animals contained little of these two components of the cholinergic system [2,16–18]. Neither the significance of the presence of the cholinergic system nor the meaning of the striking species differences is known.

Although the ACh metabolism of innervated tissue has been investigated in many laboratories, little progress has been made in understanding the structural organization, metabolism and function of ACh in placenta. In a desire to examine similarities and/or differences of the cholinergic system in nervous and non-nervous tissue and to obtain more insight into the placental handling of ACh, it was of interest to study the ability of this organ to take up ACh from the surrounding medium or extracellular sites into the intracellular compartment. Preliminary results of some of the findings described in this paper have been reported [19,20].

METHODS

Tissue sources. Fresh human placentas derived from uncomplicated term pregnancies by vaginal delivery or Caesarean section were obtained from a local hospital and transported to the laboratory in an ice chest. Guinea pig placentas were taken from guinea pigs when they were 50–60 days pregnant, and rat placentas were obtained from Sprague–Dawley rats close to term.

Tissue preparation. For human placenta, a method which recommended free-hand dissected villous tissue fragments was adopted [21]. This procedure allowed the required number of fragments to be cut at random from the exposed villous tissue after slicing the placenta disk at about one-third of its total thickness parallel to the decidua basalis. The tissue pieces (4- to 6-mm) were collected in ice-cold Krebs–Henseleit medium (KHM) of pH 7·4 which was continuously gassed with 5% CO_2 in oxygen. This solution will also be referred to as the "standard medium" and

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had the following composition in mM: NaCl, 118; KCl, 4·8; KH_2PO_4, 1; $CaCl_2$, 2·5; $MgSo_4$, 1·2; $NaHCO_3$, 27·2 and glucose 11·1. The solution contained 10 μM paraoxon (American Cyanamid, Princeton, N.J.) to inhibit enzymatic breakdown of ACh by AChE. The fragments were repeatedly washed with KHM until the wash fluid no longer appeared blood stained. At this point, one could easily eliminate connective and vascular tissue and thus use a more homogeneous villous material preparation than slices could provide.

In some experiments, it was necessary to modify the standard medium. A medium buffered with 50 mM Tris–HCl proved unsuitable for the experiments because it resulted in low uptake rates compared to KHM and other buffered media. However, a phosphate-buffered solution (20 mM of either Na or K secondary phosphate adjusted to pH 7·4 with 1 N HCl) was suitable and was used in those experiments where either Na^+ or K^+ concentrations were modified.

When the Na^+ concentration was modified, sucrose was used in equivalent amounts to maintain isosmolarity. When K^+ was raised above the standard medium concentration, Na^+ was reduced by a corresponding amount. In K^+-free medium, NaH_2PO_4 was used instead of KH_2PO_4. Guinea pig and rat placentas were sliced with a Stadie–Riggs microtome because their composition was much more homogeneous than that of human placenta.

Incubation medium. Because of the sponge-like nature of the placental tissue and the variability of tissue water distribution and morphological appearance from placenta to placenta, it was essential to measure the extracellular water (ECW) space in every experiment and deduct its size from any uptake values obtained. Therefore, inulin (carboxyl-^{14}C) (Mallinckrodt, St. Louis, Mo., sp. act. 1·71 mCi/g) was added to standard and modified media in a concentration of 25 nCi/ml.

The [^{14}C]inulin-containing solution was used to dissolve the carrier ACh (ACh iodide, Sigma Chemical Co., St. Louis, Mo.), to which in most of the experiments acetyl (*N*-methyl-[^{3}H])choline chloride (Amersham–Searle, Arlington Heights, Ill.; sp. act. 250 mCi/m-mole) and in some experiments acetyl-[^{3}H]-choline iodide (New England Nuclear, Boston, Mass.; sp. act. 49·5 mCi/m-mole) was added to provide 50 nCi/ml of medium.

Incubation of tissue. Quadruplicate samples, each containing six fragments taken at random from the pooled tissue and comprising 75–100 mg, were incubated in a final volume of 5 ml KHM in 25-ml Erlenmeyer flasks. The room atmosphere was displaced with a stream of 5% CO_2 in oxygen, and the flasks were immediately sealed with silicone rubber stoppers. They were then incubated in a gyratory water-bath shaker at 37° (New Brunswick Scientific, New Brunswick, N. J.). The pH at the end of the experiment was 7·45 to 7·49. In experiments with guinea pig and rat placentas slices comparable in weight to the fragments were used under identical conditions. All tissues except rat placenta were subjected to a pressure-blotting procedure which employed accurately controlled pressure to selectively reduce ECW and relatively increase intracellular water (ICW). The pressure exerted has previously been evaluated in relation to intracellular osmotic pressure and was less than 2 per cent of that value [21]. All six fragments of one experimental flask were then transferred to a tared glass vial and their wet weight was determined. After lyophilization overnight, the dry weight was determined which allowed the calculation of the tissue water (TW) content.

Measurement of radioactivity. The dry tissue was homogenized in a total volume of 2 ml of 5% trichloroacetic acid (TCA) by means of a motor-driven Teflon pestle in a glass homogenizer (Tri-R Instruments, Rockville, N. Y.). The homogenate was left at room temperature for at least 1 hr. After centrifugation, an aliquot of 0·5 ml of the supernatant was transferred into counting vials and 10 ml of a dioxane based counting solution was added [22]. Radioactivity was measured in a model 3380 liquid scintillation spectrometer equipped with a model 544 absolute activity analyzer for automatic external standardization (Packard Instruments, Downers Grove, Ill.).

Data calculation. Percentages of TW, ECW as measured by [^{14}C]inulin space, ICW and the concentrations of [^{3}H]ACh in ICW in relation to ^{3}H-ACh in the medium (ratio $C_i : C_o$) were calculated using the formulas described [21]. All required calculations were performed with a computer program prepared for a CDC central computer. Statistical evaluations were done with Student's *t*-test and by completely randomized analysis of variance, for which the confidence limit was set at 0·05. Unless otherwise indicated, all data shown represent the mean value ± one standard error of eight or more determinations from two or more placentas.

RESULTS

Stability of ACh. It was important to establish that intracellular radioactivity was present as ACh under the experimental conditions because *N*-methyl-[^{3}H]-choline generated by enzymatic or spontaneous hydrolysis might be incorporated into various metabolites and cellular components. This could lead to an uptake suggesting concentrating ability of the tissue but actually representing metabolic conversion [23]. Three methods were used to establish the stability of ACh and the presence of radioactivity as ACh in ICW under the experimental conditions:

(1) Homogenate of thoroughly washed placenta fragments was incubated with [1-^{14}C]ACh and [1-^{14}C]-acetyl-β-methylcholine in the presence of various concentrations of paraoxon ranging from 0·1 to 10 μM. The hydrolysis of these choline esters was examined with a radiometric method [24] and was found to be reliably and completely inhibited by 5 and 10 μM paraoxon.

(2) After incubation in [^{14}C]inulin-free standard medium for 2 and 4 hr, tissue fragments were lyophilized as usual and extracted with acetonitrile containing 2% TCA [25]. The purified aqueous extract was lyophilized and subsequently dissolved in a small volume and quantitatively spotted for high voltage paper electrophoresis. Authentic choline and ACh (30 μg of their iodide salts) either alone or in combination with their isotopic variants (acetyl-[^{3}H]choline and methyl-[^{14}C]choline, which were also used alone) were

separated on adjacent tracks of the same electropherogram (CAMAG HVE System, Camag, New Berlin, Wis.; Whatman 3 MM paper 19 × 40 cm, 100 V/cm for 25 min in buffer pH 2·0 [26]). The paper was exposed to iodine vapor, staining the 30 μg containing authentic standards, which appeared well separated (2·5 to 3-cm distance between upper staining limit of authentic ACh and lower staining extension of choline). Subsequently 1-cm paper sections deriving from the strips with tissue extracts and from the authentic radiolabeled reference compounds were examined for radioactivity by liquid scintillation spectrometry. Authentic radiolabeled [^{14}C]choline overlapped by about 3–5 per cent into the [^{3}H]ACh despite the good separation suggested by iodine vapor staining. This lack of complete separation has been described repeatedly by many laboratories. On the paper strips derived from tissue extracts of placenta fragments incubated with methyl-[^{3}H]ACh under standard conditions, radioactivity was found only in the region which corresponded to the electrophoretic mobility of authentic acetyl-[^{3}H]ACh.

(3) In some experiments, fragments were incubated with acetyl-[^{3}H]choline and analyzed by the usual procedure. The uptake ratios obtained were somewhat lower (20 per cent) than those obtained with methyl-[^{3}H]choline, but they confirmed that concentrative uptake of radiolabeled ACh was occurring regardless of which part of the molecule was labeled.

Time course of uptake of [^{3}H]ACh into ICW. When 5×10^{-5} M ACh was present in KHM, radioactivity was taken up into ICW in a time-dependent manner. This concentration of ACh was lower than the tissue concentration. If the value of 250 nmoles ACh/g of fresh tissue [4] was used to estimate cellular ACh concentrations, a value of 250 μM resulted. Such an assumption is probably incorrect because it would be based on an even distribution of ACh. The results on the size of the ICW compartment, in agreement with other values reported [21,27], indicated 20–45 per cent of the total tissue wet weight. If ACh was restricted to this space only, ICW concentrations would be in the range of 550–1250 μM because most of the ester seems to be in an intracellular "bound" form [4,7]. Therefore, uptake at 5×10^{-5} M occurred against a concentration gradient, and this concentration was used routinely in subsequent studies. In typical experiments, the ratio of the concentration of radioactivity per ml ICW (C_i) over the concentration per ml KHM (C_o) at 37° reached 1·2 after 30 min, 1·9 after 60 min, 2·4 at 120 min and 3·7 at 240 min and did not increase any further thereafter (Fig. 1). Movement of [^{3}H]ACh into the ICW compartment at 0° was markedly lower; it reached a ratio of 0·7 after 240 min and remained at this level (Fig. 1). In studies of choline uptake into synaptosomes, incubation at 0° has been used to estimate the contribution of non-saturable diffusion to the total uptake [28]. Therefore, the ratios obtained at 37° were corrected by deducting the values obtained at 0° in all future experiments (net concentration ratios). Other investigators who studied ACh uptake into brain slices used the concentration ratios obtained at high outside ACh levels to correct for diffusion [29]. There was no significant difference between the two methods in our experiments once the ACh concentration in KHM was at least 100 mM.

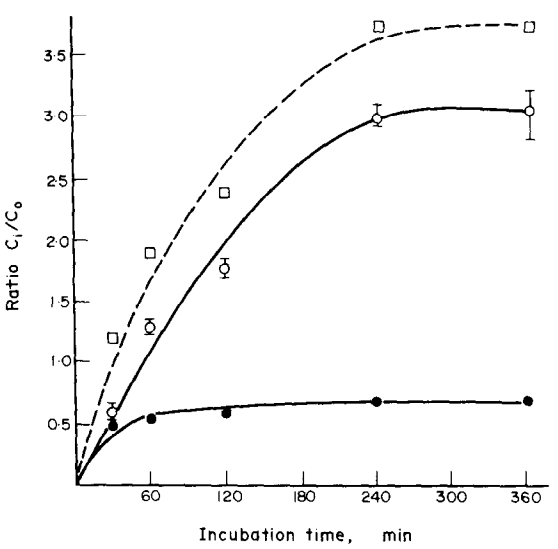

Fig. 1. Time course of uptake of [^{3}H]ACh into human placenta fragments. Fragments were incubated at 0–2° (●——●) and 37° (□- - - -□) in standard medium containing 50 μM [^{3}H]ACh for the time periods indicated. Ordinate: corrected concentration ratios (dis./min/ml of intracellular water: dis./min/ml of medium, [^{14}C]inulin extracellular water space deducted). The third curve (○——○) shows the net concentration ratios calculated by deducting the ratios obtained at 0° from those obtained at 37

Some recent experiments in embryonic and developing tissues have resulted in a striking enhancement of the uptake of several amino acids by simply preincubating the tissue for several hours in a balanced medium [21,30]. It was of interest to examine whether this phenomenon would also occur when ACh was to be transported. However, although stimulation of amino acid uptake by preincubation in KHM to levels comparable to those reported could be easily obtained, no such effect on ACh uptake was observed.

Effects of ACh concentration. The accumulation of [^{3}H]ACh by fragments of human placenta occurred over a wide range of concentrations. The concentration ratios obtained at either 0° or 37° were almost identical for a given temperature regardless of whether ACh in KHM was 50 nM or 0·5 mM. Since the sp. act. of [^{3}H]ACh increased proportionally as the concentration of carrier ACh decreased while the amount of [^{3}H]ACh per unit volume was kept constant at all concentrations (50 nCi/ml of KHM), approximately equal ratios meant that ever increasing amounts of ACh were moving into ICW as the ACh concentration in KHM was raised. There was indeed linear uptake over a wide concentration range but eventually the curve fell off when ACh was higher than 10 mM (Fig. 2A), revealing a typical slope indicative of a saturable and a non-saturable component. If the data obtained after 120 min of incubation during the linear phase of [^{3}H]ACh accumulation were plotted according to Schuberth and Sundvall [29], a straight line resulted when the reciprocal of the ratio of the active uptake component (R_a) times outside concentration (C_{ext}) was plotted against the reciprocal of C_{ext} (Fig. 2B). This indicated that the ACh uptake followed kinetics predicted by the

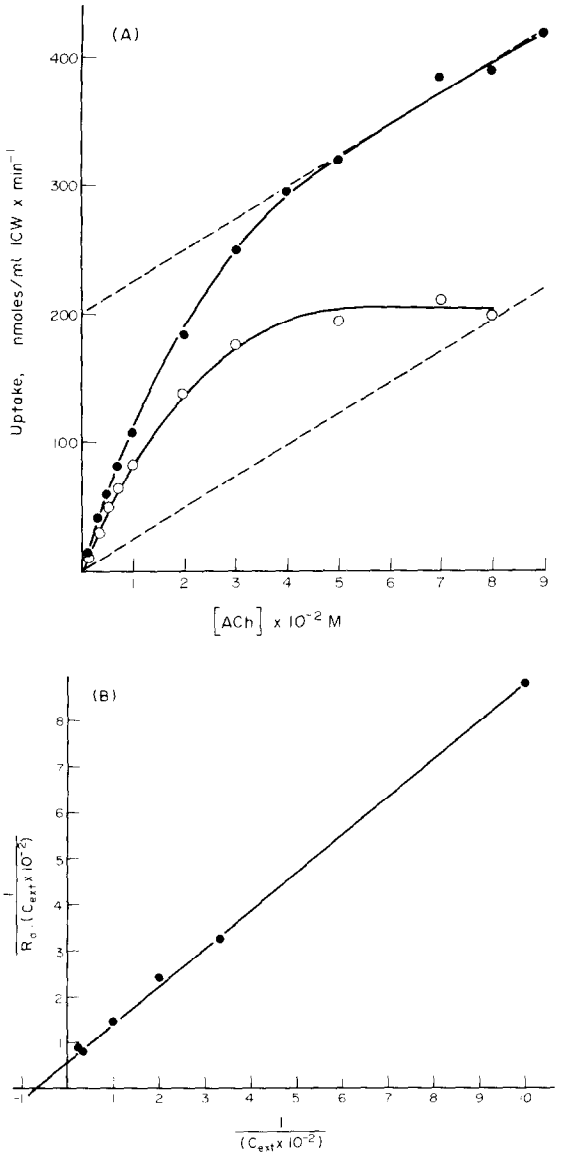

Fig. 2. Effects of ACh concentration on uptake of [^{3}H]ACh into human placenta fragments. Fragments were incubated with [^{3}H]ACh concentrations ranging from 1 to 80 mM. (A) Ordinate is the uptake expressed in nmoles/ml of intracellular water × min^{-1} based on specific activity of [^{3}H]-ACh and calculated from ratios corrected for extracellular water. Dashed line (– – –) indicates contribution of non-saturable diffusion estimated from slope resulting from ACh concentrations higher than 40 mM and by extending the line to intersect with the ordinate. Total uptake (●— —●) and saturable uptake (○ – – –○) corrected for the contribution of non-saturable diffusion by deduction of values depicted in lower dashed line. (B) Same data analyzed according to Schuberth and Sundvall [29] by plotting the reciprocal of the active component of the concentration ratio (R_a) multiplied by the concentration of ACh in the incubation medium (C_{ext}) against the reciprocal of C_{ext}. The straight line indicated compliance with the Michaelis–Menten equation.

Michaelis–Menten equation with an apparent K_m (× 10^{-2} M) of 1·49 ± 0·35 (mean ± S. D., n = 3) and V_{max} of 280 ± 68 nmoles/ml of ICW × min^{-1}.

Effects of temperature. The concentrative uptake of

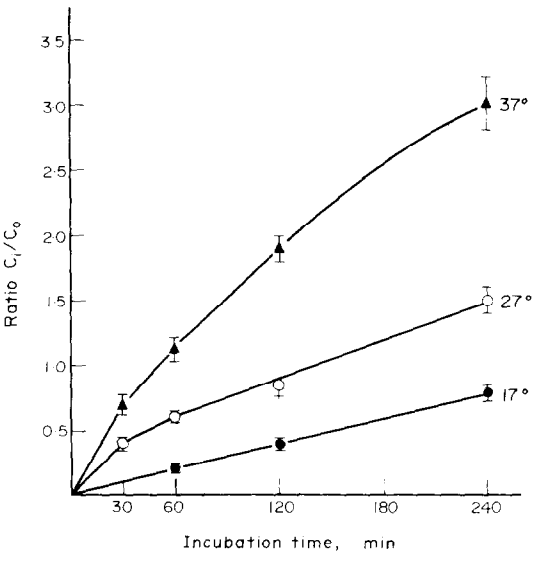

Fig. 3. Effects of temperature variation on uptake of [^{3}H]-ACh into human placenta fragments. Fragments were incubated with 50 μM [^{3}H]ACh at 17° (●– – –●), 27° (○ – – –○) and 37° (▲– – –▲) for the time periods indicated. Ordinate: net concentration ratios (see Fig. 1).

ACh into ICW showed a marked temperature dependence. When the incubation temperature was raised from 17 to 27° and further to 37°, the amount of [^{3}H]ACh taken up just about doubled at each time interval examined (Fig. 3). At 0° the ICW radioactivity of ACh did not equilibrate with the KHM radioactivity. The ratio reached values of 0·5 to 0·7 dependent on the concentration of ACh and remained at this level during incubations of up to 6-hr duration (Fig. 1).

Effects of oxygen deprivation. The uptake of ACh against a concentration gradient was markedly reduced by lack of oxygen (Fig. 4). Strict anaerobic conditions had to be maintained, however, in order

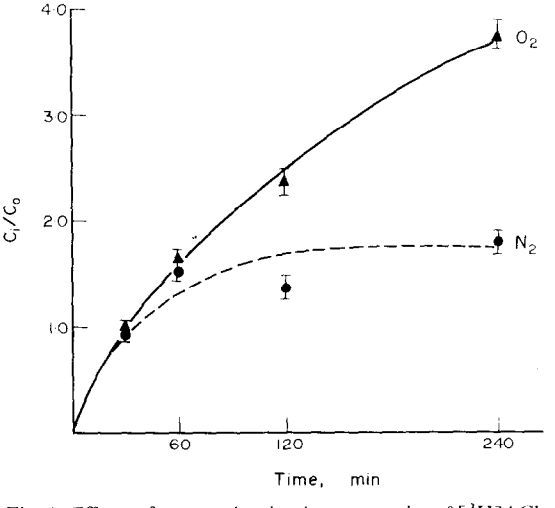

Fig. 4. Effects of oxygen deprivation on uptake of [^{3}H]ACh into human placenta fragments. Fragments were incubated with 50 μM ^{3}H-ACh in standard medium gassed with 5% CO_2 in oxygen (▲– – –▲) or 5% CO_2 in nitrogen (●– – –●). Ordinate: net concentration ratios (see Fig. 1).

to observe this effect. If the fragments were collected in KHM saturated with 5% CO_2 in oxygen and were subsequently transferred to Erlenmeyer flasks, which were only charged with N_2 at the beginning of the incubation period, no reduction of the ratio C_i/C_o was observed. Instead, higher values than in oxygenated controls resulted on several occasions. On the other hand, if the fragments were collected in KHM gassed with 5% CO_2 in nitrogen and always kept in solutions which were also saturated with this gas mixture, the concentration ratios reached about the same values as the O_2 controls after 30 and 60 min, but then the uptake ceased. The ICW concentration did not increase any further after incubation periods of 120 and 240 min, at which times it reached 58 and 48 per cent respectively, of the CO_2/oxygen-gassed flasks (Fig. 4).

Effects of glucose. Complete omission of glucose had no effect on the concentration ratios obtained at any of the time intervals examined (Fig. 5). However, if glucose was replaced by an equimolar concentration of 2-deoxyglucose, ACh uptake began to fall off after 120 min and was reduced to 50 per cent of glucose-containing controls after 240 min.

Effects of drugs which disrupt electron transport and/or uncouple oxidative phosphorylation. The presence of sodium cyanide (NaCN, 5×10^{-3} M and 1×10^{-3} M) resulted in a profound decrease of the uptake of [^{3}H]ACh as reflected by concentration ratios which were much lower after 4 hr than those of controls (Table 1). The drugs were added 15 min prior to ACh. An examination of the time course of the uptake inhibition revealed that NaCN produced a 40 per cent reduction within the first 30 min and that the inhibition progressively increased (values not shown). 2,4-Dinitrophenol (DNP, 1×10^{-3} M, 1×10^{-4} M, 2×10^{-5} M) had no effect after 30 min, but it reduced the concentration ratios at every time interval thereafter. The degree of inhibition by DNP was related to the concentration of this drug (Table 1). Inhibition of uptake was still significant at 2×10^{-5} M DNP.

Table 1. Effects of NaCN and 2,4-dinitrophenol on ACh uptake by human placenta fragments*

Drug	Molar concentration 5×10^{-3}	1×10^{-3}	1×10^{-4}	2×10^{-5}
NaCN	29	32		
DNP		22	37	75

* Placenta fragments were incubated in standard medium in the presence of NaCN and DNP in the concentrations specified. Values are expressed as per cent of the net concentration ratios (dis./min/ml of intracellular water: dis./min/ml of medium, corrected for 0 diffusion and extracellular water) obtained from control fragments after 4 hr of incubation.

Table 2. Effects of ouabain on ACh uptake by human placenta fragments*

Ouabain concn (M)	Incubation time (min) 30	60	120	240
1×10^{-3}				45
1×10^{-4}	36	37	47	47
$2{\cdot}5 \times 10^{-5}$			61	52

* Placenta fragments were incubated in standard medium in the presence of ouabain for the time period and in the concentration indicated. Values are expressed as per cent of the net concentration ratios (dis./min/ml of intracellular water: dis./min/ml of medium, corrected for diffusion and extracellular water) obtained from control fragments.

Effects of ouabain on ACh uptake. Ouabain in concentrations of 1 mM, 0·1 mM and 25 μM reduced the concentration ratios obtained to 35–60 per cent of the control values, and the drug's effects were well established within 30 min (Table 2). This observation implied that Mg^{2+}-dependent Na^+ and K^+ activated ouabain-sensitive adenosine triphosphatase (ATP phosphohydrolase, EC 3.6.1.3, ATPase), which has

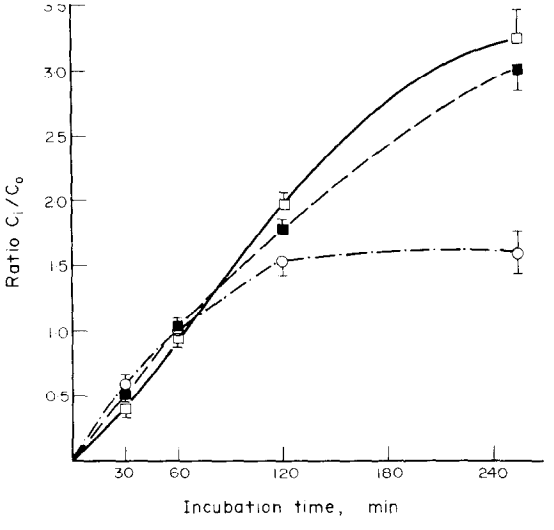

Fig. 5. Effects of 2-deoxyglucose on uptake of [^{3}H]ACh into human placenta fragments. Fragments were incubated in standard medium containing glucose (□——□), no glucose (■– – –■) or equimolar amounts of 2-deoxyglucose (○···○). Ordinate: net concentration ratios (see Fig. 1).

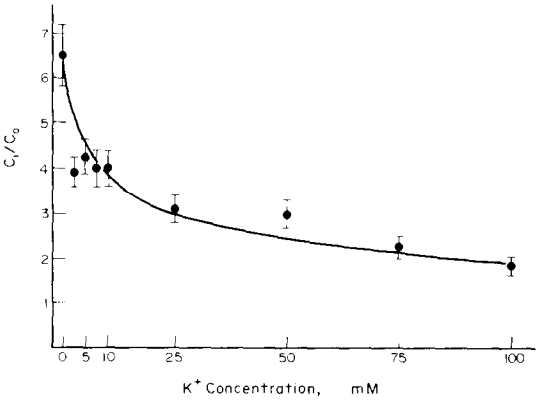

Fig. 6. Effects of variation of K^+ concentration on uptake of [^{3}H]ACh into human placenta fragments. Fragments were incubated in modified medium containing the KCl concentrations indicated. Ordinate: net concentration ratios after 4 hr (see Fig. 1).

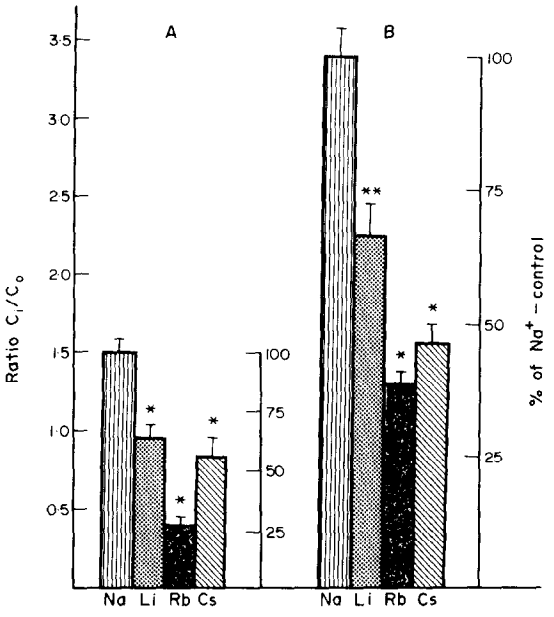

Fig. 7. Effects of Li^+, Rb^+ and Cs^+ ions on uptake of [^{3}H]ACh into human placenta fragments. Fragments were incubated in modified medium in which NaCl was replaced by equimolar concentrations of the ion under investigation. Left ordinate: net concentration ratios (see Fig. 1). Right ordinate: net concentration ratio expressed in terms of per cent of NaCl controls. (A) 2-hr incubation. (B) 4-hr incubation. Key: * $P < 0.001$; and ** $P < 0.005$.

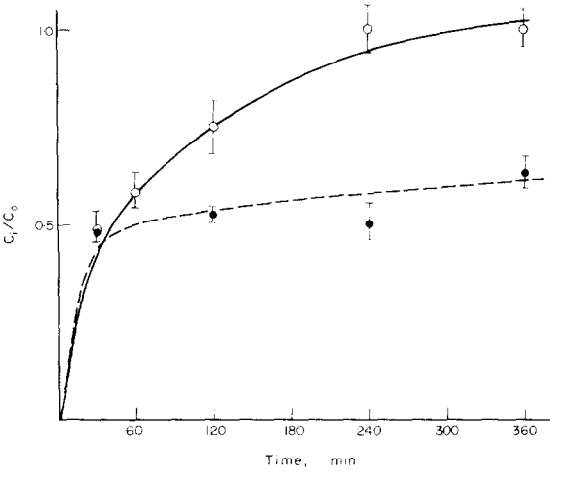

Fig. 8. Time course of uptake of [^{3}H]ACh into slices from guinea pig placenta. Slices were incubated with 0·5 μM ^{3}H-ACh in standard medium at 0–2° (●——●) and 37° (○——○). Ordinate: concentration ratio corrected for ^{14}C-inulin extracellular space.

been identified in human term placenta [31], exerted some influence on the ACh uptake.

Effects of alterations in the ionic environment. Gradual or complete removal of Na^+ and replacement by an isosmotic amount of sucrose had no significant effect on concentrative uptake of ACh. Variation of the calcium or magnesium concentration between 0 and 8 mM likewise was without effect on the concentration ratios. Maximal accumulation of [^{3}H]ACh in ICW occurred in potassium-free medium (Fig. 6). When KCl was gradually raised while NaCl was simultaneously reduced by an equimolar amount to maintain isosmolarity, similar concentration ratios were obtained with 2·5, 5, 7·5 and 10 mM KCl in the modified KHM. However, once KCl reached 25 mM a significant reduction of the uptake occurred, which was even more obvious when the K^+ concentration was increased to 100 mM. Several other members of the alkali-metal group affected ACh uptake (Fig. 7A and 7B). When the NaCl in KHM was completely replaced by an equimolar concentration of lithium (LiCl), which in some respects closely resembles Na^+ [32], the concentrative uptake of [^{3}H]ACh was significantly depressed. This was witnessed by concentration ratios which were reduced by 37 per cent after 2 hr and 34 per cent after 4 hr. Rubidium (Rb^+) closely resembles K^+ in some of its biologic characteristics [32] and caused effects quite similar to those described for high concentrations of K^+. Replacement of NaCl by 118 mM RbCl resulted in a drastic loss of ACh-concentrating ability. After 2 hr, uptake was reduced by 73 per cent compared to control values and by 62 per cent after 4 hr. The presence of an equimolar concentration of cesium (CsCl) in KHM instead of NaCl led to a reduction of the concentration ratios which was intermediate in its severity between Li^+ and Rb^+. The inhibition amounted to 44 per cent after 2 hr and 54 per cent after 4 hr.

Species variability of ACh uptake by placenta. Since common laboratory and domestic animals contained ACh in much lower concentrations [2,5,16–18], it was of interest to examine the ability of two other hemochorial placentas from guinea pig and rat to take up ACh against a concentration gradient. Because of the much lower endogenous ACh levels, the concentrations of ACh in the incubation medium were reduced to 50 and 500 nM. No concentrative uptake was detectable in guinea pig placentas (Fig. 8). Slices incubated at 0–2° reached ECW corrected concentration ratios of 0·5 to 0·6 after 120 min, which

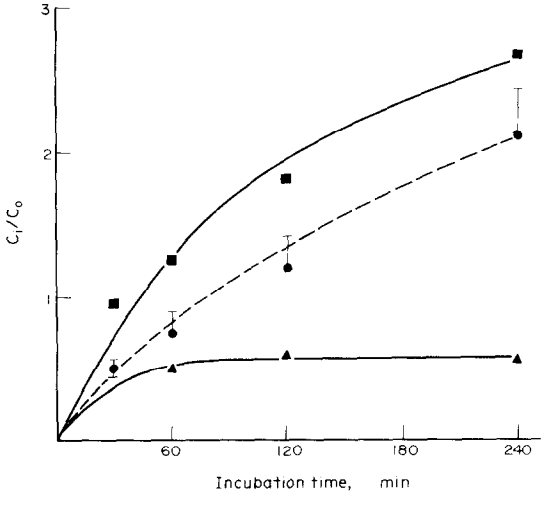

Fig. 9. Time course of uptake of [^{3}H]ACh into slices from rat placenta. Slices were incubated at 0–2° (▲——▲) and 37° (■——■) with 0·5 μM ^{3}H-ACh in standard medium for the time periods indicated. Net concentration ratios (●—— ●). For further details see Fig. 1.

remained constant thereafter. At 37° slow equilibration of the concentrations in ICW and in the incubation medium occurred. The concentration ratio reached 1·0 within about 180 min and did not increase any further. Rat placenta slices, on the other hand, showed a modest concentrating ability compared to human placenta (Fig. 9). At 0–2° the ECW corrected concentration ratio reached 0·5 to 0·6, which was very similar to both other species. However, at 37° uptake of [^{3}H]ACh occurred which led to a net concentration ratio of 2·0 within 240 min.

DISCUSSION

The observations concerning the uptake of ACh in three different species revealed striking variations between the placentas of man and rat on the one hand and guinea pig on the other hand. The results obtained with human term placenta suggested that this tissue had the capability to take up ACh into ICW against a concentration gradient. Placentas from guinea pigs lacked the ability to accumulate [^{3}H]ACh intracellularly beyond the point of equilibration with the surrounding medium, while rat placenta slices showed a modest uptake only.

The uptake by human placentas had at least two components. One was the movement of ACh, which occurred at 0°, reached a concentration ratio of about 0·6 to 0·7 and was attributed to diffusion only. The other component exhibited a marked temperature dependence with a Q_{10} of 2·0 and reached a diffusion and inulin space-corrected intracellular net concentration higher than that in the medium within 30–60 min.

As a third potential component, the process of exchange diffusion against an existing concentration gradient must be considered. By this process radioactive ACh would exchange with ACh from the tissue. Radio labeled ACh would thus appear intracellularly without really increasing the total ICW ACh concentration, while nonlabeled endogenous ACh diffused out of the cell. This event would be the more significant the lower the concentration of ACh in the medium was, i.e. when the sp. act. of [^{3}H]ACh was high. Under those circumstances the exchange diffusion of a small quantity of ACh would lead to an exchange influx of an equally small amount of radiolabeled ACh. Because of its high sp. act. this [^{3}H]ACh movement would bring about the transfer of a relatively large amount of radioactivity into ICW. Thus, radioactivity in ICW could become higher than in the surrounding medium, suggesting active transport against a concentration gradient. The contribution of exchange diffusion to the total ACh uptake in brain slices at concentrations of 10 or 80 nM was assumed to be significant and resulted in tissue : medium ratios of 15–20 [33]. The likelihood of a marked contribution of ACh diffusion in human placenta seemed to be ruled out by the observation that there was not much difference in the concentration ratios obtained when ACh in the medium was varied between 50 nM and 500 μM. Also, concentrative uptake of 50 nM [^{3}H]ACh was observed in human and rat placentas while the concentration ratios in guinea pig placentas consistently remained at or below 1·0, indicating slow equilibration by diffusion. If exchange diffusion was an important factor in explaining what appeared to be a time- and temperature-dependent concentrative [^{3}H]ACh uptake into ICW, it would also be expected to occur in placentas of other species when the outside concentration was as low as 50 nM. Morphological differences among the placentas of the three species examined, which would allow exchange diffusion of the charged ACh molecule only in human and rat placentas, seemed unlikely because all species examined have placentas of the hemochorial type and contain similar cellular elements [8].

The uptake of [^{3}H]ACh by human placenta had some properties which were similar to the fate of exogenous ACh in nervous tissue under similar experimental conditions [29,33–35]. The concentrative uptake showed characteristics which are generally attributed to active transport and which were reflected by intracellular concentrations three to four times higher than those in the medium and by the energy requirements to achieve such concentrations. Continuous functioning of the concentrative ability for several hours was clearly dependent on cell metabolism and synthesis of ATP. This conclusion was vindicated by the following experimental effects on ACh transport. It was decreased by the presence of NaCN, a nonspecific inhibitor of electron transfer through heme proteins to oxygen, which is a powerful depressor of cell ATP [36]. Concentrative uptake was markedly depressed by DNP, which uncouples oxidative phosphorylation by inhibition of electron transport in mitochondria and thus depletes cell ATP [37]. When K^+ concentrations of modified media were high, which has been observed to cause a fall of the intracellular levels of ATP [38], uptake was markedly reduced. Similar effects were observed during ACh and carbachol uptake experiments in mouse and rat brain cortex slices [29,33,35,39].

Although complete omission of glucose did not have any significant effect on the concentration ratios obtained, the presence of an equimolar amount of 2-deoxyglucose led to a marked reduction of uptake. It appeared as if the placenta used a stored source of metabolic energy, such as glycogen, which was broken down and supplied the glucose to maintain ATP levels despite lack of exogenous glucose in the medium. Therefore, ACh accumulation was not impaired. However, if 2-deoxyglucose was present, this compound probably competed with the endogenously created glucose for phosphorylation by hexokinase, thus leading to a depletion of ATP. This, in turn, brought about a decrease of the ACh uptake as witnessed by the reduced concentration ratios. The delay of about 2 hr until the onset of a decline in ACh accumulation in the 2-deoxyglucose-containing samples may have its explanation in the extraordinary ability of the placenta to function under conditions which in other tissues rapidly reduce energy production, such as DNP or hypoxia. It has been shown that human term placenta contained large stores of glycogen which could be mobilized by glycogen phosphorylase during experimental hypoxia or other treatments which decreased intracellular ATP levels [40,41]. The results concerning ACh uptake suggested that endogenous glycogen did supply glucose as a source of energy for active transport. Under aerobic

conditions enough glucose was generated in glucose-free medium to leave ACh transport unimpaired throughout the period of observation. Glycogen phosphorylase probably also provided glucose under strictly anaerobic conditions, thus still allowing concentration ratios greater than unity. This interpretation of our data was supported by a recent report which described how human term placenta took up [^{14}C]α-aminoisobutyric acid actively while incubated anaerobically or in the presence of DNP. It was shown that the required energy derived from glycogen mobilization [37].

The highest concentration ratios were obtained in K^+-free medium. This observation agreed with the results of Polak [35] obtained during experiments on ACh uptake by rat brain slices, but it did not match the findings of Liang and Quastel [33]. When brain slices were incubated in a medium containing high concentrations of K^+ they were found to lose their ability to accumulate carbachol. This was believed to be due to the elimination of the resting membrane potential resulting from depolarization by high K^+, thus abolishing the electrochemical gradient which provided the driving force for the passive accumulation of the cationic carbachol [39]. The cationic nature of ACh did not seem to be responsible for its accumulation by human placenta, because the presence of an equimolar concentration or a 10-fold molar excess of the quaternary compound tetramethylammonium did not alter the concentration ratios.

The inhibitory effect of ouabain is probably not related to the inhibition of Na^+, K^+-ATPase by this drug, because in that case one would expect removal of Na^+ to influence ACh accumulation. It appears more likely that the decrease is due to an inhibition of glycolysis, which seems to be an important pathway for the generation of metabolic energy and ATP in human placenta. In human erythrocytes, which depend almost exclusively on glycolysis as an energy source, ouabain decreases adenosine diphosphate (ADP) formation by inhibition of Na^+, K^+-ATPase. The ADP is rate limiting for phosphoglycerate kinase and subsequent ATP production. This enzyme has been shown to be an important control point linking cation transport and glycolysis [42].

The functional significance of the concentrative uptake of ACh in placenta is as unexplained as that observed in brain slices, where ACh appeared to be transported by the same uptake system as choline. Either compound competitively inhibited the uptake of the other [43]. It was speculated that the active uptake of ACh by brain slices in the presence of a cholinesterase inhibitor may indicate the presence of an emergency system with the ability to free the cholinergic receptor from ACh when AChE is inhibited [43]. The radioactive ACh taken up by preparations *in vitro* was diffusely distributed all over the nerve cells [35].

The subcellular localization of the large concentrations of ACh in human placenta is not well characterized, and the evidence supporting its "bound" nature only indirect [4]. In view of the low AChE activity of placenta homogenates [13,14] and the lack of histochemically demonstrable hydrolysis of acetylthiocholine in the placental trophoblast cells [15], it is tempting to suggest that ACh uptake demonstrated in the placenta fragments might play a role in the transport of previously released ACh back into the ICW compartment or the suspected tissue storage site which encloses "bound" ACh. Attempts in our laboratory to isolate subcellular organelles containing "bound" ACh by differential centrifugation have not been successful so far. The acid extraction used in the present analyses would reveal the total radioactive ACh in ICW. Based on our current knowledge of "bound" ACh in human placenta it cannot be decided whether the cell plasma membrane or a storage vesicle membrane—if it existed— was the rate-limiting step in the observed ACh uptake.

The species differences in the ability to take up ACh reflected further the known differences of the placental ACh system. Guinea pig* and rat placenta† contained little ACh compared to the striking concentrations in man. Although the significance of these differences is still unknown, the current results underline the care necessary when extrapolating experimental findings which involve the cholinergic system of the placenta.

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