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CHARACTERIZATION OF CHOLINE TRANSPORT AT MATERNAL AND FETAL INTERFACES OF THE PERFUSED GUINEA-PIG PLACENTA

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SUMMARY

1. Unidirectional influx and efflux of choline into the syncytiotrophoblast were investigated from both maternal and fetal circulations of the perfused guinea-pig placenta by using a single-circulation paired-tracer (extracellular reference and test substrate) dilution technique.

2. Cellular uptake of [^{3}H]choline at 0·05 mM was (mean percentage ± S.E. of mean, $n = 14$ placentae) 51 ± 2 and 49 ± 2, on maternal and fetal sides, respectively. Kinetics of unidirectional influx (0·05–4·0 mM-choline) indicated the existence of saturable and non-saturable components on both sides: on maternal and fetal interfaces the K_m (mM) values were respectively, 0·12 and 0·13, the V_{max} (μmol min^{-1} g^{-1}) values, 0·08 and 0·07 and the apparent linear transfer constants (min^{-1} g^{-1}) 0·11 and 0·12.

3. Efflux of [^{3}H]choline from the placenta back into the ipsilateral circulation (backflux) was generally fast (20–60 % in 5–6 min) and asymmetric with the fetal:maternal ratio usually above unity. Transplacental specific choline transfer in the dually perfused placenta, when observed, was small (< 10 % of the injected dose) following tracer injections in either direction based on the 5–6 min collection of the contralateral circulation (at 0·05 mM-choline). Placental retention of [^{3}H]choline at the end of the 5–6 min period was about 25 % of the injected dose when the tracers were injected from either circulation.

4. Analogues of choline such as hemicholinium-3, thiamine, ethanolamine and *N*,*N*-dimethylethanolamine inhibited choline unidirectional influx, whereas betaine and acetate had no effect.

5. The absence of the normal sodium gradient (perfusate sodium was replaced by Tris or by lithium) did not inhibit choline transport. The metabolic inhibitors dinitrophenol (1·0 mM) and potassium cyanide (1·0 mM) were essentially ineffective (up to 40 min perfusion). The sulphydryl reagent *N*-ethylmaleimide did not appear to inhibit the influx, in comparison with its effect on [^{3}H]choline backflux which was greatly accelerated, resulting in a dramatic reduction in placental net uptake of the label.

6. Our findings show that choline transport into the placenta is a rapid carrier-mediated process occurring at both maternal and fetal sides of the trophoblast, at physiological blood concentrations. This cellular uptake is possibly related to the synthesis of acetycholine, which is known to occur in human placental tissue. Specific

transplacental transfer of choline was a very slow process under the conditions of our experiments and this contrasted with the observed fast and high uptake into the trophoblast.

INTRODUCTION

Choline in plasma is an essential source of choline for the brain and other tissues, and the importance of choline as a nutrient in adult and fetal life has been reviewed by Zeisel (1981). In nerves, choline uptake is mainly related to cellular acetylcholine (ACh) synthesis and has been studied in the squid giant axon (Hodgkin & Martin, 1965) and brain synaptosomes (Marchbanks, 1968). In the erythrocyte membrane a choline transport system of unknown function has been very well characterized (see review by Martin, 1977; Deves & Krupka, 1981). In epithelia such as the intestinal mucosa (see review by Rose, 1980) choline is also transported by a specific carrier-mediated process, however, here transepithelial (lumen-to-blood) transfer of choline, rather than cellular utilization for metabolic processes, appears to be dominant.

The components of an ACh synthesizing system in the human placenta (which lacks the capacity to synthesize choline) have been clearly defined even though there is no evidence for the presence of nerves of any type (Comline, 1946; see review by Sastry & Sadavongvivad, 1979). The functions of this active ACh synthesis in human placenta, are still unknown, but it has been proposed that the placental cholinergic system may play a role in the regulation of fetal growth and, in particular, the regulation of amino acid transport (Rowell & Sastry, 1981; see Yudilevich & Sweiry, 1985). Plasma choline concentration in human, rat and rabbit neonates are 4–7-fold that in the adult (Zeisel, Epstein & Wurtman, 1980). The human placental choline concentration is high, 1·25 mM (Zeisel *et al.* 1980; Welsch, 1976*a*) compared to maternal plasma, 8–16 μM (Bligh, 1952). Accumulation of choline against such a concentration gradient, and its incorporation into ACh has been demonstrated in human placental fragments (Welsch, 1976*a*, 1978).

The present work was aimed at characterizing choline transport into the trophoblast via a carrier system which could be present on either the maternal and/or fetal sides, and also to study the directionality of transplacental transfer from either circulation. Such investigations required the intact perfused placenta in which intracellular metabolic functions are also preserved. We therefore performed experiments similar to those which, in our laboratory, have allowed the characterization of placental transport for amino acids (Yudilevich & Eaton, 1980; Eaton, Mann & Yudilevich, 1982), sugars (Yudilevich, 1976; Yudilevich, Eaton, Short & Leichtweiss, 1979) and calcium (Sweiry & Yudilevich, 1984*b*). Preliminary accounts of this work have been reported (Sweiry & Yudilevich, 1984*a*; Yudilevich & Sweiry, 1984).

METHODS

Perfused placental preparations

White Dunkin-Hartley guinea-pig dams in their last week of gestation were sedated with an intraperitoneal injection of 10 mg diazepam (Valium, Roche Laboratories) and 20–30 min later, anaesthetized with sodium pentobarbitone (Nembutal, Abbott Laboratories; 20–25 mg kg^{-1}) administered intravenously via a permanent limb vein cannula. The dam was then placed in a physiological saline (0·9% NaCl, w/v) bath maintained at 37 °C.

Two types of perfused placental preparations were employed: the *in situ* singly perfused placenta, in which the fetal circulation was replaced by an artificial perfusion, with the maternal circulation remaining intact as originally described (Money & Dancis, 1960; Reynolds & Young, 1971), and secondly, the isolated dually perfused placenta which has been fully described (Leichtweiss & Schröder, 1971; Yudilevich *et al.* 1979). Briefly, a placenta was isolated and artificially perfused open-circuit through both maternal and fetal circulations and then transferred to a small organ bath containing physiological saline (0·9% NaCl, w/v) and maintained at 37 °C. Placentae were perfused using a peristaltic pump at a rate of 3 ml min^{-1} into the fetal circulation of the singly perfused placenta and into both circulations of the fully isolated placenta. The mean perfusion pressures were generally less than 50 mmHg and in the fully isolated preparation were usually similar on the two sides. Further surgical details for both types of preparations have been reported recently (Eaton *et al.* 1982; Sweiry & Yudilevich, 1984*b*).

Perfusates

A Krebs–Ringer solution of the following composition was used: NaCl, 120 mM; KCl, 5·3 mM; $MgSO_4$, 1·2 mM; KH_2PO_4, 0·5 mM; $NaHCO_3$, 25 mM; $CaCl_2$, 2·5 mM; glucose, 5·5 mM; dextran (40000) 40 g l^{-1} and bovine serum albumin (Cohn fraction V; 1 g l^{-1}). The various other experimental perfusates are described elsehwere in this paper. All perfusates were maintained at 37 °C and gassed with 95% O_2/5% CO_2 to a pH of 7·4. All chemicals were purchased from Sigma Chemical Co.

Analysis of paired-tracer experiments

The measurement of unidirectional cellular transport of a substrate at the guinea-pig trophoblast membrane by the single-circulation paired-tracer dilution technique has been described previously for sugars (Yudilevich *et al.* 1979), amino acids (Yudilevich & Eaton, 1980; Eaton & Yudilevich, 1981; Eaton *et al.* 1982) and for calcium (Sweiry & Yudilevich, 1984*b*). Briefly, in either the singly perfused or the dually perfused placenta a 100 μl bolus containing a mixture of L-[^{14}C]glucose, an extracellular marker in the placenta (Yudilevich *et al.* 1979), and [^{3}H]choline, was rapidly (1–2 s) injected into one of the arterial inflows (donor side). This was immediately followed by collection of the venous effluent in the ipsilateral circulation, as twenty-five to thirty successive four-drop (about 120 μl) samples over 70–80 s. A final sample was then accumulated for a further 4 min. Additionally, in the dually perfused placenta samples were obtained in the contralateral circulation (6 min collection) in order to investigate transplacental transfer.

(a) Time course of choline cellular uptake, U, was determined from the ipsilateral side venous concentrations: $U = (1 - [[^3\text{H}]\text{choline}]/[\text{L-}[^{14}\text{C}]\text{glucose}]$. Fig. 1 shows that a maximal uptake value, U_{max}, could be estimated.

(b) Unidirectional choline influx, v (μmol min^{-1} g^{-1}), was calculated from the fractional maximal uptake, U_{max}, flow F (ml min^{-1} g^{-1}) and the perfusate unlabelled choline concentration, C (mM): $v = -F.\ln(1 - U_{max}).C$.

(c) Efflux of [^{3}H]choline from the trophoblast towards the injection side, backflux (b), was estimated as a percentage of U_{max}: $b = (1 - U_T/U_{max}) \times 100$. The over-all uptake, U_T, was estimated from the integrated tracer recoveries, including the final 4 min accumulated sample: $U_T = 1 - [\text{total } [^3\text{H}]\text{choline}]/[\text{total L-}[^{14}\text{C}]\text{glucose}]$. When the uptake pattern shows an increasing phase (Fig. 1) towards a maximal value (i.e. maximal uptake is not the initial uptake) then it is necessary to discard the initial samples from the calculation of backflux as otherwise they will make an artifactual contribution to the measurement of backflux.

(d) Transplacental transfer of both reference and test tracers was measured in the perfusate collected on the contralateral side over a period of 6 min. The total amount of L-[^{14}C]glucose recovered was an index of the passive 'leakage', which is highly variable in the dually perfused placenta. Therefore, specific transplacental transfer was estimated after correction for this pathway and the excess [^{3}H]choline, if any, served as an index of specific transplacental choline transfer. The use of L-glucose to define leakage pathways is based on our previous work which has clearly shown high uptakes of D-sugars (Yudilevich *et al.* 1979) and of L-amino acids (Eaton & Yudilevich, 1981; Eaton *et al.* 1982) relative to L-glucose marker.

(e) Tissue retention of labelled choline (% dose) was estimated from the total recoveries of the two tracers, in the two circulations, over the 5–6 min sampling period: [^{3}H]choline retention = total [L-[^{14}C]glucose] − total [[^{3}H]choline].

Preparation of radioactive samples for counting. The venous effluent samples, diluted aliquots of

the bolus injection mixture, ^{14}C and ^{3}H isotope standards, and background samples, were all prepared for liquid scintillation counting by the addition of 2·0 ml Readysolve H.P. scintillant cocktail (Beckman) to all samples and counted in a Beckman 7500 beta-counter. Samples and all standards were counted concurrently to a standard deviation of 1 % or less at the peak concentrations and generally about 3 % at lower activities. Since all samples were treated identically, it was unnecessary to apply individual quench corrections. The data were analysed using a Basic program on a Commodore CBM microcomputer.

Chromatographic analysis of radioactivity in venous effluent. These experiments were designed to test for potential presence of metabolites of [^{3}H]choline in the venous effluents. In dually perfused (0·05 mM-choline) placenta a large dose of [^{3}H]choline (50 μCi) was injected as a bolus (1–2 s) intra-arterially into either the maternal or fetal circulation and the venous effluents from the two sides of the placenta were immediately collected at intervals as follows: 0–30 s, 30–60 s, 60–105 s, and finally a 4 min cumulative sample. Treatment of these samples was as follows. Aliquots of 1·0 or 0·5 ml of the samples were precipitated with 4·0 ml absolute ethanol, and the supernatant was evaporated under a stream of N_2 to dryness and resuspended in 50 % ethanol. Aliquots of these samples were then spotted on 0·1 mm cellulose-coated plastic sheets (Merck 5577, B.D.H. Chemicals Ltd., Poole, Dorset) and eluted using a mixture of *n*-butanol : ethanol : acetic acid : water (8 : 2 : 1 : 3). The ^{3}H-activity distribution on the plates was analysed using a chromatographic scanner (Berthold LB 2760) and the R_f values of a [^{3}H]choline standard were compared with that obtained for each of the samples. The results from three placentae confirmed that the R_f values for the [^{3}H]choline standard coincided with the ^{3}H activity from the venous samples, indicating no detectable metabolism of [^{3}H]choline within the time of a paired-tracer experiment; R_f values were (mean $\pm$ S.D., n = number of samples analysed): [^{3}H]choline standard = 0·25 $\pm$ 0·02 (n = 6); ipsilateral side samples = 0·23 $\pm$ 0·03 (n = 12); contralateral side samples = 0·25 $\pm$ 0·02 (n = 12).

Radioactively labelled molecules. These were [methyl-^{3}H]choline chloride (80 Ci/mmol) and L-[1-^{14}C]glucose (47 mCi mmol^{-1}), purchased from New England Nuclear, Dreieich, F.R.G.

RESULTS

Choline uptake patterns at maternal and fetal sides

Fig. 1 illustrates [^{3}H]choline uptake *vs.* time for maternal and fetal side paired-tracer experiments. On both blood–tissue interfaces of the placenta a maximal plateau uptake, U_{max} is apparent and this is rapidly followed by [^{3}H]choline backflux (which results in a decreasing apparent uptake). In the control experiment (perfusate contained 0·05 mM-unlabelled choline) U_{max} values were similar: 50 % and 58 % at the maternal and fetal sides, respectively. The backflux appeared to be higher at the fetal (69 %) than at the maternal (17 %) interface. Fig. 1 also illustrates self-inhibition of [^{3}H]choline uptake. The control data (0·05 mM-choline) from twenty-one runs in fourteen dually perfused placentae are shown in Table 1. The unidirectional influx of choline was equal; however, there was a significantly higher [^{3}H]choline backflux at the fetal interface than at the maternal interface. Table 1 also shows that at 0·05 mM-choline about 25 % of the injected dose of [^{3}H]choline was still retained in the placental tissue at the end of the paired-tracer run (5–6 min).

The fetal side U_{max} and backflux measured in the singly perfused placenta, also at 0·05 mM-choline (Table 2) were similar to those measured for the fetal side of the dually perfused preparation (Table 1). Retention here could not be corrected for the transplacental transfer and hence the value given in Table 2 was estimated from the difference between the recoveries (5–6 min) of the two tracers in the ipsilateral (fetal) circulation.

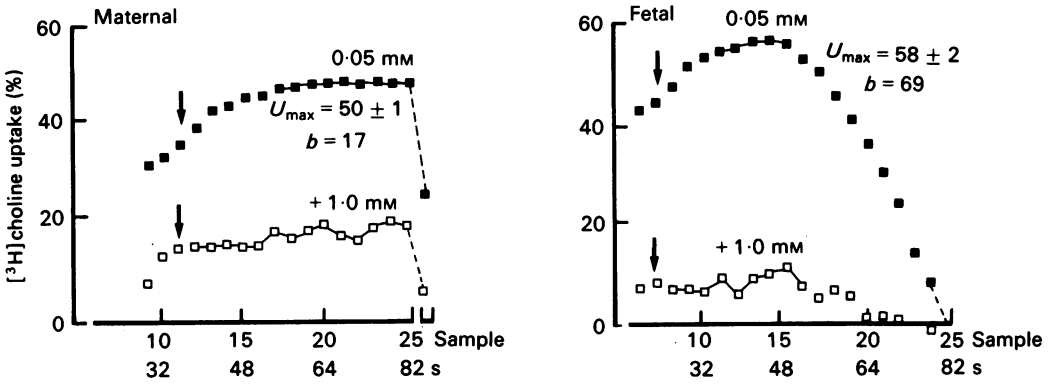

Fig. 1. Choline uptake and its self-inhibition. Percentage uptake of [^{3}H]choline is plotted against the sample collection fraction, or time, following the tracer bolus injection at both maternal (left panel) and fetal (right panel) sides of the dually perfused placenta. The patterns represent 'control' uptake in the presence of 0·05 mM-unlabelled choline in the perfusate and after perfusion with 1·05 mM-choline. Unidirectional maximal uptake (U_{max}, mean ±S.D.) is estimated from the plateau samples (joined data points) before the uptake starts to decline. The backflux b quantifies the apparent reduction in the uptake which follows the maximal uptake value (see text). The arrows indicate the peak of the respective dilution curves of the extracellular marker, L-[^{14}C]glucose (not shown here; see Fig. 1 of Sweiry & Yudilevich, 1984*b*). The values for U_{max} and b shown in the Figure are those for 'control', 0·05 mM-unlabelled choline.

TABLE 1. Summary of paired maternal and fetal [^{3}H]choline transport parameters in the dually perfused placenta in control experiments and in the presence of *N*-ethylmaleimide (5·0 mM). The latter values are given in square brackets. All perfusates contained 0·05 mM-unlabelled choline

	Maternal injection	Fetal injection	*P*
U_{max}(%)	51±2	49±2	n.s.
	[29±6]	[43±2]	
Influx (nmol min^{-1} g^{-1})	28·9±2·9	27·3±2·6	n.s.
Backflux (% U_{max})	22±3	33±3	< 0·01
	[81±7]	[75±4]	
Backflux ratio*: fetal/maternal	2·0±0·34		
Transplacental excess (% dose)	2±1	2±2	n.s.
Tissue retention (% dose)	25±2	26±2	n.s.
	[5±3]	[4±1]	

Values are mean ±S.E. of mean, for control, n = 21 paired runs obtained in fourteen dually perfused placentae, and for *N*-ethylmaleimide, n = 3–4 placentae. Significance value (P) calculated from paired t test; n.s. = not significant ($P > 0·05$). *The value given for the fetal/maternal backflux ratio is not based on the above mean value but is the mean of the ratios of twenty individual paired control runs (see Fig. 6).

Kinetics of unidirectional choline influx and inhibition by hemicholinium-3

In the kinetic experiments placentae were perfused successively with various concentrations of choline (0·05–16·0 mM) and in all cases the paired-tracer experiment was performed 4 min later. The tracer injectates contained unlabelled choline at a concentration equal to that of the perfusate under study. Inhibition of [^{3}H]choline

uptake by hemicholinium-3 (HC-3) was also investigated (0·05–1·0 mM) in the *in situ* placenta. Fig. 2 (left panel) illustrates these results. It can be seen that HC-3 was a better inhibitor of choline uptake than choline itself. The influx of choline was saturable within the concentration range shown (0·05–1·0 mM), and a weighted rectangular hyperbola fitted to the data points (Fig. 2, right panel) gave a K_m of 0·14 mM and a V_{max} of 0·13 μmol min^{-1} g^{-1}. Comparable values were obtained when these data were analysed by means of a Hanes plot (Mulflih & Widdas, 1976; Sepúlveda & Smith, 1978): K_m = 0·18 mM and V_{max} = 0·16 μmol min^{-1} g^{-1}.

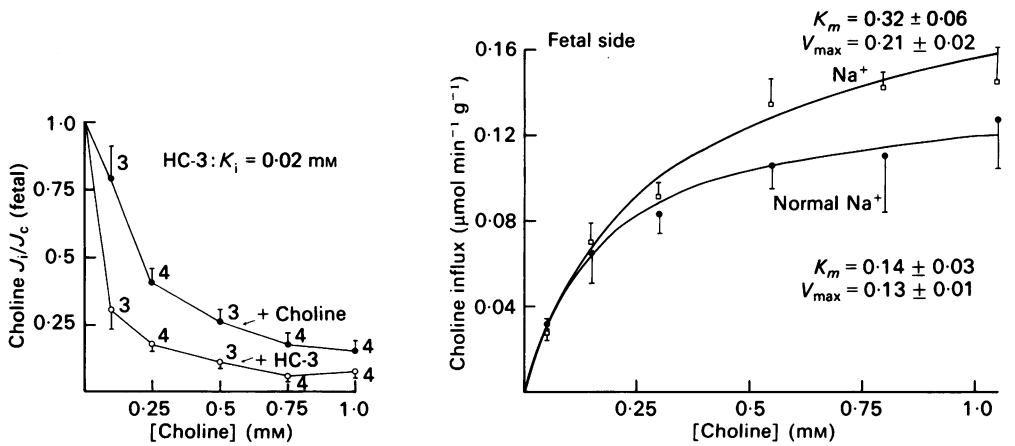

Fig. 2. Inhibition of [^{3}H]choline uptake and saturation kinetics of unidirectional choline influx. These experiments were performed on the fetal side in the singly perfused placenta and within the lower (up to 1·0 mM) range of choline concentrations. Left panel: self-inhibition of choline uptake and its inhibition by hemicholinium-3 (HC-3). The ordinate represents the ratio of the choline influx measured in the presence of the inhibitor (J_i) to the control influx (J_c) at 0·05 mM-choline. The higher inhibition produced by HC-3 was represented by a K_i of 0·02 mM (see text). Right panel: Saturation kinetics of unidirectional choline influx under conditions of normal sodium (143·1 mM), derived from data in left panel, and when the sodium concentration in the perfusate was reduced to 25·0 mM with the remainder replaced by lithium. The curves are rectangular hyperbolas obtained by a direct fit of the mean influx measured at each concentration and weighted for the reciprocal of their respective standard error. The data points are represented as mean ± S.E. of mean, n = 3–4 placentae. The estimated kinetic parameters are also shown.

To estimate the inhibitory constant (K_i) for HC-3 we employed the 'velocity ratio' method (see Neame & Richards, 1972) since inhibition by HC-3 was not studied at concentrations lower than 0·05 mM. In the 'velocity ratio' method, the uninhibited rate of choline influx may be related to the inhibited rate, assuming the inhibition is competitive: $K_i = iv_i K_m/(v-v_i)(S+K_m)$, where i is the competitor (HC-3) concentration, v_i the influx due to 0·05 mM-choline, S is the substrate concentration (0·05 mM-choline) and K_m is the affinity constant for the substrate. When the K_m of 0·14 mM obtained on the fetal side (Fig. 2) was substituted in the above equation, the resulting inhibitory constant for HC-3 (at 0·1 mM-HC-3) was 0·02 mM, i.e. seven times lower than the K_m for choline.

Evidence for a non-saturable component of choline influx

There was no obvious non-saturable component of choline influx present within the concentration range 0·05–1·0 mM; however, when the range of choline concentration was extended up to 16·0 mM, a non-saturable influx became apparent (Fig. 3). By means of a Hanes plot analysis the saturable influx could be substracted from the total influx and the remaining fraction gave a straight line of slope 0·13 min^{-1} g^{-1} (Fig. 3). The carrier-mediated component gave a K_m of 0·23 mM and a V_{max} of 0·24 μmol min^{-1} g^{-1}.

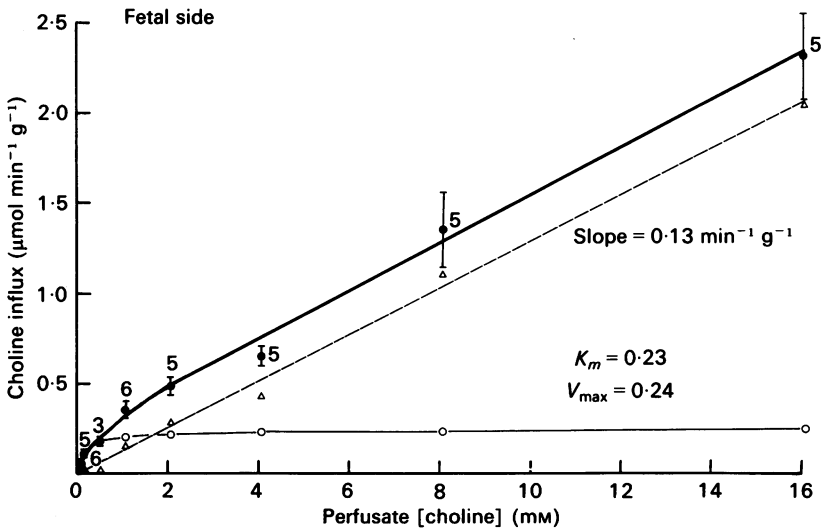

Fig. 3. Evidence for a linear non-saturable component of choline influx. In these experiments in the singly perfused placenta preparation, the range of choline concentration in the perfusate was extended to 16·0 mM. The heavy line was fitted by eye to the experimental data (●). The lower curve (○) was drawn based on the kinetic parameters (values are shown) obtained from a Hanes plot analysis. The non-saturable component of choline influx was fitted to the open triangles. The slope of this line is shown; correlation coefficient = 0·99; y intercept = −0·02. All values are mean ±S.E. of mean, n = number of placentae.

Since we were interested in comparing the two sides of the placenta, we repeated the kinetic experiments in the dually perfused placenta and again the presence of a non-saturable component appeared (Fig. 4). Furthermore, almost identical kinetic constants were found on the maternal and fetal sides of the trophoblast (Fig. 4).

Inhibition of choline influx by choline analogues

In order to characterize the choline carrier further we investigated the effects of some molecules structurally related to choline on [^{3}H]choline unidirectional influx at the maternal and fetal sides (Fig. 5). Each molecule was added to the perfusate at 1·0 mM and 4 min later a paired-tracer experiment was performed. Inhibition was about 70% with choline, its demethylated analogues, *N*,*N*-dimethylethanolamine (NNDME), and ethanolamine. However, oxidized choline, betaine (a normal

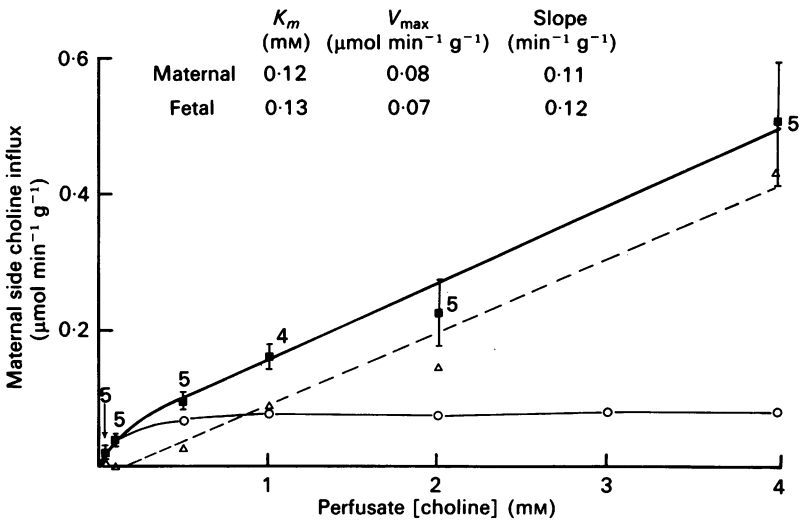

Fig. 4. Choline influx kinetics at the maternal and fetal sides of the dually perfused placenta. Saturable and non-saturable components appeared on both sides and the kinetic parameters are shown. Data are illustrated only for the maternal side and the analysis and symbols are similar to those described in Fig. 3.

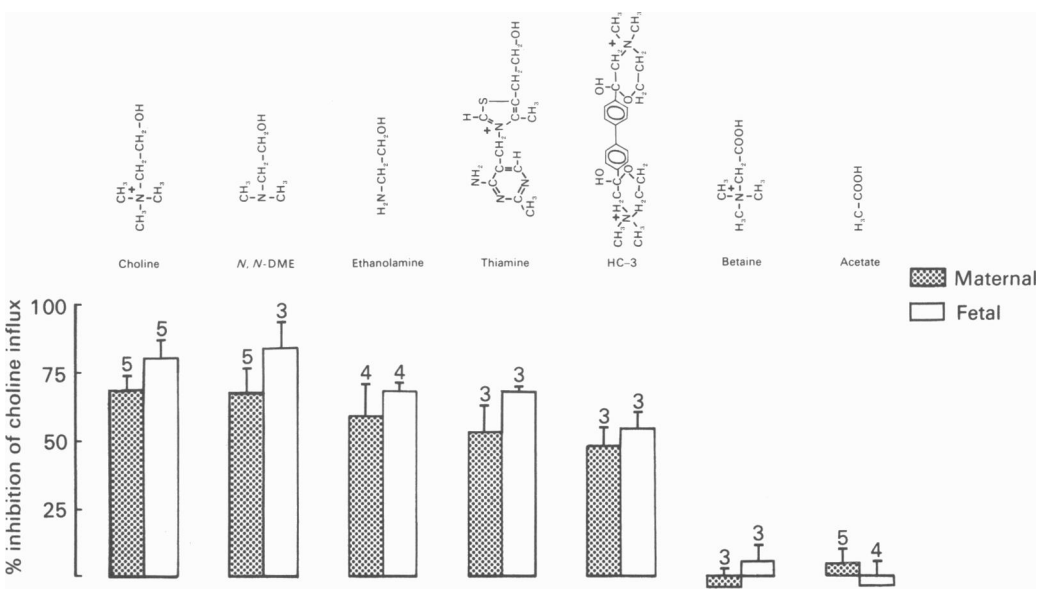

Fig. 5. Specificity of choline influx at the maternal and fetal sides in the dually perfused placenta. The molecules (structures are shown) were added to the perfusate (both circulations) at a concentration of 1·0 mM. The ordinate represents the inhibition of choline influx at 0·05 mM-unlabelled choline by the inhibitor (see Fig. 1 for an example of the inhibitory effect of choline). Values are mean ±S.E. of mean, n = number of placentae.

metabolite of choline) was ineffective and the same was found for acetate (another precursor in the synthesis of ACh). The larger analogues of choline, HC-3 and thiamine (a water-soluble vitamin) were very effective inhibitors of choline influx. At the high concentration used in these experiments the differences in affinities were not apparent. However, at lower concentrations HC-3 was distinctly a more effective inhibitor compared to choline itself (Fig. 2).

Effects of sodium-free perfusates and lithium perfusion

Two types of experiments in the singly perfused placenta were designed to test the sodium dependency of choline uptake. NaCl (120 mM) and $NaHCO_3$ (25 mM), control perfusate, were replaced by (a) equimolar quantities of Trizma HCl (145 mM) or (b) only NaCl was replaced by equimolar LiCl. In all of these experiments 0·1 mM-adenosine was added to the perfusates, including controls. This was done in order to prevent increases in vascular resistance due to lack of sodium. A total of eight singly perfused placentae were used. In some of these experiments the sodium (and potassium) ion concentration in successive venous effluents was measured (using a flame photometer) after switching to the sodium-free perfusate. It was found that after 4 min the sodium- and potassium-ion concentrations were (mean $\pm$ S.E. of mean, $n = 3$–5 placentae) 6·6 $\pm$ 2·8 mM and 5·7 $\pm$ 0·6 mM, respectively. The kinetics of unidirectional choline influx in the low-sodium, high-lithium conditions were measured (Fig. 2, right panel). There was no inhibition of choline influx and a K_m (mM) of 0·32 $\pm$ 0·06 and a V_{max} (μmol min^{-1} g^{-1}) of 0·21 $\pm$ 0·02, could be compared to a K_m of 0·14 $\pm$ 0·03 and a V_{max} of 0·13 $\pm$ 0·01 obtained in experiments with perfusates containing a normal concentration of sodium.

When sodium was replaced by Tris, experiments were performed at only 0·05 mM-choline and the results showed that the [^{3}H]choline maximal uptakes (%) were unchanged ($n = 3$–4 placentae): control, 59 $\pm$ 3; Tris, 48 $\pm$ 3. The parallel experiment with sodium replaced by lithium also showed no effect: U_{max} values (%) were 60 $\pm$ 3 in normal sodium, and 54 $\pm$ 3 in low sodium.

In the latter group of experiments we could not observe significant changes in the [^{3}H]choline efflux as estimated from the backflux (%) parameter (mean $\pm$ S.E. of mean, $n = 3$–4 placentae): control, 32 $\pm$ 3 and Tris, 30 $\pm$ 7; control, 43 $\pm$ 5 and low sodium, high lithium 41 $\pm$ 4.

Effect of increased choline concentration and HC-3 on backflux at maternal and fetal sides

At a choline concentration of 0·05 mM the fetal side [^{3}H]choline backflux was 20–55% and usually higher than the maternal side backflux 0–45% (Fig. 6). This asymmetry was more apparent at higher choline concentrations (2·0–4·0 mM) since the fetal side backflux was markedly increased, whereas at the maternal side the backflux appeared not to change (Fig. 6, right panel).

The effect of equimolar (0·25 mM) choline or HC-3 on [^{3}H]choline backflux (%) was studied in four-singly perfused placentae. HC-3 produced a marked inhibition of the backflux (16 $\pm$ 6) whereas choline was ineffective (47 $\pm$ 9) compared to control value of 46 $\pm$ 7.

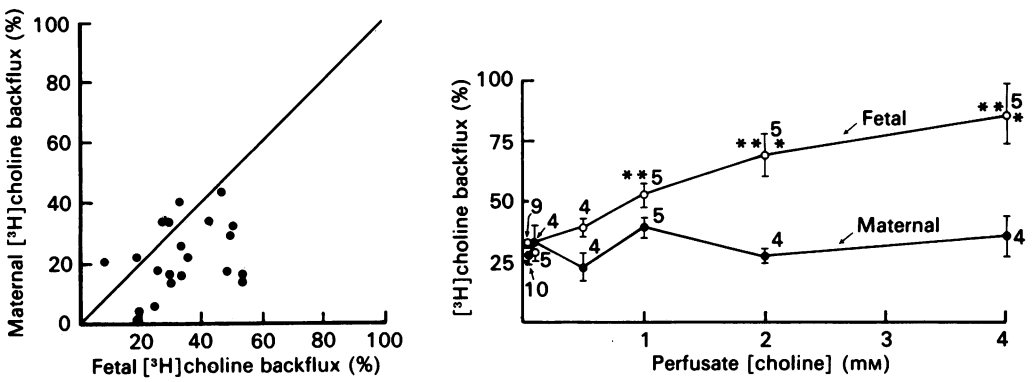

Fig. 6. Efflux of [^{3}H]choline from the trophoblast towards the injection side (backflux). These experiments were performed in the dually perfused placenta preparation. Left panel: backflux for successive paired tracer injections, one in the maternal side (ordinate) and the other in the fetal side (abscissa). The data were obtained at 0·05 mM. The line represents a ratio of unity. Results from twenty-one paired runs in fourteen placentae. Right panel: similar backflux measurements obtained at different concentrations of unlabelled choline in the perfusate. The asterisks refer to statistical differences ($P < 0·02$, unpaired t test): * on the same side with backflux at 0·05 mM-choline (control); ** with the backflux on the contralateral side at the same concentration.

Effects of 2,4-dinitrophenol, potassium cyanide and N-ethylmaleimide on choline influx and efflux

Perfusion with 2,4-dinitrophenol (2,4-DNP; 1·0 mM) or potassium cyanide (1·0 mM) for periods of up to 40 min produced no inhibitions in the unidirectional uptake and only some effect on backflux of [^{3}H]choline (Table 2). Furthermore, support for the ineffectiveness of these drugs is reflected in the similarity of the [^{3}H]choline retention (Table 2).

The effect of the sulphydryl reagent, *N*-ethylmaleimide (NEM), was investigated in the dually perfused placenta (Table 1). Choline uptake was measured before and after the addition of 5·0 mM-NEM to perfusates on both circulations. Fig. 9 represents an example of the effect of NEM on the choline uptake pattern on maternal and fetal sides. The initial uptake was not greatly affected and the major change appeared to be a high increase in the tracer efflux represented by the rapid downslope of the uptake curves. This rapid apparent efflux was confirmed by the estimated retention of [^{3}H]choline over the 5–6 min period, which was greatly reduced (Table 1.)

Transplacental transfer

In both maternal-to-fetal and fetal-to-maternal directions the simultaneously measured L-[^{14}C]glucose and [^{3}H]choline in the contralateral circulation of the dually perfused placenta was variable and ranged from zero to about 60% of the injected dose. However the recoveries of the two tracers measured simultaneously were always very similar, and only in placentae with low leakage did there appear to be a small

TABLE 2. Effect on choline transport of inhibition of aerobic metabolism. These experiments were conducted in the *in situ* perfused placenta. In each preparation three paired-tracer experiments were performed successively after 10, 25 and 40 min perfusion with the inhibitor shown (1·0 mM). Since the effect of the inhibitors did not significantly change with time, the measurements at all time intervals were averaged

	Control	DNP	KCN
U_{max}(%)	60±7 (8)	60±4 (6)	52±5 (6)
Backflux (% U_{max})	43±14 (8)	29±7 (6)*	29±9 (6)*
Retention† (%) dose)	42±9 (8)	47±7 (6)	31±7 (6)

Results are given as mean ±S.D., n = number of runs from four singly perfused placentae (controls) and two singly perfused placentae each for DNP and KCN.

* $P < 0·05$ compared to respective controls.

† This is not retention as calculated in the dually perfused placenta since transplacental choline is not measured.

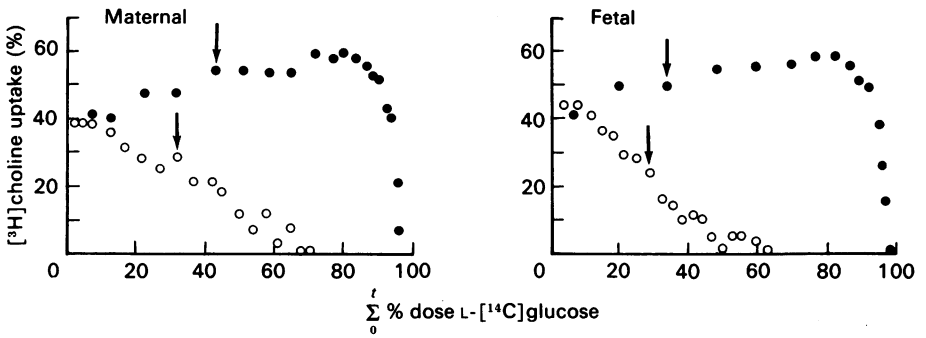

Fig. 7. Effect of *N*-ethylmaleimide (NEM) on choline uptake at maternal and fetal sides of the dually perfused placenta. ●, control [³H]choline uptake in the presence of 0·05 mM-unlabelled choline in the perfusate; ○, [³H]choline uptake following 10 min (maternal side) and 18 min (fetal side) perfusion with NEM (5·0 mM) on both sides of a placenta. The abscissa represents the accumulated recovery of the extracellular marker up to each point. The arrows indicate the peak of the respective dilution curves of the extracellular marker (not shown).

excess of [³H]choline (< 10% of the injected dose). The latter could indicate some specific transplacental transfer of choline even though the mean excess over the complete range of leakage was not different from zero (Table 1).

DISCUSSION

Kinetics of unidirectional choline influx

Our results have demonstrated, for the first time, unidirectional choline influx at both maternal and fetal surfaces of the trophoblast. The influx measurements provided evidence for the existence of two separate components of choline influx: a carrier-mediated saturable process and a non-saturable process. It was shown that the saturable component conforms to Michaelis–Menten kinetics with symmetric transport parameters on maternal and fetal sides. The non-saturable component

(apparent above 1·0 mM) could be attributed to passive diffusion or to transport by another carrier whose affinity for choline is extremely low.

The only other available choline transport data in the placenta are from studies using human fragments, in which the net intra/extracellular concentration ratios were found to be greater than unity within 3–5 min and about 4 after 15 min (Welsch, 1976*a*). The kinetic parameters reported also showed both linear and saturable components (K_m = 0·35 mM); the non-saturable influx was already apparent at 0·2 mM compared to about 1·0 mM in our intact placenta preparation.

K_m values of 0·11 and 0·6 mM have been measured for choline transport (1–5 min uptake) at the brush-border side of the intestine of the chick (Herzberg & Lerner, 1973) and guinea-pig (Kuczler, Nahrwold & Rose, 1977), respectively. Here again the presence of a non-saturable component was detectable. In the erythrocyte membrane choline uptake exhibited low affinity constants (K_m) of 0·02–0·03 mM (Martin, 1968; 1972), and in brain synaptosomal preparations a K_m of 0·28 mM has been reported (Marchbanks, 1968). The original observation in the squid giant axon indicated a K_m of 0·6 mM (Hodgkin & Martin, 1965).

Specificity of the choline carrier

In these experiments the inhibitory capacity (on labelled choline uptake) of choline itself was compared to that of structurally related molecules (Fig. 5). The affinity for the carrier remains high even when the molecule lacks one or two methyl groups, e.g. the choline precursors dimethylethanolamine and ethanolamine. In contrast, betaine (an end metabolite of choline) showed no affinity for the carrier. Also, no inhibition was observed by acetate (a precursor in the synthesis of ACh) which, on the other hand, appears to exhibit a high uptake (J. H. Sweiry & D. L. Yudilevich, unpublished observations). Hence acetate possibly has its own transport system. These results would suggest that the presence of a carboxyl group (e.g. in acetate and betaine) renders the molecule untransportable by the choline carrier. In the intestine all these analogues produced a similar range of inhibitions (Herzberg & Lerner, 1973). The inhibition of choline influx by thiamine (Fig. 5), another water-soluble vitamin, is particularly interesting since we have been unable to demonstrate placental uptake of thiamine (J. H. Sweiry & D. L. Yudilevich, unpublished observations). Thiamine inhibition has also been reported for the renal tubular excretion of choline in the chick (Rennick, 1958).

The largest analogue of choline tested, HC-3, is a characteristic competitive inhibitor of choline uptake in almost any tissue and this has also been shown for choline accumulation in human placental fragments (Welsch, 1976*a*). Our results demonstrate that the choline carrier has a higher affinity for HC-3 (K_i = 0·02 mM) than choline itself. However, we did not investigate whether the interaction was competitive or non-competitive.

Effects of sodium-free perfusion

The insensitivity to sodium of choline influx has been demonstrated in our experiments both by complete replacement of sodium with Tris and by partial sodium replacement with lithium (Fig. 2). This finding is compatible with the results reported by Welsch (1976*a*, 1978), who showed that omission of sodium in the incubation medium (replaced with sucrose) resulted in an increased level of intracellular choline.

This effect also coincided with an important decrease in the incorporation of [^{3}H]choline into ACh (Welsch, 1978), suggesting that sodium is important for the synthesis of ACh in the placenta. In the intestine, the transport of choline has been shown to be a sodium-independent process (see review by Rose, 1980) in the chick (Herzberg & Lerner, 1973), guinea-pig (Kuczler *et al.* 1977) and hamster (Sanford & Smyth, 1971), and in rat brush-border membrane vesicles (Kessler, Acuto, Storelli, Murer, Muller & Semenza, 1978). In the red cell choline fluxes do not appear to be sodium-dependent provided sodium is replaced with magnesium or calcium (Martin, 1972). On the contrary, choline transport in nervous tissue is definitely highly sodium-dependent (Marchbanks, 1968).

Our results on lack of inhibition when sodium was replaced by lithium also demonstrate that lithium does not interact with the choline carrier system in the placenta. This is an important finding since lithium is used as a therapeutic agent and has been shown to inhibit choline transport in red cells (Martin, 1972) and also in synaptosomes, at low concentrations (see Martin, 1977).

Effects of inhibitors of aerobic metabolism

When oxidative phosphorylation and/or electron transfer was inhibited by DNP or KCN the unidirectional influx of choline was little affected even after 40 min of perfusion with the inhibitors (Table 2). In contrast, there was a small but significant inhibition of the backflux of choline from the trophoblast. Welsch (1976*a*, 1978) observed a 65% inhibition of choline accumulation by placental fragments after 30 min of incubation with these inhibitors. In the intestine (Kuczler *et al.* 1977; Sanford & Smyth, 1971) and synaptosomes (Marchbanks, 1968) there was also a small effect of these metabolic inhibitors on choline accumulation.

Effect of the sulphydryl group reagent, NEM

Our results on both sides of the trophoblast showed that a major effect of NEM appeared to be a dramatic reduction in the retention of [^{3}H]choline (Table 1). It is important to note that [^{3}H]choline retention measurements are independent of the uptake pattern (Fig. 7). In line with our usual interpretation of these uptake patterns, it could be suggested that unidirectional influx (represented by the initial maximal uptake) was not highly inhibited and that the rate of backflux was greatly increased, hence resulting in a low net uptake over the 5–6 min. However, it is not possible to speculate further regarding the inhibitory effect of NEM on choline transport in the placenta. Choline transport in erythrocytes (Martin, 1971) and brain synaptosomes (Diamond & Kennedy, 1969) was irreversibly inhibited by sulphydryl reagents, and according to the interpretation of Martin (1971), later confirmed by Deves & Krupka (1981), only the inward-facing conformation of the choline carrier reacts with NEM. An additional factor to be considered in the placenta is the metabolic inhibitory effects of NEM since it has been shown to inhibit cholineacetyltransferase (Roskoski, 1974). A reduced metabolic utilization of choline would be accompanied by an increase in the efflux if the transport mechanisms were not affected.

It is interesting that in contrast to its effects on choline transport (Fig. 7), Sweiry (1983) found that, in similar experiments, NEM did not produce a decreasing $^{45}Ca^{2+}$ uptake pattern but maintained the uptake and resulted in a greatly increased (more than doubled) retention of $^{45}Ca^{2+}$ by the trophoblast (see Table 1 of Sweiry, 1983).

Backflux of choline

Choline backflux (i.e. the efflux of [^{3}H]choline towards the ipsilateral circulation) appeared, in general, to be higher at the fetal than at the maternal side of the trophoblast and could be related to the capacity of the placenta to maintain a fetal-to-maternal concentration gradient, as has been suggested for certain other substrates (Eaton & Yudilevich, 1981; Sweiry & Yudilevich, 1984*b*). This asymmetry was exaggerated when the perfusate choline concentrations were simultaneously raised (up to 4·0 mM) in both circulations since only the fetal side backflux was increased (Fig. 6). The latter finding, which could be interpreted in terms of choline counter-transport (Martin, 1968, 1971) being more sensitively activated at the fetal than at the maternal interface, has some resemblance to the asymmetric counter-transport effect for leucine observed by Cheeseman (1981) in the basolateral, but not on the brush-border, side of the intestinal epithelium.

In contrast to choline, HC-3, a known non-transportable competitive inhibitor of choline in various tissues, produced a marked inhibition of tracer choline efflux. This trans-inhibition of choline efflux by HC-3 was also found in the erythrocyte (Martin, 1968) and in nervous tissue (Marchbanks, 1982). It has been proposed that HC-3 binds to the carrier on the outer side thus immobilizing the carrier.

Transplacental choline transfer

Some excess of choline over the reference marker was only apparent at low leakage and this possibly represents the contribution of a specific carrier-mediated efflux from the trophoblast towards the contralateral circulation. It could be speculated that this represents the normal *in vivo* condition, although our experiments would not shed light on the mechanism(s) of transplacental transfer of choline.

In contrast to the present results, previous work from this laboratory has shown that glucose (Yudilevich *et al.* 1979) and amino acids (Eaton & Yudilevich, 1981; see review by Yudilevich & Sweiry, 1985) are recovered in the contralateral circulation, in marked excess over the inert reference tracer at any degree of leakage. On the other hand, the results with the choline cation are reminiscent of the finding for the calcium cation (see Fig. 7 of Sweiry & Yudilevich, 1984*b*) and the folate anion (J. H. Sweiry & D. L. Yudilevich, unpublished observations).

Choline cellular uptake and its relation to synthesis and/or transport of ACh

The fate of the labelled choline was not investigated, but rapid incorporation into metabolic pathways such as synthesis of ACh could occur in the guinea-pig placenta (which contains ACh, see Welsch, 1976*b*) as it has been observed in human placental fragments (Welsch, 1976*b*, 1978). With the techniques developed in this study, it would be of interest to investigate, in the intact placenta, the relationship between choline and ACh transport systems (Hodgkin & Martin, 1965; Welsch, 1976*b*) since ACh may play an important role in placental metabolism and fetal development.

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