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STUDIES ON ACCUMULATION AND METABOLIC FATE OF [*N*-Me^{3}H]CHOLINE IN HUMAN TERM PLACENTA FRAGMENTS

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Abstract Fragments from human term placenta accumulated [*N*-Me-^{3}H]choline against a concentration gradient. Uptake was linearly related to incubation time and temperature. Analysis of the kinetics of choline accumulation revealed the concurrent existence of a diffusional component occurring both at low temperature as well as high (50 mM) choline concentration and a carrier-mediated transport which had characteristics predicted by the Michaelis–Menten equation showing a $K_m = 3.46 \times 10^{-4}$ M and a V_{max} of 75 nmoles/ml intracellular water × min^{-1}. Net concentration ratios, corrected for diffusion and extracellular water, were larger than 1.0 within 5 min and about 4.0 after 15 min. Hemicholinium-3 was a competitive inhibitor of choline uptake with a K_i of 0.45 mM. [^{3}H]Choline accumulation was decreased by conditions known to lower intracellular ATP levels. Thus, 2,4-dinitrophenol (1 mM), sodium cyanide (5 mM) and anaerobic incubation reduced [^{3}H]choline accumulation 36, 54 and 33 per cent respectively. Ouabain (0.1 mM) also decreased the concentration ratios by 50 per cent. Modification of the ionic environment led to an increase of 36 per cent in the amount of tritium in intracellular water when Na^+ was reduced to one half of the usual 145 mM or 150 per cent when it was completely omitted and replaced by an osmotically equivalent amount of sucrose. Li^+ was without effect, while high K^+ (>25 mM), Rb^+ and Cs^+ (145 mM) depressed [^{3}H]choline accumulation. The metabolic fate of [^{3}H]choline was studied. Following a 5-min incubation with 5 μM [^{3}H]choline 95 per cent of the radioactivity was acid-soluble and 5 per cent remained in the acid-insoluble fraction. After 30 min the distribution was 88 and 12 per cent, respectively. Paper high voltage electrophoretic analysis of the acid-soluble material showed that after 5 min 55 per cent of the ^{3}H had a mobility equal to authentic choline, 35 per cent equal to acetylcholine, 6 per cent to phosphorylcholine and 1 per cent to betaine. After 20 min it was 25 per cent in choline, 60 per cent in acetylcholine, 10 per cent in phosphorylcholine and 2 per cent in betaine. A chloroform–methanol extract from the acid-insoluble residue revealed a linear increase of ^{3}H-content suggesting incorporation of [^{3}H]choline into phospholipids.

The uptake and metabolism of choline have been investigated in a variety of tissues among them excitable tissues such as the mammalian and invertebrate brain and its subcellular components [1–9], the diaphragm [10], sympathetic ganglia [11], the squid axon [12] as well as in nonexcitable tissues such as erythrocytes [13], the kidney [14] and in cells derived from neuroblastoma and maintained in culture [15–16]. Little seems to be known about the fetal ability to synthesize choline *de novo* and its requirements for this important base. The role of the placenta for the supply of choline derived from the mother or its synthetic abilities are not established. However, large concentrations of free choline reach the human placenta via the maternal blood, which contains about 7–16 nmoles/ml [17, 18] and perfuses the mature placenta at the rate of about 500 ml/min [19]. Therefore, free choline in the blood could be an important source for placental and fetal needs of this compound if there was a means of transfer existing. Once in the placental parenchymal cells choline could be metabolized by a variety of enzymes, among which would be choline acetyltransferase (acetyl-CoA-choline *O*-acetyltransferase, E.C. 2.3.1.6, ChAc) which occurs in high concentrations in the noninnervated human placenta [20, 21]. Metabolic conversion to acetylcholine (ACh) could have important bearing on placental function if the speculations concerning permeability control and regulation of transport could be proven to be correct [21–23]. Therefore it was of interest to study the accumulation of choline by human placenta and follow its metabolic fate in this tissue. This communication reports the characteristics of choline uptake by placenta fragments *in vitro*, the effects of variations in the ionic environment and some aspects of choline metabolism in term placenta.

MATERIALS AND METHODS

Tissue sources, preparation and solutions. Human placentae derived from uncomplicated term pregnancies by vaginal delivery or Caesarean section were obtained immediately after removal from the body and transported to the laboratory in an ice-chest. They were placed into a plastic tray surrounded by crushed ice. After slicing the placenta disk at about 1/3 of its total thickness parallel to the decidua basalis, small pieces of villous tissue (4–6 mm) were free-hand dissected [24] and collected in ice cold Krebs–Henseleit medium (KHM) of pH 7.4 which was continuously gassed with 5% CO_2 in oxygen. This solution had the following composition in mM: NaCl, 118; KCl, 4.8; $CaCl_2$, 2.5; $MgSO_4$, 1.2; KH_2PO_4, 1; $NaHCO_3$, 27.2; glucose, 11.1. The tissue was ready for use within 60–90 min after obtaining the specimen. Only when the conversion of [^{3}H]cho-

line into various metabolic products was measured was 10 μM paraoxon present in the standard Krebs Henseleit medium as an inhibitor of cholinesterases. When the ionic environment was modified and Na^+ or K^+ were to be omitted, 1 mM HEPES (*N*-2-hydroxyethylpiperazine *N'*-2 ethane sulfonic acid, Sigma Chemical Company, St. Louis, Mo.) was used as a buffer and adjusted with ammonium hydroxide to pH 7.4. KH_2PO_4 and $NaHCO_3$ were omitted from the HEPES-buffered solutions. When the NaCl concentration was modified, sucrose was used in twice the amounts to maintain isosmolarity. When K^+ was raised above the usual 4.8 mM in the incubation medium, Na^+ was reduced by an equimolar amount.

Tissue incubations, corrections for extracellular water space and measurement of radioactivity were performed as described [24, 25]. The incubation solution containing inulin at 25 nCi/ml (inulin-carboxyl-[^{14}C], Mallinckrodt, St. Louis, Mo., and California Bionuclear Corp., Sun Valley, Calif., sp. act. (SA) 1.71 mCi/g) was used to dissolve carrier choline iodide (Sigma Chemical Company, St. Louis, Mo.) to which choline chloride (methyl-[^{3}H], Amersham-Searle, Arlington Heights, Ill., S.A. 16.5 Ci/mmole) was added to provide 50 nCi/ml medium.

Isolation and identification of [^{3}H]choline-containing metabolites. Placenta fragments were incubated for 5, 10, 20 and 30 min. in inulin-free KHM containing 5 μM [^{3}H]choline. The amount of radioactivity was increased to 400 nCi/ml KHM of which 5 ml was used for each assay. At the end of the incubation period the flask content was rapidly filtered through glass fiber filters (Whatman GF/B, 2.4-cm diameter) placed in Millipore filtering devices and mounted on Erlenmayer flasks with vacuum attachment. The fragments were trapped and washed rapidly three times with 5 ml of ice-cold KHM containing 5 μM carrier choline. The tissue was then homogenized in 1 ml of ice-cold 10% trichloroacetic acid (TCA) in hand-operated glass homogenizers (Tenbroeck type, Kontes, Vineland, N.J.). The sample was transferred to conical 12 ml tubes and the homogenizer rinsed twice with 0.5 ml cold 5% TCA. To the homogenate were added 100 μg each choline, ACh and phosphorylcholine (PhCh) as carrier. Following centrifugation the supernatant was transferred to 12-ml conical centrifuge tubes with ground glass necks and glass stoppers. TCA was removed by shaking four times with two volumes of water-saturated ether. The tubes were briefly gassed with dry nitrogen to evaporate any remaining ether. A known amount of the acid-soluble extract was removed at this point and lyophilized overnight just as the remaining sample was. One of the samples was dissolved in 50 μl of acidic buffer (1.5 M acetic acid–0.75 M formic acid, pH 2.0 [26]). Duplicate aliquots of 5 μl were removed and radioactivity determined in a dioxane base scintillation fluid [27] using a model 3380 liquid scintillation spectrometer equipped with a model 544 absolute activity analyzer (Packard Instruments, Downers Grove, Ill.). Another aliquot of 20 μl was used for analysis by high voltage electrophoresis. This sample was applied to dry Whatman No. 1 paper strips 3 × 40 cm on a 2-cm wide part of the starting line which was drawn 5 cm away from the anodal side of the paper. The electropherogram was moistened with the acidic buffer by placing Whatman 3 MM paper on top of it to within 15 mm on either side of the starting line. Thus buffer diffused slowly towards the start where the purified tissue extract (or standards) had been applied. The moist strips were placed on the flat bed of the electrophoresis cell (CAMAG HVE-System, Camag, New Berlin, Wisc.) and 100 V/cm were applied for 25–30 min. Mixtures of standards ACh (20 μg), choline (20 μg), PhCh (100 μg) and betaine (200 μg) were separated on adjacent strips during the same run. The electropherograms were dried until only moist and exposed to I_2 vapors. All iodine-stained spots were marked in pencil and the paper allowed to hang in a fume hood overnight. On the next day the strips were cut into 1-cm long sections beginning 2 cm anodal from the starting line towards the cathode. These pieces were placed into counting vials and 2 ml of water were added. The vials were vigorously shaken for 30 min on a reciprocating shaker to elute the radioactivity from the paper. Thereafter 15 ml of dioxane scintillation fluid were added and radioactivity was determined.

The second aliquot of the acid-soluble lyophilized extract was redissolved in 0.5 ml of 10 mM phosphate buffer pH 6.6, a 25-μl aliquot removed for radioactivity determination and the remainder subjected to treatment with tetraphenylboron (Sigma) in 3-heptanone (Eastman Kodak) (5 mg/ml, 1 ml per extraction [28] to remove choline and ACh by liquid cation exchange, while leaving behind PhCh and betaine [29]. The tubes were vigorously mixed and then centrifuged to break the emulsion. Eight hundred μl of the upper organic layer was transferred to tubes containing 800 μl 0.4 N HCl and choline and ACh reextracted into the aqueous layer. Radioactivity was measured in this sample and also in the originally extracted phosphate buffer. The latter would be indicative of radioactivity which was not attributable to choline and ACh and thus did not complex with tetraphenylboron.

The acid-insoluble material was also examined for its content of radioactivity. First lipid material was extracted by washing the pellet twice with 2 ml of chloroform–methanol (2:1) and pooling the supernatant obtained after centrifugation. The remaining pellet was then solubilized in 1 ml of Soluene (Packard Instruments) and the radioactivity determined in a toluene base scintillation fluid.

Control samples of placenta fragments were processed in a similar manner. They contained an equivalent amount of tissue but they were not incubated with [^{3}H]choline. Rather, the paraoxon pretreated fragments were homogenized in TCA prior to the addition of 100 μg of the carrier standards plus about 400,000 dis/min of [^{3}H]choline and 500,000 dis/min of [1-^{14}C]acetyl-ACh.

Data calculations. The distribution of total tissue water (TW) between extracellular water (ECW) as measured by [^{14}C]inulin space and intracellular water (ICW) as well as the concentrations of ^{3}H in ICW in relation to ^{3}H in the medium (Ratio C_i:C_o) [24] were calculated with a computer program prepared for a CDC central computer. Statistical evaluations were performed with Student's *t*-test and by completely randomized analysis of variance for which the confidence limit was set at $P < 0.05$.

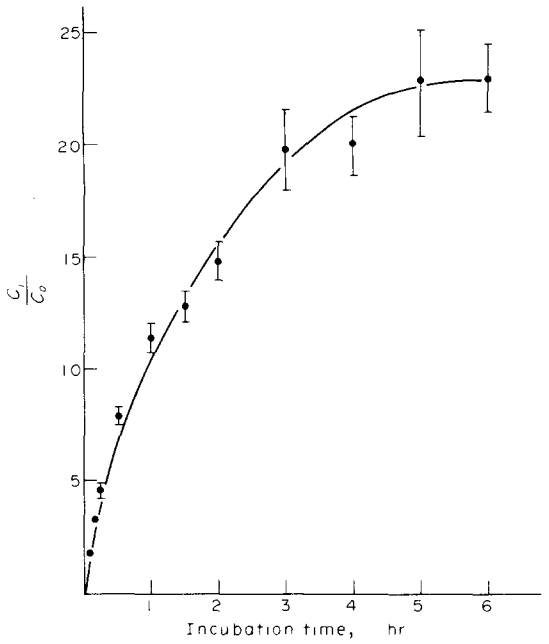

Fig. 1. Time course of accumulation of [^{3}H]choline by human term placenta fragments. Fragments were incubated in standard medium containing 5 μM [^{3}H]choline for the time periods indicated. Ordinate: corrected concentration ratios (dis/min/ml intracellular water: dis/min/ml medium, after deduction of [^{14}C]inulin extracellular water space). Each point shows the mean ± S.D. of 8–12 determinations from 2–3 placentae.

RESULTS

Time course of [^{3}H]choline accumulation. The concentration of free choline in fresh term placenta upon arrival in the laboratory was 1260 ± 236 nmoles/g tissue (mean ± S.E., n = 7; unpublished observations, made by phosphorylating endogenous choline in the presence of choline kinase and γ-[^{32}P]ATP to form [^{32}P]PhCh [30]). Following a 2-hr delay period for dissecting and handling the tissue fragments as outlined above, there was no significant increase in the concentrations of free choline. When the tissue was kept on ice for 24 hrs following the initial choline determination, choline levels were increased by about 80 per cent. It is difficult to decide from these preliminary observations whether free choline was generated during the initial storage period but immediately metabolized again. Obviously, the placenta behaved very differently from brain tissue where choline metabolism has been studied in more detail and where the free choline concentration increased 5-fold at room temperature within 15 min after the interruption of blood flow [31]. When placenta fragments were incubated with 5 μM [^{3}H]choline radioactivity appeared rapidly in the ICW compartment. This concentration was chosen because it was quite close to the concentration of free choline reported in human blood plasma at the time when these experiments were begun (1–2 μg/ml = 7–14 nmoles/ml [17]). Accumulation was linearly related to incubation time for 15–30 min, then slowed down and reached steady state values in about 3 hr. ICW concentrations of ^{3}H exceeded those in the incubation medium within 3–5 min and the concentration ratio rose about 4.0 within 15 min, 7.5 in 30 min, 11.5 in 60 min and to 20.0 and higher in 3 hr (Fig. 1). Fragments from some placentae (about 10 per cent of the specimens examined) took up [^{3}H]choline much more rapidly than the majority of the specimens. Differences in the mode of delivery (vaginal vs Caesarean section) were not the cause for these variations. It remains to be established whether these variations might be related to the choline content of the placenta. Regardless of the quantitative differences, qualitatively the plots of concentration ratios vs incubation time, and a variety of modifications in the incubation conditions were well reproducible when expressed in terms of per cent of control values.

The movement of [^{3}H]choline into ICW was markedly altered by variations in the incubation temperature. The concentration ratios obtained in a representative experiment at 0°, 17°, 27°, and 37° are shown in Fig. 2. With 5 μM [^{3}H]choline at 0° equilibration by diffusion was reached between 20–30 min, and the ratio C_i/C_o remained 1.0 thereafter for several hours. This diffusion contribution was deducted from all concentration ratios already corrected for ECW based on [^{14}C]inulin space, and the resulting values are referred to as net concentration ratios. The value of the ratio attributable to diffusion was indistinguishable from the one obtained in the presence of high choline concentrations at 37° (see following paragraph).

Effects of choline concentration. Upon variation of the choline concentration between 0.05 μM and 5 mM the ratios obtained were not different from 0.05 μM to 75 μM, but then began to decrease and reached values around 1.0 at 10 mM. This indicated saturability of the [^{3}H]choline accumulation. When 50 mM [^{3}H]choline was incubated with placenta fragments concentration ratios of about 1.0 resulted, a value

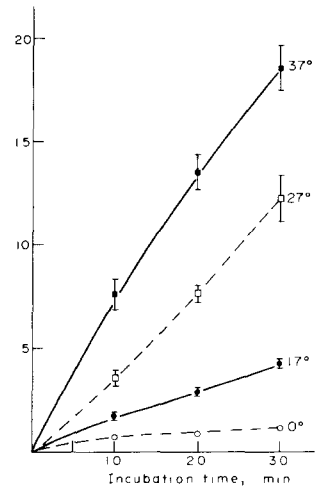

Fig. 2. Effects of various incubation temperatures on accumulation of [^{3}H]choline by human term placenta fragments. Fragments were incubated in standard medium containing 5 μM [^{3}H]choline at 0° ○——○, 17° ●——●, 27° □- - -□ and 37° ■——■ for the time periods indicated. Ordinate: corrected concentration ratios (see Fig. 1). Each point is the mean ± S.D. of 4 determinations from one placenta.

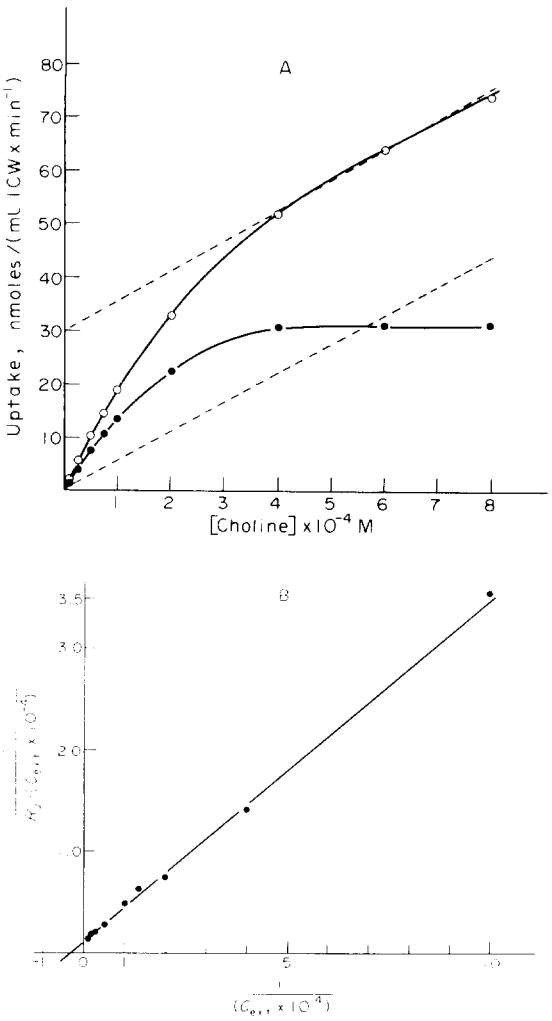

Fig. 3. Effects of choline concentration on accumulation of [^{3}H]choline by human term placenta fragments. Panel A: Fragments were incubated with [^{3}H]choline concentrations ranging from 0.1×10^{-4} M to 8×10^{-4} M. The radioactivity present in the intracellular water compartment was calculated after correction for extracellular water. Ordinate: Accumulation expressed in nmoles ml intracellular water $\times$ min^{-1} based on the sp. act. of [^{3}H]choline. Dashed line indicates contribution by non-saturable diffusion estimated from slope resulting from choline concentrations higher than 4×10^{-4} M and by extending this line to intersect with the ordinate. Total accumulation ○ ○ and saturable accumulation ● ● corrected for the non-saturable component by deduction of the values depicted in the lower dashed line.

Panel B: Saturable accumulation analyzed according to Schuberth *et al.* [1] by plotting the reciprocal of the saturable active component of the concentration ratio (R_a) multiplied by the concentration of choline in the medium (C_{ext}) against the reciprocal of C_{ext}.

which was essentially identical to the ratios obtained at 0 with 5 μM. Either approach has been used by other investigators to assess the contribution of non-saturable diffusion [1, 32]. Subsequently a narrower concentration range was examined and revealed a slope typical for a dual uptake mechanism with a non-saturable and a carrier-mediated saturable component (Fig. 3, panel A). If the net concentration values obtained during the linear uptake phase were calculated by deducting the non-saturable component and were plotted according to Schuberth *et al.* [1] a straight line resulted. In this graphical analysis the reciprocal of the saturable, and presumably active, uptake component (R_a) times outside concentration of choline (C_{ext}) was plotted against the reciprocal of C_{ext} (Fig. 3, panel B). The line which was obtained indicated that the saturable choline uptake followed kinetics predicted by the Michaelis Menten equation with an apparent K_m ($\cdot 10^{-4}$) 3.46 ± 0.72 M and a V_{max} of 7.5 ± 1.5 nmoles ml ICW^{-1} min^{-1} (mean ± S.E., n = 5). Based on the observations obtained from intact placenta fragments with eight choline concentrations ranging from 0.05 to 10 μM, there was only one choline transport system as was originally reported to exist in brain slices [1, 2, 4] while more recent studies on subcellular brain fractions have revealed a high and low affinity choline uptake system in synaptosomes [6–8, 32].

Effects of oxygen and glucose deprivation. When placenta fragments were collected, washed and incubated in KHM saturated with 5% CO_2 in nitrogen, [^{3}H]choline accumulation in ICW was not different from control samples gassed with 5% CO_2 in oxygen after 15 min of incubation (Fig. 4) but the concentration ratios were significantly reduced after 30 min. Omission of glucose or replacement by equimolar 2-deoxyglucose had no effect after 15 and 30 min of incubation. These results did not appear surprising in view of the observation that active uptake of amino acids by placenta slices was not abolished following 1.6 hr of anaerobic incubation [33] and were in

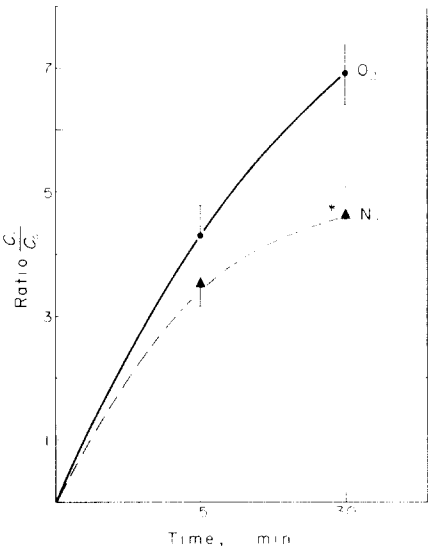

Fig. 4. Effect of anaerobic incubation conditions on accumulation of [^{3}H]choline by human term placenta fragments. Fragments were incubated in standard medium gassed with 5% CO_2 in oxygen ● ● or 5% CO_2 in nitrogen ▲ ▲ containing 5 μM [^{3}H]choline for the time periods indicated. Ordinate: net concentration ratios after deduction of extracellular water space and diffusional component. Each point is the mean ± S.D. of quadruplicate determinations from 4 placentae. Value marked with asterisk differs significantly from O_2 control ($P < 0.01$, one-tailed t-test).

Table 1. Effects of metabolic inhibitors on accumulation of [^{3}H]choline by human term placenta fragments

Incubation time (min)	Metabolic inhibitor (M) 2,4-DNP 1×10^{-3}	2,4-DNP 1×10^{-4}	NaCN 5×10^{-3}	NaCN 1×10^{-3}	Ouabain 1×10^{-4}
15	35.77 ± 3.03 (3)	29.80 ± 3.12 (3)	53.73 ± 4.99 (4)	37.27 ± 7.97 (4)	50.52 ± 6.67 (4)
30	64.93 ± 8.49 (4)	41.36 ± 12.50 (4)	59.17 ± 5.91 (5)	62.11 ± 6.39 (4)	49.75 ± 2.89 (4)

Placenta fragments were incubated in standard medium to which the inhibitors in the concentrations indicated were added 15 min prior to the addition of [^{3}H]choline. Data are expressed in terms of per cent inhibition produced at the incubation times specified. Values are means ± S.D., and the numbers in brackets are the number of placenta specimens which were examined in quadruplicate samples. Net concentration ratios of control values were 3.38–7.56 (5) at 15 min and 5.58–13.84 (5) at 30 min.

agreement with the results on ACh uptake where it required incubation times of more than 1 hr for the manifestation of the effects of 2-deoxyglucose [25].

Effects of various metabolic inhibitors. When oxidative phosphorylation and/or electron transport was inhibited with 2,4-dinitrophenol (DNP, 1 mM and 0.1 mM) or NaCN (5 mM, 1 mM), both of which deplete intracellular ATP levels, accumulation of [^{3}H]choline was markedly reduced (Table 1). Ouabain (0.1 mM) also decreased the amount of radioactivity in ICW drastically. This suggested that Mg^{2+}-dependent Na^+ and K^+ activated ouabain-sensitive adenosine triphosphatase (ATP phosphohydrolase, E.C. 3.6.1.3., Na^+-K^+-ATPase) which has been identified in human term placenta [34] could be involved in choline accumulation.

Effects of variations in the ionic environment. Gradual reduction or complete omission of Na^+ and replacement by an osmotically equivalent amount of sucrose led to a dramatic increase in [^{3}H]choline accumulation (Fig. 5), which amounted to 36, 60 and 150 per cent of the 145-mM NaCl-containing solution when Na^+ was 72, 22 and 0 mM, respectively. The concentration ratio-enhancing effect of complete lack of Na^+ was also present when the choline concentration was lowered to between 0.1 and 10 μM. When the effects of variation of K^+ concentrations (which were always paralleled by an equimolar change in Na^+ in the opposite concentration direction such that overall no change would result in osmolarity) were examined, a continuous decline of [^{3}H]choline uptake was observed (Fig. 6). The most profound

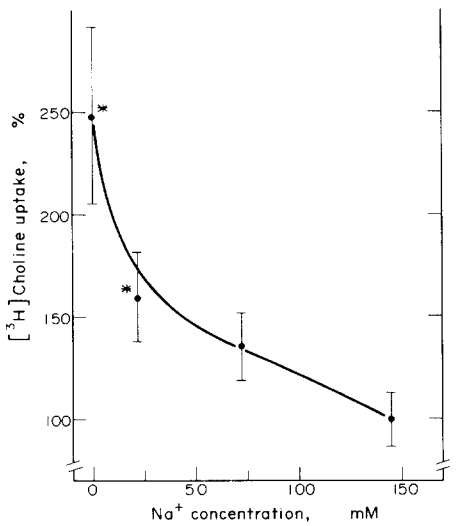

Fig. 5. Effects of variations in Na^+ concentration on accumulation of [^{3}H]choline by human term placenta fragments. Fragments were incubated in HEPES buffered medium containing 5 μM [^{3}H]choline for 30 min with the Na^+ concentrations indicated in the abscissa. Ordinate: Per cent [^{3}H]choline uptake, where the net concentration ratio of control values (145 mM NaCl and 4.8 mM KCl) of 4.13 ± 0.53 was set to be 100 per cent. NaCl was replaced by twice the concentration of sucrose to maintain isosmolarity. Each point shows the mean ± S.D. of at least 16 determinations obtained from at least 4 different placentae. Values marked with asterisk are significantly different from control ($P < 0.05$).

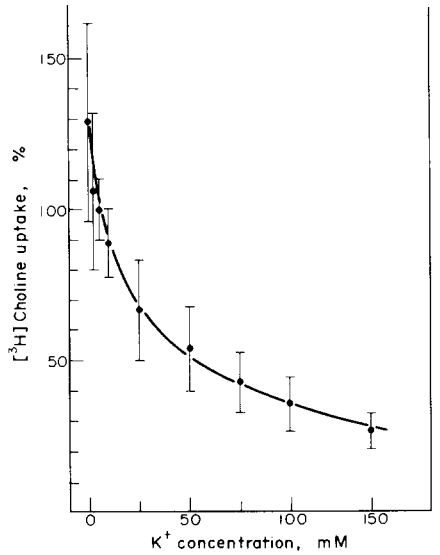

Fig. 6. Effects of variations in K^+ concentration on accumulation of [^{3}H]choline by human term placenta fragments. Fragments were incubated in HEPES buffered medium containing 5 μM [^{3}H]choline for 30 min with the K^+ concentrations indicated in the abscissa. Na^+ was changed by an equimolar amount such that NaCl and KCl equaled 150 mM. Ordinate: Per cent [^{3}H]choline uptake where the net concentration ratio of control values (145 mM NaCl and 4.8 mM KCl) of 4.41 ± 0.16 was set to be 100 per cent. Each point shows the mean ± S.D. of 12 determinations from 3 different placentae. All values with $K^+ > 10$ mM are significantly different from the control value ($P < 0.05$).

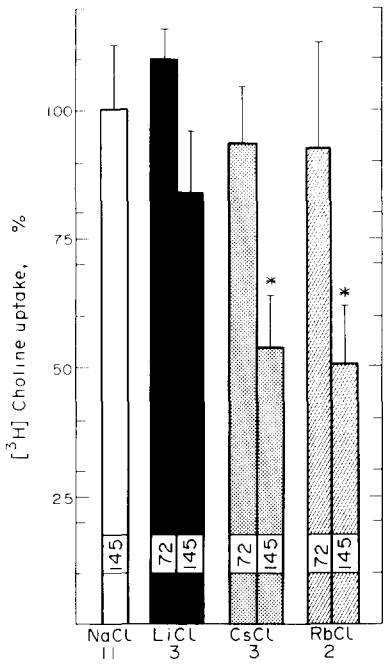

Fig. 7. Effects of Li^+, Cs^+ and Rb^+ on accumulation of [^{3}H]choline by human term placenta fragments. Fragments were incubated in HEPES buffered medium containing 5 μM [^{3}H]choline for 30 min. NaCl was partially or completely replaced by LiCl, CsCl or RbCl as indicated in the abscissa. Vertically positioned numbers in bars give the mM concentration of the ions examined. Ordinate: Per cent [^{3}H]choline uptake where the net concentration ratio of control values (145 mM NaCl and 4.8 mM KCl) of 4.33 ± 0.15 was set to be 100 per cent. Each bar shows mean ± S.D. (or variance if $n = 2$) of the numbers of placentae examined in quadruplicate samples stated below the respective salts. Values marked with asterisk are significantly different from control ($P < 0.05$).

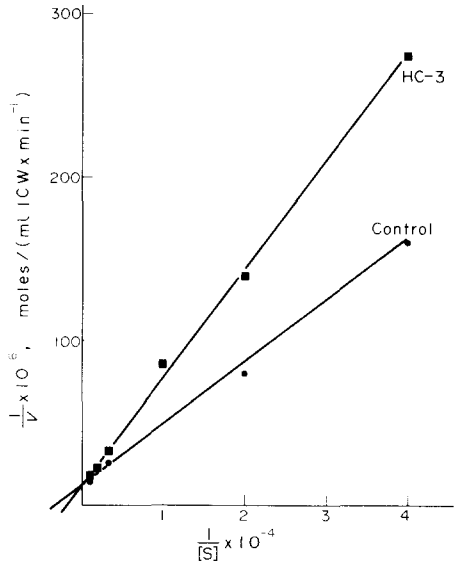

Fig. 8. Effects of hemicholinium-3 on the accumulation of [^{3}H]choline by human term placenta fragments. Fragments were incubated in standard medium (with or without 1×10^{-4} M HC-3) containing [^{3}H]choline concentrations varying between 0.25×10^{-4} M and 7×10^{-4} M. Abscissa: Reciprocal of molar choline concentration $\times 10^{-4}$; Ordinate: Reciprocal of amount of choline taken up into the intracellular water compartment. Each point is the mean of 12 determinations from 3 placentae.

depression under the experimental conditions chosen occurred at 150 mM KCl and 0 mM NaCl. Thus, the stimulating effect of lack of NaCl with osmotic make-up by sucrose was completely reversed in the presence of high concentrations of KCl. Partial or complete replacement of Na^+ by lithium (LiCl), which in some respects closely resembles Na [35] was without significant effect on the [^{3}H]choline accumulation (Fig. 7) compared with control levels of NaCl, but LiCl inhibited the stimulation of [^{3}H]choline accumulation observed in the absence of NaCl in a sucrose-containing environment. Complete replacement of NaCl by cesium (CsCl) or rubidium (RbCl) which resembles K^+ closely in some of its biologic characteristics [35] resulted in a significant reduction of the amount of ^{3}H label in ICW with values reaching about 50 per cent of control NaCl concentrations after 30 min of incubation (Fig. 7). The RbCl-caused decrease was similar to the one produced by high concentrations of KCl.

Effects of hemicholinium-3 (HC-3). HC-3 has been widely used in experiments which have examined choline uptake and metabolism in a variety of tissues. From these studies the drug has become well known as a competitive inhibitor of choline transport carrier systems. The placenta proved to be no exception. Choline uptake by the fragments was inhibited by HC-3, and when the effect was analyzed graphically with a Lineweaver Burk plot [36] the lines obtained for control and HC-3-treated fragments had the same intercept on the ordinate but different intercepts on the abscissa which was indicative of competitive inhibition (Fig. 8). When accumulation was measured

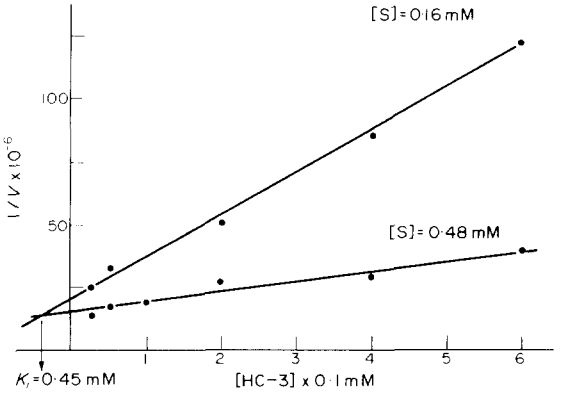

Fig. 9. Dixon analysis of inhibition of [^{3}H]choline accumulation by hemicholinium-3 in human term placenta fragments. Fragments were incubated in standard medium containing two different concentrations of choline [S] in the presence of various concentrations of hemicholinium-3 [HC-3] as indicated in the abscissa. Ordinate: Reciprocal of amount of choline taken up into the intracellular water compartment (moles/ml × min^{-1}) after 15 min incubation. The data describe a straight line for each concentration of [^{3}H]choline. The point of intersection of the two lines gives K_i on the abscissa and $1/V_{max}$ where they intersect with the ordinate. Each point is the mean of 8 determinations from 2 placentae.

Table 2. Distribution of ^{3}H-label following incubation of human term placenta fragments in [^{3}H]choline

Time min	Acid-soluble dis/min	%	Acid-insoluble dis/min	%
5	69443 ±6184 (3)	94.3	4195 ±230 (3)	5.7
10	116840 ±23120 (4)	92.9	8895 ±1255 (4)	7.1
20	175480 ±37703 (4)	90.5	18460 ±2728 (4)	9.5
30	201901 ±18227 (4)	88.0	27388 ±3177 (4)	12.0

Fragments were incubated in 5 ml standard medium containing 2 μCi [^{3}H]choline for the time periods specified. Tissue was homogenized in TCA and distribution of radioactivity measured between the acid-soluble fraction and the acid insoluble precipitate which was extracted with chloroform–methanol, (2:1 v/v). Each value shows the mean ± S.D. of duplicate determinations from the number of placentae indicated in brackets. Data are also expressed in terms of per cent distribution of the total radioactivity in each homogenized sample.

with 2 choline concentrations below and above K_m in the presence of various concentrations of HC-3, analysis of the data obtained by means of a Dixon plot [36] revealed a K_i of 0.45 mM (Fig. 9).

Fate of [^{3}H]choline in placenta fragments. Following a 5-min incubation in paraoxon containing KHM, 95 per cent of the ^{3}H label was found in the acid-soluble fraction (Table 2) and 5 per cent was in the acid-insoluble residue where in turn the radioactivity was almost completely soluble in chloroform–methanol. This suggested an association of label with lipid metabolites. Between 5 and 30 min the acid-soluble portion decreased gradually to 88 per cent, while at the same time the chloroform–methanol extract from the acid-insoluble residue increased to 12 per cent of the total radioactivity in a fashion which was linearly dependent on the incubation time. This was indicative of a time-dependent incorporation of [^{3}H]choline into lipid material. Very little radioactivity was found at any of the incubation times in the acid-insoluble residue once extracted with the lipid solvent.

Further attention was focused on the acid-soluble fraction which would contain the precursor [^{3}H]choline and [^{3}H]ACh, the metabolite of major interest. A typical example of an electropherogram of placenta extract and of authentic standards is shown in Fig. 10. Recovery of radioactivity after elution from the paper was 93.90 ± 5.59% (mean ± S.D., n = 32) of the amount applied to the starting line. PhCh moved only about 2 cm. Larger amounts of authentic substance were required for PhCh and betaine to produce iodine staining than for ACh and choline.

When the distribution of radioactivity among the different metabolites was calculated it became apparent that [^{3}H]choline was rapidly converted to several products. Most prominent was the labelling of ACh. After 5 min incubation 36 per cent of the ^{3}H was found in the ACh region (Fig. 11). This increased to 45 per cent after 10 min and reached a plateau of 60 per cent after 20 min. Concomitantly, [^{3}H]choline had dropped to 55 per cent of the total acid-soluble ^{3}H after 5 min, 44, 28 and 24 per cent after 10, 20 and 30 min, respectively. The only other metabolite which increased significantly during the 30 min incubation period was PhCh, amounting to 7 per cent after 5 min and increasing to 17 per cent after 30 min. There was always ^{3}H label in that area of the electropherogram where authentic betaine would migrate but the increase in the labelling of this component was not significant over the 30-min period. The areas corresponding to betaine and ACh were eluted in a separate experiment, the eluates from all the paper strips tentatively assigned to each compound were combined, lyophilized and again subjected to electrophoresis. Again the radioactivity migrated a distance

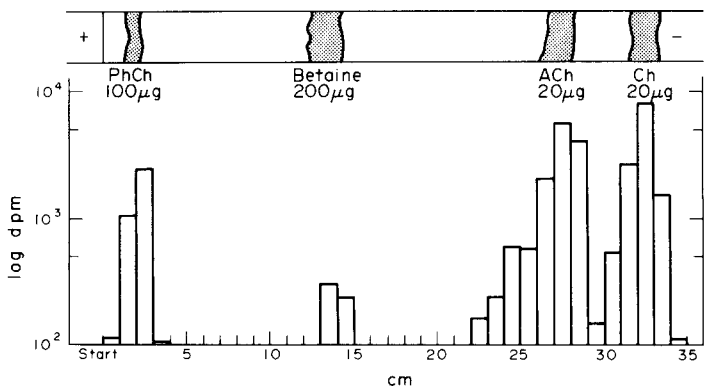

Fig. 10. High voltage electrophoresis of acid-soluble placenta extract and of authentic standards. Lyophilized acid-soluble radioactivity derived from incubation with 5 μM [^{3}H]choline was dissolved in formic acid acetic acid buffer pH 2.0 and applied to dry Whatman No. 1 paper strips, 3 × 40 cm. For further details see Methods. Authentic substances were run on parallel strips. Abscissa: migration distance in cm. Ordinate: log dis/min eluted from consecutive 1-cm sections of electrophoresis paper. This run derived from a sample incubated for 20 min. Comparison strip in upper part of the figure shows location of iodine vapor stained authentic standards PhCh, betaine, ACh and choline (Ch).

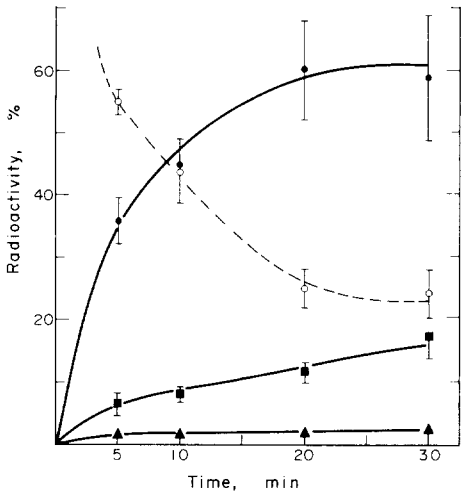

Fig. 11. Distribution of ^{3}H-label following high voltage electrophoretic separation of acid-soluble placenta extract. Radioactivity was eluted from acid-soluble extracts derived from incubation with 5 μM [^{3}H]choline which were subjected to high voltage electrophoresis. Abscissa: Incubation time of fragments in min. Ordinate: Per cent of total radioactivity recovered from eluted electrophoresis paper attributable to each of the 4 identified compounds. Each point is the mean ± S.D. of duplicate determinations from 3 placentae at 5 min and 4 placentae at all other times. ○ ○ Choline, ● ● Ach, ■ ■ PhCh and ▲ ▲ betaine.

equal to the authentic standards, producing a sharper peak of radioactivity associated with ACh than in the first run.

When a part of the acid-soluble fraction was subjected to liquid cation exchange with tetraphenylboron, 8 per cent of the radioactivity did not complex with this compound (2 experiments) after 5 min of incubation in [^{3}H]choline. This increased to 18 per cent after 30 min. These values agreed quite satisfactorily with the percentage of radioactivity attributable to PhCh and betaine based on the electrophoretic separation (about 9 and 19 per cent respectively, Fig. 11).

When [^{3}H]choline and [^{14}C]ACh were added to placenta homogenates in TCA and subjected to tetraphenylboron liquid cation exchange or electrophoresis about 5 per cent of ^{3}H but only less than 2 per cent of ^{14}C did not complex with tetraphenylboron. On the electropherograms some label was always found in the area of PhCh. These observations implied that a certain amount of ^{3}H got into PhCh without the involvement of choline kinase but by spontaneous base exchange. Negligible radioactivity remained in the acid-insoluble fraction. In regards to PhCh labelling this [^{3}H]choline base exchange would mean that the observed values could include a certain percentage of incorporation which was nonenzymatic.

DISCUSSION

Several observations support the interpretation that the choline accumulation observed in human term placenta fragments met conventional criteria of an active transport. All conditions which decreased the intracellular levels of ATP reduced the uptake of choline, supporting the contention that [^{3}H]choline movement against its concentration gradient was dependent on continuous cell metabolism and generation of ATP. The presence of DNP which uncouples oxidative phosphorylation by inhibition of electron transport in mitochondria thus depleting ATP [33] or the presence of NaCN, a non-specific inhibitor of electron transfer through heme proteins to oxygen which causes a profound depression of ATP [37], led to a marked inhibition of ^{3}H accumulation (Table 1). Anaerobic incubation also reduced [^{3}H]choline uptake although the effect was not significant prior to 30 min. These observations can be explained by the ability of the placenta to use the glycolytic pathway extensively [38, 39] and to maintain active transport during hypoxia [33].

The net concentration ratios of ^{3}H in ICW exceeded the value of the surrounding medium very rapidly. However, a value larger than unity did not necessarily indicate choline accumulation against a concentration gradient because metabolic conversion of [^{3}H]choline could explain such a distribution of radioactivity without participation of active transport. The physiological conditions concerning the concentrations of free choline in the maternal blood and placenta favored the idea that an active transport process coupled to the expenditure of metabolic energy could be operating because choline would have to move against a steep concentration gradient if free choline in the blood was used as a source of choline by placenta or fetus. Using the choline values of 7–16 μM reported in blood plasma of man [17, 18] and our preliminary measurements of 1250 nmoles/g term placenta (= 1250 μM) there is a concentration difference of about 80–180-fold against which choline would have to be transferred in the *in vivo* situation. The real free choline concentration in the placenta may even be higher. This is because the above estimate was based on an even distribution while it is not unreasonable to expect most of the choline to be in the ICW compartment, which makes up 20–45 per cent of TW in human term placenta [24, 40].

Metabolic conversion was an important aspect to be considered. Net concentration ratios after 10 min were 3.0 and higher, varying with the placenta specimens (Figs. 1, 2 and 4). Using the [^{3}H]distribution (Table 2) and the labelling in the precursor and metabolites (Fig. 11) both of which derived from paraoxon-exposed tissue (where ACh was able to accumulate) the net concentration ratios of free [^{3}H]choline were still in excess of 1.0. Since all other experiments were performed in paraoxon-free incubation medium it can be expected that a significant portion of newly synthesized [^{3}H]ACh would be hydrolyzed by acetylcholinesterase. Thus, more of the ^{3}H would be present as free [^{3}H]choline in ICW than the data with paraoxon present showed.

Marked effects on [^{3}H]choline accumulation were observed when the ionic milieu in the bathing solution was altered. Since high K^+ is commonly used to depolarize cells, it could be argued that the [^{3}H]choline accumulation observed with physiological K^+ concentrations was a cation phenomenon for which K^+ concentration differences provided the

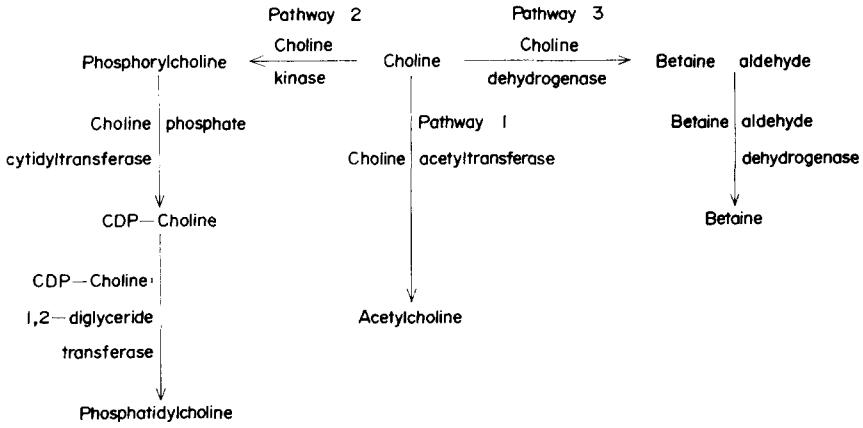

Fig. 12. Metabolic pathways of choline.

electrochemical gradient and thus the driving force. High K^+ also decreased [^{3}H]choline uptake in brain slices significantly [2, 4]. It is important at this point to stress the striking differences between brain tissue and placenta. The human placenta is a non-excitable tissue and its resting membrane potential in fresh term placentae was only 0–20 mV, and disappeared altogether in less than 3 hr [41]. These measurements discount the probability than an electrochemical gradient was responsible for the [^{3}H]choline accumulation. Additional support against this interpretation derived from the observation that an equimolar concentration or a 10-fold molar excess of the quaternary compound tetramethylammonium did not alter the concentration ratios. To an unknown degree the depression of choline accumulation by high K^+ could also be related to intracellular levels of ATP, because it has been reported that high K^+ lowered ATP [42].

Although ouabain inhibited the [^{3}H]choline accumulation this had probably no direct relationship to Na^+-K^+-ATPase and its function in cation transport because the choline cation will not bind to and substitute on this enzyme for Na^+ or K^+. Rather, the effect may be related to the significant use in the placenta of the glycogenolytic pathway. Ouabain inhibits ADP formation by inhibition of Na^+-K^+-ATPase. ADP is a crucial substrate of phosphoglycerate kinase during the synthesis of ATP via the glycolytic pathway. This enzyme has been shown to be an important rate-limiting point linking cation transport and glycolysis in erythrocytes which depend almost exclusively on energy provided by glycolysis [43].

An unusual feature compared to the different tissues in which choline uptake has been studied was the stimulation of ^{3}H accumulation in the absence of Na^+. A similar effect has been observed by one group of investigators who studied guinea pig brain synaptosomes [3]. The actions of K^+, Li^+ and Cs^+ were more comparable to the changes which these ions brought about in choline carrier systems of other tissues [2–4, 8, 15]. The carrier mediated transport in placenta had also in common with all other tissues that it was competitively inhibited by HC-3.

It is interesting to compare the metabolic fate of radioactive choline in the variety of tissues where its uptake has been studied. The results obtained in the placenta (Fig. 11, Table 2) in the presence of paraoxon suggested that synthesis of [^{3}H]ACh was the major product of enzymic activity (Fig. 12, Pathway 1). Pathway 2, the choline kinase catalyzed synthesis of [^{3}H]PhCh, was also quite prominent. This metabolite also provided the precursor for the biosynthesis of those products which appeared in the acid-insoluble but chloroform–methanol-soluble lipid extract. This fraction probably contained phosphatidylcholine. Pathway 3, leading to the synthesis of betaine was not very prominent in the placenta, while this compound was the main metabolite in the kidney [14]. When brain slices were incubated with radiolabelled choline in the absence of a cholinesterase inhibitor, very little ACh was formed and most of the radioactivity was present as unchanged choline [1, 4], while synaptosomes synthesized substantial amounts of radioactive ACh in the presence of physostigmine [5].

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