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CHOLINE METABOLISM IN HUMAN TERM PLACENTA—
STUDIES ON *DE NOVO* SYNTHESIS AND THE EFFECTS OF SOME DRUGS ON THE METABOLIC FATE OF [*N*-METHYL-^{3}H]CHOLINE

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Abstract—Fragments from human term placentae were incubated with [^{3}H]methyl-labeled methionine or *S*-adenosyl methionine as methyl donors and ethanolamine or phosphatidyldimethylethanolamine as methyl acceptors. Analysis of acid-soluble and lipid-soluble extracts by high voltage electrophoresis or thin-layer chromatography, respectively, gave no indication for the synthesis of products which would be expected to contain [^{3}H]label in the choline (Ch) moiety. These findings led to the conclusion that the placenta could not perform the methylation steps required for *de novo* synthesis of choline. Short incubations (1, 2.5, 5 and 10 min) with [^{3}H]Ch (5 μM, 0.8 μCi/ml) and paraoxon (10 μM) as an inhibitor of cholinesterases revealed that [^{3}H]Ch was quite rapidly incorporated into [^{3}H]acetylcholine (ACh) and that 18 per cent of the acid-soluble radioactivity was associated with ACh after 1 min. In the lipid-soluble fraction the major labeled product was identified as phosphatidylcholine while a minor amount of [^{3}H](< 5 per cent) was found in lysolecithin. Paraoxon was without effect on [^{3}H]Ch uptake into fragments. However, its omission significantly—yet much less than expected from innervated tissues—decreased [^{3}H] in ACh after 10 min (28 vs 44 per cent) and 20 min (37 vs 62 per cent) and increased the radioactivity remaining as free [^{3}H]Ch. 2,4-Dinitrophenol reduced [^{3}H]Ch uptake and inhibited preferentially the incorporation of tritium into phosphorylcholine (5 min: 2.5 vs 5.4 per cent; 10 min: 3.0 vs 9.7 per cent; 20 min: 6.5 vs 15.6 per cent; and 30 min: 7.5 vs 20.0 per cent). Removal of Na^+ ions accelerated the uptake of [^{3}H]Ch, but it depressed the synthesis of [^{3}H]ACh. In Na^+-free medium the percentage of acid-soluble radioactivity associated with ACh was always significantly lower (2.5 min: 9.8 vs 23.3 per cent; 5 min: 13.0 vs 35.9 per cent; 10 min: 19.7 vs 44.9 per cent; and 20 min: 26.8 vs 62.3 per cent). Thus, although there was no indication for a high-affinity, Na^+-dependent [^{3}H]Ch uptake in placenta, ACh synthesis was markedly affected by lack of Na^+ ions.

The importance of the placenta for the supply of choline (Ch), a prominent constituent of lipid-soluble and several water-soluble metabolites, to meet placental and fetal needs is not well established. Choline containing phospholipids cannot cross the placenta readily [1], and the ability of the fetal liver to synthesize Ch appears to be limited. Therefore a source for this important lipogenic base may be the placenta itself or Ch produced in the maternal liver, an organ which has the highest synthetic capability in the body [2], and the placenta is involved in the transport of this Ch. We have recently shown in our laboratory that free Ch was rapidly taken up against a concentration gradient from extracellular water into the intracellular water compartment when human term placenta fragments were incubated *in vitro* [3]. This suggested that free Ch, circulating in human blood in concentrations of 7–16 μM [4, 5], may be one of the sources of Ch required by placenta and fetus. Our study also demonstrated that the amine was rapidly incorporated into several esterified products among which acetylcholine (ACh) was by far the most prominent [3]. The latter product is a prominent constituent of the human placenta, where its functions are poorly defined. It has now been confirmed by gas chromatographic analysis that the high ACh-like activity observed more than 40 yr ago by bioassay of human placenta extracts is indeed due to the presence of ACh. The concentrations measured in this organ which has no innervation were reported to be in excess of 100 nmoles/g of fresh tissue at term [6]. It is also well established that the human placenta contains a very active choline acetyltransferase (acetyl-CoA-choline *O*-acetyltransferase, EC 2.3.1.6, ChAc) that exhibits as characteristic gestational period-dependent fluctuations [7] as the ACh tissue levels do [6]. Furthermore, it has been shown that the enzyme kinetics and ionic requirements of placental ChAc were indistinguishable from the brain enzyme [8].

The present paper reports experiments designed to examine the ability of the placenta to synthesize Ch *de novo*. Furthermore, it describes the identification of lipid-soluble metabolites synthesized during incubations with [^{3}H]Ch and the effects of paraoxon, 2,4-dinitrophenol (2,4-DNP) and Na^+ ions on the metabolic fate of [^{3}H]Ch *in vitro*.

MATERIALS AND METHODS

Tissue sources, preparation and solutions were essentially identical to the descriptions given elsewhere in detail [3, 9]. Since the measurement of concentration ratios was not the aim in this series of experiments, no labeled inulin was added. When an inhibitor of cholinesterases was indicated, 10 μM paraoxon was present in the incubation medium.

All aspects of isolation and identification of [^{3}H]Ch-containing metabolites were identical to those described, except for some modifications in the high voltage electrophoresis (HVE) separation of the acid-soluble fraction as follows [3]. The sam-

ples to be analyzed were applied to the starting line drawn 5 cm away from the anodal end of a 20 × 40 cm sheet of Whatman No. 1 paper divided into seven tracks. The dry sheet was dampened to within about 0.6 cm on either side of the starting line with a brush, thus allowing buffer (100 ml pyridine and 100 ml formic acid to 4 l water, pH 3.6 [10]), to diffuse toward the starting line and concentrate the applied sample. HVE was performed at 1800 V (45 V/cm) for 70 min. (CAMAG HVE-system, New Berlin, WI). Although the migration of Ch and ACh was much slower than in formic acid–acetic acid buffer [3], the extra time required with this particular buffer brought about sharper spots of Ch and ACh and thus better separations.

In preparation for thin-layer chromatography (t.l.c.) of the chloroform–methanol (2:1; v/v) extract, the lipid-soluble material which included the Ch-containing polar phospholipids was purified as described by Hajra *et al.* [11]. The final residue was dried and reconstituted in 100 μl chloroform of which 25 μl was applied to Silica gel plates (Silica gel 60, no. 5763, Brinkmann, Westbury, NY). The plates were developed in chloroform–methanol–glacial acetic acid–water (50:32:11:3), and the individual phospholipids were localized by I_2 vapor staining of authentic standards (Supelco, Bellefonte, PA). Initially, radioactivity on t.l.c. plates was determined by scraping consecutive 1-cm bands of plate coating into counting vials. However, when it became apparent that only two phospholipids were labeled, the scraping was restricted to those areas of the plate. Radioactivity was eluted from the gel by extraction with 3 ml methanol, and tritium was determined in a toluene base scintillation fluid with a model 3380 liquid scintillation spectrometer equipped with a model 544 absolute activity analyzer (Packard Instruments, Downers Grove, IL).

Paper chromatographic analysis of acid-soluble material

HVE was not capable of separating cytidyldiphosphocholine (CDP-Ch) from phosphorylcholine (PhCh) in either buffer. In order to assess the distribution of label between those two metabolites, a descending paper chromatographic system on Whatman No. 1 was used [12]. Cochromatography of authentic standards allowed the localization of CDP-Ch and PhCh, and radioactivity was determined in aqueous eluates obtained from corresponding areas of the sample tracks.

De novo *synthesis of Ch*

In these experiments 300 mg of placenta fragments was incubated in a final volume of 3 ml Krebs–Henseleit medium. The precursors used were ethanolamine and phosphatidyldimethylethanolamine (1.0 mM) as acceptors of labeled methyl groups from *l*-methionine[methyl-^{3}H] or *S*-adenosyl-*l*-methionine (methyl-^{3}H, 1 μCi/ml 0.1 mM) as methyldonors. Alternatively, [2-^{14}C]ethanolamine (1 μCi/ml) was used with unlabeled methyl donors.

After incubations for 0, 30, 60, 120 and 180 min in duplicate, the tissue fragments were separated from the incubation medium by rapid filtration as described for the Ch uptake studies and washed repeatedly with ice-cold incubation medium containing carrier precursors but free of radioactivity [3]. The samples were homogenized with 2 ml of ice-cold water in ground-glass homogenizers (Tenbroeck type, Kontes, Vineland, NJ). Homogenizers and pestles were rinsed three times with 1 ml each, resulting in a total of 5 ml homogenate. Two ml of this homogenate was mixed with 0.3 ml of 50% trichloroacetic acid (TCA) to precipitate protein and obtain an acid-soluble fraction, while another sample of equal volume was extracted with 8 ml chloroform–methanol to constitute the lipid-soluble fraction. Both extracts were purified as described and subjected to HVE or t.l.c. analysis. For the acid-soluble fraction, authentic standards of dimethylethnolamine, Ch, PhCh and ACh were coelectrophoresed on adjacent tracks. In order to allow an accurate localization of the potential phospholipid metabolites, authentic standards of phosphatidyldimethylethanolamine, phosphatidylcholine, lysolecithin and sphingomyelin were cochromatographed on adjacent tracks of the same plate. Radioactivity corresponding in migration to that of known standards on electrophoresis paper or t.l.c. plate coating was eluted from paper as described before [3] and from Silica gel as indicated above.

Radiochemical sources

[Methyl-^{3}H]choline chloride (sp. act. 10.1 Ci/m-mole) and [2-^{14}C]ethan-1-ol-2-amine hydrochloride (sp. act. 55 mCi/m-mole) were obtained from Amersham/Searle (Arlington Heights, IL) while *l*-methionine[methyl-^{3}H] (sp. act. 14.6 Ci/m-mole) and *S*-adenosyl-*l*-methionine[methyl-^{3}H] sp. act. 12.25 Ci/m-mole) were purchased from New England Nuclear (Boston, MA).

RESULTS

De novo *synthesis experiments*

Ch may be generated by two different metabolic pathways. One route is the stepwise methylation of ethanolamine via its mono- and dimethyl-substituted precursors, while the other possibility is a comparable sequence of methylations occurring while ethanolamine is already bound in its phospholipid derivative phosphatidylethanolamine [13]. The latter pathway gives rise to phosphatidylcholine, from which Ch can be liberated by enzymatic action [2, 13]. Phosphatidylcholine (46 per cent) and phosphatidylethanolamine (28 per cent) are the most prominent phospholipids of the human placenta [14]. The former is synthesized by human placenta fragments as indicated by intense labeling due to incorporation of [$^{32}P_i$] into this phospholipid [15]. The precursors used for our experiments were chosen so that both possibilities were considered. Further, by analysis of acid-soluble and lipid-soluble extracts resulting from incubations with any of the precursors, one should have been able to detect products which were preferentially soluble in one or the other solvent. Although the bathing medium was not analyzed for methyl-labeled metabolites, this should not alter the results as regards the ability of the placenta to

perform the type of methylations that were under investigation. The human placenta is a sponge-like tissue with high extracellular water content (50 per cent of total water, Welsch, unpublished observations and Ref. 9). Had there been any water-soluble [^{3}H]methyl-labeled material released into the extracellular water space, then this should have shown up in the acid-soluble tissue extract.

When [^{3}H]CH_3-methionine was used as the methyl donor, there was an incubation time-related accumulation of ^{3}H-label in the acid-soluble fraction, while only negligible labeling with no increase related to incubation time was found in the purified polar phospholipid fraction. Upon electrophoresis (formic acid–acetic acid buffer), radioactivity was found exclusively 5–7 cm cathodal from the origin, which corresponded to the migration rate and Ninhydrin staining of authentic [^{3}H]methionine. With *S*-adenosyl[^{3}H]CH_3-methionine as the methyl donor, uptake of ^{3}H-label showed no appreciable correlation to time, and acid-soluble extracts contained no significant amounts of radioactivity in those areas of the electropherograms where Ch and its acid-soluble precursors or esterified derivatives were located. Similarly, t.l.c. of the polar phospholipids gave no indication for incorporation of [^{3}H]methyl groups into phosphatidylethanolamine that would have resulted in the presence of the mono-, di- or trimethyl-substituted derivatives of phosphatidylethanolamine. Essentially the same conclusions were reached when [^{14}C]ethanolamine was used as a methyl acceptor for unlabeled methyl groups of *S*-adenosyl methionine. The labeling appeared to be confined to the ethanolamine moiety, while choline containing acid-soluble and lipid-soluble metabolites carried no ^{14}C-label. Further, the use of phosphatidyldimethylethanolamine as methyl acceptor revealed no indication for any [^{3}H]CH_3 transfer to phosphatidylcholine as judged from t.l.c. isolation of this phospholipid.

[^{3}H]*CH uptake experiments*

Identification of lipid-soluble radioactivity. The observations made in our previous study showed that [^{3}H]Ch was incorporated in a time-dependent manner into lipid-soluble material [3]. The metabolite most likely to be labeled was phosphatidylcholine, and, in order to confirm the speculation about the presence of this polar phospholipid, the purified extract was analyzed by t.l.c. The appearance of a

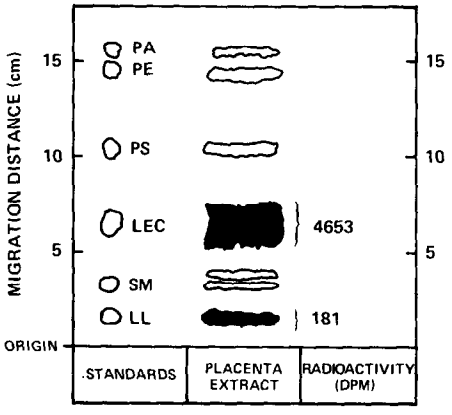

Fig. 1. Thin-layer chromatogram of lipid-soluble extract from human placenta fragments after incubation with 5 μM [^{3}H]choline. Chloroform–methanol (2:1, v/v)-soluble material was extracted from an acid-insoluble tissue pellet and purified [11]. Thin-layer chromatographic separation of polar phospholipids was accomplished in chloroform–methanol–glacial acetic acid–H_2O = 50:32:11:3, and phospholipids were visualized by I_2 vapor. Consecutive 1 cm wide bands of plate coating were scraped into counting vials for elution of phospholipids followed by liquid scintillation determination of radioactivity. Blackened areas indicate [^{3}H] label (expressed in dis./min). Radioactivity at $R_f = 0.10$ corresponded to the migration of authentic lysolecithin (LL) and at $R_f = 0.37$ to lecithin (LEC). No radioactivity was found in the region of sphingomyelin (SM). Other abbreviations used are: PA = phosphatidic acid; PE = phosphatidylethanolamine; and PS = phosphatidylserine.

Table 1. Per cent distribution of ^{3}H label among acid-soluble metabolites after separation by high voltage electrophoresis*

Time (min)	PhCh + betaine	ACh	Ch
1	2.18 ± 0.48 (3)	18.36 ± 5.00 (4)	79.58 ± 5.07 (4)
2.5	3.23 ± 0.76 (3)	23.36 ± 5.88 (4)	73.33 ± 5.25 (4)
5	4.94 ± 0.39 (4)	28.00 ± 5.43 (4)	66.39 ± 5.90 (4)
10	7.47 [5.8, 8.9] (2)	37.52 [31.8, 44.0] (2)	54.27 [49.0, 58.7] (2)

* Acid-soluble extract from placenta fragments incubated for the times indicated with 5 μM Ch (0.8 μCi/ml) and in the presence of 10 μM paraoxon was subjected to high voltage electrophoresis. Areas of the paper corresponding to the localization of authentic standards were cut out and radioactivity was eluted. PhCh and betaine were not separated in pyridine–formic acid buffer [20] but the contribution by betaine was almost negligible [3]. Each value is the mean ± S. D. of duplicate determinations from the number of placentae indicated in parentheses. At 10 min, only two placentae were examined in this series of experiments, and the individual values obtained from duplicate determinations are given in brackets.

representative t.l.c. plate derived from an extract of tissue incubated for 30 min with 5 μM [^{3}H]Ch is shown in Fig. 1. Iodine vapor staining revealed seven bands among which phosphatidylcholine was most prominent. Determination of radioactivity in the eluates of 1-cm-wide bands of the Silica gel coating showed that ^{3}H-label was associated only with two areas of the plate. Overriding among those two was the area of heaviest iodine staining, which comigrated with authentic phosphatidylcholine ($R_f = 0.37$), while a minor amount of ^{3}H was found in an area ($R_f = 0.1$) corresponding to lysolecithin. No label was found to be associated with the Ch containing sphingomyelin. Therefore, it appeared that the incorporation of ^{3}H-label into the lipid-soluble fraction was largely due to the incorporation of [^{3}H]Ch into phosphatidylcholine.

Short-term incubations with [^{3}H]Ch. Our previous measurements had revealed that Ch was taken up and incorporated quite rapidly. The most intensely labeled acid-soluble metabolite was ACh, which after 5 min accounted for 36 per cent of the ^{3}H-label [3]. When the incubation time was shortened in the present experiments (while doubling the amount of radioactivity present in the medium to 0.8 μCi/ml), it became apparent that much of this incorporation into ACh occurred already during min 1 of incubation, because 18 per cent of the acid-soluble radioactivity had electrophoretic mobility equal to authentic ACh (Table 1). This value increased to 28 per cent after 5 min and 37 per cent after 10 min, numbers not significantly different from those obtained previously.

Effects of paraoxon. It was recognized several years before ChAc was discovered that an ACh-like material was synthesized during the incubation of brain minces, provided an inhibitor of cholinesterases was present [16]. Subsequent studies *in vitro* with labeled Ch have shown that hardly any newly formed radioactive ACh could be recovered when no cholinesterase inhibitor was added [17, 18]. With respect to the human placenta, recent evidence indicated that no parenchymal acetylcholinesterase activity existed and that all enzyme activity appeared to be associated with red blood cells [19]. Our initial results were obtained in the presence of 10 μM paraoxon [3], and it was therefore of interest to compare with those data the metabolic fate of Ch when no paraoxon was added to the incubation medium. The drug had no effects on the uptake of [^{3}H]Ch from the incubation medium into both the acid-soluble and the chloroform–methanol-soluble extract. However, when the distribution of ^{3}H-label among acid-soluble products was determined by HVE, it became apparent that the percentages of tritium label in the precursor Ch and its metabolites PhCh and ACh were significantly affected by the omission of paraoxon (Table 2). More radioactivity was present as [^{3}H]Ch, while ^{3}H-labeling associated with the ACh region of the electropherogram was reduced and that migrating like authentic PhCh was increased with the longer incubation times.

Neither of the two HVE buffers used to separate the acid-soluble Ch derivatives was capable of resolving CDP-Ch from its precursor PhCh [20]. In order to obtain an estimate of how much of the radioactivity found in the PhCh area of electropherograms was actually due to PhCh and how much to CDP-Ch, acid extracts were spotted for descending paper chromatography in a solvent system [12] with which CDP-Ch remained at the starting line while PhCh had a migration corresponding to $R_f = 0.11$, Ch 0.59 and ACh 0.71. Fewer than 5 per cent of the total disintegrations recovered by elution from the two areas of interest were at the origin while the overwhelming amount had migrated corresponding to authentic PhCh.

Effects of 2,4-dinitrophenol. This compound was a powerful inhibitor of the concentrative uptake of [^{3}H]Ch as evidenced by significant reductions of the concentration ratios after 15 and 30 min of incubation [3]. A similar effect of 2,4-DNP was also reflected in the reduced content of radioactivity in the acid-soluble extracts. However, the inhibitory action of the drug on tritium appearance in the acid-soluble fraction became significant only after 10 min or more of tissue incubation, while the incorporation of [^{3}H]Ch into the lipid-soluble fraction remained unaffected for up to 20 min (Fig. 2). These observations suggested different sensitivities of the

Table 2. Distribution of ^{3}H label among acid-soluble products after incubation with 5 μM [^{3}H]choline with and without 10 μM paraoxon*

Time (min)	Ch		ACh		PhCh + betaine	
	Control	Paraoxon	Control	Paraoxon	Control	Paraoxon
5	67.32 ± 2.65† (4)	55.00 ± 1.79 (4)	27.02 ± 5.25 (4)	35.90 ± 5.37 (3)	5.47 ± 1.57 (3)	6.82 ± 1.65 (4)
10	61.05 ± 7.66† (4)	43.70 ± 4.18 (4)	28.47 ± 5.73† (4)	44.92 ± 5.29 (4)	9.77 ± 1.96 (3)	7.90 ± 0.96 (3)
20	45.82 ± 8.85† (4)	25.26 ± 2.96 (3)	36.70 ± 5.83† (4)	62.36 ± 5.25 (4)	15.65 ± 3.00† (3)	11.60 ± 1.36 (3)
30	43.87 ± 4.48† (4)	28.37 ± 8.87 (4)	33.62 ± 3.097† (4)	58.85 ± 9.81 (4)	20.05 ± 2.04† (3)	14.68 ± 3.47 (3)

* Acid-soluble extracts from placenta fragments incubated for the times specified were subjected to high voltage electrophoresis. Areas of the electropherogram corresponding to the localization of authentic standards were cut out and radioactivity was eluted. Numbers show per cent of total radioactivity recovered in each of the labeled products. Each value is the mean ± S. D. of duplicate determinations from the number of placentae indicated in parentheses.

† Value differs significantly ($P < 0.05$) from paraoxon-treated tissue.

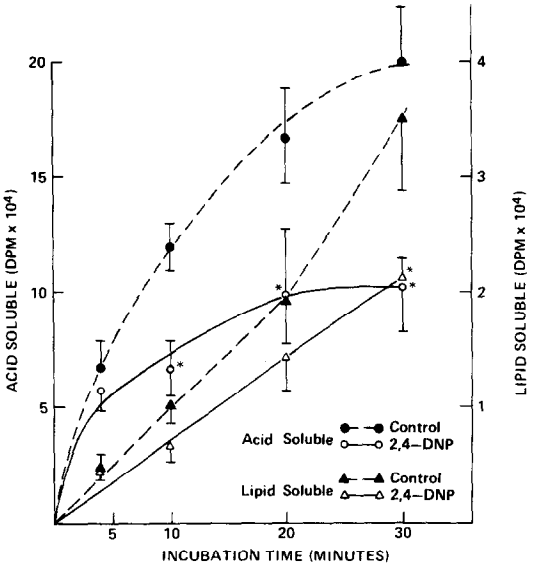

Fig. 2. Effects of 2,4-dinitrophenol (2,4-DNP) on total ^{3}H-content of acid-soluble and lipid-soluble fraction. Placenta fragments were incubated with 5 μM [^{3}H]choline for the time periods indicated on the abscissa. Total radioactivity (expressed in dis./min on the ordinates) was differentiated into acid-soluble and lipid-soluble radioactivity. Each point represents the mean ± S.D. of duplicate determinations from four 2,4-DNP and three control placentae. 2,4-DNP values marked with an asterisk differ significantly ($P < 0.05$) from the respective control values.

various metabolic pathways to 2,4-DNP. When the acid-soluble components were separated by HVE, it was found that the distribution of tritium expressed in per cent of the amount applied to the electropherogram paper did not differ as regards Ch- and ACh-associated label. However, there were significant differences at all incubation times with respect to PhCh, whose labeling intensity was significantly lower (5 min: 2.53 vs 5.46 per cent; 10 min: 3.05 vs 9.76 per cent; 20 min: 6.55 vs 15.65 per cent; and 30 min: 7.57 vs 20.05 per cent).

Effects of Na^+ ions. In the [^{3}H]Ch uptake studies it was found that reduction or omission of Na^+Cl^- and replacement by an osmotically equivalent amount of sucrose led to a marked rise in the intracellular accumulation of ^{3}H-label [3]. In view of the well-characterized association of Na^+-dependent, high-affinity choline uptake and ACh synthesis in the central nervous system [21–24], it was of interest to pursue the Na^+ effects on uptake by following the metabolic fate of [^{3}H]Ch. Fragments were incubated in solutions whose Na^+ concentrations were controlled as described [3] and which contained 10 μM paraoxon. The content of ^{3}H-label in the acid-soluble fraction was increased in fragments derived from Na^+-free incubation medium, while there was no effect on the amount of tritium extracted by chloroform–methanol.

Remarkable changes were brought about by the lack of Na^+ in regard to the distribution of ^{3}H-label among the acid-soluble constituents of the extract as revealed by HVE. The omission of Na^+ appeared to primarily affect the incorporation of [^{3}H]Ch into ACh (Table 3), whose labeling intensity was significantly reduced at all time intervals examined. Because of the decrease in ACh synthesis, free Ch-associated radioactivity was significantly higher at four out of five measuring points. Furthermore, PhCh contained more label after 10 and 20 min.

DISCUSSION

All observations made with the [methyl-^{3}H]-labeled methyl donors methionine or *S*-adenosyl methionine suggested that human term placenta tissue could not transfer such methyl groups to ethanolamine or phosphatidyldimethylethanolamine *in vitro*. No indication could be obtained that incorporation of radioactivity had occurred into Ch or any of its acid-soluble derivatives (ACh, PhCh and CDP-Ch) and the lipid-soluble phospholipid products (phosphatidylcholine, lysolecithin and sphingomyelin). When [2-^{14}C]ethanolamine was used as the acceptor of unlabeled methyl groups, the only

Table 3. Distribution of [^{3}H] label among acid-soluble products after incubation in 5 μM [^{3}H]choline with and without Na^+Cl^-*

Time (min)	Ch		ACh		PhCh + betaine	
	Control	Na^+-free	Control	Na^+-free	Control	Na^+-free
2.5	73.32 ± 4.91 (4)	83.05 ± 1.60 (3)	23.36 ± 5.51 (4)	9.88 ± 4.62† (3)	3.23 ± 0.69 (3)	3.08 ± 0.95 (3)
5	55.00 ± 1.79 (4)	80.65 ± 4.41† (3)	35.90 ± 5.37 (3)	13.05 ± 4.45† (3)	6.82 ± 1.65 (3)	4.80 ± 1.37 (3)
10	43.70 ± 4.18 (4)	63.25 ± 8.34† (3)	44.92 ± 5.29 (4)	19.78 ± 4.57† (3)	7.90 ± 0.96 (3)	11.75 ± 3.67† (3)
20	25.25 ± 2.96 (3)	48.80 ± 10.22† (3)	62.35 ± 5.25 (3)	26.86 ± 7.79† (3)	11.60 ± 1.36 (3)	18.06 ± 3.68† (3)
30	28.37 ± 8.87 (4)	50.17 ± 6.57† (2)	58.85 ± 9.82 (4)	27.60 ± 2.76† (2)	14.68 ± 3.47 (4)	16.42 ± 0.70 (2)

* Acid-soluble extracts from paraoxon (10 μM)-treated placenta fragments incubated for the times specified were subjected to high voltage electrophoresis. Areas of the electropherogram corresponding to the localization of authentic standards were cut out and radioactivity was eluted. Numbers show per cent of total radioactivity recovered in each of the labeled products. Each value is the mean ± S. D. of duplicate determinations from the number of placentae indicated in parentheses.

† Value differs significantly ($P < 0.05$) from Na^+Cl^--containing control tissues.

radioactive products were the nonmethylated derivatives phosphorylethanolamine, cytidyldiphosphoethanolamine and phosphatidylethanolamine. Therefore, it appeared from the present experiments that the human placenta could not synthesize Ch *de novo*. In this apparent inability the cholinergic system of this non-innervated, yet ACh-rich, organ may be quite similar to brain tissue. With regard to the latter, the general consensus is that the brain is dependent on the supply of Ch from a source which is probably phospholipid bound when it passes through the blood–brain barrier [13]. Only one report has recently come out claiming that brain tissue can perform methylation reactions leading to the synthesis of Ch [25]. As far as the placenta is concerned, the lack of evidence for Ch synthesis strengthened the view that the active Ch uptake system described before, may be the supplier of free Ch for placental and fetal needs [3]. An alternative means of Ch supply which would require further experimental evaluation is the uptake of Ch in a lipid-bound form or, less likely, in a water-soluble esterified form. If Ch reached the placenta in a phospholipid-bound form, then the base would have to be freed by enzymatic action. This process was found to be very rapid and significant in the rat brain, and at 37° contributed 20 nmoles of free Ch/g of brain $\times$ min^{-1} to a basal level of about 28 nmoles [26].

Based on the well-established pathways of Ch, it is not surprising to find [^{3}H]labeling of phosphatidylcholine after incubations with [^{3}H]Ch. Compared to the percentage of tritium associated with lecithin, lysolecithin contained little radioactivity at all time intervals. Since the latter phospholipid was significantly labeled by [^{32}P$_i$] during *de novo* synthesis measurements and since the concentration of lysolecithin is quite small, the small amount of [^{3}H]label associated with it may be indicative of rapid breakdown to glycerophosphorylcholine and on to free Ch [15, 14].

The incorporation of [^{3}H]Ch into newly synthesized ACh was even more rapid than suggested by the initial measurements after 5 min [3]. The distribution of radioactivity observed among the acid-soluble products after 1 min of incubation with [^{3}H]Ch was probably most seriously affected by the experimental procedure. The delay brought about by filtration and washing of the tissue fragments was similar regardless of the incubation time. Therefore, it appears reasonable to assume that shorter incubation times (1 and 2.5 min) would be relatively more affected by this unavoidable delay than samples incubated for longer times (20 and 30 min). It was not ruled out that, despite the cold washing solutions, no further conversion of Ch occurred during the washing phase. This could mean that the true value of [^{3}H]ACh at exactly 1 min was lower than 18 per cent of all acid-soluble radioactivity (Table 1).

The presence of paraoxon in the incubation medium affected the distribution of [^{3}H] label between the precursor [^{3}H]Ch and its two main esterified, acid-soluble products ACh and PhCh significantly (Table 2). However, the increase in the amount of radioactivity associated with ACh was modest compared to the observations obtained with brain slices or synaptosomes [17, 18, 27]. This finding was no longer as unexpected when one considers the evidence presented in a recent report suggesting that the parenchyma of human term placentae contained no acetylcholinesterase. All enzyme activity appeared to be associated with erythrocytes, because substrate hydrolysis was intimately correlated with the hemoglobin content of the organ which was perfused and freed of blood to variable extents [19]. The tissue fragments used in the present experiments were washed extensively. Apparently this mode of preparation was insufficient to remove all of the blood-borne acetylcholinesterase since paraoxon still caused significant changes in the metabolic fate of [^{3}H]Ch. This interpretation of our observations is supported by the description that 60 per cent of the original ACh was lost during differential centrifugation of placenta homogenates prepared from tissue minces washed and homogenized in media containing no cholinesterase inhibitor [28]. The significantly higher percentage of acid-soluble radioactivity associated with PhCh after 20 and 30 min may be due to the availability of higher intracellular Ch concentrations. In the absence of paraoxon, much less Ch was bound in ACh and thus could be available for esterification to PhCh.

The delay in onset of the effects of 2,4-DNP (Fig. 2) was in line with the previously reported actions of this metabolic inhibitor on ACh [9] and Ch uptake [3] and was probably a reflection of the ability of the placenta to use the glycolytic pathway effectively [29, 30] and maintain active transport during anaerobia and in the presence of 2,4-DNP [31]. This unusual ability of fetal tissues (of which the placenta is one) might be the explanation for why energy-dependent processes continue for surprising lengths of time without significant impairment. Nevertheless, the ATP-supported phosphorylation of [^{3}H]Ch to [^{3}H]PhCh was reduced at all time points of measurement. The lack of effect of the metabolic inhibitor on [^{3}H]Ch incorporation into the lipid-soluble fraction agreed with the observations obtained with synaptosomes where no change in the synthesis of phosphatidylcholine was measurable after 15 min of incubation with radioactive Ch [27].

In regard to the reduction of synthesis of labeled ACh (Table 3), the effects of omission of Na^+Cl^- were similar to those described in brain tissue [21–24]. Despite that similarity there remained fundamental differences between Ch uptake and ACh synthesis in brain and placenta. The latter organ revealed no indication of a high-affinity uptake system for Ch, and lack of Na^+ stimulated Ch uptake into the intracellular water compartment [3]. This was in contrast to the Na^+-dependent, high-affinity Ch uptake in rat brain synaptosomes which was intimately coupled to ACh synthesis. Nevertheless, Na^+ appeared to be important for maximal ACh synthesis to occur in the placenta.

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