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THE CHOLINERGIC SYSTEM IN TISSUES WITHOUT INNERVATION

CHOLINE ACETYLTRANSFERASE, CHOLINE AND ACETYLCHOLINE IN THE PLACENTA OF THE RHESUS MONKEY (*MACACA MULATTA*)*

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Abstract—Six near-term placentae from rhesus monkeys (*Macaca mulatta*) were analyzed for their contents of choline acetyltransferase (ChAc), acetylcholine (ACh) and choline (Ch). ChAc was measured by a radiometric assay in the presence of [1-^{14}C]acetylcoenzyme A and choline and distinguished from other acetyltransferases by a differential assay involving acetylcholinesterase or selective ion pair extraction of [1-^{14}C]ACh with tetraphenylboron. At 150 days of gestation the rhesus placenta synthesized 4.067 ± 0.737 μmoles ACh/g wet weight/hr. Ch and ACh were determined with a radiochemical method based on the phosphorylation of free Ch by choline kinase in the presence of [^{32}P]ATP. ACh was first isolated by ion pair extraction and high voltage electrophoresis. Tissue levels of Ch ranged from 737 to 3892 and ACh from 8.8 to 29.0 (nmoles/g wet weight). The rhesus monkey appears to be a suitable animal model to study the physiological significance of ACh in the placenta *in vivo*.

It has been known for a long time that the non-innervated human placenta contained large concentrations of a material with acetylcholine (ACh)-like activity [1–3]. Recent analysis by gas chromatography has established that more than 50 per cent of that activity was actually due to ACh whose concentration in the human term placenta was 112 nmoles/g of fresh tissue [4]. Soon after the discovery of choline acetyltransferase (ChAc) (acetyl-CoA: Choline *O*-acetyltransferase, EC 2.3.1.6), it was recognized that human placenta was also a very rich source of this enzyme which catalyzes the biosynthesis of ACh [5]. The kinetic characteristics of the placental ChAc appeared to be similar to the ones of the brain enzyme [6]. ChAc underwent remarkable changes during the course of human gestation [7–11] which were paralleled by corresponding fluctuations in ACh content [1–4]. Both observations indicated the highest levels toward the end of the second trimester. In contrast to the undisputed abundance of some of the components of the cholinergic system in the human placenta, the reports concerning the occurrence of ChAc and ACh in placentae of common domestic and laboratory animals have been equivocal. In a study in which ChAc was measured by determining, by means of a bioassay, ACh synthesized during a preceding incubation of placenta homogenates, it was concluded that only the placentae of higher primates (man and rhesus monkey) contained ChAc [8]. In more recent investigations, the synthesis of [1-^{14}C]ACh from [1-^{14}C]acetylcoenzyme A ([1-^{14}C]AcCoA) was revealed qualitatively in a variety of species [12]. However, further experiments in the mouse placenta have shown that the relationship of this ACh to either ChAc or carnitine acetyltransferase (acetyl-CoA: carnitine *O*-acetyltransferase, EC 2.3.1.7) was not clear [13]. In the heart, the latter enzyme could use choline as a low affinity substrate and synthesize some ACh [14].

The significance of the striking species differences as well as the functional role of ACh in the placenta remains obscure. Speculations and incomplete experimental examinations have generally focused on control of permeability and transport phenomena of the placental membranes [7, 11, 15, 16] and more recently also on the release of placental hormones [17]. For an experimental approach to identify the physiological importance of ACh in the placenta, it would be desirable to have available an animal model which contained concentrations of ChAc and ACh which were as easily measurable as those of the human placenta. Ethical considerations limit the experimental design applicable in human subjects and have restricted most well-controlled investigations to the use of perfused term placenta or to slices and homogenates obtained from this organ [see Ref. 18 for a recent review]. In view of the questions remaining as regards the activity and quantitative determination of ChAc as well as ACh in placentae of common laboratory animals, an observation made earlier by Hebb and Ratkovic [8] in a rhesus monkey appeared most promising for a more complete analysis of the cholinergic system in the placenta of that species. The present report describes the concentrations of ChAc, free choline (Ch) and total ACh in near-term placentae of six rhesus monkeys (*Macaca mulatta*).

* Some of the results of this investigation were presented at the Fall Meeting of the American Physiological Society in San Francisco, CA [*Physiologist* **18**, 444A (1975)].

MATERIALS AND METHODS

Tissue sources. Five placentae from rhesus monkeys were obtained from the Oregon Regional Primate Center in Beaverton, OR. All specimens derived from Caesarean sections which were performed on day 144 of gestation (placenta No. 1) or on day 150 (placentae Nos. 2–5). Several small tissue samples were frozen in liquid nitrogen soon after removal of the placenta, wrapped, labeled and stored deep frozen until shipped on dry ice to our laboratory, where they were stored in a deep freezer at −90° until used. Another whole rhesus placenta (No. 6) was obtained from Litton Bionetics, Inc., Kensington, MD. This specimen derived from a live birth by vaginal delivery on day 163 of gestation and was shipped on dry ice.

Preparation of tissue homogenate. For ChAc determinations, small pieces of placenta were chiseled away from the deep-frozen material. They were thawed, blotted, freed of connective tissue and chorionic plate and homogenized 1:10 (w/v) in distilled water or 10 mM EDTA, pH 7.4, containing 0.5% Triton X-100, for two 30-sec periods at full speed with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) while the sample was submerged into an ice bath. Aliquots corresponding to 1–10 mg of fresh tissue weight were used for incubation.

For choline and ACh extractions, the deep-frozen piece of tissue was weighed and transferred without any further cleaning into tubes containing ice-cold 1 N formic acid–acetone (15:85, v/v) [19] stored in crushed ice. Nine ml was used per g of frozen tissue. The samples were allowed to thaw very gradually and were homogenized as soon as the homogenizer was able to disintegrate the hard material.

These precautions were taken because of the apparent lability of ACh in human placenta, where slow freezing of the tissue and subsequent warming to room temperature caused the disappearance of all of the ACh, presumably by destroying membranes which enclosed ACh and prevented the hydrolysis of the ester by acetylcholinesterase (acetylcholine acetylhydrolase, EC 3.1.1.7, AChE) [4]. It was the intent to prevent or minimize a similar event in the monkey placenta specimens which could not be obtained except in frozen condition. The homogenate was allowed to stand in crushed ice for 30 min and was then centrifuged for 15 min at about 25,000 *g* in a refrigerated model J21 centrifuge (Beckman Instruments, Palo Alto, CA). The supernatant was decanted and suitable aliquots were lyophilized, stored in a desiccator at −90° and used for Ch or ACh analysis.

Measurement of ChAc. The activity of this enzyme was determined with two different methods. Both were based on a radiochemical procedure which measured the rate of incorporation of [1-^{14}C]acetyl from [1-^{14}C]AcCoA (New England Nuclear, Boston, MA) into newly synthesized [1-^{14}C]ACh [20]. The two methods differed in the isolation procedure which separated the precursor from the product (or products, if any, besides ACh). One technique used anion exchange chromatography with the modification recommended to improve the specificity of separation of reaction products [21]. The homogenate containing the desired amount of tissue in 50 μl was pipetted into disposable glass tubes (10 × 75 mm) and prewarmed for 2 min at 37°. The enzymatic reaction was initiated by the addition of 50 μl of prewarmed incubation mixture which resulted in the final concentration of the following components for the ion exchange method (in mM): NaCl 500; phosphate buffer, pH 7.4, 20; choline 10; [1-^{14}C]AcCoA 0.5 (sp. act. after dilution with unlabeled AcCoA from Pabst Laboratories, Milwaukee, WI, about 2 mCi/m-mole); and physostigmine 0.5. In incubation tubes which served as a measure of "unspecific" acetyl group transfer not related to ChAc activity, physotigmine was omited and AChE (type V, Sigma Chemical Co., St. Louis, MO) in an amount sufficient to hydrolyze 500 nmoles ACh/min was added instead [21]. Radioactivity eluted from columns loaded with AChE-treated samples was deducted from the values obtained in the presence of physostigmine. The difference in disintegrations between the two sets of samples was attributed to [1-^{14}C]ACh which was hydrolyzed when AChE was present [21]. Radioactivity in the aqueous effluents was determined in a toluene scintillator containing Triton X-100 (2:1, v/v; 10 ml/vial) with a model 3380 liquid scintillation spectrometer equipped with a model 544 absolute activity analyzer (Packard Instruments, Downers Grove, IL).

The second method applied to separate [1-^{14}C]-AcCoA from [1-^{14}C]ACh was that recently described by Fonnum [22] except for the use of 0.1 μCi (≃ 220,000 dis./min) of [1-^{14}C]AcCoA. In preliminary extraction experiments with authentic [1-^{14}C]ACh, it was found necessary to adjust all solutions to Fonnum's specifications in order to obtain complete extraction of ACh by ion pair formation with tetraphenylboron at pH 7.4. The samples were treated as described in detail [22].

The effects of several well-known inhibitors of ChAc were examined. Bromoacetylcholine (BrAch) and chloroacetylcholine (ClACh) were synthesized according to Chiou and Sastry [23] and dissolved only immediately prior to their experimental use because of their relative instability in aqueous solutions [24]. Further, the effect of 4-(7-naphthylvinyl) pyridine (NVP, CalBiochem, LaJolla, CA) was tested. This compound was dissolved and used in a darkroom only [25]. All enzyme incubations were run in duplicate samples.

Determination of Ch and ACh. Both quaternary amines were measured with a radiochemical method based on the phosphorylation of free choline or choline liberated by enzymatic or alkaline hydrolysis of ACh to ^{32}P-labeled phosphorylcholine (PhCh) in the presence of the enzyme choline kinase (ATP: choline phosphotransferase, EC 2.7.1.32). The approach which had to be taken included the principles of the methods described by Goldberg and McCaman [26] and Haubrich and Reid [27]. Some of the pitfalls encountered in applying the procedures developed with brain tissue to the placenta deserve mentioning. Although we were able to obtain satisfactory standard curves for Ch of ACh within a few weeks after adopting the micromethod [26] and although Ch and ACh concentrations obtained from brain extracts of microwave-sacrificed rats were in the expected range (32.0 and 26.0 nmoles/g, respectively; three animals), the assay proved to be unreliable for both the human and the monkey placenta. In the latter tissues, the molar ratios of Ch to ACh were expected to deviate

markedly from the 1:1 to 1:2 ratio encountered in total brains. The observation was made that when authentic standards of Ch and ACh were mixed and processed through stages 1 and 2 as recommended, Ch would break through in the second stage of the assay when the molar ratio of Ch:ACh was >3:1 [see Ref. 26 for details]. This happened in spite of the fact that complete conversion to PhCh occurred when each amine was examined alone in amounts up to 4 nmoles. However, a mixture of 1000 pmoles Ch with 100 pmoles ACh, for example, led to erroneously high ACh values when calculated on the basis of specific activities of the precursor [γ-^{32}P]ATP and the product [^{32}P]PhCh.*

The tissue concentrations of Ch could be determined with the assay of Goldberg and McCaman [26]. Determinations in different extracts prepared on at least two occasions agreed satisfactorily, and when known amounts of Ch were added to tissue extracts, the amount added was recovered. However, in order to determine ACh, the principle of the electrophoretic separation of Ch and ACh [27, 28] had to be incorporated into the method. After tetraphylboron ion pair extraction of the quaternary amines and their displacement into 0.4 N HCl, the samples were lyophilized. The residue was dissolved in a small volume of electrophoresis buffer and a known aliquot was streaked onto the starting line of 20 × 40 cm sheets of Whatman No. 1 paper. Standards of Ch and ACh were processed through the entire procedure to allow the construction of a proper standard curve. Authentic Ch and ACh (50 μg) were spotted on the electrophoresis paper with the addition of tissue extract to compensate for any differences in electrophoretic mobility that might be caused by such material. High voltage electrophoresis (HVE, CAMAG, New Berlin, WI) was performed by applying 1800 V (45 V/cm) for 70 min using the pyridine–formic acid buffer [27].† There was at least 1, and many times, 2 cm of paper between the tailing edge of Ch and the leading edge of ACh (as visualized by iodine vapor staining) where radioactivity was negligible. One line was drawn across the electropherogram paper, which was 2 mm above the leading edge of the stained area of ACh standards and another line 4 cm anodal to include the total ACh area. The paper was separated into individual tracks and the region containing ACh (40 × 30 mm) rolled up and eluted repeatedly (three times) with 75-μl aliquots of methanol—4 M NH_4OH = 90:10. The eluate was collected by centrifugation through the cut-off bottoms of plastic microtubes (Cole Parmer, Cat. No. 6335-10) into a fresh plastic microtube. The eluate was heated in a water bath at 75° until completely dried. During this process ($\simeq$ 1 hr) the NH_4 and heat hydrolyzed ACh. The residue in this tube was now used to determine the choline liberated from ACh.‡ Choline kinase partially purified from rabbit brain [27] and generously donated by Dr. Dean Haubrich was used in the samples which had undergone electrophoresis. The final volume was 10 μl, and the sealed tubes were incubated for 120 min at 37°. The composition of the medium was essentially identical to that described [26], and barium precipitation of excess ATP and protein was used. All remaining steps were identical to the descriptions of that method except that the micro ion exchange columns were stored under formic acid–ammonium formate and washed with water and 50 mM NaOH just prior to reuse (A. M. Goldberg, personal communication). This helped reduce the blank values from 100 to 150 cpm in the Ch assay without electrophoresis to 25–40 cpm and in the ACh assay with electrophoresis from 400–500 to 200–250 cpm.

RESULTS

Presence and activity of ChAc. When rhesus monkey placenta homogenate was incubated with [^{14}C]AcCoA and choline, [1-^{14}C]acetyl groups were rapidly incorporated into product(s) which eluted from the anion exchange columns. If the tissue was boiled prior to the incubation, values identical to the blanks inherent to the procedure resulted (0.1 to 0.2 per cent of total precursor radioactivity). Significant incorporation of label by what appeared to be an enzymatic reaction still occurred when choline was omitted from the incubation tubes, suggesting that, as previously observed in a variety of domestic and laboratory animal placentae [12], appreciable "unspecific" acetyl transfer, i.e. unrelated to ChAc, occurred. Therefore, it was necessary to modify the assay in order to improve the specificity of the isolation of [1-^{14}C]ACh. Two different methods were applied to accomplish this task. One was the differential assay of Hamprecht and Amano [21] and the other the ion pair extraction assay of Fonnum [22].

Three placenta homogenates were examined at various tissue concentrations with both methods. Radioactivity eluted from the anion exchange columns indicated that about 80 per cent of the radioactivity incorporateed into cationic or neutral materials was susceptible to AChE hydrolysis (Table 1), while 20 per cent was resistant, suggesting the presence of other metabolites.

The values obtained by ion pair extraction at pH 7.4 were very similar to the corrected values of the differential assay (Table 1) and revealed ChAc activities in the same order of magnitude as present in human term placentae [9, 10]. It appeared simpler to use the ion pair extraction for further experiments,

* This observation was also made by Drs. Alan Donelson, University of Michigan, and Dean Haubrich, Squibb Institute, personal communications.

† It was found that under our conditions the separations obtained with this particular buffer were superior to the ones obtained with formic acid–acetic acid [29] as judged by the sharp separation of radiolabeled authentic standards (with or without 50 μg of carrier amine) or of placenta extracts treated with the *N*-methyl [^{3}H]Ch where *N*-methyl [^{3}H]ACh had been synthesized, (unpublished observations).

‡ It appears important to mention that the purified choline kinase from baker's yeast which is commercially available (Sigma Chemicals, St. Louis, MO, product No. C-2761, now C-7138 with higher specific activity) was not suitable for this step. Apparently there were unidentified factors associated with the electrophoresis process which severely inhibited the subsequent phosphorylation reaction. This observation has previously been made by Haubrich [30], but was not mentioned by other investigators who purified the enzyme originating from yeast in their own laboratories [31, 32].

Table 1. Determination of choline acetyltransferase by differential assay and by ion pair extraction*

Placenta No. (incubation time)	Method	Radioactivity (dis./min)				ACh synthesis	
		Boiled tissue	Control	−Eserine + AChE	Net activity	(nmoles/g wet wt/hr)	(nmoles/mg protein/hr)
1	Column	225	24,252	4,623	19,629	6,847	37.2
2 mg (20 min)	TPB	840	19,437	753	18,597	6,487	35.2
3	Column	275	25,316	4,944	20,372	4,740	34.6
6 mg (10 min)	TPB	712	20,811	511	20,099	4,720	34.4
4	Column	225	16,712	3,605	13,107	4,573	27.2
2 mg (20 min)	TPB	840	14,116	721	13,276	4,630	27.5

* Rhesus monkey placentae were homogenized as specified in Materials and Methods. The tissue was incubated in a total volume of 100 μl with [1-^{14}C]acetylcoenzyme A and choline. Four duplicate sets of tubes were used for each specimen and each time point. Two sets contained acetylcholinesterase to hydrolyze all ACh synthesized. The incubation mixture was analyzed by anion exchange chromatography (column) or ion pair extraction with tetraphenylboron (TPB).

because the selectivity for [1-^{14}C]ACh extraction was satisfactory, thus eliminating the need for parallel samples with AChE and reducing by one half the need for expensive [^{14}C]AcCoA.

There was a reasonably linear relationship between tissue concentration and/or incubation time with regard to the amount of [1-^{14}C]ACh synthesized (Fig. 1). The analyses of the remaining three placentae (Nos. 2, 5 and 6) revealed ChAc activity ranging from 1896 to 4752 nmoles ACh synthesized/g of tissue/hr (Table 2). Since only placentae Nos. 2–5 derived from equal durations of pregnancy and ChAc in human placenta declines toward term [7–10], it did not seem appropriate to include placenta No. 1 or 6 in the calculation of a mean value. The lower value in specimen No. 6 may be due to the fact that this was the only naturally delivered term placenta. Placentae obtained by Caesarean section at 150 days of pregnancy synthesized 4.067 ± 0.737 (S. D., N = 4) μmoles ACh/g wet wt/hr.

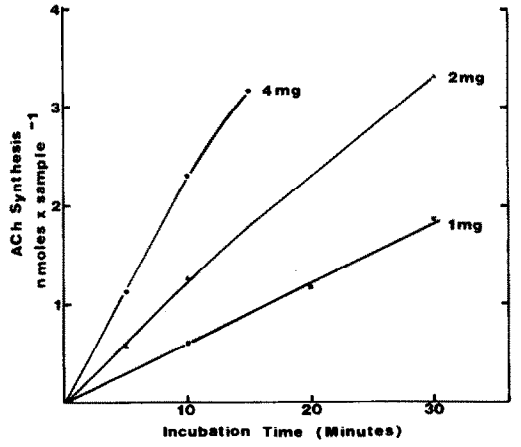

Fig. 1. Correlation between tissue concentration and incubation time with respect to [1-^{14}C]ACh synthesized by homogenate of rhesus monkey placenta. Variable amounts of tissue (placenta No. 5) were incubated in duplicate 100-μl samples with [1-^{14}C]acetylcoenzyme A and choline. After the incubation time specified samples were extracted with tetraphenylboron as described by Fonnum [22]. Values are expressed as nmoles ACh formed during incubation.

When the halogenated ACh derivatives BrACh and ClACh or NVP were added to the incubation mixtures in concentrations varying from 10 to 250 μM, a dose-related inhibition of ACh synthesis was observed (Fig. 2). BrACh was the most effective drug, causing about a 75 per cent reduction of [1-^{14}C]ACh formation at 10 μM.

Determinations of Ch and ACh. The Ch concentrations in the four specimens derived from Caesarean sections at 150 days of pregnancy were quite similar (Table 2). Placenta No. 1 was markedly different because the free choline content was high enough to

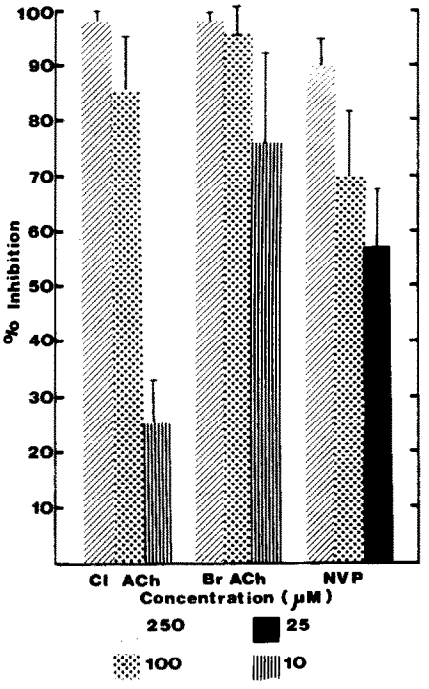

Fig. 2. Effect of ChAc-inhibiting drugs on [1-^{14}C]acetyl transfer into ACh. Homogenate (2 mg) from each placenta was incubated for 20 min with [1-^{14}C]acetylcoenzyme A and choline in the absence or presence of the drug concentrations specified. Samples were extracted with tetraphenylboron as described by Fonnum [22]. Values are expressed as per cent inhibition observed compared to control and represent mean ± S. D. from six rhesus monkey placentae.

Table 2. Concentrations of choline acetyltransferase, free choline and total acetylcholine in rhesus monkey placentae*

Placenta No.	Gestational age (days)	ChAc activity (nmoles ACh synthesized/hr)	ChAc activity (nmoles ACh synthesized/mg protein/hr)	Choline (nmoles/g)	ACh (nmoles/g)
1	144†	6439	35.0	3892 (3725–4192)	17.1 (11.8–24.2)
2	150†	4752	31.7	998 (898–1066)	16.8 (8.9–30.4)
3	150†	4326	31.6	737 (681–812)	8.8 (8.4–8.9)
4	150†	4165	26.5	793 (748–850)	29.0 (20.8–35.4)
5	150†	3025	18.2	1193 (975–1382)	24.7 (12.5–34.3)
6	163‡	1896	10.5	1601 (1452–1727)	25.7 (19.3–29.3)

* Choline acetyltransferase was determined with the liquid cation exchange method of Fonnum [22]. Free choline was measured after ion pair extraction from tissue with tetraphenylboron [26]. ACh was first isolated by high voltage electrophoresis [27]. Both quaternary amines were assayed with a radiochemical method based on formation of [^{32}P]phosphorylcholine. For both choline and acetylcholine analysis, different small pieces from each placenta were extracted on separate occasions and duplicate determinations were repeatedly analyzed. The values shown represent the mean values (range) of the tissue concentrations thus obtained from samples which were neither cleaned nor blotted prior to homogenization.
† Caesarean sections.
‡ Vaginal delivery at term.

cause staining of the electropherogram area corresponding to the migration of authentic Ch when the extracts were applied for electrophoretic separation of Ch and ACh prior to ACh analysis. For the analysis of free Ch with the method of Goldberg and McCaman [26], formic acid–acetone extract amounts equivalent to 0.5 to 2 mg tissue were sufficient to determine the Ch content.

For ACh analysis the equivalent of 10–25 mg tissue was used for each assay tube, and the tissue contents ranged from 8.8 to 29.0 nmoles ACh/g of placenta (Table 2).

DISCUSSION

The presence of high concentrations of ChAc in the near-term placenta of rhesus monkeys was ascertained by three different approaches. First, it was found that about 80 per cent of the radioactive metabolite synthesized from [1-^{14}C]AcCoA and choline was sensitive to hydrolysis by AChE. The particular preparation of the latter enzyme was highly purified and could be considered to be quite specific for the hydrolysis of ACh. Second, almost identical amounts of [1-^{14}C]ACh were synthesized when the tissue samples were analyzed by liquid cation exchange with tetraphenylboron. Under the pH conditions of the assay this reaction was highly specific for [1-^{14}C]ACh and would not extract [1-^{14}C]acetylcarnitine, a by-product of major concern in the measurement of ChAc activity in various tissues [14, 22] and a prominent metabolite in the mouse placenta [13]. The precursor [1-^{14}C]AcCoA was extracted only to the extent of 0.3 to 0.4 per cent of the total radioactivity. Additional support for the interpretation that the acetylating enzyme activity was due to ChAc derived from the effects which known inhibitors of this enzyme exerted on the synthesis of [1-^{14}C]ACh. The extent of the inhibition caused by halogenated ACh analogues was comparable to the effects reported on the ChAc of human placenta [24].

The activity of ChAc in the specimens examined (see Tables 1 and 2) was quite similar to the single value reported by Hebb and Ratkovic [8] using a bioassay. These authors measured the equivalent of 0.88 mg ACh synthesized/g of fresh weight/hr (based on ACh chloride as the standard corresponding to 4.8 μmoles). The ChAc activity of the rhesus placentae appeared to be in the same order of magnitude as that of human term placentae [9, 10] and outstandingly higher than the low or questionable synthetic capacity in the placentae of common domestic and laboratory animals [9, 13]. We had the opportunity to examine one placenta of a lemur, a lower primate, and experienced the same difficulties in measuring ChAc in that specimen (unpublished observations) as in the mouse placentae [13]. This confirmed the tentative conclusion reached by Hebb and Ratkovic [8] that high concentrations of ChAc were unique for the placentae of man and higher primates.

The tissue concentrations of free Ch in the placenta have not been examined before. The levels found in placenta No. 1 were exceptionally high compared to the other specimens. It could not be established if this was due to differences in the handling of this particular tissue sample which may have allowed the liberation of choline from other bound sources such as phospholipids.

The specificity of the ACh assay was based on two separation methods which were used to isolate this ester from the monkey placenta, i.e. ion pair extraction of Ch and ACh with tetraphenylboron [22, 33] followed by electrophoretic separation of these two amines [27]. In agreement with Haubrich *et*

al. [27, 28], we found the separation of the labeled variants of Ch and ACh by high voltage electrophoresis to be very satisfactory with no overlap of the tailing edge of Ch into ACh when the pyridine–formic acid buffer system was used. It is not possible to decide how close the ACh concentrations were to the levels which were present in the fresh placentae at the time of delivery. Unfortunately it was not possible to prepare extracts at that time, and the tissues had to be frozen in liquid nitrogen before shipment to our laboratory. It was attempted to minimize the danger of loss of placental ACh which was observed after slow freezing in human placentae [4] by rapid freezing of small pieces in liquid nitrogen and by using special precautions during thawing as described. The ACh tissue levels might have been higher had they been determined on fresh tissue.

In interpreting the data obtained, it might be most meaningful to focus attention on the high activity of ChAc as an indicator for the abundance of the cholinergic system in the rhesus monkey placenta. High ChAc has usually been found to be associated with high ACh content and vice versa. Based on these observations, it appeared that the rhesus monkey should provide an excellent animal model system to study the functions of ACh in the placenta *in vivo*.

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