HUMAN PLACENTAL CHOLINERGIC SYSTEM: OCCURRENCE, DISTRIBUTION, VARIATION WITH GESTATIONAL AGE, AND RELEASE OF ACETYLCHOLINE

BY

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DEDICATION

This thesis is dedicated to my senior brother, Silas Kayode Olubadewo, whose ambitious endeavor and achievements in life motivated my ambition to keep on climbing the ladder of academic pursuit.

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CHAPTER I

STATEMENT OF THE PROBLEM

Numerous reports have been published about the occurrence of a choline acetyltransferase (ChA)-acetylcholine (ACh)-acetylcholinesterase (AChE)-like system in the human placenta. The lack of neuronal innervation in the placenta prompted a variety of investigations resulting in diverse speculations about the functional significance of the placental cholinergic system. Of special interest is the high levels of ACh-like activity in human placenta. Investigators are also interested in knowing the similarities or differences between cholinergic systems of placental and nervous tissues. All determinations of placental ACh-like activity to date have been performed by bioassay methods which are not specific for ACh.

Although many reports were published about the levels of placental ACh-like activity, there is a negligible number of studies designed to elucidate the role of placental ACh-like substances, in which pharmacologic

manipulations were used to influence the components of the placental cholinergic system. Information on the effects of drugs on the placental ACh-like activity ought to go a long way in paving the road to a clearly defined role for placental cholinergic system. If is therefore the objective of this study to provide some basic information about human placental ACh-like activity; particularly: identification and estimation of ACh-like substances in placenta by gas chromatography and other modern chemical methods; the nature of ACh in the placenta and its storage; the site of localization of ACh; the subcellular distribution of ACh; variation of ACh concentration according to the period of gestational development and mechanisms of ACh release. These studies may provide the necessary information for postulating a working hypothesis for the role of ACh in placenta.

CHAPTER II

INTRODUCTION

A. Components of the Cholinergic System in Nervous Tissues

The various components of the cholinergic system in a cholinergic nerve terminal are illustrated in Figure II-1. The cholinergic system in the nervous tissue is composed of five components, namely:

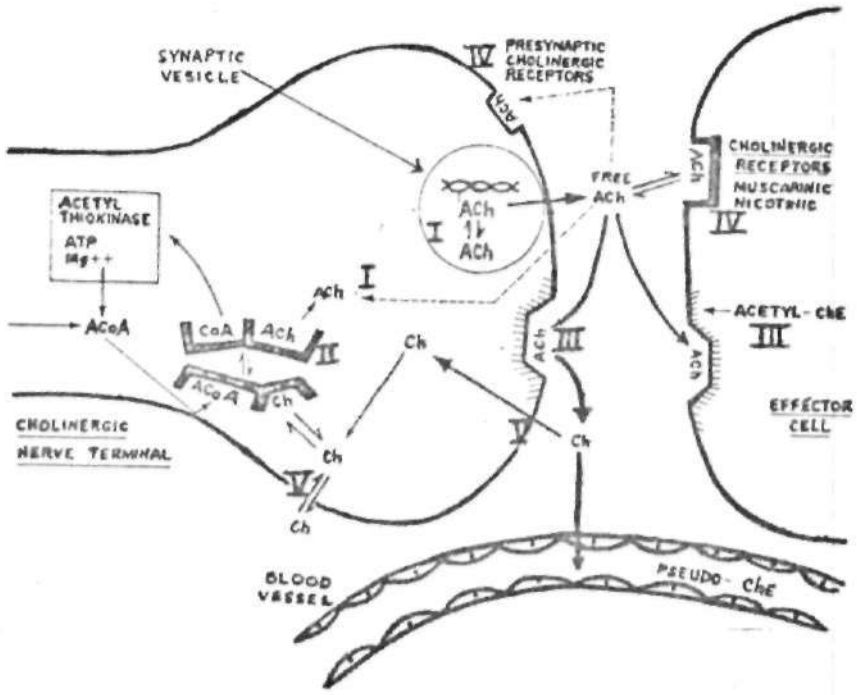
I. Acetylcholine, ACh, the neurotransmitter;

II. Choline acetyltransferase, ChA, the enzyme which catalyzes the synthesis of ACh from acetylcoenzyme A (ACoA) and choline (Ch);

III. Acetylcholinesterase, AChE, which hydrolyses ACh into choline and acetic acid;

IV. Cholinergic receptor, ChR, the macromolecular entity with which ACh interacts to produce its effects; and

V. The High-affinity Choline Carrier: This moiety is not generally considered as a


SYNAPTIC VESICLE
IV PRESYNAPTIC CHOLINERGIC RECEPTORS
ACh
ACETYL THIOKINASE
ATP
Mg++
ACoA
CoA
Ach
II
ACoA
Ch
CHOLINERGIC NERVE TERMINAL
Ch
VI
Ch
ACh
I
Ach
I
ACh
I
ACh
FREE ACh
ACh
CHOLINERGIC RECEPTORS MUSCARINIC NICOTINIC
IV
ACETYL-ChE
III
ACh
III
ACh
EFFECTOR CELL
V
Ch
BLOOD VESSEL
PSEUDO-ChE

component of the cholinergic system. It has been recently proposed as such by Whittaker and Dowdall (7).

The role of ACh in chemical transmission in the nervous tissue was convincingly demonstrated by Otto Loewi (2) who showed that the cardioinhibitory substance produced by vagal stimulation was "vagustoff" which was later isolated and characterized as ACh. Acetylcholine has been isolated and quantified from various tissues. Its concentration in typical nervous tissues are (nmoles/g tissue): brain: 20-30 (3); autonomic ganglia: 66-161 (4); and phrenic nerve-diaphragm preparation: 1.2 (5). Subcellular fractionation studies of nerve terminals have indicated that ACh is localized in the synaptic vesicles (6, 8) which are regarded as the storage sites of ACh. Acetylcholine is released spontaneously from nervous tissues incubated in physiological solutions or by electrical stimulation of cholinergic neurons. The release of ACh can be modified by varying experimental conditions, e.g., an increase in the concentration of extracellular calcium or potassium enhances amount of ACh released (9). Botulinum toxin (10) or cocaine (11) decrease the amount of ACh released from the nerve terminal.

Studies on the cholinergic receptors, ChR, began when Dale (12) demonstrated that the pharmacological actions of ACh could be described either as nicotine-like or as muscarine-like. The response to ACh occurs through molecular interactions between ACh and the receptor. The ChR whether nicotinic or muscarinic has two sites, anionic and cationic (or esterophilic) on its active surface. When proper alignment between ChR and ACh is achieved, membrane perturbation occurs, and changes in the permeability properties of the membrane ensue (1).

ACh is synthesized by the enzyme choline acetyltransferase, ChA, which was first described by Nachmansohn and Machado (13). This enzyme catalyzes the reaction between ACoA and choline with the formation of ACh and CoA. The enzyme is localized in the nerve terminal, and appears to have a similar subcellular distribution pattern to that of ACh (8). There are few potent inhibitors of ChA. The more common ones are halogenated-acetylcholines (51) and styrylpyridine analogues (15).

Acetylcholinesterase (AChE) and psuedocholinesterase (ChE) terminate the actions of ACh by hydrolysing it to choline and acetic acid (24). AChE is most commonly associated with synaptic sites and erythrocytes, while ChE

is present in the plasma.' AChE is more important than ChE with respect to ACh metabolism at synapses. Subcellular distribution studies by differential centrifugation have shown that AChE is associated with membranes in the microsomal fraction (8). This enzyme can be inhibited by many pharmacological agents which include carbamates (e.g., physostigmine) and the organophosphates (e.g., echothiophate) (24). These agents potentiate the actions of ACh.

B. The Components of the Cholinergic System in Non-nervous Tissues

The typical non-nervous tissues or organs in which the occurrence of one or more of the components of the cholinergic system has been reported are erythrocytes, placenta and spermatozoa.

The non-innervated parts of the spleen of ox and horse as well as the rabbit's corneal epithelium contain high levels of ACh, 110 and 147 nmoles/g tissue respectively (16, 17). The high content of ACh in these tissues has always been as intriguing as the occurrence of components of the cholinergic system in non-nervous tissues. The ACh of spleen and cornea is currently considered to be of neuronal origin (16, 17).

In the erythrocyte, the occurrence and functional significance of acetylcholinesterase (45) has been more extensively studied than the other two members, ACh and ChA, of the cholinergic system. Galehr and Plattner (18) first described the capacity of erythrocytes to split ACh. The ACh-splitting enzyme of erythrocytes was later identified as AChE (19). On the basis of the localization of the AChE on the outer surface of the erythrocyte membrane, it has been suggested that the enzyme participates in the regulation of active transport of monovalent cations (20). Although more than a dozen enzymes have been identified in the erythrocyte membrane, changes in pathological conditions have been found regularly only with changes in AChE concentration. For example, decreased activities of erythrocyte AChE have been found in cases of neonatal ABO disease, small-for-gestational-age babies, paroxysmal nocturnal hemoglobinuria, and "familial" reduction of AChE activity (21). The concentration of AChE of erythrocytes has also been correlated with the age of cells.

The erythrocyte ACh content (93-140 nmoles per ml of blood) first reported in 1930 by Kapfhammer and Bischoff (22) was later shown to be abnormally high by Chang and Gaddum (23). The modern and specific techniques of

ACh analysis have to be applied for the determination of ACh content in erythrocytes. The activity of ChA in erythrocytes was found to be less than that in nervous tissues (21, 45). Mathias and Sheppard (65) showed that the rate of ACh synthesis by erythrocyte ChA was much lower than the amount of ACh which was required to provide the free energy necessary for the accumulation of K^+ against a concentration gradient.

The discovery of an ACh-ChA-AChE cycle in the spermatozoa of mammals and marine invertebrates (25, 26, 28, 31) has spurred investigations of the possible functional significance of the cholinergic system in the spermatozoa. That discovery has also raised the hopes of investigators who are seeking to develop chemical male contraceptives. Saiko (25) recently demonstrated by bioassay the presence of ACh-like substances in the cytoplasm of the spermatozoa of bull, rabbit, boar and ram. Using pyrolysis-gas chromatography, Bishop (26) recently wrote a dissertation on the occurrence of ACh and ChA in the human and bull spermatozoa. He reported a value of 4.27 pmole ACh/million cells for bull spermatozoa and a value of 28.57 pmole ACh/million cells for human spermatozoa. The author also reported the discovery of propionylcholine

in the bull spermatozoa. There have been various reports on the cholinesterase-like activity in mammalian and mollusc spermatozoa (28-32). The bull and pig spermatozoan enzyme was characterized as AChE by its substrate specificity. When the spermatozoa was fractionated into head, midpiece and tail, all parts showed AChE activity, but there were species differences in its concentration in the various parts (32). When subjected to various pharmacological manipulations, the sperm cells exhibited responses (motility changes) as predicted by the effects of the same agents in nervous system. This led Nelson (33-35) to propose that the spermatozoan AChE regulates the intracellular level of ACh which in turn controls the sperm cell motility. Choline acetyltransferase activity in the rat and rabbit spermatozoa has been reported (26, 36). Although the bull spermatozoa contained less ACh than that in human spermatozoa, it was the bull sperm that contained the higher ChA activity (26). Chronic administration of trimethlphosphate to adult rats and rabbits decreased the levels of ChA in spermatozoa and also caused sterility (36). The spermatozoa of treated animals exhibited abnormal motility patterns.

The occurrence of components of the cholinergic system has long been known in the human placenta. Acetylcholine-like activity was initially reported to be much higher than that in any other tissues or organs of the body (38). According to Chang and Gaddum (23) the level of ACh was found not to be as high as that reported by Bischoff et al. (38). Chang and Gaddum also reported that they could not detect ACh-like activity in placentas of any other mammals, but Heirman (56) later claimed that the level of ACh-like substances in cow placenta was almost as high as that in human placenta. Further investigations on this issue have not been made in other laboratories. Placental cholinesterase-like activity (41-43) was proved to be due to AChE, because it preferentially hydrolyzed acetyl-β-methylcholine rather than benzoylcholine (39, 40). Histochemical studies of guinea-pig placenta (44, 45) indicated that the enzyme was localized within the syncytiotrophoblast.

Comline (46) first demonstrated the presence of a choline acetylase-like activity in the human placenta. Recently, Welsch (47) reported the synthesis of ACh from choline and acetyl-CoA by homogenates of placentas of guinea-pig, dog, mouse, cow, horse and cat. Many

investigators described the properties of partially purified ChA from placenta (48-50). Detailed kinetic studies with partially purified ChA preparations indicated that both placental and brain choline acetyltransferases synthesize ACh by the same mechanism, i.e., the Theorell-Chance mechanism (50-53).

The above review illustrates the widespread occurrence of one or more of the components of cholinergic system in tissues or organs classically regarded as non-nervous. In all cases, the activities of AChE and ChA closely resemble those in nervous tissues. However, the quantity and function of ACh appear to differ from what is known in nervous tissues.

C. Studies on Placental Acetylcholine

Placental ACh-like activity has been studied more extensively than the other members of the placental cholinergic system (23, 38, 54-58). Bischoff et al. (38) were the first to demonstrate the presence of an ACh-like substance in the human term placenta. Tney reported values of 259-370 nmoles ACh/g tissue. Haupstein (54) later reported values much greater than those found by Bischoff et al. (38). He pointed out that preterm placentas contained

higher ACh levels than term ones. Both Haupstein and Bischoff and associates agreed that the uterus contained no ACh. However, while Bischoff and associates proposed that placental ACh would initiate uterine contractions for parturition, Haupstein could find no link between placental ACh and triggering of uterine contractions.

With highly refined bioassay techniques Chang and Gaddum (23) confirmed that the human placenta contained an ACh-like substance. However, the value of 104 nmoles ACh-equivalent/g tissue quoted by these investigators was much lower than that which was previously reported. Some investigators (55) reported values for ACh as high as those found by Bischoff et al. (38). The value reported by Chang and Gaddum was more generally accepted as later investigators have confirmed (56, 58, 59, 64). The discrepancy in the reported values of placental ACh-like activity could be attributed to methodological defects and the type of placentas analyzed. For instance, the placenta used by Strack et al. (55) was kept in alcohol for over a year before its ACh content was analyzed. The major methodologic defect arose from the fact that the tissue used in the bioassay system could respond to other substances which behave like ACh in the incubation medium,

e.g., K^+ and extracted natural cholinomimetics. According to Chang and Gaddum (23) and Heirman (56), abnormally high values of placental ACh reported by earlier investigators was due to interfering substances in the bioassay set-up. The approach by Chang and Gaddum (23) commonly referred to as "parallel assay" (16) was capable of reducing interference to the minimum. Both Heirman (56) and Chang and Gaddum (23) concluded that placental ACh was not responsible for inducing uterine contractions during labor. They expressed the view that it was difficult to relate the high ACh-like levels to any known physiological function. Also difficult to reconcile was the fact that only the human placenta showed high ACh-like level.

In a series of publications from the Chinese laboratory of Chang and associates (56-62), the authors confirmed the observations of Chang and Gaddum (23) on the level of placental ACh-like substance. They reported that the quantity of placental ACh-like substance (about 110-185 nmoles/g tissue) remained constant until the time of delivery. During labor, however, a significant fall in ACh level occurred. The authors further showed that placental ACh-like activity was very low in cases of uterine inertia. On the other hand, they, and later, Reynolds and

Foster (66), demonstrated very high placental ACh levels in cases of premature separation of the placenta, hypertonic uterine contractions and premature labors. From these findings it was concluded that ACh intervenes in the initiation of delivery, just as was concluded by Bischoff et al. (38). In support of their conclusions, the Chinese group claimed significant therapeutic efficacy by periodic infusion of ACh in cases of uterine inertia and prolonged labors. In a modern clinical setting, Goodling (64) reported evidences confirming the Chinese studies. As mentioned earlier, Heirman (56) disagreed with the postulate that placental ACh-like substance initiated uterine contractions. He based his disagreement on the fact that the decline of ACh level correlated very closely with the length of time that the placenta remained in the uterus after the baby was separated.

A different line of thought was followed by Bull et al. (67), Hebb and Ratkovic (68) and Koelle (69). In their studies, they noted that placental choline acetyltransferase activity was higher during mid-trimester than at early and late periods of gestation. A similar finding was recently reported by Welsch (70). Bull and associates further noted that ChA activity was localized within the

chorionic villi in the cell layers which separate the maternal from fetal blood (67). These findings led to the postulate that placental ACh participates in the active transport of materials across the placental barrier. To date, no direct evidence confirming this proposal has been reported. By analogies to the known ability of ACh to increase membrane permeability of excitable tissues to the common extracellular cations and anions, the above postulate seems more acceptable than the involvement of ACh in triggering uterine contractions.

Other studies relevant to this review came from the Chinese group. Chang (63) studied the release of ACh by perfusion of the fetal circulation with oxygenated saline. He observed some ACh release which in no way reflected the observed changes in tissue levels of ACh. In this study, carried out over a period of 8 hours, he recorded near-zero amount of ACh release in the first 2 hours of the perfusion. Since the ACh released was into the fetal side of the syncytiotrophoblast, this report neither supported previous contention of ACh involvement in uterine contraction, nor did it support the proposal that ACh was involved in regulation of active transport of nutrients through the placenta (67-69).

Chang (57, 60) and Wen et al. (59) reported on the nature of existence of ACh in the placenta. Chang (60) demonstrated the existence of a "bound" and a "free" pool of ACh by pressing the bloody juice out of minced placenta. Wen et al. (59) concluded from their histochemical studies that ACh was localized in the placental syncytium.

In their attempts to ascribe a role to placental ACh, von Euler (71), Cuichta and Geutieri (72) and Dancis et al. (73) infused ACh or methacholine into the fetal circulation of human and guinea-pig placentas. None of the above studies showed any consistent effects of ACh on the fetal and placental vascular system. Therefore, it was inferred that placental ACh had no effect on the fetal side of the syncytiotrophoblast.

Although there is so much controversy about the quantity and function of human placental ACh, it is interesting to note that there is relatively little disagreement about the levels and functional significance of the enzymes which metabolize ACh; namely: acetylcholinesterase and choline acetyltransferase.

D. Scope of the Present Study

As outlined above, there is widespread controversy about the quantity and functional significance of ACh in human placenta. In view of the potential importance of ACh for placental function and therefore fetal development, it is of interest to examine in greater depth certain aspects of the placental ACh that could elucidate its functional significance.

All the experiments described in this dissertation have been designed to establish two clearly definable goals.

The first goal was to identify the ACh-like substances in human placenta by gas chromatography and other modern techniques. These new methods were used to study the localization, distribution, variation with gestational age and release of ACh from placenta. This information would be expected to define a base for future investigations of the role of placental ACh.

The second goal was to establish the pattern of spontaneous release of placental ACh into a physiological salt solution. Special attention was paid to the effects of potassium, calcium, cocaine and nicotine on this ACh

release pattern, and the mechanism of ACh release. It is assumed that the demonstration of a release process, and how drugs influence it, would aid in conceptualizing an _in vivo_ regulatory role for placental ACh in transport of nutrients through the placenta.

CHAPTER III

OCCURRENCE, DISTRIBUTION AND VARIATION WITH GESTATIONAL AGE OF ACETYLCHOLINE IN HUMAN PLACENTA

A. Introduction

A number of investigators have reported the occurrence of acetylcholine (ACh)-like activity in the extracts of human placenta using bioassay preparations (23, 38, 54-58). However, the components of the ACh-like activity in placental extracts were not identified.

If was shown by Comline in 1946 that there is choline acetyltransferase (ChA) in human placenta (46). The placental homogenates of the guinea pig, dog, cat, mouse, horse and cow were shown to synthesize ACh from acetylcoenzyme A and choline (47). A number of investigators have obtained partially purified preparations of ChA from human placenta (48-50). Recently, it has been shown that brain ChA and placental ChA will synthesize ACh by the same enzyme mechanism, namely, the Theorell-Chance mechanism (15, 50-52).

The presence of cholinesterases in human placenta has been indicated by a number of investigations (41-43). The human placental enzyme has been characterized as acetylcholinesterase (AChE) by Ord and Thompson (39) and by Koshakji et al. (40). Acetyl-β-methylcholine is a specific substrate for the placental AChE, which does not hydrolyze benzoylcholine to any significant degree.

The above observations indicate that the three components of the cholinergic system, ACh-like activity, ChA, AChE, are present in the human placenta. Since the placentae of man and animals lack innervation, a question arises as to whether the placental cholinergic system is in any way similar to that of the brain cholinergic system. Human placental ChA and AChE have been characterized. In the present study we are interested in the characterization of the components of the ACh-like activity of the human placenta by gas chromatography, its distribution, the nature of its occurrence and its variation with gestational age. The present study indicated that the major component of the ACh-like activity of human term placenta is ACh (112 n mol/g of wet tissue), which is localized mainly in the syncytiotrophoblast layer. The placental ACh levels during the second trimester of pregnancy were

higher than the ACh levels during the first and the third trimesters of pregnancy.

B. Materials and Methods

Source and Mode of Collection of Human Placentas

Both term and mid-gestational human placentas have been used in the following studies.

The term placentas studied were collected from Vanderbilt University Hospital Labor and Delivery (Vanderbilt L & D) Room following normal or caesarian delivery. Early and middle trimester placentas were collected from either the Nashville Planned Parenthood Center, where abortion was performed by vacuum suction, or from Vanderbilt L & D following therapeutic abortion by saline infusion.

Of the placentas used for the determination of acetylcholine levels, some were collected either immediately or as late as one or two days after they were expelled. The over-24-hour placentas were kept refrigerated at $4^{o}C$, but not frozen. Those placentas used for studying subcellular distribution and release of ACh were collected no later than 45 minutes (i.e., when still warm to the touch) after delivery.

Extraction of Placental ACh

Placental ACh was extracted and analyzed according to the method described by Schmidt et al. (3) for determining the ACh content of rat brains.

Tissue Handling and ACh Extraction

The surface of the placenta was washed free of maternal blood with saline, and the amniotic membranes were trimmed off. A portion of the placenta weighing about 5 g was cut out and transferred into the homogenization chamber of the Sorvall Omnimixer; 0.50 μmole of propionlycholine iodide (PCh) was added as an internal standard (I.S.). Then approximately 50 ml of a 2 percent solution of trichloroacetic acid in acetonitrile (2 percent TCA-in-MeCN) were added, and the tissue was homogenized for 30 seconds at the maximum speed of the Omnimixer.

The homogenate was kept at room temperature for 30 minutes and then centrifuged. Fifteen ml of the supernatant were transferred to a 50-ml centrifuge tube and about an equal volume of water was added. The mixture was extracted with two 15-ml portions of diethyl ether to remove tissue lipids and organic solvent. After each ether

wash, the mixture was centrifuged and the ether layer was aspirated off. A gentle stream of dry nitrogen gas was passed through the aqueous extract to drive off traces of ether.

A general scheme for the extraction of ACh and related compounds is shown in flow-sheet, Figure III-1.

Precipitation of Quarternary Ammonium Compounds

Approximately 2 ml of the ether-free aqueous extract were pipetted into a 13-ml conical centrifuge tube and diluted with 2 ml of water. About 20 μg of tetramethylammonium (TMA) chloride were added as coprecipitant. The mixture was briskly shaken on a vortex mixer. Quaternary ammonium compounds were precipitated by adding 0.15 ml of a potassium iodide-iodine solution ($KI-I_2$; containing 2 g KI with 1 g I_2 in 10 ml water). After the tube was shaken on a vortex mixer, it was incubated in ice for 30 minutes. The tube was centrifuged at 3000 r.p.m. for 15 minutes on the IEC model PR-2 centrifuge. The supernatant was aspirated off leaving the precipitate of quarternary ammonium compounds as iodides (precipitate QC) on the side of the tube.

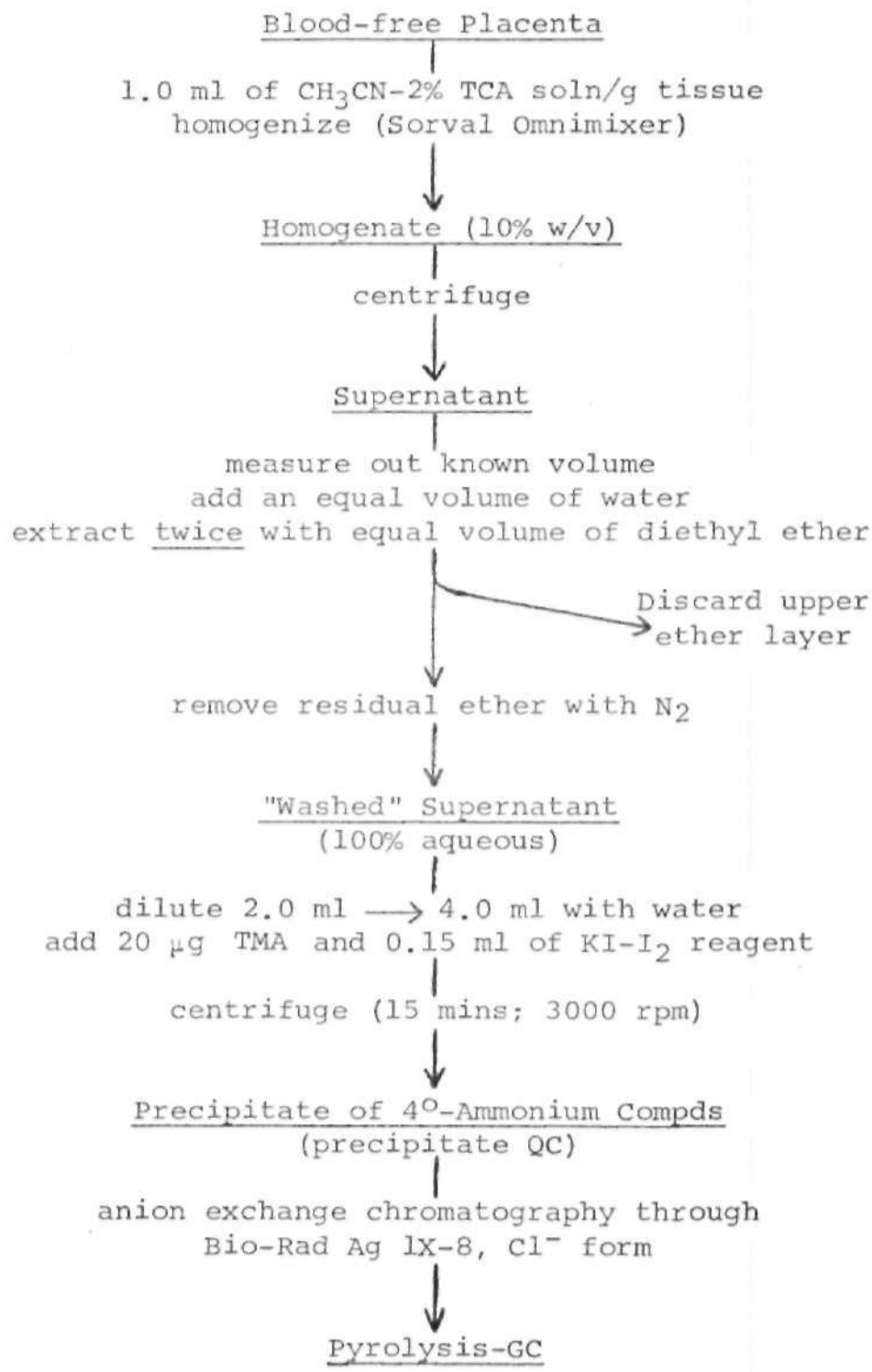


Figure III-1. General scheme for extraction of ACh and related compounds from placenta (adapted from Schmidt et al. [3]).

Pyrolysis-GC Analysis of ACh in Extract

The precipitate of quaternary ammonium compounds was dissolved by gently adding about 50 µl of a methanol-acetonitrile (MeOH-MeCN) (30:70 v/v) solution down the side of the centrifuge tube. A micro-column prepared by filling a 1.0 x 25.0 mm glass capillary tube with Bio-Rad Ag 1X - 8, Cl^- form, anion exchanger was attached to the needle of a Hamilton microliter syringe. The iodide of the dissolved precipitate was exchanged for Cl^- ions by drawing the solution through the micro-column into the syringe. This was done in order to remove iodine which causes rapid deterioration of the GC column. The clear solution was transferred to a platinum pyrolysis ribbon on a hot plate.

After the solvent was evaporated, the ribbon was screwed onto the electrodes in the pyrolyzer chamber. Then the chamber was tightly closed, and the control knob of the pyrolyzer was switched to the "purge" position. This operation allowed the carrier gas to displace air from the pyrolyzer chamber. After "purging" for about 2 or 4 minutes, the control knob was turned to "analyze"

position, and immediately thereafter the pyrolyzer heater was turned "on" for 5 seconds.

The pyrolyzed sample was simultaneously swept onto the chromatographic column where the components were separated. The column and oven conditions were:

> The 1/4-inch (O.D.) by 10-foot glass column was packed with 20 percent Carbowax 6000 on 60-80 mesh Chromosorb W, HMDS (custom prepared by Applied Sci. Labs. Inc.), pyrolyzer body temperature was 160°C, oven temperature 140°, F.I.D. 160°. The gasses were run at 80 ml/min for N_2, 200 ml/min for 0_2, and 60 ml/ml hydrogen.

The products of pyrolysis of ACh^+ Cl^- and $PrCh^+$ Cl^- were respectively dimethylaminoethyl acetate (DMAEA) and dimethylaminoethyl propionate (DMAEP).

Calculation of the Amount of ACh

The chromatogram obtained from the recorder was analyzed by calculating the ratio:

$$\frac{\text{Height of the DMAEA peak}}{\text{Height of the DMAEP peak}}$$

This ratio was multiplied by the number of nmoles of $PrCh^+$ I^- added as an internal standard per gm of tissue.

The resulting product would be equal to the number of nmoles of ACh per g of tissue.

Separation of placental ACh from other quaternary ammonium compounds by column chromatography. Amberlite-CG-50 resin (3 to 4 g) was washed with distilled water (3 x 30 ml) and the washed resin was soaked in distilled water (50 ml) overnight. The suspension of the resin was used to prepare a column (15 cm) in a 20 ml burette. The column was further washed with potassium phosphate buffer (100 ml, 0.5 M, pH 6.9). After the fluid was drained off the column, a solution (0.5 ml) of quaternary ammonium compounds (precipitate QC) from the placental extract was poured over the top of the column. After the sample was absorbed by the column, the column was eluted with potassium phosphate buffer. During the elution period about 2 ml of fluid was maintained over the column and 2 ml samples of the eluate were collected at a rate of 1 ml/min in an automatic fraction collector. The quaternary ammonium compounds in each fraction were extracted with acetonitrile. ACh, choline, and an unidentified compound (compound X) were analyzed by pyrolysis gas chromatography.

In a separate experiment, ^{14}C-ACh (0.02 μc) was subjected to separation by column chromatography. ^{14}C-ACh in the elution fractions was counted according to the procedures described elsewhere (50, 51).

C. Results

Occurrence of ACh and other quaternary ammonium compounds in placental extract. The pyrolysis gas chromatogram of a placental extract is shown in Figure III-2. During pyrolysis, the quaternary ammonium compounds are converted into their corresponding tertiary analogs. The peak B has been identified as 2-dimethylaminoethyl acetate, formed from endogenous ACh. The height of the peak could be increased quantitatively by the addition of known quantities of ACh to the placental extract. The peak B disappeared when the placental extracts were subjected to an alkaline hydrolysis (Figure III-3).

The peak A was due to 2-dimethylaminoethyl-propionate, formed from propionylcholine which was added to the placental extract as an internal standard. Peak A was absent when placental extracts were analyzed without the addition of propionylcholine. The peak C was due to

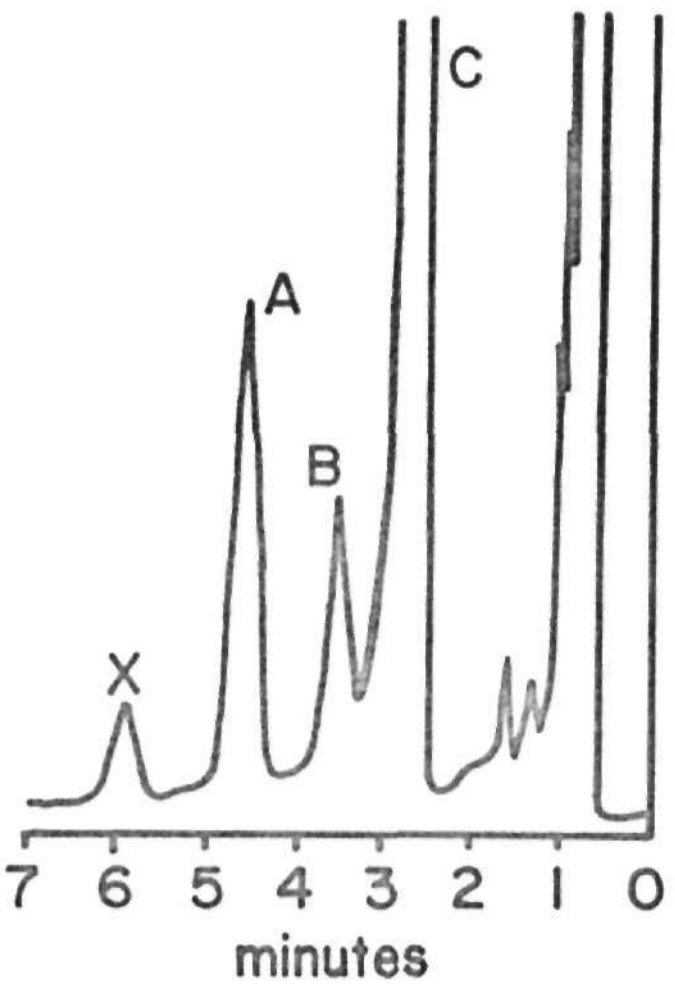
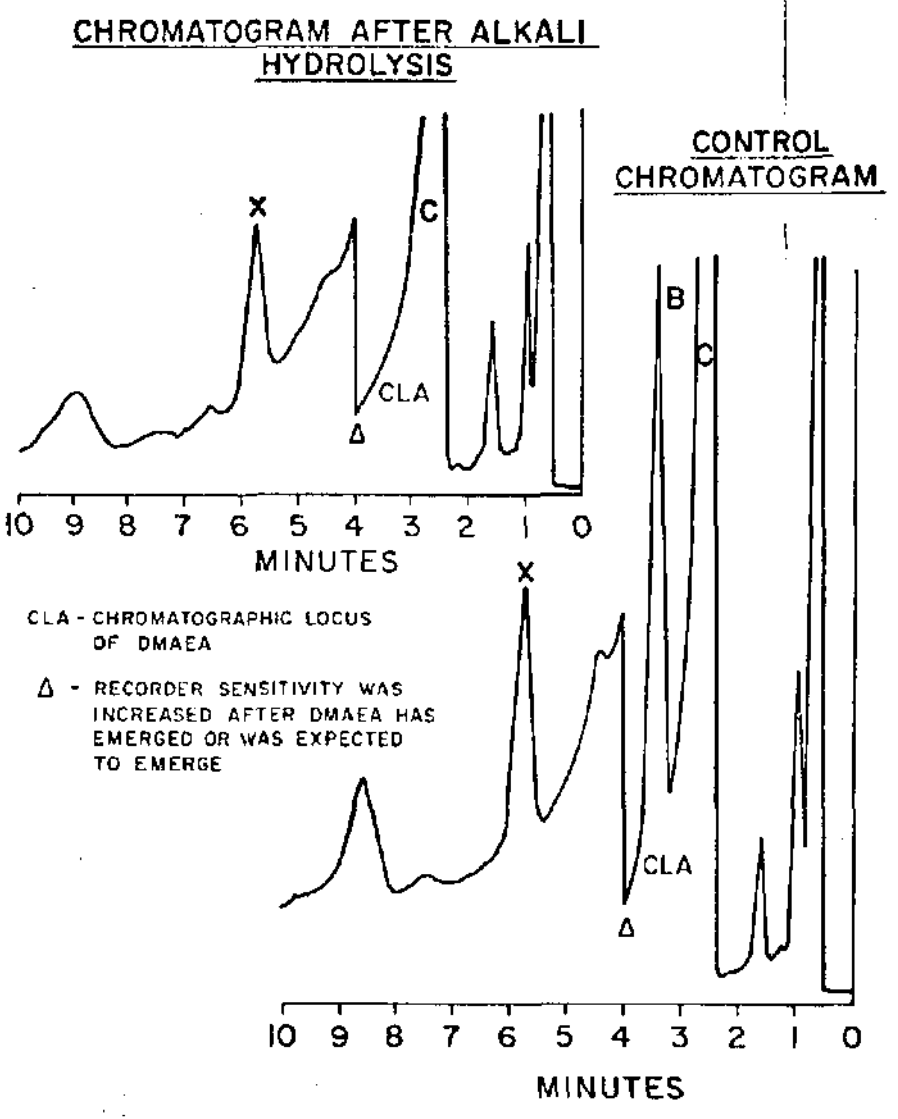


Figure III-2. Gas chromatogram of the quaternary ammonium compounds separated from the human placenta and subjected to pyrolysis. Peak A: 2-dimethylaminoethyl propionate formed from propionylcholine which was added as an internal standard. Peak B: 2-dimethylaminoethyl acetate formed from endogenous ACh. Peak C: 2-dimethyl-aminoethanol formed from endogenous choline. Peak X: an endogenous amine or a compound formed during pyrolysis which was not identified.


CHROMATOGRAM AFTER ALKALI HYDROLYSIS
X
C
CLA
Δ
10 9 8 7 6 5 4 3 2 1 0
MINUTES
X
CLA - CHROMATOGRAPHIC LOCUS OF DMAEA
Δ - RECORDER SENSITIVITY WAS INCREASED AFTER DMAEA HAS EMERGED OR WAS EXPECTED TO EMERGE
CONTROL CHROMATOGRAM
B
C
CLA
Δ
10 9 8 7 6 5 4 3 2 1 0
MINUTES

2-dimethylaminoethanol, formed from choline in the placenta. Peak C was not affected by alkaline hydrolysis.

The peak X was due to a tertiary amine, formed from an endogenous quaternary ammonium compound, and it was not identified in our studies. Alkaline hydrolysis of the placental extracts had no effect on peak X. Peak X had a consistently longer retention time than peaks A, B and C.

Separation of quaternary ammonium compounds from the placental extract by column chromatography. It is possible to separate the quaternary ammonium compounds into two components using the cationic ion exchange resin Amberlite CG-50. In the elution chromatogram, the first component was collected in fractions 6-12 and the largest amount was collected in fraction 9 (Figure III-4). When authentic ^{14}C-ACh was chromatographed under identical conditions, the largest amount appeared again in fraction 9 (Figure III-4). The pyrolysis gas chromatogram of fraction 9 exhibited a prominent peak, with a retention time of 3.8 minutes (Figure III-5a). This peak was identified as that of 2-dimethylaminoethyl acetate, which was formed from ACh during pyrolysis. Therefore, the quaternary

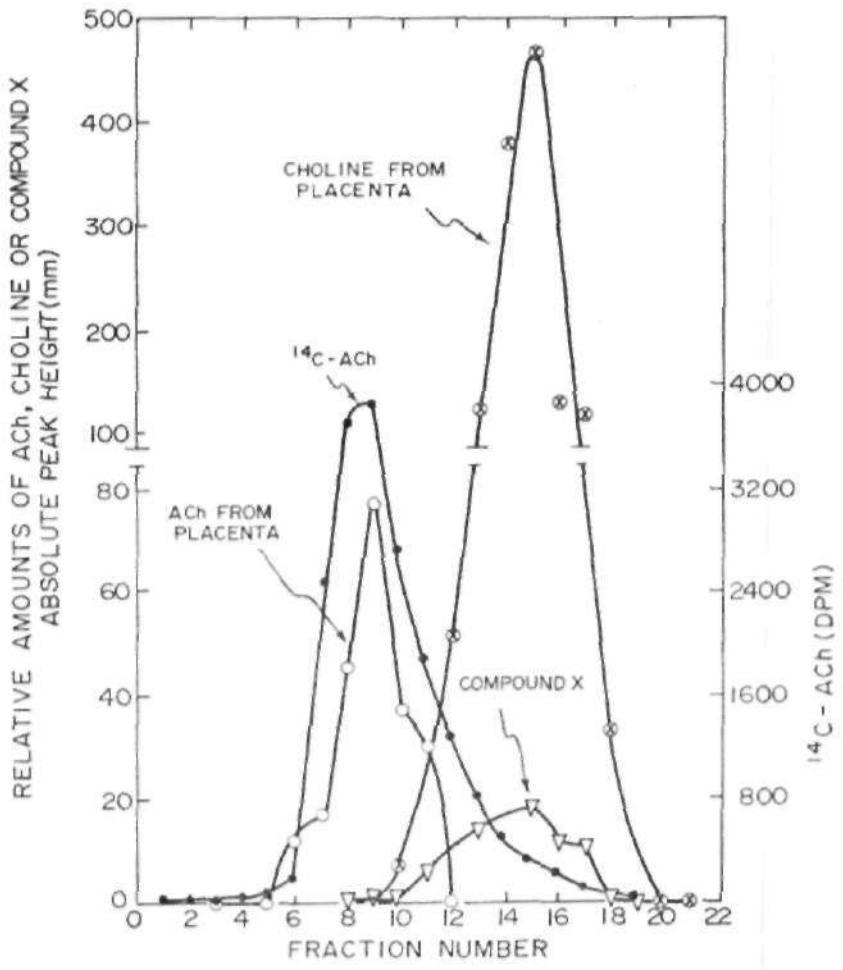


Figure III-4. Separation of the quaternary ammonium compounds from the placenta by chromatography on a column of Amberlite CG-50. The relative amounts of ACh, choline and compound X were analyzed by pyrolysis gas chromatography.

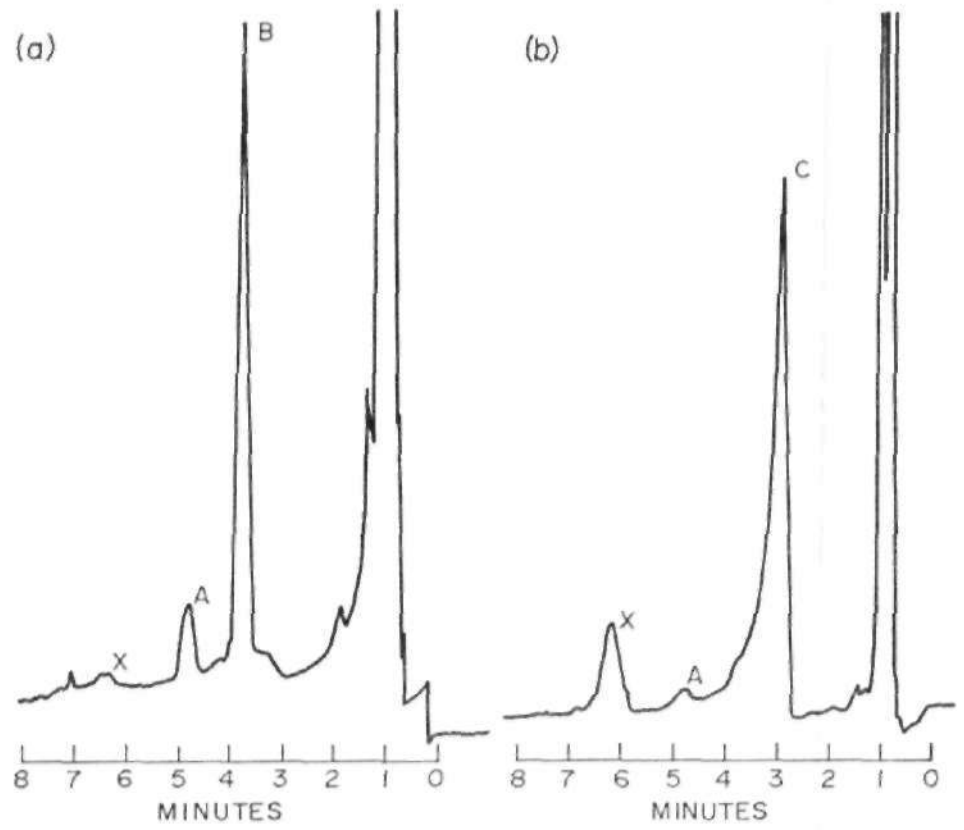


Figure III-5. (a) Pyrolysis gas chromatogram of the quaternary ammonium compounds collected in fraction 9 of Figure III-4. (b) Pyrolysis gas chromatogram of the quaternary ammonium compounds collected in fraction 15 of Figure III-4. The two chromatograms were run at different electrometer settings. Peak A: 2-dimethylaminoethyl propionate formed from the internal standard, propionylcholine. Peak B: 2-dimethylaminoethyl acetate formed from endogenous ACh. Peak C: 2-dimethylaminoethanol formed from endogenous choline. Peak X: the compound which was not identified.

ammonium compound in fraction 9 was identified as ACh. No peak for the presence of 2-dimethylaminoethanol was observed. No significant peak for the presence of the tertiary compound which might have been formed from compound X was observed. Therefore, fraction 9 contains ACh, and is free from choline and compound X.

In the elution chromatogram, a second component was collected in fractions 10-18, and the largest amount was collected in fraction 15 (Figure III-5b). The pyrolysis gas chromatogram of fraction 15 indicated the presence of two prominent peaks, with retention times of 2.9 and 6.3 minutes. Pyrolysis of an authentic sample of 2-dimethylaminoethanol (or choline) gave a single peak with a retention time of 2.9 minutes on the gas chromatogram. The peak with the retention time of 6.3 minutes was due to a tertiary amine formed from the unidentified quaternary ammonium compound in the placental extracts.

According to the above experiment, fractions 6-9 contained ACh. Choline and the unidentified compound could not be detected in these fractions. Fractions 12-18 contained choline and the unidentified compound X. Fractions 11 and 12 contained all three compounds: ACh,

choline and unidentified compound X. Therefore, the experiments support the existence of ACh in human placenta.

Content of ACh in term human placenta. The ACh contents from ten term human placentas, collected after therapeutic deliveries, are shown in Table III-1. There are significant differences in the concentrations of ACh from placenta to placenta. The reasons for these differences are not known. Some of these differences might be explained by the clinical histories of the mothers, the mode of delivery and the duration of labor, etc. According to our data from 34 deliveries, human term placentas contained 112.05 ± 7.08 n mol/g of wet tissue. The same group of placentas contained an unidentified compound X in small amounts (15.75 ± 1.83 nmoles of ACh iodide equivalents/g of wet tissue). This compound could be detected in only an occasional placenta.

Distribution of ACh in the term human placenta. The relative distribution of ACh in various concentric segments of human placenta is shown in Figure III-6a (also see Table III-2). In order to determine this, each placenta was cut into six concentric segments and the segments were

TABLE III-1

SOME TYPICAL VALUES OF ACh IN HUMAN TERM PLACENTA[a]

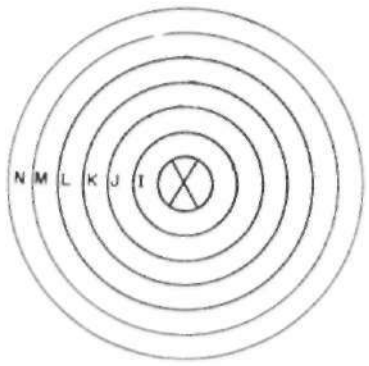
Serial Number of Placentas	ACh nmoles/g Wet Tissue Mean ± S.E. (N)[b]	Unidentified Compound in ACh Equivalents nmoles/g Wet Tissue Mean ± S.E. (N)[c]
1	61.51 ± 1.55 (5)	20.98 ± 1.96 (3)
2	116.90 ± 1.65 (7)	20.40 ± 1.35 (4)
3	141.43 ± 1.88 (3)	15.16 ± 0.37 (3)
4	131.77 ± 3.60 (3)	15.31 ± 0.26 (3)
5	77.20 ± 1.80 (4)	13.33 ± 1.10 (4)
6	91.37 ± 2.83 (3)	8.53 ± 1.02 (3)
7	51.68 ± 1.45 (4)	7.95 ± 0.04 (4)
8	93.84 ± 3.35 (3)	8.57 ± 0.15 (3)
9	129.97 ± 3.09 (3)	9.63 ± 0.37 (3)
10	91.10 ± 1.20 (3)	20.80 ± 2.12 (3)

[a]The whole placenta was extracted with acetonitrile and an aliquot of the extract was used for ACh analysis. Gestation period, 38-44 weeks. N indicates the number of aliquots analyzed from each placenta.

[b]Mean (M_1) ± S.E. for the above first group of 10 placentas: 98.66 ± 9.66 n mol/g of wet tissue. A second group of 24 term placental samples (representative radial sections) were analyzed for ACh. Mean (M_2 ± S.E. for the second group: 117.63 ± 9.06. There was no significant difference ($p > 0.05$) between M_1 and M_2. The overall mean for 34 placentas of both groups was 112.05 ± 7.08 n mol/g of wet tissue. N indicates the number of aliquots analyzed for ACh from each placental extract.

[c]Mean (M_3) ± S.E. for the above first group of placentas: 16.28 ± 2.84 n mol of ACh equivalents/g wet tissue. Mean (M_4) ± S.E. for the second group of placentas: 14.07 ± 1.68. There was no significant difference ($p > 0.05$) between M_3 and M_4. The overall mean for compound X from both groups of placentas = 15.75 ± 1.83.

A



CONCENTRIC SEGMENTS	nmol ACh/g wet tissue
N	99.09 ± 0.09
M	122.02 ± 0.07
L	120.30 ± 0.07
K	117.00 ± 0.04
J	121.72 ± 0.07
I	76.79 ± 0.01

B

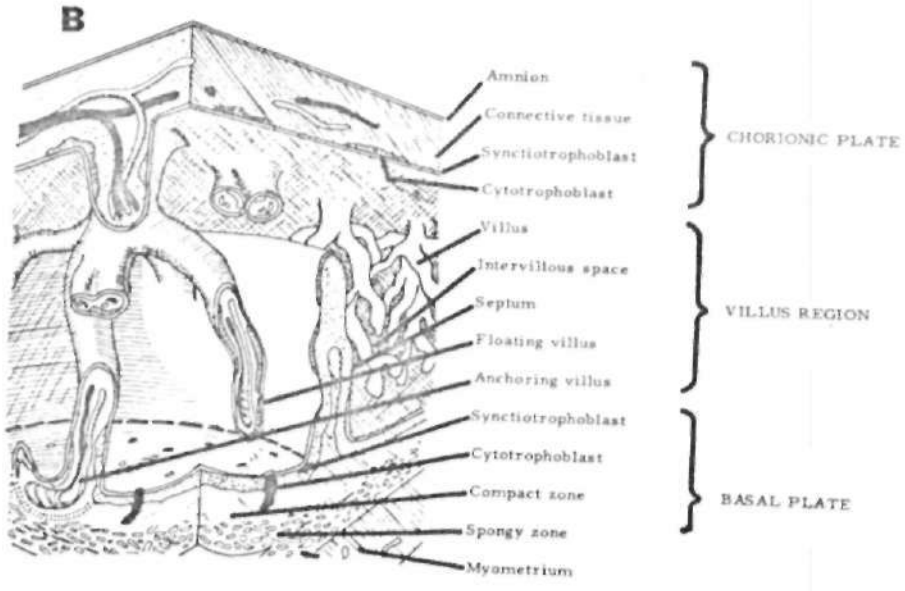


TABLE III-2--Continued

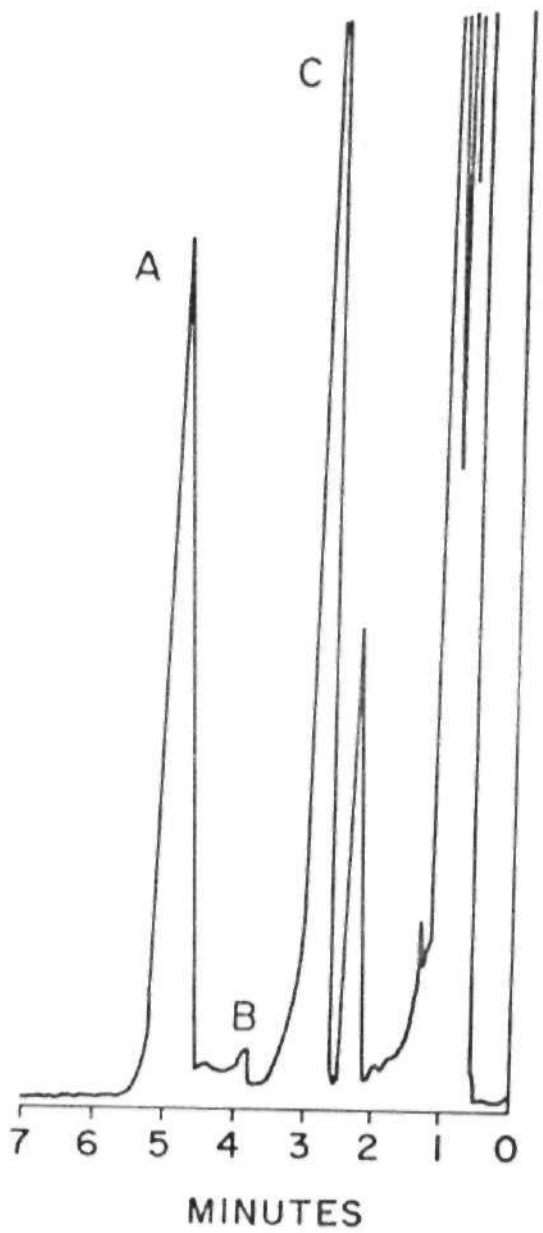
in Tissue: nmoles per gram Wet Tissue[a]		
Villi[b]	Basal Plate	Chorionic Plate
334.13 ± 9.28	252.50 ± 1.53	100.37 ± 5.37
166.22 ± 1.18	84.10 ± 2.20	73.45 ± 0.05
223.67 ± 4.81	190.00 ± 2.804	91.37 ± 3.98
65.82 ± 1.55	54.35 ± 1.46	16.29 ± 0.57
103.30 ± 5.50	72.85 ± 0.35	57.95 ± 2.45
117.08 ± 7.51	76.85 ± 2.65	40.00 ± 0.60
184.65 ± 4.45	99.98 ± 7.73	37.75 ± 2.26
68.33 ± 1.08	36.49 ± 0.75	17.82 ± 1.07
123.37 ± 1.87	68.58 ± 5.04	24.40 ± 0.23
307.27 ± 6.87	241.40 ± 28.94	191.23 ± 6.07

[a]Mean ± S.E. from a minimum of 3 or more determinations for each tissue sample.

[b]The villi contained about 3.22 ± 0.39 times higher concentration of ACh than that in the chorionic plate; the basal plate contained about 2.10 ± 0.23 times higher concentration that than in the chorionic plate.

analyzed separately for ACh. The ACh concentration in the concentric segment next to the periphery of the umbilical cord was lower than the ACh concentration in the other segments. This suggests that there are higher concentrations of ACh where the placental villi are located. In order to verify this, we have dissected floating villi, the basal plate along with anchoring villi, the trophoblast layer and the chorionic plate (Figure III-6b). The total surface area of the trophoblast in the floating villi and the anchoring villi ought to be larger than the trophoblast layer of the chorionic plate. Therefore, one can associate ACh with the syncytiotrophoblast.

The influence of cold storage (4°C) and freezing on placental ACh content. When a human placenta was frozen for 48 hours and then warmed to laboratory temperature, the entire amount of ACh had decomposed (Figures III-7 and III-8). There was no change in the concentration of the unidentified compound X, even after freezing a placenta for 168 hours (Figure III-8). When a placenta was kept for 77 days at 4°C, about 30 percent of the ACh remained in the tissues. These observations suggest that ACh was bound within membranes. Freezing breaks the membranes


A
B
C
7 6 5 4 3 2 1 0
MINUTES

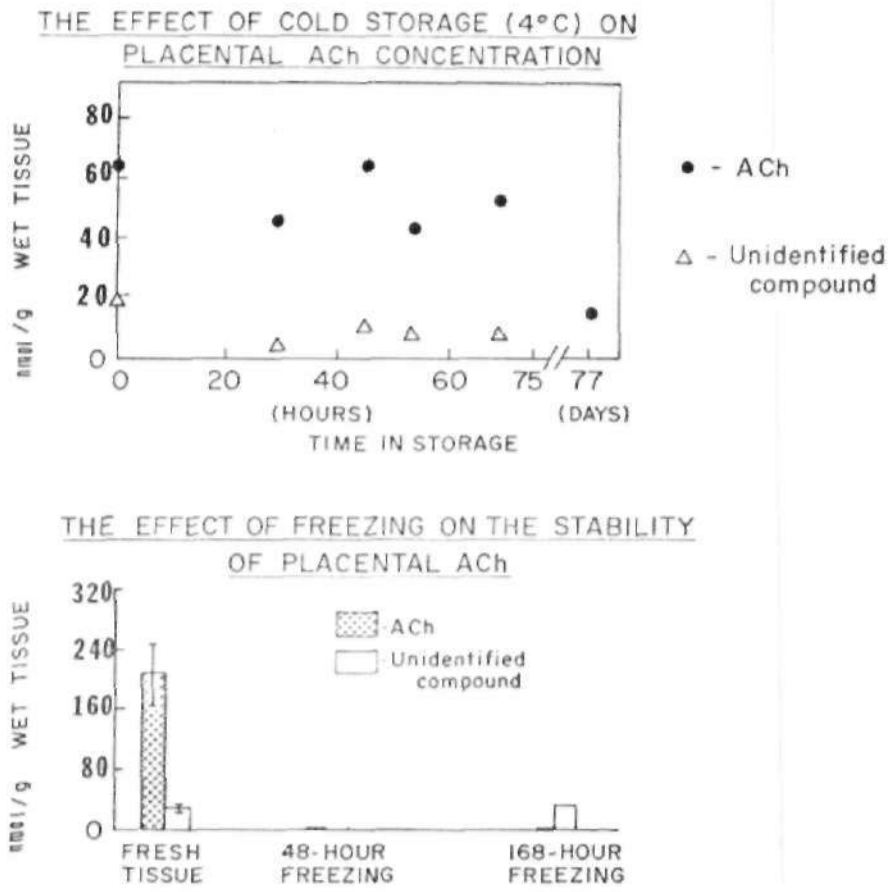


Figure III-8. The influence of cold storage, and freezing and thawing, on the stability of placental ACh. Each point is a mean ± S.E. from three determinations. When the S.E.s are too small to indicate on figures, they are omitted.

and liberates ACh, which is subsequently hydrolyzed by AChE. The unidentified compound X might not contain an ester bond, which would explain why it was not affected by freezing or AChE hydrolysis.

Variation of the concentration of human placental ACh as a function of the gestation period. Human placentas collected from 82 cases of therapeutic abortions, spontaneous abortions and therapeutic deliveries were analyzed for ACh content (Table III-3). ACh values for the first eight weeks of pregnancy could not be quoted, because the placenta is not clearly defined until the third month. The gestation period could be assessed within a range of ± two weeks. Therefore, the ACh values for four consecutive weeks of pregnancy were pooled. The values in Table III-3 indicate that the ACh concentration was lower during the first and third trimesters of pregnancy than it was during the second trimester of pregnancy. The peak values for ACh concentration were found during the 21-24 weeks of pregnancy.

TABLE III-3

VARIATION OF PLACENTAL ACh AS A FUNCTION OF GESTATIONAL AGE

Gestational Ages[a] (Weeks)	Number of Placentas	nmoles ACh per gram Wet Tissue[b]	
		Mean (± S.E.)	Range
9-12	1	129.45	--
13-16	11	342.21 (±30.57)	218.73-531.00
17-20	12	317.08 (±31.92)	181.70-542.95
21-24	4	722.90 (±63.33)	566.00-843.45
25-28	2	231.00 (±42.67)	188.33-273.67
29-32	4	249.18 (±28.32)	173.13-307.67
33-36	11	152.92 (±14.68)	103.70-285.00
37-40	25	104.75 (± 6.70)	51.68-202.35
41-44	12	87.77 (± 5.43)	50.40-122.75

[a]Gestational age could be assessed within ± 2 weeks.

[b]A representative radial section of the placenta was analyzed for ACh.

D. Discussion

According to Chang (60) the ACh-like activity of term placental extracts is equivalent to about 180-230 nmoles of ACh/g of wet tissue. Our results indicate that term placenta contains about 112 nmoles of ACh/g of wet tissue. Therefore, about 55 percent of the ACh-like activity of the placental extracts could be identified as ACh.

The unidentified compound in the term placenta was not hydrolyzed by alkali. It could not be separated from choline by ion-exchange chromatography using the ion exchange resin Amberlite CG-50. It is possible that it is an analog of choline.

Our investigations indicate that placental ACh is bound within membranes. When human placenta was frozen for 48 hours and warmed to laboratory temperature, the entire amount of ACh decomposed. When the placenta was kept for 77 days at 4°C, about 30 percent of it remained in the tissues. The formation of ice crystals in the frozen tissue destroys the membrane structure and ACh is released, which is then hydrolyzed by AChE. Similar results with brain tissue have been reported in the

literature (73, 75). Freezing and thawing of brain tissue brings about the release of tissue-bound ACh, which is destroyed by AChE. According to some estimations (50, 60), a major portion of ACh-like activity (95 percent) in human placenta is in a bound form.

The available evidence indicates that ACh is localized in the villus tissue of the human placenta. There are 20 to 30 large villus trunks which correspond to the cotyledons (lobes) arranged in a circle around the umbilical cord (75). The concentric segment next to the umbilical cord and the peripheral segment have lower concentrations of ACh than the central concentric segments. This suggests that high ACh concentrations are found at positions where villi are localized. The ACh concentrations in the various sections of the placenta could be arranged in the following order (Figure III-6b): villus tissue > basal plate (containing parts of anchoring villi) > chorionic plate. The surface areas of the trophoblast in the above sections could be arranged in the same order.

The syncytiotrophoblast layer is fully formed by about four months and the cytotrophoblast disappears (75). At about this time, high concentrations of ACh are found in the placenta. The fully developed villus contains only

three layers, syncytiotrophoblast, connective tissue and vascular fetal endothelium (75). In histological studies, certain granules can be precipitated in the syncytiotrophoblast by a special fixative (1 percent ammonium reineckate in 10 percent formaldehyde or 80 percent alcohol), which is specific for ACh-like compounds (59). These granules were localized along the free border of the syncytiotrophoblast. Electron microscopic studies have demonstrated that there are vesicles at the base of the microvilli of the syncytiotrophoblast (76, 77). The surface areas of the syncytiotrophoblast could be arranged in the following order: villus tissue > basal plate which contains anchoring villi > chorionic plate (75). The ACh concentrations in these tissues could be arranged in the same order. These observations indicate that ACh is possibly localized in the syncytiotrophoblast. However, it has yet to be demonstrated that ACh is contained in vesicles.

There was a variation in the ACh concentration with gestational age of the placenta. The highest values for ACh concentration were found during 21-24 weeks of pregnancy. The variation in ChA activity shows a pattern similar to that seen for ACh (67, 78). This pattern

remained the same when ChA activity was expressed per unit weight of the tissue or protein (67). There was peak ChA activity at about 16-20 weeks of gestation and a fourfold decrease in activity at parturition as well as at 8-13 weeks of development (78). The development of the placental cholinergic system, as indicated by ACh-ChA, follows the development of the syncytiotrophoblast (75) during the first 6 months of pregnancy. The reasons for the decrease in ChA-ACh concentrations in the term placenta are not known.

There are two principal stages in the development, an embryonic period (1-2 months) and a fetal period (2-9 months) (75). Neither the syncytiotrophoblast nor the placental cholinergic system (ChA-ACh) is fully developed during the embryonic period. Many substances (80, 81) (e.g., α-aminoisobutyric acid [78], diphenylhydantoin [82]) cross the placental barrier with relative ease. The degree of the teratogenic sensitivity of the human fetus to chemicals reaches the highest levels during this period (81). During the first part of the fetal period (2-6 months), the organs undergo little more than maturation (histogenesis) at the histological level. The size of the human fetus increases by about 250 times during this

period. Chemicals do not cross the placental barrier as easily as they did during the embryonic period; and their transport does seem to be regulated (78, 81). The placental cholinergic system is fully developed during this period. During the latter part of the fetal period, functional maturation of the fetus is achieved. The placental levels of ChA-ACh have decreased. Chemicals do seem to cross the placental barrier more easily than they did during the mid-gestation period (80, 81, 83). These observations indicate that the placental cholinergic system may play a significant role in the regulation of the transport of nutrients and chemicals across the syncytiotrophoblast and thereby regulates the fetal growth. Studies on the interrelationships between placental transport and fetal growth during the manipulation of the placental cholinergic system using pharmacological agents (e.g., inhibitors of AChE and ChA) may provide an answer for the role of ACh in the placenta.

CHAPTER IV

SUBCELLULAR DISTRIBUTION OF ACh IN HUMAN TERM PLACENTAS

A. Introduction

The successful isolation of synaptic vesicles from brain tissues by Whittaker and his associates (7, 84, 85) and de Robertis and his co-workers (6) contributed to our knowledge of the organization of cholinergic nerve terminal and storage of ACh. Hence differential centrifugation of a homogenized guinea-pig or rabbit brain (84) indicated that ACh was distributed among the subcellular structures (using Whittaker's notations) as follows (percentage of total ACh in whole homogenate):

Fraction P1 (Nuclear, Nuc):	15-20%
Fraction P2 (Mitochondrial, M_T):	50-60%
Fraction P3 (Microsomal, M_C):	0- 5%
Fraction S3 (Cytosol, high-speed supernatant):	20-30%

Simultaneous determination of ChA distribution (84) indicated a similar pattern. The subcellular distribution of

ACh in other cholinergically innervated tissues, particularly the neuromuscular junction of skeletal and smooth muscles and the autonomic ganglia has also been examined. In these tissues, ACh is identically partitioned among subcellular fractions as it is in the brain.

Investigations of the subcellular distribution of other neutrotransmitters such as serotonin (5-HT) and norepinephrine (NE) showed that these are subcellularly partitioned in a pattern similar to that of ACh.

The subcellular distribution of ACh was explained in terms of ACh storage. It was suggested that ACh storage site was in the synaptic vesicles which could be separated from fraction P2.

The information derived from these subcellular distribution studies have contributed significantly to an understanding of the organization of the cholinergic nerve terminals, and the mechanisms by which drugs interact with ACh and other neurotransmitters in nervous tissues.

In view of the high ACh content of the human placenta, and since the placenta is generally regarded as devoid of neuronal innervation, the question naturally arises: How does the ACh distribution and storage in placenta compare with distribution and storage in the

innervated tissues? The answer (or answers) to this question is expected to pave the way for further investigations into the possible interactions between certain drugs and the placental cholinergic system. Furthermore, an understanding of the mode of storage of placental ACh would provide some insight into the mechanism by which ACh is released. There are no published reports on the studies of the storage of ACh in the placenta. Hence it seemed desirable to make a more extensive study of this subject and to examine how ACh storage is related to ACh release or vice versa.

In the studies reported here, homogenates of human term placentas were fractionated by differential centrifugation. The content of ACh in each fraction was determined by pyrolysis-gas chromatography as described previously.

The observations made in these studies indicated that the distribution of placental cholinergic system appeared to be significantly different from that in the peripheral and central nervous systems.

B. Methods

Solutions Used

1. Normal saline: 0.9 percent (w/v) sodium chloride solution containing 1 x 10^{-5} M phospholine iodide.

2. Homogenization fluids:

a) 0.1 M KCl - 0.005 M Histidine buffer, pH 7.2 (86) containing 0.5 mM EDTA, 0.02 M NaCN (50) and 1 x 10^{-5} M phospholine iodide.

b) 0.32 M Sucrose solution (8) containing 0.5 mM EDTA, 0.02 M NaCN and 1 x 10^{-5} M phospholine iodide.

c) When AChE distribution was studied, EDTA, NaCN and phospholine iodide were omitted from saline and the homogenization media.

All solutions were stored at 0-4^{o}C in the refrigerator or the cold room until used.

Tissue Preparation

All operations were performed in the cold room where the temperature was 0-4^{o}C (86).

The freshly collected placenta was rinsed free of maternal blood by saline. Chorionic and amniotic membranes

were trimmed off, then the placenta was placed in a tray cooled on ice. An arterial cannula was installed, and the fetal blood was washed out by infusion of Normal Saline solution. The cleaned portion was carved out, then both the chorionic plate and the basal plate were dissected out and discarded.

The remaining portion, which was predominantly villus tissue, was washed four times in saline.

Homogenate Preparation

Approximately 25 g of the washed villus tissue was weighed out in a cooled, preweighed vessel containing 75 ml of homogenization medium. The vessel was then immersed in ice and the villus tissue was minced thoroughly. Homogenization of the tissue was performed with either the Sorvall Omnimixer homogenizer or the Potter-Elvehjem glass mortar-teflon pestle homogenizer. When the Omnimixer was employed, it was operated at its maximum speed for 20 seconds, while homogenization with the mortar-pestle homogenizer required 10-12 passes of the pestle at maximum motor speed. In either case, the homogenizer chamber-blade or mortar-pestle assembly was washed with 25 ml of fresh homogenization medium. This wash was mixed with the

homogenate to give a final 25 percent (w/v) homogenate. The combined homogenate was filtered once through four layers of surgical gauze (37) and the total filtrate volume was recorded. This filtration process removed unbroken cells and debris.

About 7 ml of the filtrate was pipetted into a 50-ml centrifuge tube. This portion was used to determine the ACh content of the entire filtered homogenate. The remaining filtrate was fractionated by centrifugation.

Differential Centrifugation

Differential centrifugation was performed with a modified method of Whitaker et al. (85). The filtered homogenate was centrifuged at 1000 x g (~3000 rpm) for 15 minutes in a type 30 fixed angle rotor on a Sorval RC-2 refrigerated ultracentrifuge. The pellet (P1) was kept aside for later ACh and protein analyses. The supernatant was centrifuged again in the Sorval RC-2 centrifuge at 14,500 x g (11,000 rpm) for 40 minutes to isolate the mitochondrial fraction. The mitochondrial pellet (P2 fraction) was kept aside for later ACh and protein assays. Isolation of the microsomal fraction, P3, was performed in the Beckman L2 Preparative Ultracentrifuge using either a

Type 40 or Type 50 Ti fixed angle rotor. Centrifugation was done at 105,000 x g (40,000 rpm) either for 60 or 90 minutes. Two experiments were performed at 167,000 x g (50,000 rpm) for about 22 hours in order to ensure that microscopic ACh vesicles are sedimented. The P3 fraction and the high-speed supernatant, S3 fraction, were kept aside until later assayed for ACh and protein.

Analysis for ACh Content of Fractions

Immediately after each of pellets P1, P2 and P3 was obtained, the pellet was resuspended in 10 ml of homogenization medium and manually homogenized with a glass mortar-teflon pestle homogeinzer. One milliliter of each suspension as well as 2.0 ml of supernatant S3 was diluted to 10 ml. The diluted suspensions or solution were used later to assay for protein, succinic dehydrogenase (SDH), ChA, and AChE activities in each fraction.

Nine milliliters of the undiluted S3 and the suspensions of P1, P2 and P3 were each mixed with 15 nmoles of $PCh^+ I^-$, as an internal standard, and about 25 ml of the 2 percent TCA-in-MeCN solution which precipitated proteins and solubilized lipoid substances. After standing in the laboratory temperature for 2 hours, the supernatant

in each case was decanted and extracted as described in Chapter III. When 0.32 M sucrose solution was used as the homogenization medium, the ether-free aqueous extract was subjected to the ion-pair formation extraction technique described later in Figure V-1 (page).

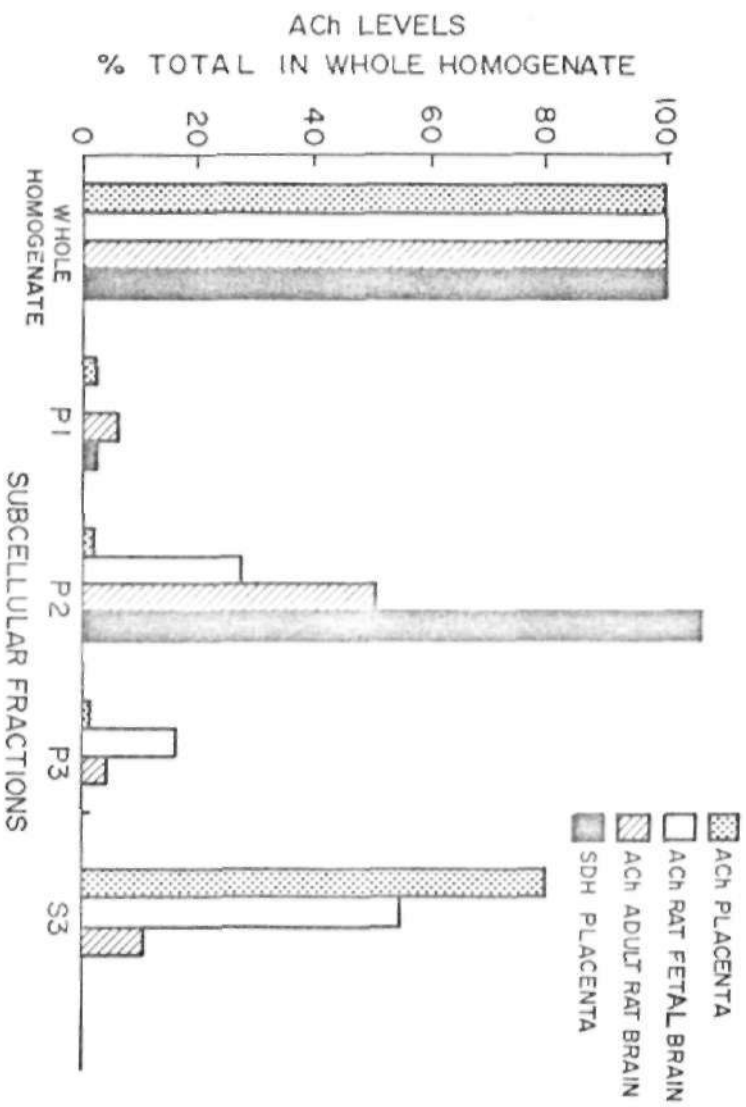
Protein assay. The protein content of each fraction was analyzed according to the method of Sutherland et al. (14). This was the unit of expressing enzymatic activities in the fractions.

SDH assay. Succinic dehydrogenase activity was assayed according to the procedure described by Pennington (87). The assay was used to evaluate the degree of purity of the mitochondrial fractions and their separation from the other fractions.

AChE and ChA assays. ChA was assayed as described by Henderson and Sastry (50), while AChE was based on the method of Chiou (90).

C. Results

The distribution of ACh in subcellular fractions obtained by differential centrifugation of homogenates of placental villus tissue preparations is summarized in Table IV-1, and shown in Figure IV-1.



SUBCELLULAR DISTRIBUTION OF ACh IN HUMAN PLACENTA, ADULT RAT BRAIN, AND FETAL RAT BRAIN

TABLE IV-1--Continued

in nmole (%)[b] per Fraction		
Mitochondrial	Microsomal	Supernatant
2.93 (7.17)	1.04 (2.55)	11.95 (27.2)
0.87 (1.30)	0.65 (0.97)	24.0 (35.8)
7.94 (0.41)	3.33 (0.17)	1097. (56.6)
25.35 (1.62)	9.37 (0.60)	950. (60.5)
11.50 (1.22)	7.06 (0.75)	860. (91.0)
14.47 (0.74)	7.05 (0.36)	1509. (76.7)
19.20 (1.28)	5.65 (0.38)	925. (61.4)
45.72 (2.19)	12.88 (0.60)	1788. (83.2)
33.47 (1.34)	14.25 (0.57)	2480. (99.2)
46.3 (1.61)	NS	2740. (98.7)

[a]Experimental Condition 1: Medium of 0.32 M Sucrose.
Experimental Condition 2: Medium of 0.32 M Sucrose, containing 0.02 M NaCN, 0.5 mM EDTA, and 10^{-5} M Phospholine Iodide.
Experimental Condition 3: Medium containing 0.1 M KCl, 0.005 M Histidine, 0.02 M NaCN, 0.5 mM EDTA, and 10^{-5} M Phospholine Iodide.

[b]Calculated as percentage of total ACh in the homogenate.

The value of ACh in each fraction was expressed both in absolute values as nanomoles (nmoles) per entire fraction as well as in percentage of total ACh in whole filtered homogenate. The percentage recovery of ACh was about 82·86 percent. Although the absolute values of ACh in each placenta vary widely from one placenta to another, the subcellular fraction with the highest content of ACh was the high-speed supernatant, S3. This fraction accounted for an average 79.30 percent of total ACh in the whole homogenate. Small amounts of ACh were found in fractions P1 and P2 both of which contained approximately equal amounts of ACh. The microsomal fraction, P3, contained only about half the ACh content of either P1 or P2.

These findings differed significantly from reported subcellular distribution of ACh in the nervous tissues (6-8, 84). In an attempt to rationalize the observed pattern of subcellular distribution of placental ACh, we studied the subcellular distribution of ACh in adult and fetal rat brains. It has been reported that the rat brain remains immature until the sixth day post-parturition (44). These studies on rat brains are useful to judge the accuracy of our techniques. If we could confirm the widely reported ACh distribution pattern in adult rat brain, then we can

conclude that the observed distribution pattern is inherent in the placenta. A comparison of the placental ACh distribution with that of fetal rat brain could then be taken as a measure of the degree of maturational development of ACh storage system in placenta. Figure IV-1 indicates that in the adult rat brain, the ACh content of the mitochondrial fraction P2 was higher than those of fractions P1, P3 and S3. Such a pattern was frequently reported by previous investigators (6-8, 85). However, for a 20-day old rat fetal brain, the ACh content of fraction S3 was higher than in the other subcellular fractions. These two studies indicated that the subcellular distribution of placental ACh was not artifactually produced because of the homogenization medium. Rather, the reported pattern was real.

In order to judge the nature of ACh content in fraction S3, it was subjected to equilibrium dialysis. The dialysis time was about 22 hours. To determine the time that equilibrium ought to be achieved between the inside and outside of the dialysis bag, a solution of pure acetylcholine iodide (Nutritional Biochemicals, Cleveland, Ohio) in 0.32 M sucrose was dialyzed for different lengths of time. As seen in Table IV-2, pure ACh diffused to

SUBCELLULAR DISTRIBUTION OF SDH, ACh, ChA ACTIVITIES
IN HOMOGENATES OF HUMAN TERM PLACENTAS

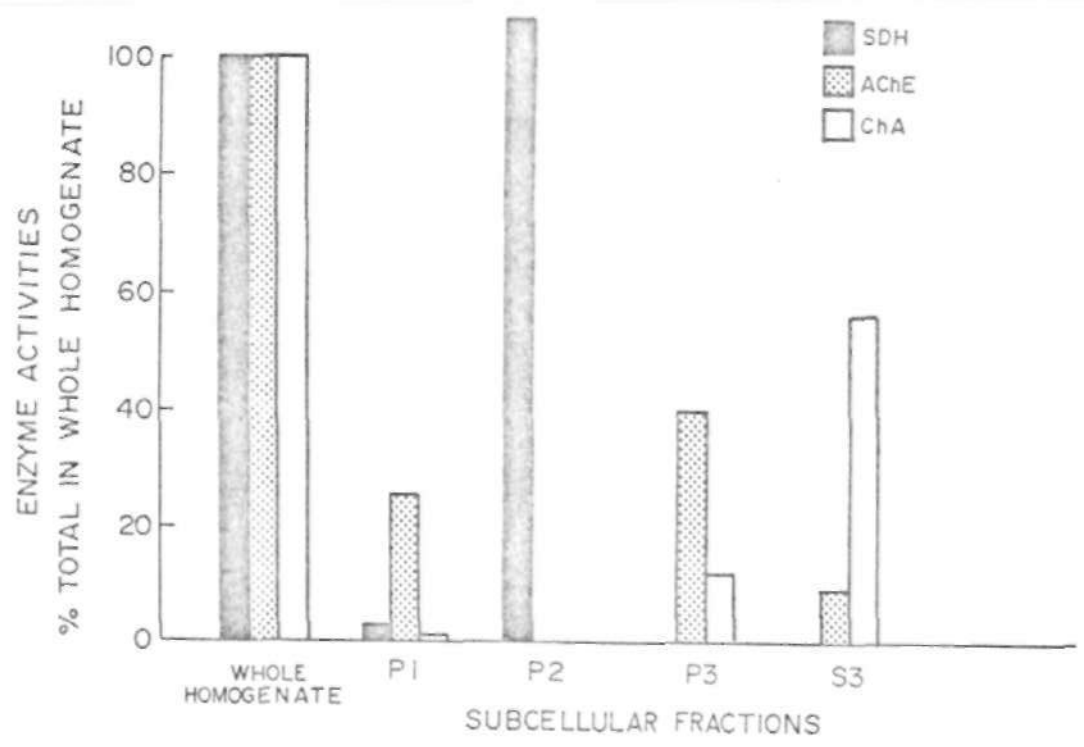


TABLE IV-2

EQUILIBRIUM DIALYSIS OF AN ACh SOLUTION IN SUCROSE AND SAMPLES OF THE HIGH-SPEED SUPERNATANT FRACTION

Description of Sample		Dialysis Time (hrs)	ACh CONC (nmoles/ml) Inside Dialysis Bag	Outside Dialysis Bag	Inside / Outside
Pure ACh soln in 0.32 M sucrose (190.4 nmoles per ml soln)		3-1/2	89.5	64.5	1.39
		6-1/2	79.5	73.3	1.09
		13-1/4	69.5	69.5	1.00
		24-1/2	69.0	69.0	1.00
Supernatant (samples from different placentas)	#1	24	4.36	2.81	1.55
	#2	22-1/3	3.13	2.44	1.28
	#3	24	4.95	3.86	1.28

equilibrium within 13 hours. However, samples of high-speed supernatants from three placentas which were dialyzed for 22 hours did not reach equilibrium. The amount of ACh (nmoles/ml) remaining inside the dialysis bag exceeded the amount outside the bag by an average of 36.70 percent. This indicates some ACh binding to proteins or other cytoplasmic macromolecules. An analysis of the concentration of protein inside and outside the dialysis bag showed that there was very little protein escaping from the bag.

In some placentas, the distribution of ChA and AChE activities were studied. As shown in Figure IV-2, the distribution of placental ChA activities appeared to be similar to the distribution of ACh. On the other hand, AChE activities appeared to be predominantly situated on the nuclear (P1) and microsomal (P3) membranes.

The degree of completeness of separation of the subcellular fractions was monitored by the determination of SDH. Figure IV-2 shows that the mitochondrial pellet, fraction P2, contained by far the highest SDH activities. This would indicate that there was minimal cross-contamination between fractions.

Influence of the Ommission of the Anti-acetylcholinesterase Agent from the Homogenization Medium

The effect of the presence or absence of an anti-acetylcholinesterase agent has been used extensively to confirm that ACh in the nerve terminals was enclosed within the synaptic vesicles (88). In homogenates of placenta numbers 1 and 2 (Table IV-1), phospholine iodide was omitted from sucrose solution. As Table IV-1 shows, the amount of ACh detected in whole filtered homogenate was only 5.44 percent of the amount detected when the anti-AChE agent was present. Further an average recovery of only 41 percent was possible. Nevertheless, the highest ACh concentration was again determined in the high-speed supernatant.

D. Discussion

A comparison of ACh distribution among subcellular structures in placenta with those in nerve terminals (6-8, 84, 88) showed that the two systems do not have much in common. In the mature nerve terminals, the bulk of the tissue ACh was confined within the P2 fraction. Based on density gradient centrifugation of the resuspended P2

fraction coupled with electron microscopic (EM) studies, the subcellular structure containing most of the ACh was identified as synaptosomes. Further analysis showed that the synaptosome was essentially a bag containing numerous synaptic vesicles which are currently regarded as the storage site of ACh. In the placenta, on the other hand, the bulk of tissue ACh was associated with the high-speed supernatant.

In the only reported study which appeared as an abstract on the subcellular distribution of placental ACh, Hagen (89) also found that the fraction S3 contained the highest amount of ACh. The paucity of reports on the subcellular distribution of placental ACh is rather perplexing, especially when one bears in mind that the huge quantity of placental ACh has always raised more questions than was ever answered.

Two of the important objectives of investigating the subcellular distribution of a given tissue constituent are: (1) to elucidate the role of subcellular structures in the biochemical and physiological functions of the substance, e.g., ACh in this case; and (2) to correlate the structural localization of the tissue constituent to the mode of its storage. In the CNS and other nervous tissues,

these two objectives have been achieved for ACh as a result of many years of intensive investigations involving global cooperation. The achievement of the above objectives had been accelerated by the fact that the biochemical and physiological functions of ACh as a neurotransmitter were precisely known. Since the biochemical and physiological functions of ACh in the placenta are as yet unknown, and are only being speculated upon, our studies have not been directed towards elucidating the role of subcellular structures in ACh functions. More important is the fact that for the first time we have shown some correlation between the structural localization of ACh and its mode of storage in the placenta. However, it is expected that the findings reported here can serve as a first step towards elucidating the overall role of ACh in placental function.

In Chapter III of this dissertation it was reported that cold storage (4^{o}C) of placenta for 72 hours did not significantly alter placental ACh level. On the other hand, freezing-thawing treatments led to total destruction of placental ACh. Stone (73, 74) had previously reported similar observations on ACh in the brain. These findings indicate some form of tissue storage for placental ACh

which has not previously been investigated. Although ACh was located in the cytoplasm, it was not located in the same compartment as that of AChE. Therefore, ACh was not subjected to AChE hydrolysis in situ. The experiments using equilibrium dialysis indicated that ACh was bound to cytoplasmic macromolecules, presumably proteins. The exact nature of such binding was not investigated.

The localization of ACh in the high-speed supernatant was not unique to the placenta. The sciatic nerve of chicken and the cerebrum of pigeon and chicken as well as insect nervous tissues were reported to have highest ACh content and ChA activities in the fraction S3 (4). Hebb (4) suggested that the results obtained with chicken and insect nervous tissues may simply reflect differences in the degree of robustness and mechanical properties of the nerve terminals. It is hard to judge whether this suggestion could be applied to the situation in the placenta.

The possibility of the existence of ACh storage granules in placental villus was not excluded by this report. Wen, Chang, and Wong (59) had histochemically demonstrated what they identified as ACh-storing granular structures in placental slices. Confirmatory or contradictory evidences have not yet been reported in other

laboratories. Hence, in view of their apparently convincing histograms obtained after infusion of ammonium reineckate and potassium dichromate, one is tempted to assume a probable existence of granular structures adapted for storage of ACh. The weakness of their study lies in the fact that ACh-reineckate precipitate could have coalesced into granular structures in the syncytiotrophoblast. Very recent EM histological studies (75-77, 91, 92) showed the existence of numerous and diverse granular structures in the syncytiotrophoblast. These granular structures do not seem to coincide with those reported by Wen et al. (59). However, since those EM studies were not specifically aimed at showing ACh storage granules, it is not yet possible to state categorically whether or not ACh storage vesicles exist in the placental syncytiotrophoblast. Furthermore, numerous granules were observed in the electronmicrographs of placentas of ungulates (92) yet these placentas were reported devoid of ACh (23).

From our subcellular distribution studies we can conclude that ACh storage granules comparable to the synaptic vesicles in neuronal terminals of nerve tissues do not exist in the placenta. This finding can possibly be explained in terms of the embryonic origin of the placenta.

If by a remote possibility storage granules did exist in the syncytiotrophoblast *in vivo*, then such granules would be immature or embryonic, and hence might be unable to survive the homogenization procedure. The underdeveloped nature of ACh storage in immature organs was further illustrated by our studies on the 20-day old fetal rat brain, where the fraction S3 also contained the highest level of ACh. It is well known that during the fetal and perinatal periods, the rat brain is immature and remained so until the sixth day after parturition (44).

Finally, the existence of ACh in the cytoplasm without being hydrolyzed is quite possible. ACh hydrolysis by AChE only occurs when ACh is free. Our experiments using equilibrium dialysis indicated some degree of ACh binding to proteins, hence that alone is sufficient to preserve placental ACh. The binding of ACh to macromolecules in placenta can be said to be comparable to the sequestration of ACh within membrane-limited granules in nerve terminals. The safety of ACh is further guaranteed by the fact that AChE is localized on the membranous fractions P1 and P3.

CHAPTER V

RELEASE OF ACh FROM PLACENTAL VILLUS TISSUE PREPARATION

A. Introduction

The ideal technique for studying the release of a pharmacologically active principle extractable from a given tissue or organ is *in situ* perfusion of the tissue or organ. *In vivo* perfusion of most organs in a human being is ethically unacceptable, hence investigators have resorted to developing animal models that could duplicate various systems in man. In some cases, no readily accessible animal model can be developed, and the human placenta is one such case (92, 93). For this reason, the human placenta is usually studied in *in vitro* systems.

The *in vitro* studies are in general limited by the fact that the system under investigation is isolated from its natural environment, however, this approach has the immense advantage that experimental conditions can be accurately controlled and manipulated.

The *in vitro* techniques commonly employed for studying the various functions of the placenta are either perfusion of the whole organ or incubation of isolated tissue preparations. The perfusion techniques currently available for studying the human placenta (94, 95) involve rather complex and cumbersome apparatus which do not necessarily reproduce the *in vivo* environment of the placenta. Tissue slices, on the other hand, are generally more easily handled. Although experiments with placental tissue slices have been reported (47, 93), in the present studies our attempts to prepare slices of the placental villus tissue (with the Stadie-Riggs tissue slicer) were unsuccessful. It was observed that owing to the anatomical organization of the placenta, thin slices of the placental villus do not maintain their shape in the incubation medium. We were able to develop an alternative system to the fragile placental tissue slices by simply cutting the villus tissue into small pieces having dimensions of between 0.5-1.0 cm in their longest axis. These tissue pieces could float in the incubation medium and each piece remained intact throughout the incubation period.

ACh exists in large quantities in the placenta. The most popular proposed function attributed to placental

ACh is that of regulating transport of nutrients and ions that pass through the placenta into the fetus (4, 67). This implies that ACh has a role in the functioning of the much talked about placental barrier. For ACh to fulfill this role, it has to be released. The mechanism by which ACh is released is certainly a basic element in the understanding of how ACh participates in the functioning of the placental barrier. There have been few reports on the release of ACh from the placenta, and in those cases only ACh-like activities were measured by bioassay (63). We have therefore embarked on a more thorough study of the pattern of the release of placental ACh into a physiological salt solution. The cumulative ACh release was measured by removing aliquots of the incubation fluid at specified time intervals. The ACh content of each aliquot was measured by pyrolysis-gas chromatography.

Nicotine is one of the active principles in the tobacco smoke which is frequently associated with low birth weights of babies born to women who are heavy cigarette smokers (27, 96). Since nicotine is well known as a cholinomimetic, it appears logical to assume that it would interact with the placental cholinergic system. The most labile component of the cholinergic system is ACh.

Therefore, one of the methods by which nicotine might produce its effects is by either enhancing or preventing ACh output. For this reason, special attention was paid to the effect of nicotine on the release of placental ACh.

Douglas (97) had proposed a generalized stimulus-secretion coupling mechanism of release of substances from various tissues. In a long series of reports, Douglas and his team showed that calcium entry into cells was an essential first step in the release processes. We have examined some aspects of the role of Ca^{2+} in ACh release from placenta. These studies were performed with the aim of establishing some of the roles of the placental villus membrane in the release of ACh.

B. Methods

The Incubation Medium

The incubation medium used for these release studies was the Krebs-Ringer bicarbonate (KRB) buffer, pH 7.2-7.4. According to the recommendations in "Manometric Techniques" (98) this buffer most closely duplicates the ionic composition of plasma. Its composition (mM final concentrations) is: NaCl 118.5; KCl 4.87; $CaCl_2$ 2.54;

$MgSO_4$ 1.185; KH_2PO_4 1.185; and $NaHCO_3$ 24.88. The pH was adjusted to 7.2-7.4 by gasing with 95 percent O_2--5 percent CO_2. All solutions contained 1 x 10^{-5} M phospholine iodide, a cholinesterase inhibitor, which was added just prior to use. This solution shall be referred to as Normal KRB solution. To prepare Ca-free solutions, $CaCl_2$ was omitted and an equivalent amount of NaCl was added. About 2.0 mM EDTA.Na_4 was added to chelate any Ca^{2+} ions released from the tissues. Solutions not used immediately were kept at 4°C in the refrigerator.

Tissue preparation. A segment of the placenta was cleared of blood as described in Chapter IV, but the Normal KRB solution was infused instead of normal saline. The cleaned portion was carved out and both the chorionic and the basal plates were trimmed off. Then the placental villus tissue was diced into small pieces (0.5-1.0 cm longest axis) using a scissors. These pieces were rapidly washed four times in fresh cold KRB solution, and kept in ice until transferred into the tissue bath.

The incubation system. The incubation system was constructed as follows: Six 50-ml tissue baths (chambers, compartments) were mounted in a water bath. Each tissue

chamber was connected through its base to a 95 percent O_2-5 percent CO_2 gas source. The water bath was heated to 37°C. A gentle stream of the O_2-CO_2 was passed into the empty tissue compartments. This gas flow would prevent backflow of fluid when 40 ml of cold Normal KRB solution were added to each chamber. The whole system was allowed to warm up to the water bath temperature.

Tissue Incubation and Sampling for ACh

Sufficient tissue pieces were added to the 40-ml of KRB solution in each chamber to displace approximately 5.0 ml of solution. These tissue pieces weighed between 2.0-2.5 g after blotting with filter paper. Following a 10-minute equilibration period, a 2-ml aliquot of the incubation fluid was pipetted from each tissue bath. These represented the zero-time samples. Subsequently 2-ml aliquots from each chamber were pipetted out at 10, 20 and 35 minutes. Samples so taken represented controls for each chamber, hence each chamber served as its own control.

Immediately following the 35-minute sample, the test drug or cation was added to five of the tissue compartments, the sixth chamber served as control during the experimental period. A 10-minute equilibration time was

allowed to elapse, after which the first experimental (i.e., drug effect) 2-ml aliquot was pipetted out. Subsequently, 3 more aliquots were taken out at 15-minute intervals. Ten nmoles of $PCh^+ I^-$ was added to each aliquot as internal standard.

The following agents were investigated at the indicated concentrations for their influence on the pattern of ACh release from the placental villus tissue preparation:

Ca^{2+} (mM)	0, 2.54 (Normal KRB), 5.94, and 7.4
K^+ (mM)	17.44, and 63.10
Nicotine (μM)	5.74, 14.90, 57.90, and 766.0
Cocaine (μM)	2.80, 181, 362, and 561
Ba^{2+} (mM)	2.54
Mg^{2+} (mM)	3.55, and 16.59

The effects of Atropine (7.7×10^{-5} - 152×10^{-4} M) and d-Tubocurarine (d-TC; 9.30×10^{-7} - 1.03×10^{-4} M) on nicotine induced increase of ACh release were studied for the purpose of elucidating the type of receptor with which nicotine interacts. Also the role of prostaglandin in the release of placental ACh was briefly investigated by incubating the tissues with indomethacin, an inhibitor of prostaglandin synthesis, and N-ethylmaleimide (NEM) which

was reported to inhibit 15-hydroxyprostaglandin dehydrogenase (99), an enzyme which degrades the prostaglandins.

Extraction and Determination of ACh in Each Aliquot

The ACh component of each aliquot was extracted by a modification of the ion-pair formation technique described ty Karlen et al. (100). The principle of this technique is based on the fact that ACh forms a stable ion-pair complex with 2,2',4,4',6,6'-hexanitrodiphenylamine (dipycrilamine, DPA) in a strongly alkaline medium. This complex is soluble in dichloromethane (DCM) and ACh is released from it by acidification.

The step-by-step procedure is described in the following flow diagram, Figure V-1.

Quantitation. The amount of ACh in each aliquot was calculated from the chromatograms as previously described. The value of ACh released was plotted against the time the aliquot was taken out. The slopes of the curves during the control and experimental periods were compared. The statistical significance of the difference between means was determined by the Student t-test.

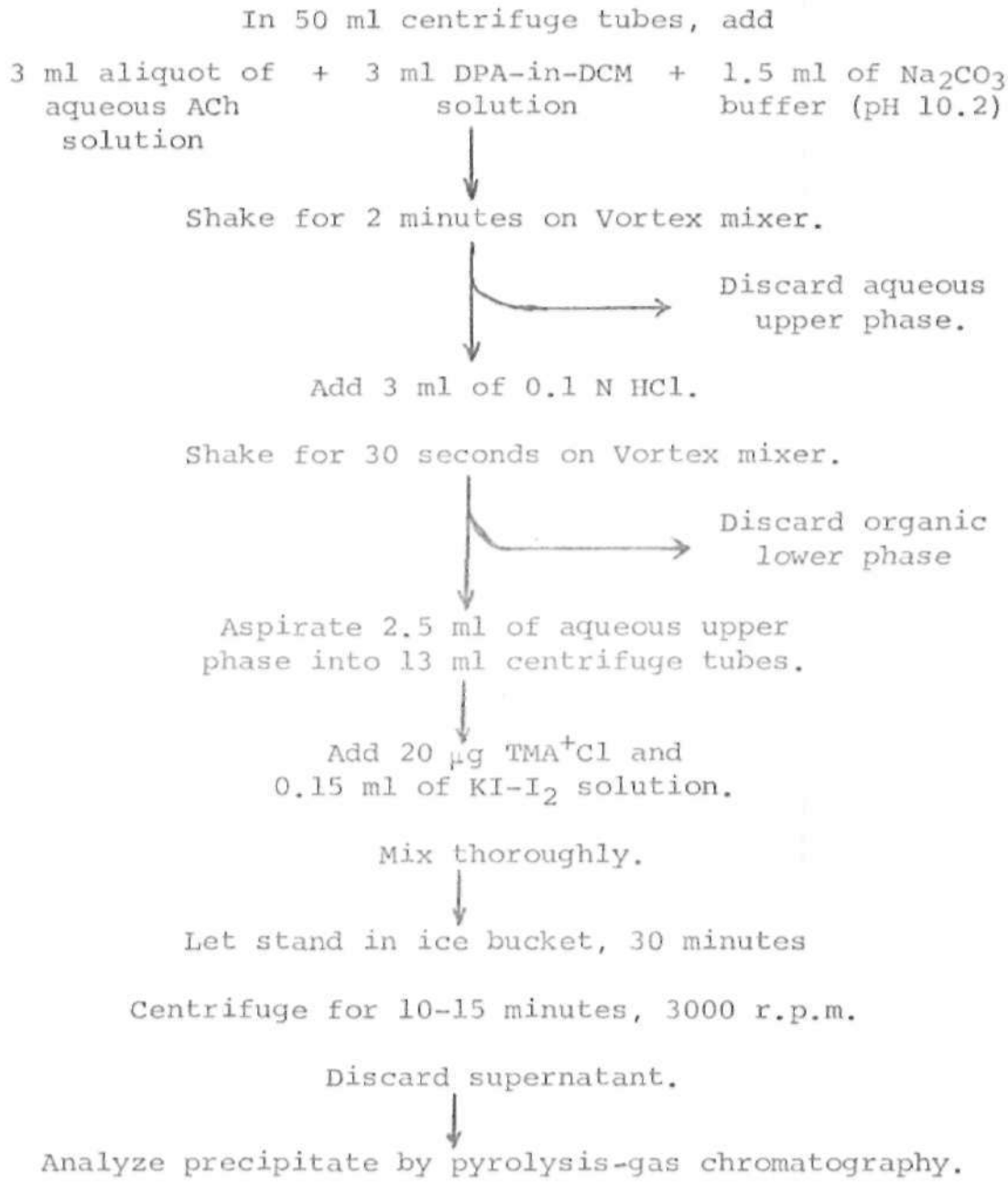


Figure V-1. Extraction of ACh from aqueous physiological solutions.

C. Results

Preliminary studies on the release of ACh into KRB solution showed that the cumulative ACh release was linear (Figure V-2) for 90 minutes. Because a linear curve can be easily analyzed and because the drug-induced changes in a linear system can be easily explained, it was decided to limit the incubation time to 90 minutes. The entire incubation period was divided into an initial 35 minutes control period followed by a 55-minute period during which drug or ion effects were examined.

Effects of K^+, Mg^{2+}, Ba^{2+}, and Ca^{2+} on ACh Release from Placenta

Potassium, K^+. Figure V-3 shows the effect of K^+ on placental ACh release. Ten minutes after raising the K^+ concentration to 17.44 mM, the amount of ACh released was elevated above the amount released in the Normal KRB medium. This increased release by high K^+ was progressively higher than in Normal KRB throughout the remaining period of incubation. The slope of the release curve (which is a direct measurement of the rate of release) with 17.44 mM K^+ was 21 percent higher than in Normal KRB. When the K^+ concentration was raised to 63.10 mM, the

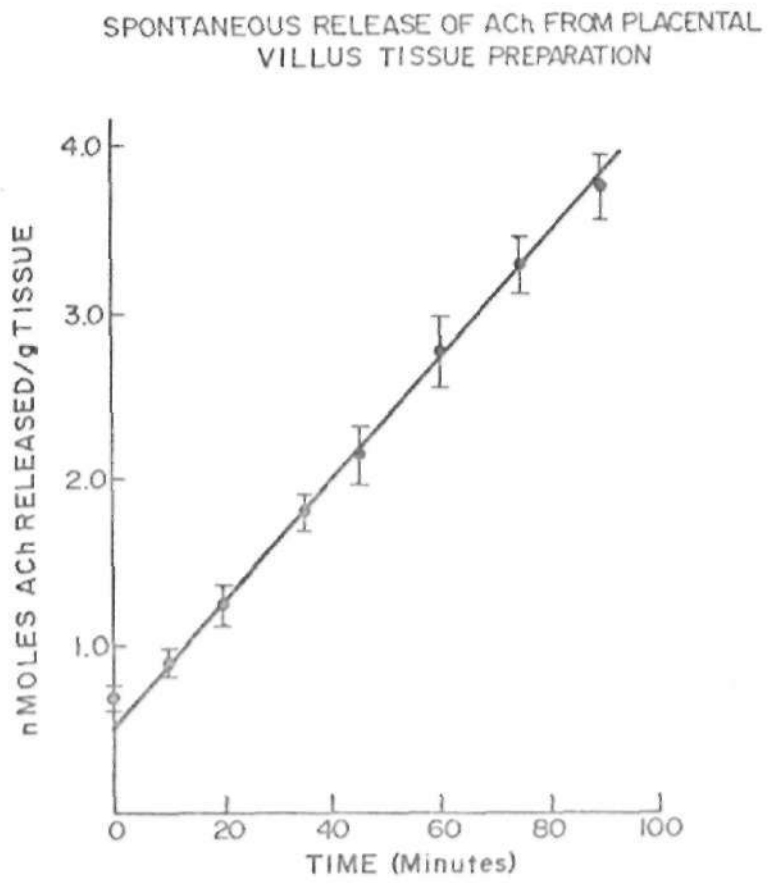


Figure V-2. Spontaneous release of ACh from placental villus tissue preparation. Pieces of placental villus were incubated in KRB solution at 37°C. Two ml aliquots of incubation fluid were pipetted out at the indicated time. ACh content of each aliquot was determined by pyrolysis-GC. Each point represents mean values (± S.E.M.) from 7 different placental villus preparations.

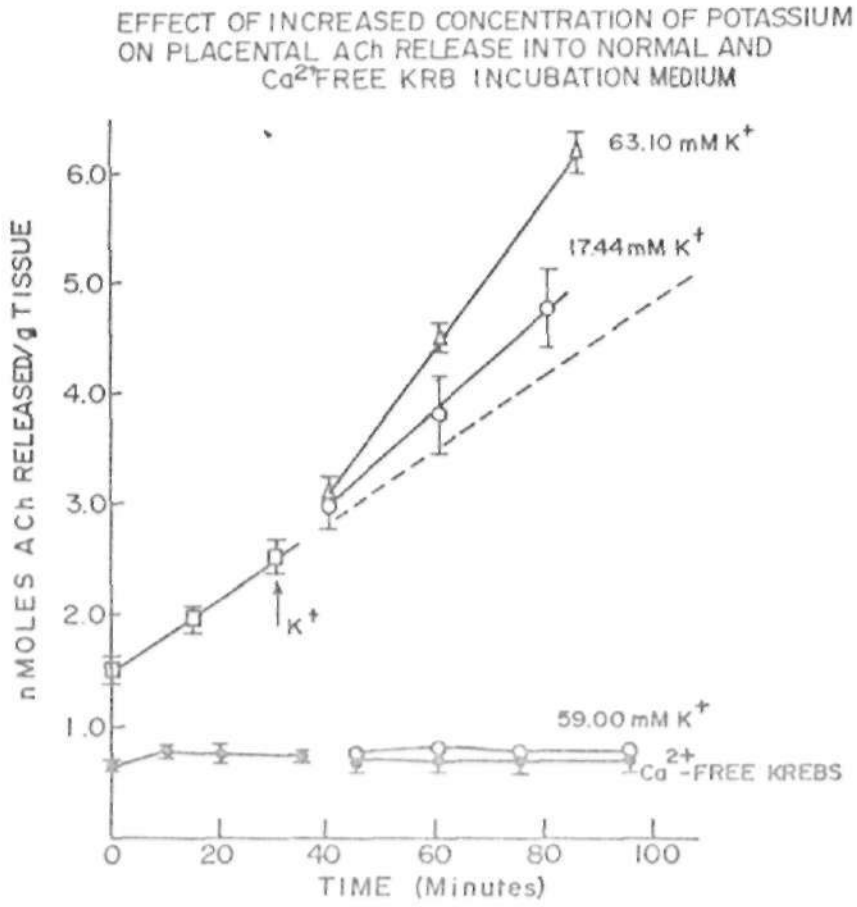


Figure V-3. Effect of increased concentration of potassium on placental ACh release into normal and Ca^{2+}-free KRB incubation medium. Tissues were incubated for 30 minutes control period. High K^+ solutions were added and 10 minutes allowed for equibration, before first K-effect sample taken. Ca-free Krebs contained 2.05 mM EDTA. Extrapolated line showed expected curve for a control containing normal KRB K^+.

quantity as well as the rate of ACh output was higher than those observed with 17.44 mM K^+. The rate of ACh release with 63.10 mM K^+ was 69 percent higher than with 17.44 mM K^+ and 103 percent higher than the control rate in Normal KRB. (See Table V-1 for rates of ACh release.)

To examine the role of calcium in the observed K^+ effects, the concentration of K^+ was increased in a Ca-free medium. In the absence of Ca^{2+} the amount of ACh released was very small (lower curve in Figure V-3) and K^+ was unable to enhance the release.

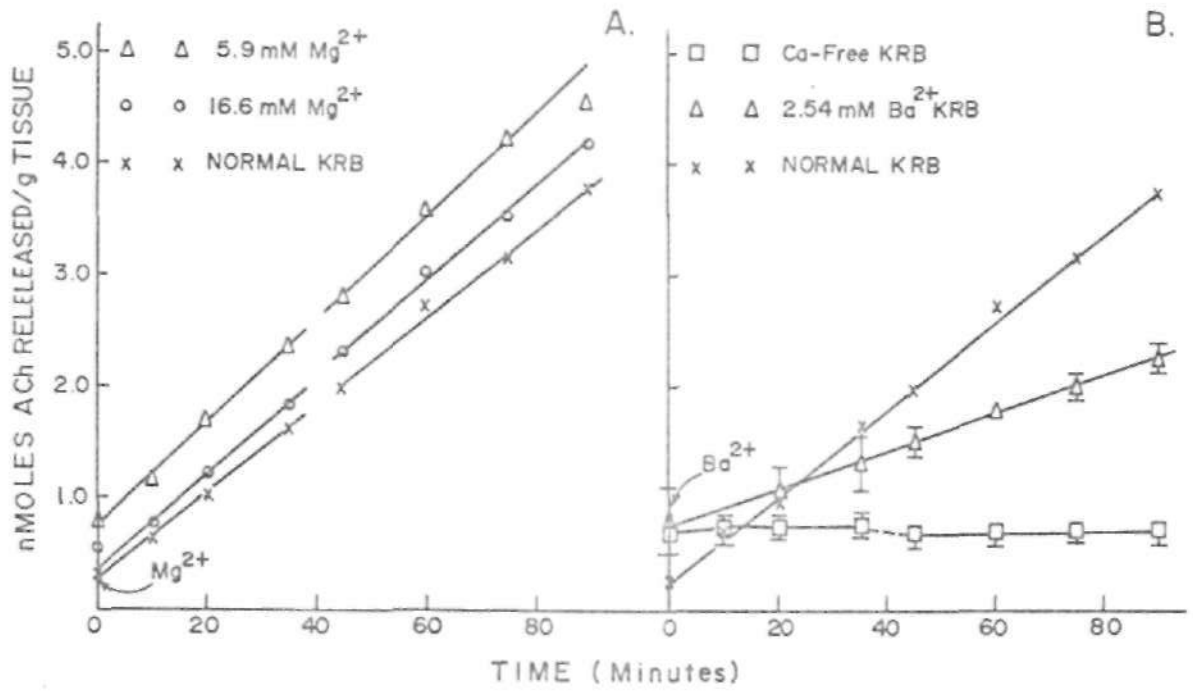
Mg^2 and Ba^{2+}. Magnesium is a well known antagonist of Ca^{2+} (102, 116). Its effect on release of placental ACh was tested in order to ascertain to what extent the placental villus resembles other tissues such as the brain slices and the adrenals. The Mg^{2+} concentration was raised to either 5.90 or 16.60 mM in the organ baths prior to the addition of the villus tissues. Figure V-4a shows that increasing Mg^{2+} concentration did not decrease ACh release from placenta. On the contrary, it tended to increase the quantity of ACh released, but has no effect on the rate of release. The average amount of ACh output in the presence of 5.90 mM Mg^{2+} was $\sim$22 percent higher than that by

Cocaine (5.61×10^{-4} M) + Nicotine (5.79×10^{-5} M)	16.72 ± 1.13	47.44; **
Cocaine (5.61×10^{-4} M) + Calcium (4.64 mM)	28.74 ± 0.13	81.54; *
Mg^{2+} 16.6 mM	38.48 ± 3.10	100.66; n.s.
Atropine (7.69×10^{-5} M)	7.34 ± 0.45	20.83; **
Nicotine + Atropine	17.80 ± 0.51	50.49; **
d-TC (1.54×10^{-6} M)	35.25	100.00; n.s.
Nicotine + d-TC	50.77 ± 1.06	144.03; **
Zero Ca^{2+}	0.00	0.00

The rate of ACh release was estimated from the slope of the curve obtained by plotting cumulative amount of released ACh (ordinate) against time of sampling (abscissa).

* $p < 0.5$; ** $p < 0.005$; n.s. = not significant.

EFFECTS OF INCREASED MAGNESIUM (Mg^{2+}) AND SUBSTITUTION OF BARIUM (Ba^{2+}) FOR CALCIUM ON RELEASE OF PLACENTAL ACh



16.60 mM Mg^{2+}, and ~31 percent higher than that released into Normal KRB. This might mean that the inhibitory concentration of Mg^{2+} was higher than 16.60 mM which might be a reflection of an apparent insensitivity of placental villus to the depressant effect of Mg^{2+}.

Douglas and co-workers (103) reported that barium would substitute for calcium in their incubation medium for studying the release of acetylcholine from superior cervical ganglion. Figure V-4b shows the result of incubating placental villus tissues in a barium (2.54 mM) KRB medium. It was noted that although a Ba^{2+} KRB medium could support placental ACh release, it was not able to release as much ACh as with Normal KRB.

Calcium, Ca^{2+}. The essentiality of Ca^{2+} for the maintenance of diverse release processes has been established (9, 97). The entry of Ca^{2+} into cells was described by Douglas and co-workers (97, 102) as the trigger or signal event for stimulus-secretion coupling processes. As seen in Figure V-5, bottom curve, a Ca-free medium was not able to support release of ACh from placental villus preparations. There was hardly any change in the amount of ACh released during the 90 minutes incubation period. In the

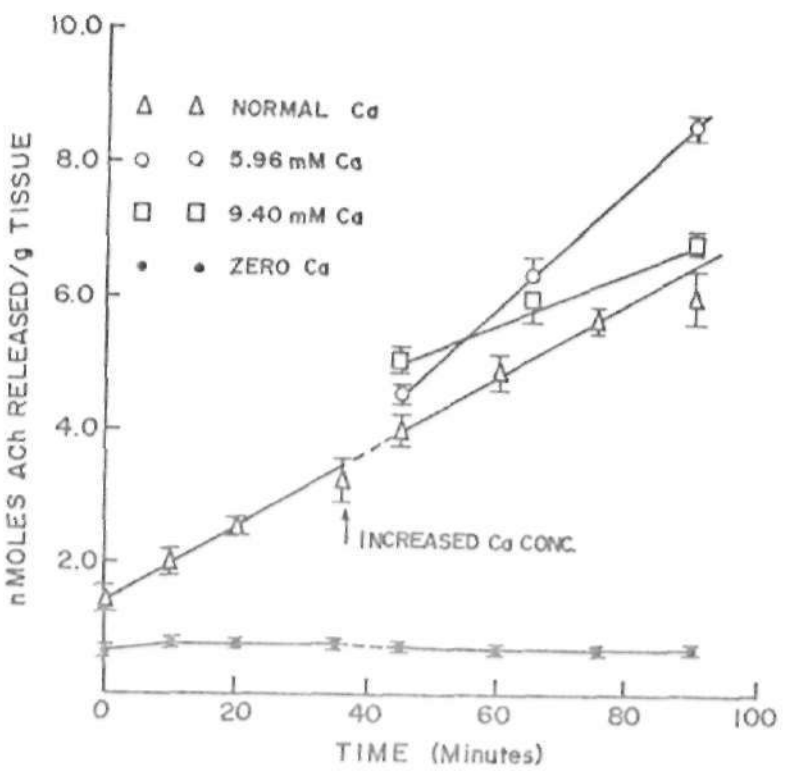


Figure V-5. Effects of Ca-free KRB and increasing KRB Ca^{2+} on placental ACh release. The Ca concentration was increased after 35 minutes control period. Ca-free medium contained 2.05 mM EDTA. Incubation conditions and sampling were described in the text. Each point obtained by averaging 8-10 experiments. Vertical bars are S.E.M.

Normal KRB medium containing 2.54 mM Ca^{2+}, the amount of ACh released increased from 1.40 nmoles ACh/g tissue at zero time to 6.00 nmoles at 90 minutes of incubation. Increasing the concentration of Ca^{2+} in the medium to 5.96 and 9.40 mM caused changes in the amount and rate of ACh output. The lower Ca^{2+} concentration increased the slope of the release curve by 57.10 percent above the control, whereas the higher Ca^{+2} concentration decreased the slope by 30.10 percent below the control. Attempts to increase the Ca^{2+} concentration above 9.40 mM were not successful because Ca^{2+} salts were precipitated. Hence it is not possible to ascertain whether or not high Ca^{2+} was actually inhibitory to ACh release.

Effect of Cocaine on Release of Placental ACh

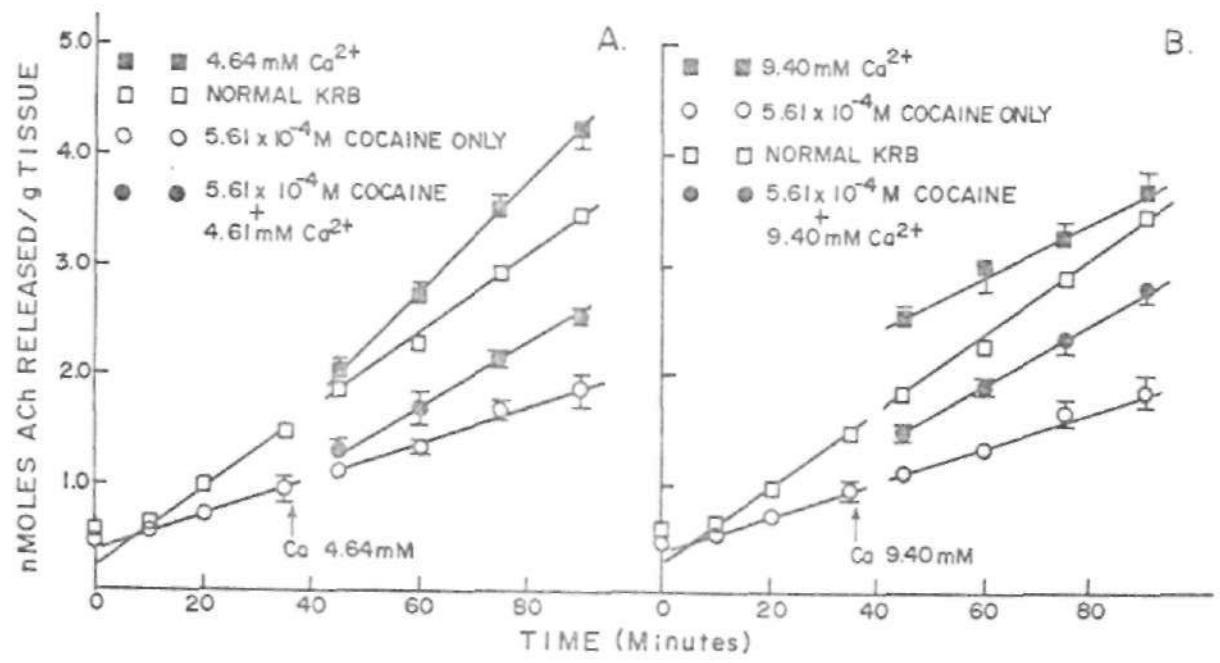
Cocaine is frequently reported as a local anesthetic (104) and a calcium antagonist (105, 106). The recent upsurge of cocaine popularity as a drug of abuse led to suspicions that pregnant drug addicts might also use cocaine. Cocaine was reported to be an antagonist of the release of ACh from the longitudinal strip of the guinea-pig ileum (107) and it is well known as an inhibitor

of the re-uptake of catecholamines (108). Figure V-7a shows the effect of cocaine on the release of placental ACh. At the two concentrations shown in the figure, 2.80×10^{-4} M and 5.61×10^{-4} M, cocaine reduced the amount as well as the rate of ACh output. After 35 minutes of incubation, the amount of ACh released in the presence of 2.80×10^{-4} M cocaine was 74.67 percent of control while the amount was only 62 percent of control in the presence of 5.61×10^{-4} M cocaine. The depression of the rate of ACh release by cocaine was also dose-related. The lower concentration of cocaine decreased the rate to 63.23 percent of that of control while the higher cocaine concentration caused the rate to fall to 47.44 percent of the control rate. At 2.80×10^{-6} M cocaine had no effect on either the quantity or rate of ACh output. Concentrations higher than 5.60×10^{-4} M cocaine interfered with the ACh extraction procedure.

Cocaine and Calcium

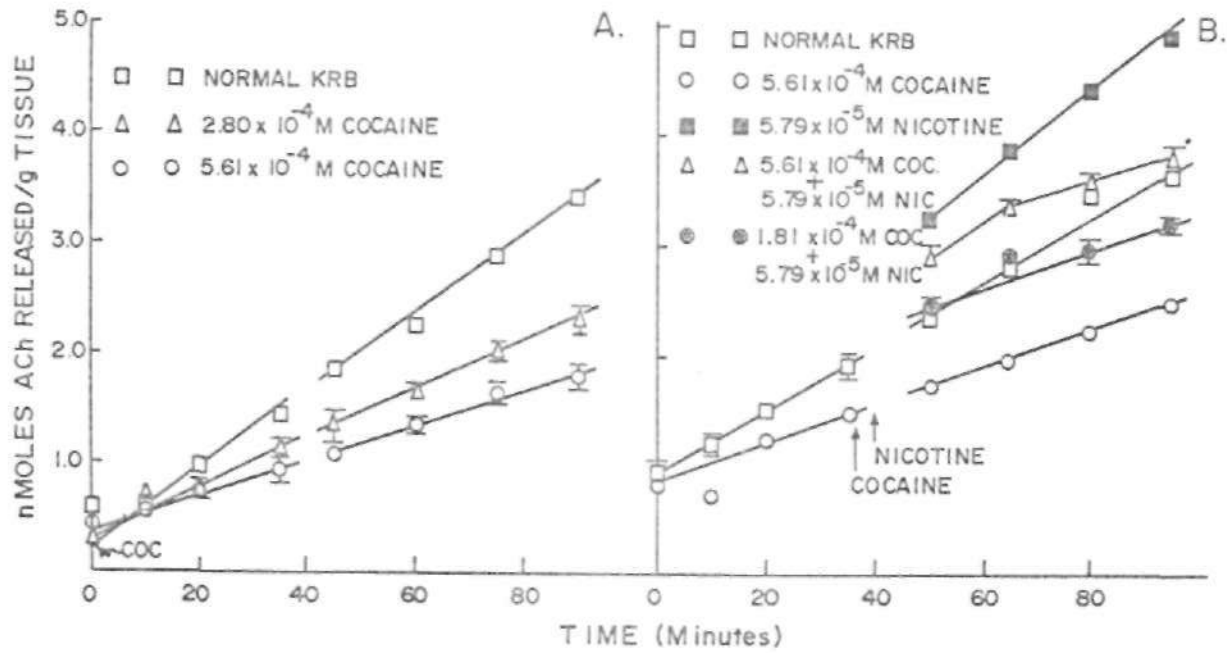
Since one of the mechanisms of action of cocaine is by antagonizing calcium movements (106), it was argued that cocaine should be able to antagonize the calcium-induced alterations of ACh release. This is indeed the

INFLUENCE OF INCREASED CONCENTRATION OF CALCIUM ON THE EFFECT OF COCAINE ON PLACENTAL ACh RELEASE



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EFFECT OF COCAINE AND COCAINE + NICOTINE ON THE RELEASE OF ACh FROM PLACENTA



case as is evident from Figures V-6a and V-6b. In the Normal KRB incubation medium (Figure V-6a) and 35 minutes following the increment of Ca concentration to 4.64 mM, the villus tissues released 3.45 nmoles ACh per g tissue whereas only 2.10 nmoles ACh were released by the same concentration of Ca in the presence of 5.61×10^{-4} M cocaine. The increased rate of release produced by Ca^{2+} was also depressed under the influence of cocaine.

A different picture was obtained with 9.40 mM Ca^{2+} (Figure V-6b). This concentration of Ca^{2+} depressed the rate of ACh release 30 percent below the rate in the Normal KRB incubation medium. However, in the presence of 5.61×10^{-4} M cocaine, 9.40 mM Ca produced an increased rate of release. This could be interpreted as a reversal by cocaine of the release depressent effect of 9.40 mM Ca^{2+}.

Cocaine and Nicotine

Figure V-7b shows the influence of cocaine on nicotine-induced increase of ACh release. The sympathomimetic action of nicotine has frequently been reported to be blocked by cocaine (109, 110). As will be reported in detail later, nicotine (5.79×10^{-5} M) significantly

increased the rate and the amount of ACh output. Since both drugs are unfavorable to pregnancy, it would appear relevant to investigate any possible interactions between them on the placental ACh.

Both concentrations (1.81×10^{-4} and 5.61×10^{-4} M) of cocaine tested significantly depressed the nicotine-induced increases of the amount of ACh released and rate of release ($p < 0.5$). The rate of ACh release by nicotine-plus-cocaine was the same as the rate of release in the presence of cocaine alone. But the amount of ACh output by cocaine-plus-nicotine was much higher than by cocaine alone. This may mean that the mechanism by which nicotine increased the rate of release of ACh is different from the mechanism by which the amount released is increased.

Effect of Nicotine on ACh Release

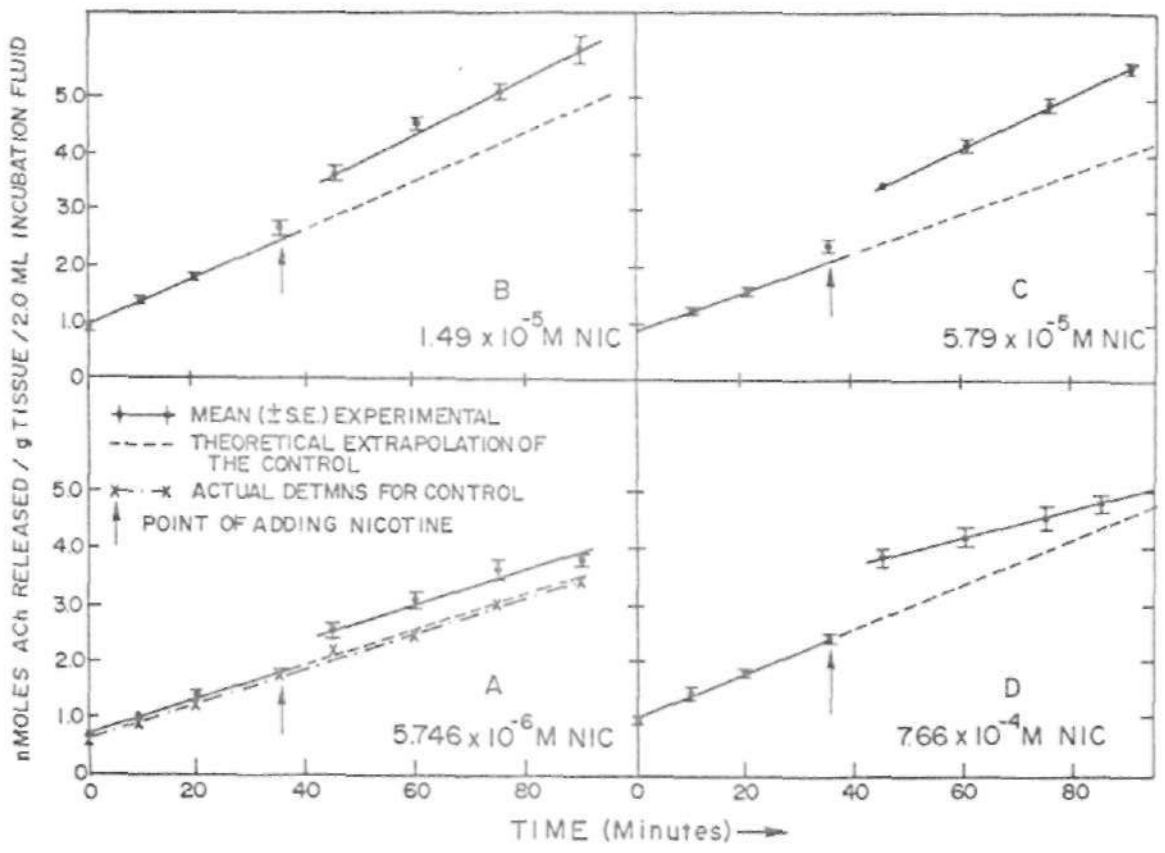
Among the frequently proposed functions of acetylcholine in the placenta, its involvement in nutrient transport regulation appears most popular. There is as yet no known mechanism by which nicotine (as a component of cigarette smoke) contributes to the production of small-for-gestational-age (SGA) babies. Hence this study was initiated in order to examine the possibility of an

interaction between nicotine and the placental cholinergic system. Such an interaction might be in such a direction as to disrupt the nutrient transport system of the placenta.

Figure V-8 illustrates the observed effects of nicotine on placental ACh release. Each point was obtained from 6-8 determinations. In panel A, 5.75×10^{-6} M nicotine produced a slight but non-significant elevation of the amount of ACh released into the incubation fluid. The slope of the release curve (i.e., the rate of release) after the addition of nicotine was the same as before nicotine addition. In panel B, the addition of 1.49×10^{-5} M nicotine to the incubation medium led to an increase of both the quantity and the rate of ACh output. The amount released after 35 minutes contact with nicotine was 25 percent higher than the amount released during the same period in the Normal KRB medium without nicotine. However, the increase in slope of output was only 9.25 percent higher than control. This increase in slope was not statistically significant ($p > 0.5$).

The addition of 5.79×10^{-5} M nicotine, panel C, to the tissues in the incubation fluid produced significant

INFLUENCE OF NICOTINE ON THE PLACENTAL ACh RELEASE vs TIME



($p < 0.005$) increases of both quantity and rate of ACh release. The rate of release by nicotine was ~40 percent higher than in Normal KRB medium without nicotine. A different picture was observed in the presence of 7.66×10^{-4} M nicotine, panel D. After 10 minutes of contact with nicotine, ACh output was much higher (35.71 percent) than that obtained in the absence of nicotine. At the end of incubation, i.e., 55 minutes contact time, the ACh content in the organ baths containing nicotine was only 11.25 percent higher than in the baths without nicotine. The overall rate of release after nicotine addition was 33 percent less than the control rate.

An analysis of the effects of different concentrations of nicotine on the ACh release is shown in Figure V-9. The amount of ACh output in control organ baths was subtracted from the values of ACh output in organ baths containing nicotine at 10 and 55-minute contact time. These differences were plotted against nicotine concentration (abscissa) on a semilogarithmic graph paper. A characteristic S-shaped dose-response curve was obtained with the output differences after the 10-minute contact time. However, a bell-shaped curve was obtained with the differences calculated for the 55-minute contact time. This

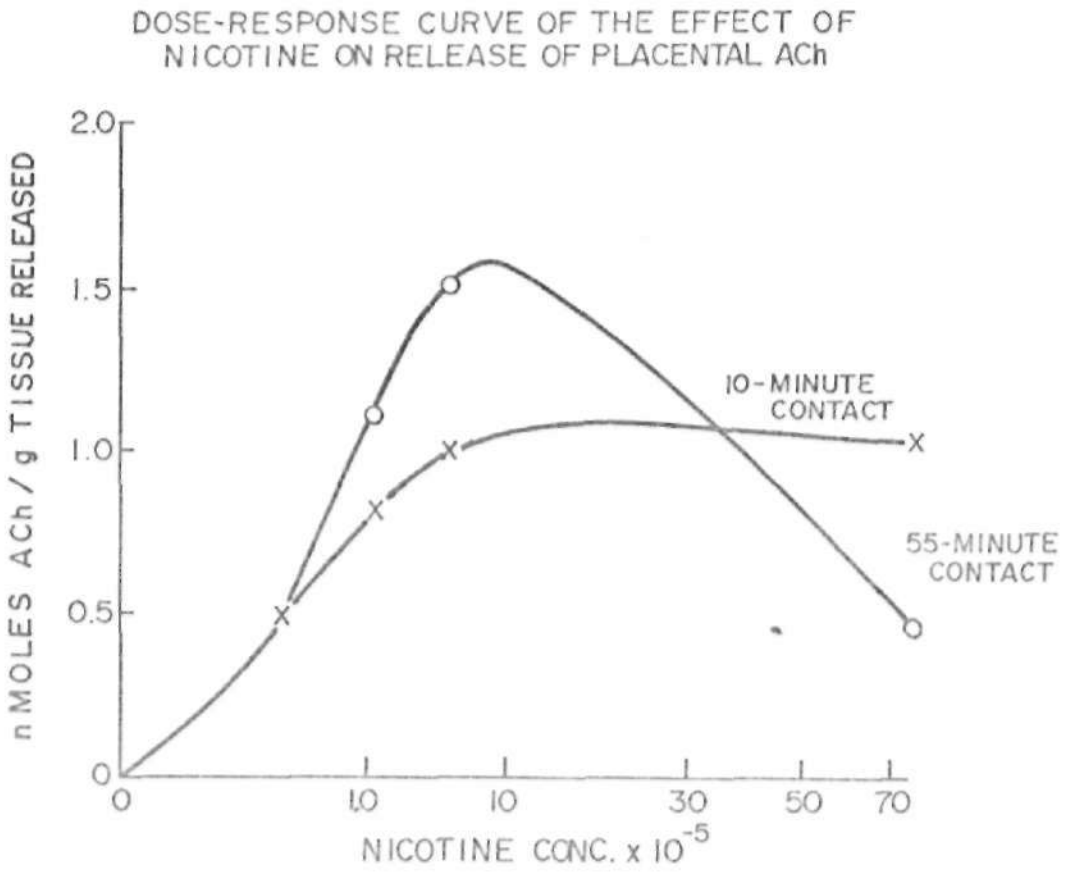


Figure V-9. Dose-response curve of the effect of nicotine on release of placental ACh. Values plotted on the ordinate represent the differences between ACh release in the presence of nicotine and the absence of nicotine. Contact time equals the length of time between nicotine addition and sampling of incubation medium.

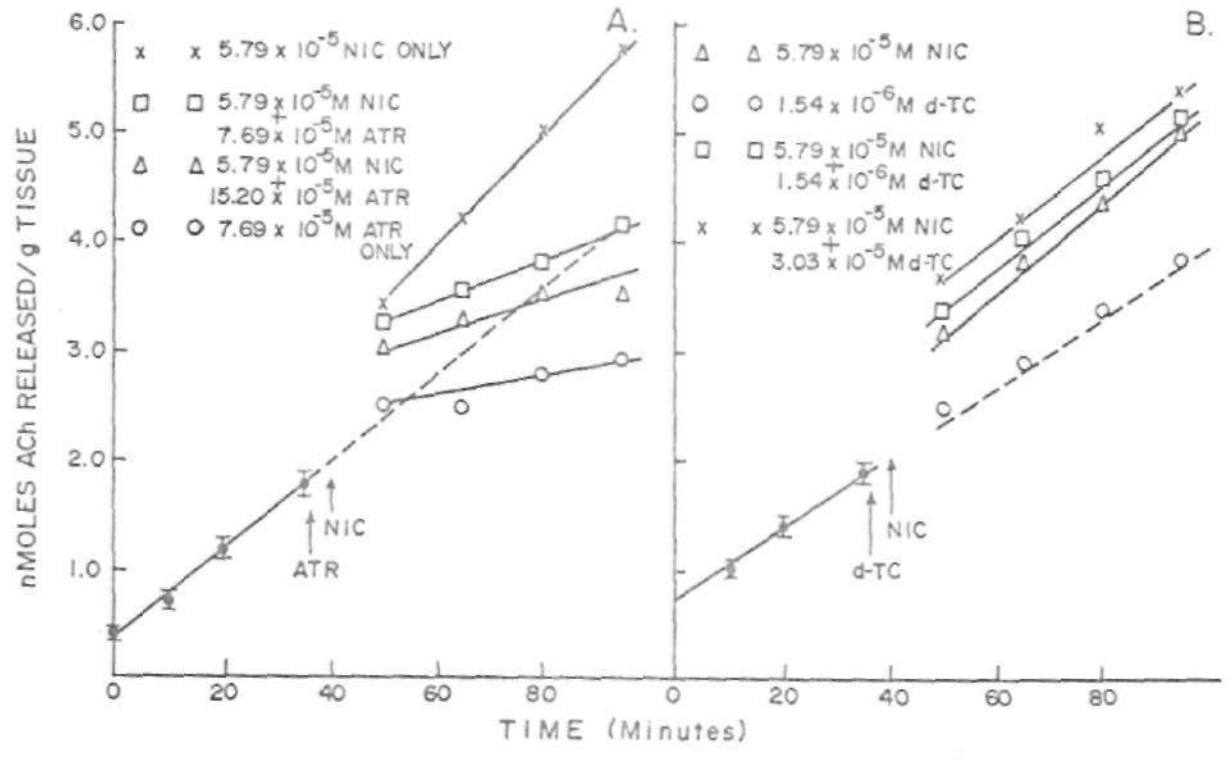
finding was interpreted in terms of a biphasic action of nicotine--an initially stimulatory action at low concentrations followed by an inhibition at the higher concentrations. This type of nicotine effect is well known in the nervous tissues and it is characteristically referred to as a depolarization block (111). It is possible that the above observation on the effect of nicotine on placental ACh release operates by the same mechanism as the depolarization block in the nervous tissues.

The influence of some anti-cholinergic drugs on the stimulatory effect of nicotine on placental ACh release have been studied.

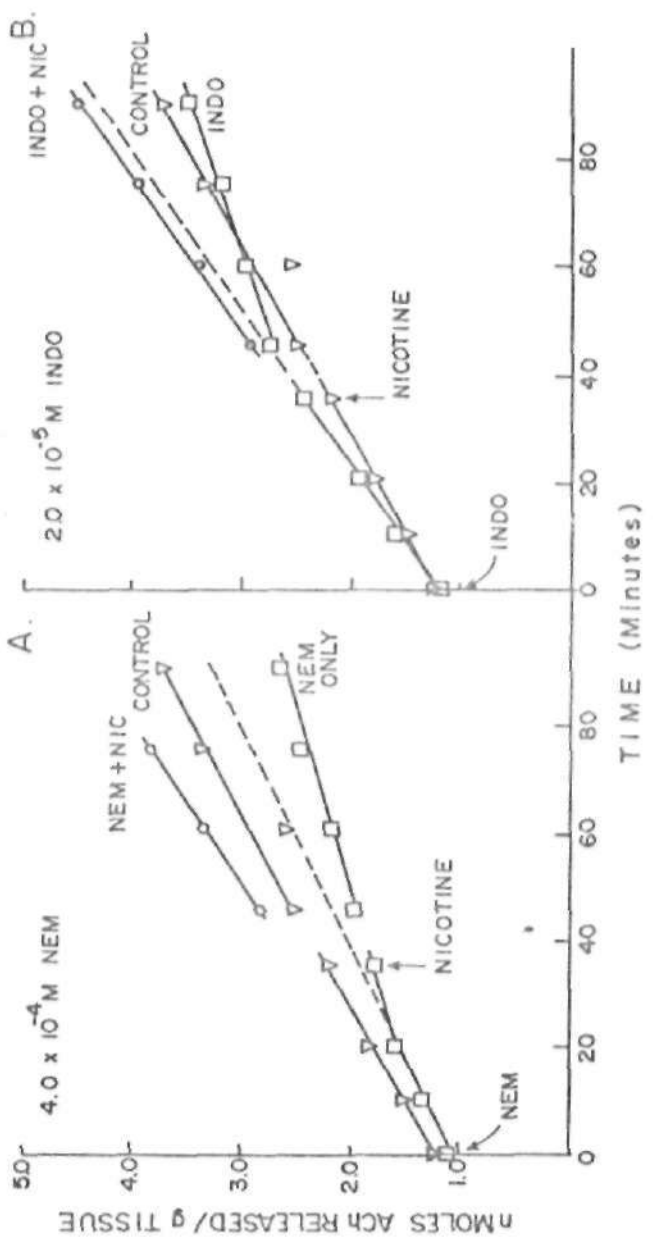
Nicotine and Calcium

In a calcium-free KRB medium containing 2.05 mM EDTA, nicotine failed to stimulate ACh release. This is shown in Figure V-12. Hence the effect of nicotine must be dependent on extracellular Ca^{2+}.

INFLUENCE OF ATROPINE AND d-TUBOCURARINE ON THE NICOTINE-INDUCED INCREASE OF PLACENTAL ACh RELEASE



THE INFLUENCE OF N-ETHYLMALEIMIDE AND INDOMETHACIN ON THE RELEASE OF ACh FROM PLACENTA



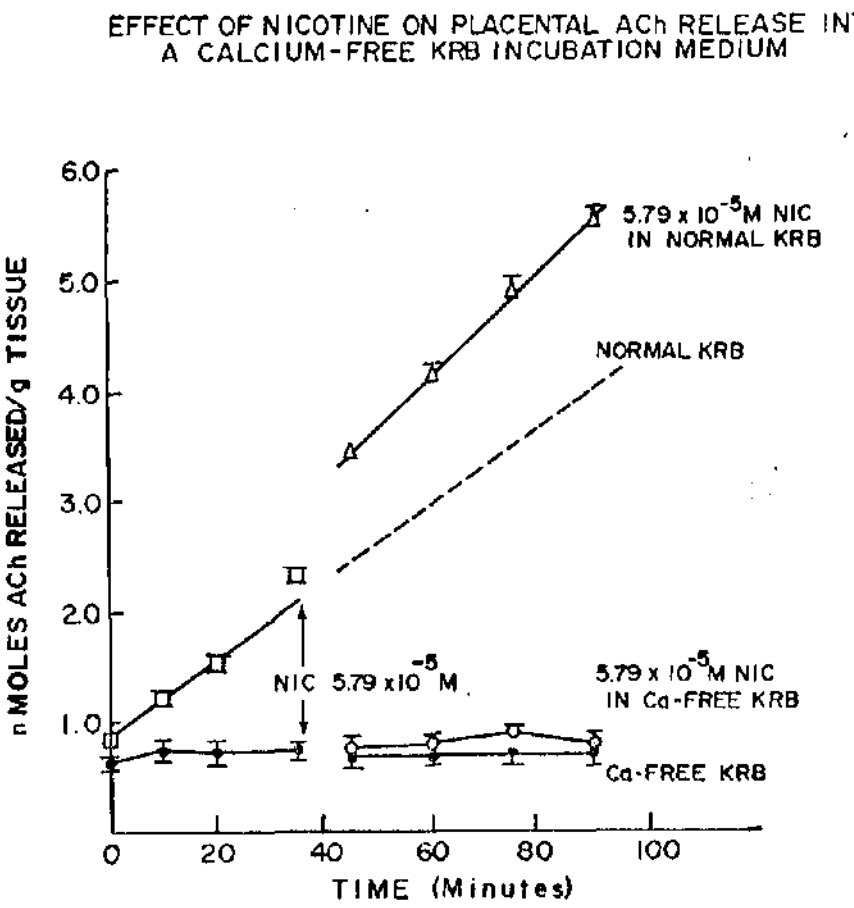


Figure V-12. Effect of nicotine on placental ACh release into a calcium-free KRB incubation medium. Experimental conditions were as in Figure V-2. Ca-free KRB contained 2.05 mM EDTA. The upper curves were the same as in Figure V-8c and were reproduced here for comparison purposes. Each point (for Ca-free KRB experiments) represents an average of 8-10 determinations.

Nicotine and d-Tubocurarine

The effect of adding d-tubocurarine (d-TC) to placental villus tissues prior to adding nicotine is shown in Figure V-10b. In the presence of d-TC alone, the output of ACh was not different from control. When nicotine (5.79×10^{-5} M) was added 5 minutes after d-TC, nicotine was again able to stimulate an increased ACh release almost as though d-TC were not present. The apparent increase of ACh output with the different concentrations (1.54×10^{-6} and 3.03×10^{-5} M) of d-TC-plus-nicotine was not statistically significant. Hence d-TC which is a potent inhibitor ($I_{50} = 7.7 \times 10^{-7}$ M) of nicotine effects at nicotinic receptor sites has no effect on the nicotine-induced increase of placental ACh release.

Nicotine and Atropine

Figure V-10a shows that atropine (7.69×10^{-5} M) by itself depressed the amount of ACh released. In fact, there was almost a complete cessation of release after the addition of this concentration of atropine. The addition of 5.79×10^{-5} M nicotine after a prior treatment with atropine caused an elevation of both amount and rate of

ACh output. However, nicotine could not stimulate an increase of ACh release in the presence of atropine to the same extent as it did when atropine was absent. The higher the atropine concentration the greater the depression of nicotine enhancement of ACh release. Therefore, it appeared that the release of ACh by nicotine might be mediated through a muscarinic-type mechanism.

These findings did not agree with the commonly reported effects of atropine and d-TC on ACh release from nervous tissue preparations (112, 113). However, the effect of ACh on a number of secretory glands is blocked by atropine. This indicates that the trophoblast may be analogous to a glandular tissue.

Nicotine and Indomethacin

It is becoming more and more widely accepted that prostaglandins modulate the release of transmitters from the autonomic ganglia (114). Indomethacin is a potent inhibitor (I_{50} = 0.75 μM) of prostaglandin synthetase, the enzyme which catalyses synthesis of prostaglandins (99). Since virtually all the components of the prostaglandin system have been reported to exist in the placenta, it was proposed that the prostaglandins might act as modulators

Nicotine and N-ethylmaleimide (NEM)

Crutchley and Piper (115) showed that N-ethylmaleimide (NEM) at a concentration of 10^{-5}-10^{-3} M inhibited catabolism of the prostaglandins by prostaglandin-15-hydroxydehydrogenase (PGDH). This meant that the prostaglandins could accumulate to high levels. Hence the effect of high levels of the prostaglandins on placental ACh release was studied in the presence of 4×10^{-4} M NEM. Figure V-11a illustrates the effect of NEM. The quantity of ACh released by NEM was slightly (though not statistically significant) lower than that in normal KRB. As with indomethacin, two rates of release were observed, thus: the rate of release of ACh during the initial 20 minutes of incubation was 62 percent higher than the rate during the remaining 70 minutes of incubation. When 5.79×10^{-5} M nicotine was introduced into the incubation medium containing NEM, a significant increase of ACh output was produced. The rate of ACh release with nicotine-plus-NEM was 117.93 percent higher than with NEM alone. However, this rate was not significantly higher than the output rate in the normal KRB medium. Again there is no clear explanation of what was observed here.

Relationship between Tissue Level of ACh and Placental ACh Release

The level of ACh in pieces of the villus tissue preparation was determined before and after incubation. As shown in Table V-2, the tissue ACh level at the end of the incubation period was on the average twice the level prior to incubation. As seen in Figure V-2, the cumulative ACh release rose from 1.40 nmoles ACh/g tissue at zero time to 6.00 nmoles ACh/g tissue at 90 minutes of incubation. The percentage increase of release during the 90 minutes of incubation was 328.43 percent. Under all types of experimental conditions, the average percentage increase of tissue ACh level was 99.89 percent (the highest increase was 153.02 percent). This suggested to us that changes in tissue ACh level was probably not the controlling factor in the observed cumulative ACh release from the placental villus tissue preparation.

D. Discussion

Our experiments have shown that when incubated in Krebs-Ringer bicarbonate (KRB) solution, placental villus tissues continuously release ACh. The demonstration

K^+ 17.44 mM	86.15 ± 1.73	178.83 ± 4.27	106.42
63.10 mM	74.00 ± 1.20	159.00 ± 3.23	115.00
Cocaine 5.61×10^{-4} M	92.80 ± 2.30	198.00 ± 2.00	113.36
2.80×10^{-4} M	153.50 ± 3.12	304.33 ± 8.91	97.61
Cocaine + Nicotine	90.75 ± 1.75	207.63 ± 4.67	128.79
Cocaine + Ca^{2+}	70.15 ± 2.85	136.83 ± 2.59	95.05
Mg^{2+} 16.6 mM	93.95 ± 6.70	176.33 ± 2.83	87.69
Nicotine + Atropine	65.10 ± 1.10	134.25 ± 6.25	106.22
Nicotine + d-TC	69.60 ± 1.33	176.10 ± 6.20	153.02
Zero Ca^{2+}	58.70 ± 7.24	59.16 ± 3.38	0

of such a release process was the principal aim of this study.

Most of our current knowledge about acetylcholine release originated from experiments on either the cat superior cervical ganglion (4, 53, 111) or nerve-striated muscle preparations (9, 111, 116). These are all nervous tissues, and information derived from them should ideally be applied to other nervous tissues. The placental villus tissue preparation is devoid of neuronal innervation, and is therefore a source of information about ACh release process from non-nervous tissues. The chief advantage of this tissue preparation is the easily measurable amount of ACh which is released. The ACh released is determined by a physico-chemical method, pyrolysis-gas chromatography, which measures the level of pure acetylcholine rather than "ACh-like substances."

There are a couple of disadvantages of the villus tissue preparation. In the first place, the villus is derived from the expelled placenta. This organ is in its terminal stages of its existence and is classically regarded as composed of "dying" tissues. Nevertheless, in preliminary studies, the physiological viability of the villus tissue preparation has been demonstrated (101).

When the villus tissue pieces were incubated with ^{14}C-α-aminobutyric acid, it accumulated the amino acid against a concentration gradient. The active transport system was sensitive to metabolic inhibitors such as nitrogen and 2,4-dinitrophenol. The second disadvantage of this tissue preparation is that the side of the syncytiotrophoblast, maternal or fetal, where ACh release occurs is questionable. Although this question was not pursued, it is assumed that owing to the way in which the tissue pieces have been prepared, large areas of the syncytium are left intact. The fetal side of this tissue is mainly capillaries, which might have collapsed due to the absence of blood pressure. Hence the bulk of ACh release is expected to be outwards on the maternal side.

In the single reported study on placental ACh release (63), the placenta was perfused with oxygenated saline. This report measured release into the fetal side. Since ACh release was determined by bioassay, much higher values than ours were reported. Furthermore, the author showed that ACh was not released to any measurable extent into the perfusion fluid until two hours after the start of perfusion. The experiment was conducted over an 8-hour period. In view of the "dying" nature of the

placenta, it would seem unlikely that the placenta could remain viable for that length of time. In that case then, the observed ACh release cannot be considered physiological.

The effects of K^+ and Ca^{2+} in increasing placental ACh release are very much like their effects on release processes in nervous tissues and hormone-secreting glands. Thus high K^+ concentration is reported to increase output of ACh from guinea-pig ileal longitudinal muscle (107), isolated synaptosomes (117), and cortical tissue slices (118). Also increased concentrations of Ca^{2+} in the incubation or perfusion of fluids leads to increases of ACh release from various nervous tissue preparations (9, 116, 117). The explanations for the enhancement of ACh release by K^+ and Ca^{2+} almost always involve nerve cell membrane depolarization. The question arising is: Are K^+ and Ca^{2+} effects on placental ACh release produced through the same mechanism(s) by which they increase ACh release in nervous tissues? By virtue of the fact that both the placenta and the nervous tissues have the same components, ACh-ChA-AChE, of the cholinergic system, one is tempted to answer the above question in the affirmative. It would, however, be erroneous to accept such an answer, because certain

subtle differences do exist between the nervous tissues and the placenta. The placenta is a non-excitable tissue with a low and transient resting membrane potential. As reported by Carstensen et al. (119), the placental membrane potential of 0-20 mV found in 91 percent of their measurements was only detectable up to 3 hours after delivery. As a result of handling of the placenta and preparation of the villus tissues used in our experiments, bioelectricity might at best be minimal during the experimental period. Therefore, the depolarization of the syncytiotrophoblast membrane could be expected to play only a minor role in the enhancement of placental ACh release by high K^+ and Ca^{2+}. A similar conclusion was reached by Douglas (97) in explaining the effects of ACh and K^+ on release of catecholamines from chromaffin cells.

A more likely mechanism for enhancement of placental ACh release by K^+ and Ca^{2+} would involve the entrance of these cations into the cytoplasm of the syncytial trophoblast. Such a process has been proposed by Douglas (97, 102) to explain the release of catecholamines from adrenal medulla. It is the basis of the "stimulus-secretion coupling" hypothesis. The same process is found to be operating in the release of diverse compounds

such as ACh from nerve endings (116), vasopressin from neurohypophysis (120), and histamine and serotonin from mast cells (97). All these substances are sequestered within membrane-limited granules, are released by exocytosis set in motion by Ca^{2+} entry. There is no clear evidence for the presence of ACh storage granules in placenta. However, there is some evidence for the bound nature of ACh in the placenta. Due to the strong positive charge on the quaternary head of ACh, one would expect that ACh is possibly bound to the negatively charged proteins (analogous to vesiculin in the nerve [7]) in placenta. Calcium entry is possibly necessary to neutralize the negatively charged proteins before ACh can be released.

Since membrane depolarization and exocytotic processes seem unlikely to account for placental ACh release, other release mechanisms must be sought. According to Douglas and colleagues (97) in all their studies, explanations for cases where secretion was successfully evoked always include an assumption of the occurrence of some changes in the chemistry of the plasma membrane. Douglas further contends that the exact nature of the molecular rearrangement occurring in membranes on exposure to

membrane-active substances (secretagogues) are still unknown. In order to accommodate their various observations on the release of catecholamines from chromaffin cells, it was proposed that various secretagogues have the capacity to "decalcify" the chromaffin membrane. Membrane decalcification was speculated by Shanes (122) concerning the effects of ACh, high K^+ and depolarizing currents on nerves and muscle. The sequence of events leading to the release of catecholamines from chromaffin cells exposed to various secretagogues (e.g., ACh) was summarized by Douglas (97) as follows:

Interaction between ACh and the plasma membrane of the chromaffin cell

↓

Altered membrane chemistry (conformation?)

↓

Increased membrane permeability

↓

Entry of sodium and calcium ions

↓

Depolarization (without impulse formation)

By following the same line of thought, the release of ACh from placenta can be described in the following way: Ca^{2+} interacts with the syncytiotrophoblastic membrane (on the maternal side?) in such a way as to stimulate calcium influx into the syncytial cytoplasm. Inside the cytoplasm, Ca^{2+} would displace ACh from its binding site. The exit of ACh is ensured by the persistent opening of "channels" which are opened as a result of the Ca-membrane interaction (decalcification?). After its release, ACh itself may interact with the syncytiotrophoblast membrane in such a way as to enhance Ca^{2+} uptake which would lead to further ACh release. Such a mechanism was described by Koelle (4, 111) as the amplifier mechanism of ACh release from nerve terminals.

Under the above scheme, stimulatory concentrations of K^{+}, Ca^{2+} and nicotine would be expected to accelerate Ca^{2+}-membrane interaction with consequent accumulation of Ca^{2+} and displacement of ACh from its binding sites. On the other hand, cocaine, atropine and inhibitory concentrations of Ca^{2+} and nicotine probably decrease either the Ca^{2+} membrane interaction or the accumulation of Ca^{2+} and the displacement of bound ACh by Ca^{2+}. In addition to the nicotine-induced Ca^{2+} mobilization, an uptake system for

nicotine probably exists whereby nicotine itself is taken up into the syncytiotrophoblast cytoplasm where it would displace bound ACh. As mentioned earlier, cocaine completely inhibited the nicotine-induced elevation of the rate of ACh release, but was not so effective in inhibiting nicotine-induced increase of the absolute quantity of ACh released. This might be due to the ability of nicotine to both mobilize Ca^{2+} and also enter into the cytoplasm to displace ACh. In this case then, the increase in rate of ACh release by nicotine is due to Ca^{2+} movement which is sensitive to cocaine (105, 106), while the absolute amount of ACh released is increased by both the Ca^{2+} movements and the involvement of nicotine in displacing ACh. Inhibition of nicotine by atropine could occur at the level of Ca^{2+} mobilization as well as displacement of bound ACh.

Although direct evidence for Ca^{2+} uptake through the syncytiotrophoblast membrane was not obtained in the present study, a couple of indirect evidences are worth mentioning. Removal of Ca^{2+} from the external medium led to near-zero release of ACh. Cocaine and amethocaine are both anesthetic agents which presumably possess the same mechanism of action. Rubin et al. (123) showed that

amethocaine strongly inhibited the uptake of $^{45}Ca^{2+}$ in perfused cat adrenal glands and cocaine had been shown to depress Ca^{2+} uptake in smooth muscles (105, 106). Hence these indirect evidences are taken to indicate that Ca^{2+} movement is important in the release of placental ACh.

One piece of evidence that looks out of place in this study is the inhibition of the rate of ACh release by 9.40 mM Ca^{2+}. Most other reports of secretions of various substances from varying types of tissues have always indicated that very high levels of Ca^{2+} (sometimes up to 20 mM) produce elevation of secretion. The contradicting result shown here can be rationalized on the basis of the fact that we measured the cumulative ACh release by Ca^{2+} over a 55-minute period. In the particular case of Douglas et al. (103), Ca^{2+}-induced ACh release was measured after a 2 or a 5-minute period of contact with Ca^{2+}. If we had measured ACh released from placental villus after 5 or even 10-minute contact with elevated Ca^{2+} concentrations, our conclusions might have agreed with those of other workers.

Although Mg^{2+} is commonly regarded as a calcium antagonist, this is not the case with the placental ACh release. This can only be explained as a case of placental insensitivity to the effects of Mg^{2+}. This is not the

first time that the placenta was shown to be insensitive to changes in the concentrations of the common extracellular cations. It has been reported that although the placenta is very sensitive to maternal hyponatremia, it is not sensitive to maternal hypokalemia, and is therefore able to protect the fetus in cases of maternal hypokalemia (124). Therefore, the result of Mg^{2+} effect on placental ACh release might well be another piece of evidence in support of the homeostatic functions of the placenta.

The role of prostaglandins in modulating ACh release from placenta is not clear from the limited studies reported here.

Comparison of Placental Cholinergic System with Cholinergic Systems in Nervous Tissues, Sperm, and Erythrocytes

It is appropriate at this point to put the organization of placental cholinergic system in clear perspective by comparing it with the organization of components of the cholinergic systems in other tissues such as nerves, sperm, and erythrocytes. Such a comparison would provide a deeper insight into the evaluation of the functional

significance of ACh in human placenta. Table V-3 summarizes the main differences and similarities between the various cholinergic systems.

Proposed functions	Regulation of transport (?) Initiation of uterine contractions (??)	Neurotransmission	Sperm motility	Ion transport (?)
	Cholinesterases (ChE)			
Type	AChE	AChE	AChE	AChE
Occurrence (μmoles ACh split per hr)	80 (per g tissue)	682 (per g cortex tissue)	0.4-1.8 per mg protein	153-440 per mg protein
Subcellular site	Microsomes (P3) Nuclear (P1)	Microsomes (P3)	?	?
Inhibitors	Eserine Organophosphates	Eserine Organophosphates	Eserine	Eserine

Acetylcholinergic Receptors (ChR)

Occurrence	Yes (?)	Yes	Yes	?
Type	Muscarinic (?)	Nicotinic Muscarinic	Nicotinic (?)	?

SOURCES OF INFORMATION: Data for placenta were obtained from this dissertation, reference numbers 4, 23, 42, 47, 50, 70.

Data for rat brain were obtained from reference numbers 3, 24, 44, 45, 111.

Data for bull and human sperms were obtained from reference numbers 26, 28, 32, 34, 35.

Data for erythrocytes were obtained from reference numbers 4, 21, 44, 45, 65.

CHAPTER VI

GENERAL DISCUSSIONS

In the foregoing reports, we have accomplished the goals set out as the scope of the present study. Thus, to recapitulate the highlights of our findings: the occurrence of an "ACh-like substance" (23, 38, 54-58) has been reaffirmed, and, by our pyrolysis-GC method, over 60 percent of placental "ACh-like activity" was due to pure ACh. Placental ACh was higher at midtrimester than at early and late trimesters. Subcellular distribution studies showed that ACh was localized in the cytoplasm of syncytiotroproblast, presumably bound to proteins. A release process was demonstrated for placental ACh and this release process was responsive to various ions and drugs in the extracellular fluids.

Since a discussion appropriate to each of the results has been made at the end of each chapter, only certain conclusions will be emphasized at this point.

The experiments summarized above provide the necessary preliminary information for developing a model for the release of ACh, and the hypothesized role of placental ACh in transport of nutrients into the fetus. This model is an attempt to relate the conclusions made from the present work with other types of studies which have bearing on this problem.

A special effort is made to explain the possible way(s) in which nicotine might interact with placental cholinergic system in order to produce small-for-gestational-age babies. It should be pointed out at this point that the development of small-for-gestational-age (SGA) babies is not an exclusive property of nicotine. Numerous other agents including maternal malnutrition, viral infections, high vascular resistance in cord blood vessels, and even carbon monixide as well as other tobacco products in cigarette smoke have been implicated in production of SGA babies. However, most reports show high correlations between production of SGA babies and intensity of maternal cigarette smoking or direct administration of nicotine. This plus the fact that an easy rationale can be constructed for an interaction between nicotine as a

cholinomimetic and a placental cholinergic system make nicotine a favorite candidate for speculations of this nature.

A widely held opinion about the transport of exogenous and endogenous substances through the placenta (81) is that most substances readily pass through the placenta. This is not surprising when one bears in mind that most such studies are carried out on term placentas, and little or no attempt is made to seek correlations between the transport processes and the developmental stages of the placenta and the fetus. Although a few reports of such correlative studies have been made (125), more extensive studies of this type are certainly needed.

Three major conclusions can be made from our present studies. Taken jointly or individually, these conclusions constitute the premises upon which a working model for ACh involvement in transplacental transport is based.

The first conclusion is that during gestation the level of placental ACh changes in such a way as to suggest participation of ACh in regulating the transport of endogenous and exogenous substances through the syncytiotrophoblast. This conclusion is based on two separate studies. From a study of diphenylhydantoin uptake by rat placenta and alpha-aminoisobutyric acid uptake by human placenta at

different gestational ages (101, 125), the curves obtained showed a trough at mid-gestational period. The study of variation of ACh levels with gestational ages of human placentas gave a curve with a hump at mid-trimester (see Chapter III of this study). By superimposing these two types of curves, an inverse relationship between placental ACh levels and uptake processes was developed. That is, as ACh concentration increases beyond a certain level, the transport of nutrients (per unit weight of the villus tissue) through the placenta is decreased and vice versa.

The fact that ACh was found to be highest within a layer of placental tissue with the highest surface area of the syncytiotrophoblast is a further support for the concept of ACh involvement in the regulation of transport of endogenous and exogenous substances through the placenta.

The second conclusion is that the site of action of ACh is the maternal side of the syncytiotrophoblast. This premise is based on a study of the organization of the components of the placental cholinergic system. As indicated in the study of the subcellular distribution of placental ACh, there are no ACh storage granules comparable to the synaptic vesicles of nerve terminals. Rather, ACh

appears to be concentrated in the high-speed supernatant fraction, and is apparently bound by some soluble macromolecules. The localization of ACh within the cytoplasm of the syncytiotrophoblast seems to be an adaptation suited for the local hormonal role of ACh. By virtue of its subcellular localization, ACh could rapidly respond to stimuli from nutrients arriving through the maternal circulation. According to reports by von Euler (71) and Geutieri and associates (72), ACh does not have any consistently measurable effect on the fetal vascular system. From a different point of view, Dancis et al. (79) showed that in situ infusion of methacholine (an ACh analogue) through the fetal circulation did not modify guinea-pig placental permeability. Therefore, placental ACh probably acts only on the maternal side of the syncytiotrophoblast.

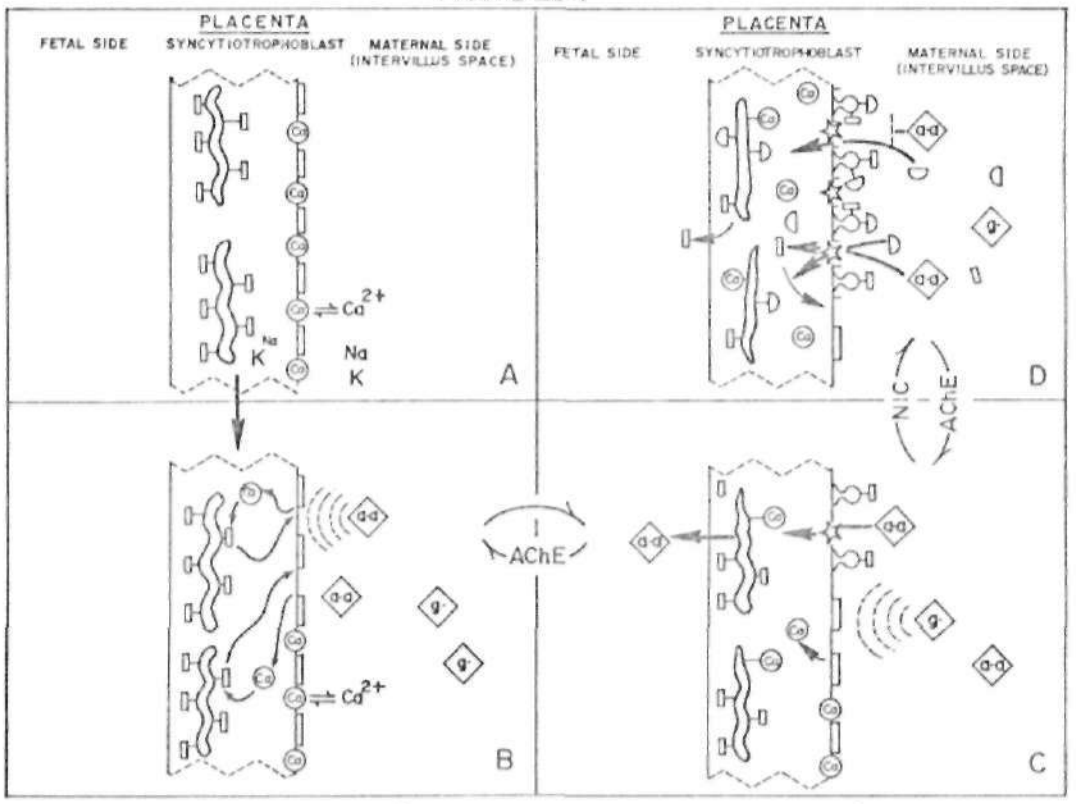
The third conclusion is that as a local hormone, ACh is released at a steady optimum rate, and that nicotine interferes with that steady ACh release either by decreasing or increasing the rate. It is assumed that any deviation from the optimum rate of ACh release would affect the ACh-membrane interaction in such a way as to disrupt the maintenance of the membrane in an optimum state for nutrient transport. That nicotine interferes with placental

ACh release has been detailed in Chapter V of the present study. Two recent reports (126, 127) indicated that the plasma level of nicotine following the smoking of a single cigarette was 186.5 nmoles/liter. Our release studies showed that the spontaneous rate of ACh release was 35 pmole/g tissue/minute. This rate was increased by 40 percent when the concentration of nicotine in the incubation system was 58 μmoles/litre. To acquire a plasma level of nicotine as high as 57.9 μmoles/litre, one would have to smoke 300 cigarettes (assuming no rapid loss of nicotine from plasma). While the consumption of so many cigarettes may seem impossible, it should be pointed out that our release studies were conducted in vitro, and it is possible that the placental cholinergic system is highly sensitive to nicotine in vivo. Further, with increasing contact time, nicotine may also exhibit antagonist effects. This contention can be supported by the results of a study of the effects of cigarette smoking on cat's blood pressure (128). The cat was given a single "puff" (equivalent to 50-ml inspiratory air) of cigarette smoke. This "puff" of cigarette smoke was found to be equipotent with the i.v. injection of 93.17 nmoles/kg of nicotine in producing hypotension and bradycardia. Therefore, it seems that

the nicotine-induced increase of placental ACh release can be appropriately compared to the nicotine-induced hypotension and bradycardia in the cat. By making such a comparison it is possible to show that a person does not require 300 cigarettes in order to produce effects equipotent with 5.79×10^{-5} M nicotine. If a person takes 10 "puffs" on each cigarette, he would have inhaled sufficient cigarette smoke equipotent with an i.v. injection of 931.70 nmoles/kg of nicotine. Therefore, to inhale cigarette smoke equivalent to 57.90 μmoles/litre nicotine, only 60 cigarettes would be required. So it is not too difficult for a heavy cigarette smoker to smoke a sufficient number of cigarettes to produce effects equivalent to 5.79×10^{-5} M nicotine, the stimulatory nicotine concentration in our study.

With these conclusions as background, it is proposed that the steps which might be involved in the control of nutrient transport into the placenta by ACh are as depicted in Figure VI-1, which illustrates a working model for "ACh stimulation-transport coupling."

FIGURE VI-1



A WORKING MODEL DEPICTING "STIMULUS-SECRETION-TRANSPORT" COUPLING IN PLACENTAL SYNCYTIOTROPHOBLAST

Description and Discussion of Figure VI-1

Figure VI-1a shows the placental syncytiotrophoblast as a hypothetical resting state. ACh is shown attached to some macromolecular units. Membrane calcium (Ca) is seen in place on the membrane and also in equilibrium with extracellular calcium ions (Ca^{2+}). Rows of cholinergic sites are shown on the maternal side of the syncytium.

In Figure VI-1b, the arrival of a nutrient molecule is accompanied with Brownian movement which causes the displacement of membrane Ca. The displaced Ca interacts with the macromolecule that binds ACh and displaces ACh. Free ACh then migrates outwards and is released to the maternal side of the syncytiotrophoblast.

Figure VI-1c shows an interaction between the released ACh and the cholinergic sites on the membrane. This ACh-membrane interaction causes membrane perturbation which either leads to more Ca mobilization and ACh release (the "amplifier-type" mechanism of release), or activation of a nutrient carrier system. The carrier system then picks up the stimulating nutrient molecule and transports it into the cytoplasm of the syncytiotrophoblast from where it diffuses to the fetal circulatory system.

The above descriptions cover events occurring under the optimum conditions for release. The binding of ACh to the membrane cholinergic sites is a reversible process. Free ACh is rapidly hydrolyzed by AChE and ChE. To restore the membrane condition to the situation in Figure VI-1b, the membrane of the syncytiotrophoblast oscillates continuously between conditions a to d.

Since there are different classes of nutrient molecules required by the fetus, the carrier systems would be of different types. However, each carrier system can only be activated through the same sequence of events described above, i.e.: Stimulus ⟶ Ca mobilization ⟶ ACh release ⟶ Carrier system activation ⟶ Transport.

In view of the fact that xenobiotic and nutrient uptake processes were not included as one of the objectives of the present investigations, the postulation of a nutrient or a xenobiotic carrier system can only be justified by the widespread literature reports on the subject (79-81, 83, 99, 101, 125, 129).

Nicotine can disrupt the optimum situation described in various ways, namely:

(1) Nicotine causes many units of membrane Ca to be dislodged (133, 134).

(2) This leads to increased displacement of ACh thus increasing ACh release.

(3) Nicotine itself may be transported into the syncytial cytoplasm where it displaces more ACh which in turn is released.

The overall results of these effects of nicotine is to make excess ACh available for interaction with the cholinergic sites on the membrane. Nicotine by itself can mimic ACh on the membrane. Thus an inflated ACh-membrane (as well as nicotine-membrane) interaction occurs with consequent non-specific bindings. Even though the resultant membrane perturbations may lead to activation of the carrier system, nutrient molecules cannot reach the carrier site because of the non-specifically bound ACh and nicotine. Therefore, a deficiency of nutrient supply to the fetus would occur.

The inhibitory effect of atropine also fits into the above scheme. Atropine is probably taken up into the cytoplasm of the syncytiotrophoblast. There it may interact with the ACh-binding macromolecules to produce conformational changes which tend to reduce the effective

Ca-macromolecule interactions which normally lead to ACh release. Atropine may also prevent an "amplifier-type" release of ACh by binding onto the cholinergic sites on the membrane. By either or both of the above mechanisms, atropine can depress the nicotine-induced ACh release.

On a different level, the effect of cocaine can also be explained. Cocaine is a membrane stabilizer and a calcium antagonist. Therefore, as a membrane stabilizer, cocaine may increase the free-energy required to dislodge the membrane Ca. Furthermore, free Ca may be antagonized at the site of Ca-macromolecule interaction, thus leading to a decreased ACh release.

The speculations above might be looked upon as a form of "Stimulus-Secretion-Transport" coupling phenomenon. The idea of coupled transport is not new. The transport of H^+, Na^+, K^+, glucose, and amino acids in the renal tubules (130), as well as amino acids and Na^+ in intestine (131) have been shown to exhibit a form of coupling. In the intestinal transport system, the uptake of amino acids is said to be accompanied by (coupled to) the secretion of H^+ and Na^+. Similarly, in the renal tubular system, the reabsorption of Na^+, glucose, and amino acids is coupled

to the secretion of H^+. These two coupled transport processes have never been shown to be preceded by any particular form of stimulus. (This does not exclude the possibility of such a stimulus though.) What is considered new in the idea of a "stimulus-secretion-transport coupling" is that the transport of a nutrient molecule is preceded by a secretory process (ACh release in this case) which is set in motion by a stimulus (the arrival of the nutrient molecule). This idea is an extension of "stimulus-secretion coupling" (97). The verification of the idea of a "stimulus-secretion-transport coupling" must certainly await many more years of investigation.

As a sequel to the above discussion, a couple of major lines of approaches which would be worthwhile investigating will be outlined. On the question of ACh levels in placenta, there is little that needs to be done. However, it is suggested that more placentas should be analyzed for the purpose of determining the ACh levels. The clinical histories of the placental donors should be compiled. An attempt should then be made to tabulate and classify the various factors which relate ACh levels to clinical histories. Compounds which are responsible for about 40 percent of ACh-like activity in placenta have

not been identified. They may be identified in future investigations.

The question of the nature of existence of ACh at the subcellular level needs to be probed to greater depths. To ascertain the presence or absence of ACh storage granules, it is suggested that the procedure of the isolation of synaptic vesicle by freon infusion described by Whittaker's group (7) be adapted to the placenta. Also synaptic vesicles have been isolated by disrupting tissues in hypotonic solutions and performing density gradient centrifugation on the disrupted tissues. In the event that storage vesicles are shown to be absent in placenta, it is interesting to isolate and characterize the protein with which ACh is bound in the cytoplasm. Whittaker's group (7) has shown that ACh is probably bound to a progein called "vesiculin" which has been isolated from synaptic vesicles of cholinergic terminals. It is of interest to determine whether a protein of that nature exists in the placenta.

The release of placental ACh has been demonstrated. It is suggested that the mechanism of ACh release can be further probed by the use of a wider variety of drugs. For instance, a new school of thought about the release of ACh and other releasable substances from tissues

postulated that release occurs by inhibition of the membrane Na-K-Mg-ATPase (107, 132). Hence drugs which modulate this membrane enzyme should be used to modify placental ACh release. More important to the present study is the transport of nutrients through the placenta. It would be most interesting to see a demonstration of a direct correlation between changes in transport processes and pharmacological manipulations of the placental cholinergic system.

CHAPTER VII

SUMMARY

The studies reported here have elucidated various aspects of human placental acetylcholine (ACh).

Placental ACh was extracted into a solution of 2 percent trichloroacetic acid-in-acetonitrile (TCA-in-MeCN). The ACh was isolated and then analyzed by a pyrolysis-gas chromatographic method.

The value of ACh contents in 34 human term placentas (36-44 weeks of gestation) was 112 ± 7 nmoles/g wet tissue (range 50-285 nmoles). This value was approximately 50-60 percent of the value reported for "acetylcholine-like substance" by Chang and Gaddum in 1933. The term placenta was cut into either radial or concentric segments. ACh content of each segment was analyzed. The distribution of ACh in the radial segment was fairly uniform. Of the concentric segments, the segment closest to the umbilical cord was lowest, while the others have identical values of ACh content.

The ACh level of the term placenta remained fairly unchanged over a period of 72 hours when the placenta was kept at 4°C. However, placental ACh was totally destroyed when the placenta was frozen prior to ACh determination.

The placenta was dissected into chorionic plate, villus tissue and basal plate. ACh content of each tissue was analyzed, and compared to that of whole placenta. The tissues can be arranged in order of increasing ACh content as follows:

$$\text{Chorionic Plate} < \text{Basal Plate} = \text{Whole Placenta} < \text{Villus Tissue}$$

Since the villus tissue has the highest surface area of syncytiotrophoblast, it suggested that ACh exists predominantly in the syncytiotrophoblast.

The variation of the placental ACh at different gestational ages of the placenta was investigated. It was found that placental ACh during the mid-trimester (14-25 weeks of gestation) was much higher than during the first (0-12 weeks) and last (26-42 weeks) trimesters. This peak concentration of placental ACh corresponds well with the fetal period of histogenesis and early functional maturation.

The subcellular distribution of placental ACh was studied. Homogenates of placental villus tissues were separated by differential ultracentrifugation into four primary fractions, namely: nuclear (P1); mitrochondrial (P2); microsomal (P3); and high-speed supernatant (S3). The ACh content of each fraction as well as of the whole homogenate was determined. The subcellular fractions can be arranged in order of decreasing ACh content as follows:

$$S3 \gg P1 = P2 > P3$$

From the results of equilibrium dialysis of samples of the high-speed supernatant, it was suggested that ACh was stored bound to some soluble macromolecules in the cytoplasm of the syncytiotrophoblast.

A release process was demonstrated for placental ACh. The rate of spontaneous ACh release was 35 pmole/g tissue/minute. This release process was modified by changes in the concentrations of cations and addition of drugs in the incubation medium. The elevation of K^+ concentration led to increased ACh release. Increasing the extracellular Ca^{2+} level to 5.96 mM was also stimulatory to ACh release, however, at a concentration of 9.40 mM

Ca^{2+} significantly depressed the rate of release by about 30 percent below control. Substitution of Ba^{2+} for Ca^{2+} led to a sustained but decreased ACh release. In the absence of Ca^{2+}, ACh release was negligibly small, and neither K^{+} nor nicotine could stimulate the release. It was therefore concluded that Ca^{2+} was essential for the placental release of ACh.

The effect of various concentrations of nicotine on the placental ACh release process was investigated. Nicotine showed a biphasic effect on the rate of ACh release as follows:

at 5.75×10^{-6} M, there was no effect;

at 5.79×10^{-5} M, nicotine produced about 40 percent elevation of the rate of release;

at 7.66×10^{-4} M, nicotine depressed the rate by about 30 percent.

The stimulatory effect of nicotine was significantly depressed by atropine, but not by d-tubocurarine. This led to the suggestion that the nicotine effect was probably of the muscarinic type.

Cocaine was by itself inhibitory to ACh release. Cocaine antagonized both the stimulatory and inhibitory

effects of Ca^{2+}, and also depressed the nicotine-induced increase of ACh release.

The results obtained in the present investigation were placed in perspective with other studies related to this subject, and three major conclusions were made. These conclusions are: (1) during the period of gestation, the level of placental ACh changes in a way that suggests participation of ACh in regulating the transport of endogenous and exogenous substances through the syncytiotrophoblast; (2) placental ACh is stored bound to cytoplasmic macromolecules, and the site of action of ACh is most likely the maternal side of the syncytiotrophoblast; and (3) as a local hormone, ACh is released at a steady optimum rate and that nicotine interferes with that steady ACh release either by decreasing or increasing the rate. Based on these conclusions, a working model was constructed to show possible ways in which placental ACh might act on the syncytiotrophoblast to regulate the transport of substances through the placenta.

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