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Relationship of acetylcholine in the placenta to the onset of labor

George F. Hoffmeister
University of Nebraska Medical Center

THE RELATIONSHIP OF ACETYLCHOLINE IN THE PLACENTA TO THE ONSET OF LABOR

George F. Hoffmeister

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The purpose of this paper is to demonstrate the presence of acetycholine in the placenta and to discuss the relationship of acetycholine to the onset of labor. As early as 1907 Dixon and Taylor[1] noted that placental extracts increased the tonus and increased the rhythmical contracti s of the pregnant uterus. They concluded that the placenta produces a chemical substance which, with the ripening of the placenta, is liberated and by contracting the uterus might induce the onset of labor.

Historical

Bohm (1885)[2] first extracted a physiologically active substance from the human placenta. He determined values of 50 mg. of choline per placenta, or in an verage 500 gm. placenta 100 ga mmas* of acetylcholine activity per gram of pl centa. Haupstein (1932)[3] noted values of 74 to 459 mg. per Kg. or 74 to 459 gammas p r gram in placental samples. Chang and Gaddum (1933)[4], while determining Ach** levels in various animal and human tissues, detected concen-

*gamma = 0.001 mg. or 0.000001 gm.
**Ach will hereafter indicate acetylcholine.

trations as high as 28 gammas per gram of human placenta. The finding of such high quantitie of Ach in an organ without innervation is surprising, since Ach is associated with nervous activity and is released by nerve endings. However nervous tissue does not show such large amounts of acetylcholine present. MacIntosh (1940)[5] found levels from 0.4 to 18 gammas per gram in peripheral nerves, 0.02 to 4.0 gammas per gram in spinal cord in white and grey matter, respectively, and 0.2 to 7.0 gammas per gram in brain. High levels of Ach in the placenta have been confirmed by several workers (6,7,8,9,15) and clinical application of the use of Ach for ineffective labor has been tried (18).

Chang, Wen, and Wong (1935)[6] demonstrated granules believed to be precursors of Ach in the syncitial layer and Langhans layer of the villus. These granules are precipitated by special fixatives (1% ammonium Reineckate ether in 10, formaldehyde or in 80% alchohol) and stained by aniline acid-fuchsin. Since Ach activity can be demonstrated in extr ts of villous tissue only and can not be detected in liquor amnii, amniotic membrane, or umbilical cord, they concluded that Ach is formed by the cells of chorionic epithelium of the villi. They also found that Ach is present in the placenta

in both a free form and a reserve form. The reserve form is not present in tissues stored in the ice box for two to four hours. They noted that placenta of guinea pig, rabbit, and dog, although containing Ach, do not show the reserve form.

Chang (1935)[7] describes the extraction of the free and reserve forms. The free Ach can be readily extracted by alchohol or saline, providing precautions are taken to prevent cholinesterase activity by use of eserine and by handling at ice cold temperatures. The reserve Ach is liberated by incubating the placenta at 37° C. for three to four hours. Upon incubation the total Ach level rises to as much as two and one half times the level of free Ach. In 1936 Chang[8] demonstrated by perfusion of fresh placentas with saline that incubation in the presence of eserine at 36° C. produced an increase in Ach level of both the perfusion fluid and the placental tissue. The addition of potassium to the perfusion solution caused a rise in the perfusate Ach but a decrease in tissue Ach. From these experiments Chang concluded that there is Ach-genesis in the placenta in vitro in the presence of eserine. Chang, Lee, Meng, and Wang (1942)[9] showed that for Ach-genesis the

presence of cholinesterase is required, but the factor causing the increase in Ach seems to be something other than cholinesterase since grinding, freezing, and drying destroy this factor, but leave the cholinesterase intact for this phenomenon to take place. Krantz and Carr (1949)[10] state that choline is acetylated in the presence of water-soluble preparations from acetone-dried rabbit brain in the presence of citrate yeast juice, choline, Mg^{++}, K^{+}, and adenosine triphosphate:

citrate ⟶ oxalacetate + "active" acetate

"active" acetate + choline ⟶ acetylcholine

They suggest that the enzyme catalyzing the synthesis of Ach is citrogenase and Mg^{++} is probably its prosthetic group. Perhaps citrogenase is the intracellular factor for which Chang, et al[9] were searching. elsh (1943)[11] demonstrated that synthesis of Ach in vitro is an aerobic process, requiring room temperature or higher, with air available and substrate present.

Theories of the Role of Ach in Labor

Chang, Lee, and Meng (1940)[12] suggested that Ach may play a local role in the normal mechanism of labor. This was wholly theory, and Chang himself warns that theoretical deduction based on studies of a tissue which is discharged as being of no more use to the

body must be guarded against. However, in 1949, Chang[13] further strengthened this theory by demonstrating an increased blood level of Ach toward term and with the onset of labor. He noted that increased levels of Ach gradually appear in the maternal blood toward the second trimester of gestation and that a further increase appears at the onset of labor and that it disappears 48 hours or more after labor. Twenty normal subjects of both sexes failed to show such evidence of increased blood levels of Ach.

Walker and Henderson (1934)[14] had shown that Ach blood levels were highest in the third to sixth months of pregnanc- and gradually diminished toward term. These findings agree with the presence of the granules described above which were found to be more plentiful in early or abortive placentas than in older placentas. Walker and Henderson found that choline chloride content of cord blood was higher in cases of labors less than ten hours duration than in those over ten hours. The dur tion of labor increased directly with the blood pressure (up to 130 mm. Hg. in primiparas and 120 mm. Hg. in multiparas) and th t choline chloride varies directly with this increase in pressure (up to the stated limits). They found gre ter concentrations of choline chloride in umbilical cord blood than in

mat rnal blood of the same patients. This would indicate that the Ach was being forme d on the fetal side of the placental barrier rather than that the Ach was being carried to the plac enta fro the uterus, which was active during labor. Walker and Henderson suggest two func tions of c oline chloride in labor: (1) The neutralizatio of the action of adrenaline; and (2) the stimulation of the uterus.

Eagle (1941)[15] noted the average levels of Ach in pregnant women fro 207 days before term until term is 13.1 gammas/c.c., during labor is 15.0 gammas/c.c., and just after delivery is 24.6 gammas/c.c. The avera e in non pregnant females was 14 .6 gammas /c.c. and in miles w s 13.4 gammas/c.c. Steffanelli and Petronio (1948)[16] noted that the Ach level of blood and the placenta in eleven healthy pateients increased during labor and gradually decreased fro the tenth day on.

Chang, Ien, and Wong (1935)[6] found that Ach levels in the placenta were high in cases of abortion, placenta praevia, and osteomalacia and low in cases of pulmonary tuberculosis and uterine inertia. They established that the action of Ach on the uterus is oxytocic. Bell and Playfair (1937)[17] used Ach in the treatment of twenty-three cases of uterine inertia.

They used 0.2 gms. Ach intramuscularl every three hours. They concluded that Ach is of definite value in the treatment of uterine inertia, and in their experience proved more successful than the other preparations used at that time, including estrin, pitocin, pituitrin, pituchinol, and quinine. They used Ac only when sedation and mild stimulative measures had failed.

Reynolds and Foster (1939, 1940)[18,19,20] further strengthened the theory of the role of acetylcholine in labor by demonstrating the effects of estrogens on the acetylcholine content of the uterus. They found that in rabbits at term the acetylcholine content of the uterus was twice that of any preceding day. They found that estrogens had a pronounced cholinergic action on the uterus of the rabbit and that they increased the Ach content of the uterus within one hour. By twelve hours after injection the Ach content was distinctly less nd a second injection at that time again raises the Ach content. Since estrogens in the human gradual y rise to a peak at term and drop after labor we mig t conclude th t they are the trigger

which sets off the chain of events of Ach production in the placenta and thus increasing Ach in the uterus which, at certain critical level, produces its oxytocic effect on the uterus, thus initiating the onset of labor. Reynolds and Foster state that direct experiments, in which rabbits at term are treated with eserine, atrophine, or nicotine, may supply the essential proof of the role of Ach in the uterus during labor.

Methods of Determination of Acetylcholine

There are several methods of demonstrating the presence of acetylcholine in tissue extracts. Chang and Gaddum (1933)[4], used several different tests and, in most cases, obtained the same result. Their method of extraction of Ach from tissue has been almost universally employed since they described it in 1933. The tissue is minced nd placed in 2 c.c. of 10% trichloracetic acid per gram of tissue. This is allowed to stand for about two hours. Perry (1949)[21] has demonstrated that a maximum field of over 85% of the Ach is accomplised by trichloracetic acid in any period between 1 1/2 to 4 1/2 hours and that it

is 60% destroyed in 19 hours. After this period of extraction Chang and Gaddum filtered off the precipitated proteins and extracted the Ach by one of two methods: (1) Extract with ether until the solution is only faintly acid; or (2) neutralize and reconcentrate in vacuo until 1 c.c. = 1-10 grams of tissue.

The tissue extract is then tested by one of several tests. Chang and Gaddum compared the result of four methods of bioassy or determining tissue Ach:

(1) Frog rectus abdominis contracts when stimulated with Ach. They state that frog rectus is not affected by histamine, adenosine, or calcium, but is stimulated by potassium.

(2) Dorsal muscle of leech contract with Ach, but is not affected by histamine, calcium, adenosine, or potassium.

(3) Rabbit heart beat is decreased by Ach and potassium, but stimulated by histamine and calcium.

(4) Rabbit intestine is inhibited by Ach, but also several other of the substances present.

Besides the methods for bioassay of Ach used by Chang and Gaddum, they used five methods of pharmacological differentiation of Ach from other substances whic leave little doubt in one's mind that the substance measured was anything other than Ach:

(1) If the action of an extract is increased by eserine, the effect is not due to choline or any other substance, except Ach. (This is true for frog rectus and leech muscle, but not true for rabbit intestine.)

(2) The activity should disappear when the extract is mixed with blood (due to high cholinesterase content of red cells), but if the blood has been previously treated with eserine this reaction should be greatly slowed.

(3) The substance should be unstable in alkaline solution and heat.

(4) The substance is antagonized by atropine or nicotine.

These tests are used to differentiate histamine, adenosine, and potassium.

(5) They concluded that the effect must be due to Ach, when the results obtained in each case were the same, when the ctivity of the extracts is estimated quantitatively in terms of Ach using several different tests.

In determining placental Ach, they used the latter test, comparing the action of the extract on frog rectus and rabbit gut. Since the results compared quantitatively in terms of Ach, they concluded that the substance measured was Ach.

Since the method of Chang and Gaddum requires concentration of the material for detection by the frog rectus, which is not extremely sensitive to Ach, several other methods have been used.

Method of bioassay	Minimal Ach detectable in gammas/c.c.
Frog rectus abdominis	0.02
Frog heart	0.01
Dorsal leech muscle	0.002
Heart of Venus mercenaria	0.00005 to 0.001

Sensitivity of some biological methods for the determination of acetylcholine (Modified from Tower and McEachern (1948))[22]

Table I

Because of the high sensitivity of the Venus mercenaria (Table I) and the availability of material for determination by this method in the Pharmacology Department at the University of Nebraska, School of Medicine, it was decided that this method should be very satisfactory for the purposes of this paper.

The Venus heart is insensitive to potassium, histamine, and adrenalin (Tower and McEachern[22]). It is insensitive to pH changes between 5 and 8.5 and is insensitive to anticholinesterases, eg. physostigmine, prostigmine, and di-isopropyifiurophosphate. Welsh (1943)[11] agrees that the Venus heart is unaffected by substances other than Ach in tissue extracts. Welsh and Taub (1948)[23] found that radical departures from the normal concentration of the common ions of the sea water solution used for bathing the heart have little effect on its activity. They found that the treatment of the solution with anticholinesterases (eserine, neostignime, or DFP) may potentiate the action two to five times.

Prosser (1940)[24] determined that Ach acts on the pacemaker and conductive mechanism of the myogenic Venus heart and that it leaves the contractility mechanism intact. He states that stimulation of the visceral ganglion causes inhibition in diastole resembling the effect of Ach. Downing, Dunn, and McIntyre (1950)[25] determined the effect of Ach on the contractility and ECG changes in the Venus heart. They found that Ach reduces the ECG emf more than the contractility thus making possible the detection of 0.000025 gamma/c.c.

Method

Placental samples were obtained at delivery, rapidly examined by the attending physician, a small sample cut and quick-frozen in a sample bottle with the aid of solid CO_2. These samples were numbered and case history kept on each. The plan was to demonstrate a gradual increase in Ach content of the placenta before term, by assay of samples obtained from abortions and early Cesarian sections, and a certain level in placentas at term indicating that there was a critical level of Ach production which, when absorbed by the uterus, "set off" the beginning and brought about the conclusion of parturition. Quick-freezing was accomplished in most cases within two minutes after delivery of the placenta to halt the action of cholinesterase which is plentiful both in the placent (Torda 1942[26]) and in the blood. The samples were stored in a common deep freeze unit at below freezing temperature until used for assaying.

The tissue was weighed in samples of 0.5 to 2 gm. while still frozen and added to 20 c.c. of sea water, made acid to pH 1.5 with trichloracetic acid. The sea water formula was obtained from Tower and McEachern (1948)[22] and simulates closely

the extracellular fluid of the Venus, a s lt water clam. (Table II)

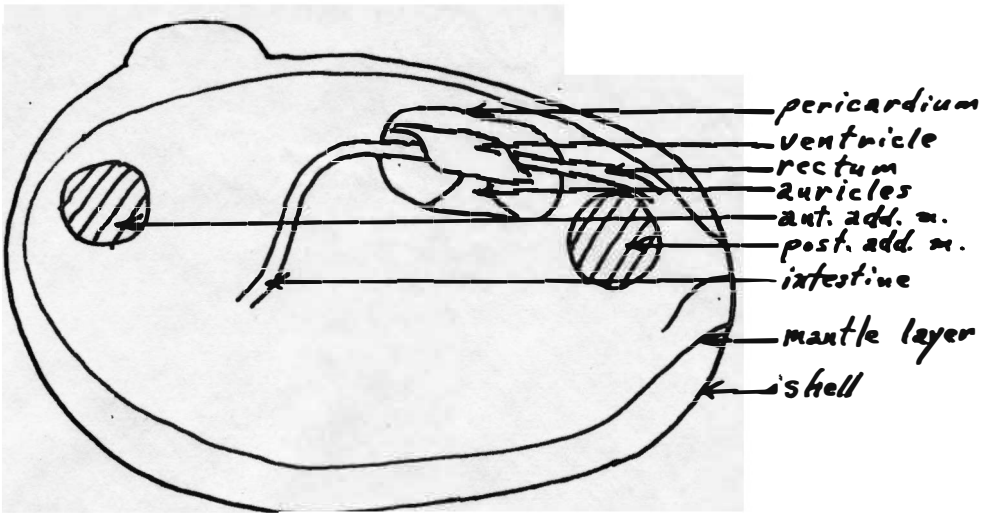
NaCl	30 gms./liter
$MgSO_4 \cdot 7H_2O$	5.1
$CaCl_2 \cdot 2H_2O$	1.25
KCl	0.9
$NaHCO_3$	0.2
Glucose	0.25
$NaH_2PO_4 \cdot H_2O$	0.695(1 c.c./L. 0.5M)
Na_2CO_3	0.053(0.5 c.c./L. 0.5M)

Sea Water Formula

Table II

The tissue was homogenzised in the acid sea water by means of a Waring blender which was rinsed out with and additional 10 c.c. of acid. The solution was then allowedto stand for two hours in the acid for maximal extraction of Ach (see above, Perry 1949[21]). The precipitated proteins were then filtered out and the filtrate neutralized with 1 N $NaHCO_3$ to pH between 5.5 and 6.5 (thus keeping the solution slightly acid to prevent alkaline-hydrolysis of Ach and remaining within the limits of activitv of the clam heart). The solution was then tested immediately or stored in the ice box until testing for a maximum of two hours. There was no need to concentrate the solution in vacuo, since the clam heart is so much more sensitive than the frog rectus.

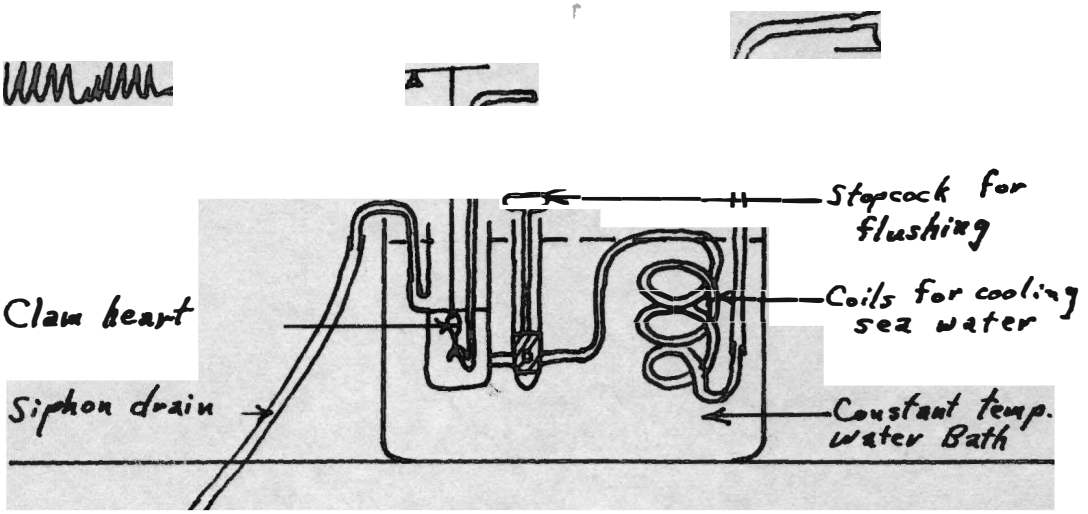
The clams were stored dry in an icebox at about 5° C, at which temperature they remain alive for about two weeks. For removing the heart for testing, the shell is cracked on either side by a light blow with a hammer and the shell removed by severing the attachments of the anterior and posterior adductor muscles on each side. The clam may then be set up in a portion of the shell for convenience and the heart dissected free. The heart is approached through the dorsal side, where it is covered by a thin layer of tissue and its pericardium. The gut will be seen passing through the heart, which appears pink. For greater range of motion with systole, the gut may be severed by entering the gut outside the pericardium with a small pair of scissors and cutting the gut from the inside, taking care not to cut or stretch the heart. The pericardium is then opened and a ligature placed around one end of the gut. The heart is dissected free, taking care not to injure either the ventricle or the bilateral auricular wings. A ligature is then placed around the gut at the oppsite end of the heart and the gut cut outside each ligature freeing the heart, which may then be placed in the testing apperatus.



Diagramatic Sketch of the Position
of the Heart of Venus merceneria

Diagram I

One ligature is tied directly to the end of a glass rod which is immersed to the bottom of the testing well. Theother ligature is attached to the writing lever counterbalanced with 250 mg. total. The testing well is immersed in a constant temperature water bath kept at between 10° and 20° C. The well holds 35 c.c. of solution so that the heart is continually bathed in sea water solution except when the well is drained by siphon for flushing out solutions. The writing lever records contractions on slowly rotating smoked kymograph paper, which is then shellaced for permanency.



Simplified Sketch of Apparatus
for Testing Solutions with the Clam Heart

Diagram II

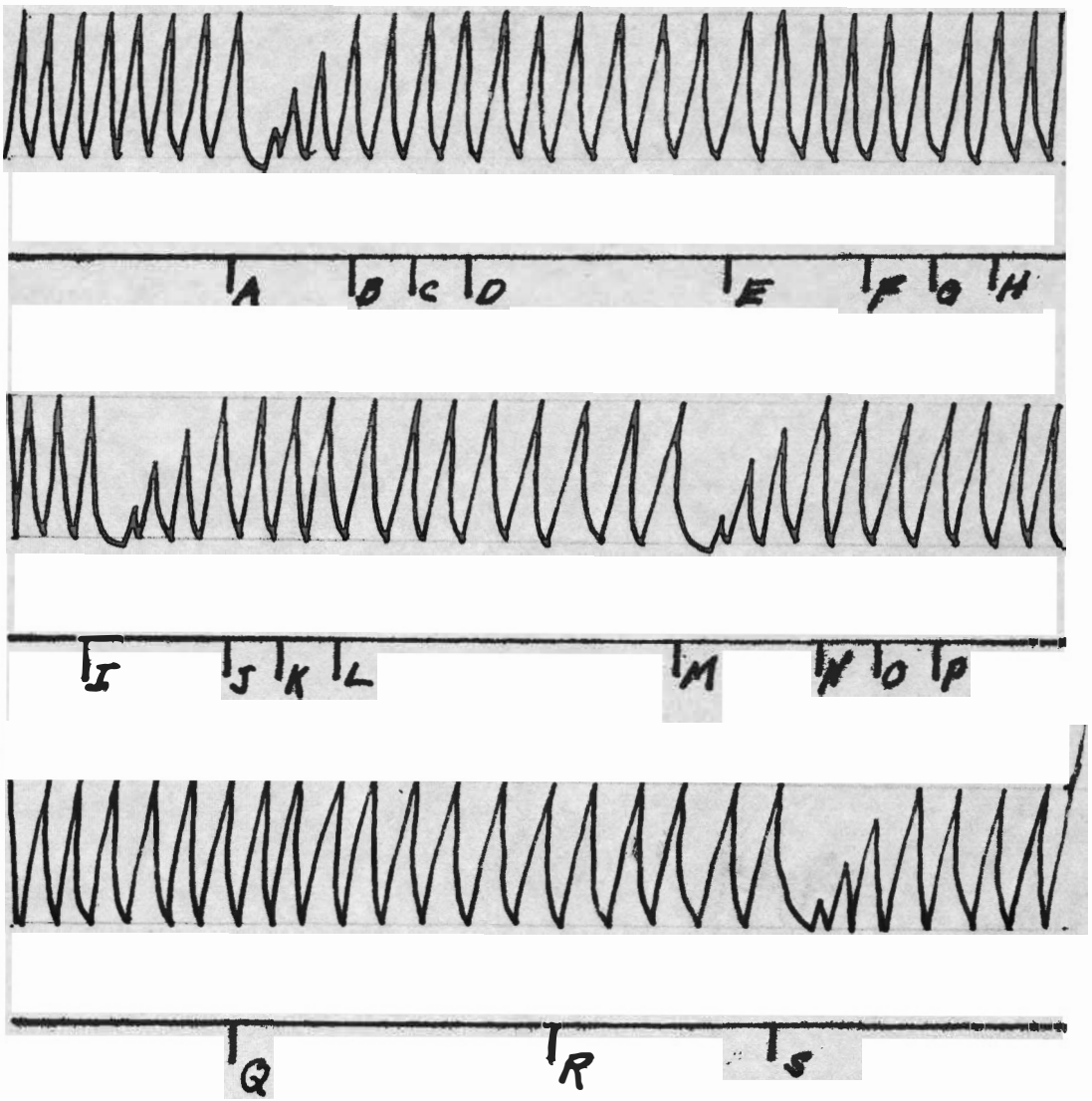
Results

The clam heart is stabilized and ready for testing after about 30 minutes. It is first standardized against known concentrations of Ach. The sensitivity of clams in this experiment was usually in the range of 1:350,000,000 to 1:700,000,000 or 0.0028 to 0.0014 gammas per c.c. respectively.

The solution tested as controls and unknowns and the results were as follows:

(1) Trichloracetic acid sea water neutralized to pH 5.5-6.5.

No reaction, either stimulative or depressive.



Drawings of Typical Reactions of Venus Mercenaria to Solutions Tested

A, I and S = Ach standard 1:350,000,000
BCD, FGH, JKL, and NOP = Flushing with sea water
E = Testing for activity in extraction solution
M = Ach added to tissue and extracted - conc. 1:350,000,000
Q and R = Absence of activity of placental extracts
S = Heart still reacts to Ach standard 1:350,000,000

Diagram III

2. Ach 1:1000, 0.5 c.c. added to 20 c.c. extraction solution and unknown placenta tissue sample, homogenized, filtered, and neutralized to pH 5.5-6.5. Final dilu-tion of 1:350,000,000 gave same amount of depression as standard solution. Thus there was no loss of Ach by the extraction process, but no additional Ach from the placental tissue.

3. Extracted placental tissue, filtered and neutralized to pH 5.5-6.5 showed no effect of Ach. There was no effect on the heart at all. (Diagram III)

The dilutions of these extracts are calculated in the following examples:

2 gm. placenta/30 c.c. extraction sol. + 3 c.c. base for neutralizing

2 gm. placent /33 c.c. sol. = 0.06 gm placenta/c.c.

One c.c. of this extract added to 35 c.c. of sea water bathing the heart =

0.06 gms. placenta/36 c.c.= 0.00166 gm. placenta/c.c.

In order to produce an effect on the heart, 0.0028 gamma Ac /c.c. are required (sensitivity of heart). Therefore 0.0028 gamma Ach per 0.00166 gm. of placent or 1.68 gammas per gram of placenta were not present.

Since amounts as low as 1.68 gammas per gram were not present, even lower concentrations were looked for.

10 c.c. of extract (0.06 gm. placenta/c.c.) added to 35 c.c. of sea water bathing the heart =

0.06 gms. placent /45 c.c.= 0.0133 gms. placenta/c.c.

0.0028 gammas Ach per 0.0133 gms. placenta = 0.21 gammas/gm.

Thus it is determined that there may be Ach in the placenta, but in concentrations less than 0.21 gammas/gm. These results were disappointing after attempting to confirm the results of Chang and Gaddum (1933)[1] who found 28 gammas per gram or over 100 times as much Ach as was found by this method.

Conclusion

The only conclusion one can draw from the result of this determination is that Ach is not present in the placent in quantities as great as 0.21 gammas per gram. However this conclusion seems highly improbable in the light of all the previous research on the subject; therefore, several reasons for failure have been examined.

By the addition of Ach to samples and running these controls through the extracting process and testing, it may be concluded that there is no loss of Ach from that point on. Therefore the fault must be in the process of handling the placental sample before extracting. The time lost from

delivery until complete freezing of the placenta was, in most cases, less than two minutes. Since none of the previous workers had described the process of handling the sample or the time involved before acidifying or eserinizing it, it was assumed that two minutes was not too long.

Upon a search of the literature for the time required to destroy Ach, it was found (Torda, 1942[26]) that placental tissue perfused and washed out contains cholinesterase sufficient in 1 mg. to decompose $6/10^3$ gamma of Ach at 35° in one second. Thus 1 gram of placenta will destroy 6 gammas in one second. Therefore in five second the amount of Ach in one gram of placenta, as described by Chang and Gaddum (1933)[4] is no longer present.

The reserve Ach which is described by Chang, et al (1935)[6] would not be present in using this method since the placenta samples are frozen, thus preventing the citrogenase activity in the synthesis.

Since the time element from time of delivery to time of complete inhibition of cholinesterase activity is so essential in determinations of Ach, it is recommended that future workers use several methods for preparation of the sample:

1. Small samples of about 0.5 to 1 gm. may be cut from the placenta as soon as it is delivered, making a total of about 2 gms. and dropped immediate-ly in 20 c.c. of 10% trichloracetic acid sea water. This sample may then be homogenized in a blender
and the extraction process carried out.

2. Small samples as described above may be placed immediately in eserine solution and then handled rapidly beginning the extraction process.

3. Small samples may be cut about 5 mm. thick and immediately sandwiched between two blocks of solid CO_2 for quick-freezing. In this manner the samples may be stored for future use as was done in this experiment.

Another possibility for failure in this problem may be that previous workers have not measured Ach at all, but have described as Ach some other substance present in tissue extracts such as histamine, adenosine, Ca, or K.

Best and McHenry (1931)[27] state that the simplest extraction of tissue contains histamine. The presence of histamine may be determined by the effect on the atropinized cats blood pressure. Chang and Gaddum (1933)[4] used the atrophine test as one of their pharmacologic differentiations of Ach from

other substances and leave no doubt that the substance they obtained was Ach.

From the findings of other workers, one must con-clude that Ach is present in the placenta in fairly large amounts at term. That Ach content of the placenta plays a role in the normal mechanism of labor can not be definitely proved by findings at this time but may be shown in the future by the use of experimental animals treated at term with eserine, atropine, or nicotine as described by Reynolds and Foster (1940)[20], and by intra-myometrial or uterine artery injections of Ach in preg-nant animals to produce the onset of labor.

D'Incerti and Confalonieri (1951)[28], after examining the effects of various drugs on pregnant uteri at term exposed by Ceasarian section, state that the responses of the uterus to the autonomic mediators (Ach and adrenalin) are not strong enough to suggest that they have a decisive influence on the dynamics of parturition.

Hofbauer (1946)[29] discusses the possibility of the role of Ach formed by the placenta as the normal mechanism preventing eclampsia. He postulates that in pregnancy the activity of postpituitary principles is heightened. It has been shown that the powerful vasodilator activity of Ach has an antagonistic

action on the pitressin effect on the arteriolar tree. In normal pregnancy Ach is found in the normal placenta, but in severely preeclamptic patients the placental Ach is greatly decreased. Thus one wonders if Ach in the placenta may not have two roles: (1) Maintaining a hormonal balance by antagonism of postpituitary principles, and (2) hormonal control of the onset of active labor.

Summary

1. Ach has been found in large amounts (28 gammas/gm) and is probably manufactured by villus cells in vivo and in vitro at 37° C.

2. Clinically Ach has been useful in treatment of uterine inertia.

3. No Ach was found in placentas tested using the "Venus" method of determination. Concentrations of 0.0133 gm. placenta/c.c. of solution would have demonstrated as little as 0.21 gamma/gm.

4. Reasons for failure to demonstrate Ach in the placenta are discussed. The most probable reason is the length of time between delivery and freezing of the sample, thus allowing activity of cholinesterase to hydrolyze Ach present.

5. It is concluded that Ach is present in the placenta, it may play a role in the normal mechanic of labor, and may play a role in the mechanics of eclampsia.

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