SOME STUDIES ON AMNIOTIC FLUID

being a thesis presented

by

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DECLARATION

I hereby declare that the following thesis is a record of the results of experiments carried out by me. It is my own composition and it has not previously been presented in application for a Higher Degree.

The experiments were carried out in the Physiology and Biochemistry research laboratories of St. Salvator's College, St. Andrews under the joint direction of Dr. S. Bayne and Dr. D. Thirkell.

CERTIFICATE

We hereby certify that Isabel C.A. Greig has spent 6 terms in Research Work under our direction and that she has fulfilled the Conditions of Ordinance No. (51) (St. Andrews) and that she is qualified to submit the accompanying thesis in application for the degree of Master of Science.

UNIVERSITY CAREER

I entered St. Salvator's College of the University of St. Andrews as an Assistant in Physiology in October, 1960. The researches described in this thesis was begun in 1960 and continued until the termination of my appointment in 1963.

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CONTENTS

VOLUME

Amniotic fluid appears at an early stage in gestation and its existence in the ovum at four and a half days was demonstrated by Hertig and Rock (1942). The amount of fluid increases progressively during pregnancy and, according to Reynolds (1949), is believed to reach a maximum at 28 to 30 weeks and thereafter decreases, although the traditional view was that amniotic fluid continued to increase right up to term.

The amount present at term varies from one pregnancy to another even in the same woman. The range of normal appears to be from 500 to 1000 ml. although 2000 ml. has been considered to be within normal limits; - for instance Hamilton and Boyd (1955) quote 1800 ml. as the average amount at term. It has been usual to consider large volumes as normal where the foetus is normal and has no deformities, and hydramnios to be present where there are foetal deformities but this can only be considered a broad general division for numerous cases exist where the volumes of fluid are well over 2000 ml. and clearly hydramnios is present, yet the foetus is normal.

The simplest method of measurement is the collection of fluid at artifical rupture of the membranes. Spontaneous rupture rarely occurs under conditions where collection is possible. Even collection via a catheter, however, is highly inaccurate as some fluid is always lost and it is frequently contaminated with blood and urine.

Cox and Chalmers (1953) during their work on the transfer of radioactive substances across the membranes, carried out puncture of the amniotic sac at Caesarian section before incision of the uterine wall. Their cases were of elective section at term for conditions such as pelvic disproportion and they found values shown in the following table.

Table I Volumes of amniotic fluid at different stages of pregnancy

14 weeks	100 ml.
18 weeks	255 ml.
32 weeks	630-720 ml.
34 weeks	390 and 1290 ml.
36 weeks	350,600,1350 and 1500 ml.

There is clearly a wide scatter of results.

Values of volume very early in pregnancy have been obtained by Hanon and his co-workers (1955) who measured the contents of the amniotic sac in two early gestations at therapeutic abortion. At 6 weeks the volume was 4.8 ml. and at 12 weeks 33 ml.

Extremely small volumes of amniotic fluid do occur. For example, an association between renal agenesis and small volumes has been reported. Wagner and Fuchs (1962) recorded a case during therapeutic abortion at 16 weeks where the foetus had only one hypoplastic kidney and the urethra was atretic. The volume of amniotic fluid was found to be 27 ml. whereas the normal amount at this stage is about 250 ml.

Dilution techniques for measuring volume have been used. Hunter and Plentl (1954) used radioactive substances such as radio-active iodinated albumin, but in view of the possible harmful effects of radiation on the foetus Elliott and Inman (1961) used Coomassie Blue in a series of 129 cases. This approach had been used, as early as 1933 by Dieckmann and Davis with Congo Red, while Albano (1937) used Phenolphthalein. Albano questioned the validity of dye dilutions which showed diminutions of volume towards term, suggesting that some dye might be taken up by the foetal gut thus giving falsely low values. Lambiotte and Rosa (1949) carried out a series of investigations injecting inulin diluted with a few mls. of normal saline into the amniotic sac and withdrawing 5 mls. of fluid after 1 hour. Inulin is considered non absorbable, (at least in this length of time). They found volumes of 400 to 1200 mls. at term. However, as they did not do a series throughout pregnancy, the question of diminution towards term remains uncertain. Tsuda (1955) using Congo red and Evans blue suggested that volume increases to 28 weeks, thereafter decreasing slightly, while Fujii, Miyamoto, Arai, and Nomiyama (1951) share this view.

Returning to Elliott and Inman's series of 129 cases, 59 of these were normal pregnancies, 35 patients had pre-eclamptic toxaemia, and 35 had essential hypertension. They found that in normal women there was a maximal volume at 38 weeks, averaging 1000 ml., which thereafter declined at a rate of 145 ml. per week until at 43 weeks it was about 250 ml. In pre-eclamptic cases the

peak volume was reached before 37 weeks and was about half that of normal pregnancy. A similar decline in volume occurred but the lowest volume was reached before 43 weeks. They correlated the size of the placenta with fluid volume and found that in normal cases larger volumes of amniotic fluid were associated with either very large or very small placentae. This effect could not be demonstrated in hypertensive or pre-eclamptic women. The authors suggest that when the placenta is small the balance between secretion and absorption tends to favour the accumulation of amniotic fluid, whereas when it is large, the placenta may develop areas of calcification or infarction or become oedematous. However, it might be argued that such pregnancies though normal clinically, and resulting in the birth of a normal infant, are not strictly normal physiologically.

In hypertension associated with pregnancy the greater the birth weight the greater the amount of fluid, but multiparous hypertensive patients often had oligohydramnios. In pre-eclampsia oligohydramnios was common, especially in older patients.

Summary

There is wide variation in the volume of amniotic fluid whatever methods of measurement are used.

Difficulties exist in attempting to define a "normal" volume.

The question of a maximum volume occurring around 28 weeks is still a matter of debate.

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PRESSURE

Attempts have been made to determine an average value for the pressure within the amniotic sac. Clearly it cannot be a static value but will depend upon the state of contraction of the uterine muscle. During a Braxton Hicks contraction, for example, pressure will increase temporarily.

The range of pressures met with has been studied by Rosenfeld and Lapan (1950) who measured the intra-amniotic pressure in 30 patients using needles introduced through the uterine wall before Caesarian section. The values varied from 60 to 300 mms. water. They also measured the pressures within the venous sinuses. (see Table II).

Table II

Venous Sinus Pressure	Amniotic Fluid Pressure
210 mms. water	150 mms. water
60 " "	60 " "
230 " "	210 " "
140 " "	290 to 340 mms. water
230 " "	180 to 200 " "
210 " "	195 mms. water

Hellman, Triconi and Gupta (1957) measured the pressure in the amniotic fluid and in the inter-villous spaces in eight cases. They found variations in the mean resting pressures of both, but the minute to minute changes in amniotic fluid pressure were less

than those of the inter-villous spaces. (see Table III).

Table III

Pressure in inter-villous blodd	Pressure in amniotic fluid	Pressure after Pitocin	
		Inter-vill.	A.F.
140 mms. water	425 mms. water		
154 " "	120 " "		
104 " "	195 " "	570 mms.	600 mms.
120 " "	270 " "	192 "	354 "
138 " "	68 " "	360 "	410 "
212 " "	417 " "		
240 " "	205 " "		
265 " "	330 " "		

Lindgren (1959) studied amniotic fluid pressure in relation to rupture of the membranes and found that, contrary to previous ideas, the pressure did not alter as the cervix dilated.

Hendricks, Tyler, Quilligan and Tucker (1959) showed that the inter-villous pressure on the maternal side of the placenta was in equilibrium with the foetal side, thus differing from the views of Hellman et al.

Heyns and Samson (1962) in studying the effect of maternal abdominal decompression on foetal oxygenation inserted fine polythene catheters through the cervix when it was 3-4 cms. dilated. They found the resting pressure to be 12-15 mms. Hg. Occasionally

it was 20 mms., but never above 30 mms. During the first stage of labour the pressure mounted rapidly to 40 mms. during contractions whereas in the second stage of labour pressure rose to 50-70 mms. Hg.

There has been considerable interest in the pressure required to rupture the membranes and a number of in vitro experiments have been performed. The amnion has been found to be more resistant than the chorion and Danforth, McElin and States (1956) made a critical study of the results given by Embrey (1954). In vitro the amnion requires a pressure of the order of 300 dynes cm^2 to rupture it.

The actual mechanism of rupture was originally thought to be due to the sudden increase in pressure at the most dependent point where the membranes are not reinforced by uterine wall but this theory does not explain premature rupture before the cervix is dilated. At artifical rupture it has often been noted how tough the membranes are and it seems unlikely that they would resist the frequent contractions which occur throughout pregnancy only to break down under the early contractions of labour which are not much greater in strength or duration than those to which they have been subjected, unless there is another factor at work.

So far, what this factor might be is not clear. The mechanical effect of the descending head pushing a wedge of fluid in front of it may play a part, but in his book "le Liquide Amniotique" Dr. Hanon suggested that there is some evidence for the activity, at term, of an enzyme which reduces the resistance of the membranes

and Mme. Coquin-Carnot showed the presence of lipase in the cervix of mice. Clearly, if such an enzyme were present locally in women at term lowering of the resistance of the membranes near the cervix would allow a small contraction to effect rupture.

Summary

Average values of pressure in the amniotic sac have been obtained and compared with the placental inter-villous pressures.

The important subject of the mechanism of rupture of the membranes requires further study.

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ORIGIN

The question of where amniotic fluid is formed and how it circulates is a complex one. For many years, indeed, since classical times amniotic fluid was thought to be simply a cushioning layer around the foetus. It was believed to arise largely from foetal urine and to be totally inert and static. Modern views are radically different but it is interesting to consider the evidence for the classical point of view.

The idea that amniotic fluid consisted principally of foetal urine was based on the findings that both uric acid and urea were present in it. Some animal experiments had shown that substances injected into the foetus in utero appear in the foetal urine. Thirdly, cases of oligo hydramnios in assoication with maldevelopment or absence of the renal tract have been reported. Fourthly, urine is present in the bladder of the new born infant and micturition often takes place immediately after delivery.

The presence of urea does not point only to foetal urine for it is capable of passing across biological membranes by dialysis. Fluid appears in the developing ovum from the earliest stages of gestation while the renal tract is only fully developed much later. Portes and Granjon (1948) showed that "Tenebryl" a radio-opaque substance when injected into the amniotic sac outlined the maternal urinary tract but not that of the foetus, suggesting that there may be differences in functioning ability even when the foetal

renal tract is apparently developed. The presence of oligo-hydramnios in renal tract deformities is also by no means the rule. Sometimes the amount of amniotic fluid is normal and sometimes there is hydramnios. Imperforate penile urethra in the foetus can be present where the amount of fluid is normal.

Hanon et al in the first edition of "le Liquide Amniotique" compared the urine produced by the first micturition of an infant with a sample of amniotic fluid. Amniotic fluid contained protein while the urine did not. There was 2.4 gms./litre of sodium chloride in the urine but 6 gms./litre in amniotic fluid. Both these findings suggest a second source of protein and electrolytes. They also quote an interesting calculation based on the observation that the speed of replacement of amniotic fluid calculated from experiments using heavy water is 2 hours 54 minutes. (The subject of circulation will be dealt with in greater detail later). The amount of foetal urine in 24 hours which would be required to replace the amniotic fluid would be 500-1000 ml. The least conservative estimate of kidney function suggests it could not secrete much more than a tenth of this amount. 500 ml. would represent in weight about a sixth of the body weight at term.

A common finding in experiments where radio-opaque material has been injected into the amniotic sac has been outlining of the foetal digestive tract. It has been established that the foetus swallows amniotic fluid and though the toxic elements of foetal urine may be small repeated ingestion would result in some degree of electrolyte imbalance and, in time a toxic effect on the foetus and

although Vernix caseosa protects the skin a constant bath of urine alone would certainly cause damage.

In general, it seems probable that micturition in utero can and sometimes does occur but it is not the main factor in the formation of amniotic fluid.

A recent observation by Neumann and Favier (1963) was that of an infant with an imperforate urethra who was born looking normal but developed a large fluid filled sac in the first few minutes after birth. This suggests that there is not even accumulation of urine in the bladder to any extent before birth. Arising out of this is the possibility that there might be reabsorption from the bladder wall. So far there has been no work published on this in man but the phenomenon has been studied in adult animals. Ogawa (1954) experimented on absorption of radio-active isotopes from the bladder in rats, guinea pigs and dogs, while Myiamoto used mice.

It has been suggested that the amniotic fluid may be secreted directly by the amniotic epithelium or that it may pass across the membranes from the interstitial fluid or the blood of the mother.

Keiffer (1926) in his histological studies described vacuoles which can be seen forming within the cells of the membrane, enlarging and finally bursting to shed their contents into the amniotic cavity. At the same time fatty granules appear in the cells. This secretion alone however, would hardly be enough to explain the rapid renewal of amniotic fluid.

Danforth and Hull (1958) described a number of technqiues which they used to study amniotic structure. They used sections mounted as a whole and then studied the cells after teasing them out or after digestion. The unfixed tissue was studied by phase contrast and by electron microscopy. Their results confirmed Keiffer's observations but they also pointed out the presence of a brush border similar to that of the renal tubules. A structure of this kind might well be associated with active transport of fluid across the cells. Further, Hanon et al (1955) reported the presence of carbonic anhydrase in the membranes which would support the possibility of active transport. Danforth also describes a network of intracellular canaliculi like those of gastric epithelium. Candiani (1958) using histochemical methods and an electron microscope, described the ultra-structure of the cells as follows: "The free surface presents a series of cytoplasmic expansions like rows of minute villi. Thus the active surface of the cell is considerably increased. The structure appears to be the same in the early embryo and at term. On the basal surface an intra cytoplasmic lacunar system can be seen".

Further work remains to be done in the field of the ultra-structure of the membranes but it seems likely that fluid can be secreted by these cells though this can only be a minor contribution to the total volume of amniotic fluid, and cannot explain the rapid turnover.

There is some evidence for a maternal origin. Substances injected into the maternal circulation can be recovered from the fluid. These include coloured agents such as indigo sulphate, and chemically detectable substances such as potassium ferrocyanide, sodium thiocyanate and sodium hyposulphite. Isotopically labelled materials e.g. heavy water also appear in it. On the other hand many substances do not pass the amniotic barrier, glucose inulin and "Tenebryl" being examples. Molecular size does not seem to be the determining factor. This will be dealt with again in the section on circulation.

Paths of reabsorption are thought to be by the digestive and respiratory tracts of the foetus. Radio-opaque substances injected into the amniotic cavity can be seen to outline the gut thus confirming that the foetus swallows while in utero. Soon afterwards the urinary tract of the mother can be seen to show radio-opacity. No other system in mother or foetus appears to be affected. Histological proof of the presence of amniotic fluid in the lungs has been found but they do not usually become opaque during amniography. Direct absorption across the membranes also seems a possibility but so far there has been no demonstration of this.

Summary

No foetal sources are alone sufficient to account for the volume of amniotic fluid formed.

Foetal urine and secretion by membrane cells might be additional sources.

Transfer across the membranes seems the most likely explanation.

There is histological and enzymic evidence for active transport across membrane cells - perhaps similar to that of the renal tubules.

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CIRCULATION

For many years, as mentioned in the earlier section, amniotic fluid was thought to be static although clinical observation after premature rupture had shown that the fluid continued to be formed in considerable quantities. The concept of the circulation of amniotic fluid is relatively new and has been demonstrated by the use of harmless foreign chemicals and substances normally found in the fluid labelled with 'heavy'isotopes. In some animal experiments radio active materials have been used.

Boucek and Benton (1932) using phenol sulpha naphthalene, saffron or cochineal, injected these substances into the amniotic sac of the pregnant rat. Phenol sulpha naphthalene was recovered from the maternal circulation within 30 to 60 minutes. The authors pointed out that the time at which it appeared in the mother's blood might be even earlier than this since the test substance had to be in fairly high concentration before it could be detected. "Experimenting ourselves on hydramnios at 7-8 months" state Hanon and his colleagues, "we found that phenol sulpha naphthalene leaves the amniotic cavity very slowly. This characteristic enabled us to measure the volume of hydramnios using a dilution method. The volumes obtained corresponded with those obtained 48 hours later using mannitol. Five days later phenol sulpha naphthalene was still present in the fluid and when the membranes ruptured with the onset of spontaneous labour, there was about 25% of the original quantity injected still

present." (Hanon et al 1963).

Albano (1933) studied what he called "retrograde ovular function", i.e. the elimination or excretion of substances used by the developing ovum. He also used phenolsulpho-naphthalene which he injected into the amniotic sac of women from 20 weeks until term. He found that after 24 hours only 30% of the amount injected remained, while after 48 hours only traces remained. The result was the same, whatever the stage of gestation. There is clearly some experimental difference here but it may well be due to the type of patient studied as conditions of circulation in hydramnios must be far from normal.

Rosa (1952) tried to define the actual mechanism of passage. He used "Ombradyl", (a radio-opaque material), glucose and inulin. "Ombradyl" injected into the maternal circulation did not make the foetal renal tract radio-opaque. Instead, when injected into the amniotic cavity it rapidly outlined the foetal digestive tract and then the maternal renal tract. He therefore concluded that the pathway for removal of amniotic fluid was from the foetal gut via the foetal circulation and from there through the placenta to the maternal circulation. This is clearly the route taken by this particular substance but does not preclude the possibility of alternative pathways. "Ombradyl" when injected into the maternal circulation does not appear in the amniotic fluid.

Glucose introduced into the amniotic sac, rapidly induces maternal hyperglycaemia, though it is slight and of short duration

and is not accompanied by glycosuria. A maternal blood sugar which is elevated does not appear to affect the amniotic fluid. According to Rosa these findings suggest an active but selective absorption by the amniotic membrane of water and glucose and certain other dissolved substances.

Savignac (1953) in conditions similar to Rosa's used "Diodrast" another radio-opaque substance, and confirmed Rosa's results with "Ombradyl".

However, Bubani (1958) using glucose, failed to demonstrate transamniotic passage in either direction.

McCaughy, Corey, Scoggin, Bobbit and Thornton (1959) studied the transfer of urea, using the fact that the levels in foetal and maternal serum are the same (about 23 mg./litre) whereas they are slightly higher in the amniotic fluid. They found evidence of direct transfer across the membrane.

Grynfogel, Hutchinson, Kelly and Plentl (1962) believe that the urea of amniotic fluid is derived, for the most part, from the foetal circulation without invoking the vexed question of foetal micturition, but direct passage from the mother is negligible. Their experiments were carried out using labelled urea in the monkey. The recent work of Hutchinson, Kelly, Friedman and Plentl (1962) should also be mentioned. They measured the exchange of labelled urea between mother and foetus in the monkey and found a rapid transfer from the maternal to the foetal serum but movement to the

amniotic fluid was slower. The ratio of amniotic fluid urea derived from the maternal circulation compared with that from the foetal circulation was 3:1.

There has also been a considerable amount of work on the permeability of the membranes in vitro. Andreas and Schmidt (1960) removed the membranes in their entirety and then studied their permeability to a number of substances. Under their conditions they found the membranes allowed the passage of substances whose molecular weight was less than 30,000. The presence of electrolytes increased the speed of passage.

Garby (1957) had studied the permeability of the isolated amnion and arrived at the conclusion that minute "pores" are present which have a diameter of 100 Å. and allow the passage of materials up to the size of albumins.

Interesting though work on isolated membranes may be, it is clear that no very definite conclusions can be drawn from it as to exactly what takes place in the living organism.

Possibly the most productive line of research has been that using isotopically labelled tracers (either radioactive or "heavy" isotopes). These have been mentioned already but it is of interest to return to some of this work.

In pregnant women and in animals, attention has mainly been directed to the passage of labelled material from the maternal to the foetal circulation, i.e. the problem of transfer across the

placenta. Nevertheless some work has been concerned with their passage into amniotic fluid and the speed of renewal of the fluid.

The earliest work published was that of Flexner and Gellhorn (1942) who used guinea pigs as their subjects and administered heavy water and labelled sodium in the form of $^{24}NaCl$. The animals were tested at all stages of gestation from the third week until term. Heavy water was injected into the maternal circulation and the amniotic sac was opened 30 minutes later. Other animals were injected with $^{24}NaCl$ and the amniotic sac opened after 3 hours. They reported that the speed of turnover of the liquor lay between 0.9 and 1.7 times per hour, on average a little more than one. Put in another way, the speed with which the heavy water appeared in the amniotic fluid was such that a volume of water equal to that of the amniotic fluid is replaced at least once an hour at all stages of gestation. They reported that the transfer of sodium was about 50 times slower which seems a curious finding in view of the results of Vosburgh, Flexner, Corvie, Hellman, Proktor and Wilde (1948) who used similar techniques in women and measured the speed of passage of water and sodium from the maternal circulation to the amniotic fluid. They found that the whole of the amniotic fluid is renewed in 2 hours 54 minutes, while the transfer of sodium is five times slower. It could be shown that every hour 34.5% of the water and 6.9% of the sodium were replaced.

Further, Plentl and Hutchinson (1953) and Plentl (1954) using heavy water in women at term showed that the replacement is 600 ml. per hour. Comparable results were obtained by Cox and Chalmers (1953[a]) who carried out investigations on patients about to undergo elective Caesarian section for conditions such as disproportion. Injections were made immediately before operation and on incision of the uterine wall, all the fluid was aspirated and the volume measured. The results showed that the average value for sodium exchange was 19.1% per hour. The authors pointed out that equilibrium between the level of labelled serum in the blood and amniotic fluid was reached in five hours. The average amount of sodium transferred was 0.5 gm./hour. Although the methods used give no direct evidence of the mode of transfer from the maternal circulation to the amniotic fluid, the results suggest that the speed of passage is too great for it to take place through the urinary system of the foetus. Some cases were noted in which labelled sodium appeared 5 minutes after its intravenous injection into the mother.

A similar experiment on sodium transfer was carried out on three cases of hydramnios (Cox and Chalmers 1953[b]) in each of which the volume of amniotic fluid was about 5 litres. The foetuses were alive but on delivery had multiple deformities. 2.8% of the labelled sodium was exchanged per hour.

In two cases of pre-eclamptic toxaemia which were studied the passage of sodium was faster. The conclusion drawn from the series of experiments was that in normal cases the replacement of all the sodium of the amniotic fluid takes place in 5 hours but in as much as 336 hours in cases of hydramnios. Sodium injected into the maternal circulation appears, on average, in the amiotic fluid less than 6 minutes later.

Pinson (1952) studied the passage of water labelled with deuterium on tritium from the mother to the foetus. He also concluded that the whole of the amniotic fluid could be replaced in minutes or at most a few hours.

Neslen, Hunter and Plentl (1954) used a more complex approach. ^{22}Na and ^{24}Na were employed simultaneously, one being injected into the maternal circulation and one into the amniotic fluid. The speed of exchange was found to be almost the same in each direction. They also reported that the speed of transfer of D_2O was almost five times greater than that of sodium or potassium. They believe the electrolytes of the liquor to be in dynamic equilibrium with the maternal plasma, each one exchanging at its own characteristic rate. (e.g. 0.31 moles of water/hour, 0.014 moles of sodium/hour, 0.00041 moles of potassium per hour).

From other experiments both on women and monkeys, the authors conclude that the system behaves as if it were made up of three compartments, mother, foetus, and amniotic fluid. 25 to 50% of the water exchange takes place via the foetus and placenta,

while the rest is through direct exchange between the maternal body fluids and the amniotic fluid.

Gray and Plentl (1954) endeavoured to demonstrate their ideas using a system of three compartments ingeniously connected by pumps, which simulated the conditions in vivo. It was using this device that they arrived at their conclusions.

Paul, Enn, Reynolds and Chinard (1956) experimenting on rabbits at term [21] used water containing D_2O injected into maternal circulation and water containing T_2O into the amniotic sac. Their results showed that approximately half the water exchange was via the foetus and the placenta, the rest was direct exchange across the membranes. Morin, Hanon, Coquin-Carnot and Roux (1960)[22] have demonstrated the presence of carbonic anhydrase in the membranes. It is well known that this enzyme plays an important part in renal tubular exchange and it might do so here.

Famiani and Amici (1954) think that Whartons jelly might be involved in fluid exchange because of its chemical composition and its lacunar structure. If this were so to a significant extent, one might expect that there would be some correlation between lengths of cord and amount of liquor. A recent article in the British Medical Journal by Malpas (1964) discussed the relationship between cord length and placental weights, but unfortunately no mention was made of amounts of amniotic fluid.

The movements of labelled protein in mother and foetus have also been studied. Candiani (1957 and 1958) studied the

movement of albumin labelled with Iodine-131 in rats, guinea pigs, normal and hydramniotic patients. In normal women at term (4 cases), the labelled protein injected intravenously, was found in the amniotic fluid after 6 hours and reached a maximum in 12 hours. Similar albumin injected into the amniotic sac, appeared in the maternal blood 12 hours later. Numerous controls showed that the radioactivity measured was actually that of the protein i.e. the iodine had not become separated from its protein. In two cases of hydramnios radioactivity appeared in the fluid 3 hours after injection into the maternal circulation and reached its maximum 18 hours later. Polvani (1958) obtained similar results in the rat. Cottafavi and Mentasti (1959) administered labelled albumin by mouth and it appeared in the amniotic fluid 15 minutes later, but it could be argued that in this case some breakdown might have occurred.

Feletig (1957) showed that 150 mg. of protein enter th liquor in an hour. He believes that they are derived from desquamation of cells from the surface of the membranes and foetus.

Summary

Circulation of the amniotic fluid is now definitely established.

Values for rate of replacement are quoted above and suggest a rapid turnover.

The mechanism of transport of fluid from mother to foetus has not yet been established.

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CHARACTER AND COMPOSITION

Amniotic fluid like lymph and cerebrospinal fluid belongs to the extra-cellular compartment of the body and resembles to some extent extra-cellular fluid. When freshly withdrawn from the body amniotic fluid is colourless, slightly opalescent fluid. The degree of opalescence depends on the amount of suspended matter in it. The particles are mostly those of sebaceous material and desquamated cells.

Uranga, Imaz and Gascon (1950) published the following series of values for the physical constants.

TABLE IV

Density	1.006 (± 0.002)
Viscosity	1.
Freezing point	-0.5°C. (± 0.06°)
Osmotic pressure	5.800 (± 0.2) atm.
pH	7.43

Mannherz (1949) had found that the pH of fluid kept airtight, lay between 6.9 and 7.1. Rooth, Sjostedt and Caligara (1961) measured the pH of samples taken at different stages of pregnancy, and they found it to be 7.16 in early pregnancy (average of 17 samples) and 6.85 at term (average of 26 samples). They suggest that the pH is less than that of blood because of the relatively high CO_2 tension and amount of fixed acid accompanied by a smaller

buffering ability.

CHEMICAL COMPOSITION

Many analyses of the composition of amniotic fluid have been made. It is a complex mixture of organic and inorganic materials, the latter being of more importance from the osmotic point of view.

INORGANIC CONSTITUENTS

Uranga, Imaz and Gascon (1950) reported that amniotic fluid consisted of 96.4% water and that the dry residue was 1.18% (± 0.06) while the ash was 0.73%.

The principal inorganic constituents were as follows:-

TABLE V

Chloride	0.35 gms./100 ml.	(± 0.05)
Phosphorus	0.0038 "	(± 0.002)
Sodium	0.30 "	(± 0.02)
Potassium	0.018 "	(± 0.009)
Calcium	0.010 "	(± 0.009)
Magnesium	0.0028 "	(± 0.0008)

Other authors have made particular studies of certain elements. Cantarow (1933) reported a value for calcium of 5.46 mgs. % while Merritt (1931) gave 6.59 mgs. %. Mannherz (1949) reported the alkaline reserve as lying between 32 and 62 volumes % of bicarbonate. The majority of values were between 32 and 41 volumes %.

Hanon and his co-workers (1955) investigated the ionic pattern of 10 specimens from normal patients at term and their results are shown in Table VI :-

TABLE VI

Chloride	0.365 gms.%
Alkaline reserve	35 vols.%
Phosphorus	0.0027 gms.%
Sulphur	0.0037 gms.%
Potassium	0.019 gms.%
Sodium	0.29L gms.%
Calcium	0.0072 gms.%
Magnesium	0.0022 gms.%
Freezing point	-0.51°C

These figures have been confirmed in general by several recent papers: Battaglia, Prystowsky, Smission, Hellegers and Bruns (1959), Partensky (1960), Mischel (1960), Westin, Lind and Teger-Nilsson (1960) and Schreiner, Buhlman and Held (1961).

ORGANIC CONSTITUENTS

Protein

Amniotic fluid is relatively poor in protein. Uranga, Imaz and Gascon (1950) reported a level of 0.21 gm.% (±0.08) and Hanon et al (1955) found average values of 0.26 gms.%. The various protein fractions have been studied by paper electrophoresis. Hanon et al (1955) stated that ten samples of fluid obtained at

term showed 5 fractions with speeds of migration similar to those of the protein fractions of serum. Although speed of migration is not an absolute criterion of the identity of the proteins, it allows comparison with serum and gives a relative indication of molecular size.

The relative concentrations of the protein types were as follows:-

TABLE VII

	Albumins	54%
$\alpha 1$	Globulins	7%
$\alpha 2$	Globulins	6.5%
β	Globulins	12.5%
γ	Globulins	20%

Albumin/globulin ration = 1.15

The accepted average value for blood serum are worth comparing with these.

TABLE VIII

	Albumin	60%
$\alpha 1$	Globulin	5%
$\alpha 2$	Globulin	7.5%
β	Globulin	12.5%
γ	Globulin	15%

Comparison shows some correlation between their relative fractions. The total protein of serum is however, about 30 times higher.

Using the same technique, Palliez, Bizerte, Cotteel and Delecour (1954) obtained two main components, one with a speed equal to that of serum albumin and another group with speeds equal to that of the globulins, but they did not identify the components beyond all doubt.

However, it can be said that the major protein constituent of amniotic fluid is identical with serum albumin. Besides albumin four other fractions can be distinguished with electrophoretic speeds like those of the serum globulins.

Mentasti (1959), Brezezinski, Sadowsky and Shafrir (1961), have recently published studies of the protein fractions of amniotic fluid, with similar findings.

In the early months of pregnancy, Westin (1960) and Vergiever, Stroup, Sheff and Westphal (1962), found protein levels of 5-7 gms./litre. Such levels have only previously been found in pathological pregnancy, for example some cases of hydramnios according to Mentasti (1959) and Hanon et al (1961).

The technique of immunoelectropheresis has been applied to amniotic fluid by several workers. The most interesting results are those of Lambiotte and Salmon (1962a) who showed the presence of at least two antigenic substances peculiar to amniotic fluid and more recently (1962b) that these components can pass into the

maternal circulation during labour. These substances cannot be β_2A macroglobulin or β_2M macroglobulin as Massayef (1960) had shown these to be consistently absent from amniotic fluid which contained most of the proteins present in serum. Hottinger and Strebel (1960) gave similar results.

Amino-acids and Peptides

Amniotic fluid contains numerous amino-acids. Claudattos and Ionnesco-Garnattea (1933) found an amino-acid level of 5.2 - 6.9 mg.%. Crumpler, Dent and Linden (1950) more recently carried out comparative studies of the amino-acid levels in the foetal and maternal blood at birth. They measured the amino-acid nitrogen and isolated the individual acids by paper chromatography. The amino-acid nitrogen was almost always greater (in the ratio of 3/1) in the foetal than the maternal blood. They suggested that the placenta may be concentrating amino-acids thus aiding foetal protein synthesis. The inverse ration (4β1) observed in four cases of toxaemia might be the result of alteration in placental function.

Palliez, Bizerte, Cotell and Delecour (1954) studied the isolation, separation and identification of the amino-acids of liquor. They used the technique of ion exchange chromatography. Separation into five main groups occurred and these were later elucidated by paper chromatography.

Group 1, was essentially the free amino-acids of which the most important quantitatively were glutamic acid, glycine and alanine. Next in amount came valine, leucine, lysine, histidine and arginine.

Serine; aspartic acid, cystine, hydroxyproline, proline, tyrosine and methionine were present in smaller amounts. Traces of amino-butyric acid, threonine, and β-alanine were also found.

Group 2, the second fraction contained a certain amount of free taurine but was largely made up of polypeptides. After hydrolysis, chromatography showed the presence of their constituent amino-acids. The last three fractions were made up of peptides with a very acid character. They were rich in glutamic and aspartic acid. β-Alanine and amino-butyric acid were also detected.

Qualitative studies of amino-acids have also been made by Candiani (1958), Orlandi, Bottiglioni, and Torsello (1958) and Partensky (1960).

Sugars

The presence or absence of reducing sugars was a matter of debate for many years. It is now generally accepted that reducing sugars are normally present. (Mohs. (1931), Cantarrow et al (1933) and Makepeace, Freemont, Smith, Daily and Carrol (1931)), Mayayoshi, Ichijo (1934) measured the level of reducing sugar in 14 cases at term and found an average value of 20 mgs.%; 10 mgs.% of this was fructose, the rest glucose. However, the level of reducing sugar is higher in early pregnancy and may reach levels of 90 mgs.%. Masuko (1940) showed that amniotic fluid obtained from 30 cases at rupture of the membranes gave a positive Seliwanoff test for fructose. Magnin et al (1952) measured the sugar present in 24 specimens recovered from the vagina during labour. The values

were between 25 and 55 mgs.% most being between 30 and 35 mgs.%. The authors did not specify the types of reducing sugar.

Sozansky (1958) gave the value for normal pregnancy as 250 mg./litre but again, did not specify the sugars. 40 to 720 mg. per 100 cc. fluid was reported by Caminti and Serluca (1956) and this was not related to the maternal blood sugar.

Huggett (1955) in experiments upon rabbits showed the presence of glucose and fructose but reported that there was a difference in their rate of entry into amniotic fluid, fructose entering more slowly. Bubani (1958) on the other hand could not detect any glucose at all !

Acid-base balance of amniotic fluid

Rooth, Sjostedt and Caligara (1961) suggested that the pH of amniotic fluid is less than that of blood because of the relatively high CO_2 tension, large amount of fixed acids and smaller buffering action.

Schreiner, Buhlman and Held (1961) measured the pH and CO_2 pressure in patients between 11-14 weeks and between 36-41 weeks. Specimens were obtained by trans-abdominal puncture. The pH was found to increase from 7.1 in the first trimester to 6.98 in the last trimester. The pCO_2 however, merely showed a small increase at the end of pregnancy and the average value was 55-57 mm. Hg.

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SOME ENZYME STUDIES

It was shown by La Due, Wrobleski and Karmen (1954) that the levels of the enzyme glutamic oxaloacetic transaminase in homogenised samples of liver, myocardium and skeletal muscle were higher than those of other organs similarly treated. Shortly after this it was shown that on death or damage of cells in the myocardium, the enzyme is liberated into the serum.

The estimation of serum glutamic oxaloacetic transaminase (S.G.O.T.) was soon introduced into clinical practice as an aid to the diagnosis of acute myocardial infarction. The level of (S.G.O.T.) in the serum of normal healthy individuals varies from 5-40 units/ml. and does not vary by more than 10 units from day to day. It is also apparently independent of the time of day the blood samples are taken and the amount of exercise or nature of the subject's meals.

Since the enzyme is found widely distributed in the body, it seems likely that necrosis of other tissues might produce the same effect. Wrobleski and La Due (1955), showed that it was raised in liver lesions although it is usually the serum glutamic pyruvic transaminase which is of interest here. Merrill, Lumley, Stone, Grace and Meneely (1956) reported a rise after damage to skeletal muscle and Chinsky, Shmagranof and Sherry (1956) after pancreatic damage.

The levels of S.G.O.T. in maternal serum during pregnancy have

been measured. Borglin (1958), Dubach and Stamm (1958) and Persson (1959) reported no significant alterations during normal pregnancy.

However, it seemed possible that in conditions where there is damage to the uterine wall, placenta, or foetus the levels in the amniotic fluid might change.

Accordingly, a series of S.G.O.T. levels of amniotic fluid from normal patients and those who were suffering from a number of disorders i.e. pre-eclamptic toxaemia, posmaturity, and hydramnios were measured, and are reported below.

Since the time that this was carried out there have been a number of papers on the subject.

Persson (1960), reported on Serum glutamic oxaloacetic transaminase (S.G.O.T.) and Serum glutamic pyruvic transaminase (S.G.P.T.) levels in the uterus, placenta and foetal and maternal serum in normal pregnancy.

TABLE IX

4th - 6th month

Enzyme type	Maternal serum	Uterine wall	Foetal serum	Placenta
S.G.O.T.	24 units/ml.	11,000 u/ml.	94 units/ml.	7900 u/ml.
S.G.P.T.	23	3,800	70	4300
	Delivery	Retro placental serum		
S.G.O.T.	31	112 u/ml.	77	10,800
S.G.P.T.	21	63	48	5,300

He did not report on the levels in amniotic fluid. However, Mentasti (1958) reported that S.G.O.T. was constantly present in the fluid at levels lower than that in maternal or foetal blood. It was also reported that in meconium-stained amniotic fluid the levels were higher.

Kubli (1961) on the other hand found an average value of 33 units, the same or higher than that in maternal blood. The level was raised in intra-uterine foetal death but was not affected by the presence of meconium. No relationship between pathological conditions and S,G.O.T. levels could be demonstrated.

Other Enzymes

An extensive review was made in 1962 by Lapan and Friedman (1962) of the average levels of a number of enzymes. They reported as follows:

Cholinesterase.- 0.2 units, lower than the maternal serum level (0.6 units /ml.).

Tributyrinase is absent from amniotic fluid. It is present in maternal serum at a level of 0.6 units/ml.

Alkaline phosphatase.- 5.6 units. This is lower than in maternal serum which contains 11.9 units/ml.

Lactic dehydrogenase. - 375 units/ml. in amniotic fluid but only 177 units/100 ml. in maternal serum.

Phosphohexose isomerase. - 89.3 units/ml. in amniotic fluid but 37.4 units/ml. in maternal serum.

Alkaline phosphatase was investigated by Bubani (1958) who found levels of from 2.3 to 83.2 King-Armstrong units/ml. in a series of 15 patients. He could find no correlation with maternal blood levels. Where the amniotic fluid was stained with meconium, the enzyme level was high. This fitted in with a suggestion that alkaline phosphatase levels rose with foetal distress. This view is not held by Petry and Damminger (1956) who correlates alkaline phosphatase with the state of the membranes. He believes that the chorion decidua complex is the source of the enzyme, not the placenta or maternal blood. Mischel (1960) gives levels of between 2 and 55 King Armstrong units/ml., the levels rising to 255 King Armstrong units/ml. in the presence of meconium.

Acid phosphatase

This enzyme has been measured by Kresser (1963) and he showed higher amniotic fluid levels than serum levels, during delivery although throughout pregnancy the level varied greatly. There was very little or none in the first few months. The enzyme present was mostly of type II i.e. the prostatic type and it is possible it may have originated in the amnion or the foetal urine.

Histamine

Cerasuolo and Cilento (1959) showed that histamine is present and that the levels were higher in patients with pre-eclamptic toxaemia. They suggested this might be because of a breakdown in the placental barrier in cases of toxaemia where placental infarction is frequently found.

Trypsin inhibiting activity of amniotic fluid

Woraschk (1962) reported that the trypsin inhibiting power of amniotic fluid was 35 times less than that of serum, but in some pathological cases it was increased which he attributed to a simultaneous protein increase. The trypsin inhibitors are thought to be the same as those in serum, but to be present in such low concentration as to exert no effect on the coagulation system in amniotic fluid embolism.

This does not agree with the findings of Albrechtson and Trolle (1955). This subject is also dealt with by Stichbury (1959) who showed that the average antiprotease power of amniotic fluid was 41 mg./ml. while that of neo-natal urine was only 3.4 mg./ml. similar to that of adults. The antiprotease power of maternal serum was 760 mg./ml. Thus amniotic fluid had 12 times greater antiprotease power than urine but it was only 1/19 that of plasma.

Experimental Material

Specimens of amniotic fluid obtained from patients at Craigtoun Hospital who had undergone high rupture of the membranes, were collected in polythene bottles. They were subsequently placed in a refrigerator. Specimens which were contaminated with blood were discarded. In cases where it was possible specimens of maternal blood were also taken and any haemolysed blood samples were discarded. Estimations of enzyme level were then performed.

There were a number of difficulties involved in obtaining suitable specimens. Firstly, only a few high ruptures were

performed and the material from low ruptures was no use for enzyme studies as it was contaminated with vaginal material. Secondly, liaison between the Hospital and the Physiology department was a little erratic and some specimens had to be discarded as they had been collected more than 48 hours before examination.

The original idea of examining specimens of foetal and maternal blood and of the placenta from each case had to be abandoned because of the liaison difficulties and also because the Hospital had only one refrigerator which was also used for storing blood for transfusion and was therefore not always available for keeping biological specimens.

Method

Amniotic fluid samples were centrifuged to remove all cellular material and other debris.

The S.G.O.T. activity was then determined using the Boehringer test pack.

The principle of the test is that glutamic oxaloacetic transaminase catalyses the reversible reaction

glutamate + oxaloacetate = aspartate + α ketoglutarate.

In this reaction which proceeds from right to left, the S.G.O.T. activity is determined by measuring photometrically the rate of increase of the oxaloacetate formed per minute. This increase is assayed in a coupled indicator reaction catalysed by malic dehydrogenase.

$$\text{oxaloacetate} + \text{DPN-H} + H^{+} \rightarrow \text{malate} + \text{DPN}^{+}$$

The amount of DPN-H used in the reaction is proportional to the amount of oxalate formed at the same time.

Reagents

1. Phosphate/aspartate (0.1 M phosphate buffer pH 7.4) 0.2×10^{-2} M aspartate. 0.3 ml. of chloroform were added to prevent growth of micro-organisms.
2. α Keto glutarate. 0.2 M solution of the sodium salt.
3. Reduced DPN-H 1.2×10^{-2} M dissolved in the phosphate aspartate buffer.
4. Malic dehydrogenase 0.5 enzyme/ml.

Procedure

A blank containing amniotic fluid only was run with each series to eliminate any possible light absorption from amniotic fluid itself.

The blank contained 2.5 ml. reagent 1.
0.5 ml. amniotic fluid.

The reaction mixture contained
2.3 ml. reagent 1.
0.05 ml. DPN-H
0.05 ml. Malic dehydrogenase.
0.50 ml. Amniotic fluid.

This was gently mixed and placed in a constant temperature bath at 25°C. After 15 minutes 0.1 ml. α keto glutarate was added and the mixture poured immediately into a 1 cm. cell. 2 Minutes later, serial readings of absorbency at two minute intervals at a wavelength of 360 mμ against the blank were taken.

Calculation

According to Wrobleski, La Due and Karmen (1954) 1 unit is the quantity of S.G.O.T. present in 1 ml. of serum which changes the DPN-H absorbency at 23°C and 366μ within 1 minute by 0.001.

Thus if 0.5 ml. of fluid were used

$$A\,\frac{340}{1\text{ min}} \times 200 = A\,\frac{366}{1\text{ min}} \times 3780$$

where 1.89 is the factor corresponding to the ratios of DPNH at 340 and 360μ.

Results

Normal cases as controls	Amniotic fluid enzyme level/ml.
3	13.2 units
	14.0 "
	17.5 "
Pre-eclamptic toxaemia	
3	8.6 units
	30.4 "
	11.4 "
Post maturity	
6	4.7 units
	15.2 "
	3.8 "
	14.0 "
	9.8 "
	5.2 "
Hydramnios	
1	nil units
Meconium stained liquor	
2	8.6 units
	11.4 "

Conclusions

From such a small sample it is clearly impossible to draw many valid conclusions. However, one or two points can be made:

1. Serum glutamic oxaloacetate is present in normal amniotic fluid.
2. It is present in amniotic fluid in cases of pre-eclamptic toxaemia and post-maturity.
3. The absence of serum glutamic oxaloacetate in one case of hydramnios suggests further examination of such cases might be worthwhile.
4. No gross abnormality of enzyme level was seen in specimens stained with meconium.
5. The enzyme levels in cases of post-maturity appear to fall into a low and a high group (3-5 and 10-15 units).
6. The abnormally high value in one case of pre-eclamptic toxaemia suggests further examination of such cases might be worthwhile.

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FOETAL ERYTHROCYTES IN THE MATERNAL CIRCULATION

The presence of foetal red cells in the maternal circulation has only an indirect connection with amniotic fluid. However, in view of findings in relation to cases of hydramnios this piece of work is included.

The occurrence of foetal red cells in the maternal blood has been described on a number of occasions following the discovery by Kleihauer in 1957 that the haemoglobin of adult red cells in a fixed smear was readily eluted by an acid phosphate buffer while that of the foetal cells was not.

Hull (1959)[1] reported that 21% of post partum women had foetal cells in their circulation.

Frazer and Raper (1962)[2] pointed out that foetal cells could also be demonstrated in maternal blood during pregnancy. Their study concerned a series of patients at 34 weeks only where they found positive films in 19 out of 240 women. (7.8%).

Levi, Clarke, Gueritat, Walter and Raper (1961) found 19% of cases studied showed cells between 6 months and term but they could not demonstrate their presence earlier than the 5th month of pregnancy.

Brown (1963)[3] collected specimens from 165 patients who had experienced no complications in pregnancy or labour. She reported that in 83 cases foetal cells were seen in small numbers but she could not relate the figures to parity or duration of labour.

The phenomenon has largely been considered from the standpoint of 'A.B.O.' blood group incompatibility. When the groups of mother and baby were compatible in a series of 132 cases, 70 cases were found to have foetal red cells in their circulation while 62 did not. However, in ABO incompatibility foetal cells could still be demonstrated if they were looked for soon enough after delivery. Brown (1963) found them in 13 out of 33 cases which was a higher incidence than that reported by Frazer and Raper (1962) (16%) or by Cohen and Zuelzer (1962)[4] (15%).

The escape of foetal red cells has been suggested as the mechanism of rhesus sensitisation.

It has been shown that foetal cells can persist for more than a week after delivery by Dr. Goodall, Queens College, Dundee (private communication) and he has found cells even up to 30 days after delivery. It is therefore possible that a sudden large leak at delivery may result in a slowly developing sensitisation which may only become apparent at the next pregnancy.

It is interesting to note that, although it might be expected that the violent uterine activity associated with labour would result in a partial breakdown of the placental barrier, no such activity is apparently required to force foetal red cells across the barrier. They can pass through in the course of a normal pregnancy.

This raises questions about the nature of the leak across the barrier.

Is it uni-directional ? Is it affected by local conditions such as increase in amniotic fluid pressure ?

If a positive correlation between amniotic fluid pressure and the presence of a large number of foetal cells in the maternal circulation could be shown, it might be possible that the transient rise of pressure in the uterine contractions which normally take place during pregnancy might be sufficient to force across a few cells.

Brown (1963) has reported that large placentae appear to be correlated positively with escape of cells into the maternal circulation.

The present investigation was confined to finding whether foetal cells could be found earlier than 34 weeks and whether conditions resulting in high amniotic fluid pressure affected the transfer of cells.

Material and Methods

Simple blood films were prepared from a series of ante-natal and post-natal patients from Craigtoun Hospital and from Dr. R. McIntyre's ante-natal clinic.

The numbers available in this way were somewhat more limited than these described by previous workers.

The slides were allowed to dry and then treated in the following manner recommended by Dr. Goodall.

Principle. - Haemoglobin A, precipitated by drying and 80% ethanol, becomes readily soluble in citric acid-phosphate buffer pH 3.2 - 3.4. Precipitated foetal haemoglobin is very slowly soluble under these conditions.

Procedure. - The dried slide was fixed in 80% ethanol for 5 mins., rinsed with tap water and dried.

2. The slide was then incubated in citric acid-phosphate buffer pH 3.3 at 37°C for 3-5 mins.

Buffer Solution A = 0.2 M $Na_2 HPO_4$

Buffer Solution B = 0.1 M citric acid

(Mix 26.6 ml. A with 73.4 ml. B and check with pH meter). The slide was placed vertically in the buffer which had previously been warmed to the required temperature so that there were no bubbles to spoil the surface of the slide. The slide was lifted out after 1 minute and again after 3 minutes, inverted and replaced to achieve a slight degree of mixing.

3. The slide was then rinsed with tap water.

4. It was next stained for 3 minutes with acid Haemotoxylin Erlich and rinsed, then counter stained for 3 minutes with Erythrosine "Merck" (0.1%) in water.

It was found that it was most important to have the buffer at exactly the right temperature, otherwise the cells took up too much stain afterwards and it was difficult to produce true ghost cells to contrast with the red foetal cells.

The counterstain May Grunewald originally used was found to be difficult to wash out. This has also been found by Dr. Goodall who found Erlich's haemotoxylin and erythrosine to be much more satisfactory.

5. The slide was then examined under the microscope. The number of foetal cells visible in two longitudinal traverses of the slide was recorded. When the stained film is one cell thick, and the cells are evenly distributed the approximate number covered in 2 traverses is 120,000 cells.

Results

27 ante-natal patients were examined

7 cases were earlier than 30 weeks, the earliest being 19 weeks.

1. Foetal cells found in 19 cases = 70.3% (ante-natal cases).
2. Foetal cells present as early as 19 weeks.
3. Average number of cells per 2 traverses of slide = 2.

30 post-natal cases were examined

1. Foetal cells found in 23 cases = 76%
2. Average number of cells per 2 traverses of slide = 2.

3 cases of gross hydramnios at 33 weeks were examined

In the first case 15 cells per 2 traverses were seen suggesting a large leak.

In the second case no cells were seen.

In the third no cells were seen.

Conclusions

1. Foetal red cells can appear in the maternal circulation as early as 19 weeks.
2. Though the series is perhaps too small to draw definite conclusions, it seems that a much higher percentage of ante-natal patients show positive smears than in Frazer and Raper's series. (70% as against 7%).
3. Post-natal cases show 76% as against 50.9%(Brown).
4. No correlation has so far been demonstrated between rise of pressure and transplacental leak except in one case of hydramnios. As the other two cases showed no cells it suggests that pressure alone is not the causative factor but clearly, more investigations should be done.
5. It is possible that differences of staining technique may account for the difference in results.

Discussion

The techniques of staining fixed blood films to demonstrate the presence of foetal red cells are subject to difficulties and the "two traverse" scanning method clearly has sources of error in their estimation. The results suggest that foetal cells may pass into the maternal circulation commonly rather than rarely both before and after delivery. So far the movement of cells has only been shown from the foetus to the mother. It would be interesting to see whether cells pass in the opposite direction. This could perhaps

be done by much the same technique only looking for ghost cells in a stained film of foetal blood.

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THE LIPID FRACTION OF AMNIOTIC FLUID

According to Hanon (1961) the total amount of lipid present in normal amniotic fluid is 48 mg./100 ml.

Work in the field of investigation of the various lipids involved appears to have been rather fragmentary and largely related to certain clinical interests, e.g. oxytocic activity and its possible role in the mechanism of labour; steroids in the liquor in normal and diabetic pregnancy.

The position with regard to oxytocic lipids was briefly reviewed by Hawkins (1962) in the introduction to his paper. For a number of years extracts of amniotic fluid have been known to cause contraction of the isolated guinea pig uterus. The active principle called "eutocin" by Hanon has been shown to be soluble in lipid solvents, especially ether. It was distinguishable from histamine, acetyl choline, choline and pituitary pitocin. Eliminating cholesterol and phospholipids from the extract did not affect its activity.

Ferraris (1958) confirmed these findings and showed that the active substance would stimulate isolated strips of non-pregnant human uterus.

Hawkins (1962) reported the mean ether extractable lipid content of amniotic fluid in early pregnancy (10-20 weeks) as 0.13 mg./ml. and of late pregnancy as 0.64 mg./ml. The oxytocic activity of the early extract was 14 units/mgm., that of the later extract 3.6 units/mg.

Hawkins suggests that these lipids resemble those found by Pickles (1958) in work upon endometrium and the menstrual flow.

The original ether extracts could be further broken down by partition between methanol and heptane. The methanol fraction was several times as active as the heptane fraction. Partition chromatography on silica gel demonstrated the presence of four active substances. One of these appeared to be a free acid.

It is therefore clear that some of the lipids present in amniotic fluid have oxytocic action though whether they have any function in the course of pregnancy and labour is still not known.

Cortisol and cortisone were estimated in specimens from normal and diabetic patients by Baird and Bush (1960) using a chromatographic method. They found concentrations of 2.6 μg./100 ml. of cortisol and 1.3 μg./100 ml. of cortisone in normal fluid and 2.2 μg./100 ml. and 1.4 μg./100 ml. respectively in the fluid from diabetic patients.

Hoet and Osinski (1954) published a paper on the chromatography of the steroids of normal and diabetic patients. They stated that no steroids were found in normal fluids but that 25-50 μg. 17-hydroxy-cortico-steroids and cortisone were present in specimens from diabetic patients.

Orlandi, Bottigliani and Torsello (1958) investigated the presence of 17-ketosteroids and the total corticoids of amniotic fluid. They found 17-ketosteroids in all of 25 samples and measured total corticoids in 5 samples and suggested that

17-ketosteroids are normal components of the liquor and are not influenced by the stage of pregnancy or the sex of the foetus.

Klopper (1959) reported the isolation of pregnanediol from amniotic fluid.

Helmy and Hack (1962) carried out a survey and comparison of the lipids in maternal and cord blood and amniotic fluid. Their three amniotic fluid samples were found to be qualitatively similar on chromatographic analysis. Ethanolamine and choline plasmalogens were present as were sphingomyelin and neutral lipid. Some additional substances, probably glycolipid and inositide were seen. There was a large amount of lecithin and possibly some monoglyceride.

As can be seen from the above results it appears that no quantitative study of the lipid components has so far been carried out.

It was therefore decided to try to isolate the lipids from a specimen of amniotic fluid and separate the fraction into its various components in quantitative fashion. It was hoped to be able to compare the results found in normal samples with those from patients with hydramnios.

Material and Method

The amniotic fluid was obtained from patients in Craigtoun Hospital undergoing high rupture of the membranes for induction of labour. The specimens were refrigerated immediately and used within 48 hours.

Extraction of Lipid

The lipids were extracted by adding 300 ml. of amniotic fluid in 20 ml. portions drop by drop to 15 times its volume of boiling ethanol/ether (3:1 V/V).

The precipitated protein was removed by filtration through fat-free filter paper and the lipid recovered from the solvent by evaporation to dryness in a rotary evaporatore at 37°C. The lipids were then dissolved in a small volume of light petroleum (b.p. 60-70°C) and stored in the refrigerator.

Separation of the Lipids

Separation of the lipids was carried out on a silicic acid column according to the method of Hirsch and Ahrens (1958).

Apparatus. - A glass column was used with a quickfit joint at the top and a sintered glass filter at the base. A water condenser surrounded the entire length of the column, as temperature control is essential for the absorption of lipids on silicic acid is diminished with rise in temperature, thus causing more rapid elution. Water jacketing also prevents vapourisation of the solvent in the column which would otherwise occur and disturb the packing of the column and hence the separation. Several reservoirs of total capacity 1000 ml. were placed above the column with a 500 ml. separating funnel at the top.

COLUMN PREPARATION

The sintered disc was covered by a circular disc of fat free filter paper. 10 gms. of silicic acid were weighed and dusted into the column which was gradually filled, the sides being tapped from time to time to help the silicic acid to settle as even packing is of great importance. Air pressure was then applied to the top of the column at 2-3 lbs./square inch, using a pump with a pressure stabilising cylinder, until the column showed no further sign of shrinkage.

The column was then washed with 10 mls. ether, 30 mls. acetone/ether (1:1 V/V) and finally with 20 mls. of ether.

Suction applied to the base of the column speeded up the process but care was taken to release the suction gradually for air intake at the base of the column would disrupt the packing.

Then light petroleum (b.p. 60-70^{o}C) was run through the column for 10-12 hours so that the column equilibrated with the first eluting solvent to be used.

CHARGING THE COLUMN

The solution of lipid in light petroleum (b.p. 60-70^{o}C) was taken to dryness and a known weight redissolved in a minimum volume of the same solvent. Meanwhile, the solvent with which the column was being finally washed had run through so that its upper level had almost reached the top of the silicic acid. The system was therefore drained using a pipette, and pressure applied until the level was exactly that of the top of the silica. The experimental material was applied by pipette to the top of the column.

Elution of the column. - The reservoirs were replaced above the column and filled with light petroleum (b.p. 60-70°C) to which 1% anhydrous ether had been added. Elution was carried out with solutions of increasing polarity to produce orderly migration of the different lipid classes down the column.

The fractions were collected in an automatic fraction collector in 10 ml. samples. The contents of two tubes were pooled so that the fractions were 20 ml.

To increase the speed of collection pressure of 5 lbs./square inch was applied to the head of the column as before.

Treatment of fractions.- Each 20 ml. fraction was evaporated to dryness, redissolved in ethanol and taken to dryness again in weighed flasks. The weight of each fraction was thus found and an elution diagram by weight constructed. (Figure 1). Analysis of fractions was then carried out.

A specimen of normal amniotic fluid was used (300 ml.). 20 ml. portions of fluid were added to 400 ml. boiling ethanol-ether. The weight of lipid applied to the column was 0.213 mg. and the eluting solvents used were, in order, as follows:-

1% ether - this elutes hydrocarbons, waxes and cholesterol esters.

4% ether - this elutes triglycerides.

8% ether - this elutes free fatty acids, and cholesterol.

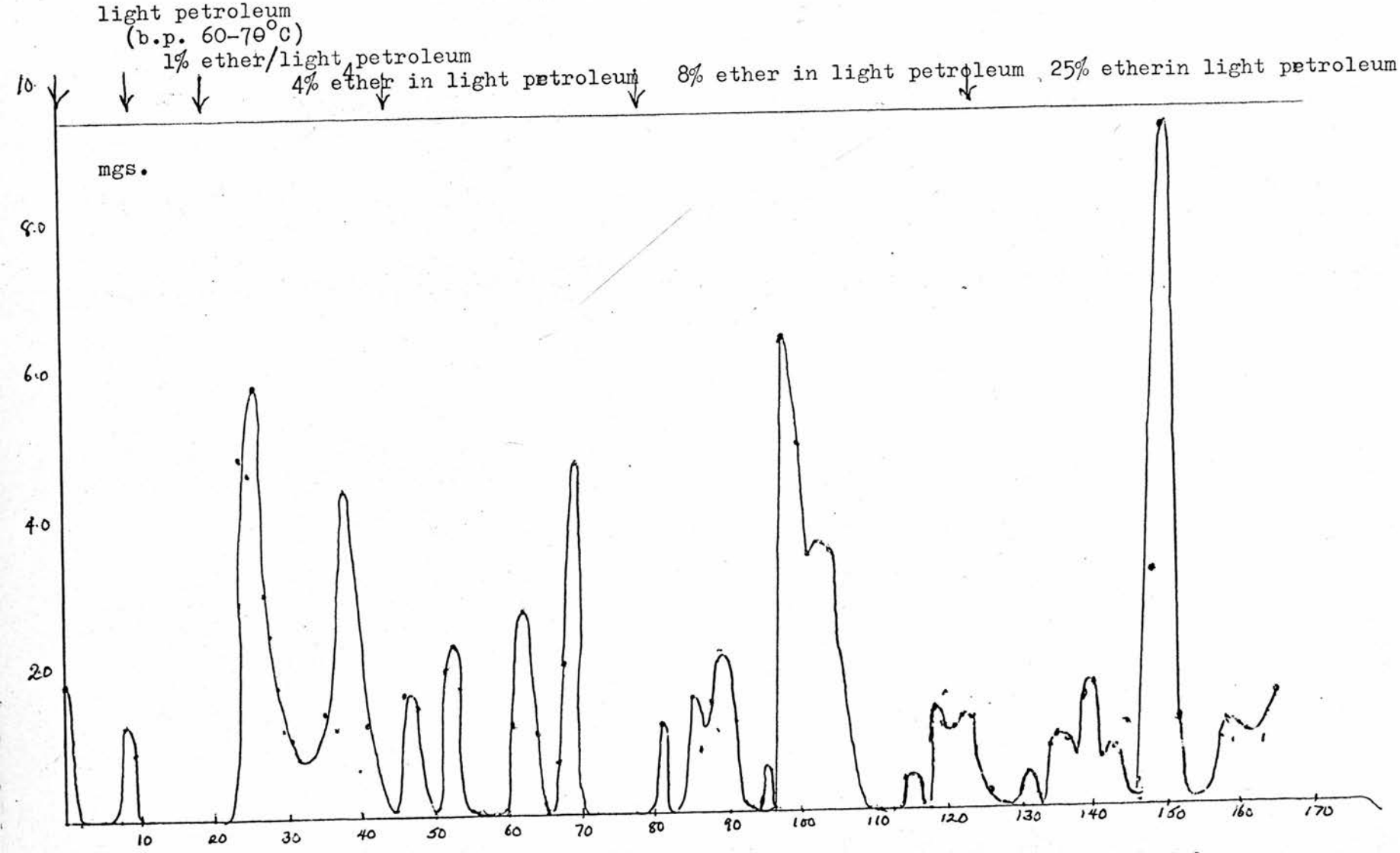
25% ether - this elutes diglycerides.

100% ether - this elutes monoglycerides.

methanol - this elutes phospholipids.

FIGURE I

ELUTION DIAGRAM FOR LIPIDS



The elution diagram is shown in Fig. 1.

It was hoped to identify the components of each fraction by specific tests, such as the Lieberman-Buchardt reaction for sterols and by thin layer chromatography.

Results

Lieberman-Burchardt reaction

Fraction	1	Negative.
"	2	Negative.
"	3	Slightly positive as for cholesterol.
"	4	Positive as for cholesterol.
"	5	Negative.
"	6	Negative.
"	7	Deep red. Positive as for ergosterols.
"	8	" " " "
"	9	Negative.
"	10	Slightly positive as for cholesterol.
"	11	" " " "
"	12	Negative.
"	13	Negative.
"	14	Negative.
"	15	Positive as for ergosterols.
"	16	Blackish colour. Negative (?)
"	17	" "

These results suggest the presence of cholesterol and other sterols but exact identification was not possible by this method.

Thin Layer Chromatography

Chromatography of the fractions was attempted on glass plates coated with a thin layer of silica Gel G (Merck).

After each fraction was spotted on to the plate about 1½ inches from the edge, it was placed in a small tank containing solvent. The solvent used was cyclohexane/ethyl acetate (7:3 V/V or 9:1 V/V) and when the solvent had run more than three quarters of the way up the plate, the solvent front was marked, the plate removed and allowed to dry. It was then inspected under Ultra-violet light before spraying with stain.

The main difficulty encountered was in the technique of staining. It was almost impossible to create a sufficiently fine spray not to cause blotching of the surface of the plate.

One of the commonly used stains for steroids is concentrated sulphuric acid but this was most difficult of all to spray and its use was abandoned. Matthews (1963) described the use of a mixture of vanillin, sulphuric acid and ethanol. The proportions suggested were 0.5% vanillin in H_2SO_4 - ethanol (4:1 V/V). The reagent has to be freshly prepared daily. The development of maximum colour was obtained by heating to 100°C for 5 minutes.

Fractions 3,4 and 5 were investigated by the method (see diagram No. I) and the result indicated that these fractions were of similar composition. Fraction IV was run and stained with vanillin. The colours seen (see diagram No. II) were consistent with those stated by Matthews (1963) to suggest the presence of

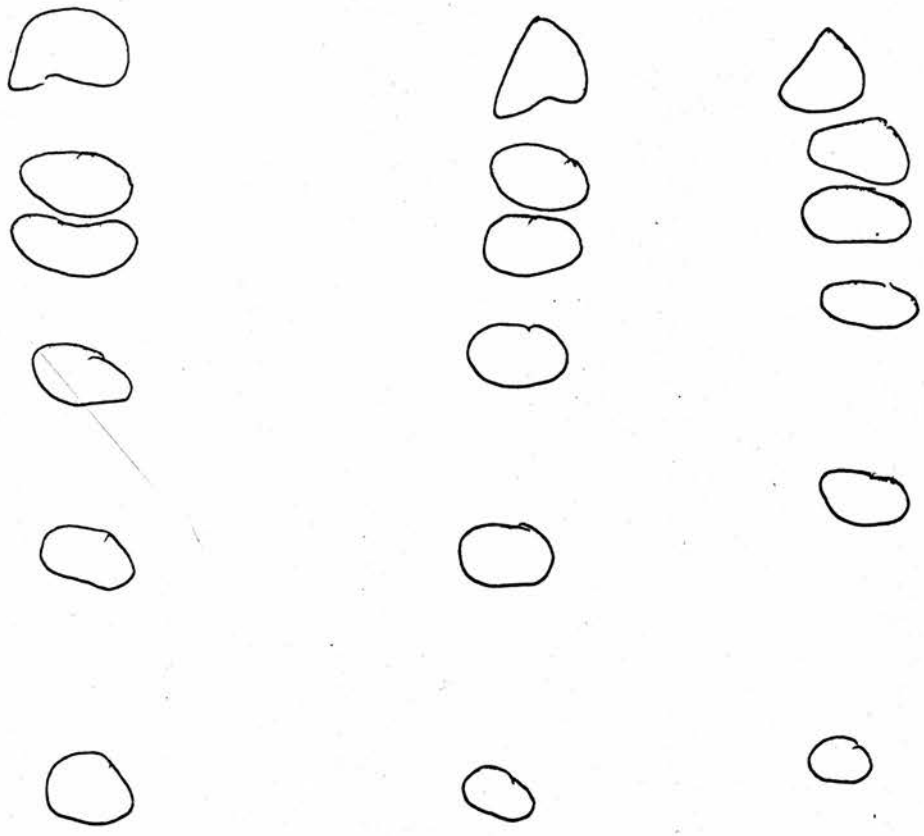
FRACTIONS III, IV, and V.

(under Ultra-violet light)

Tracing from glass plate used in thin layer chromatography.

Solvent: cyclo-hexane/ ethyl acetate 7:3 v/v.

solvent front.



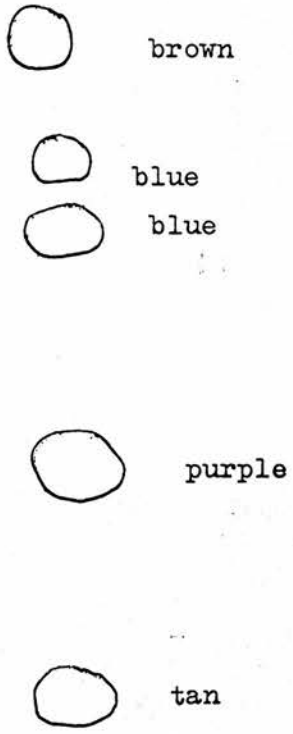
III IV V

FRACTION IV.

Stain : Vanillin.

Solvent : cyclo- hexane/ ethyl acetate 7:3 v/v.

solvent front.



pregnenolone, allopregnane, and ethynyl oestradiol-3-methyl ether.

The association of R_f values with the colours produced using vanillin would therefore be the most satisfactory way of investigating the components of each fraction.

In view of the similarity of Fractions III, IV and V, infra-red spectra of a range of the fractions were performed.

Infra-red spectra

It was thought that there might be some information to be gained by recording the infra-red spectra of a number of the fractions to determine whether some of the fractions which appeared separate as they came from the column might, in fact, contain the same substances, that is that the phenomenon of trailing might have occurred. Infra-red spectra of fractions 1,2,3,4,5,6,7,8,10,13,14,15,16,18 and the last fraction were recorded from 5 to 15μ.

In addition a specimen of vernix caseosa was obtained for comparison without treatment on the column. Bands due to traces of solvent were also identified by comparison.

The infra-red spectrum of cholesterol was also recorded under similar conditions to those of the fractions i.e. thin film between rock salt plates dissolved in Nujol where necessary.

All spectra showed a remarkable similarity indicating that very little separation had occurred on the column.

Features of the spectrum of cholesterol, particularly peaks at 11.95, 12.55 and 13.7μ were observed in most of the fraction spectra indicating the presence of this substance. In spite of the

apparent separation shown by clear peaks in the elution diagram, chromatography and infra-red spectra show no great difference between the fractions.

This is in agreement with Hawkins (private communication 1964) who experienced similar difficulties attempting separation of lipid materials from amniotic fluid on silicic acid columns.

However this technique has considerable possibilities for elucidation of the lipid fractions in amniotic fluid and probably some experiments with different eluting solvents or varying strengths might make separation much more successful.

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