ABSTRACT OF THESIS

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Title of Thesis Studies on the Protein in Amniotic Fluid

1. A review of the literature has shown that the major soluble antigens in amniotic fluid at term are of serum type, and that of these antigens albumin, transferrin and group-specific component are mainly of maternal origin. It was not clear whether this conclusion applied to the protein in amniotic fluid earlier in gestation, nor had extensive studies been made into the nature of the non-serum protein in amniotic fluid. The object of the work presented in this thesis was to investigate the nature and origin of the soluble protein in amniotic fluid throughout pregnancy.

2. An immunological investigation showed that when rabbits were immunised with amniotic fluid, the majority of their immune response was directed against serum protein. The major antigens in the fluid were found to include albumin, α_1-antitrypsin, group-specific component, and transferrin.

3. A method was developed for phenotyping group-specific component (Gc) in amniotic fluid and in serum by antibody-antigen crossed electrophoresis. Maternal serum and amniotic fluid was collected from each of 56 pregnancies at between ten and thirty-eight weeks of gestation, and in each case the Gc phenotype of the fluid was the same as the maternal phenotype. In 11 of these cases, a sample of fetal serum was also obtained, and in four cases in which the fetus was discordant from the mother, the Gc phenotype of the fluid was the same as the maternal phenotype. From this it was concluded that the majority of the Gc in amniotic fluid was of maternal origin, from ten weeks of pregnancy onwards.

4. The concentrations of albumin, α_1-antitrypsin, Gc, and transferrin were measured in amniotic fluid and in maternal and fetal serum. The concentration patterns of the four proteins in amniotic fluid were very similar and maximal concentrations were found between twenty and thirty weeks, showing a general similarity to the total protein concentration. When the concentrations of the individual proteins in amniotic fluid were expressed as ratios of the concentrations in maternal or fetal serum, a general similarity was found between the ratios of albumin, α_1-antitrypsin, Gc, and transferrin. These concentration ratios were much less than that found for α-fetoprotein. From this it was concluded that the albumin, α_1-antitrypsin, and transferrin in amniotic fluid were mainly of maternal origin from about ten weeks of gestation onwards.

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STUDIES ON THE PROTEIN IN AMNIOTIC FLUID

Roger G. Sutcliffe

A thesis presented for the degree of Doctor of Philosophy of the University of Edinburgh in the Faculty of Medicine

September, 1972

CONTENTS

SUMMARY

1. A review of the literature has shown that the major soluble antigens in amniotic fluid at term are of serum type, and that of these antigens albumin, transferrin, and group-specific component are mainly of maternal origin. It was not clear whether this conclusion applied to the protein in amniotic fluid ealier in gestation, nor had extensive studies been made into the nature of the non-serum protein in amniotic fluid. The object of the work presented in this thesis was to investigate the nature and origin of the soluble protein in amniotic fluid throughout pregnancy.

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5. Rabbit antisera against amniotic fluid were absorbed with adult male human serum to search for antigens of non-serum origin in amniotic fluid. After a considerable course of immunisation, antibodies against two proteins were found. An α_2-protein was found in amniotic fluid and decidua throughout pregnancy, and in amnion and chorion early in pregnancy. The protein was absent from maternal and fetal serum. Because the protein was consistently found in decidua both by antibody-antigen crossed electrophoresis and immunofluorescence, decidua was thought to be an origin of this protein. A β-mobility protein was also found. This was present in maternal and fetal serum at concentrations similar to those in amniotic fluid. Comparisons of the concentration gradient of this protein with that of Gc and α-fetoprotein suggested that the β-protein in amniotic fluid was mainly of non-serum origin, at least up to thirty weeks of pregnancy.

6. During the course of concentration gradient studies on amniotic fluid α-fetoprotein, it became apparent that this fetal serum protein might be of value for the prenatal diagnosis of anencephaly. This possibility was examined in 22 pregnancies leading to anencephaly or anencephaly combined with spina bifida, where it was possible to get amniotic fluid in the third trimester. In these cases the concentrations of α-fetoprotein in the amniotic fluid were found to be grossly elevated above control levels.

In five cases of spina bifida after thirty-two weeks, the concentration of α-fetoprotein in the amniotic fluids were indistinguishable from the controls. However, a single fluid was fortuitously obtained at 13 weeks of gestation from a hysterotomy where the fetus had a myelocele spina bifida. In this case the concentration of α-fetoprotein in the fluid was over 4 times as great as the highest normal control.

These results suggested that it will be possible to diagnose anencephaly prenatally early enough to carry out termination of pregnancy. Such a conclusion may not hold in the case of spina bifida, where there is considerable variation in the extent of the lesion.

7. Investigations were made into the specific activities of α-1,4-glucosidase, hexosaminidase, acid phosphatase, and the heat-stable and heat-labile fractions of alkaline phosphatase.in amniotic fluid. Various specific activity patterns were found during gestation, none of which were similar to the protein concentration in amniotic fluid. Particularly notable were the specific activity maxima found in the cases of heat-labile alkaline phosphatase and α-1,4-glucosidase between thirteen and eighteen weeks of gestation, and for the alkaline and acid phosphatases at term. The gradients of the enzyme specific activities

between amniotic fluid and maternal or fetal serum were compared with proteins known to be of maternal or fetal serum origin (using Gc and α-fetoprotein, respectively). The analysis showed that the enzymes studied could not be regarded as being simply of serum origin throughout pregnancy.

ACKNOWLEDGEMENTS

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INTRODUCTION

1. The Medical Uses and Potentials of Amniotic Fluid Analysis

Although amniocentesis was first suggested and performed by Lamblin 1881 and by Henkel in 1919, the technique did not acquire practical value until the 1950's. Bevis (1950, 1953, 1956) was the first to claim that the severity of haemolytic disease of the newborn (rhesus isoimmunisation) could be predicted from the concentration of iron or of bilirubin in the amniotic fluid. This claim was confirmed by Walker (1957), Mackay (1961), and Liley (1961, 1963) and amniotic fluid analysis became increasingly used for estimating the severity of isoimmunisation during pregnancy. At the present time, transabdominal amniocentesis is carried out on cases at risk for rhesus isoimmunisation between about twenty weeks of gestation and term (Walker, 1970). The bilirubin concentration in the fluid is usually measured by spectrophotometry (Liley, 1961), and the concentration used to monitor the disease.

The successful diagnosis of rhesus disease, together with the relatively low risks of transabdominal amniocentesis (Scrimgeour, 1972) stimulated attempts to diagnose other genetic and non-genetic conditions prenatally. The first prenatal genetic analyses in man were made by Serr (1955) Fuchs and Riis (1956) and James (1956), who showed that the sex of a fetus could be predicted from the sex-chromatin of uncultured amniotic fluid cells. Riis and Fuchs (1960) also suggested that chromosomal karyotypes could be made from cultured amniotic fluid cells, although it was not until 1965 that the first successful report was made by Klinger (1965). More extensive work was made by Steele and Breg (1966) and Jacobson and Barter (1967), the latter workers identifying a D/D translocation carrier *in utero*.

These studies enabled Nadler (1968), Valenti and Kehaty (1969) and Valenti, Scutta and Kehaty (1968) to carry out prenatal chromosome studies in cases which were at high risk for Down's syndrome. Nadler (1968) reported the first prenatal diagnosis of Down's syndrome which was followed by therapeutic abortion and subsequently confirmed by cytogenetic analysis of fetal tissue. Since these initial reports, cytogenetic studies on cultured amniotic fluid cells have become routine in several centres, providing screening facilities for cases at high risk for chromosome aneuploidy, and facilities for sex determination in cases where the mother is a possible or proven carrier of an X-linked condition.

The adrenogenital syndrome was the first inborn error of metabolism to be diagnosed in utero. Jeffcoate et al (1965) showed that this disease could be diagnosed shortly before birth by measuring the concentrations of pregnane-diol and 17-ketosteroids in amniotic fluid supernatant. The ability to diagnose this disease at the earliest possible time is important as affected infants respond best to treatment during the first few days of life. The work of Jeffcoate et al (1965) was confirmed by Fuchs (1967) and Nicholls (1969), although Merkatz et al (1969) and New (1970) found that the technique could not be used successfully in early or mid-pregnancy. Further developments, such as a more rigorous classification of the adrenogenital syndromes* may increase the precision of prenatal diagnosis, while the injection of hydrocortisone into the fetus (Nicholls 1970) may also provide a means of treatment in utero.

*Adrenal hyperplasia type I is accompanied by the most severe changes in steroid metabolism, and therefore could be regarded as the most likely type to be diagnosed by amniocentesis; yet it was a case of type I disease that Merkatz et al (1969) attempted unsuccessfully to diagnose in this way. It should be born in mind, however, that mild and severe forms of adrenal hyperplasia type I have been described (Galal et al 1969).

New (1970) attempted the prenatal diagnosis of a case of adrenal hyperplasia type V (McKusick 1971).

Subsequently, it has been shown that cell-free amniotic fluid could be used to diagnose Tay Sachs disease prenatally (O'Brien et al 1971; Friedland et al 1971), and perhaps also methylmalonic acidaemia (Morrow et al 1970). Nadler and Messina (1969) also claimed that Pompe's disease could be diagnosed by supernatant analysis, but in a further paper this was refuted (Nadler et al 1970). Matalon and et al (1970) made similar claims in the case of Hurler's syndrome, however, further work by Brock et al (1971) and then by Matalon, Dorfman and Nadler (1972) denied this. It is fairly clear that the majority of inborn errors of metabolism are best diagnosed prenatally by studies on cultured amniotic fluid cells (Brock, 1972). The limitation of this approach lies in whether the disease is expressed in cultured cells.

Apart from the diagnosis of rhesus isoimmunisation, there are a number of diagnostic problems in obstetrics which have benefited or might benefit from amniotic fluid studies. Since most of these problems are more physiological than genetic, experiments on the supernatant of amniotic fluid (and possibly also on uncultured cells) becomes the approach of choice. Many attempts have been made to devise amniotic fluid tests which would provide reliable estimates of fetal maturity, and thus define the optimal time for delivery. In this field, the established approach has been to measure the concentrations of lipid staining cells in the amniotic fluid, together with the concentrations of electrolytes, urea and creatinine (Brosens and Gordon, 1966; Chan et al 1969; Lind et al 1969; Wyatt et al 1969; Lind and Billewicz 1971; Gauthier et al 1972;

However, it must be concluded that, at present, the precision

of these methods available fall considerably short of that required, if they are to be of clinical value. The narrow selection of amniotic fluid constituents which have been studied so far probably accounts for the lack of progress in this field.

One of the reasons for needing a reliable estimate of fetal maturity, is to avoid the delivery of babies who are liable to respiratory distress syndrome. This syndrome is partly due to an absence of the low surface-tension 'surfactant' lipids from the pulmonary alveolar surfaces (Adams et al 1965; Chu et al 1965). This causes a disproportionate increase in the surface-tension of the smallest alveoli, which forces the air out and causes alveolar collapse (West, 1970). Thus, a number of workers have studied the supernatant lipids of amniotic fluid, in order to see if surfactant-type lipids (lecithins) could be found. The work of Biezenski et al (1968) and Nelson (1969) showed that such lipids were present, and that they could be of pulmonary origin. Recent work by Gluck et al (1971) and Bhagwanani et al (1972) suggests that the measurement of amniotic fluid lecithin : sphingomyelin ratios are of value in predicting the immediate liability of a fetus to respiratory distress syndrome when delivered.

The pregnancy of the diabetic mother is particularly difficult to manage during the last two months, owing to the high risk of sudden, quite unheralded fetal death after about thirty-eight weeks of gestation (Farquahar, 1965; Baird, 1969). To counter this problem, the babies of diabetic mothers are delivered between thirty-six and thirty-eight weeks. This measure results in an increased incidence of premature babies, some of which are severely affected with respiratory distress syndrome. If the concentrations of lecithin and sphingomyelin in

amniotic fluid prove to be of value for diagnosing the liability of these babies to respiratory distress syndrome, then an important advance will have been made. However, there is a clear need to be able to monitor the progress of the pregnancy of the diabetic mother in order to gain warning of impending fetal death. No advance has been made along these lines, partly because the cause of death is not understood (see Baird, 1969), but also because the studies to date have concentrated on the measurement of insulin (for example) in maternal blood. Wide-ranging empirical studies on amniotic fluid would clearly be of value here but the limited knowledge of amniotic fluid solutes has so far prevented this.

A further potential of amniocentesis lies in the prenatal diagnosis of congenital malformations. A number of attempts have been made to find tests which could diagnose anencephaly and spina bifida, though little success has been made to date. A further discussion of this problem is found on pp.72-73.

Further advances in the use of amniocentesis for the diagnosis and management of abnormal pregnancies, depend to a considerable extent on gaining a further insight into the biology of amniotic fluid macromolecules, and especially of protein. Protein has the advantages of a relatively low rate of diffusional turnover, and considerable tissue specificity. Knowledge of the quantitative and qualitative protein changes which occur in the amniotic fluid during pregnancy would provide:

1. An increased range of amniotic fluid measurements, which could be tested for clinical potential.
2. Data which may throw light on the manner in which protein enters

the amniotic fluid, and in particular the extent to which the protein in amniotic fluid is of maternal or fetal origin.

* * *

The following sections review the embryology of the human amniotic cavity, and the maintainance and turnover of the amniotic fluid. Then, the quantitative and qualitative nature of human amniotic fluid supernatant protein is discussed, with special reference to work which sheds light upon its origin. This review allows a number of distinct problems to be posed, and investigations into these are reported and discussed in the following sections.

2. The Embryology of the Amniotic Cavity

From very early in gestation until birth, the human fetus is surrounded by amniotic fluid. This fluid lies within the amniotic cavity which appears by the end of the first week of gestation as a space within the inner cell mass of the blastocyst (Hertig and Rock, 1945). By the second week (Fig.1a), the amniotic cavity separates the epidermal surface of the embryonic disc from the extraembryonic mesoderm. The extraembryonic mesoderm develops within the blastocyst cavity to fill the spaces found between the trophoblast and the amnion, the edge of the bilaminar disc, and the primary yolk sac (Hertig and Rock, 1941; Hamilton and Boyd, 1960). Soon after this, most of the mesoderm soon degenerates and only remains as a covering of cells over these tissues (Fig. 1b). The fluid space which results from this degeneration is known as the extraembryonic coelom (Hamilton,

Boyd and Mossman, 1964), and by about fifteen days, the embryo is suspended in the coelom, being connected to the trophoblast simply by a connecting (umbilical) stalk. At about this stage the embryo undergoes neurulation (Fig.1c), and is covered on its epidermal surface by the amniotic cavity and on its endodermal surface by the primary and then the secondary yolk sacs. Soon, (Fig.1d) the amnion grows over the developing head, tail and sides of the embryo and closes in upon the connecting stalk and the secondary yolk sac. This constricts the yolk sac until part of it is included in the embryo (to develop into the gut and allantois), and the remainder lies outside the embryo as the yolk sac (Hamilton, Boyd and Mossman, 1964).

Fig.2a is a diagram to show the arrangement of the conceptus at about ten weeks of gestation. Fig.2b shows the membranes, placenta, and umbilical cord of a ten week fetus. The amniotic cavity has been filled with water, and the extraembryonic coelom is clearly visible between the amniotic and chorionic membranes. The yolk sac can also be seen in the coelom. Further expansion of the amniotic cavity between ten and fourteen weeks of gestation leads to the obliteration of the extraembryonic coelom, so that in the later stages of pregnancy it is represented only by the space between the amnion and chorion, and contains the remnants of the yolk sac.

Up to about ten weeks of gestation, the conceptus is implanted in the wall of the uterus and is surrounded by parietal uterine decidua. During this time the trophoblast (chorion) is covered with villi which become vascularised as they grow into the parietal decidua. However, during the third and fourth months of pregnancy

Figure 1 Diagrams to illustrate the development of the amniotic cavity. Details are found on pp.6-7.

a. The embryo during the second week of gestation

b. The development of the extra embryonic coelom

c. Growth of the amnion over the head fold of the embryo

d. Growth of the amnion over the ventral surface of the embryo, and the exclusion of the yolk sac into the extra-embryonic coelom.

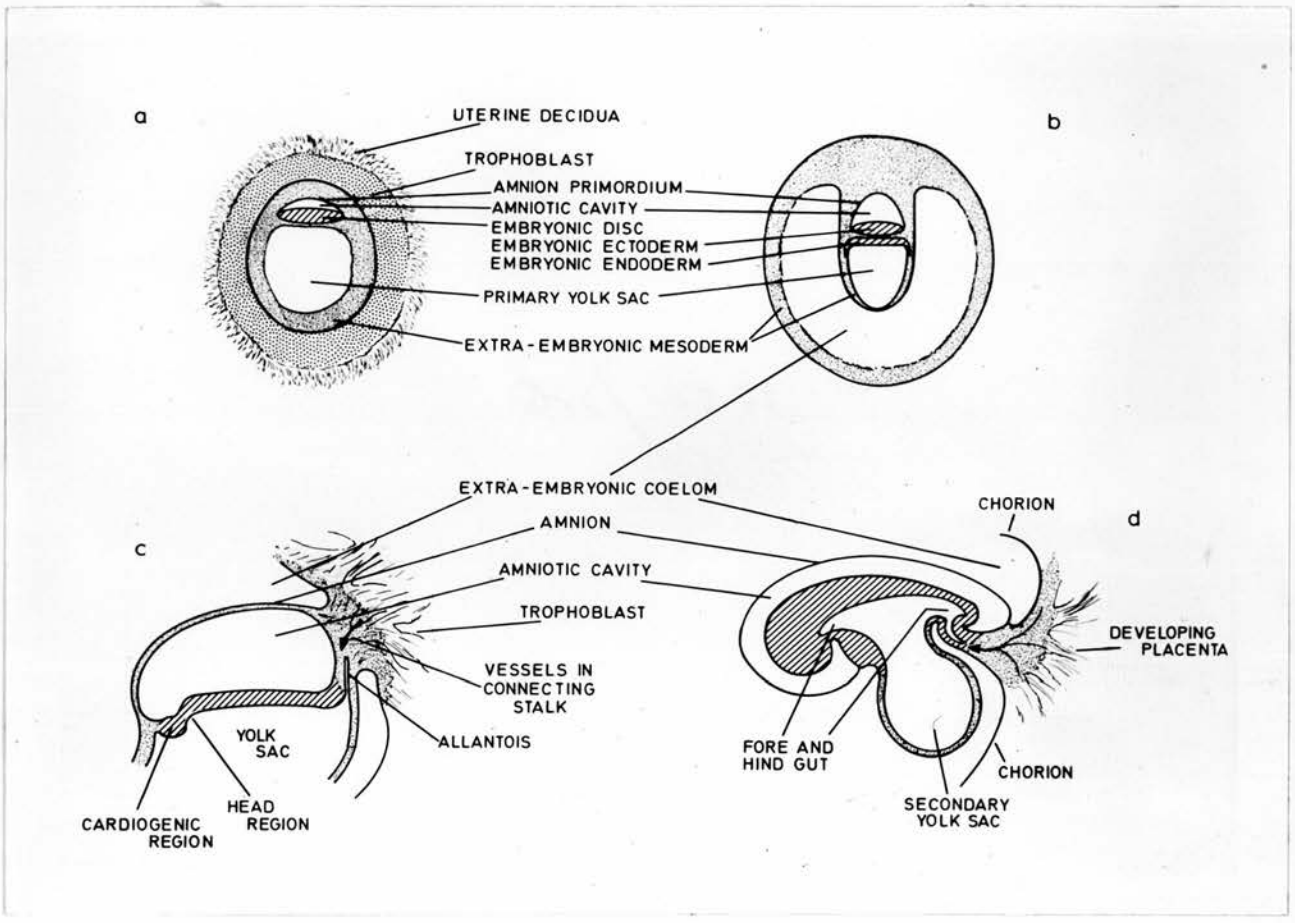
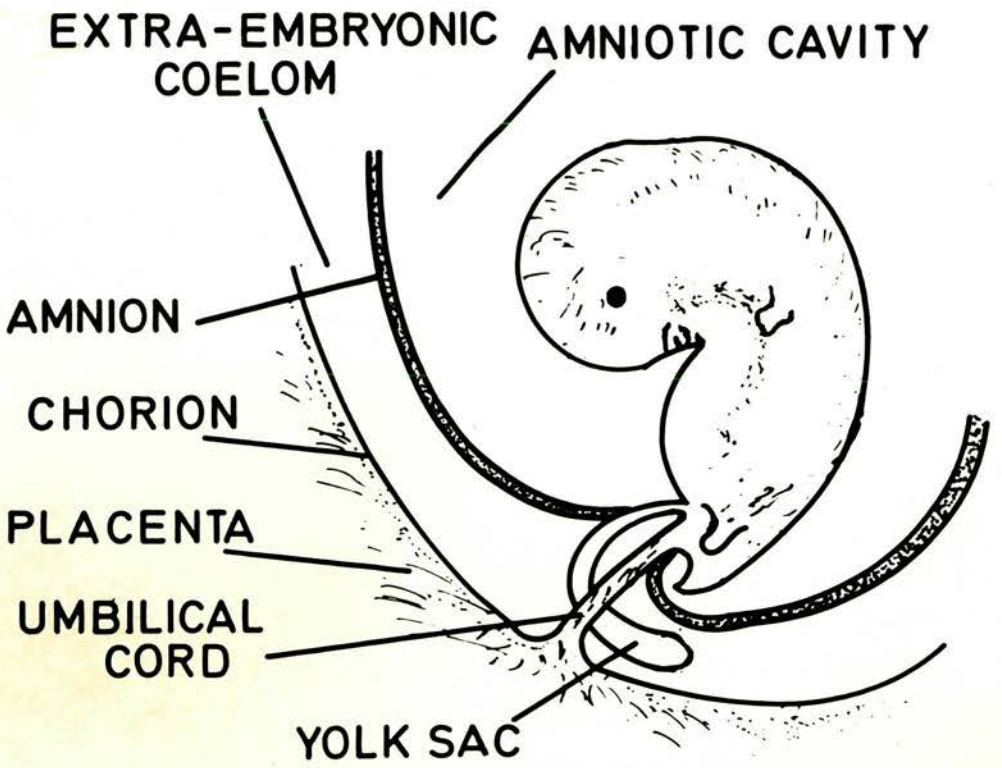

a
UTERINE DECIDUA
TROPHOBLAST
AMNION PRIMORDIUM
AMNIOTIC CAVITY
EMBRYONIC DISC
EMBRYONIC ECTODERM
EMBRYONIC ENDODERM
PRIMARY YOLK SAC
EXTRA-EMBRYONIC MESODERM
b
EXTRA-EMBRYONIC COELOM
AMNION
AMNIOTIC CAVITY
TROPHOBLAST
VESSELS IN CONNECTING STALK
ALLANTOIS
YOLK SAC
HEAD REGION
CARDIOGENIC REGION
c
CHORION
d
DEVELOPING PLACENTA
FORE AND HIND GUT
CHORION
SECONDARY YOLK SAC

Figure 2 The conceptus at ten weeks of gestation. For details

see p.7.

a. Diagram of a ten week fetus, drawn to show the arrangement of the amniotic cavity and the extra-embryonic coelom.

b. Dissection of the membranes of a ten week fetus. The fetus is out of sight at the top of the umbilical cord. The open amniotic sac has been filled with water, and the extraembryonic coelom is seen as the space between the pale amniotic membrane, and the glistening vascular chorion lying behind and to the left. Note the location of the yolk sac in the extraembryonic coelom.


EXTRA-EMBRYONIC COELOM
AMNIOTIC CAVITY
AMNION
CHORION
PLACENTA
UMBILICAL CORD
YOLK SAC

a

2

b

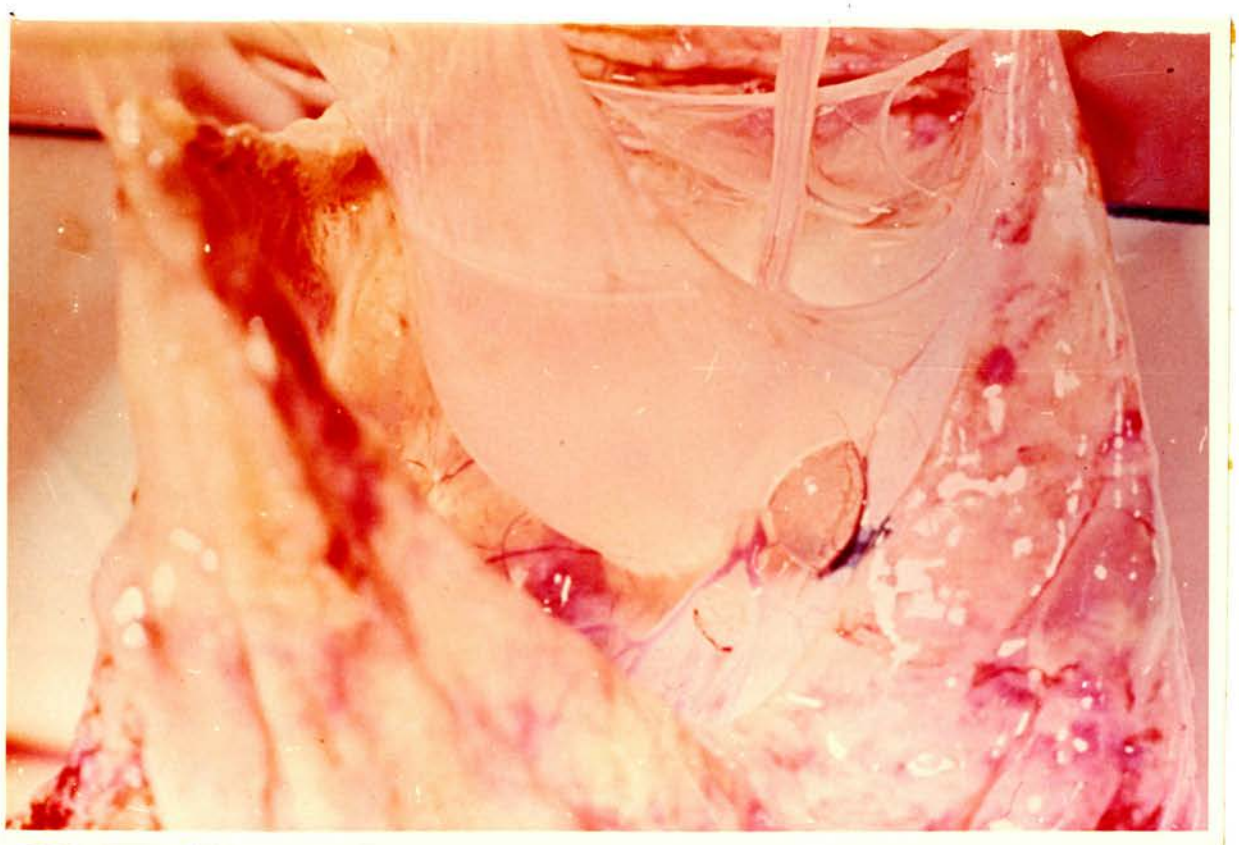
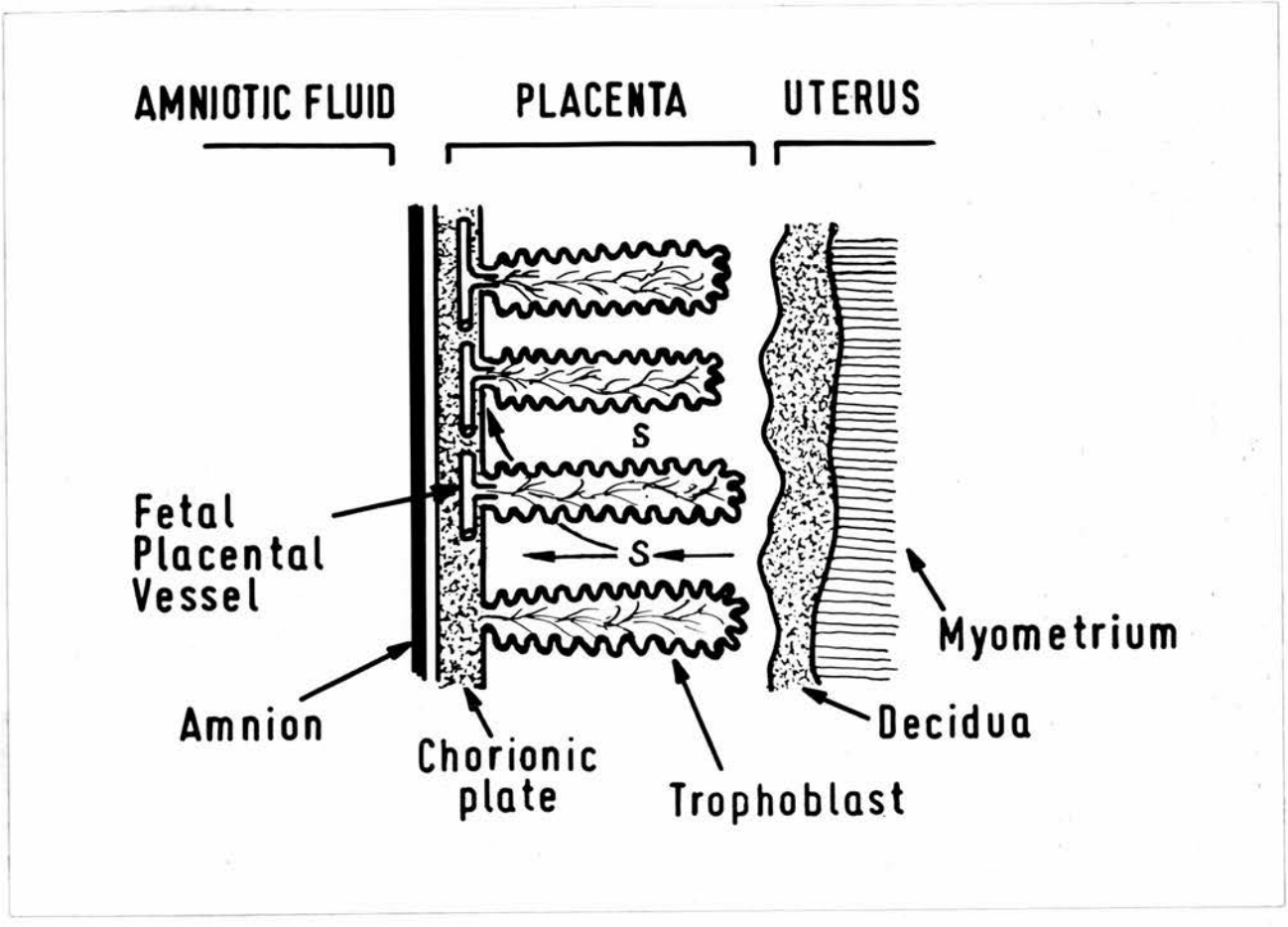


Figure 3 The relations between the amniotic fluid, the maternal serum and the placenta. Maternal blood can penetrate the spaces (S) between the placental villi and flows against the chorionic plate. The arrows show the direction of flow. See discussion on pp.104-105.


AMNIOTIC FLUID
PLACENTA
UTERUS
S
S
Fetal
Placental
Vessel
Amnion
Chorionic
plate
Trophoblast
Decidua
Myometrium

the conceptus grows to occupy the entire uterine cavity. At this stage the trophoblastic villi which are adjacent to the implantation site develop into placental tissue, whilst the others atrophy. Thus by the end of the fourth week of pregnancy the amniotic cavity is bounded by the placental and the reflected membranes, with the placenta and uterus, respectively, as deeper relations. Fig.3 is a diagram showing the relations between the placental membranes, the placental villi, the maternal and fetal blood spaces and the decidua. The effect of these relations on the nature of amniotic fluid solutes is discussed on pp.104-105.

3. Amniotic Fluid Volume and Turnover

a. Volume

The volume of amniotic fluid is known to increase from less than 30 ml at eight weeks to about 100 ml at fourteen weeks and 300 ml at eighteen weeks of gestation (Wagner and Fuchs, 1962; Rhodes, 1966; Abramovitch, 1968; Gillibrand, 1969; Lind, 1970; Nelson, 1972). It is not clear what the volumes are between twenty and thirty weeks but by dye dilution studies on fifty-nine normal pregnancies from thirty-seven weeks of gestation onwards, Elliot and Inman (1961) showed that maximal amniotic fluid volumes occurred at about thirty-eight weeks (in nine cases the mean = 1032 ml and the range = 533-1512 ml) and that volumes declined thereafter to a mean of 250 ml at forty-three weeks. Gadd (1966) used similar methods to show that between thirty-two and thirty-seven weeks the volume ranged from between 450 and 1100 ml with no apparent trend; whereas after thirty-seven weeks volumes fell, to lie between nearly zero and 500 ml at forty-three weeks.

A number of studies have thrown light on the factors which control the rate of amniotic fluid turnover. Hutchinson et al (1959) have shown that there is direct exchange of water between the amniotic fluid and both the mother and the fetus from twelve weeks of pregnancy until term. They calculated that the respective water fluxes in and out of the amniotic fluid (irrespective of source) were about 75 ml/hr at twelve weeks, 181 ml/hr at twenty weeks and 412 ml/hr at term. The fluxes of water into the fluid from the mother and the fetus were approximately equal, except in a single case studied at twelve weeks, where the contribution from the mother was thirty times that from the fetus. Although this data suggests that the water in amniotic fluid turns over very rapidly, this probably occurs by the simple exchange of water between the amniotic fluid and the surrounding tissues. Of considerable interest is the extent to which the turnover occurs as discrete net fluxes of water across individual tissues, since it is by the management of these fluxes that the total volume of the amniotic fluid will probably be controlled. Net fluxes of water may occur across semipermeable membranes under the influence of hydrostatic or osmotic pressures, or they may occur as irreversible, 'bulk' transfers of water, as occurs for example in fetal micturation and swallowing. The following sections will discuss the extent to which various fetal tissues and functions contribute net fluxes of water either into and out of the amniotic fluid, and the extent to which these individual fluxes control the total volume of the amniotic fluid. Because of the paucity of published data, a detailed examination is only possible for the most advanced stages of pregnancy. A subsequent section will outline the possible factors which operate

earlier in pregnancy.

b. Factors affecting amniotic fluid volume and turnover toward term

i. Fetal Swallowing

It has been clear for many years that the fetus can swallow amniotic fluid. Davis and Potter (1946) and Scott and Wilson (1957) pointed out that fetal swallowing must be the cause of the lanugo and amniotic fluid cell debris which is found in the faeces of newborn babies, and of the radio-opaque material found in the gastrointestinal tract after amniography. Pritchard (1965) measured the bulk turnover of amniotic fluid by injecting I^{131} labelled albumin and Cr^{51}-labelled red cells into the amniotic cavity about twenty-four hours before delivery. He found that the volume of fluid swallowed by normal babies at term was between 210 ml and 760 ml per 24 hours, the mean volume being 450 ml per 24 hours. This was in close agreement with the findings of Rosa (1951), suggesting that fetal swallowing alone could bring about the complete turnover of the amniotic fluid in two to three days (assuming an amniotic fluid volume of less than about 1.5L, Elliot and Inman, 1961; Gadd, 1966 and Gillibrand, 1969).

In a discussion on the role of fetal swallowing in the control of liquor volumes, Abramovitch (1970) pointed out that the rate of water turnover (Hutchinson et al 1959) is far in excess of the rate of fetal swallowing at term (Pritchard, 1965). From this he suggested that swallowing may play a minor role in the control of liquor volumes. However, most of the water turnover in amniotic fluid is probably due to the diffusion of water in and out of the amniotic sac, across passive tissue membranes. Although net fluxes

of water can occur across such membranes under the influence of external hydrostatic and osmotic pressures, the membranes themselves are unlikely to be able to regulate these fluxes beyond gross changes in permeability. Conversely, fetal swallowing may occur at variable rates, and is an unbalanced process which causes a net efflux of water from the amniotic fluid. Swallowing may therefore be of considerable regulatory importance, even though its contribution to the total rate of water turnover is small.

A number of studies have shown that abnormally large volumes of amniotic fluid (polyhydramnios) occur more frequently in cases of fetal malformation, and it has been suggested that in many of these cases, the malformation prevents normal volumes of liquor being swallowed in utero. Jeffcoate and Scott (1959) surveyed the records of 169 patients with polyhydramnios and found that in 54 (32%) of the cases there was evidence of defective fetal swallowing. Of these 54 cases, 34 were anencephalic or iniencephalic, 16 had gastro-intestinal atresias or other similar obstructions, and 4 had hydrops fetalis which was accompanied by gross oral oedema. In a similar recent study, Gadd (1970) found that 24% of patients with polyhydramnios had an anencephalic baby, and 5% had babies with obstructions of the alimentary tract. Jeffcoate and Scott (1959) suggested that the anencephalic and iniencephalic fetuses were unable to swallow, either because their brain malformation was extensive enough to interfere with the neural control of swallowing, or because the cervical abnormality was severe enough to cause mechanical obstructions to swallowing. They suggested that the cases of anencephaly which were not associated with polyhydramnios were mild enough to permit swallowing.

McLain (1963) injected radio-opaque material into the amniotic sac to observe the rate of fetal swallowing, and found the rate to be reduced in cases of polyhydramnios. The swallowing rates of anencephalic fetuses have also been measured directly. Pritchard (1965, 1966) observed that in six cases, the rate of swallowing was between 0 and 13 ml per 24 hours. Abramovitch (1970) found that in six out of eight cases the rate of swallowing was less than 24 ml per 24 hr., whilst in the two other cases there were rates of 79 and 237 ml per 24 hr. These data show clearly that the rate of swallowing is severely reduced in most cases of anencephaly.

Abramovitch (1970) found no correlation between the rate of swallowing of anencephalic fetuses and the volume of their respective amniotic fluids, and suggested that fetal swallowing played little part in the production of polyhydramnios. However, it must be added that the measurements of swallowing were made after the excessive volume of liquor had been established, so that a role for swallowing in the genesis of polyhydramnios cannot be ruled out. Nevertheless, it may be that the increased volumes of amniotic fluid are due to the transudation of cerebro-spinal fluid (CSF) or other fluids out of the exposed brain. In this case, the volume of amniotic fluid might still bear some relation to the severity of the lesion, as has been suggested by Jeffcoate and Scott (1959); however, this would not so much be due to defective swallowing, as to the extent to which the brain was exposed. Potter (1961) considers it unlikely that CSF would materially contribute to the fluid volume; however, it would not necessarily be prevented by the collagen membrane which Benirschke and McKay (1953) found to be covering the exposed parts

of the anencephalic brain. Evidence which supports the possibility of fluid transudation from the brain is discussed on p.80.

Few studies have been made on the physiology of polyhydramnios associated with normal fetuses. Pritchard (1966) measured the swallowing rates in three such fetuses at term, by injecting Cr^{51} labelled red cells into their amniotic fluid. He found that two fetuses swallowed normal quantities of amniotic fluid, whilst the third swallowed much less than normal. This suggested that polyhydramnios in normal fetuses may either be due to oversupply of amniotic fluid water, or to a reduced rate of swallowing. However, work by Hutchinson et al (1959) on two cases of polyhydramnios at term, suggests that the situation may be more complex. Although there was a reduction in the rate of transfer of water from the amniotic fluid into the fetus, there was also a marked reduction in the rate of exchange of water across the placenta between the mother and the fetus. They found that although more water than usual was passing out of the fetus into the fluid, the rate of water efflux from the fluid into the maternal serum was at least as great as in the control patients at term. The causal connection between the reduced water fluxes between the two circulations and the genesis of polyhydramnios remains unsolved.

ii. Fetal Urine

Abramovitch (1970), discussing the extent to which fetal urine may be considered as a major source of amniotic fluid, pointed out that no firm conclusion was possible, since no clear evidence existed on the rate of voiding in utero. He suggested that at term the fetus might void 450ml/24hrs in order, perhaps, to balance

the effect of swallowing. However this may well be an overestimate, as it ignores other sources utilising hydrostatic energy (e.g. umbilical cord transudation). The situation early in gestation is still less clear. Urine has been found in the fetal urinary bladder from eleven weeks of gestation onwards (Abramovitch, 1968, 1970), though attempts to deduce voiding rates from bladder capacities are hazardous. Jeffcoate and Scott (1959) reviewed reports of oligohydramnios associated with agenesis or obstruction of the fetal urinary tract. In a total of 295 cases of severe urinary tract anomaly, there was "firm or presumptive" evidence of oligohydramnios at delivery in 100 cases. In the majority of the remaining cases, no reference to the volume of amniotic fluid was made. There have been cases of renal agenesis reported in which there were normal or large volumes of liquor. However, in three such cases (Jeffcoate and Scott, 1959; Bain and Scott, 1960) the fetus was anen- or inien-cephalic, and thus the water may well have been associated with the cranial rather than the urinary lesion. Despite these exceptions (see Abramovitch, 1970), cases of bilateral renal agenesis and urinary tract obstruction have been reported with normal volumes of liquor and no other detectable abnormality. Abramovitch (1970) has pointed out that the additional liquor may be due to changes in the permeability of the umbilical cord and the fetal membranes, or to other apparently minor changes associated with renal agenesis, such as hypoplastic lungs (Potter, 1961). Alterations in the fetal swallowing rate must also be considered.

iii. Umbilical Cord Transudation

The radioactive tracer experiments of Hutchinson et al (1959)

and of Plöntl (1961) showed that the umbilical cord can transfer water from the fetal circulation into the amniotic fluid. These conclusions confirmed those of Runge (1927, 1929), Runge et al (1928), and were later amplified by Feliks (1968a, b). Feliks showed that water and a variety of small molecules could permeate the cord, and from his data it may be calculated that the cord passed 30-40 ml water per hour into the amniotic fluid at term under the influence of fetal blood pressure. Feliks (1968b) also found that the permeability of the cord for water and for other small molecules (except chloride) increased as the cord diameter increased, and suggested that this was due to the thicker cords being more hydrated. In subsequent investigations, the degree of cord hydration was studied, and Feliks' suggestions were supported (Patrick and Walker, 1969). The notably thick umbilical cords, which are commonly found in infants of diabetic mothers, are also very hydrated (Patrick and Walker, 1969); and it may be that the well-known association between maternal diabetes and polyhydramnios is due to a change in umbilical cord permeability.

Parry and Abramovitch (1970) investigated the possibility that the umbilical cord might regulate water exchange between the fetus and the amniotic fluid by studying the cord's histology. They found that the cells on the surface of the cord were relatively unspecialised in appearance, and that gaps occurred between the 'type I' cells which occur particularly at the fetal end of the cord. They suggested (as did Tait, 1875 -1876) that much of the water exchanged passed through these gaps, and that the cells on the surface of the cord played little role in controlling this exchange. Parry and Abramovitch pointed out that the endothelium of the cord vessels may

be of importance in regulating water exchange.

iv. Water Fluxes across the Fetal Skin and Membranes

It is thought that, in the absence of hydrostatic pressure gradients, net fluxes of water across membranes are always associated with active sodium transport (Ussing, 1964; Stein, 1967). This sodium transport is usually, but not always associated with an electrical potential difference across the membrane. Thus the finding that at term there is no potential difference across the amnion (Garby, 1957; Mellor et al 1969) strongly indicates that this tissue cannot transport salt and water actively. Observations made on early samples of amnion by Lind et al (1972) produced similar results. Lind et al (1972) also made direct studies on the transport of sodium ions across fetal skin and amnion by radioisotope flux measurement. They concluded that no active transport of sodium occurs across these tissues during the second trimester of pregnancy. Collectively, these data indicate very strongly that the fetal skin and amnion are not able to regulate the flux of water between the amniotic fluid and other compartments.

v. The Precision of Influx Regulation at Term

The number of possible net sources of amniotic fluid water raises the question of whether there is any inherent precision in the flux rates from individual sources. One as-yet unremarked observation suggests that there is considerable precision, although the location of its control is not clear. At term, Lind et al (1969) and Roopnarinesingh and Morris (1970) have both found a close correlation between the concentrations of creatinine in amniotic fluid and in maternal serum, despite the concentration in

amniotic fluid being 2-5 times that in maternal serum. Since fetal urine is the major source of amniotic fluid creatinine, it follows that the concentration of creatinine in maternal serum controls the concentration in amniotic fluid via the fetal serum and urine. The concentration of creatinine in amniotic fluid is unlikely to control that in maternal serum, since the difference in volumes of the two compartments is so great. This suggests that the rate at which the fetus excretes creatinine is closely regulated (probably by the glomerular filtration rate). A further implication of this finding is that there is a closely controlled relationship between the quantity of creatinine voided and the rate of supply of water from both the urine and from other sources (e.g. umbilical cord). This particularly applies at term, when the bulk turnover of amniotic fluid is relatively rapid. If the net influx of water into the amniotic fluid was very variable, then no proportionality would be found between the creatinine concentrations in the maternal serum and the amniotic fluid.

c. Sources of amniotic fluid water in the first half of pregnancy

All the evidence cited above refers to the latter half of pregnancy. Polyhydramnios is not usually detectable early in pregnancy; and in cases of renal agenesis, it is not clear whether the oligohydramnios of late pregnancy was present before twenty weeks. Thus, the association between renal agenesis and oligohydramnios may not be used as evidence for a major fetal urinary contribution to amniotic fluid early in pregnancy. Although urine has been found in the bladders of fetuses from eleven weeks of gestation onwards, (Abramovitch, 1968, 1970), there is no direct evidence of the rate at which urine is voided. Calculations suggest that between 7 and

17ml are voided per 24 hours, however the premisses upon which the calculations were based are arguable (Abramovitch, 1970). Between seventeen and twenty-one weeks of gestation, the concentration of sodium and chloride in amniotic fluid is approximately twice that in fetal urine, and nearly equal to that in fetal and maternal plasma (Abramovitch, 1970). However, this does not argue against fetal urine as a major net source of amniotic fluid water, because the rate of sodium chloride influx from other sources has not been fully investigated.

Lind and Hytten (1970) and Lind et al (1972) have claimed that amniotic fluid before twenty weeks of pregnancy is "an extension of the fetal extracellular space" and that its volume and other characteristics are controlled by the fetus. However, it is disputable whether there is evidence to support this claim. The fact that there is a close correlation between the volume of amniotic fluid and the fetal length or weight cannot be used to argue that the fetus controls the volume and composition of amniotic fluid.

Lind et al (1972) rightly point out that some net influx of water into the amniotic sac must occur to counteract the efflux of water resulting from the hydrostatic pressure of the uterus on the amniotic sac. The net influx must also balance the effect of fetal swallowing, although this is a much smaller factor than at term (Pritchard, 1966; Abramovitch, 1970). However, it is not clear that there is a net flux of water from the fetus into the amniotic fluid in addition to the voiding of urine. Since Lind et al (1972) were unable to find in vitro evidence that a net flux of water might occur as a result of sodium transport across the fetal skin and membranes (p. 16), it

may be that fetal sources of water which are independent of fetal urine only enter the amniotic sac under the hydrostatic force of the fetal blood pressure. Whether the fetal blood pressure is sufficiently high enough early in pregnancy to cause a significant contribution is not known.

It is possible that the placental membranes might provide a pathway for net water transport. The energy for this transport would come from the pressure of maternal blood circulating against the chorionic plate, between the roots of villi. The development of the placenta and its membranes, together with possible changes in the maternal placental circulation might be responsible for some of the increase in fluid volume during the first six months of pregnancy. Although the point has not been investigated early in pregnancy, it seems very likely that the hydrostatic pressure gradient across the placental membranes due to the maternal circulation under the chorionic plate will exceed that across the fetal skin, so that this maternal source is worthy of some consideration. Some indirect evidence will be discussed later (p.118) which suggests that permeability changes occur in the fetal membranes during the first six months of pregnancy.

4. Amniotic Fluid Protein

a. Quantity

The protein in amniotic fluid may be considered as existing in a number of different forms. It may be part of an intact cell (e.g. Nelson and Emery, 1970), or it may be a free cellular organelle (Salafsky and Nadler, 1971), or it may be soluble protein. This

review is concerned with the soluble protein in human amniotic fluid, although in many of the papers cited cellular organelles have not been stringently removed by centrifugation.

Cantarow et al (1933) showed that the mean protein concentration in term amniotic fluid was less than one tenth of that in maternal serum. Derrington and Soothill (1961) compared the concentrations of various serum proteins in amniotic fluid at term with the concentrations found in maternal and fetal serum. They found that the amniotic fluid : maternal serum ratios for albumin ranged from 1/12 to 1/40; for transferrin (siderophilin) : 1/12 to 1/56; for γ-globulin : 1/32 to 1/144; and for caeruloplasmin : 1/64 to 1/128. The corresponding protein concentration ratios for amniotic fluid : fetal serum were for albumin : 1/10 to 1/64, for transferrin : 1/4 to 1/28, for γ-globulin : 1/56 to 1/192, and for caeruloplasmin : 1/16 to 1/24.

Queenan et al (1970) showed that there were changes in the protein concentration in amniotic fluid as pregnancy proceeded. Although there was a considerable scatter of results, they found that the protein concentration increased from a mean of 3.5mg/ml at twelve weeks of gestation to a maximum of 6.6mg/ml at about twenty-five weeks. After twenty-five weeks there was a marked fall in concentration to about 3mg/ml at thirty-five weeks. Between thirty-five and thirty-nine weeks there was little further change in concentration. No consistent changes in the protein concentration were found in cases of mild or moderate rhesus iso-immunisation, although in very severe cases more random patterns with some very high values were observed as pregnancy proceeded. The reasons for the changes in

amniotic fluid protein concentration were not clear, partly because the nature of the protein had not been adequately studied. Queenan et al (1970) suggested that the fall in protein concentration after twenty-six weeks was due either to fetal swallowing or to dilution following an increase in amniotic fluid volume.

Gitlin and Biasucci (1969) have measured the concentrations of various serum proteins in a small series of amniotic fluids before term. However, the data are not extensive enough to allow a firm conclusion to be drawn about the quantitative behaviour of serum proteins in amniotic fluid.

b. Serum origin of amniotic fluid protein

Paper, disc, and cellulose-acetate electrophoresis have shown that the mobilities of the major proteins in amniotic fluid are similar to those in serum, and various authors have claimed that the protein in amniotic fluid is either of maternal or fetal serum origin (see Table 1). All the studies in Table 1 reported amniotic fluid protein bands corresponding to serum albumin, and α_1, β, and γ-globulin. The α_2 globulins in amniotic fluid were present in very low concentrations, and were best detected by cellulose-acetate electrophoresis (Wild, 1961), or by concentrating the fluid prior to paper electrophoresis. The majority of the work was carried out on fluids taken between thirty-six weeks of gestation and term. Only McKay et al (1958) and Brzezinski et al (1964) studied earlier samples. In most of the studies, the electrophoretic patterns were scanned by an integrating densitometer to express the percentage of the total stained protein which was found in each band. In this way it was established that the percentage of albumin in amniotic fluid protein exceeded that in maternal serum (Table 1). Immunoelectrophoresis

TABLE 1

Electrophoretic studies on Amniotic fluid protein

Author		Method*	No. of Amniotic fluids studied during each trimester				Conclusion++
			1st	2nd	3rd	Term	
Barbanti	(1956)	PE		✓	✓	✓	Similar to MS
Palliez et al	(1956)	PE				✓	Amniotic epithelium secretion
McKay et al	(1958)	PE	7				AF A:G >MS suggest maternal origin of protein
Mentasti	(1959)	FME		✓	✓	✓	$\gamma G/A \uparrow$ after 6 month suggest fetal serum origin
Abbas & Tovey	(1960)	PE Dialysis				✓	AF A:G >MS AF A:G = MS dialysate
Strebel	(1960)	IEs	3	4	3	12	Serum protein in AF
Derrington & Soothill	(1961)	IEa				14	Most AF protein is of serum type
Wild	(1961)	PE CE				40	AF A:G > MS
Brzezinski et al	(1961)	PE MBE				23	A:G of FS ⩾ AF ⩾ MS ∴fetal origin
Viergiver et al	(1962)	PE			1	13	A:G of FS>AF>MS
Brzezinski et al	(1964)	MBE	3	11			A:G of AF>FS. Bis-albuminaemia in fetus and AF indicates fetal origin - see text p.33
Heron	(1966)	PE			4	103	AF A:G ratio increases from 1.0 at 36 wks. to 1.8 at 43 wks.
Usategui-Gomez et al	(1966)	DE				12	Serum Protein in AF
von Kleist et al	(1968)	IEa	——12——		2	2	Serum and non-serum proteins
Castelazo-Ayala & Karchmer	(1968)	PE			——28——		Serum protein : in normal & abnormal pregnancies

TABLE 1 (contd.)

Author	Method*	No. of Amniotic fluids studied during each trimester				Conclusion++
		1st	2nd	3rd	Term	
Vernier et al (1969)	IEs				9	A:G of AF > MS
Fischbacher & Quinlivan (1970)	IEs				✓	Serum protein in AF

* Key

DE = Disc Electrophoresis

FME = Free micro-electrophoresis

IEs = Immunoelectrophoresis using antisera against human serum

IEa = Immunoelectrophoresis using antisera against amniotic fluid

✓ = number unspecified

++A:G = Albumin / Globulin concentration ratio

$\gamma G/A$ = γGlobulin / Albumin "

AF = Amniotic fluid

MS = Maternal serum

FS = Fetal serum

using antibodies against human serum protein confirmed that the major amniotic fluid proteins were indeed serum proteins (Strebel, 1960; Derrington and Soothill, 1961; Vernier et al 1969; Fischbacher and Quinlivan, 1970). However, it remained a possibility that there were non-serum proteins in amniotic fluid, which were not detected by the insensitive methods of paper or cellulose acetate electrophoresis, and would not be detected by immunoelectrophoresis using antisera against human serum protein. A number of immunological studies have been made to examine this possibility.

c. Non-serum protein in amniotic fluid

Derrington and Soothill (1961) raised rabbit antibodies against term amniotic fluid protein, and absorbed the resultant antiserum with human serum to remove the antibodies against human serum protein. They then tested the absorbed antiserum against human amniotic fluid by immunoelectrophoresis to search for non-serum proteins. Because they found no residual precipitin lines, Derrington and Soothill concluded that the vast majority of protein in term amniotic fluid was of serum type. It fell to Salmon et al (1962) to describe the first non-serum protein in human term amniotic fluid. The origin of this protein was shown by immunofluorescence to be the epithelial cells of the amnion. Subsequent studies showed that this protein had an α_1 mobility (Lambotte and Salmon, 1962; Lambotte, 1966), and that it was a glycoprotein with an unusually high content of reducing sugar (Lambotte and Uhlenbruck, 1966; Lambotte and Gosselin-Rey, 1967). A similar protein was found in bovine amnion, and the unusual nature of these glycoproteins prompted Lambotte and Uhlenbruck (1966) to call them Amniomucoids.

A specific amnion protein of α_1-mobility has also been found in rabbit amniotic fluid and in neonatal rabbit sera up to the twentieth day of life (Lambotte, 1963), though this protein was not detectable in maternal sera. Of considerable interest is the finding of Lambotte et al (1963) that rabbit α_1-amnion protein will evoke an antibody response when injected into the mother rabbit. Further, Lambotte et al found some multiparous animals who had developed antibodies against rabbit α_1-amnion protein in the absence of experimental immunisation. This observation prompted speculation about the role of such immune phenomena in the genesis of congenital abnormality and abortion. Lambotte (1965) immunised a number of adult female rabbits with rabbit amniotic fluid protein and counted the number of still-births which occurred in subsequent pregnancies. In the immunised group of rabbits, 64.5% of fetuses were stillborn, whereas the rate in primaparous control rabbits was 0%, and in multiparous control rabbits was 4.4%. Fetal death could also be brought about by injecting a control pregnant rabbit with serum from a sensitised rabbit. Fetal death was unaccompanied by visible congenital abnormalities, and those fetuses which were live born were apparently normal. However, it is not clear that this observation can be simply applied to the case of the multiparous rabbits who have spontaneously acquired antibodies against amniotic fluid protein, since in these cases the rate of fetal death was not linked to the presence of the antibodies.

A similar immunological study of the non-serum proteins in human amniotic fluid was made by von Kleist et al (1968). These workers studied twelve fluids of between six and sixteen weeks

gestation, two fluids between sixteen weeks and term, and two samples of amniotic fluid at term. Some, or all of these samples were used to immunise two rabbits, and the resultant antisera were absorbed with human serum and tested for antibodies against non-serum protein. Four non-serum protein precipitin lines were detected by immuno-electrophoresis. One α_1 mobility protein was identified as α_1-fetoprotein. Two α_2-proteins were found in late liquor samples and were found to be present in amnion. A β-mobility protein was also found, though this was shown to be due to contaminating haemoglobin.

These preliminary studies used relatively insensitive qualitative immunoelectrophoretic methods to detect new antigens. There is a clear need to extend these studies, using more sensitive and quantitative techniques. This will enable a further understanding of the factors causing the changes in amniotic fluid protein concentration observed by Queenan et al (1970), and will provide potentially important data on the contribution of tissue-specific antigens to the amniotic fluid of normal and abnormal fetuses.

d. Origin of the serum proteins in amniotic fluid

i. Simple quantitative studies

A number of quantitative and qualitative studies have been made on the origins of the serum proteins in amniotic fluid. McKay et al (1958) pointed out that the increased proportion of albumin in the fluid was typical of the protein of the interstitial fluids, and concluded that a maternal serum origin was likely during the first trimester. Abbas and Tovey (1960) reasoned that if the serum protein in amniotic fluid was of maternal origin, then it must enter the amniotic sac by diffusing through the fetal membranes. They

therefore asked whether the differences between the maternal serum and the amniotic fluid patterns were due to the diffusion process. They found that at term, the pattern of amniotic fluid protein was very similar to that obtained by dialysing maternal serum through the fetal membranes, and concluded that the amniotic fluid might be a simple dialysate of maternal serum. However, as Wild (1961) has pointed out, the equivalent experiment of diffusing fetal serum through the surface of the fetus (umbilical cord) has not been carried out, so that a fetal contribution is not excluded. Indeed, because the percentage of albumin in fetal serum protein is greater than in maternal serum (McKay et al 1958; Brzezinski et al 1961), Brzezinski et al (1961) thought that the protein in amniotic fluid was of fetal origin.

Mentasti (1959) was among the first to suggest that the protein in amniotic fluid resembled fetal serum protein more closely than maternal serum protein. Using free microelectrophoresis he found that after the sixth month of pregnancy, there was an increase in the percentage of γ-globulin and a decrease in the percentage of albumin in both fetal serum and amniotic fluid protein. No such change was detected in maternal serum. This finding has not been re-examined, and more recent work on the origin of albumin and γ-globulin in amniotic fluid suggests that the conclusion is questionable (Derrington and Soothill, 1961; Gitlin et al 1964).

Wild (1961) found a correlation between the concentrations of bilirubin and protein in amniotic fluid, and suggested that since the bilirubin was of fetal origin, the albumin (which binds the bilirubin) must also be of fetal origin. He suggested that the

umbilical cord was the most likely route for the complex to enter the amniotic sac. It must be added that in severe cases of rhesus disease, this proportionality is not found as there is an increase in the ratio of bilirubin : protein (Morris et al 1967; Fort, 1971). Although Wild's observations are of considerable interest, it has yet to be demonstrated that bilirubin actually passes out of the fetus bound to albumin. It is fairly clear that most of the bilirubin in amniotic fluid and fetal serum is unconjugated (see Fort, 1971) and that about 99% is bound to albumin, (Odell et al 1969; Mandelbaum et al 1967). However, the covalent linkage between albumin and bilirubin is not strong. Bilirubin may be displaced from the protein by low pH, anionic drugs, and some naturally occurring anions, including bile acids and fatty acids (see Odell et al 1969). If the umbilical cord is a major source of amniotic fluid bilirubin, then the polyanionic mucopolysaccharides of Wharton's jelly may displace bilirubin from the albumin. If this occurs, the bilirubin will pass much more quickly into the amniotic fluid than will albumin, because of the differences in molecular size. Once in the amniotic fluid, the bilirubin would again be able to bind to albumin, irrespective of the protein's origin.

Derrington and Soothill (1961) argued that if serum proteins entered the amniotic fluid by ultrafiltration, then proteins of similar molecular weight would diffuse into the amniotic fluid at approximately the same rate. Thus the concentration ratio of such proteins in amniotic fluid would be similar to the ratio in their serum of origin. Comparison of the concentration ratio in amniotic fluid with the ratio in maternal and fetal serum should therefore

provide an indication of the origin of the amniotic fluid proteins, provided that the ratios in maternal and fetal serum were sufficiently different. They therefore compared the ratio of transferrin (siderophilin) : albumin and the ratio of caeruloplasmin : γ-globulin, and found that the ratios in term amniotic fluid were significantly more similar to the ratios in maternal serum than to the fetal serum ratios. They concluded that at term these proteins were more likely to be of maternal rather than fetal origin.

Usategui-Gomez et al (1966) expressed the concentrations of transferrin and caeruloplasmin in amniotic fluid as percentages of the total protein, and compared the percentages in amniotic fluid with those in the maternal and fetal serum. They found that the percentages of the two proteins in amniotic fluid were very similar to those in maternal serum, and over twice as great as the percentages in fetal serum. From this they concluded that the proteins were of maternal origin. However, a similar pattern was not found for other serum proteins, and although this may be explained on the bases of molecular weight, other interpretations could be advanced.

Of considerable interest is a preliminary report on the haemopexin and albumin in amniotic fluid. Haemopexin is a β-glycoprotein which binds free haem in the serum. Muller-Eberhard and Bashore (1970) found that in cases of Rh isoimmunisation, the ratio of haemopexin : albumin in amniotic fluid fell during severe fetal haemolysis. The concentration of haemopexin in adult and neonatal serum falls when haemolysis occurs for any reason (Muller-Eberhard et al 1968; Sears, 1969). This is believed to occur as a result of the haemopexin-haem complexes being removed from the serum by the liver (Muller-Eberhard et al 1969). It is not

clear at present whether the change in amniotic protein ratios found in severe Rh disease are due to haem in the amniotic fluid precipitating haemopexin, or whether the ratio change is caused by changes in the serum. The latter possibility may be more likely, and is of interest as it poses the question of whether amniotic fluid haemopexin is of maternal or fetal serum origin. Since starch gel electrophoresis has failed to reveal genetic variants of human haemopexin (Stewart and Lovrien, 1971), quantitative studies will have to be made on the concentration changes of haem and haemopexin in fetal and maternal serum.

α-Fetoprotein has been detected in amniotic fluids between six and thirteen weeks by Gitlin and Boesman (1966) and throughout gestation by Seppälä and Ruoslahti (1972). This fetal serum protein is synthesised in the fetal liver, yolk sac and gastrointestinal tract from the sixth week of gestation (Gitlin and Boesman, 1967; Gitlin et al 1972), and since the concentration of α-fetoprotein in maternal serum is much lower than that in amniotic fluid (Gitlin and Boesman, 1966; Ruoslahti and Seppälä, 1972), it has been suggested that the protein must enter the amniotic fluid direct from the fetus. However, although it is not clear how much of a contribution the yolk sac makes to the α-fetoprotein in amniotic fluid, what contribution it does make after 10 weeks of gestation will be independent of the fetus, since the vitelline vessels have been obliterated by this time. Gitlin and Boesman (1966) found that the α-fetoprotein in fetal urine was often greater than that in the amniotic fluid early in gestation, and suggested this was a major source of the protein. They pointed out that if urine was a major source, then

alternative sources were probably contributing albumin to the amniotic fluid, because the concentration ratio of albumin to α-fetoprotein in amniotic fluid was 12 to 80 times greater than that in fetal urine. This discrepancy in relative concentrations is even more significant if the yolk sac is contributing α-fetoprotein to the amniotic fluid. However, it is not clear that the fetus contributes urine to the amniotic fluid at this early stage of pregnancy (p. 17), and the albumin and α-fetoprotein may diffuse out of other fetal tissues at different rates. Of considerable interest is the finding of a large concentration gradient for α-fetoprotein between the fetal serum and the amniotic fluid. Gitlin and Boesman (1966) found this to be of the order of 182 : 1 to 625 : 1 between nine and thirteen weeks of gestation. This may indicate the relative impermeability of the fetal tissues to the flux of fetal serum protein into the amniotic fluid.

Casper and Benjamin (1970) have reported that, whilst they could detect immunoreactive insulin in amniotic fluids from twenty weeks of gestation until term, none was present in the single fluid sample studied at sixteen weeks of pregnancy. They suggested that the appearance of insulin in the amniotic fluid between sixteen and twenty weeks of gestation was due to the onset of insulin secretion by the fetal pancreas. However, insulin has been detected in fetal plasma as early as eleven weeks of gestation (Adam et al 1969); and by fifteen weeks the concentration in fetal plasma exceeds that in maternal plasma (Thorell, 1970). Thus, the failure to find insulin in a single sample of fluid at sixteen weeks of gestation does not constitute evidence that the insulin in amniotic fluid is of fetal origin.

Casper and Benjamin (1970) also found that insulin was absent from the amniotic fluids of three fetuses who died *in utero* during the third trimester of pregnancy. From this, they argued that the insulin in amniotic fluid was "mainly or entirely fetal in origin". However, insulin is a labile molecule, prone to loss of activity by reduction of its disulphide bonds (Buse *et al* 1962; Katzen *et al* 1962). Furthermore, the placenta is known to contain potent insulinase activity (Goodner *et al* 1959), and it may be that the absence of insulin in the fluids of dead fetuses is due to insulin degradation (this is especially likely as the fetuses had been dead *in utero* up to 3 weeks before amniocentesis). This possibility could have been simply investigated by adding insulin to the amniotic fluid sample and testing the effect of incubation at 37°C; it is not clear that this was tested by their recovery experiment.

In a further attempt to study the relationship of the amniotic fluid insulin to that in maternal serum, Casper and Benjamin measured the insulin in single samples of amniotic fluid taken from third trimester patients before, and at various times during oral glucose tolerance tests. They claimed that a peak in the concentration of insulin in amniotic fluid only occurred at one hour after the test dose, whilst that in maternal serum lasted over three hours. However, the elevation of the mean insulin concentration in amniotic fluid one hour after the test dose was due mainly to the very high levels in two of the six fluids studied. Further observations are needed to clarify this point. If a concentration peak does occur at this time, then the decline during the second hour provides evidence of the lability of amniotic fluid insulin.

It has been shown that glucose and tolbutamide have much greater effects on the neonatal serum insulin concentration if the mother is diabetic (Baird and Farquhar, 1962; Velasco and Paulsen, 1969; Cole et al 1970). Thus, if the data of Casper and Benjamin (1970) on the changes in amniotic fluid insulin concentrations are supported, comparison of the fluid insulin responses to glucose and tolbutamide loads in normal and diabetic mothers may throw light on the origin of amniotic fluid insulin.

Human placental lactogen has been found in term amniotic fluid and in maternal serum, but is virtually absent from umbilical cord blood (Tallberg et al 1965). As this protein is thought to be synthesised only in the placenta it must enter the amniotic fluid by diffusing through the placental or reflected membranes. The relative concentrations of the protein in amniotic fluid and maternal serum have not been measured (Seppälä, personal communication).

ii. Evidence from genetic, immunological and radioisotopic markers

The use of genetic variants is one of the methods of choice for investigating the origin of a protein in amniotic fluid. The method was first used by Seppälä, Ruoslahti and Tallberg (1966) to determine the origin of transferrin in amniotic fluid. These workers studied the transferrin (Tf) phenotypes in amniotic fluid and maternal and cord sera from pregnancies at term. In twenty-seven cases the Tf phenotypes in the maternal and cord sera were both concordant for the common type : CC, however, in two cases there was discordance between the maternal and fetal phenotypes and in both cases the amniotic fluid Tf phenotype was that of the mother. Similar work was carried out on the α_2 group-specific component (Gc) by Ruoslahti et al (1966) and Usategui-Gomez and Morgan (1966).

The frequency of variants is much higher for Gc than for Tf so that it is easier to find feto-maternal discordance. Both groups of workers found that in term amniotic fluid, the Gc phenotype was that of the mother. The haptoglobin (Hp) in amniotic fluid must be maintainly of maternal origin since it is absent from 90% of fetal sera (Schultz and Heremens, 1966). However, Usategui-Gomez et al (1966) showed that haptoglobin was only present in term amniotic fluids when the maternal phenotype was 1-1. They pointed out that the Hp 2-1 and 2-2 forms have higher molecular weights than Hp 1-1, and that this may have prevented them from entering the amniotic sac. The effect of molecular weight on protein permeation will be further considered on p.

The ABH antigens are known to occur in amniotic fluid (Putkonen, 1930; Freda, 1958; Przestwor, 1964; Turowska and Bromboszcz, 1967; Harper et al 1971). In a series of twenty-two term pregnancies, Freda (1958) found that the fetal blood group antigens were expressed in the amniotic fluid when the fetus was a secretor and the maternal antigen was expressed when the mother was a secretor. Both phenotypes were usually expressed when there was discordance for the blood group antigens if both mother and fetus were secretor-positive. Przestwor (1964) and Turowska and Bromboszcz (1967) found that the occurrence of blood group substances in the amniotic fluid was correlated with the fetal secretor phenotype. In a recent study of sixty-seven pregnancies between nine and twenty-four weeks of gestation, Harper et al (1971) found that the phenotype of soluble blood group substances in the amniotic fluid was determined by the fetus, and their presence controlled by the fetal secretor phenotype. The reason for the discrepancy between the results of Freda (1958)

and Harper et al (1971) is not clear. It may represent a difference between amniotic fluids early and late in gestation, or it may be that the technique of Harper et al (1971) was not sensitive enough to detect a maternal contribution.

Because the secretor locus is linked to that of myotonic dystrophy (Mohr, 1954; Renwick et al 1971) it has been suggested that linkage analysis could be of importance for the prenatal diagnosis of myotonic dystrophy (Renwick, 1969). However, linkage analysis will be useful in only about 10-15% of pregnancies at high risk (Renwick, 1969; Smith, 1972), and about 10% of the diagnoses made will be erroneous because of recombination.

Using a rather insensitive method, Brzezinski et al (1964) claimed to have found a case of fetal bisalbuminaemia in which the amniotic fluid contained the variant albumin, whilst the maternal serum did not. Because the variant protein was said to be clearly evident in the fetal and fluid samples, it was suggested that the albumin in amniotic fluid was of fetal origin. The fetus in this case was in the sixth month of gestation, and the extra albumin band appeared on electrophoresis as a small superimposed peak close to the normal albumin peak. However, no evidence was provided that it was antigenically related to albumin, and unfortunately no paternal serum was available to show that the trait was heritable. Further, although electrophoresis showed that the amniotic fluid also had an unusual protein peak in a similar position to that of the albumin variant in serum, there were only two protein peaks of slower mobility, and one of these was also superimposed on the albumin peak. This pattern was very different from that of other amniotic fluids, which

all showed the globulin peaks as three separate entities well behind albumin. For these reasons, the claim of Brzezinski et al (1964) is based on unsatisfactory evidence. It may be that other explanations underlie their findings, and this is further discussed on p.58

It would be laborious to attempt to repeat the work of Brzezinski et al (1964), as bisalbuminaemia is a rare genetic variant, but another approach has been successful at term and could be developed further. Dancis et al (1960, 1961) and Gitlin et al (1964) labelled albumin with I^{131}, and injected it into women at three and nine months of pregnancy, respectively. They both found that the concentration (counts per ml) of labelled albumin in amniotic fluid was less than in fetal or maternal serum; however, Gitlin et al (1964) showed that the specific activity of the albumin (counts per mg albumin) in term amniotic fluid was 3-5 times greater than that in fetal serum. If the amniotic fluid albumin had been of fetal origin its specific activity would not have exceeded that in fetal serum; thus a substantial fraction of the albumin in amniotic fluid at term must be of maternal origin.

The specific activities of albumin and of γ-globulin in amniotic fluids at three months gestation were less than those found in corresponding maternal and fetal sera* (Dancis et al 1960), and the same was found for 7 S γ_2-globulin (i.e. Fc fragments) at term (Gitlin et al 1964). Although these results may indicate that the

*Specific activities measured by Dancis et al (1960) were cpm per mg protein. The individual concentrations of albumin and γ-globulin were not measured.

proteins are of fetal origin, they are compatible with the hypothesis of a maternal origin. Early in pregnancy the proteins may diffuse more slowly into the amniotic fluid than into the fetal serum, so that the specific activity of albumin in an amniotic fluid at three months may be lower than in the fetal serum. At term the rate of 'bulk' turnover of amniotic fluid is far greater than at three months (see section on fetal swallowing), whilst the amniotic fluid protein concentrations are similar (Queenan et al 1970). Thus at term there must be a rapid flux of protein into the amniotic fluid; this may account for the high specific activity of albumin found in amniotic fluid at term by Gitlin et al (1964). Evidence will be presented on the concentration of serum proteins in amniotic fluid which may indicate that the permeability of the membranes are lowest early in pregnancy (p.117).

Radioactive labelling experiments may not be necessary for investigating the origin of amniotic fluid Ig G, since specific serotypes may be measured. However, the mere measurement of the titres of Rh-antibodies (Usategui-Gomez and Stearns, 1969) or of poliomyelitis antibodies (Lipton and Steigman, 1957) is not sufficient, because the concentration of Ig G in amniotic fluid (at term) is much less than in the fetal and maternal sera (Derrington and Soothill, 1961). If the titre of a specific Ig G antibody in amniotic fluid was expressed as a fraction of the total Ig G in the maternal and the fetal sera, a more meaningful result would be obtained. It must be stressed that the radioactive or immune labelling experiments described above can only prove that the majority of a protein is of maternal origin since the protein is injected into or synthesised in the mother. Since the direct injection of

radioactive protein into the human fetus is out of the question, the use of genetic markers and tissue specific proteins are the only direct ways of searching for fetal serum proteins in the amniotic fluid.

e. The selective effect of molecular weight on the serum proteins present in amniotic fluid

Molecular weight is probably the major physical parameter determining whether a serum protein can permeate into the amniotic fluid. However, the shape and surface properties of the protein will also control its relative concentration. Pre-albumin, albumin, α_1-antitrypsin, α_2-caeruloplasmin, group-specific component, haptoglobin 1-1, haemopexin, transferrin, $\beta_{1\ A-C}$-globulin, plasminogen, Ig G_1 and Ig A are all serum proteins which have been detected in amniotic fluid (Strebel, 1960; Gitlin and Biasucci, 1969; Muller-Eberhard and Bashore, 1970). The heaviest of these proteins is α_2-caeruloplasmin, which has a molecular weight of approximately 160,000 (Schultz and Heremans, 1966). In contrast, α_1-lipoprotein (mol. wt.* : 435,000), α_2 macroglobulin (mol. wt. : 820,000), β-lipoprotein (mol. wt. : 3.2×10^6), α_2-macroglobulin (mol. wt. : 820,000), and Ig M (mol. wt. : 1×10^6) are absent from the fluid (Strebel, 1960; Gitlin and Biasucci, 1969).

It is clear that the majority of the haptoglobin in amniotic fluid is of maternal origin, and that only the Hp1-1 form enters the fluid in quantities detectable by disc electrophoresis (see p. 32: Usategui-Gomez et al 1966). This may be accounted for by the

*abbreviation: mol. wt. = molecular weight; data from Schultz and Heremans (1966).

differences in molecular weight. The molecular weight of Hp 1-1 is 85,000 (Jayle and Boussier, 1955), whereas Hp 2-1 and 2-2 form polymers of molecular weights up to 1 x 10^6 (Usategui-Gomez et al 1966; Giblett, 1969).

The finding that the fetal secretor phenotype is expressed in the amniotic fluid (p.32 ; Harper et al 1971), is of interest not only for its medical genetic implications, but also for its biological ones. Secretor positive individuals are believed to secrete soluble blood group substances from most exocrine glands (see Giblett, 1969). Human endometrium and parietal decidua are secretory tissues (see Hamilton et al 1964), and might well be expected to secrete blood group substances (Freda, 1958). However, it appears from the genetic studies early in gestation that the maternal phenotype is not expressed in detectable quantities in the amniotic fluid. The reason for this may be that the molecular weights of the soluble blood group substances are about 300,000 (Giblett, 1969), and thus do not easily cross the fetal membranes. The fetal tissue of origin of these substances remains unsolved.

f. Routes of entry for serum protein into the amniotic fluid

i. The fetal membranes

This is the only pathway available for proteins which enter direct from the maternal serum or the uterus (e.g. haptoglobin and group-specific component). It would seem that the placental (as opposed to reflected) membranes may transmit much of the maternal serum protein found in amniotic fluid, because maternal blood flows freely against the chorionic plate (Fig.3). However, in vitro studies have shown the placental and reflected portions of the amnion

are equally permeable to albumin (Garby, 1957).

Keiffer (1926) and Palliez et al (1956) claimed that the amniotic epithelium secreted lipids and possibly other material into the amniotic fluid, though the in vitro work of Garby (1957) suggests that the permeation of I^{131} albumin through the membrane occurs merely by passive diffusion through pores in the amnion. The permeability of albumin through the amnion was found to be about 0.013 times that of sodium and chloride ions and 0.026 times that of creatinine (Garby, 1957). The possible contribution of amnion protein to the amniotic fluid is discussed on pp.23-24.

ii. Fetal entry routes

Possible routes include the urine, the gastrointestinal, buccal and bronchial secretions, the umbilical cord and the fetal skin. However, although there is evidence to support the use of some of these routes, the size of their contribution is not known. Albumin and α-fetoprotein have been found in fetal urine between six and thirteen weeks of gestation, and the possible significance of the high fetal serum : amniotic fluid concentration gradient for α-fetoprotein has been discussed on p.29 . Gastrointestinal and bronchial secretions are known to contain some serum protein (Schultz and Heremans, 1966) and may contribute protein towards term. This may especially apply to lung secretion, since evidence from amniotic fluid lipids suggests that pulmonary secretion occurs toward term [illegible] and Potter and Bohlender, 1941).

It is not clear whether serum protein permeates through the human umbilical cord into the amniotic fluid, despite the interesting speculations of Wild (1961). However, fluorescent antibody staining

has shown serum protein to be present in human Wharton's jelly at term (Bardawil, Toy and Hertig, 1958), and albumin has been shown to pass through sheep cords <u>in vitro</u> (Barcroft, Danielli, Harper and Mitchell, 1944).

The concentration of the proteins in fetal serum does not remain constant during gestation, and this must be considered when discussing a possible fetal contribution to the serum protein in amniotic fluid. Gitlin and Biasucci (1969) found that the concentration of fetal serum γG-immunoglobulin, β_{IC}-globulin, C^1 esterase inhibitor, transferrin, haemopexin, and α_2-macroglobin increased linearly by about ten-fold between ten and about thirty to forty weeks of gestation, and a similar five-fold increase was found for albumin (Gitlin and Boesman, 1966). At term, the concentrations of these proteins were approximately the same as in maternal serum. However, Gitlin and Biasucci (1969) found that some protein concentrations remained uniform from early in gestation (α_1-antitrypsin), or increased by only two- to three-fold (caeruloplasmin, plasminogen) or actually fell (α-lipoprotein, and possibly γA-immunoglobulin). In the case of only three proteins were the concentrations in fetal serum greater than in maternal serum. These proteins were albumin (Mendenhall, 1970), α_2-macroglobulin (Gitlin and Biasucci, 1969) and α-fetoprotein (Gitlin and Boesman, 1966). Only in the case of α-fetoprotein was the concentration gradient appreciable, and this serum protein is the only one for which clear evidence of a fetal contribution into the amniotic fluid exists.

5. Enzymes in Amniotic Fluid Supernatant

Table 2 summarises the enzymes which have been studied in amniotic fluid at the various stages of pregnancy. Some of the studies were made either to carry out prenatal diagnoses of inborn errors of metabolism, or to enquire into the origin of amniotic fluid enzymes. Most of the studies, however, were made in the hope that ways of monitoring fetal distress or prematurity might emerge. The work in the latter category was rarely thorough enough for there to be any hope of success.

The recent studies of O'Brien et al (1971) and Friedland et al (1971) have shown that Tay-Sachs disease may be diagnosed in utero between the fifteenth and twenty-sixth weeks by measuring the specific activity of the A form of hexosaminidase in amniotic fluid supernatant, as well as cultured and uncultured cells. The initial attempt to diagnose Pompe's disease prenatally by measuring α-1, 4-glucosidase in amniotic fluid was encouraging (Nadler and Messina, 1969); however subsequent studies (Nadler, Bigley and Hug, 1970) showed that normal α-1, 4-glucosidase activity may be present in the amniotic fluids of fetuses affected with the disease. However, the limited quantity of control data makes it difficult to assess the biological significance of this finding. Fluharty et al (1971) have reported that the α-1, 4-glucosidase in amniotic fluid is less sensitive to inhibition by turanose than is the enzyme deficient in Pompe's disease. They also found that the fluid enzyme was more labile, and that its pH optimum was more neutral. The turanose inhibition and pH profiles were confirmed by Salafsky and Nadler (1972). These studies strongly suggest that the α-1,4-glucosidase in amniotic fluid is not the same

as that which is absent in Pompe's disease, though it must be added that these kinetic differences may be due to the very different protein environments of the crude extracts used in the enzyme studies.

A few quantitative studies have been made into the origin of amniotic fluid enzymes. Salafsky and Nadler (1971) have attempted to study the particle binding of enzymes in amniotic fluid supernatant. They subjected two amniotic fluids from the second trimester of pregnancy to differential centrifugation, and claimed that over 85% of the alkaline phosphatase and α-1,4-glucosidase in amniotic fluid was particle bound, whereas only 6% of the hexosaminidase was bound. Yet, although electron microscopical analyses showed that sub-cellular organelles were present in amniotic fluid, it is not clear that the enzymes were located within these particles, rather than being merely adsorbed either onto their surfaces, or onto the surfaces of the general rissue fragments present.

Geyer and Schneider (1970) have measured the specific activities of a number of enzymes in term amniotic fluid and in various fetal tissues. On the basis of the relative enzyme specific activities, Geyer (1970) suggested that the enzymes in amniotic fluid came from amnion. However, the lactate dehydrogenase and malate dehydrogenase isoenzyme patterns did not fully confirm this.

The possibility that serum might constitute a source of amniotic fluid enzymes as well as other proteins has not been thoroughly examined. Tornqvist et al (1971) found that during the second trimester of pregnancy the specific activity of the diamine oxidase in amniotic fluid is somewhat less than in maternal serum, whilst at term there was no significant difference. The specific activity of

the enzyme in fetal serum was considerably less than in either maternal serum or amniotic fluid. Aminoguanidine is an inhibitor of diamine oxidase, and the same workers injected 0.2 mg/Kg of this intra-muscularly into the mothers in ten individual pregnancies during the second trimester. They found that there was a ten-fold decrease in enzyme specific activity in the maternal serum, and a five-fold decrease in the amniotic fluid. The significance of these results is not clear, since the inhibitor may have entered the amniotic fluid independently of the enzymes.

TABLE 2

Summary of the enzymes studied in amniotic fluid supernatant

Enzyme	Authors		
	Studies at term	Studies before term	(trimester)
Acid phosphatase	Galerne et al (1970) Geyer and Schneider (1970) Geyer (1970) Seelich and Gomolka (1952)		
Alkaline Phosphatase			
total	Ahmed and King (1959)		
"	Geyer and Schneider (1970)		
"	Geyer (1970)		
"	Mischel (1960)		
		Nadler and Salafsky (1971)	(2nd)
placental fraction	Roopnarinesingh et al (1972)	Roopnarinesingh et al (1972)	(3rd)
total	Seelich and Gomolka (1952)		
Aldolase	Antonini et al (1957) Zelnicek and Povarek (1961) Paysant et al (1969)	Paysant et al (1969)	(3rd)
Aminotripeptidase	Brosnens (1966)		
α-Amylase	Geyer and Schneider (1970) Geyer (1970) Paysant et al (1969)	Paysant et al (1969)	(3rd)
Arylaminopeptidase	Ryback et al (1971)		
Carboxypeptidase	Brosens (1966)		

TABLE 2 (contd)

Summary of the enzymes studied in amniotic fluid supernatant

Enzyme	Authors		
	Studies at term	Studies before term	(trimester)
Cholinesterase	Geyer and Schneider (1970) Geyer (1970) Woyton et al (1969)	Woyton et al (1969)	(3rd)
Creatine kinase	Streiff et al (1970)		
Cytochrome C oxidase (absent)		Salafsky and Nadler (1971)	(2nd)
Diamine oxidase (pp.41-42)	Southren et al (1965) Swanberg (1950) Tornqvist et al (1971)	Southren et al (1965) Swanberg (1950) Tornqvist et al (1971)	(3rd) (2nd)
Diastase	Maeda (1924)		
α-Fucosidase		Wiederschein et al (1971)	(3rd)
α-Galactosidase (trace)		Wiederschein et al (1971)	(3rd)
β-Galactosidase (absent)		Wiederschein et al (1971)	(3rd)
β-Glucuronidase	Toschi, P. (1964)		
Glucose-6-phosphate dehydrogenase	Geyer and Schneider (1970) Geyer (1970)		

TABLE 2 (contd)

Enzyme	Authors		
	Studies at term	Studies before term	(trimester)
α-1,4-Glucosidase (p.3, 40-41)		Fluharty et al (1971)	(2nd)
		Nadler and Messina (1969)	"
		Nadler et al (1970)	"
		Salafsky and Nadler (1971)	"
		Salafsky and Nadler (1972)	"
Glutamate dehydrogenase (absent)		Salafsky and Nadler (1971)	"
γ-Glutamyl transpeptidase	Galerne et al (1970)		
Hexosaminidase (p.3, 41)		O'Brien et al (1971)	(2nd + 28wks)
		Friedland et al (1971)	(2nd)
		Salafsky and Nadler (1971)	(2nd)
Histaminase	Uuspaa (1951)		
Kimnogen	Delhaye et al (1966) Wiegershausen et al (1967)		
Lactate dehydrogenase	Antonini et al (1957) Geyer and Schneider (1970) Geyer (1970) Paysant et al (1969) Randow et al (1965) Woyton et al (1969)	Paysant et al (1969) Woyton et al (1969)	(3rd) (3rd)

TABLE 2 (contd)

Enzyme	Authors		
	Studies at term	Studies before term	(trimester)
Leucine amino-peptidase	Beecham et al (1962) Geyer and Schneider (1970) Geyer (1970)		
Lipase	Maeda (1924)		
Malate dehydrogenase	Geyer and Schneider (1970) Geyer (1970) Zelnicek and Povarek (1961)		
α-Mannosidase		Wiederschein et al (1971)	(3rd)
Monoamine oxidase (present) (absent)	Brzezinski et al (1962)	Salafsky and Nadler (1971)	(2nd)
5-Nucleotidase	Ahmed and King (1959)		
Pepsinogen	Maeda (1924) Wagner (1961)		
Phosphohexose isomerase	Antonini et al (1957)		
Pyrophosphatase	Ahmed and King (1959)		

TABLE 2 (contd)

Enzyme	Authors		
	Studies at term	Studies before term	(trimester)
Ribose-5-phosphate isomerase	Antonini et al (1957)		
Succinicdehydrogenase (Absent)		Salafsky and Nadler (1971)	
Transaminases			
Alanine & Aspartate G.P.T. and G.O.T.	Geyer and Schneider (1970) Geyer (1970) Geyer and Schneider (1970) Geyer (1970) Paysant et al (1969) Randow et al (1965) Woyton et al (1969) Zelnicek and Povarek (1961)	Paysant et al (1969) Woyton et al (1969)	(3rd) (3rd)
Ornithine-keto acid		Shih and Schulman (1970)	(2nd)

THE AIMS OF THIS STUDY

The introduction has reviewed work showing that the major soluble antigens in amniotic fluid at term are of serum type, and that of these antigens albumin, transferrin, and group-specific component are mainly of maternal origin. However, it is not clear whether this conclusion applies to the proteins in amniotic fluid earlier in pregnancy. Likewise, little is known about the nature and origin of non-serum protein in amniotic fluid.

The object of the work presented in this thesis was to investigate the nature and origin of amniotic fluid protein throughout pregnancy. It was also an intention to examine any finding which might appear to be of value for the prenatal diagnosis of fetal disease.

IMMUNOLOGICAL STUDIES ON THE NATURE AND ORIGIN OF AMNIOTIC FLUID PROTEIN

MATERIALS AND METHODS

a. Introduction to the measurement of antibody-antigen complexes

A variety of direct and indirect methods can be used to observe antibody-antigen reactions *in vitro*. The selection of an appropriate method depends partly on whether quantitative or qualitative data are needed; and also on the solubility of the antigen, the completeness of the antibody (i.e. its ability to form insoluble antibody-antigen complexes *in vitro*), and the required sensitivity. The major methods fall into one of two categories. In the first, the insoluble antibody-antigen complexes are studied in 1mm thick agar or agarose gels. In the other, the quantity of antigen available to complex with antibody is measured indirectly, by measuring either the extent to which it competes for antibody binding sites with a purified, radiolabelled standard form of the same antigen (radioimmunoassay), or by measuring the amount of complement which is fixed during the formation of immune complexes between the antigen and immunoglobulins A and G.

In agar or agarose gel systems, the antibody-antigen complex exists as a precipitate which may be stained with protein stains after the soluble protein has been washed out with saline. The basic requirements for this approach is a *soluble* antigen (so that it can diffuse through the gel), and a complete antibody (so that the immune complexes are insoluble and thus form visible precipitates).

One of the simplest gel precipitation methods is the double diffusion method of Ouchterlony (1948) which is used mainly for studying the structural relationships between antigens using antisera of limited specificity. In this method, the antibody solution (antiserum) and appropriate antigen are placed in separate wells at between 5mm and 2cm apart in agar (or agarose) gel. An immune precipitate occurs after the antibody and antigen have diffused through the gel to meet each other. The position of the precipitate line depends upon the relative mobilities and concentrations of the proteins. Too high a concentration of antigen will result in the non-appearance of a precipitate, since most immune complexes are soluble in antigen excess. In the case of horse antibodies the complex is also soluble in antibody excess, but this is not true of rabbit or sheep antibodies.

Multiple reactions in the double diffusion system are indicated by the number of precipitation lines between the antigen and antiserum wells. In such cases it is usually not possible to make detailed studies on the individual bands without the aid of immunoelectrophoresis (Grabar and Williams, 1953; Scheidegger, 1955). The principle of immunoelectrophoresis is the same as in double diffusion, except that the antigens are first separated out by electrophoresis. After the electrophoretic step, the antiserum is then poured into a small trough which is cut in the gel close beside and parallel to the protein tract. The antibodies then diffuse into the gel and complex with antigen to form arcuate precipitates which approximately describe the concentration locus along one side of the antigen spot in the gel. The individual precipitates of different proteins cross over each other without interference (being reactions

of non-identity). Because the complex forms close to the outer border of the antigen, the precipitate's outer edge is usually smooth. Its inner edge is also smooth because the precipitate is soluble in antigen excess. Rather sharper lines can be obtained if horse antiserum is used, since the immune complexes are soluble in both antibody and antigen excess.

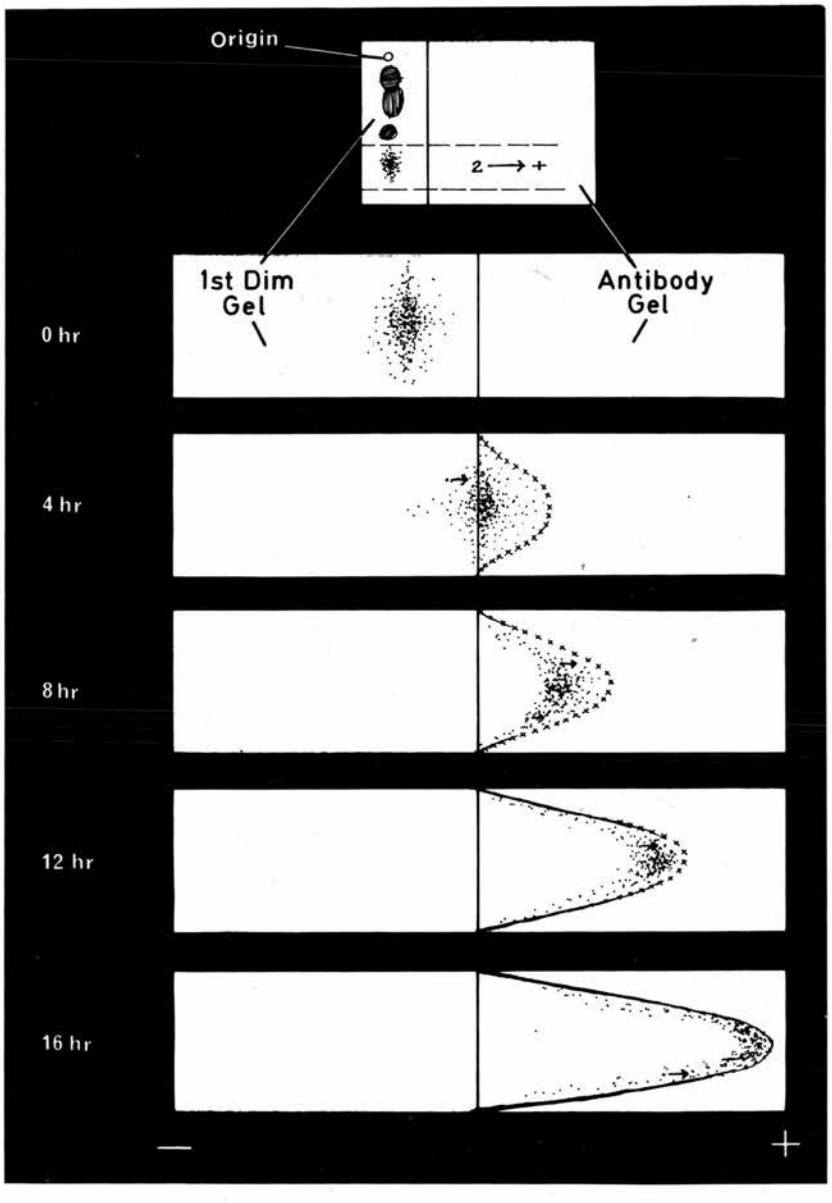
Unlike the Ouchterlony and immunoelectrophoretic methods, there are gel systems which are quantitative, or both quantitative and qualitative. With the exception of Oudin's diffusion velocity method (Oudin, 1948), they employ the following principle. A small zone of antigen is allowed to migrate (by either diffusion or electrophoresis) into a gel containing antibody. This migration results in a spreading of the antigen and a successive lowering of its concentration in the gel. When the antigen concentration is low enough for it not to be in excess, the antibody will complex with it and precipitate. Thus the area contained within the precipitate increases in proportion to the quantity of antigen present, provided a standard concentration of antibody is present in the gel. This is the basis of the radial immunodiffusion test (Mancini et al 1965) in which the sample is placed in a small well in the antibody gel, and diffuses radially to form a ring precipitate around the well during the following 3 days.

In multivalent systems, radial immunodiffusion is not appropriate, because the many concentric precipitates are difficult to distinguish. Antibody-antigen crossed electrophoresis (Ressler, 1961; Laurell, 1965; 1966; Freeman and Smith, 1970) resolves this problem, by first separating the antigens by electrophoresis into a track in a simple agarose gel without antibody. An agarose gel containing antibody

Figure 5 Diagram to show the development of immune precipitates in antibody-antigen crossed electrophoresis. See pp. 46-47.

The top diagram shows the hypothetical distribution of four antigens after a sample has been subjected to electrophoresis in the first dimension. The antibody gel has been laid on the right-hand side of the plate, and the system is now ready for the second dimension electrophoresis in the direction marked by the arrow. The strip of gel outlined by the dashed lines either side of the stippled antigen is considered in the lower diagrams.

The lower diagrams show the migration of the stippled antigen into the antibody gel at various times. The crosses show the areas in which antibody-antigen precipitation is prevented by the excess of antigen following. The solid line shows the development of the precipitate.


Origin
2 → +
1st Dim Gel
Antibody Gel
0 hr
4 hr
8 hr
12 hr
16 hr
—
+

is then laid beside the antigen track, and the antigens are driven into the antibody gel by further gentle electrophoresis (the so-called second dimension step) in a direction perpendicular to the first electrophoretic step (the so-called first dimension step). As an antigen migrates into the antibody gel (Fig. 5) the low concentration of antigen in front does not precipitate with antibody because the antibody-antigen complexes are dissolved by the majority of the antigen which follows (this occurs in the regions marked by the crosses in the diagramatic tracks of Fig. 5). Because of this process, the antigen only precipitates when most of it is spread out in a thin rocket-shaped band. The area under this band is directly related to the antigen concentration, and indirectly proportional to the concentration of antibody in the second dimension gel. Fig.5 shows that the majority of the antigen is involved in the formation of the rocket-shaped precipitate. This should be contrasted with the immune precipitate of immunoelectrophoresis which involves only a fraction of the total antigen present. It is this feature that makes quantitative antibody-antigen crossed electrophoresis (AACE) far more sensitive than immunoelectrophoresis.

Both gel methods are of considerable importance, as they allow electrophoretic studies to be made on genetic protein variants. AACE also permits individual proteins to be measured with confidence as separate peaks in agarose gel. Complement fixation, on the other hand, is a more sensitive method although it cannot be easily used to study individual proteins in the absence of monovalent antisera. It can, of course, be used to search for tissue specific antigens by using absorbed antisera. Radioimmunoassay is of no value in the search for

new antigens, since it requires a purified radio-labelled standard form of the antigen to be available. It is of most value when well characterised antigens (e.g. hormones) are to be measured at low concentrations.

b. Preparation and injection of antigen, collection and adsorption of antisera

Amniotic fluid was obtained from three sources. Samples from early in gestation were taken from hysterotomy sacs, and those from about 20-38 weeks were taken by amniocentesis at the clinic for patients with rhesus iso-immunisation. Collections of amniotic fluid at term were made using an amnioscope during artificial rupture of the membranes. The specimens of amniotic fluid were centrifuged at 125g for 10 mins., and were then concentrated x 10 by pressure filtration at 4°C using Sartorius membrane filters (pore size:5-15μ) at 130 p.s.i. During the concentration procedure a thick deposit of amniotic fluid solute accumulated on the filter and this was vigorously resuspended before the material was stored at -20°C.

Adult male New-Zealand white, and Dutch-cross rabbits were injected subcutaneously with 0.5 - 1.0 ml of x10 concentrated amniotic fluid at approximately three weekly intervals. The first three injections were dispersed in Freund's complete adjuvant (Difco). A week after the third injection, the rabbits were bled from an ear vein, and the antiserum was tested against amniotic fluid by immuno-electrophoresis and AACE. When antibodies of sufficient strength were present in the rabbit sera, injections were spaced to maintain

TABLE 3

	Tank buffer	Gel buffer
Diethylbarbituric acid	13.8 g	11.07 g
Na barbitone	87.6 g	70.07 g
Ca lactate	3.84 g	10.24 g
Methiolate	-	$^{1}/_{10,000}$*
Water	10L	10L
pH	8.6	8.6

*Final concentration of methiolate in buffer

the yield of antibody.

To search for antibodies against non-serum proteins using AACE, the antisera against amniotic fluid were absorbed with pooled adult (male) human serum. The following routine was used. To 15 ml of rabbit antiserum was added 20µl pooled adult human serum (PAHS). This was then centrifuged at 12,000 g for 10 mins at 4°C, and the supernatant was removed. To this supernatant was added 40µl PAHS, and the mixture was recentrifuged. This was repeated with respectively 80µl, 120µl, and 150µl of PAHS; and thereafter with about 8 cycles of 150µl PAHS. This gradual increase in PAHS concentration ensured that all the complete anti-PAHS antibodies were removed by the centrifugation. If the entire 1.61 ml PAHS had been added at once, some of the complete anti-PAHS antibodies would probably have been in antigen excess and so would not have precipitated. The methods for testing the completeness of the adsorption process is outlined on p.52.

c. Immunoelectrophoresis

Immunoelectrophoresis was carried out in 1% agar gels on 76 mm glass microscope slides by the method of Hirschfeld (1959,1960,1962). The concentrations of the buffer systems actually used in the gels and in the electrophoresis tanks are shown in Table 3 . However, the stock solution of gel buffer was made at double the final concentration. The agar gel was then made by boiling 2 gm of agar per 100 ml of water for 10 minutes, and then adding an equal volume of double strength gel buffer at 65°C.

d. Antibody-antigen crossed electrophoresis (AACE)

AACE was carried out using barbitone-calcium lactate buffer systems of the final concentrations shown in Table 3. Agarose gel at a final concentration of 1% were made up in the same way as agar gels, except that the 2% agarose/H_2O solution was boiled for approximately 45 minutes (at room pressure). After the equal volume of double strength stock gel buffer had been added and mixed, 10ml aliquots of the final gel solution were poured onto individual 3.25 inch square glass cover slides (Kodak) and allowed to set on a levelling table. The remainder of the liquid agarose gel was put in a stoppered tube in a water bath at 60-61°C.

If two-dimensional AACE was to be carried out, a single well of between 1.5 and 8µl capacity was punched in one corner of the gels after they had set (Fig. 6a). A wider bore Pasteur pipette made a suitable punch. The gels were then placed in Shandon (Kohn) tanks either on specially fitted cooling plates, or in a 4°C cold room. The tanks were filled with the appropriate buffer (Table 3) and the gels were connected to the tank buffers by filter-paper wicks. The sample-wells were then loaded and electrophoresis was begun in the 1st dimension at 6 v/cm along one margin of each gel (Fig. 6a). If the gels were laid on cooling plates in tanks at room temperature, their surfaces were slowly flooded by condensation occuring above them. This was avoided by suspending a long glass plate about 7 mm above the gels.

After about 2½ hours, the first dimension electrophoresis was stopped, and the gels were removed from the tanks. The gel to the side of the first dimension protein track was removed and replaced

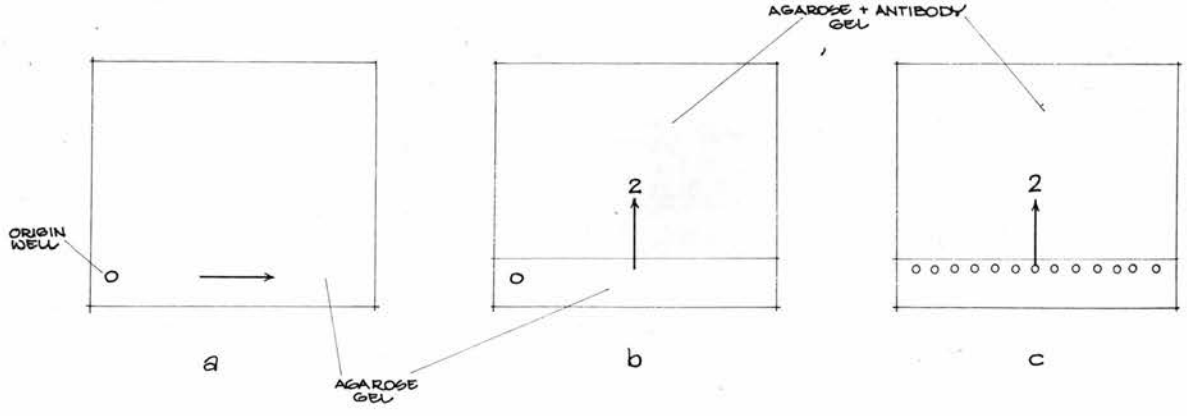
Figure 6 Antibody-antigen crossed electrophoresis (AACE)

a. Two-dimensional AACE : 1st dimension. The arrow shows the direction of the electrophoresis toward the anode.

b. Two-dimensional AACE : 2nd dimension. The antibody gel has been laid beside the 1st dimension track, and electrophoresis is carried out toward the anode in the direction of the arrow.

c. One-dimensional AACE. Many wells are cut in agarose gel, just behind the antibody gel. Electrophoresis is carried out in the direction of the arrow.

for details see pp.50-51.


AGAROSE + ANTIBODY GEL
ORIGIN WELL
2
2
a
b
c
AGAROSE GEL

with a volume of antiserum gel in proportion to the volume of gel removed (Fig. 6 b). The antiserum gel was made by adding a volume of antiserum to the liquid agarose gel which had been incubated at 60-61°C. The proportion of antiserum depended upon its specific antibody titre, although proportions in excess of 40% were difficult to use. After the second dimension gels had set, they were returned to the electrophoresis tanks for the second dimension electrophoresis. This was carried out for over 15 hours at 1.5 v/cm in a direction PERPENDICULAR to the first (Fig. 6 b), so that the proteins migrated toward the anode into the antibody gel.

For single dimension AACE, the first dimension electrophoresis step was unnecessary, usually because a monospecific antiserum was available. Thus, up to thirteen sample wells could be cut behind the antibody-gel, as show in Fig. 6 c. Electrophoresis in the second dimension was then carried out as usual at 1.5 v/cm.

At the end of the second dimension electrophoresis, the gels were removed from the tanks and immersed in several changes of physiological saline for between 24 and 48 hours. The gels were then drained, and numbered by slipping numbered pieces of paper between each gel and its glass plate. Filter-paper was then placed on top of each gel, which were then dried into a thin transparent film either at room temperature, or at a temperature less than 60°C. The dried gels were stained using 1% amido black in 50 : 40 : 10 methanol : water : acetic acid. The gels were destained in the same solvent without amido black, and were then dried.

For quantitative studies, the areas under the individual protein curves were measured by planimetry, using a light box and transparent

millimetre squared graph paper. Linearily studies were carried out on all the proteins measured, and these are reported in the individual sections following.

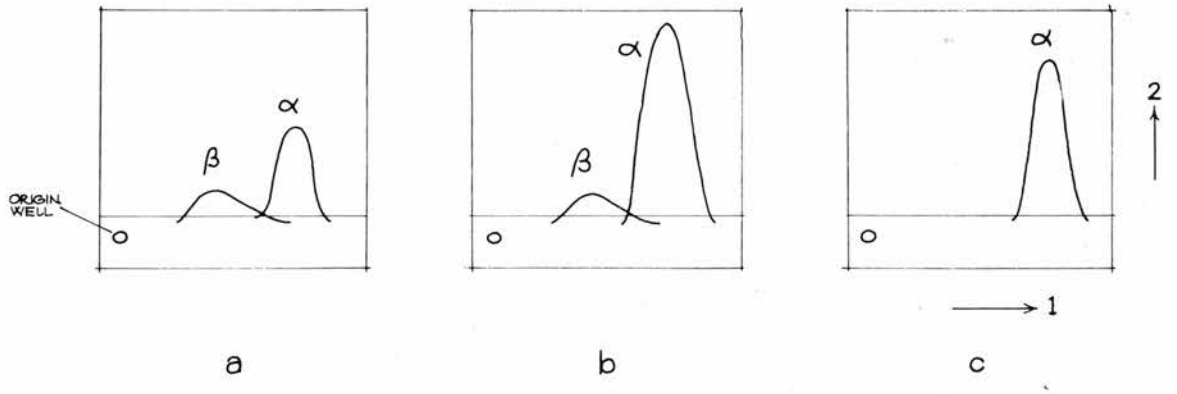
e. Testing the completeness of antibody absorption using AACE

After an antiserum against amniotic fluid protein (for example) had been absorbed with adult serum, it was tested by AACE against amniotic fluid, to search for non-serum proteins in amniotic fluid. When such proteins were found, it was important to show that they were not serum proteins which were precipitating because the antiserum had not been completely absorbed. This was tested by one or two-dimensional AACE, and is illustrated in Fig. 7. A known volume of the amniotic fluid sample was run both alone, and also with an excess of the absorbing antigen (i.e. adult serum) in the same well. Finally, the absorbing antigen was run alone. If the size of the precipitate found for the amniotic fluid sample was unaffected by the addition of an excess (8-10μl) of absorbing antigen, and if no peaks were found when the absorbing antigen was run alone, then the amniotic fluid antigen was regarded as being of non-serum type.

Figure 7 Testing the completeness of antibody absorption by two dimensional AACE.

Rabbit antibodies against hypothetical tissue extract A have been absorbed with serum, and Fig.7a shows that two proteins (α and β) are detected when a known volume of extract A is tested against the absorbed antiserum by AACE. In Fig.7b the same volume of extract and a sample of serum are placed in the same well, and subjected to AACE. The result is that the α peak increases in height whilst the β remains as in Fig.7a. This suggests that the antibodies against the α-protein have not been totally absorbed, and this is supported in Fig.7c where the addition of serum alone into the origin well results in a protein peak.

By the same reasoning, the β protein is not found in serum.


ORIGIN
WELL
β
α
o
a
β
α
o
b
α
o
c
2
1

THE MAJOR ANTIGENS IN AMNIOTIC FLUID

a. Introduction

It is not clear whether the major antigens in amniotic fluid before term are of serum or non-serum type. To investigate this question, amniotic fluids from various stages of pregnancy were concentrated and were injected into rabbits. The following sections describe the nature and quantitative behaviour of the major amniotic fluid antigens to which the rabbits responded.

b. Materials and Methods

i. Rabbit antisera were raised against x 10 concentrated amniotic fluids from various stages of gestation, using the methods summarised on pp. 48-49. Table 4 summarises the number of amniotic fluids injected, together with their corresponding gestational ages.

Antibody-antigen crossed electrophoresis (AACE) was used to study the specificities of the immune responses. Full methods for AACE, and for the absorption and testing of the rabbit antisera are given on pp. 50-52.

ii. Identification of antigens

Commercially produced monospecific antisera were obtained from Behringwerke AG for the identification of α_1-lipoprotein, α_1-antitrypsin, α_1- easily precipitable glycoprotein, α_2- HS glycoprotein, group-specific component (Gc), and haemopexin. Group specific component also showed its characteristic heterogeneity (Fig. 18), and α_1-lipoprotein was usually associated with a rapidly migrating degradation product. Albumin and transferrin were identified by their characteristic positions, and by their relatively intense precipitates.

TABLE 4

Summary of the rabbits injected with amniotic fluid protein, and of the appearance of antibody specifities

Rabbit	Breed	Antigen Injected	Maintenance dose (average)	Major Antibody Specificity		Minor Antibody Specificity	
				Nature	Time of appearance	Nature	Time of appearance (months)*
5Y4	Dutch Cross	Amniotic fluid at term, concentrated x 10	1ml/20 days	Serum protein	4–6 weeks	α_2-decidual protein β-protein	3(+), 5(+++) 4(+), 6(-)
6X1	" "	"	"	" "	6–9 weeks	α_2-decidual protein β-protein	5(++) 5(++)
6M6	" "	Amniotic fluid from between 12 and 20 weeks of gestation concentrated x 10	"	" "	7–9 weeks	β-protein	6(++)
5X5	" "	"	"	" "	6–8 weeks	none detected	6(-)
1	New Zealand White	Amniotic fluid from between 20 and 24 weeks of gestation concentrated x 10 note (A)	"	" "	5–6 weeks	none detected	4(-)(see note B)
5	"	37 week hydramnios specimen, normal baby delivered	"	" "	7–8 weeks	α_2-decidual	4(+)
6	"	"	"	" "	6–7 weeks	none detected	- (see note C)

Notes

(A) Mainly from a hysterotomy at 24 weeks.
(B) Injections stopped after 4 months because of lack of antigen
(C) Injections stopped after 5 months

*
(-) = Antibodies not detected.
(+) = Antibodies just detected, but too weak for detailed studies.
(++) = Antibodies detectable in quantities just adequate for detailed studies by AACE.
(+++) = Antibodies present in quantities adequate for detailed studies by AACE.

iii. Quantitation

The concentrations of albumin, α_1-antitrypsin, Gc, and transferrin were measured in amniotic fluid, in maternal and fetal sera, and in fetal urine by two dimensional AACE, using experimentally induced rabbit anti-amniotic fluid antisera. Adequate peaks were obtained for these serum proteins by using a 4cm wide second-dimension gel which contained 10-15% antiserum.

Between 2 and 4µl of amniotic fluid, and 1-2µl of 1/10 saline-diluted maternal serum and term fetal serum were used for each gel. Fetal sera obtained early in gestation were diluted up to 5 times with physiological saline.

The areas under the peaks were measured by planimetry. Fig.8 shows that the areas under the peaks for albumin, α_1-antitrypsin, Gc, and transferrin were proportional to the volume of amniotic fluid added. The concentrations of albumin, α_1-antitrypsin and transferrin were calculated from a 'technicon' standard serum. Gc was expressed in arbitrary units (mm^2) as no standard was available.

Figure 8 Linearity tests for serum proteins by AACE.

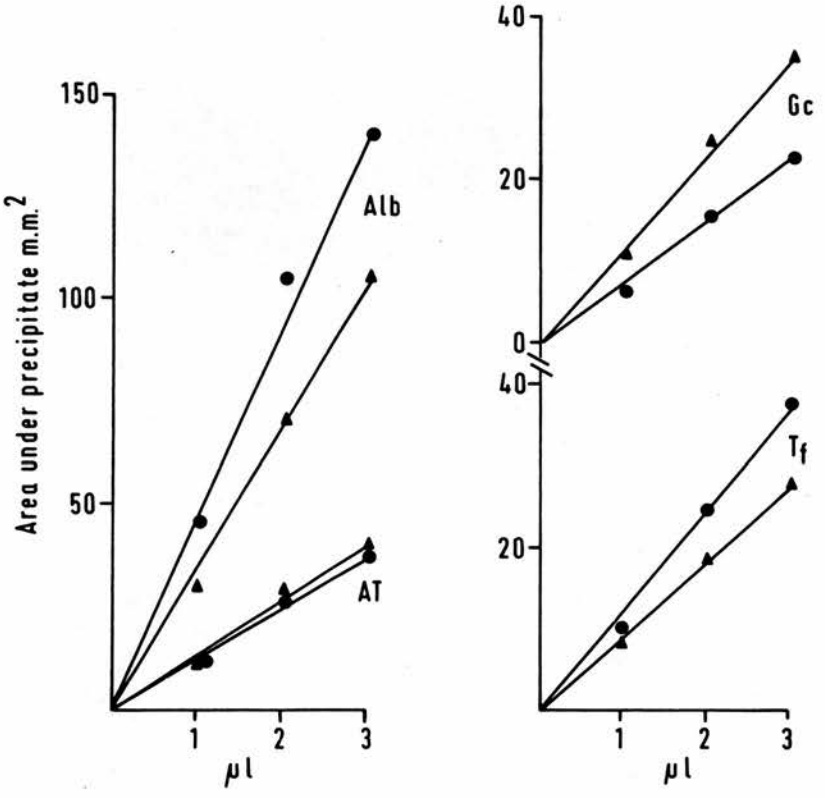
Varying volumes of amniotic fluid were subjected to AACE, and the areas under the protein precipitates (ordinates) were plotted against the volume of amniotic fluid applied (abscissa), for each protein.

Alb = Albumin

AT = α_1-antitrypsin

Gc = group-specific component

Tf = Transferrin


Area under precipitate m.m.^{2}
150
100
50
Alb
AT
1
2
3
µl
40
20
0
Gc
Tf

c. Results

The antisera which had been raised in rabbits against amniotic fluid were tested by AACE. Fig. 9 a shows the precipitation lines which were observed when a sample of amniotic fluid of twenty-four weeks of gestation was studied by AACE, using antiserum from a rabbit which had been immunised with the same fluid. Fig. 9 b shows the pattern observed in the case of a maternal serum at nineteen weeks of pregnancy using the same antiserum. A number of distinct precipitates were found, each corresponding to a separate protein. In order to prove that all the amniotic fluid proteins visible in Fig. 9 a were of serum type, the antiserum was absorbed with pooled adult male human serum. When the absorbed antiserum was tested by AACE against amniotic fluid, none of the precipitates shown in Fig.9a were present. This result was found in all the antisera studied, and indicates that the majority of the antibodies raised against amniotic fluid react with serum protein. Non-serum antigens could only be detected by using higher concentrations of antiserum, and these are described on pp. 66-71.

The strongest precipitates were identified as albumin, α_1-anti-trypsin, and transferrin by position and by the use of specific antisera. α_2-Group-specific component (Gc) was also found to be a prominent protein, showing characteristic heterogeneity (Fig.18). A number of unidentified proteins were also detected by AACE using rabbit anti-amniotic fluid antisera. Small quantities of easily precipitable α_1-glycoprotein, α_2-HS glycoprotein, haemopexin and the β_{1A-C} component of complement, were also identified by specific antisera, or, in the latter case, simply by its degradation product.

TABLE 5

The mean concentrations of the major serum proteins in amniotic flu
expressed as ratios of their mean concentrations in maternal and fe
serum, both early in gestation and at term.

	13-18 weeks of gestation		At Term	
	$MS/_{AF}$	$FS/_{AF}$	$MS/_{AF}$	$FS/_{AF}$
Albumin	9.6	3.2	25	27
α-Antitrypsin	18	4.4	35	17
Gc	15	2.1	32	15
Transferrin	14	2.0	47	15

MS = Maternal serum

FS = Fetal Serum

AF = Amniotic fluid

The concentrations of albumin, α_1-antitrypsin, Gc, and transferrin were measured in amniotic fluid by AACE and are shown in Figs. 10 - 13. The concentrations of all four proteins rose linearily from ten to twenty weeks, after which there was a plateau followed by a usually rapid fall in concentration between thirty weeks of gestation and term. The mean concentration ratios for these proteins between the maternal or fetal serum and the amniotic fluid are summarised in Table 5. The table shows that the protein concentrations in the sera exceed that in the amniotic fluid, and that the gradient between the fetal serum and the amniotic fluid is less than that between the maternal serum and the fluid. It is also clear that these gradients increase during gestation. To investigate possible relationships between the concentrations of the proteins in amniotic fluid with those in maternal serum, the respective concentrations of albumin, α_1-antitrypsin, and transferrin were expressed as a ratio to the Gc concentration and the ratio for each protein in amniotic fluid plotted against that for each protein in maternal serum (Figs. 14-16). By expressing the protein concentration as a ratio to Gc some of the variation of the data is reduced, as the measurement is independent of amniotic fluid dilution. Figs.14-16 show that in each case there is a correlation between the relative concentrations of the proteins in amniotic fluid with those in maternal serum.

d. Discussion

In this study, rabbits were challenged immunologically with amniotic fluid from various stages of gestation, in order to determine the nature of the amniotic fluid protein. The fact that the majority

Figure 9a AACE of a sample of 24 week amniotic fluid using antiserum from rabbit 1 (Table 4).

Figure 9b AACE of a sample of maternal serum from a patient in the 19th week of pregnancy.

A = Albumin

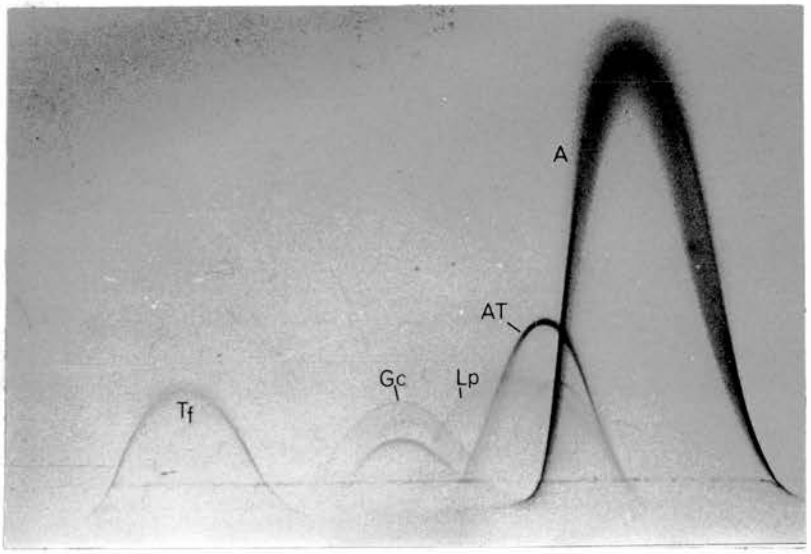
AT = α_1-Antitrypsin

Lp = α_1-Lipoprotein

Gc = Group-specific component

Tf = Transferrin

9a



9b

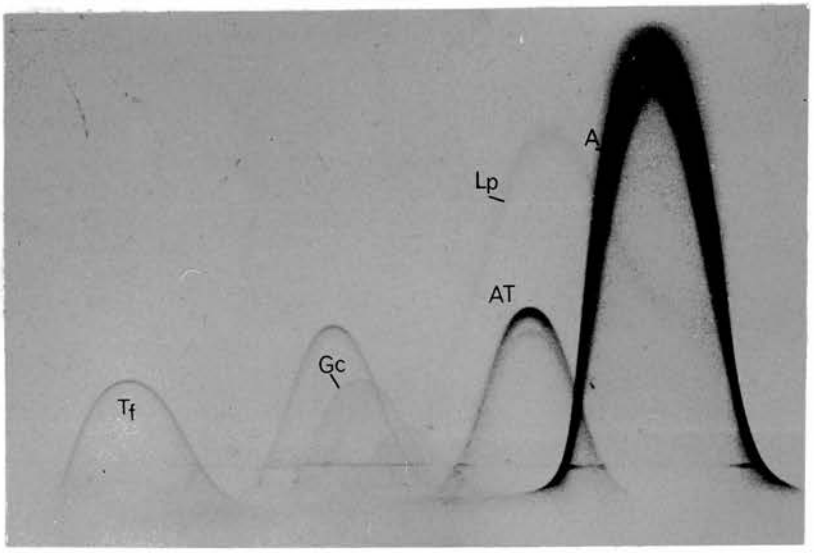


Figure 10 The concentration of albumin in amniotic fluid, measured by two-dimensional AACE. Concentrations expressed in mg/ml.

Figure 11 The concentration of α_1-antitrypsin in amniotic fluid, measured by two-dimensional AACE. Concentrations expressed in mg/ml.

Symbols for Figs.10-13

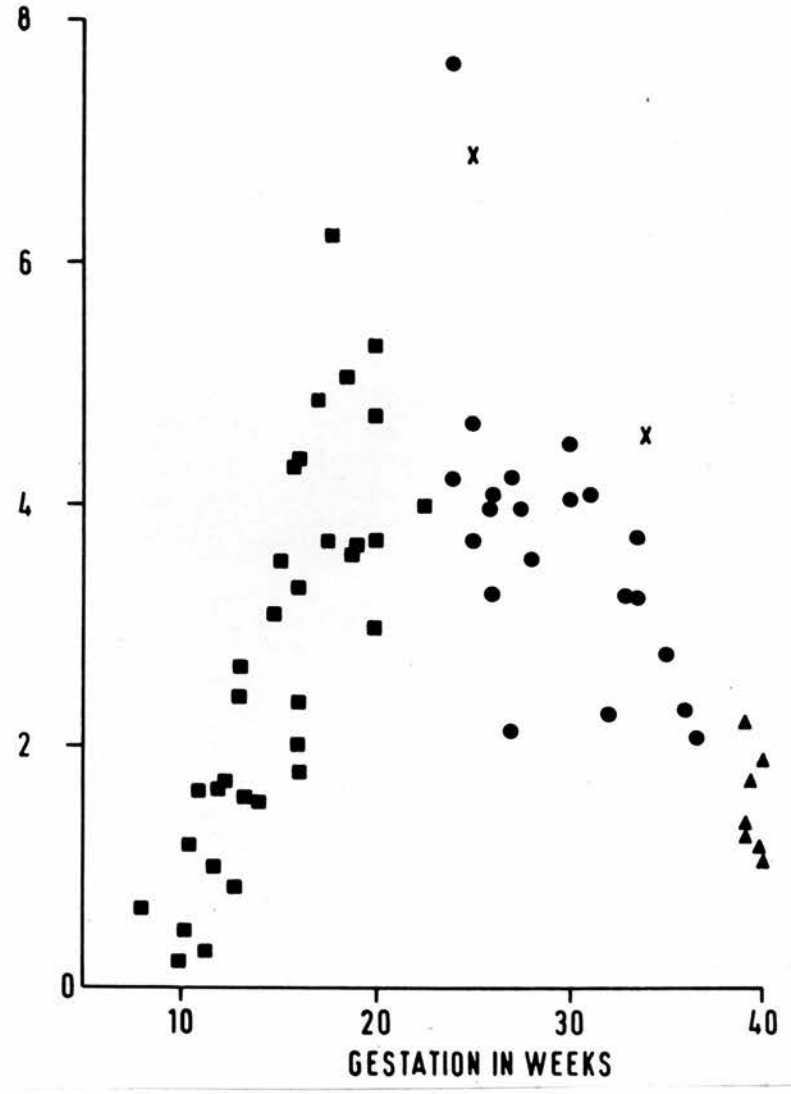
■ = Hysterotomy

● = Amniocentesis

▲ = Arteficial Rupture of Membranes, Normal pregnancy

X = Arteficial Rupture of Membranes, Anencephaly

10


8
6
4
2
0
10
20
30
40
GESTATION IN WEEKS

11

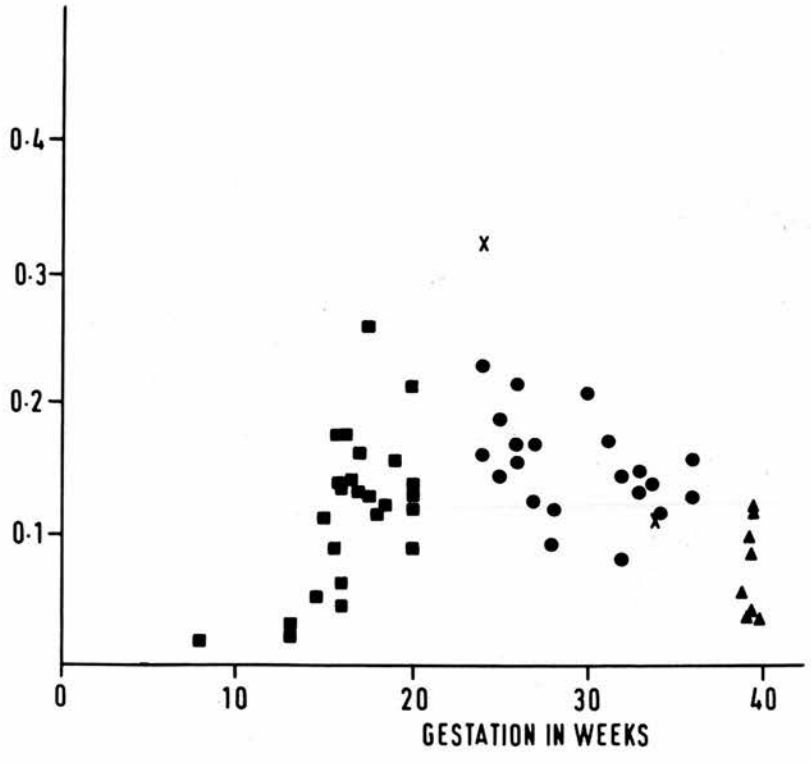
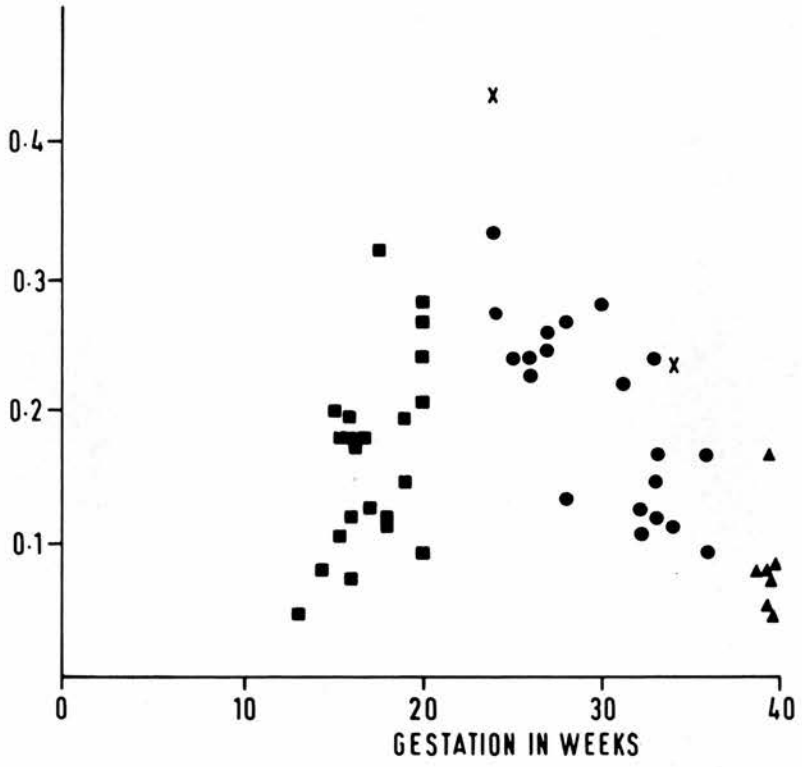

0·4
0·3
0·2
0·1
0
10
20
30
40
GESTATION IN WEEKS

Figure 12 The concentration of group-specific component in amniotic fluid, measured by two-dimensional AACE and expressed in arbitrary units.

Figure 13 The concentration of transferrin in amniotic fluid, measured by two-dimensional AACE. Concentrations expressed in mg/ml.

12


0·4
0·3
0·2
0·1
0
10
20
30
40
GESTATION IN WEEKS

13

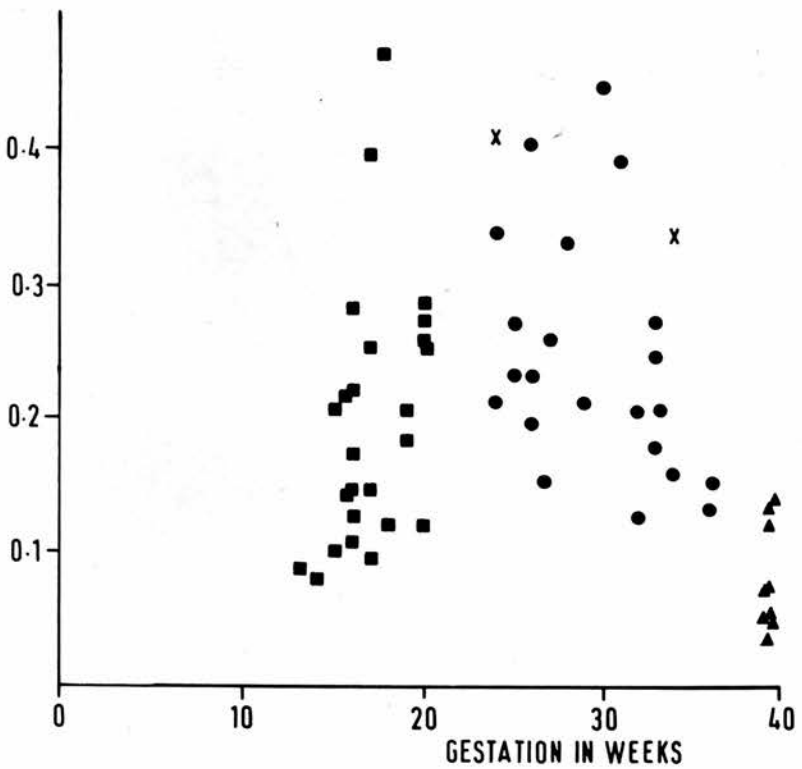
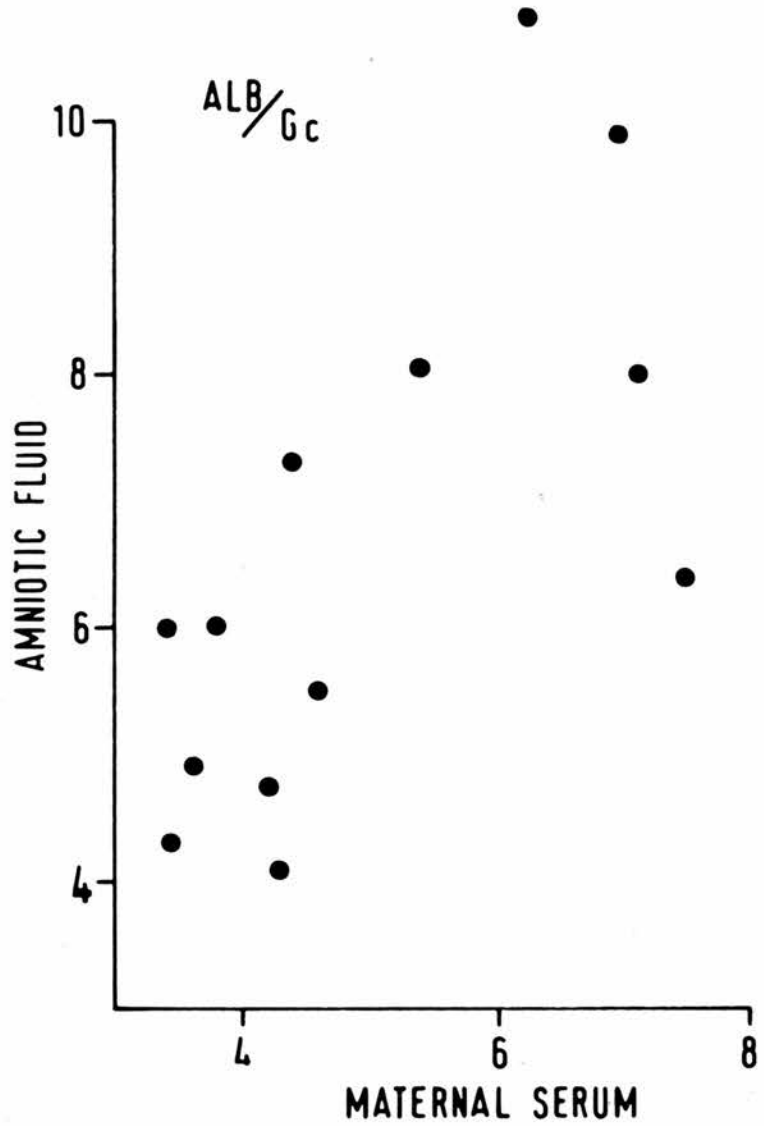

0·4
0·3
0·2
0·1
0
10
20
30
40
GESTATION IN WEEKS

Figure 14 The ratio of the concentration of albumin : Gc in maternal sera, plotted against the ratio of albumin : Gc in their respective samples of amniotic fluid.

Figure 15 The ratio of the concentration of α_1-antitrypsin : Gc in maternal sera, plotted against the ratio of α_1-antitrypsin : Gc in their respective samples of amniotic fluid.

14


ALB/Gc
10
8
6
4
AMNIOTIC FLUID
4
6
8
MATERNAL SERUM

15

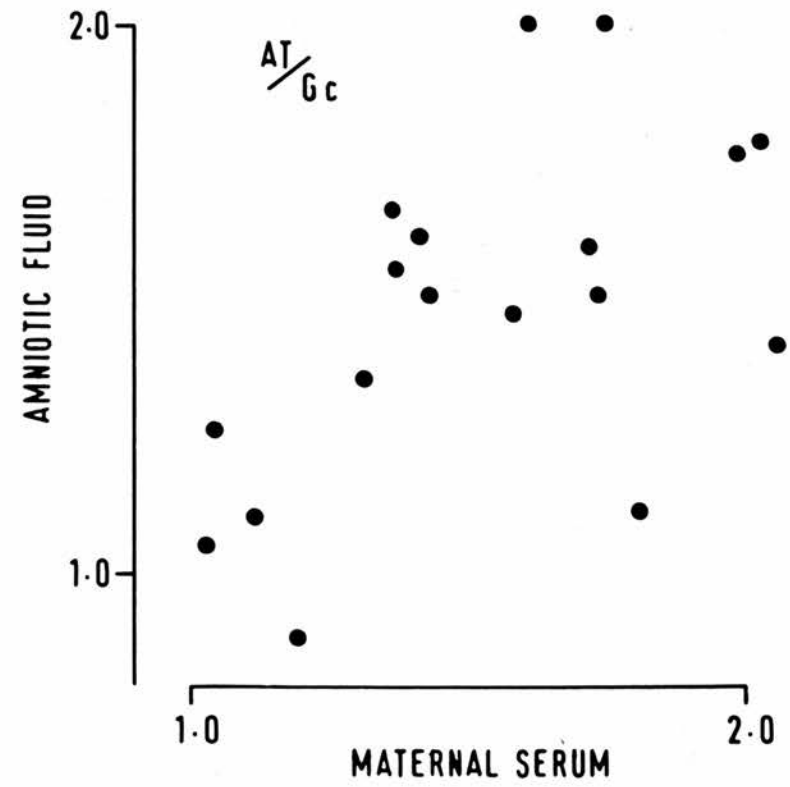
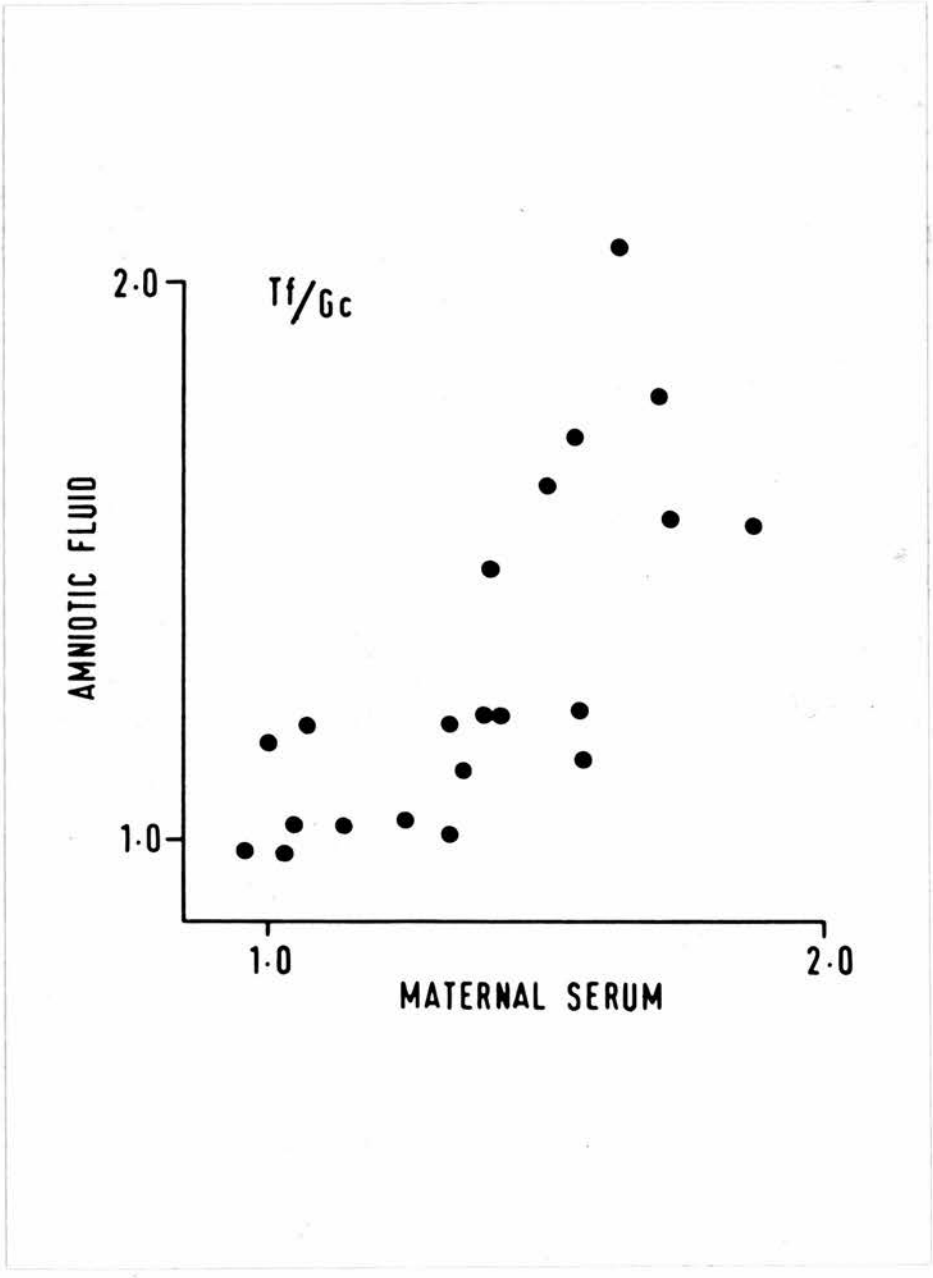

AT/Gc
2·0
1·0
AMNIOTIC FLUID
1·0
2·0
MATERNAL SERUM

Figure 16 The ratio of the concentration of transferrin : Gc in maternal sera, plotted against the ratio of transferrin : Gc in their respective samples of amniotic fluid.


Tf/Gc
2·0
1·0
AMNIOTIC FLUID
1·0
2·0
MATERNAL SERUM

of AACE precipitates were abolished by adsorbing the anti-amniotic fluid antisera with adult human serum suggests that the majority of the 'complete' rabbit antibodies reacted with serum proteins. Figs. 10-13 show that before about thirty-five weeks the concentrations of four of the major serum proteins in amniotic fluid change in a manner similar to those of the protein concentration measured by the biuret (Queenan et al 1970) and Lowry methods (Fig. 48). The fact that after thirty-five weeks the fluid protein concentration remains approximately level, whilst the concentration of albumin and the other serum proteins continue to fall (Figs. 10-13), suggests that there is an increasing contribution of non-serum protein into the amniotic fluid after thirty-five weeks. Enzymological evidence has been put forward (pp. 114-116) for a tissue contribution to the protein of amniotic fluid, both early and later in gestation, though no immunological evidence has so far been found (pp. 117-118) to support this.

The origin of the serum proteins in amniotic fluid has been the subject of a number of enquiries. At term, the studies on amniotic fluid albumin, Gc, haptoglobin, transferrin and IgA leave little doubt that there is a major maternal contribution at this time (pp. 31-34). Yet, conflicting evidence exists for the serum proteins early in gestation. Gitlin and Boesman (1966) reported α-fetoprotein to be present in amniotic fluid and fetal urine in fetuses up to thirteen weeks of gestation. They showed that the ratio of albumin : α-fetoprotein in fetal urine was less than that in amniotic fluid, suggesting that there was an alternative (perhaps maternal) source of the amniotic fluid albumin. Their findings have been extended in this thesis

(Fig. 33). However, Brzezinski, Sadovski and Shafrir (1964) have suggested that the albumin in amniotic fluid is of fetal origin. They claimed to have found a case in which a normal mother had a bisalbuminaemic fetus, which expressed its extra albumin band in both fetal serum and amniotic fluid. These observations were made by descending boundary electrophoresis: a non-specific technique which could offer no evidence that the extra protein peak was a variant form of albumin. Significantly, the fluid in question appeared to be lacking β and γ-globulins. The major β-globulin in amniotic fluid is transferrin, and in our experience (Fig. 13) this protein is invariably present in fluids at six months of gestation. It is therefore unlikely that the β-globulins were absent, and probable that they were present in the most slowly migrating protein band. This being so, the α_1 and α_2 bands would be displaced to lie close behind albumin so that these three bands would become superimposed. Such antefacts of migration rate can occur as a result of protein concentration procedures, and would produce novel protein patterns.

The following section (pp.61-65) shows that the Gc in amniotic fluid is of maternal origin throughout gestation, and that it enters the fluid by crossing the amnion and chorion. The close similarity between the concentration pattern of amniotic fluid Gc and the patterns of albumin, α_1-antitrypsin, and transferrin, suggests that all four proteins are mainly of maternal origin throughout gestation. Two other lines of evidence support this suggestion.

Table 5 shows the concentration gradients for the serum proteins between the sera and the amniotic fluid. Early in gestation, the concentration ratios lie between 9.6 and 18, and these gradients

increase to between 25 and 47 at term. The rather smaller gradient found early in pregnancy is found for all the proteins, including Gc which is known to be of maternal origin (pp.61-65). In contrast, α-fetoprotein (AFP) has a fetal serum : amniotic fluid ratio of about 200 : 1 at between 13 and 18 weeks of pregnancy*. This is despite the fact that AFP is detectable in early fetal urine, whereas the other serum proteins, except albumin, are not detectable in urine by AACE.

An experiment was carried out to test the possibility that the four major serum proteins in amniotic fluid were mainly of maternal origin between twenty and thirty weeks of gestation (when their concentrations in amniotic fluid are maximal). The concentrations of albumin, α_1-antitrypsin and transferrin in maternal serum and amniotic fluid were expressed as a ratio of the Gc concentration in the respective samples. Figs.14-16 show that there was a positive relationship between the protein ratios in the respective maternal sera and amniotic fluid samples. Thus, variations in the concentrations of maternal serum proteins result in similar changes in the amniotic fluid proteins. In all three cases, there was an approximate 1 : 1 proportionality between the relative protein concentration changes in maternal serum and amniotic fluid. Such a proportionality was also found by Derrington and Soothill (1961) for the transferrin : albumin and ceruloplasmin : γ-globulin relationships between maternal serum and amniotic fluid at term. Collectively, these findings suggest that a large proportion of the amniotic fluid serum protein is of maternal origin. In the light of the genetic studies on Gc, it is difficult to suggest that a large fetal serum contribution occurs.

* see p.77

However, the presence of albumin in fetal urine (Gitlin and Boesman, 1966; and Fig. 33) and in umbilical cord (p. 39) shows that this protein may have dual origin early in pregnancy.

THE ORIGIN OF AMNIOTIC FLUID GROUP-SPECIFIC COMPONENT

a. Introduction

Although it has been shown that the majority of the protein in amniotic fluid is of serum type, it is not clear whether it is of fetal or maternal origin before term. Group-specific component (Gc) is a polymorphic α_2-globulin which is present in amniotic fluid throughout gestation (Fig.12). The relatively high frequency of heterozygotes for Gc has made this an ideal candidate for genetic studies into the origin of serum proteins in amniotic fluid, and it has already been shown that the Gc in amniotic fluid *at term* is of maternal origin (Ruoslahti *et al* 1966; Usategui-Gomez and Morgan, 1966). This section describes an investigation into the origin of the Gc in amniotic fluid before term, by comparing the Gc phenotype of the fluid with that of the corresponding maternal serum.

b. Introduction to Methods

Group-specific component (Gc) was first identified immuno-electrophoretically by Hirschfeld (1959) because of its characteristic heterogeneity in human serum. It is a polymorphic α_2-glycoprotein, with two common alleles Gc^1 and Gc^2. The frequency of heterozygotes for Gc (Gc 2-1) is relatively high in most populations (Giblett, 1969) being approximately 38% in Edinburgh (p.65). Serum phenotyping for Gc is usually carried out by immunoelectrophoresis (Hirschfeld, 1959; Reinskou, 1963) or, more occasionally, by starch gel or acrylamide gel electrophoresis (Bearn *et al* 1964; Kitchin and Bearn, 1966). However none of these techniques are quantitative, and difficulties can arise

when the Gc is in low concentration or substantially degraded (Nerstrøm, 1963; Nerstrøm et al 1964). Previous phenotyping studies on the Gc in term amniotic fluid have employed immunoelectrophoresis and have used concentrated fluid (Ruoslahti et al 1966; Usategui-Gomez and Morgan, 1966). In our hands, immunoelectrophoresis could often be used for reliable Gc phenotyping of amniotic fluid (see Fig.17) but each sample had to be run three or four times for results of acceptable quality. Quite frequently, samples of fluid were found which produced consistently uninterpretable patterns, because the Gc, α_1-antitrypsin and albumin precipitates became continuous. As this effect was probably an artefact due to the concentration procedure, an alternative method of phenotyping was developed. AACE was the method chosen, since Gc was detectable using 2-4µl of unconcentrated amniotic fluid (Fig. 9a). The reasons for the increased sensitivity of AACE compared to immunoelectrophoresis are discussed on p.47 It has previously been pointed out that antigen-antibody crossed electrophoresis (AACE) shows Gc as a heterogeneous protein (Kitchin, 1965; Weeke, 1970).

c. Development of AACE for phenotyping Gc

AACE was carried out at 4°-8°C on 3.25 inch square glass plates using a 1% agarose gel and the buffer systems of Hirschfeld (1960) as described on p.50. In routine experiments, the sample was electrophoresed in the 1st dimension for up to 2½ hours at 6v/cm, along one margin of the gel. After this, the gel beside the protein track was replaced with 1% agarose gel containing 5-15% of rabbit antiserum (anti-amniotic fluid). The second dimension electrophoresis was then

carried out perpendicular to the first at 1.5v/cm for over 15 hours.

Fig.18 shows the immune precipitates of the three common Gc phenotypes obtained by AACE. In these experiments, 2µl of amniotic fluid was placed in the antigen well and run in the 1st dimension for 2 hours at 6v/cm. No rapidly migrating (α_1) components of Gc were found in any of the amniotic fluids tested, though these were often found in haemolysed sera, as has been previously reported by Nerstrøm (1963) and Nerstrøm et al (1964). Figs.19 a and 19 b show the Gc pattern obtained from such a sample. The α_1-Gc degradation product is clearly visible, and prolonged electrophoresis in the 1st dimension was needed to separate it from the α_2-Gc proteins. However, after $2\frac{1}{2}$ hrs at 6v/cm, the anodal end of the gel began to shrink, presumably as a result of endosmosis. This vaused the cathodal protein trails of albumin and the α_1-proteins which are visible in Fig.19 a. To prevent this, the 1st dimension electrophoresis was interrupted after $2\frac{1}{4}$ hrs and the gel ahead of the albumin band was replaced with fresh gel solution. After this had set, the electrophoresis was continued for a further $2\frac{3}{4}$ hrs, after which the second dimension gel was poured in the usual way. Fig. 19 b shows that the gel-replacement technique prevented the albumin and α_1-protein trails, and simplified the phenotyping of the Gc protein.

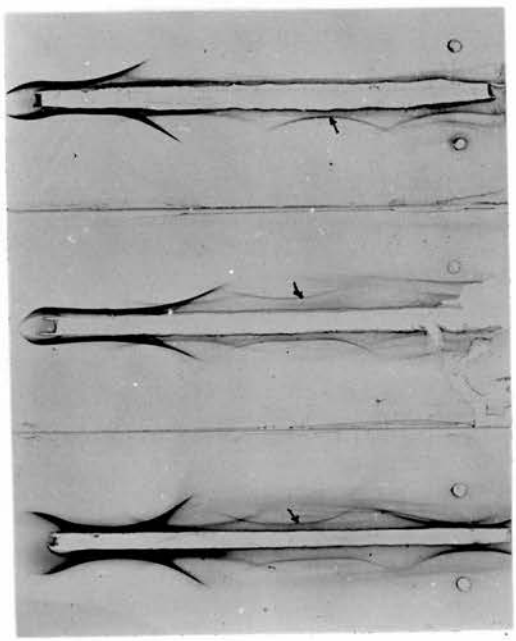
These results show that AACE is a potent tool for carrying out Gc phenotyping in the presence of haemolysis, or when the concentration of Gc is as low as, for example, in amniotic fluid. Concentrated amniotic fluid seems to be an ideal antigen for eliciting a multivalent rabbit antiserum with relatively high titres of anti-Gc antibodies. This is probably because the majority of soluble amniotic fluid

Figure 17 Gc phenotyping of amniotic fluid by immunoelectrophoresis.

10 x concentrated amniotic fluid was studied using the methods on p.49.

Unabsorbed antiserum from rabbit 5Y4 was used in the troughs.

Top run	upper well	-
	lower well	- Gc 2-2
Middle run	upper well	- Gc 1-1
	lower well	- Gc 2-1
Lower run	upper well	- Gc 1-1
	lower well	- Gc 2-1



17

Figure 18 Sections of AACE gels showing the three common Gc phenotypes (arrowed peaks).

Figure 19 Fetal serum proteins subjected to prolonged AACE. Run (a) was carried out without 1st dimension gel replacement; run (b) was made with gel replacement. Note Gc^1 and Gc^2 peaks, and the α_1 - Gc degradation product (D.P.)

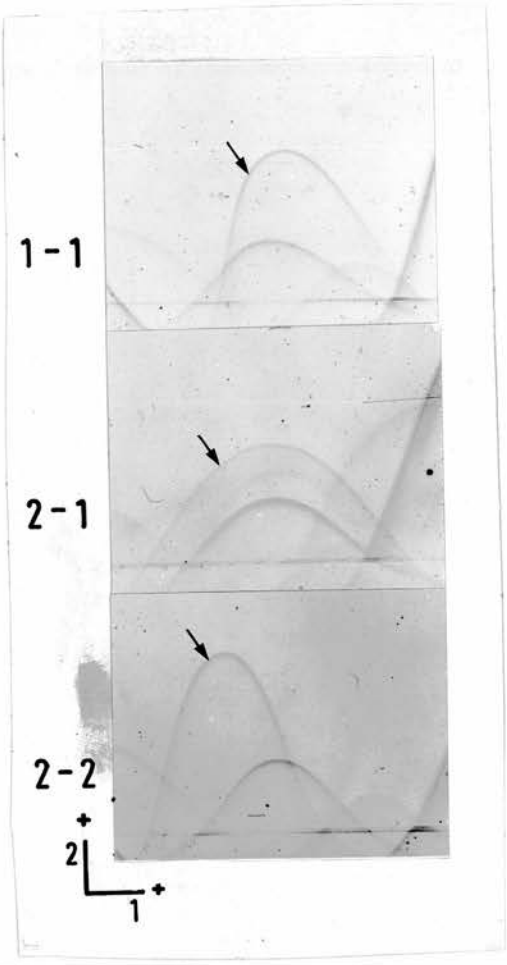
A = Albumin

AT = α_1-Antitrypsin

Gc = Group-specific

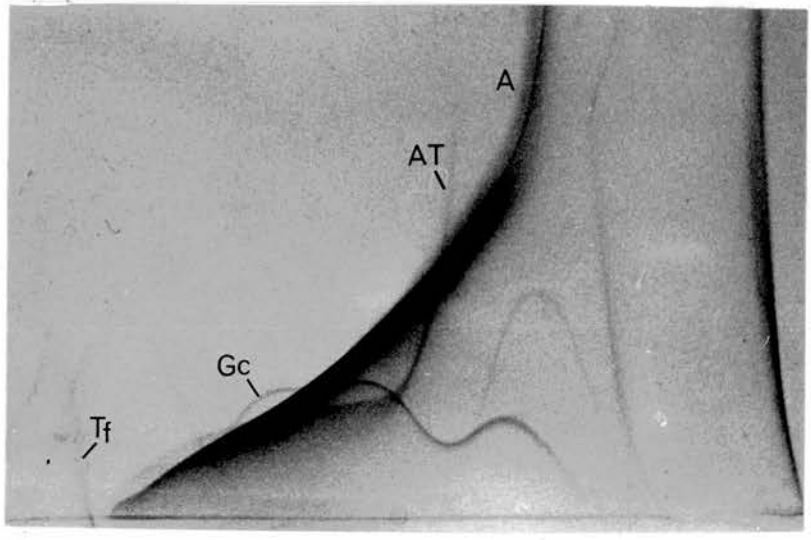
Tf = Transferrin

18



19

a



b

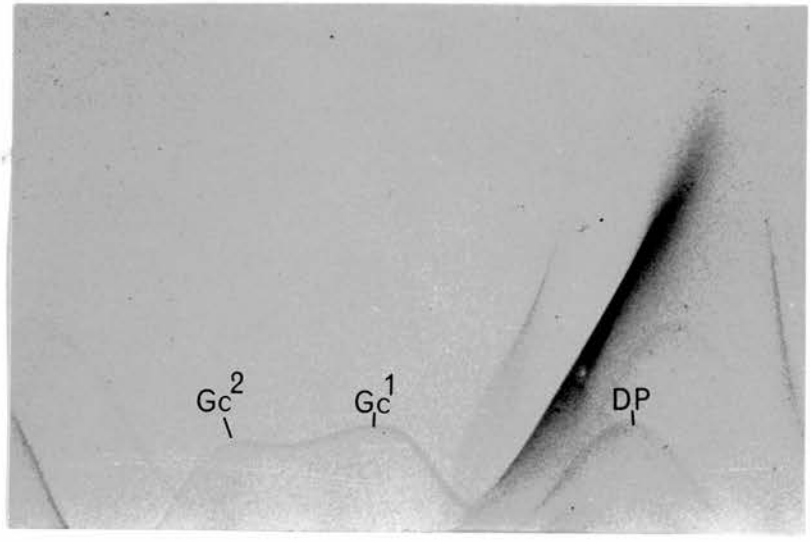


TABLE 6

The number of pairs studied in which concordance or discordance for Gc phenotype was found between the amniotic fluid and maternal serum, broken down according to gestational age and maternal Gc phenotype.

Gestation weeks	Concordant pairs Maternal Gc phenotype			Discordant pairs All phenotypes
	1-1	2-1	2-2	
10-18	11	8	2	0
19-27	5	5	2	0
28-38	14	8	1	0
Phenotype frequency	.54	.38	.09	

TABLE 7

Showing the Gc phenotype of the amniotic fluid in cases of discordance between maternal and fetal serum.

Case	Gestation	Gc phenotype		
		Maternal serum	Amniotic fluid	Fetal serum
1	15	1-1	1-1	2-1
2	16	1-1	1-1	2-1
3	16	1-1	1-1	2-1
4	24	1-1	1-1	2-1

antigens are those of serum protein (Derrington and Soothill, 1961; Von Kleist et al 1968; see pp.53-60) whose molecular weights are mainly less than about 170,000 (see pp.36-37, and Usategui-Gomez et al 1966). Since Gc has a molecular weight of about 50,000 (Schultze and Heremens, 1966) it will be enriched in amniotic fluid relative to the higher molecular weight proteins, and will therefore occupy a greater proportion of the rabbit's immune response when obtained from amniotic fluid rather than from serum.

d. Results

The Gc phenotypes observed in amniotic fluid and maternal serum are shown in Figs. 18-19. Table 6 shows that concordance of phenotype was found in all of the fifty-six maternal serum-amniotic fluid pairs studied. Amongst the cases summarised in the top line of Table 6 were eleven in which a sample of fetal serum was available for phenotyping. In four of these cases, discordance was found between the fetal and maternal phenotypes, and in each case, the phenotype of the amniotic fluid Gc was that of the mother (Table 7) Fig. 19a and b both show the fetal serum phenotype in case 2 in Table 7, the amniotic fluid phenotype in this case is shown in the top panel of Fig. 18.

e. Discussion and Conclusions

Polymorphism studies on Gc protein have shown that the protein in fetal serum is synthesised by the fetus from ten weeks of gestation onwards (Melartin et al 1965). Thus, in cases where the fetal phenotype is discordant with the mother's, the amniotic fluid Gc would be concordant with the maternal phenotype if it is of maternal origin, but discordant if it is of fetal origin. In the latter case, the expected frequency of discordance between the fetal and maternal phenotypes is equal to the heterozygote frequency in the population (Elandt-Johnson, 1971). From the frequency of heterozygotes (Table 6) approximately 38% discordance in at least one of the gestational groups is expected if there is a major fetal contribution to the Gc of amniotic fluid. Table 6 shows that no discordant cases were found. Furthermore, in four cases between ten and eighteen weeks of gestation, in which the fetal phenotype was known to be discordant from the maternal type, discordance was found between the fetal and amniotic fluid phenotypes.

From these results it is concluded that throughout gestation the Gc in amniotic fluid is of maternal origin, and that it enters the fluid by passing through the placental or reflected membranes. This finding sounds a cautionary note for any attempts to carry out prenatal diagnoses of genetic disease either by direct screening of serum proteins, or by linkage analysis using serum protein polymorphisms.

NON-SERUM ANTIGENS IN AMNIOTIC FLUID

a. Introduction

Although it is clear that the major antigens in amniotic fluid are of serum origin (pp.53-60), the search for minor antigens is of importance since some of them may be tissue specific, and would therefore provide a way of measuring the contribution of a particular tissue to the protein in amniotic fluid. Previous immunological studies on the non-serum proteins in amniotic fluid have been carried out by Lambotte and colleagues and von Kleist et al (1968) (see pp.22-24). These workers found α_1 and α_2 amnion-specific proteins in amniotic fluid by using immunofluorescence and immunoelectrophoresis, although they made no attempt at quantitative studies. In the experiments to be described in this section, rabbit antisera against human amniotic fluid were absorbed with serum, and the antibodies against non-serum protein were sought by AACE*.

b. Materials and Methods

Rabbits were immunised with amniotic fluid according to the schedule on p.48 and Table 4. After the rabbits had been bled, the antiserum was absorbed with pooled adult male human serum as described on p.49. The resultant absorbed antiserum was then tested against 10x concentrated and unconcentrated amniotic fluid by AACE. The method for AACE is described on p.50.

Many of the antisera had very weak antibodies against non-serum antigens, so that high concentrations of antiserum had to be occasionally used in the second dimension AACE gel. This necessitated prolonged

* Antibody-antigen crossed electrophoresis

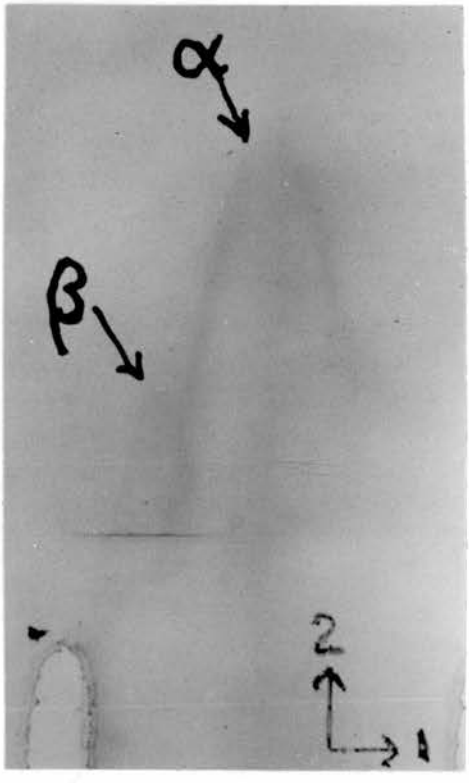
washing of the gel, and sometimes resulted in distortions of the second dimension gel. To prevent the distortions confusing the results of quantitative studies, a number of standard antigen preparations were run in each gel, and tests were made to ensure that the areas under the immune precipitates were proportional to the quantity of the antigen applied to the gel. Such tests are reported in the results.

c. Results

As noted in Table 4, antibodies against non-serum protein in amniotic fluid appeared considerably later than those against serum protein. Only three rabbits developed antibodies against non-serum proteins in concentrations which were of use for specificity studies. AACE was used to investigate the specificities of these antisera, using amniotic fluid as antigen. In two antisera (5 Y 4, 6X1, see Table 4) two precipitates were found when amniotic fluid was used as antigen (Fig.20). After three months, the antibodies to the more cathodally migrating protein virtually disappeared in rabbit 5 Y 4, whilst the antibodies against the anodal protein became stronger. Fig.21a shows an AACE experiment to investigate the mobility of the anodal protein using 5Y4 antiserum absorbed with pooled adult human male serum. To compare the mobility of the anodal protein with serum protein, an immunoelectrophoretic run has also been carried out in agarose gel beside the 1st dimension of the AACE run. The mobility of the anodal protein is close to that of Gc 1-1, and it was therefore referred to as an α_2-protein. Fig. 21a is also noteworthy as two precipitates are visible: one under the other. This was not found in other samples of amniotic fluid (see Fig. 21b).

Figure 20 Two dimensional AACE of amniotic fluid (10μl) using absorbed 5Y4 antiserum. The first dimension was carried out for 50 minutes. Two peaks were found, the most anodal is labelled α and the slower migrating is labelled β.

The arrows indicate the direction of the anode for the first and second dimensions.



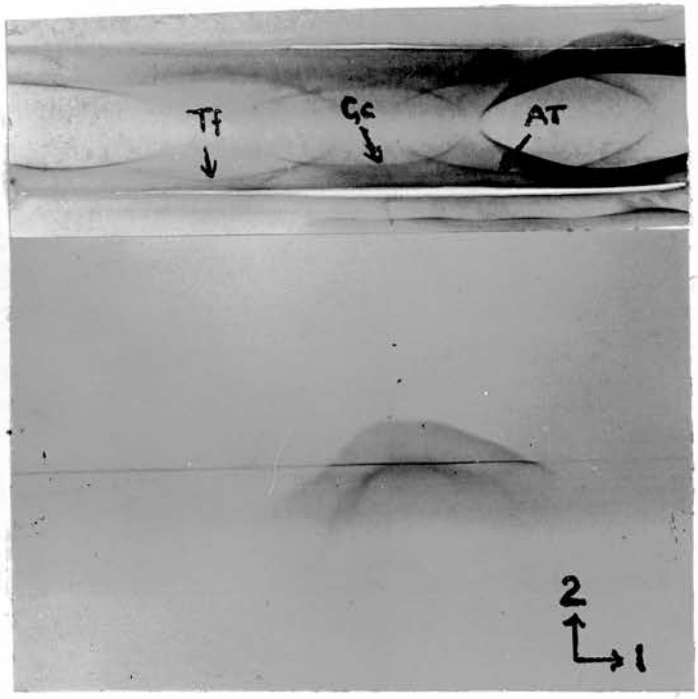
20

Figure 21 The mobility of the α-protein in amniotic fluid, tested by two-dimensional AACE using absorbed antiserum 5Y4.

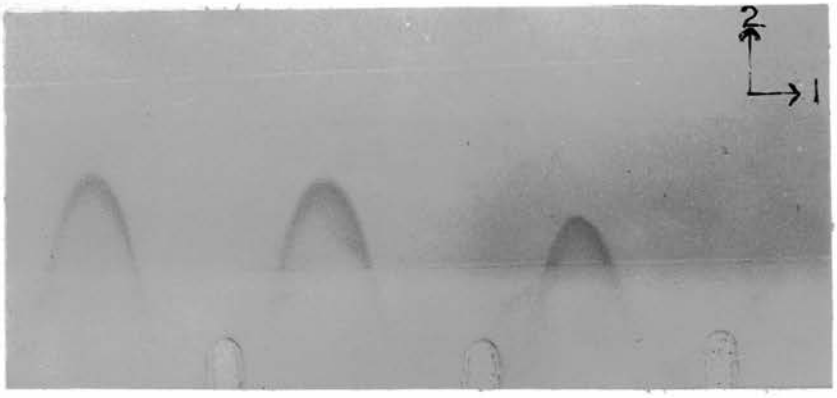
a) Shows the mobility of the α-protein compared with an immunoelectrophoresis slide of serum protein. The immunoelectrophoresis was carried out in the agarose of the AACE plate, parallel to the first dimension run, and was therefore under identical conditions. After the first dimension step, the immunoelectrophoresis gel strip was removed to allow the pouring of the antibody gel. After the strip had been removed, an immunoelectrophoresis trough was cut in it in the usual way. The mobility of the α-protein corresponded to Gc 1-1, and it was therefore called an α_2-protein

b) AACE of the α_2-protein using absorbed 5Y4 antiserum. The first dimension was run for 50 minutes.

21



a



b

It was essential to show whether the α_2-protein was a serum protein, and this was tested by two-dimmensional AACE in Fig.22. The absence of a precipitate when term maternal serum was run alone (track c) shows that the absorption of the antiserum was complete, and that the protein is not detectable in maternal serum at term.

Experiments were next carried out to investigate the possible tissue origin of the α_2-protein in amniotic fluid, by making 30% w/v saline extracts,from various tissues and running the samples against absorbed antiserum in AACE. α_2-Protein was found in two samples of uterine decidua obtained at sixteen weeks of pregnancy (at hysterotomy), and in a sample of decidua at term (obtained at caesarian section). The protein was also found in traces in amnion and chorion early in gestation (15-18 weeks), however, it was not detectable in these tissues at term. The protein was not detected in maternal or fetal sera at any stage of pregnancy, or in umbilical cord, placenta, or in fetal skin and liver. Fig.23 shows a two dimensional AACE in which an extract of 16 week decidua and amniotic fluid are run separately (tracks a and c respectively) and together (track b). The increase in peak height in track b indicates a reaction of identity. If the proteins in the two samples had not been identical the two precipitates would have been found separately in track b.

Immunofluorescent studies were made to attempt to see if the α_2-protein was located within the cells of the decidua. The method of indirect fluorescent labelling was employed, using FITC-labelled sheep anti-rabbit -globulin (Burroughs Wellcome). The labelled sheep antibodies were used to locate the regions in which the specific rabbit anti-α_2-protein antibodies had been bound to the surface of frozen sections of decidua. The control sections were exposed to the serum of rabbits which had not been immunised. When the sections were viewed under the fluorescent microscope, fluorescence was only found in the slides which had been exposed to the rabbit-α_2-protein antibodies, virtually none was visible in the controls. A clear green fluorescence was found in the former sections in about 20-40% of cells, and the

distribution of staining did not correspond to the edges of the uterine glands. This experiment was carried out on two samples of decidua (Obtained at 16 and 40 weeks gestation respectively), and showed that the α_2-protein was located within the cells of the decidua. However, no photographs were available of the results, and the data was very limited owing to the shortage of the strong 5Y4 antiserum which was used in these experiments.

After about six months of immunisation, the antiserum against α_2-protein became strong enough to carry out quantitative measurements. One-dimensional AACE was used, with an antibody concentration of 18% in the second dimension gel. Fig. 24a shows that the area under the precipitate is proportional to the volume of amniotic fluid added. The relative concentration of the α_2-protein was therefore measured in a number of amniotic fluids, using 4-8μl of fluid per well. The results (Fig. 24b) show that the concentration of α_2-protein in amniotic fluid is maximal between twenty and thirty weeks of gestation.

The second protein found in amniotic fluid using absorbed antisera was mentioned briefly on p.67 and was shown in Fig.20. Its mobility was tested by AACE and was compared with a parallel immunoelectrophoretic run in agarose gel. The protein was found as an asymmetric β-mobility peak, migrating a little ahead of transferrin (Fig.25). The antiserum used in all the studies on the β-protein was obtained from rabbit 6M6 some seven months after the initial immunisation. The strength of the antiserum and the quantity of β-protein in amniotic fluid was only great enough to produce small AACE peaks of up to 30mm^2 area, which stained up clearly but rather faintly. The intensity of the peak could be improved only by increasing the percentage of antiserum in the antibody gel, and this reduced the peak area to an extent that made detailed studies impossible.

The specific antiserum was tested for the completeness of absorption in the usual way (Figs. 7, and 22) and it was found that the addition of adult male human serum to amniotic fluid caused no detectable increase in the size of the amniotic fluid protein peak. However, when the serum was subjected to AACE by itself, a very small precipitate was found, yet the protein was present in maternal serum and fetal serum at much higher concentrations. It was also detected in 30% saline extracts of fetal liver, chorion, and placenta. It was not detected in amnion, umbilical cord, or skin; nor was it present in washed adult or fetal red cells.

Quantitative studies were made on the β-protein by one dimensional AACE. 6-10μl of amniotic fluid were placed in the wells and 8% absorbed antiserum in the antibody gel. Fig.2b shows that the height of the β-protein peak increased in proportion to the quantity of amniotic fluid in the well. However, the peaks stained weakly and the system was therefore less than ideal. Figs. 27a and b show the relative concentrations of the β-protein in amniotic fluid and in maternal and fetal sera. Although the data is not extensive, the concentration of β-protein in amniotic fluid and maternal serum is maximal between about eighteen and thirty weeks of gestation. The concentrations of β-protein in maternal serum were slightly greater than in amniotic fluid until about thirty weeks, when the difference became more apparent. The concentration of the β-protein in the sera of three non-pregnant adults did not exceed 3 units when expressed in the units of Figs.27a and b.

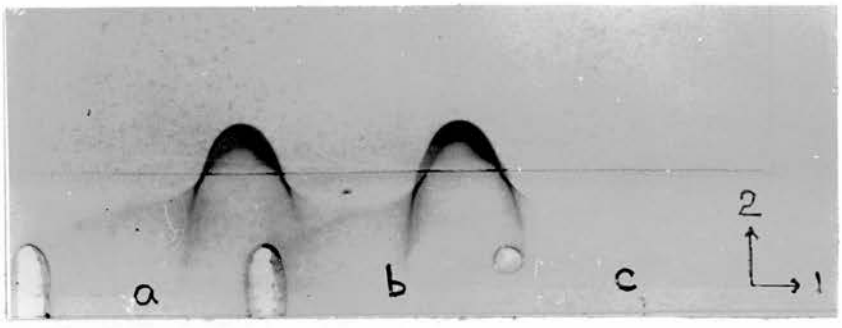
Figure 22 Testing the absorption of serum 5Y4 by AACE.

run a) 6µl of 30% w/v decidual extract

b) " " " " " + 8µl serum

c) 8µl serum

Figure 23 The presence of α_2-protein in decidua, demonstrated by two-dimensional AACE. 6µl of 30% w/v extract of decidua, and 8µl of 20 week amniotic fluid were used.



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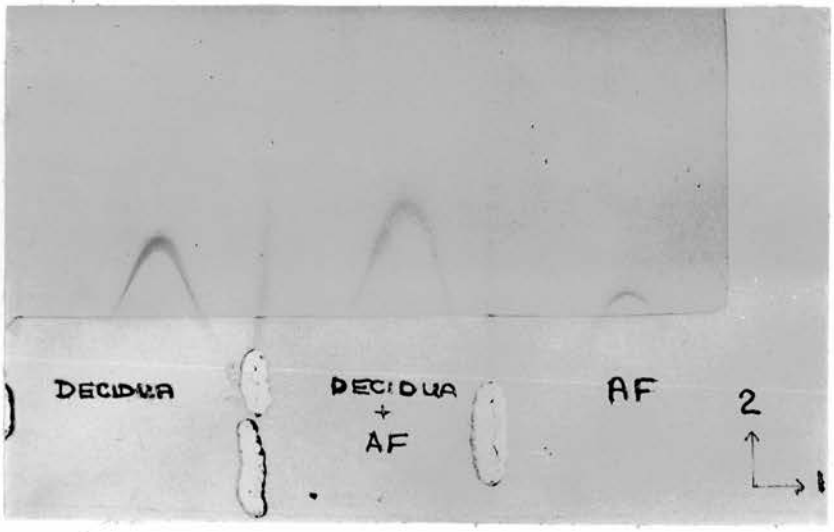
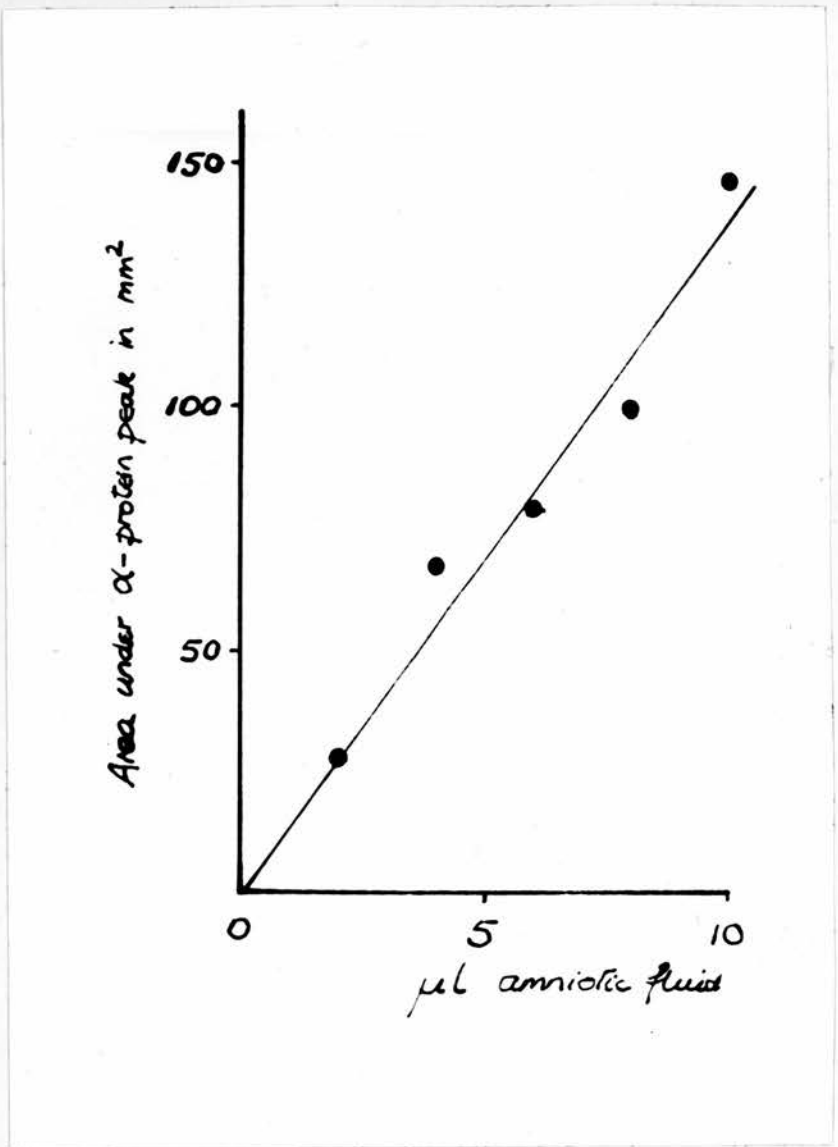


23

Figure 24a Measurement of the α_2-protein concentration by one dimensional AACE. The graph shows that there was a proportionality between the volume of amniotic fluid added and the area under the immune precipitate.

b) The concentration (arbitrary units) of α_2-protein in amniotic fluid throughout gestation.

a


150
100
50
0
5
10
Area under α-protein peak in mm²
μl amniotic fluid

b

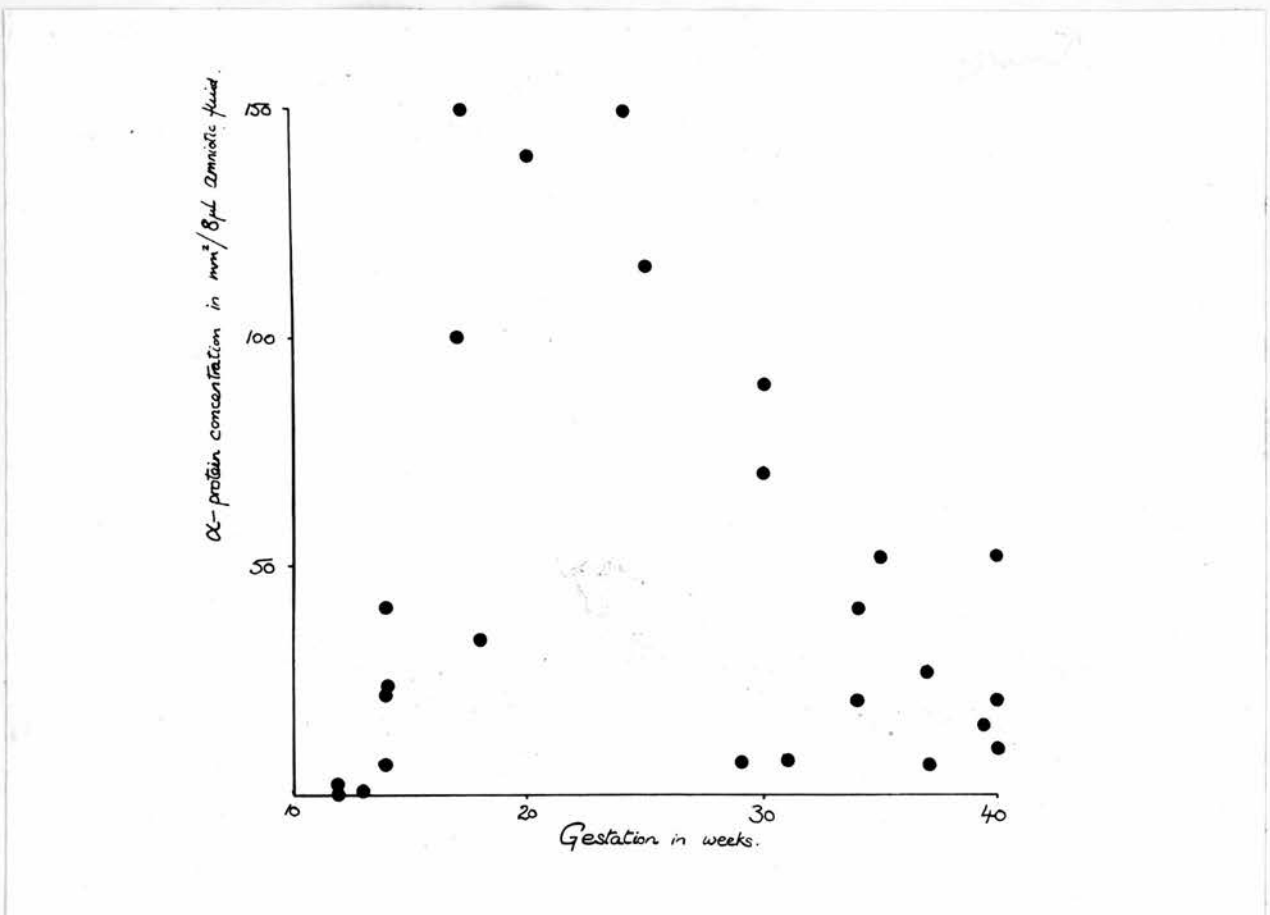
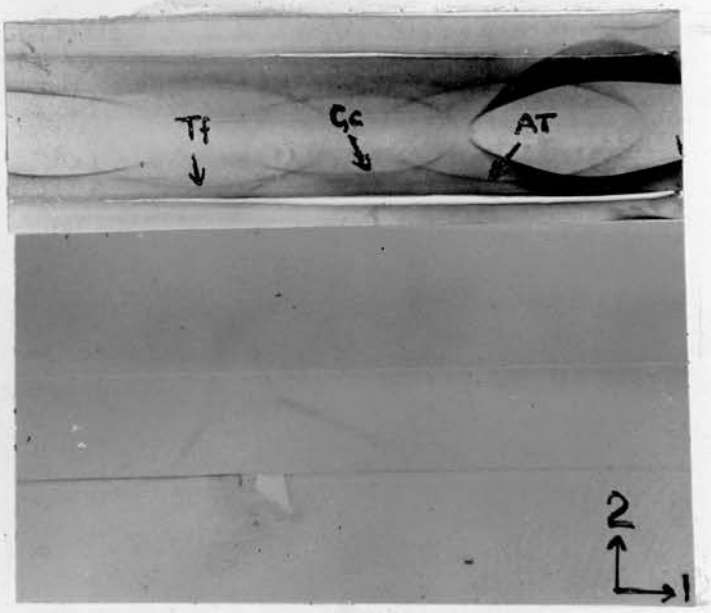

α-protein concentration in mm²/8μl Amniotic fluid.
150
100
50
10
20
30
40
Gestation in weeks.

Figure 25 The mobility of the β-protein in amniotic fluid, tested by two dimensional AACE. This experiment was carried out in parallel wi that in Fig. 21a and the details are found there. The mobility of the β-protein was similar to that of transferrin.

Figure 26 Measurement of the β-protein concentration by one dimensional AACE. The graph shows that there was a proportionality between the volume of amniotic fluid added and the area under the immune precipitate.

25


Tf
Cc
AT
2
1

26

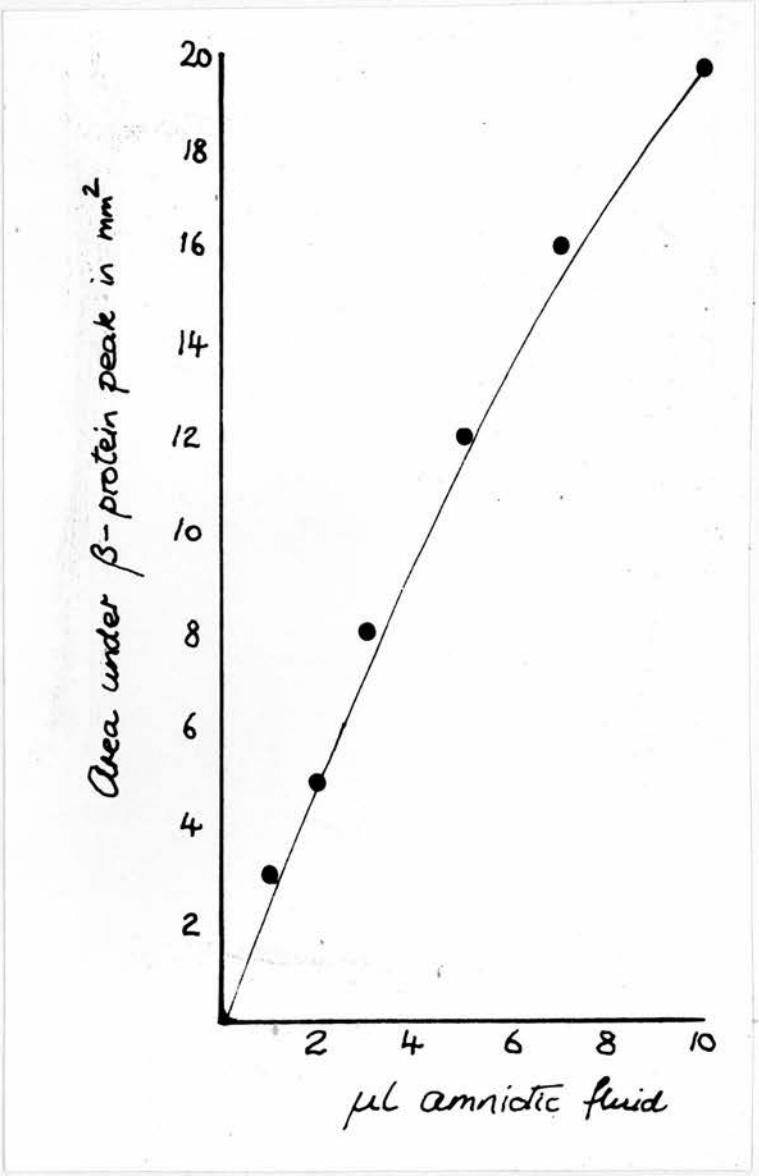
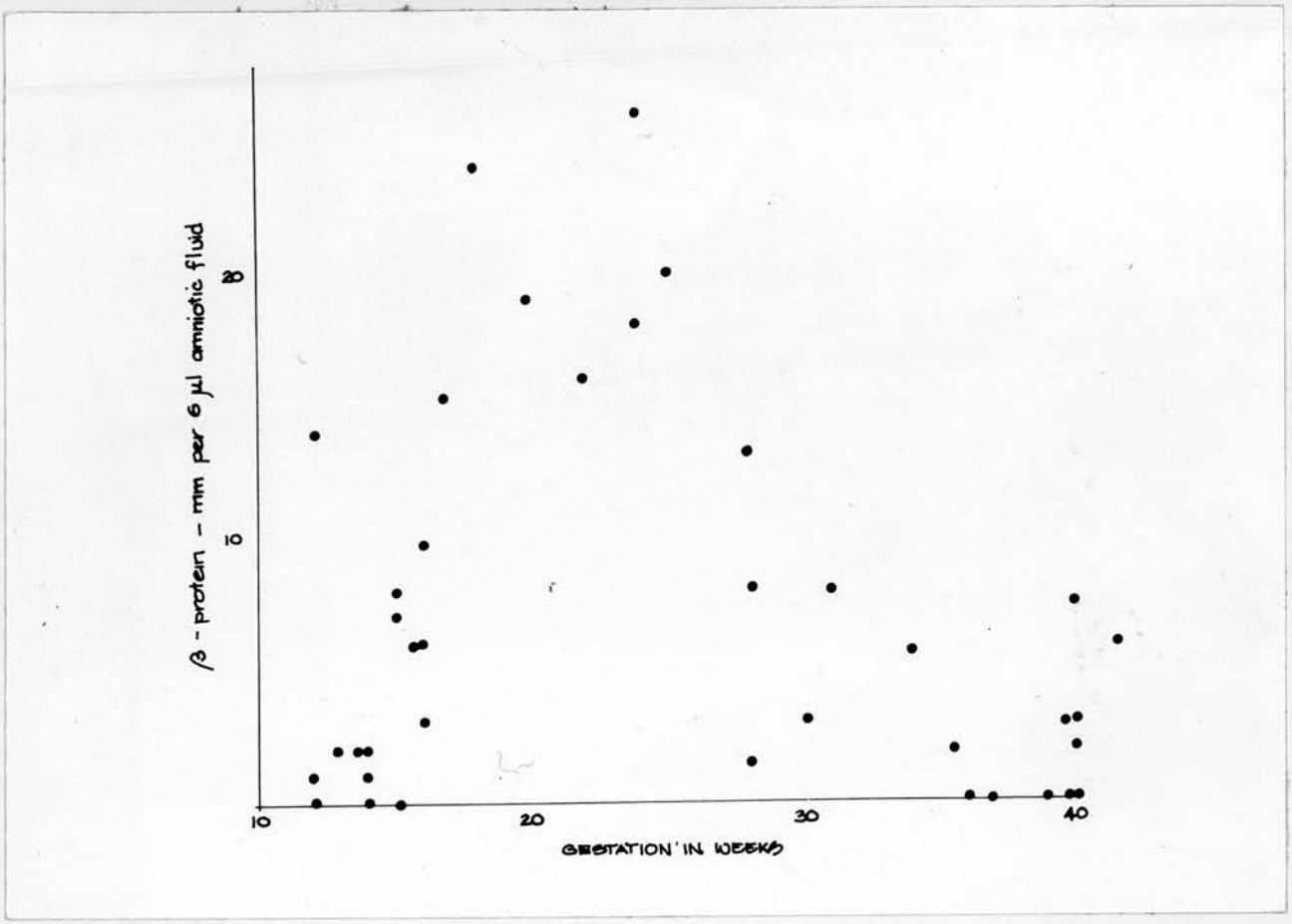

Area under β-protein peak in mm²
20
18
16
14
12
10
8
6
4
2
2
4
6
8
10
μl amniotic fluid

Figure 27 a) The concentration (arbitrary units) of β-protein in amniotic fluid throughout gestation.

b) The concentration (arbitrary units) of β-protein in maternal and fetal serum at various stages of pregnancy. The units in Figs. 27a and b are the same.

● = Maternal serum

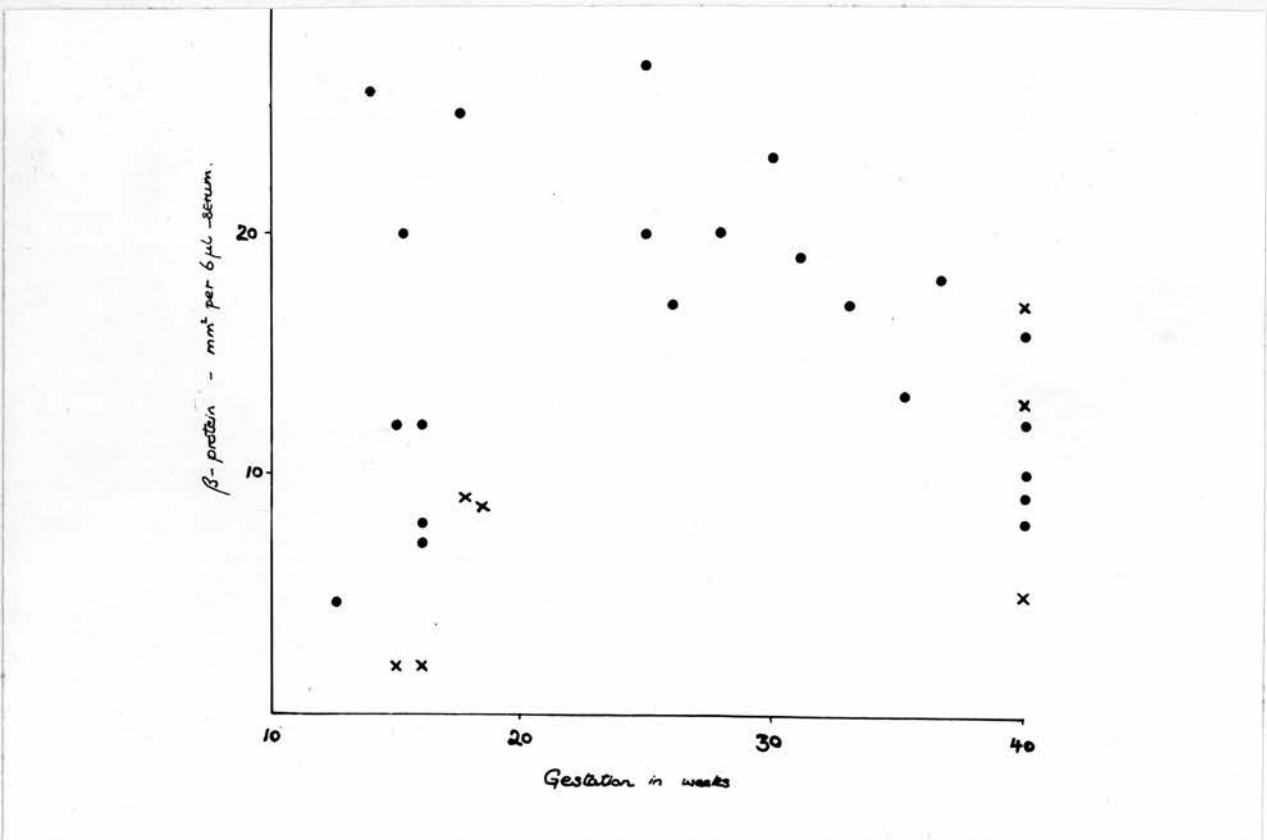
× = Fetal serum


β - protein - mm per 6 µl amniotic fluid
10
20
30
40
GESTATION IN WEEKS

a

27

b


β-protein - mm per 6 µl serum.
10
20
10
20
30
40
Gestation in weeks

DISCUSSION

The results show that when the rabbit antisera against amniotic fluid were absorbed with adult male human serum, two specificities remained. The faster migrating (anodal) protein had an α_2-mobility, and was found in amniotic fluid and decidua both early and late in gestation, and also in amnion and chorion early in gestation. The concentration of the protein in amniotic fluid was maximal at between twenty and thirty weeks of pregnancy, and the protein was absent from maternal and fetal serum. The α_2-amnion proteins described by von Kleist *et al* (1968) were present at maximal concentrations in term amniotic fluid. The amnion protein found by Lambotte and colleagues (pp.22-23) was of α_1-mobility and was therefore only a little more rapidly migrating than the α_2-proteins of von Kleist *et al* (1968) and Fig.25. Lambotte and colleagues demonstrated the presence of their protein in amnion by immunofluorescence, but neither they nor von Kleist *et al* invested the possibility of a decidual origin for the proteins.

Is it possible that all three studies have been investigating the same protein? At present no definite answer is available. The quantitative behaviour of the α_2-protein described here (Fig.24b) is different from that described by von-Kleist *et al* (1968). The absence of the α_2-protein from term amnion and chorion (p.68) is probably not complete, but as the concentration must be below that detectable by AACE, this suggests that the protein is not identical to that described by Lambotte and colleagues.

The studies on the α_2-protein in this thesis have shown that it is present in uterine decidua both early and late in pregnancy. Although this suggests that the decidua is a source of α_2-protein,

it does not rule out alternative sources in the membranes. This is because a tissue synthesising a protein need not store it intracellularily at any appreciable concentration. *In vitro* studies using radio-active amino acids would be essential for a final decision on the source of this protein. At present the α_2-protein described in this thesis is referred to as α_2-decidual protein only because it was consistently found in decidua, and because immunofluorescent studies showed it to be located within the cells of this tissue.

The β-mobility protein described in the results was difficult to study because it as present in low concentrations. A fluid protein of similar mobility was found to be haemoglobin by von Kleist *et al* (1968); however, the protein described here was absent from washed adult and fetal red cells, and must have a different identity. The origin of the β-protein has not been determined. Before thirty weeks it is present in maternal and fetal serum at concentrations not greatly in excess of the concentrations in amniotic fluid. This is [illegible] to the concentration gradients of amniotic fluid proteins which are known to be of serum origin. Table 7 shows that the gradient for Gc (known to be of maternal origin) increases from a mean of 15 at thirteen to eighteen weeks to a mean of 32 at term; and calculations (p.77) show that the gradient for α-fetoprotein (fetal serum origin) is in the order of 1:206. This data suggests that much of the β-protein in amniotic fluid is synthesised somewhere in the tissues surrounding the amniotic fluid, and this hypothesis has been supported by the finding of β-protein in placenta and chorion. Yet, the theoretical difficulties in determining the tissue origin of a protein (see above) make a conclusion impossible from the present data.

The major problem in attempting to search for antigens of non-serum origin in amniotic fluid lies in the weak nature of the rabbits' immune response to them. Longer periods of immunisation, together with complement fixation methods may increase the potential of this type of study.

THE PRENATAL DIAGNOSIS OF ANENCEPHALY AND SPINA BIFIDA

a. Introduction

Anencephaly and spina bifida cystica are the most common of the congenital malformations of the central nervous system, with a combined incidence of up to 7 in a thousand in the U.K. (Elwood, 1970; Renwick, 1972). The risk of either disorder is increased to 1 in 20 in a family where there is already an affected sib (Carter et al 1966) and to 1 in 10 or above in a family where there are two affected sibs (Carter and Roberts, 1967). One of the prerequisites of antenatal diagnosis, the identification of pregnancies with enhanced risks, is thus satisfied. The other prerequisite, a marker molecule which will indicate an affected fetus early enough to allow termination of pregnancy, has so far not been found. Various attempts have been made before now to find marker molecules in amniotic fluid which would indicate the presence of fetuses with anencephaly and/or spina bifida (Lambert and Pennington, 1965; Emery et al 1972; Hall, 1972). Cassady and Cailliteau (1967), Lee and Wei (1970), and Stewart and Taylor (1964) have all reported that bilirubin or a bilirubin-like molecule with an extinction coefficient at 450 nm is present in the amniotic fluid surrounding anencephalic fetuses at or near term. This finding suggested that enhanced leakage or transudation of fetal blood components was occurring either directly into the fluid or into the fluid via the C.S.F., and that detection of fetal blood components in the fluid might be of use for the prenatal diagnosis of anencephaly. This possibility gains extra support from the indications that in anencephaly, the vascular tissue on the floor of the skull is virtually

exposed to the amniotic fluid (Willis, 1958).

For any such blood components in amniotic fluid to be of diagnostic use, they would have to be known to be of fetal rather than of maternal origin. Thus, α-fetoprotein (AFP) was the obvious protein to study. It was a simple matter to test this hypothesis by comparing amniotic fluids from normal and abnormal fetuses, since methods had already been developed to study the AFP concentration gradient between fetal serum and amniotic fluid.

Originally discovered by Bergstrand and Czar (1957), AFP is virtually an exclusive product of the conceptus, being synthesised in the yolk sac, liver and gastrointestinal tract from as early as six weeks of gestation (Gitlin et al 1972). It is known to reach maximum concentration in fetal serum at about 13 to 15 weeks and then to decline towards term (Gitlin and Boesman, 1966). It has been detected in amniotic fluid throughout gestation (Gitlin and Boesman, 1966; Adinolfi and Gardner, 1967; Seppälä and Ruoslahti, 1972), although its low concentration at term lead to the failure of Smith et al (1971) to find it.

The following sections report an investigation into the concentrations of AFP in the amniotic fluids of normal fetuses and of fetuses affected with a variety of CNS malformations. The most common of these malformations was anencephaly.

b. Materials and Methods

i. Introduction

Immunological assays are essential for the measurement of AFP concentrations in body fluids. Gitlin and Boesman (1966) used radial-immunodiffusion and an absorbed rabbit antiserum to measure

AFP in fetal serum and amniotic fluid. This technique was not sensitive enough to measure the concentration of AFP in amniotic fluid at term with confidence. The highly sensitive technique of radio-immunoassay has recently been applied to the measurement of AFP in amniotic fluid and also maternal serum by Ruoslahti and Seppälä (1972) and Seppälä and Ruoslahti (1972).

The relative insensitivity of radial-immunodiffusion and the considerable technical difficulties (p.48) of radio-immunoassay lead us to develop a technique for measuring AFP by antibody-antigen crossed electrophoresis (AACE). After this had been developed a similar technique was reported by Nørgaard-Pedersen (1972).

ii. Materials

Samples of amniotic fluid were obtained either at termination of pregnancy, or at delivery, or by transabdominal amniocentesis. They were centrifuged for 5 minutes at 125 g, and the clear supernatants were removed. These supernatants were stored at -25°C for periods up to 3 years. Since the effect of prolonged storage on AFP is not known, each sample from a pregnancy leading to a congenital malformation was matched with a control sample of the same approximate gestation, which had been stored for an equivalent length of time. Congenital malformations were classified as anencephaly (with or without spina bifida), spina bifida and hydrocephaly (with or without spina bifida). Most of the control samples were obtained from terminations of pregnancy or from amniocenteses carried out during the management of rhesus-incompatible pregnancies.

iii. Methods

AFP was measured by one-dimensional antibody-antigen crossed electrophoresis using rabbit anti-serum which was initially kindly provided by Dr J.V. Clark and subsequently bought from Behringwerke AG.

Since these antisera were monospecific, it was not necessary to carry out the initial electrophoretic step of 2-dimensional AACE. The commercial antiserum was used at a final concentration of 0.25% in a 1% agarose gel. Amniotic fluid (5-15 µl) was placed in the sample wells and electrophoresis carried out overnight. The sensitivity limit of the assay was about 1 µg/ml, though values under 3 µg/ml were somewhat unreliable. Absolute values of AFP were calculated with reference to a standard supplied by Professor G. Abelev. If a Behringwerke standard is used the values in Figures 1 and 2 must be multiplied by 0.7. Protein was measured by the method of Lowry et al (1951).

The concentrations of AFP in fetal serum and urine were measured in the same way as for amniotic fluid, except that in the case of fetal serum a prior dilution of between x50 and x100 was carried out with physiological saline.

Fig.28 shows that increases in AFP concentration in the samples causes a smooth linear increase in the area under the precipitates. At least two standard AFP samples were run in each AACE plate in order to provide internal standards.

c. Results

Amniotic fluid α-fetoprotein concentrations in normal and abnormal pregnancies

Fig.29 shows the concentration of AFP found in normal amniotic fluids between eleven weeks of pregnancy and term. Maximal concentrations were found in amniotic fluid at 13 weeks of gestation, and these declined rapidly between 15 and 20 weeks. Subsequently there was a steady fall in concentrations, so that the quantities of AFP in

amniotic fluids at term were close to the minimum detectable by AACE. The mean concentration of AFP in the control fluids from the period 25 to 42 weeks of gestation was 3.1 μg/ml the range being from 0 to 10.5.

In 22 pregnancies leading to anencephaly or anencephaly combined with spina bifida, where it was possible to get amniotic fluid in the third trimester, the concentrations of AFP in the fluid were grossly elevated. All were above 12 μg/ml while some ranged as high as 200 μg/ml (Fig. 30). In another 9 'anencephalic' pregnancies where fluid was obtained only after 35 weeks gestation, the distinction was less clear though 5 of the samples had strongly raised AFP concentrations.

In five spina bifida cases after 32 weeks (three of which were associated with hydrocephaly) the concentration of AFP in the amniotic fluids were indistinguishable from the controls. However, a single fluid was fortuitously obtained at 13 weeks of gestation from a hysterotomy where the fetus had a myelocele spina bifida.* In this case the concentration of AFP in the fluid was 350 μg/ml, which was over 4 times as great as the highest normal control (Fig. 31).

Since many of the control samples used in this study were obtained from pregnancies past 20 weeks in which there was a risk of rhesus incompatibility, it was important to establish what effect this had on amniotic fluid AFP concentrations. Two pregnancies were studied where at least four successive amniocenteses had been necessary to monitor fetal status. In both the degree of isoimmunisation was

*personal communication from Dr M.M. Nelson whose careful studies of abortion material made this observation possible.

TABLE 9

α-Fetoprotein concentrations in amniotic fluids from pregnancies in which the fetus was affected with various conditions. Gestation refers to the time at which the fluid was obtained.

Condition	Gestation (wks)	α-Fetoprotein (μg/ml.)
Rh isoimmunisation necessitating three post-natal exchange transfusions. Live-born infant, progressing well	24	1.6
	26	1.2
	28	0.5
	30	1.1
	32	0.4
	33	0
	34	0
Rh isoimmunisation necessitating two intra-uterine and three post-natal exchange transfusions. Live-born infant progressing well.	24	1.1
	25	0.8
	26	1.3
	27	1.2
	28	1.3
Rh isoimmunisation necessitating intra-uterine exchange transfusion. Stillbirth.	24	2.2
Rh isoimmunisation. Hydrops fetalis. Stillbirth.	27	6.5
Rh isoimmunisation. Stillbirth	35	1.7
Rh isoimmunisation (anti-E). Stillbirth	29	0
Duodenal atresia. Live birth.	36	4.2
Renal hypoplasia. Neonatal death.	21	5.1
Stunted embryo. Termination.	12	34.2
Rubella contact. Termination.	22	4.9
Ectopia vesica. Termination.	16	23.6
Exomphalos. Termination.	10	13.7
Diabetic mother. Hydramnios. Normal infant.	35	2.0
Prediabetic mother. Hydramnios. Mongol infant.	39	6.3

sufficient to require postnatal transfusions, and in one of the pregnancies intrauterine transfusions had also been necessary. In neither case was there any indication of a raised AFP level (Table 9), nor was there in four cases of rhesus isoimmunisation leading to intrauterine death. A variety of other conditions where there seemed a possibility of elevated amniotic fluid AFP were tested, but in none was an abnormal level found (Table 9).

α-Fetoprotein in maternal and fetal serum and in fetal urine

AFP was not detectable in any of the maternal sera obtained at the delivery of normal or anencephalic babies. The limit of sensitivity of the method used was 1.0 μg/ml.

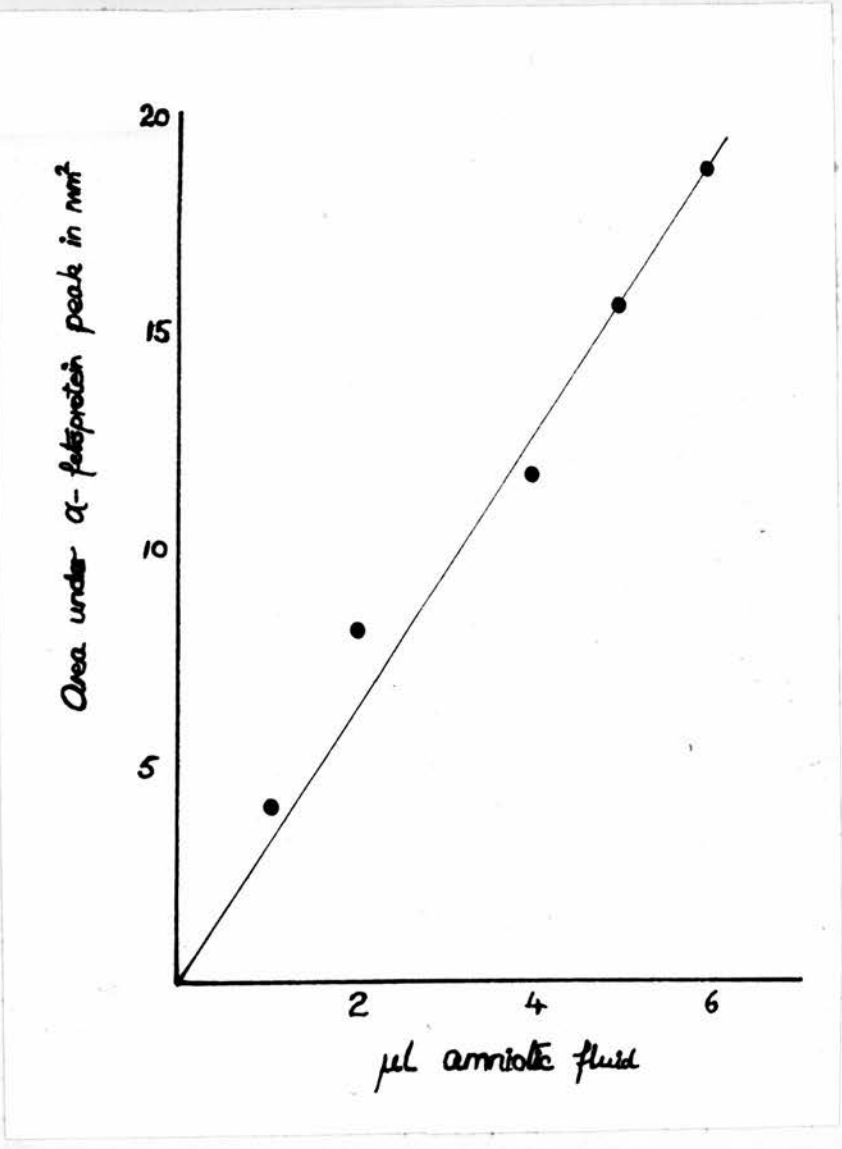
Fig. 32 shows the concentration of AFP in fetal sera and urine from various stages of gestation. Between 13 and 18 weeks of gestation, the concentration of AFP in fetal serum ranged between 0.54 and 10.8 mg/ml, with a mean of 7.6 mg/ml. During the same period, the concentration of AFP in amniotic fluid ranged between 21.8 and 87.2 μg/ml, with the mean value being 36.9 μg/ml. Thus the mean AFP concentration ratio between the fetal serum and the amniotic fluid was of the order of 206 : 1.

Fig. 33 summarises the ratios of the concentrations of albumin and α-fetoprotein which were calculated for a number of samples of amniotic fluid and of fetal serum and urine between thirteen and eighteen weeks of gestation. Unfortunately in the case of this figure, no samples of serum, urine, and fluid were available from the same fetus. However the figure shows clearly that the mean ratio of albumin to α-fetoprotein is greater in amniotic fluid than in fetal urine or serum by a factor of 10 or more.

Figure 28 The relationship between the volume of amniotic fluid subjected to AACE (abscissa) and the area under the α-fetoprotein precipitate (ordinate).

Figure 29 α-Fetoprotein (AFP) concentrations in amniotic fluid at various stages of gestation. ● Normal samples; ▲ samples obtained during monitoring of pregnancies at risk for rhesus isoimmunisation, but where bilirubin concentration was low; O, unexplained hydramnios; X unexplained stillbirth.

28


Area under α-fetoprotein peak in mm²
20
15
10
5
2
4
6
μl amniotic fluid

29

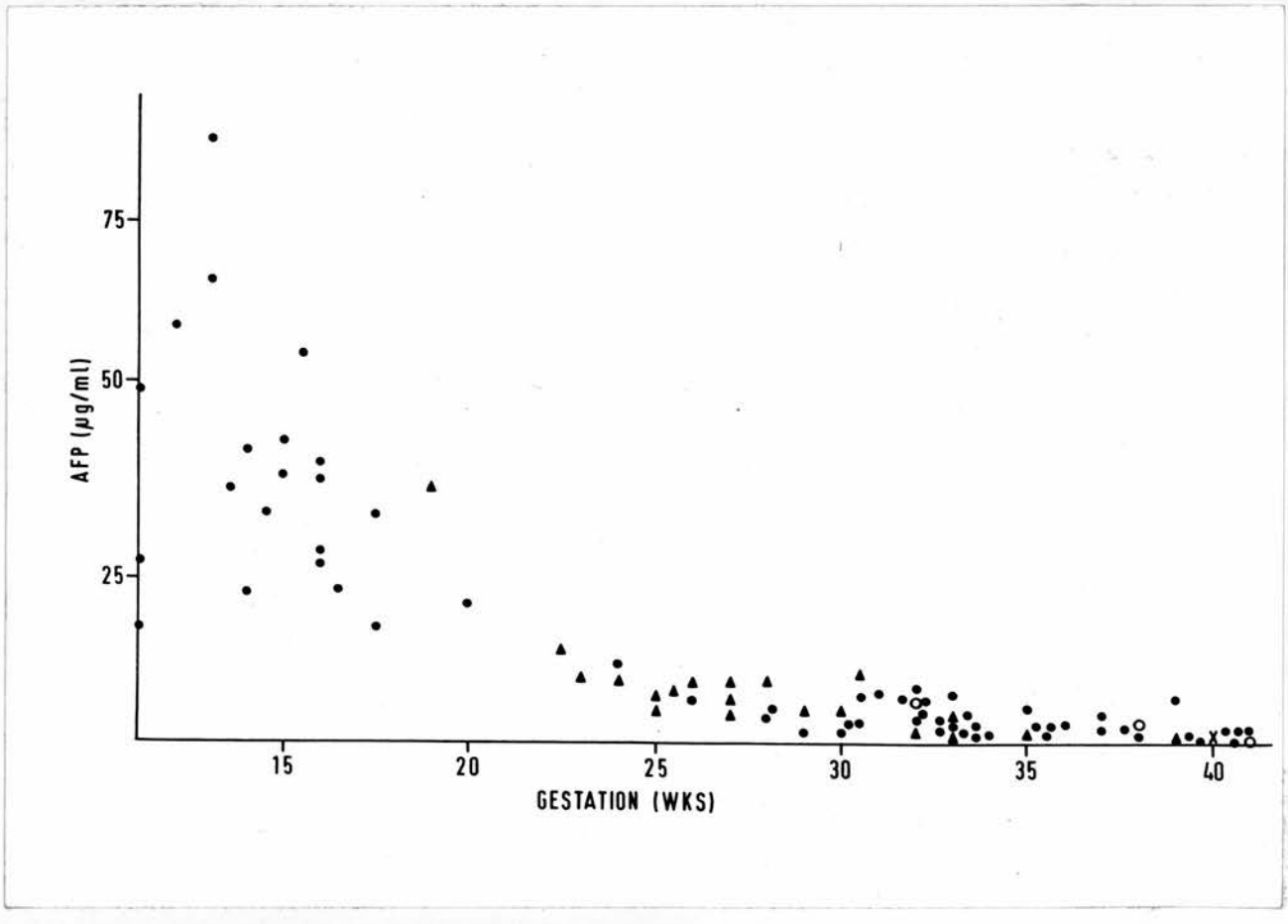
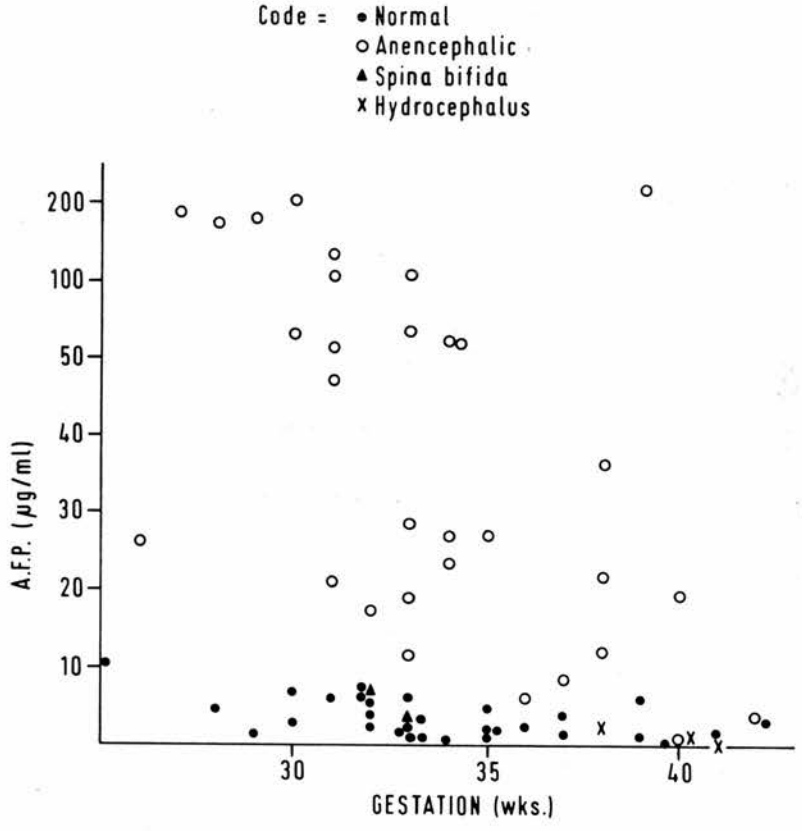

AFP (μg/ml)
75
50
25
15
20
25
30
35
40
GESTATION (WKS)

Figure 30 The concentration of α-fetoprotein (AFP) in the amniotic fluid of normal pregnancies, and of pregnancies leading to anencephaly, spina bifida and hydrocephaly between twenty-five weeks of gestation and term.

Figure 31 The concentration of α-fetoprotein (AFP) in the amniotic fluids of normal pregnancies between ten and twenty five weeks, and of a pregnancy at thirteen weeks in which the fetus was affected with a myelocele spina bifida.

30


Code = • Normal
○ Anencephalic
▲ Spina bifida
x Hydrocephalus
A.F.P. (µg/ml)
200
100
50
40
30
20
10
30
35
40
GESTATION (wks.)

31

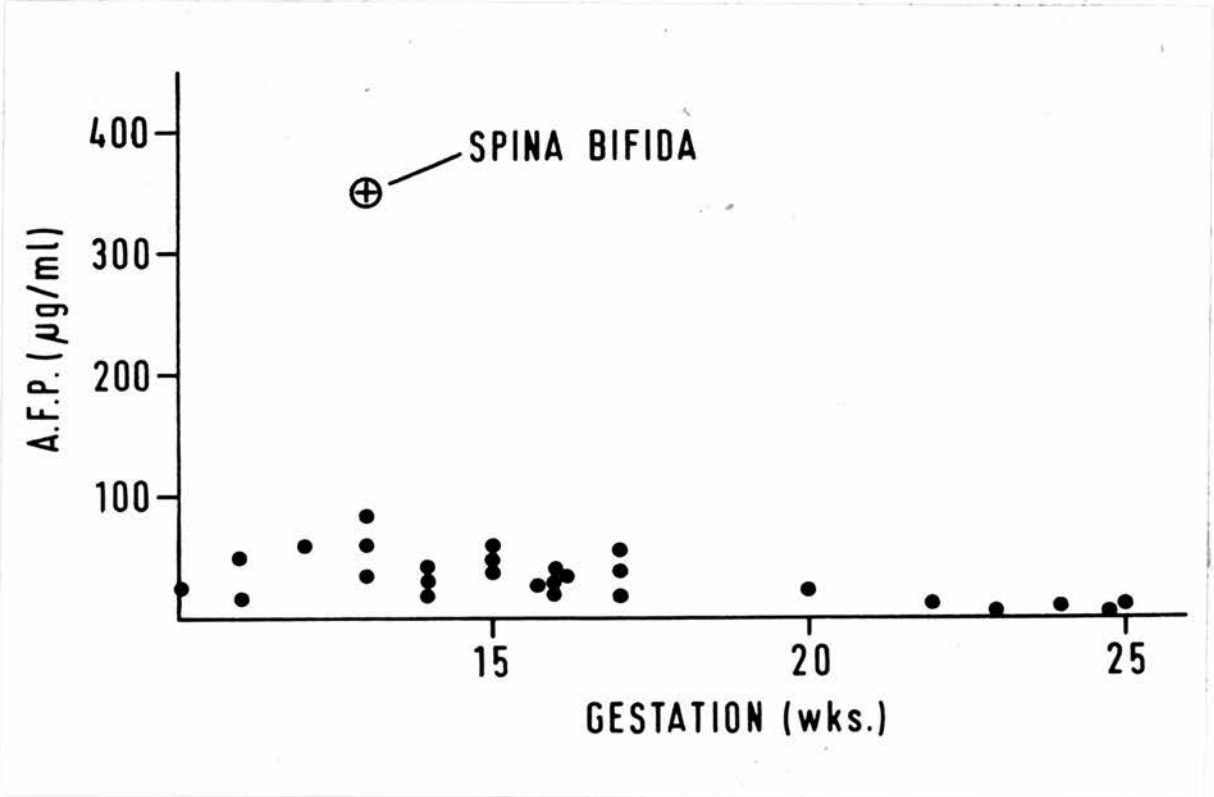

SPINA BIFIDA
A.F.P. (µg/ml)
400
300
200
100
15
20
25
GESTATION (wks.)

Figure 32 The concentration of α-fetoprotein in fetal serum and urine from various stages of gestation.

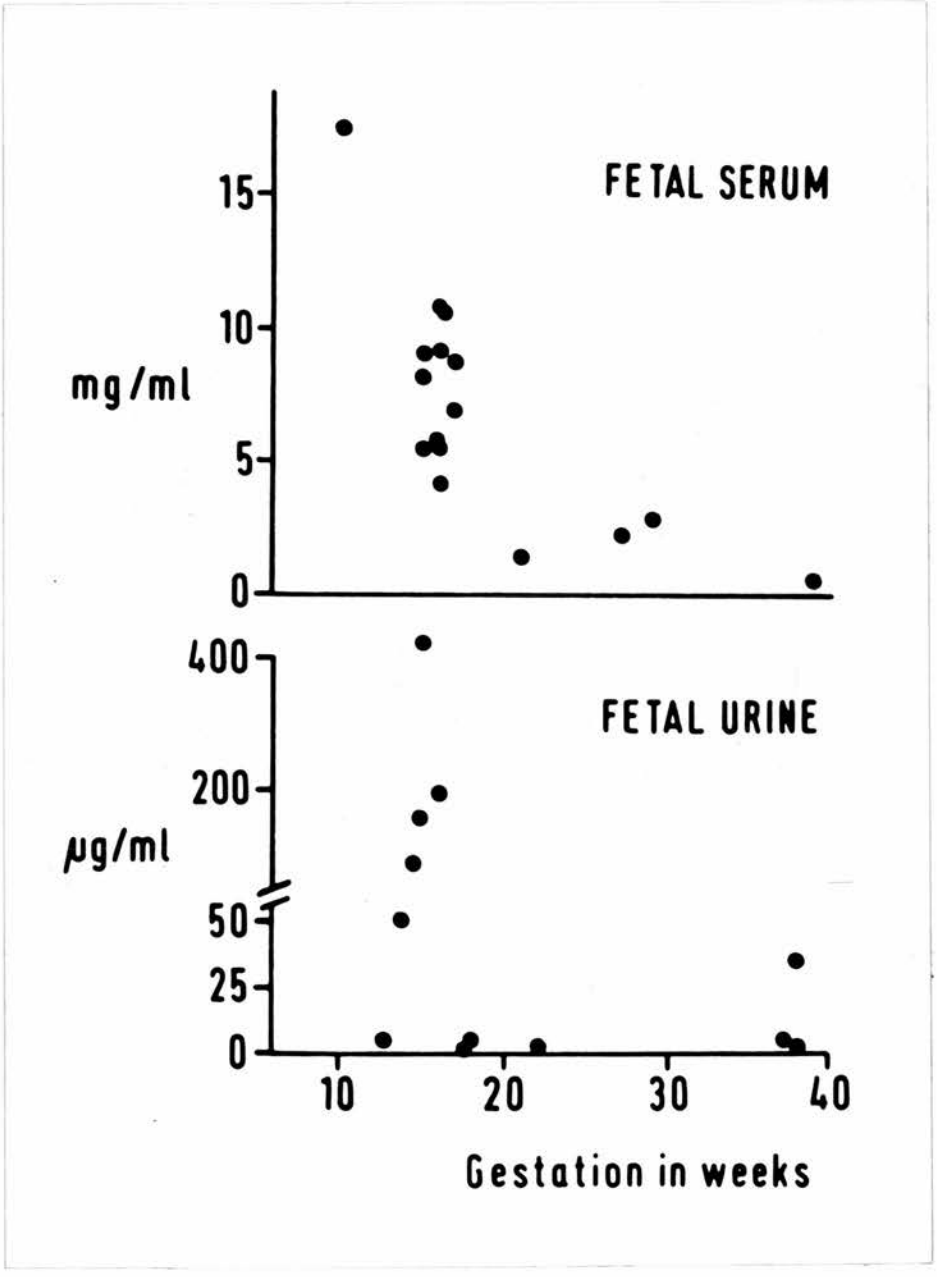

FETAL SERUM
15
10
5
0
mg/ml
FETAL URINE
400
200
50
25
0
µg/ml
10
20
30
40
Gestation in weeks

Figure 33 The ratio of the concentration of albumin to the concentration of α-fetoprotein in samples of fetal serum, fetal urine, and amniotic fluid from hysterotomies between 13 and 18 weeks of gestation.

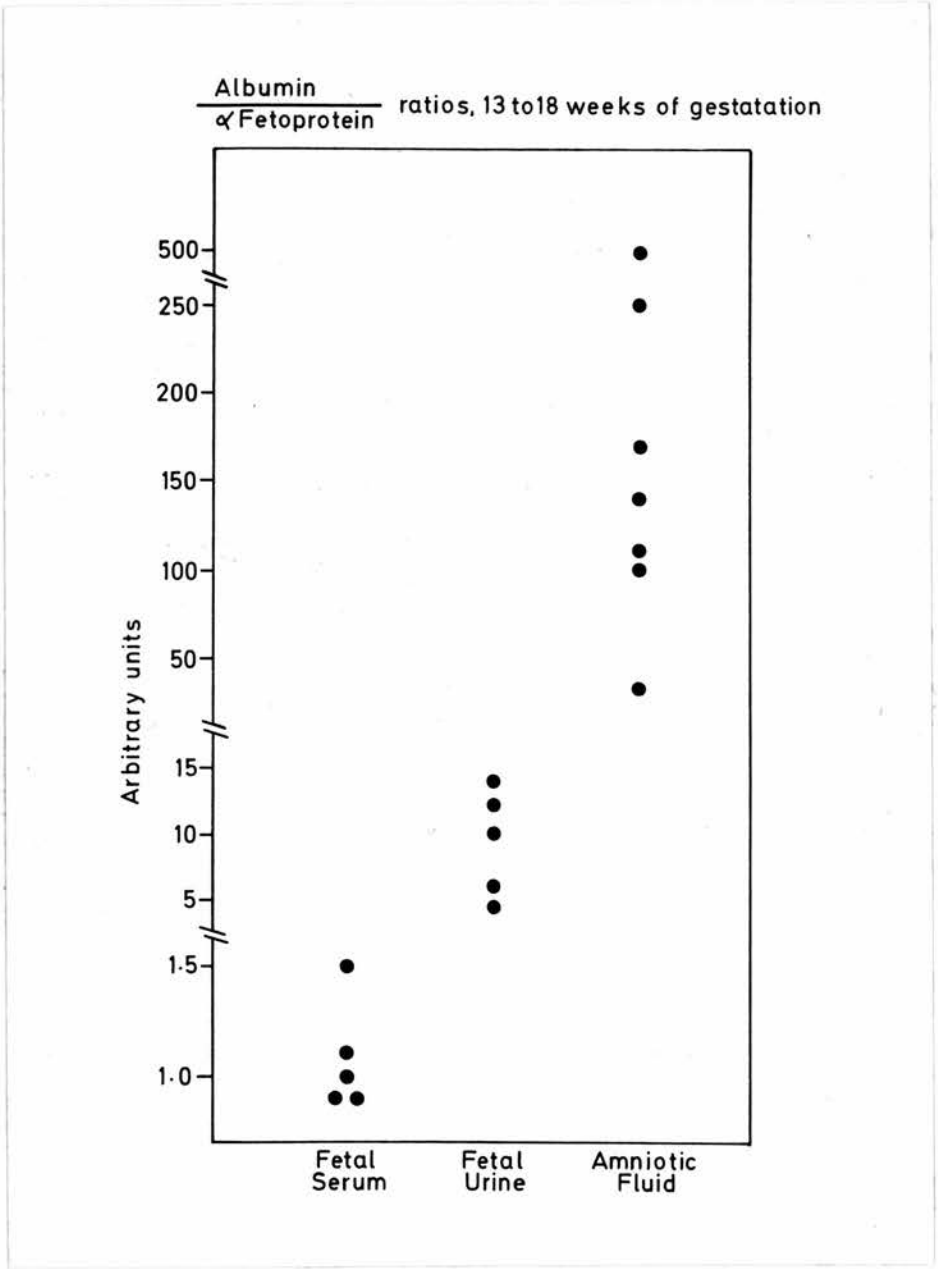

Albumin / α Fetoprotein ratios, 13 to 18 weeks of gestatation
Arbitrary units
500
250
200
150
100
50
15
10
5
1·5
1·0
Fetal Serum
Fetal Urine
Amniotic Fluid

Figure 34 The effect of incubation at 37°C on the concentrations of α-fetoprotein, albumin, α_1-antitrypsin, group-specific component, and transferrin.

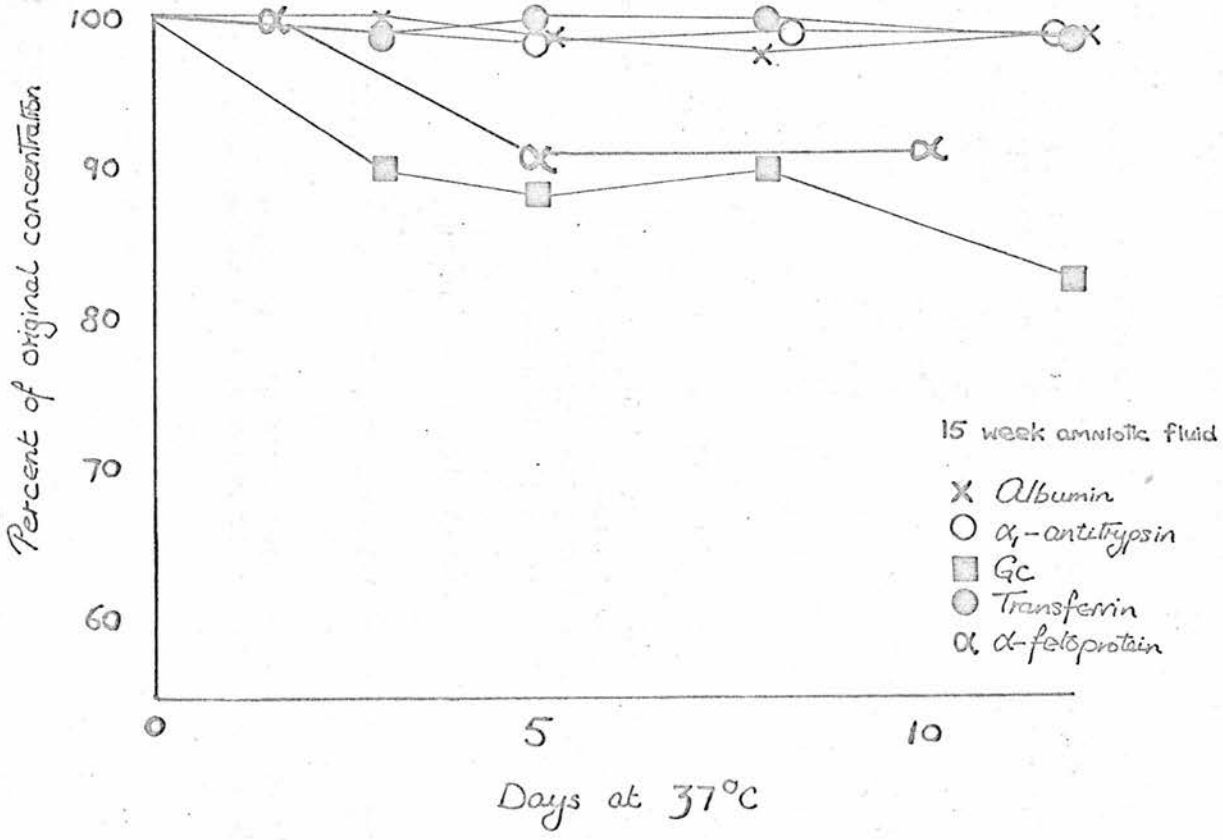
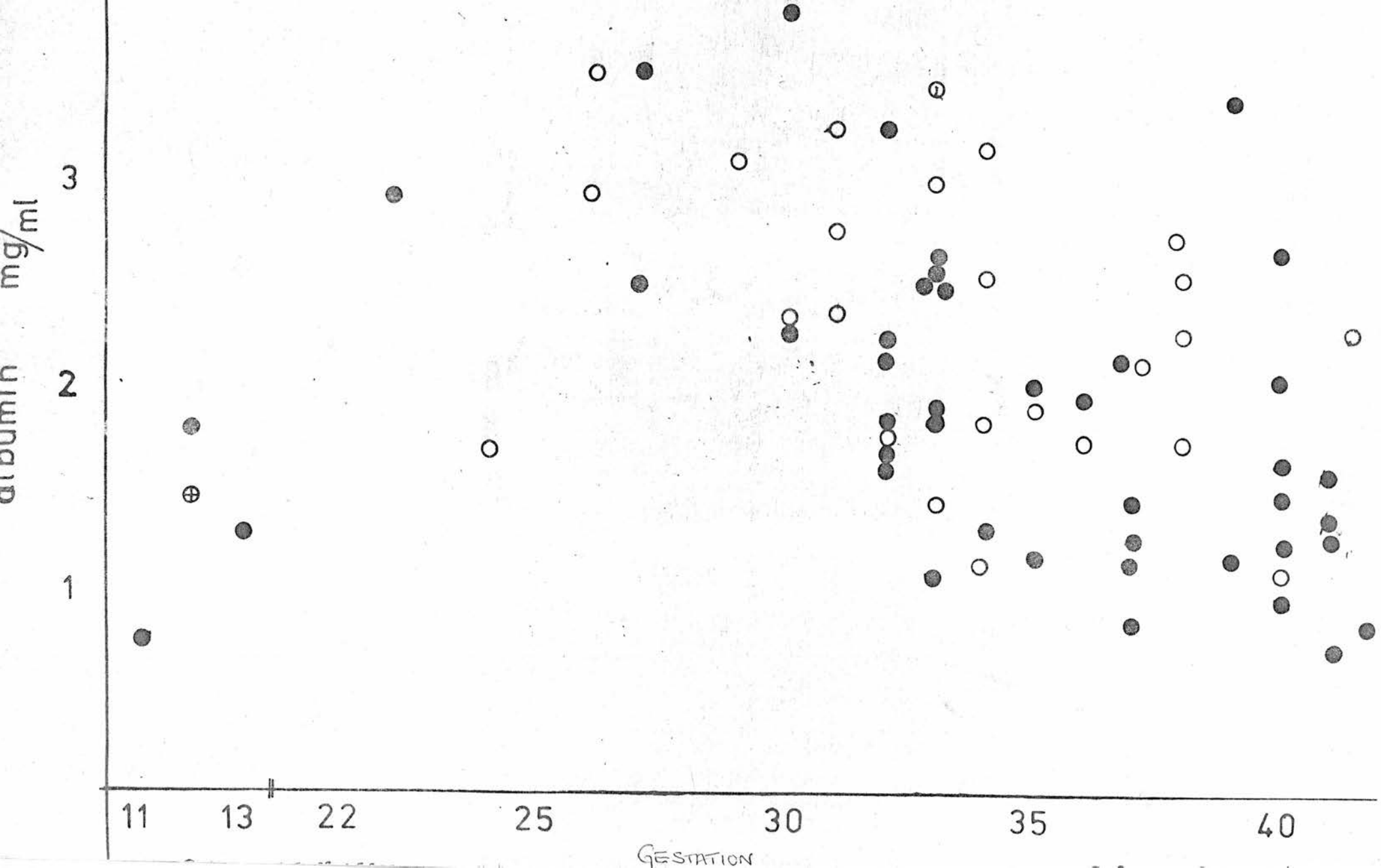

100
90
80
70
60
Percent of original concentration
0
5
10
Days at 37°C
15 week amniotic fluid
Albumin
α1-antitrypsin
Gc
Transferrin
α-feloprotein

Figure 35 The concentration of albumin in amniotic fluids from normal and anencephalic pregnancies, measured by one-dimensional AACE. Compare with Figure 30.


albumin mg/ml
3
2
1
11
13
22
25
30
35
40
GESTATION

d. Discussion

Fig.30 shows clearly that in cases of anencephaly during the third trimester, the AFP concentrations in amniotic fluid greatly exceed those in matched controls. This suggests that anencephaly may be detectable by fluid analysis early enough to allow termination of pregnancy. There are three reasons to support this suggestion. Firstly, even though there are high concentrations of AFP in amniotic fluids from normal fetuses before 20 weeks (Fig.29), several of the anencephalic fluids between 27 and 35 weeks of gestation had AFP concentrations well above the entire normal range. Secondly, in the single case of spina bifida at thirteen weeks of gestation there were grossly elevated concentrations of AFP. It must be emphasised here that there are different types of spina bifida, and that myelocele and total myeloschisis are more likely to be associated with extrusion of fetal proteins into the fluid than are meningomyelocele, meningocele or spina bifida occulta. The thirteen week case studied here was a definite myelocele (personal communication from Dr M.M. Nelson), whilst the later cases were probably all meningomyelocele or meningocele. This may indicate that unclosed neural plate malformations will be diagnosable antenatally. The third reason for believing that AFP may be of value for the early prenatal diagnosis of anencephaly lies in the AFP concentration ratio between the fetal serum and amniotic fluid. It is reasonable to assume that in anencephaly, the raised AFP concentration in amniotic fluid is due to the protein diffusing out of the blood vessels in the exposed portions of the cranial contents. The driving force for this diffusion would be represented by the normal AFP concentration gradient being the fetal serum and amniotic fluid. The fact that this gradient is about

200 : 1 between the thirteenth and eighteenth weeks of gestation strongly suggests that during this time there would be an elevation in the concentration of amniotic fluid AFP, provided that a sufficient cranial lesion was present.

Is it possible that the reduced rates of fetal swallowing in anencephaly (Pritchard, 1965, 1966; Abramovitch, 1970; p.12) cause the elevation of AFP concentrations by reducing its rate of turnover? Although it is clear that AFP is a fairly stable protein at 37°C (Fig. 34), and that it will therefore be particularly liable to the effects of bulk fluid turnover, the reduced rate of swallowing in anencephaly cannot account for the specific alteration in AFP concentrations. The first reason for this is that the maximum difference in swallowing rates between normal and anencephalic fetuses will be presumably at term, since it is then that the normal fetus is thought to be swallowing at its fastest. Thus, if the absence of swallowing was the major factor in causing the elevated amniotic fluid levels, the effect would be greatest at term; yet Fig.35 shows that the AFP concentrations in anencephalic fluids at term approach those of the controls. Further evidence against this theory comes the albumin concentration in amniotic fluid. Because swallowing is unselective, it would be expected to have the same effect on similar proteins. Albumin and AFP are both fairly stable (Fig.30) and have similar molecular weights (mol. wt. albumin : 65,000 - Schultz and Heremens, 1967; mol. wt. of AFP : 75,000, Gitlin & Boesman 1967) Despite these similarities, there are no great differences between the concentrations of albumin found in normal and in anencephalic amniotic fluids (Fig. 34). This indicates that the altered

concentration of AFP in anencephalic fetuses is not primarily due to lack of swallowing.

The finding that a specific fetal protein is present in the fluid of anencephalic fetuses suggests that the protein is transuding into the fluid either from brain tissue, or from the choroid plexus (possibly via the CSF), or from the many small blood vessels which are exposed within the remnants of the cranium. Such protein transudation suggests that increased leakage of water may also occur. This may go far to account for the polyhydramnios which is observed in anencephaly (see pp.12-13).

ENZYMES IN AMNIOTIC FLUID: A Study of Specific Activity Patterns during Pregnancy

a. Introduction

Amniotic fluid analysis has been shown to be of value in the prenatal diagnosis of both genetic and non-genetic conditions. Tay-Sachs disease can be diagnosed between fifteen and twenty-eight weeks of gestation by measurement of hexosaminidase A in amniotic fluid (O'Brien et al 1971; Friedland et al 1971). Bilirubin measurement has become routine for the diagnosis and assessment of rhesus isoimmunisation (see, for example, Robertson, 1969), and the measurement of fluid phospholipids has been claimed to be of value in determining the liability of a fetus to respiratory distress syndrome at birth (Gluck et al 1971; Bhagwanani et al 1972).

A greater understanding of the origin and quantitative behaviour of the proteins in amniotic fluid would help in attempting to extend the diagnostic potential of amniocentesis. This section reports an investigation into the specific activity patterns of a number of amniotic fluid enzymes during pregnancy, and discusses their potential usefulness in the diagnosis of fetal maturity. The factors which contribute to the changes in enzyme specific activity during gestation are discussed in a subsequent section.

The enzymes chosen for study were α-1,4-glucosidase, hexosaminidase, acid phosphatase, and the heat-stable and heat-labile fractions of placental alkaline phosphatase. The lysosomal enzymes α-1,4-glucosidase and hexosaminidase were of particular interest since enzymes with their substrate-specificities are absent in Pompe's disease and Tay Sachs disease, respectively. Acid phosphatase was

investigated because it is also a lysosomal enzyme, and thus its specific activity pattern could be compared with those of α-1,4-glucosidase and hexosaminidase. The heat-stable fraction of alkaline phosphatase is synthesised specifically in the placental trophoblast (see Hunter, 1969), and is often referred to as placental alkaline phosphatase. This enzyme was included in the study in order to examine the relationships between the placenta and the amniotic cavity. The heat-labile (non placental) fraction of alkaline phosphatase was included out of interest, since the stable fraction had already been measured.

b) Materials and Methods

i. Amniotic fluid

Amniotic fluid was obtained from three sources. Samples from early in gestation were taken from hysterotomy sacs, and those from about 20-38 weeks were taken by amniocentesis at the clinic for patients with rhesus iso-immunisation. Collections of amniotic fluid at term were made using an amnioscope during artificial rupture of the membranes. In all cases, the duration of pregnancy was measured from the first day of the last menstrual period. All samples which were visibly contaminated by blood or meconium were excluded from the series. In the patients having repeated amniocentesis only the initial specimen was collected. Specimens with a ΔOD which fell outside the A1, B1, B2, C1 and C2 zones of Robertson (1969) were excluded.

The samples of amniotic fluid were centrifuged for 45 minutes at 27,000 g at 4°C, and the clear supernatant was removed. Enzyme assays were carried out within two days of the sample being collected, the material being stored at 4°C during this period.

ii. Routine assay methods

Hexosaminidase was assayed in a system of 0.1 M citrate buffer pH 4.25, 5.3mM p-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside and 5µl of amniotic fluid in a final volume of 0.15ml. Acid phosphatase was assayed in a system of 0.1 M citrate buffer pH 4.9, 10.5mM 'Sigma 104' phosphatase substrate (p-nitrophenyl phosphate), 0.4% formaldehyde and 20-40µl amniotic fluid in a final volume of 0.15 ml. The formaldehyde inhibited any red cell acid phosphatase present. α-1,4-Glucosidase was assayed in a system of 0.18 M

citrate pH 4.9, 12.8mM p-nitrophenyl-α-D-glucopyranoside and 10μl of amniotic fluid, in a final volume of 0.15 ml. Alkaline phosphatase was assayed in a system of 62mM sodium carbonate buffer pH 10.75, 12.5mM $MgCl_2$, 32.5mM 'Sigma 104' phosphatase substrate and 5-20μl amniotic fluid in a final volume of 50μl. To determine the specific activity of the placental (heat-stable) alkaline phosphatase, an aliquot of amniotic fluid was incubated at 65°C for 25 minutes, after the method of Hunter (1969). The difference in specific activity between the unheated and heated amniotic fluid aliquots represented the heat-labile alkaline phosphatase. In all assays a control without amniotic fluid and a control without substrate were included.

The assay solutions were incubated for 2 hours at 37°C under saturated vapour pressure to minimise evaporation. The reactions were then stopped by the addition of 0.4M glycine-NaOH buffer pH 10.3, to a final volume of 1.0ml. The optical density of the solutions was measured spectrophotometrically at 400 mμ. The enzyme specific activities were calculated by subtracting the sum of the controls from the full systems, and using the molar extinction coefficient of p-nitrophenol (1.775×10^4 under these conditions) to express the results in μ moles substrate hydrolysed per hour per ml amniotic fluid. The protein concentration in amniotic fluid was measured by Lowry's method (Lowry et al 1951), and it was used to calculate specific activity in μ moles per hour per gm of protein.

iii. Inhibition of red cell acid phosphatase

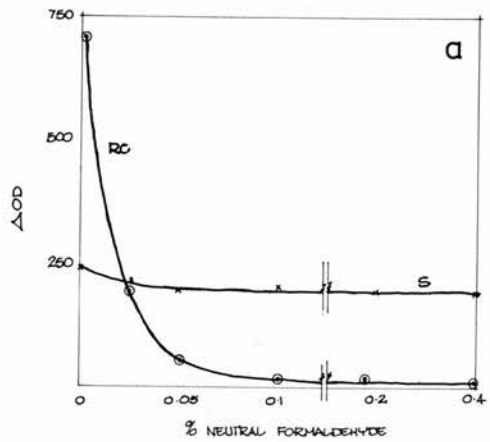
Red cell acid phosphatase is a different enzyme from lysosomal acid phosphatase which is found in other tissues. For example, whilst the red cell enzyme is polymorphic in humans (Hopkinson et al 1963)

no such variation is found in the lysosomal form. The activities of the two enzymes may be differentiated by a number of specific inhibitors. Neutral formaldehyde is a potent inhibitor of red cell acid phosphatase (Abul-Fadl and King, 1949), and it was therefore used to differentiate between the enzymes in this study. Fig36a shows the effect of neutral formaldehyde at various concentrations on the specific activity of red cell acid phosphatase. 0.4% Formaldehyde was found to almost completely inhibit the red cell enzyme whilst having little effect on the acid phosphatase in serum. An experiment was therefore made to find whether 0.4% neutral formaldehyde would selectively inhibit red cell phosphatase which had been added in varying quantities to an amniotic fluid obtained at term. Fig36 b shows that the effect of adding formaldehyde to the assay system is to selectively inhibit the red cell enzyme which had been added to the amniotic fluid. Although the inhibition is very strong it is not complete: since the curve in the presence of formaldehyde still increases with increasing red cell enzyme. However, the effect of formaldehyde is clearly great enough to show whether there is a significant red cell enzyme contribution to the acid phosphatase in amniotic fluid.

The specific activity of red cell acid phosphatase was measured as the difference between the specific activities of acid phosphatase in the presence and absence of 0.4% neutral formaldehyde. Fig37 shows the percentage of acid phosphatase in amniotic fluid which was of the non-red cell type. There appears to be a small contribution from red cell enzymes early in gestation, which disappears toward term.

Figure 36 a) The effect of neutral formaldehyde on the activity of red cell (RC) and serum (S) acid phosphatase.

b) The effect on the activity of acid phosphatase in amniotic fluid of adding various volumes of red cell lysate, assayed in presence and absence of 4% neutral formaldehyde (4% HCHO). 50µl of term amniotic fluid was present in all the assays, and 0-20µl of red cell lysate (at a concentration of $^{1}/_{70}$ v/v) was added, to test the extent to which the red cell phosphatase was inhibited.


750
500
250
ΔOD
a
RC
S
0
0·05
0·1
0·2
0·4
% NEUTRAL FORMALDEHYDE

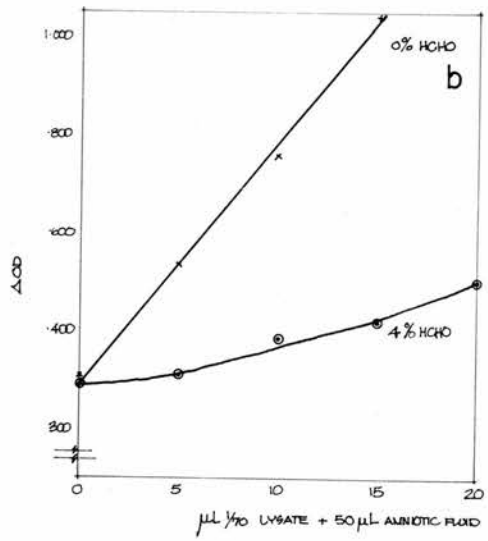
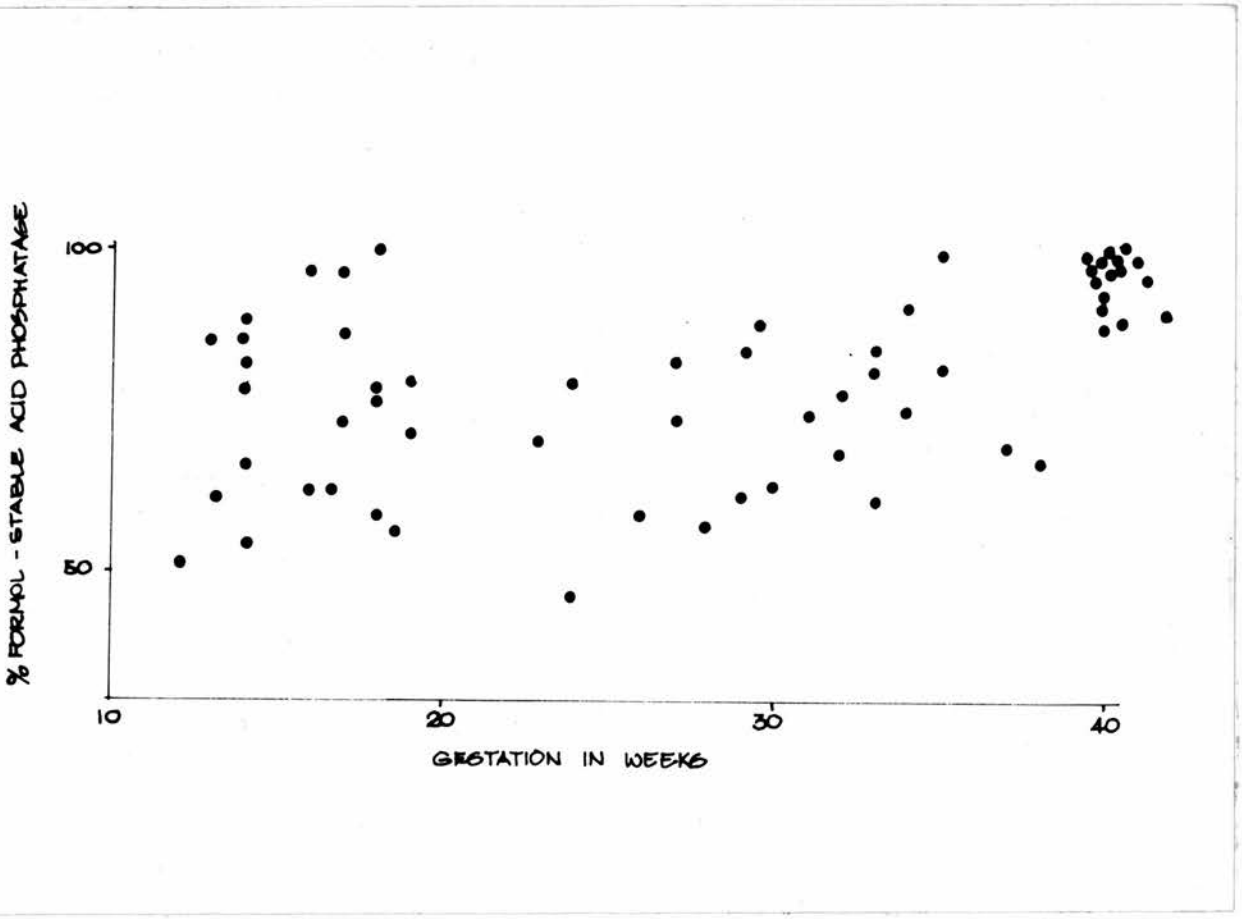

1·000
·800
·600
·400
·200
ΔOD
0% HCHO
b
4% HCHO
0
5
10
15
20
μL 1/10 LYSATE + 50 μL AMNIOTIC FLUID

Figure 37 The specific activity of formol-resistant (non-red cell) acid phosphatase in amniotic fluid, expressed as the percentage of the total acid phosphatase specific activity in amniotic flu


% FORMOL - STABLE ACID PHOSPHATASE
100
50
10
20
30
40
GESTATION IN WEEKS

iv. The measurement of placental alkaline phosphatase

A number of different forms of alkaline phosphatase occur in various human tissues (see Suzuki et al 1969), and the increase in phosphatase specific activity in the serum of pregnant women is known to be due to the appearance of a placental form (Beck and Clark, 1950; Boyer, 1961; McMaster et al 1964). This placental form of alkaline phosphatase has been found to be particularly resistant to heat denaturation (McMaster et al 1964; Hunter, 1969) and to be particularly strongly inhibited by phenylalanine (Suzuki et al 1969). These properties have lead to the use of either phenylalanine or heat to distinguish between the placental and non-placental forms of the enzyme in pregnant human serum.

Experiments were made to investigate the best system for measuring the placental and non-placental forms of alkaline phosphatase in amniotic fluid. Placental homogenates (in saline) served as a source of placental alkaline phosphatase and adult male serum as a source of non-placental alkaline phosphatase. Fig.38a shows the effect of varying concentrations of DL-phenylalanine in the assay system on the activity of the placental (P) and the serum (S) enzymes. At 18mM DL-phenylalanine, the placental phosphatase activity was inhibited by 75% whereas the male serum enzyme was subject to very little inhibition.

To test the heat-stability of the enzymes, aliquots of the serum and the placental extract were either kept at 4°C or were incubated at between 40 and 80°C for various periods of time. The aliquots were then tested for phosphatase activity. Fig.38b shows the effect of 25 minutes incubation at between 47° and 78°C. The serum phos-

phatase was almost entirely inactivated after 25 minutes at 65°C, whereas at the same temperature the placental enzyme had only lost 16% of the activity which was found in the aliquot kept at 4°C. The placental phosphatases are highly labile when exposed to temperatures above 70°C for 25 minutes.

The heat and phenylalanine sensitivities of the alkaline phosphatases in amniotic fluid were investigated next. The upper curve in Fig.38c shows the heat-stability profile of the enzymes in an amniotic fluid at term. There is a gradual loss of activity between 4 and 65°C, and a more rapid loss between 70° and 80°C. If the enzymes in amniotic fluid had similar stabilities to those in serum and placenta, the loss of activity between 70° and 80°C should be due to placental alkaline phosphatase, whilst the non-placental forms should have been inactivated by 65°C. Since phenylalanine selectively inhibits the placental form, the heat stability profile could be further investigated by assaying the enzyme activities in the presence of phenylalanine after the incubations were ended. Fig.38c shows the heat stability profiles of amniotic fluid alkaline phosphatases measured in the presence (+ Øala) and absence (-Øala) of 18mM DL-phenylalanine in the assay system. The curves show that a phenylalanine resistant form of the enzyme is progressively inhibited between 4 and 65°C, and that the loss of activity between 70 and 80°C is due to a phenylalanine-sensitive enzyme. The results are consistent with the hypothesis that the non-placental forms of alkaline phosphatase in amniotic fluid are selectively inhibited by incubation for 25 minutes at 65°C. This incubation period was therefore used to measure the placental

TABLE 10

Michaelis constants and pH optima of the enzymes

ENZYME	Km[+]	pH optimum
hexosaminidase	2.6	4.25
α-1,4-glucosidase	3.3	4.9
acid phosphatase	0.8[*]	4.9
total alkaline phosphatase	6.0	10.75

[+] expressed as mM substrate

[*] substrate inhibition above 10mM substrate

fraction of alkaline phosphatase in amniotic fluid and serum. The same system has been used by Hunter (1969) to measure placental alkaline phosphatase in maternal serum. Roopnarinesingh et al (1972) assayed placental alkaline phosphatase in amniotic fluids at term using 55°C as the inactivation temperature. This may give an over-estimate as the inactivation of the non-placental forms may be incomplete.

v. Enzyme kinetics

pH optima

To ensure that the enzymes were assayed at optimal pH, the effect of changing the pH was observed for each enzyme. Fig.39 shows the effect of pH on the activities of each enzyme. The pH optima are summarised in Table 10. Routine assays were carried out at the pH optima.

Michaelis constants

To ensure that the enzymes were assayed at optimal substrate concentrations, the effect of changing the substrate concentration was observed for each enzyme. Fig.40 shows the relationships between the substrate concentrations and enzyme activities. Note that in the case of acid phosphatase, there was inhibition of enzyme activity at substrate concentrations above10mM p-nitrophenyl phosphate. Each enzyme was routinely assayed under conditions of saturating substrate concentrations, except in the case of hexosaminidase, where the sparing solubility of the substrate precluded saturating conditions.

Effect of altering the quantity of enzyme present, or the length of the assay time

For the enzyme assays to the reliable, there should be a linear relationship between the quantity of the enzyme added to the

assay system and the quantity of substrate hydrolysed. There should also be a linear relationship between the length of time for which an assay is run and the quantity of substrate hydrolysed. Fig.41 shows that for each enzyme, the assay systems provided linear results both in terms of time and the quantity of enzyme added.

Figure 38 Inhibition studies on alkaline phosphatase. Details on pp.86-87.

(a) The effect of phenylalanine on the enzymes in placenta (P) and serum (S).

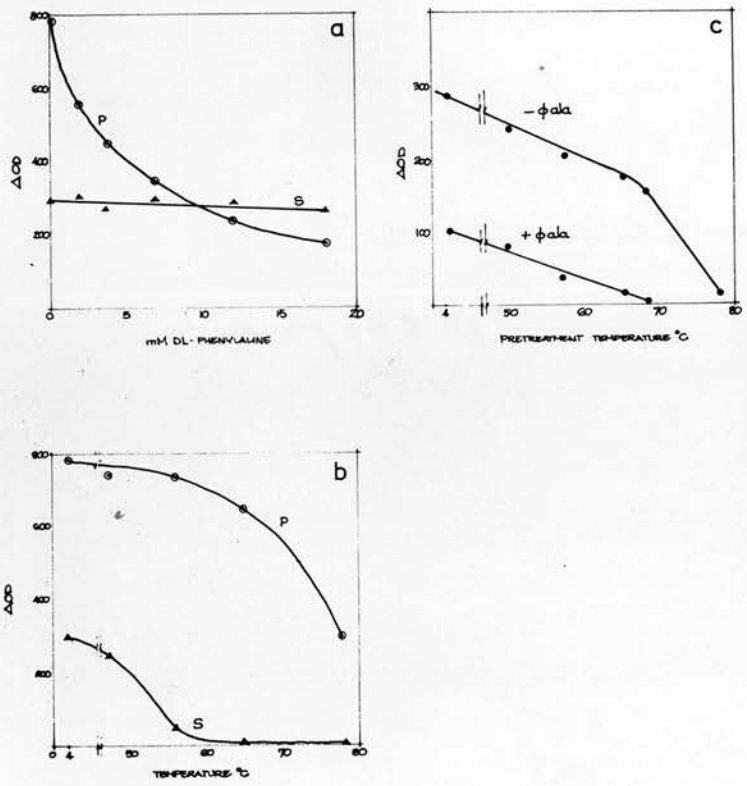
(b) The effect of temperature on the enzymes in placenta (P) and serum (S)

(c) The effect of temperature on the enzyme in term amniotic fluid, assayed in the presence (+ ala) and absence (- ala) of 18mM DL-phenylalanine.

Figure 39 The effect of pH on the activities of amniotic fluid enzymes.

(a) hexosaminidase, (b) α-1,4-glucosidase

(c) acid phosphatase, (d) total alkaline phosphatase


a
P
S
ΔOD
mM DL-PHENYLALINE
b
P
S
ΔOD
TEMPERATURE °C
c
-φala
+φala
ΔOD
PRETREATMENT TEMPERATURE °C

38

39

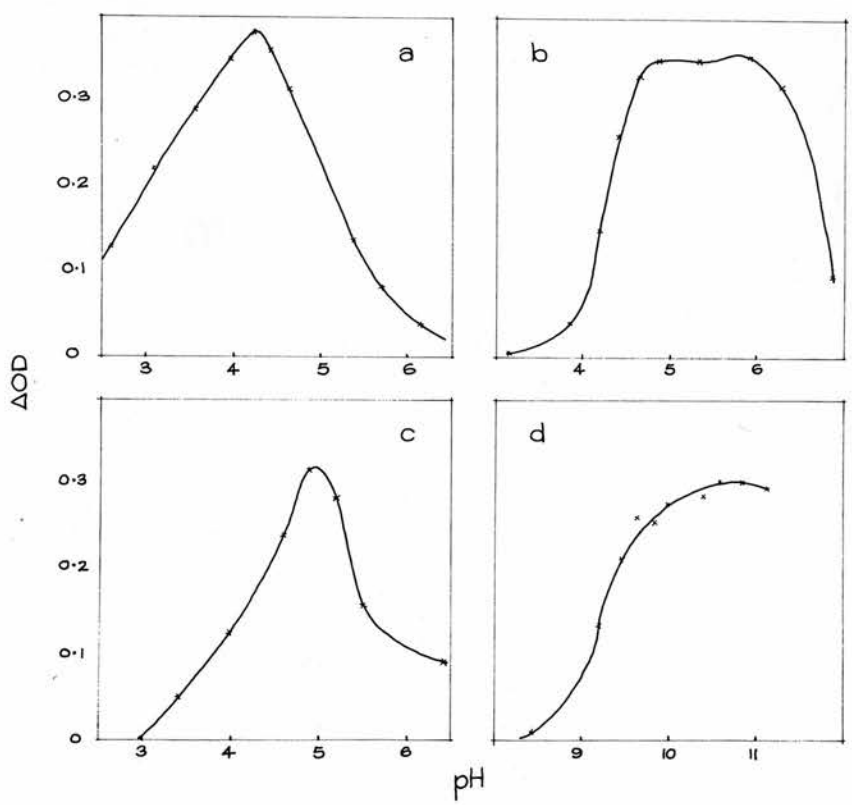

a
b
c
d
ΔOD
pH

Figure 40 The effect of substrate concentration on the activities of amniotic fluid enzymes. a) Lindwever-Burke plots of hexosaminidase (Hex) and α-1,4-glucosidase (Glu). b and c) Michaelis-Menten plots of (b) acid phosphatase, and (c) alkaline phosphatase.

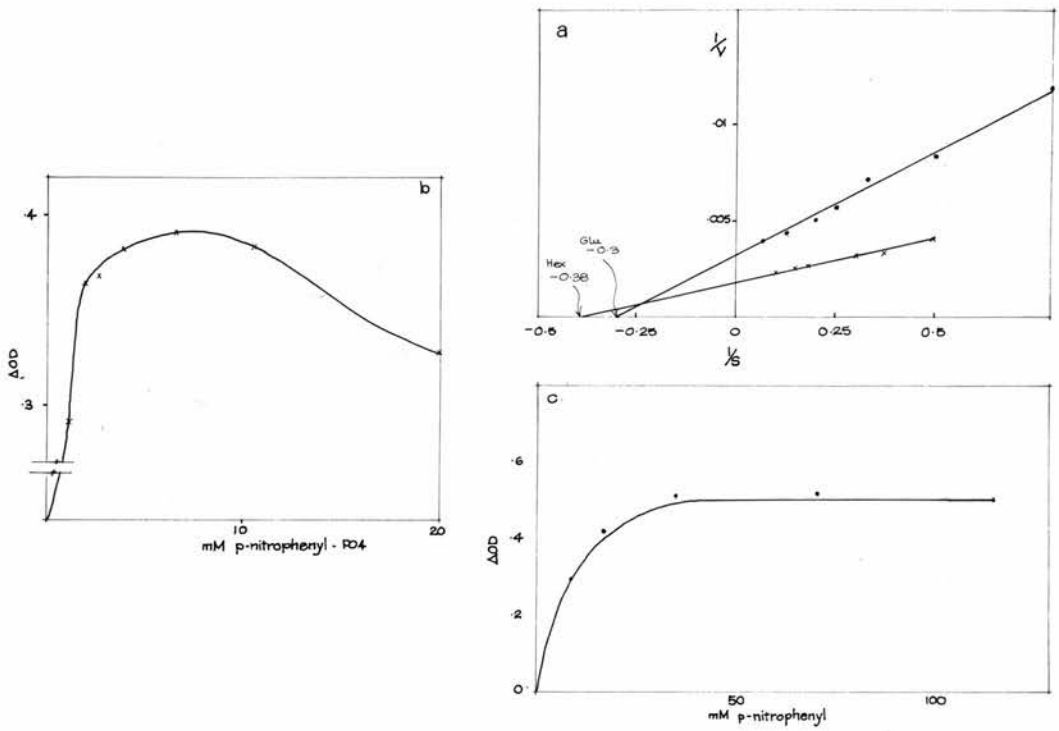
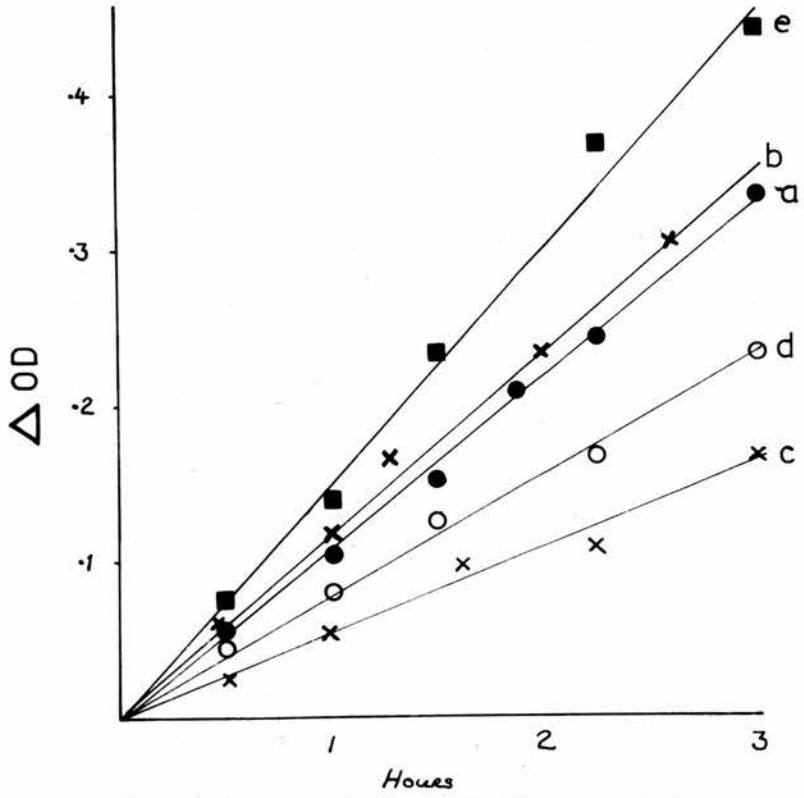

a
1/V
Glu
−0·3
Hex
−0·38
−0·5
−0·25
0
0·25
0·5
1/S
b
ΔOD
·3
·4
10
20
mM p-nitrophenyl - R04
c
ΔOD
0
·2
·4
·6
50
100
mM p-nitrophenyl

Figure 41a The effect of changing the time of incubation on the quantity of substrate hydrolysed by (a) hexosaminidase, (b) acid phosphatase, (c) α-1,4-glucosidase, (d) heat-labile alkaline phosphatase, and (e) heat-stable alkaline phosphatase. The volumes of amniotic fluid added to the assays were (a) 5μl, (b) 40μl, (c) (d) and (e) 10μl. All the assays were carried out on term amniotic fluid, with the exception of α-1,4-glucosidase, which was assayed using a 16 week sample of fluid.

For assaying conditions see pp. 83-84.

Figure 41b The effect of changing the volume of amniotic fluid added to the assay systems of (a) hexosaminidase, (b) acid phosphatase, (c) α-1,4-glucosidase, (d) heat-labile alkaline phosphatase, (e) heat-stable alkaline phosphatase.

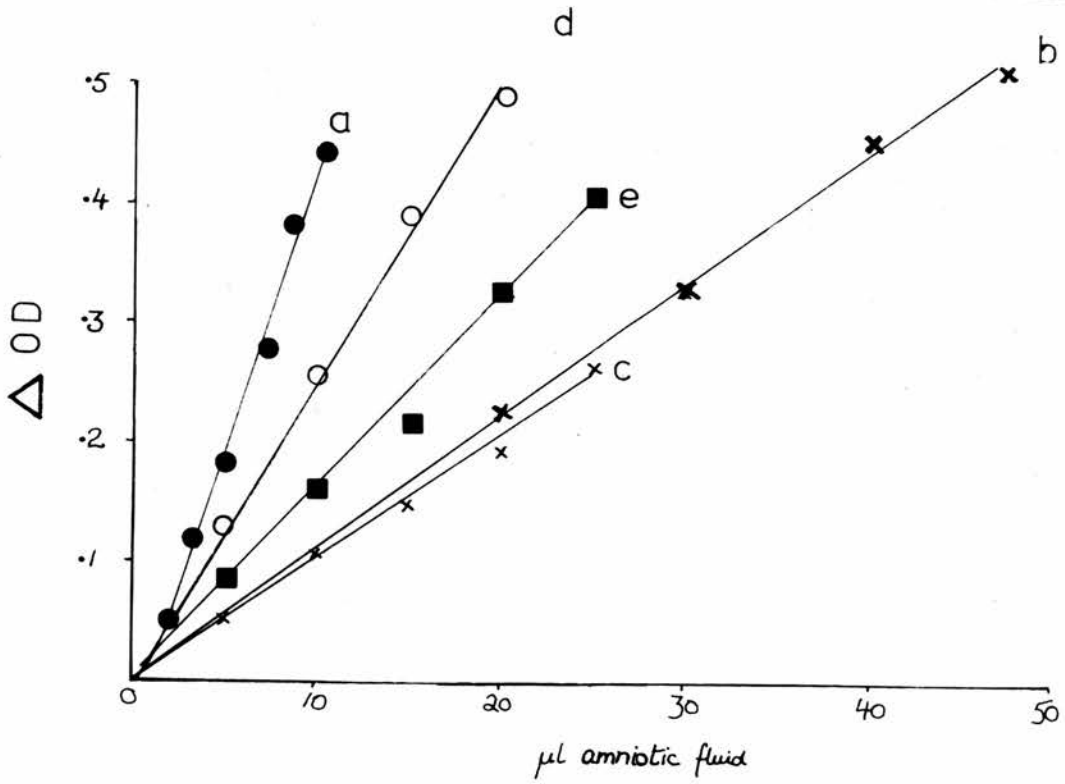
For assaying conditions see pp.83-84.


e
b
a
d
c
·4
·3
·2
·1
Δ OD
1
2
3
Hours

a

41

b


d
b
a
e
c
·5
·4
·3
·2
·1
Δ OD
0
10
20
30
40
50
μl amniotic fluid

c. Results

α-1,4-Glucosidase

Figs.42a and b show the patterns of specific activity on volume and protein bases respectively. A peak of activity was found between thirteen and eighteen weeks of gestation. After this period consistently low specific activities were observed.

Heat-Labile Alkaline Phosphatase

Figs.43a and b show the patterns of specific activity on volume and protein bases respectively. An initial peak in activity occurred between thirteen and eighteen weeks, and during this time a close relationship was found between the specific activities of heat-labile alkaline phosphatase and α-1,4-glucosidase in individual amniotic fluids (Fig44). Heat-labile alkaline phosphatase was found at very low levels between eighteen and thirty-six weeks of gestation, after which there was a considerable rise (accompanied by a wide scatter). When enzyme specific activity is expressed on a protein basis (Fig.43b) both peaks of activity are exaggerated, and the rise toward term occurs earlier.

Acid Phosphatase (Figs.45a and b)

Little change in specific activity per ml amniotic fluid was observed before about thirty-two weeks. After this time there was an increase in specific activity, together with considerable variation between individual values. Fig.45b shows that a minor peak in specific activity per gm protein occurs between thirteen and eighteen weeks of gestation, as well as the more obvious peak at term.

Hexosaminidase (Figs.46a and b)

No clear pattern emerges from the enzyme specific activity per ml amniotic fluid, although the lowest specific activities are found before fourteen weeks of gestation. However, Fig.46b shows the specific activity per mg protein is minimal between twenty and thirty weeks.

Placental Alkaline Phosphatase (Figs.47a and b)

A gradual increase in specific activity was observed between ten and thirty weeks, after which the specific activity rose more rapidly. Between thirty and forty weeks, the specific activity of placental alkaline phosphatase in amniotic fluid increases by 3 to 3.5-fold. Expression of activity on a protein basis lead to an exaggeration of the rise in the latter half of pregnancy.

Figure 42 Amniotic fluid α-1,4-glucosidase, expressed (a) per ml amniotic fluid, and (b) per mg protein, plotted against gestational age.

Symbols for Figs.42-43, 45-47

■ = Hysterotomy

● = Amniocentesis

▲ = Arteficial Rupture of Membranes, Normal pregnancy

X = Arteficial Rupture of Membranes, Anencephaly

α-1,4 GLUCOSIDASE μ mole/hr/ml AMNIOTIC FLUID
1·0
0·5
0
1·9
1·4
1·3
10
20
30
40
GESTATION IN WEEKS

a

α-1,4 GLUCOSIDASE μ mole/hr/mg protein
0·6
0·4
0·2
0
10
20
30
40
GESTATION IN WEEKS

b

Figure 43 Amniotic fluid heat-labile alkaline phosphatase, expressed (a) per ml amniotic fluid, and (b) per gm protein, plotted against gestational age.

a

HEAT LABILE ALKALINE PHOSPHATASE
μ mole/hr/ml AMNIOTIC FLUID
1·0
0·5
0
10
20
30
40
GESTATION IN WEEKS

b

1827
HEAT LABILE ALKALINE PHOSPHATASE
μ mole/hr/mg protein
800
500
200
150
100
50
0
10
20
30
40
GESTATION IN WEEKS

Figure 44 The relationship between amniotic fluid α-1,4-glucosidase and heat labile alkaline phosphatase between ten and twenty weeks of gestation, expressed as μmole/hr/ml amniotic fluid.

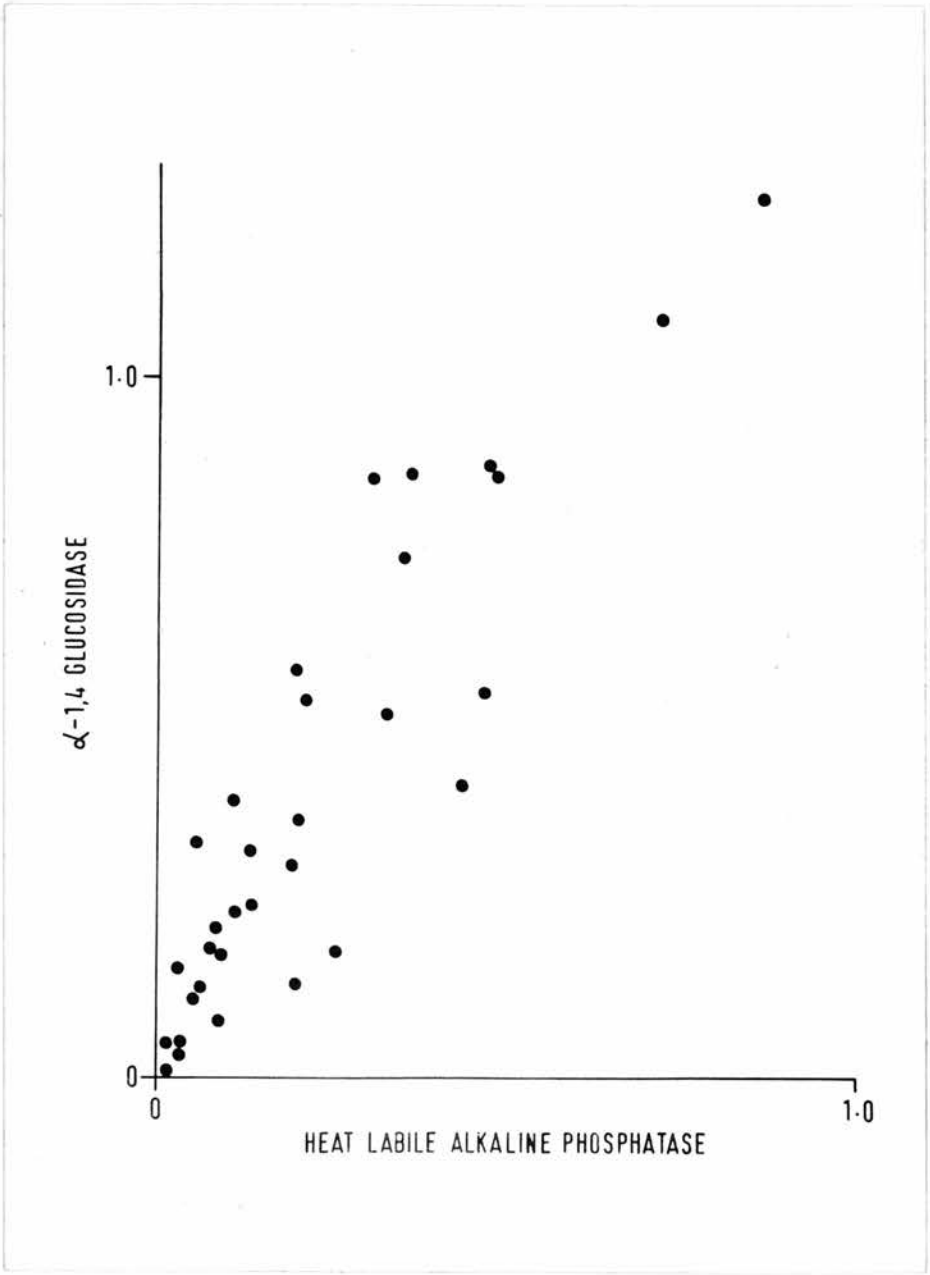

1·0
0
0
1·0
α-1,4 GLUCOSIDASE
HEAT LABILE ALKALINE PHOSPHATASE

Figure 45 Amniotic fluid acid phosphatase, expressed (a) per ml amniotic fluid, and (b) per gm protein, plotted against gestational age.

a
ACID PHOSPHATASE μ mole/hr/ml AMNIOTIC FLUID
0·4
0·2
0
0·9
10
20
30
40
GESTATION IN WEEKS

b
ACID PHOSPHATASE
μ mole/hr/gm protein
200
150
100
90
50
0
648
443
319
10
20
30
40
GESTATION IN WEEKS

Figure 46 Amniotic fluid hexosaminidase, expressed (a) per ml amniotic fluid, and (b) per mg protein, plotted against gestational age.

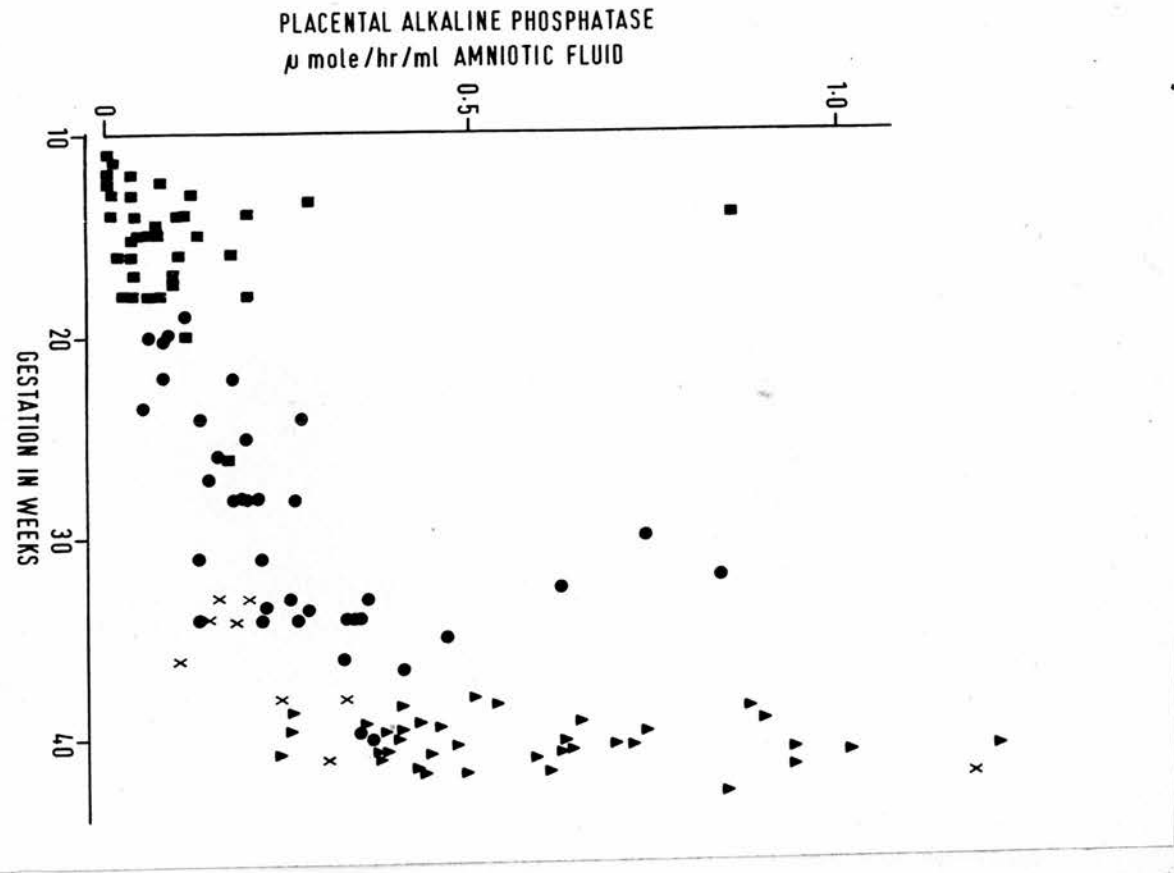
a

N Ac β-HEXOSAMINIDASE μ mole/hr/ml AMNIOTIC FLUID
2·0
1·0
0
10
20
30
40
GESTATION IN WEEKS
x 4·0

b

NAc β-HEXOSAMINIDASE
μ mole/hr/mg protein
1·5
1·0
0·5
0
10
20
30
40
GESTATION IN WEEKS

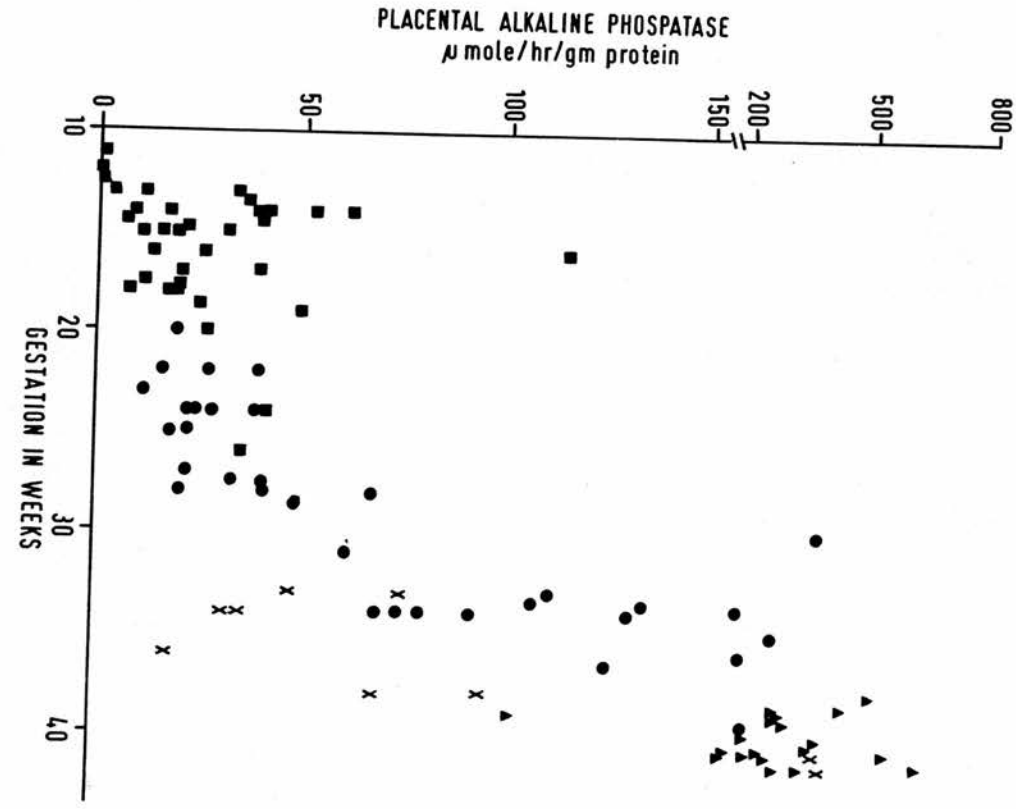
Figure 47 Amniotic fluid placental alkaline phosphatase, expressed (a) per ml amniotic fluid, and (b) per gm protein, plotted against gestational age.


PLACENTAL ALKALINE PHOSPHATASE
μ mole/hr/ml AMNIOTIC FLUID
0
0·5
1·0
10
20
30
40
GESTATION IN WEEKS

a

47

b


PLACENTAL ALKALINE PHOSPATASE
μ mole/hr/gm protein
0
50
100
150
200
500
800
10
20
30
40
GESTATION IN WEEKS

d. Discussion

The results show that complex changes of enzyme specific activity occur in the amniotic fluid as gestation proceeds. Generally, hexosaminidase had the highest specific activity in amniotic fluid and, with the exception of α-1,4-glucosidase, all the enzymes were at their highest specific activities at term.

Between thirteen and eighteen weeks of gestation, there is a peak in the specific activities of α-1,4-glucosidase and heat-labile alkaline phosphatase. The close correlation between the values for these enzymes in individual amniotic fluids (Fig.44) suggests that their activity peaks are due to the same developmental event. The effects of this event are not limited to these two enzymes, for there is a simultaneous increase in the specific activity of hexosaminidase and also minor activity peaks for the other enzymes.

Between twenty and thirty weeks of gestation, the specific activities of α-1,4-glucosidase and heat-labile alkaline phosphatase fall to their lowest levels, whilst those of the other enzymes remain unchanged. After thirty weeks, the specific activities of hexosaminidase and α-1,4-glucosidase remained unchanged, whilst those of acid phosphatase, placental alkaline phosphatase, and (somewhat later) heat-labile alkaline phosphatase rise to their maximal levels. Roopnarinesingh et al (1972) also found that the specific activity of placental alkaline phosphatase increased during this time, though these workers used 56°C as their inactivation temperature and so may have also been measuring a labile fraction of alkaline phosphatase. The considerable scatter of the specific activities of these phosphatases at term may be partially due to errors in estimating gestational age, or to the varying proximity of the onset of labour.

Such errors would have their greatest effect upon enzymes undergoing rapid changes in specific activity. The lack of correlation between the levels of the three enzymes in the individual amniotic fluids after 37 weeks does not seem to be due to technical problems. Meconium stained amniotic fluids were excluded from the series as they contain excessive quantities of (heat-labile) alkaline phosphatase (Geyer and Schneider, 1970). It was possible that the samples of amniotic fluid obtained at artificial rupture of the membranes were not typical of the rest of the fluid. This point was investigated in three patients at the induction of labour. In these patients, ten consecutive 20 ml samples of amniotic fluid were taken for analysis while it drained from the amnioscope. In no case was significant variation found in the protein concentration or in any of the enzyme specific activities.

In this study no significant correlations were found between the amniotic fluid protein concentration and the enzyme specific activities. However, the specific activities on a protein basis were plotted against gestational age because this caused an exaggeration of the early and late peaks of enzyme specific activity. This occurs because the protein concentration reaches a maximum at approximately twenty-five weeks of gestation (Queenan et al 1970; Fig.48). Although the biological basis of this expression of specific activity is questionable, the exaggeration it brings about may be valuable if the measurements are to be used for maturity testing or other studies during the last trimester of pregnancy.

The patterns of enzyme specific activity reported here show that complex factors control the composition of amniotic fluid, and the

possible nature of these factors is dealt with in a subsequent section (pp. 95-100). Yet, whatever the biological implications of these findings, the trends observed during the latter half of pregnancy suggest that the measurement of enzymes in amniotic fluid may be of use in the assessment of fetal maturity. In particular, the changes in phosphatase activity, on both volume and protein bases, appear to merit further investigation.

3. POSSIBLE ORIGINS OF AMNIOTIC FLUID ENZYMES

a. Introduction

A number of studies have suggested that the majority of the protein in amniotic fluid is of serum type. Paper electrophoresis has been used by a number of authors (see Table 1) to show the similarity between the amniotic fluid protein and serum protein. Derrington and Soothill (1961) immunised rabbits with amniotic fluid obtained at term, and found by immunoelectrophoresis that the antibody response was directed solely toward serum protein. In the work of von Kleist et al (1968) and that of our own (p.53) rabbits were immunised with amniotic fluid from various stages of gestation, and the majority of their immune responses were directed toward serum protein throughout gestation.

Although proteins of non-serum origin must constitute a minor fraction of the total amniotic fluid protein, their study is of interest for a number of reasons. Knowledge of the way in which local tissues contribute to the fluid may increase understanding of the formation and maintenance of the amniotic fluid. Secondly, the identification of proteins which come from tissues of fetal origin should be of value in the antenatal diagnosis of some genetic disease (O'Brien et al 1971; Friedland et al 1971; Brock and Sutcliffe, 1972 a and b) and finally data collected throughout pregnancy may be useful in the assessment of fetal maturity.

Immunological and enzymological techniques can be used in attempts to find tissue protein in the amniotic fluid. In the previous section the activity patterns of a group of amniotic fluid enzymes were investigated, and it was suggested that these measurements might be

used for the assessment of fetal maturity. In this section, the biological implications of the results are considered by firstly discussing the extent to which the enzymes are of serum origin, and then considering possible tissue origins of amniotic fluid enzymes.

b. Materials and Methods

The assay systems used have been described on p.83. The maternal and fetal sera were diluted respectively up to x10 and x5 with physiological saline before assay. Extracts of amniotic fluid cells were made using the methods of Sutcliffe and Brock (1971). In order to follow the release of cellular enzyme small serial samples of the supernatant were collected during the course of sonication. The maximum specific activities of released enzyme were used to calculate the ratio of supernatant to cellular enzyme in samples of amniotic fluid at term.

c. Results

i. Amniotic fluid protein concentration

Fig.48 shows the distribution of protein concentrations in amniotic fluid found throughout pregnancy. Although there is considerable scatter of results, there was a rise in mean protein concentration between ten and twenty-five weeks. The quadratic curve of best fit was calculated for this period by multiple regression analysis, and is shown in Fig. 48 . Between twenty-five and thirty-two weeks the protein concentration falls by half and remains at this level until term. Fig. 49a shows the rate of accumulation of protein in the amniotic fluid between ten and twenty-five weeks of gestation. It was constructed by taking the product of the mean amniotic fluid volume for each weekly period between ten and twenty-five weeks of gestation and the weekly protein concentration as calculated from the quadratic curve of best fit. The mean volumes were taken from the combined data of Wagner and Fuchs (1962); Gadd (1966), Rhodes (1966), Gillibrand (1969) and Abramovitch (1968). The difference between the products for consecutive weeks was taken as the rate of protein accumulation per week.

ii. Enzymes in serum and urine

The specific activities of hexosaminidase, acid phosphatase, α-1,4-glucosidase and the heat-labile and placental alkaline phosphatases were measured in maternal and fetal sera, and in fetal urine. The results are shown in Table 11. The specific activity of heat-labile alkaline phosphatase could not be measured in maternal serum at term because of the exceedingly high levels of placental alkaline phosphatase.

Figure 48 The protein concentration in amniotic fluid supernatant measured by Lowry's method. The best fitting quadratic curve for the data between ten and twenty-five weeks of gestation was plotted. It is of the form:

$$P = 1.266G + 0.026G^2 - 9.822$$

where P = protein concentration, and G = gestation in weeks*.

■ = Hysterotomy

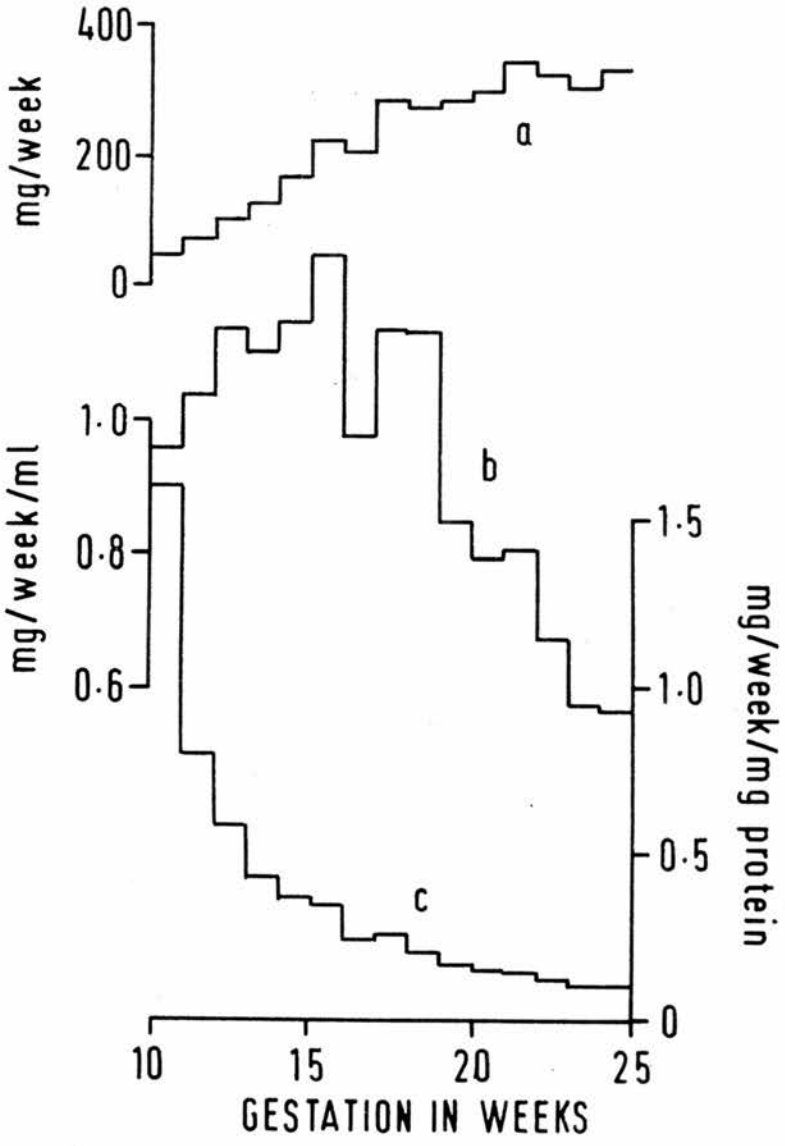
● = Amniocentesis

▲ = Arteficial Rupture of Membranes, Normal pregnancy.

× = " " " Ancephaly.

Figure 49 Histograms of amniotic fluid protein influx calculated from the regression curve in Fig.48 and the mean amniotic fluid volumes in the literature. The graphs show the rate of protein influx expressed per week (a), per week per ml amniotic fluid (b) and per week per mg amniotic fluid protein (c).

*Grateful thanks are due to Miss S. Holloway, BSc., for computing the quadratic curve of best fit.


400
200
0
mg/week
a
1·0
0·8
0·6
mg/week/ml
b
c
1·5
1·0
0·5
0
mg/week/mg protein
10
15
20
25
GESTATION IN WEEKS

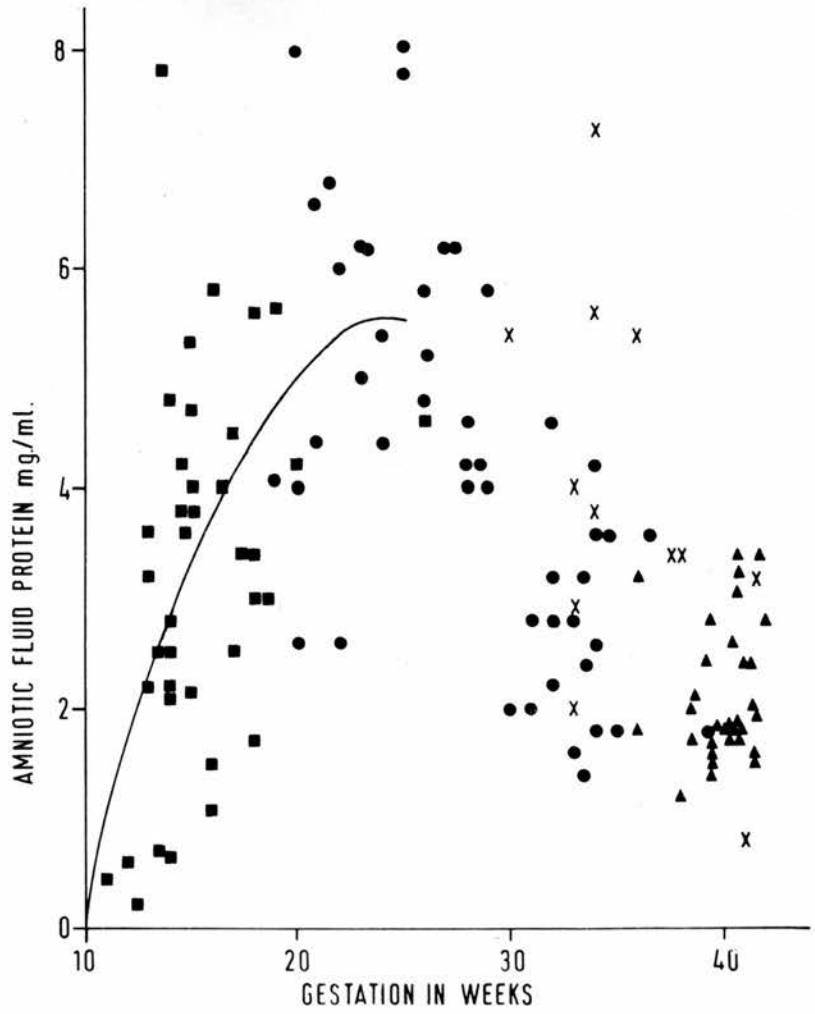

8
6
4
2
0
AMNIOTIC FLUID PROTEIN mg/ml.
10
20
30
40
GESTATION IN WEEKS

Figure 50 The liberation of amniotic fluid cell enzymes during sonication. The maximum specific activities were used to calculate the enzyme specific activities in amniotic fluid.

Figure 51 The decay of enzyme activity in a sixteen week amniotic fluid incubated at 37°C. The enzymes are placental alkaline phosphatase (▼), heat-labile alkaline phosphatase (■), acid phosphatase (◆), hexosaminidase (●) and α-1,4-glucosidase (▲).

PROTEIN
mg/ml
4·0
1·0
ALKALINE
PHOSPHATASE
5
3
1
ACID
PHOSPHATASE
m μ mole/hr
30
20
10
β - HEXOSAMINIDASE
100
60
20
0
50
100
150
200
SONICATION TIME IN SEC.

50

51

PERCENT OF INITIAL ACTIVITY
100
50
0
5
10
DAYS AT 37 °C

TABLE II

Enzymes* in Maternal and Fetal Serum and Fetal Urine

	Gestation weeks	No.	hexosamin-idase	Acid phosphatase	Alkaline phosphatase		α-glucos-idase
					Heat labile	Placental	
Maternal Serum							
	9-11	10	2.46 1.72-3.42	0.48 0.35-0.75	0.54 0.42-0.81	0.10 0.07-0.12	.07
	13-18	11	3.00 2.05-4.08	0.5 0.34-0.79	0.65 0.39-0.81	0.33 0.12-0.82	.08
	39-41	16	8.33 5.21-13.0	0.55 0.41-0.78	†	11.90 4.60-22.6	-
Fetal Serum							
	13-18	10	2.20 1.2-7.23	0.87 0.40-1.90	4.43 2.65-6.7	0.26 0-0.66	0.26 0.13-0.42
	30-38	7	1.28 0.27-1.79	0.60 0.44-0.79	2.33 1.0-3.54	0.3 0-0.45	- 0.11-0.15
	39-41	11	2.2 1.70-3.82	1.64 0.70-2.51	2.91 1.31-5.23	0.38 0.21-0.83	-
Fetal Urine					**Total**		
	13-18	11	0.47 0.15-0.90	0.56 0.28-1.10	0.08 0-0.18		0.03 0-0.07
	22.40	6	0.26 0.05-0.37	2.20 0.45-4.38	0.14 0.04-0.13		0.25 0.10-0.45

* Mean and range of observations, expressed as μmoles substrate hydrolysed per hr per ml at 37°C.

† Accurate measurement not possible due to high levels of placental alkaline phosphatase.

Enzymes in amniotic fluid cells

The liberation of enzyme from amniotic fluid cells during the course of sonication is shown in Fig.50. Between 40 and 150 seconds of sonication were required for maximum enzyme liberation. From the results the quantity of cellular enzyme was calculated and expressed as the specific activity of enzyme (mole/hr) per precipitate from 1ml of amniotic fluid (Table 12). In each case the quantity of enzyme present in 1ml amniotic fluid supernatant was measured as mole/hr per ml supernatant. The ratio of the supernatant to cellular enzyme was then calculated. In each case the quantity of enzyme in the supernatant exceeded that in the cell fraction (Table 12).

TABLE 12

Ratio between the enzyme specific activities in the supernatant and cellular fractions of amniotic fluid

Enzyme	Case	Amniotic fluid enzyme content		
		Supernatant*	Cells‡	Supernatant/Cell Ratio
Hexosaminidase	D	1.44	0.67	2.15
	E	2.00	0.56	3.57
	T	1.45	0.64	2.27
Acid phosphatase	D	0.21	0.02	10.5
	E	0.24	0.03	8.00
	T	0.13	0.03	4.33
Heat labile alkaline phosphatase †	D	1.11	0.02	56.0
	E	0.87	0.03	29.0
	T	0.75	0.02	37.5

*μmole/hr per ml supernatant

‡μmole/hr per precipitate from 1 ml amniotic fluid

† measured as heat labile alkaline phosphatase in supernatant, and total alkaline phosphatase in cells.

d. Discussion

Immunological methods have shown that most of the protein in amniotic fluid is of serum type (Derrington and Soothill, 1961; von Kleist et al 1968; p.53). Thus the protein concentration determined by Lowry's method measures the approximate concentration of serum protein in the amniotic fluid. Figure 43 shows that this protein concentration rose from ten weeks of gestation to reach a maximum between twenty and thirty weeks, and then declines until term. These findings are in good agreement with the work of Queenan et al (1970), who used the biuret method for protein measurement.

In contrast to the protein concentration, the specific activities of the amniotic fluid enzymes studied in the previous section (p.90) showed little or no tendency to rise initially and reach a maximum between twenty and thirty weeks. In most cases we found maximal enzyme specific activities at term and/or between thirteen and eighteen weeks of gestation. These obvious differences lead one to question the extent to which serum can be a major source of these enzymes in amniotic fluid.

For an amniotic fluid enzyme to be of serum origin, three conditions must be fulfilled. Firstly, it must be able to pass into the amniotic sac from the serum. Secondly, its specific activity pattern during gestation should be predictable from the concentration changes of other serum proteins in the amniotic fluid. Finally, the gradient of enzyme specific activity between the serum and the amniotic fluid should be similar to the concentration gradients of proteins which are known to be of serum origin.

i. Molecular Weight

Previous studies (Usategui-Gomez et al 1966; Gitlin and Biasucci, 1969; see p.36) suggest that serum proteins which have molecular weights of less than about 170,000 can permeate into the amniotic fluid. Four of the enzymes studied here have molecular weights under 170,000. Robinson and Stirling (1968) found the molecular weight of hexosaminidase to be 100,000. Placental alkaline phosphatase is dimeric at physiological pH, and has a molecular weight of 116,000 (Sussman and Gottlieb, 1969). The three forms of acid phosphatase which have been found in human placenta have molecular weights respectively of 35,000; 105,000; and over 200,000 (Di Pietro and Zengerle, 1967). No data is available for human α-1,4-glucosidase, however the enzymes purified from bovine and from rat liver have molecular weights of 107,000 and 114,000 respectively, (Bruni et al 1969; Jeffrey et al 1970). Human heat labile alkaline phosphatase exists in a number of distinct forms and has not yet been purified.

ii. The effect of lability on expected enzyme specific activity patterns

Enzymatic proteins are more labile than the majority of serum proteins, and most of the enzymes studied here progressively lose activity when incubated in vitro at 37°C (Fig.51). This observation makes it important to decide whether the specific activity pattern of a labile enzyme of serum origin will differ from that of the amniotic fluid protein concentration. Because of the difficulty of extrapolating from in vitro lability data to the in vivo situation, a theoretical examination of enzyme lability will be made, and its result will be compared with the pattern expected for a stable enzyme of serum origin.

Stable amniotic fluid enzymes of serum origin would have patterns of specific activity per ml parallel to the protein concentration (provided the enzyme levels in serum remain constant). However, the phenomenon of enzyme lability will reduce the rate of accumulation of measurable enzyme, so that the specific activity of a very labile enzyme is controlled by its rate of influx into the amniotic fluid. The rate of influx of the enzyme will be similar to that of the other serum proteins, in proportions controlled by molecular weight and other characteristics. Before about 25 weeks the rate of influx of protein into the amniotic fluid will be in proportion to its rate of accumulation (see Fig.49 line a), since removal of protein by fetal swallowing is negligible at this time. (By 20 weeks of gestation the fetus swallows less than 5% of the total volume of liquor in twenty-four hours, Abramovitch, 1970).

Since the total specific activity of a highly labile enzyme will be in proportion to the rate of influx of protein into the amniotic fluid, division of the rate of influx of protein by the mean total fluid volume and the mean total fluid protein content respectively will provide patterns which are in proportion to a labile enzyme's specific activity per ml amniotic fluid and per mg protein. The results of this calculation are shown as histograms in Fig.49 lines b and c respectively. The influx of total protein on a volume basis is maximum at about sixteen weeks whilst expression on a protein basis results in a maximum between ten and eleven weeks of gestation. This result suggests that in the case of labile enzymes early in gestation, the mere demonstration of a difference between the patterns of enzyme activity and of protein concentration in the amniotic fluid is insufficient to allow the conclusion that the

enzyme comes from a source other than serum. This conclusion applies particularly to α-1,4-glucosidase, heat labile alkaline phosphatase, and hexosaminidase, all of which show considerable increases between ten and fifteen weeks of gestation. The analysis cannot, however, be extended beyond about twenty-five weeks of pregnancy because the increasing rate of fetal swallowing (Pritchard, 1965) will increase the rate of protein efflux from the fluid, and so prevent an accurate assessment of influx.

iii. Analysis of concentration gradients

If an enzyme is primarily of serum origin, the ratio of its specific activities between serum and amniotic fluid should be similar to the concentration ratios of other proteins which are found in amniotic fluid, and which are known to be exclusively of serum origin. The α_2 group-specific component (Gc) in amniotic fluid is known to be of maternal serum origin throughout gestation (p.61-65) and so can be used as an approximate measure of the maternal serum : amniotic fluid protein concentration gradient. Similarly, α-fetoprotein may be used as a measure of the fetal serum : amniotic fluid protein concentration gradient, since it is practically absent from the maternal serum (Ruoslahti and Seppälä, 1972). The mean concentration gradient for Gc has been found to increase during gestation from 15 at between 13 and 18 weeks of gestation, to 32 at term (Table 5). In the case of α-fetoprotein the mean gradient between thirteen and eighteen weeks has been found to be 206 (p. 77) : a magnitude which is similar to that found earlier in gestation by Gitlin and Boesman (1966).

Table 13 shows the mean specific activities observed for the enzymes in amniotic fluid at term and between thirteen and eighteen

TABLE 13

The mean specific activities* observed for the amniotic fluid enzymes, and those calculated from the maternal and fetal serum enzyme activities summarised in Table 11

	No.	Mean* observed	Means* expected		Observed / total expected
			Maternal serum or fetal serum origin		
13-18 weeks gestation					
hexosaminidase	36	1.06	0.20	0.01	5.0
acid phosphatase	35	0.06	0.03	<0.01	1.5
heat-labile alkaline phosphatase	30	0.24	0.04	0.02	4.0
placental alkaline phosphatase	29	0.09	0.02	< 0.01	3.0
α-1,4 glucosidase	30	0.54	0.01	< 0.01	27.0
Pregnancies at Term					
hexosaminidase	25	1.72	0.26	-	6.6
acid phosphatase	25	0.23	0.02	-	11.5
heat-labile alkaline phosphatase †	25	0.60	0.02	-	30.0
placental alkaline phosphatase	25	0.65	0.37	-	1.8

* μmole substrate hydrolysed/hr/ml amniotic fluid.

† using maternal serum data from thirteen to eighteen weeks gestation, see Table 11

weeks of gestation. The expected specific activities have been calculated from the maternal and fetal serum enzyme specific activities (Table11), and the maternal or fetal serum : amniotic fluid protein gradients as outlined above. The term data has not been tested on the hypothesis of a fetal serum origin since the α-fetoprotein gradient at term is very high (Gitlin and Boesman, 1966). The results show that in all cases the specific activities observed in amniotic fluid are in excess of those calculated on the hypothesis of a serum origin for these enzymes. Particularly striking are the low levels which should be expected on the hypothesis of a fetal serum origin.

The validity of the analysis in Table13 must be examined, since surface properties and molecular weight are of major importance in determining the rate of diffusion of individual proteins. The molecular weights of most of the enzymes studied are greater than those of Gc and α-fetoprotein.* Thus the concentration gradients of the latter proteins may be underestimates of the expected specific activity gradients of the enzymes. If this is so, the conclusion that many of the enzymes are of non-serum origin is made more likely. However, an independent line of evidence suggests that the analysis is of value, at least in the case of placental alkaline phosphatase at term, for which an expected to observed ratio of 1.8 was calculated. Between thirty weeks of gestation and term, the concentration of major serum proteins in amniotic fluid falls by approximately half (Figs. 10-13,48). Therefore, if the amniotic fluid placental alkaline phosphatase is of serum origin, its specific activity would not rise at the same rate as in the maternal serum. Between thirty and forty

* molecular weight of Gc = 50,000 (Schultz and Heremens, 1966)
of α-fetoprotein = 75,000 (p.79)
of enzymes : see p.101

weeks of gestation the specific activities of the enzyme in both amniotic fluid (Fig.47a) and in maternal serum (Hunter, 1969) increase by 3 to 3.5 fold. This suggests that there is approximately twice the expected enzyme specific activity in term amniotic fluid, and that the enzyme is therefore not in simple equilibrium with that in maternal mixed venous serum. This bears out the conclusion from Table 13.

To what extent do the calculations for placental alkaline phosphatase provide an insight into the relationships between the amniotic fluid and the placenta? Placental alkaline phosphatase is located in the placental trophoblast (Ahmed and King, 1959; Wislocki and Padykula, 1961; Hunter, 1969), so that in haemochorial placentae it is not possible for the enzyme to diffuse directly from actively exchanging trophoblast through the membrane into the amniotic fluid, without passing into the maternal serum (see fig.3. p.7, and Hamilton and Boyd, 1960). Thus placental alkaline phosphatase might be expected to behave in amniotic fluid as a maternal serum protein. However, as discussed above, the enzyme does not behave in amniotic fluid as though it was simply of maternal serum origin.

There are two possible explanations for these findings. The first is that the enzyme specific activity in mixed venous blood is significantly less than that in the maternal placental blood. The difference in the specific activity of placental alkaline phosphatase between the arterial and venous sides of the maternal placental circulation may be calculated from the in vivo decay of the enzyme rate post-partum, and the rate of circulation of maternal blood through the placenta. The post-partum clearance rate of placental alkaline phosphatase is approximately 1 L/day (from Hunter, 1969).

Footnote to p.106

c = Clearance = 1L/day

v = Blood volume = 5L

r = Rate of blood flow through placenta = 500 ml/min

V = Specific activity of enzyme in placental venous blood

A = " " " " " " arterial blood

If there was only one complete circulation of blood through the placenta in one day, then:

$$V = A + \frac{cA}{v}$$

Total number of blood circulations through the placenta in one day $= \frac{r}{v}$

For $\frac{r}{v}$ circulations per day:

$$V = A + \frac{cA}{v} \times \frac{v}{r}$$

$$= A\left(1 + \frac{c}{r}\right)$$

$$\therefore \frac{V}{A} = 1 + \frac{c}{r}$$

$$r = 0.5 \times 60 \times 24 \text{ L/day}$$

$$= 720$$

$$\therefore \frac{V}{A} = 1 + \frac{1}{720} = < 0.002$$

The maternal circulation rate through the term placenta is about 500 ml/min. (Assali et al 1960). From this, the maternal veno-arterial ratio for placental alkaline phosphatase may be calculated as being only of the order of 1.002 to maintain the specific activity of the enzyme in serum.* This small difference shows that the mixed venous serum adequately reflects the enzyme activity of circulating placental blood. Only in the event of maternal venous pooling beneath the chorionic plate might serious discrepancies arise. Perhaps a more acceptable explanation for the excessive quantities of placental alkaline phosphatase in amniotic fluid, is that parts of the trophoblast are closely applied to the chorionic plate so that an enzyme diffusion pathway exists which is independent of maternal serum. Such trophoblast would not be carrying out transport functions as it would be 'starved' of maternal serum. The extent to which the specific activity of placental alkaline phosphatase exceeds that expected on the basis of the maternal gradient argument above may therefore reflect the extent of trophoblast 'starvation'.

iv. Alternative tissue sources of enzyme

Having shown that maternal serum is neither a major source of α-1,4-glucosidase, hexosaminidase or of placental or heat labile alkaline phosphatase between 13 and 18 weeks, nor of hexosaminidase, acid phosphatase, or heat-labile alkaline phosphatase towards term, alternative tissue sources must be discussed. Any of the fetal or maternal tissues which are adjacent to the amniotic fluid or which are able to secrete protein into the fluid must be regarded as potential sources. These include fetal serum, urine, skin and respiratory and alimentary secretions, as well as umbilical cord (a potential source of mucopolysaccharide), placenta, amnion,

* see opposite page

chorion and amniotic fluid cells. Maternal protein of non-serum origin can only enter the amniotic fluid from the uterine decidua and myometrium.

It is possible that amniotic fluid cells contribute to the fluid enzymes. However, it is difficult to understand how they could cause the changes in enzyme activity observed between ten and twenty weeks of gestation, since Nelson and Emery (1970) have shown that the concentration of amniotic fluid cells is maximal at term. The relationship between the enzyme specific activities in the cells and the supernatants of three term amniotic fluids was therefore studied. Table 12 shows that in one ml of amniotic fluid, there was more enzyme activity in the supernatant fluid than in the cells. In the case of heat-labile alkaline phosphatase there was between 29 and 56 times as much enzyme in the supernatant as in the cells, whereas the ratio for acid phosphatase was 4-10, and for hexosaminidase 2-4. From these results it is clear that at term the minority of amniotic fluid enzymes is cellular, and that the cells cannot be a major source of alkaline phosphatase or acid phosphatase. It remains to be decided whether the supernatant : cell ratio for hexosaminidase indicates that cells are a major source of this enzyme.

A similar supernatant : cell analysis has not been made on amniotic fluid early in gestation. However, there are reasons to suggest that amniotic fluid cells are an insignificant source of supernatant enzyme between thirteen and eighteen weeks of gestation. Firstly, the cell concentration is very low at the time of the early specific activity peaks of hexosaminidase, α-1,4-glucosidase, and heat-labile alkaline phosphatase. Further, between twenty and

thirty weeks, when the cell concentration is increasing at its maximal rate (Nelson and Emery, 1970) no obvious increase in enzyme specific activity occurs.

The similarity between the early activity peaks of α-1,4-glucosidase and heat-labile alkaline phosphatase suggests that the same tissue is responsible for liberating both enzymes. However, it is not known whether this tissue is maternal or fetal. Genetic studies should provide the clearest answer to this question, yet both successful (Nadler and Messina, 1969) and unsuccessful (Nadler et al 1970) prenatal diagnoses of Pompe's disease have been reported using the measurement of supernatant α-1,4-glucosidase. However, it is difficult to decide on the biological significance of this observation owing to the lack of extensive control data, and possible complications due to unrecognised fetal heterozygosity. Of considerable interest is the finding of Salafsky and Nadler (1971) that the majority of amniotic fluid α-1,4-glucosidase at sixteen weeks is bound to free cellular ogranelles. These organelles must be of fetal origin which suggests that the enzyme is also of fetal origin. Paradoxically, the same workers (Salafsky and Nadler, 1972) have also claimed that the α-1,4-glucosidase in amniotic fluid is not kinetically identical to that of fetal tissue or amniotic fluid cells. At present, no firm conclusions can be reached about the origin of the α-1,4-glucosidase of amniotic fluid. However, it must be stressed that if the enzyme is of maternal origin, then it must be synthesised within the uterus, since its diffusion pathway into the amniotic sac must be independent of maternal serum.

In contrast to α-1,4-glucosidase, heat-labile alkaline phosphatase is found at high specific activities both early and late in

gestation. Electrophoresis of various human tissue extracts have shown that at least four distinct forms of the phosphatase exist (see Suzuki et al 1969), so that it is unwise to regard the amniotic fluid enzyme as a single entity, or to assume that the same entity participates in both the early and late peaks of activity. Indeed, since α-1,4-glucosidase is not elevated at term, it seems that the phosphatase peak at term is not due to the same process of tissue break-down as occurred earlier in gestation.

There is a lack of similarity between the patterns of α-1,4-glucosidase, hexosaminidase and acid phosphatase during the last eight weeks of gestation. Although all three enzymes are probably of lysosomal origin and are ubiquitously distributed in body tissues, acid phosphatase alone shows a marked increase in specific activity toward term. This suggests that the increased levels of acid phosphatase are not due to local tissue changes, and argues against the suggestion of Seelich and Gomolka (1952) that the placenta is a major source of amniotic fluid acid phosphatase. These workers claimed that the ratio of specific activityof acid phosphatase and placental alkaline phosphatase in the amniotic fluid agreed well with that in the placenta; however, Ahmed and King (1959) were unable to confirm this. The absence of an elevation of acid phosphatase in maternal or fetal serum in the last eight weeks of pregnancy suggests that the placenta as a major source of acid phosphatase.

It is of interest to note (see Table11) that the specific activity of acid phosphatase in fetal urine is considerably greater than that of either hexosaminidase or α-1,4-glucosidase. Further,

the specific activity of acid phosphatase in fetal urine is considerably greater than in most amniotic fluids, so that fetal urine may be tentatively advanced as a major source of amniotic fluid acid phosphatase during the last eight weeks of gestation.

e. Conclusion

This study shows that the majority of the enzymes investigated are probably not of fetal or maternal serum origin. The complex patterns of enzyme activity found between thirteen and eighteen weeks of gestation, and during the last two months of pregnancy, suggest that significant quantities of tissue protein are released into the amniotic fluid. However, the sources of non-serum protein in the amniotic fluid remain in doubt.

GENERAL DISCUSSION

i. The Nature and Origin of Amniotic Fluid Protein

It has been shown by Queenan *et al* (1970) and also in this thesis (Fig. 48) that the soluble protein in amniotic fluid reaches maximal concentrations at about twenty-five weeks of gestation. To understand the significance of the changes in amniotic fluid protein concentration it was necessary to investigate the nature and origin of the protein. Although Derrington and Soothill (1961) had shown that the majority of the soluble proteins in amniotic fluid *at term* were typical serum proteins (e.g. albumin, transferrin), the nature of the protein earlier in gestation had not been thoroughly examined, and it was possible, for example, that the peak in protein concentration at twenty-five weeks was due to an influx of protein from other sources. This question was investigated immunologically in the present study, by raising rabbit antibodies against amniotic fluid protein. It was found that when amniotic fluid protein from various stages of gestation was injected into rabbits, the majority of the immune response was directed against serum proteins. This confirmed the preliminary findings of von Kleist *et al* (1968). The major proteins were found to be albumin, α_1-antitrypsin, group-specific component and transferrin, and the changes in concentration of these proteins in amniotic fluid were very similar to the changes in the concentration of the total soluble protein. These observations showed that throughout gestation the majority of the soluble protein in amniotic fluid was of serum type, and experiments were therefore made to investigate the origins of these serum proteins in amniotic fluid.

Previous studies on amniotic fluid at term had shown that the group-specific component (Gc), transferrin, and albumin are mainly of maternal origin (Ruoslahti et al 1966; Tallberg et al 1966; Usategui-Gomez and Morgan, 1966; Gitlin et al 1964). The present study investigated the origin of Gc in amniotic fluids from earlier stages of pregnancy, and showed that it was of maternal origin from ten weeks of gestation onwards. A close similarity was found between the concentration pattern of amniotic fluid Gc during pregnancy, and the patterns of albumin, α_1-antitrypsin and transferrin, and this suggested that the other serum proteins were also mainly of maternal origin throughout gestation. This possibility was supported by two additional facts. First, the concentration gradients of these proteins between fetal serum and amniotic fluid were much less than that for α-fetoprotein (which comes from the fetus) both between 13 and 18 weeks of gestation and at term. Secondly, when calculations were made of the ratios of the concentration of Gc to the concentration of either albumin, α_1-antitrypsin, or transferrin in amniotic fluid and maternal serum, there was a proportionality between the ratios which suggested that in addition to Gc, the other serum proteins were also of maternal origin (p.59. figs.14-16).

Although these data suggest that the majority of amniotic fluid serum protein is of maternal origin throughout pregnancy, there is very likely to be a small fetal contribution. The evidence for this is the presence of small quantities of α-fetoprotein in amniotic fluid, and the presence of albumin in fetal urine early in gestation (Gitlin and Boesman, 1966; and Fig.33). Further support comes from the presence of serum protein in umbilical cord matrix (see p.39).

The presence of serum protein in the umbilical cord has been confirmed in this laboratory by injecting thoroughly washed cord extracts into rabbits and finding that the majority of the immune response was directed toward serum protein. Similar experiments have shown the presence of serum proteins in the tracheal aspirates of new born babies, and these proteins will presumably enter the amniotic fluid with the pulmonary surfactant lipids.

Previously, the only proteins of non-serum origin which have been found in amniotic fluid were the amnion proteins described by Lambotte and colleagues (p.22) and von Kleist et al (1968). In the present study, immunological and enzymological evidence has been advanced to show that other proteins present in amniotic fluid are of non-serum origin. Immunological investigations using adsorbed anti-sera revealed an α_2-protein which was consistently found in decidua and which was not detectable in serum, and whose concentration in amniotic fluid changed in a similar way to the total protein and serum protein concentrations. A β-mobility protein was also detected in amniotic fluid by immunological methods. This protein was present at lower concentrations in adult male serum than in fetal or maternal serum, and it was also found in extracts of placenta and chorion. The concentration gradients of this protein between amniotic fluid and the maternal and fetal sera were found to be less than those of known maternal serum (Gc) and fetal serum (α-feto-protein) markers at least before thirty weeks of pregnancy, and this suggested that much of this protein enters the fluid independently of the maternal and fetal sera.

It is not clear whether the α_2-decidual protein has been previously described in amniotic fluid, since the possibility of a

decidual contribution to the protein in amniotic fluid had not been considered by other workers. The absence of the protein in amnion obtained at term strongly suggests that the decidual protein is not the same as the amnion proteins described by other workers. Furthermore, the amnion proteins described by von Kleist et al (1968) were present in maximal concentrations in amniotic fluid at term, whereas the decidual protein was at maximal concentrations at about twenty-five weeks of pregnancy. No references have been found to a protein similar to the β-mobility protein described in this thesis.

Another way of investigating protein contributions to amniotic fluid is to study the specific activity patterns of amniotic fluid enzymes. Many tissue enzymes are of course present in appreciable concentrations in serum and it was expected that this would to some extent obscure their route of entry into the amniotic fluid. But there seemed a reasonable chance that a detailed study of the changes in enzyme specific activities with advancing gestation both in amniotic fluid and in serum would throw light on primary and secondary origins. The specific activities of a group of amniotic fluid enzymes were therefore measured from ten weeks of gestation until term and a number of rather distinctive specific activity patterns were found. α-1,4-Glucosidase showed a peak in specific activity between thirteen and eighteen weeks of gestation. Heat-labile alkaline phosphatase had an early peak of specific activity which was similar to that of α-1,4-glucosidase and an additional specific activity peak after thirty-seven weeks. Acid phosphatase showed a peak of specific activity after thirty-two weeks, while the specific activity of hexosaminidase remained unchanged during most of pregnancy, although the lowest levels were found before fourteen weeks.

The other enzyme studied was placental alkaline phosphatase, and it showed a gradual increase in specific activity between ten and thirty weeks, after which time a more rapid increase occurred.

The specific activity patterns of the amniotic fluid enzymes contrasted sharply with the concentration patterns of the total soluble protein and of the individual serum proteins in amniotic fluid. Although this suggested that the enzymes were not of serum origin, it was possible that amniotic fluid enzyme lability and changes in specific activities of the enzymes in the serum might account in part for the patterns which had been observed. Since a theoretical treatment of enzyme lability (p.101) showed that a peak in amniotic fluid enzyme specific activity before twenty weeks could conceivably occur in an enzyme of serum origin, a quantitative comparison was made of the enzyme specific activities in amniotic fluid and in the maternal and fetal sera. The basis of this analysis was that if the enzymes were of serum origin, the ratios of their specific activities between the amniotic fluid and the maternal and fetal sera would be similar to the concentration ratios for proteins known to be of maternal or fetal serum origin.

Amniotic fluid group-specific component and α-fetoprotein were therefore chosen as marker proteins as these were known to be respectively of maternal and fetal serum origin. The result of the comparison strongly suggested that some of the enzymes mainly diffused into the amniotic fluid independently of the serum. Between thirteen and eighteen weeks of gestation, the specific activities of hexosaminidase, α-1,4-glucosidase, and the placental and heat-labile alkaline phosphatases in amniotic fluid were found to be in excess

of those expected on the hypothesis of serum origin; although a significant fraction of the acid phosphatase activity during this period could be attributed to its influx from maternal serum. In term amniotic fluid, hexosaminidase, heat-labile alkaline phosphatase and acid phosphatase appeared to originate from the surrounding tissues, whilst fetal urine was found to be a potential source of acid phosphatase. The placental alkaline phosphatase in term amniotic fluid had a mean specific activity which was the closest to that expected on the hypothesis of a serum origin. However, there was evidence to indicate that its specific activity was also in excess of that expected for an enzyme of serum origin, and the possible implications of this was discussed in terms of the relationships between the placental trophoblast, the maternal serum, and the chorionic plate.

It is clear that this attempt to enquire into the origin of amniotic fluid enzymes rests upon the comparison of dissimilar proteins, and that this could be misleading. Yet analysis of the dissimilarities shows that the concentration gradients of Gc and AFP are probably underestimates of the gradients of enzyme specific activity which are predicted on the hypothesis that the enzymes are of serum origin, since Gc and AFP are smaller and more stable than the enzymes (see p. 104, and Figs. 34 and 51). Furthermore, there was independent evidence available for placental alkaline phosphatase which closely supported the gradient calculations. It is therefore concluded that whilst this approach is very approximate, it is of value in assessing the likelihood that a particular protein in amniotic fluid is of serum origin. Clearly the approach is quite inapplicable to small or non-protein solutes.

Although the enzymological evidence suggested that there was a release of tissue protein into the amniotic fluid between thirteen and eighteen weeks of gestation and again at term, the only amniotic fluid proteins which were found immunologically to be of tissue origin had maximum concentrations at about twenty-five weeks. In this context it must be added that the sensitivity of the immulogical methods used is probably far below that of the enzymological assays (in terms of μg protein detected). It is highly likely that other proteins would be detected by the more sensitive complement fixation test, and that these proteins would exhibit other concentration patterns. Such experiments would be necessary to confirm the general conclusions of the enzyme studies, and in particular to investigate the tissues responsible for the contribution of α-1,4-glucosidase and heat-labile alkaline phosphatase early in pregnancy.

A number of factors are likely to contribute to the changes in the serum protein and total protein concentrations in amniotic fluid. The fall in concentration between thirty and forty weeks of gestation is probably due to increased rates of fetal swallowing, micturation and umbilical cord transudation. The increase in protein concentrations between ten and twenty weeks of gestation occurred for all the proteins measured, with the exception of α-fetoprotein, which showed a fall in concentration similar to that in fetal serum. The increase in the concentration of Gc in amniotic fluid during this period suggests that there is an increase in the protein permeability of the membrane barrier between the maternal serum and the amniotic fluid, since Gc has been shown to be mainly of maternal origin throughout pregnancy. This increase in permeability would result in a more rapid entry of

maternal serum proteins into the amniotic fluid.

To what extent could these changes in permeability have effects on water fluxes between the maternal serum and the amniotic fluid? The increase in the permeability of the placental membranes to macromolecules will result not only in an enhanced permeability to water, but also in the water fluxes being increasingly controlled by hydrostatic pressure gradients. Since the placenta lies between the uterine decidua and the amniotic sac, it is probable that the pressure of blood in the intervillous spaces and under the chorionic plate will exceed the pressure in the amniotic fluid if the maternal placental blood is to circulate. In this way a hydrostatic pressure gradient would exist across the placental membranes which would bring about a net flux of water from the maternal circulation into the amniotic fluid. At present there is no evidence on this question, although Lind and Hytten (1971) and Lind et al (1972) suggest that the fetus controls the total volume of the amniotic fluid, and that the amniotic fluid is an extension of the fetal extracellular space during the second trimester of pregnancy (p. 18). This has been partly disproved in the present study by demonstrating that Gc and probably other major amniotic fluid antigens are mainly of maternal origin. Yet, the finding of α-fetoprotein in amniotic fluid shows that fetal serum does made some (probably small) contribution. Certainly, the increase in volume between ten and twenty weeks cannot be due to a gross influx of fetal serum, since the concentration of α-fetoprotein in amniotic fluid falls during this time, and its fetal serum : amniotic fluid gradient remains very high.

ii. Medical Implications

The point of immediate interest from this study is that anencephaly and possibly spina bifida may be diagnosed antenatally by amniotic fluid α-fetoprotein measurements, at a stage early enough to permit therapeutic abortion. Theoretical reasons have been advanced to suggest that early diagnosis should be possible in anencephaly, and the question can only now be resolved by studying cases at risk early in pregnancy. In the case of spina bifida, the usefulness of α-fetoprotein may be limited to those severe cases in which the vertebral canal is exposed to the amniotic fluid. It seems unlikely that the technique could be used to diagnose less gross forms of spina bifida. This is a drawback, since it is these latter cases which constitute the most serious and unhappy paediatric problems and would therefore be best subjected to therapeutic abortion.

The use of α-fetoprotein for the diagnosis of some congenital abnormalities of the central nervous system emphasises the value of immunological studies on amniotic fluid. It should be possible to assess the protein contribution of specific tissues to the amniotic fluid by measuring tissue-specific antigens, as has been illustrated by the work reported here on α_2-decidual protein and by the work of Lambotte et al (p.22) and of von Kleist et al (1968) on amnion proteins. Preliminary studies by Sutcliffe and Brock (unpublished) have shown that tissue specific proteins are present in the aspirates of neonatal tracheal secretions, and although these proteins were not detectable in unconcentrated amniotic fluid at term by antibody-antigen crossed electrophoresis, it seems quite possible that their presence will be demonstrated by complement fixation tests. The measurement of

specific lung components in amniotic fluid will help in further investigating the origin of amniotic fluid lipids, and will perhaps provide new ways of assessing pulmonary maturity.

It is possible that detailed description and measurement of the non-serum proteins in amniotic fluid will provide further tests of fetal maturity. Indeed, in amniotic fluid, changes have been observed toward term in the specific activities of some enzymes of mainly non-serum origin, and it has been suggested that these may be of value in estimating fetal maturity (pp.81-94). This possibility was further investigated by comparing the enzyme specific activities in amniotic fluid with the maturity of the fetus as estimated by menstrual history or paediatric examination (Sutcliffe et al 1972). The results of this study were equivocal because of the difficulty in assessing the significance of paediatric tests of maturity, and possibly because the amniotic fluid samples were obtained at artificial rupture of the membranes.

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Human serum normally contains only the conjugated bile salts, glycocholate, glycochenodeoxycholate, taurocholate, and taurochenodeoxycholate (Sandberg *et al.*, 1965; Panveliwalla *et al.*, 1970), with a total concentration below 5 μmol/l. In serum from jaundiced patients the total bile salt concentration may rise to values exceeding 250 μmol/l (Panveliwalla *et al.*, 1970). The conjugated chenodeoxycholates and free acids that we have now shown to inhibit brain respiration *in vitro* often account for more than half of the total bile salts and may perhaps exert an inhibitor action *in vivo*.

These observations may be relevant to the genesis of coma during hepatic failure, for it is known that during hepatic coma the O_2 uptake of human brain is below that of normal subjects (Wechsler *et al.*, 1954; Fazekas *et al.*, 1956). Although several of the metabolites that are retained in extracellular fluid during hepatic coma may inhibit respiration by rat brain slices *in vitro* (Walshe *et al.*, 1958; Lascelles & Taylor, 1968), only bile salts do so at concentrations close to those found pathologically. Ammonia, for example, will inhibit respiration by rat brain slices only when in a concentration of approx. 1000 times that of plasma, even though the plasma ammonia concentration in hepatic coma often parallels the degree of coma. These difficulties stand in the way of accepting a role for bile salts in causing hepatic coma. First, it is not known whether bile salts pass into cerebrospinal fluid; secondly, many jaundiced patients have raised plasma concentrations of bile salts without coma supervening; thirdly, the correction of hepatic coma by treatment with high-carbohydrate regimes cannot be easily explained if bile salts are solely responsible for the fall of O_2 uptake. For these reasons we are increasingly attracted to the possibility that more than one factor is involved.

The inhibition of respiration achieved by the conjugated bile salts parallels their detergent properties. Thus Hofmann (1963) has shown that for representative polar and non-polar compounds the conjugated deoxycholates and chenodeoxycholates have a lower critical micellar concentration and higher saturation ratio than the cholate conjugates. It is possible that the bile salts in hepatic coma permit relatively easy access to the brain of other respiratory toxins that would not themselves inhibit brain respiration in so low a concentration. In this context it is noteworthy that Lascelles & Taylor (1968) were able to show, with brain slices, potentiation of inhibition of O_2 uptake by combinations of metabolites each of which was present in a concentration known not to be inhibitory.

Finally, hepatic coma is often accompanied by acute renal failure. There can be no doubt that bile salts can penetrate the glomeruli and be reabsorbed by the renal tubules. If free chenodeoxycholate were formed in hepatic disease it might perhaps inhibit renal O_2 uptake and contribute to the development of acute renal failure.

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Prenatal Diagnosis of Anencephaly

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The prenatal diagnosis of a number of inborn errors of metabolism and chromosome disorders is now possible through biochemical and cytogenetic assays on cultured amniotic-fluid cells. These techniques do not allow recognition of most congenital mal-

formations (unless associated with a chromosomal aberration), where both the genetics and cause are obscure. The commonest of the congenital malformations of the central nervous system are anencephaly and spina bifida, which have incidences of up to 3.6 in 1000 in the U.K. (Elwood, 1970). Since the risk of either disorder increases dramatically in families where there are already affected sibs, a proportion of high-risk pregnancies can be readily identified (Carter *et al.*, 1966; Carter & Fraser-Roberts, 1967).

In anencephaly there are some indications that vascular tissue on the floor of the skull is exposed to the amniotic fluid (Willis, 1958). This might allow leakage of foetal blood and could explain the reports of elevated amniotic-fluid bilirubin concentration in this disorder (Cassady & Cailliteau, 1967; Lee & Wei, 1970). We therefore decided to measure the concentration of a specifically foetal protein in the fluid. α-Fetoprotein was chosen since it is an exclusive product of the conceptus, being synthesized in the yolk-sac, liver and gastrointestinal tract from as early as 6 weeks of gestation (Gitlin *et al.*, 1972). It is known to reach maximum concentration in foetal serum at about 13–15 weeks and then to decline towards term (Gitlin & Boesman, 1966). There have been contradictory reports about its presence in amniotic fluid (Gitlin & Boesman, 1966; Smith *et al.*, 1971), although by using a sensitive radioimmunoassay method Seppälä & Ruoslahti (1972) were able to detect it at all stages of gestation.

In the present study α-fetoprotein was measured by one-dimensional antibody–antigen crossed electrophoresis (Brock & Sutcliffe, 1972). In normal pregnancies α-fetoprotein concentration in amniotic fluid was maximal at 13 weeks of gestation, declined rapidly between 15 and 20 weeks and then fell steadily towards term (Fig. 1). In 22 pregnancies leading to anencephaly or anencephaly combined with spina bifida, where it was possible to get amniotic fluid in the third trimester, α-fetoprotein concentrations were grossly elevated. All were above 12 μg/ml, and some ranged as high as 200 μg/ml (Brock & Sutcliffe, 1972). When concentrations were expressed per mg of protein or per mg of albumin the distinction between anencephalic and normal fluids remained clear. In another nine 'anencephalic' pregnancies where fluid was obtained only after 35 weeks' gestation the distinction was less clear, although five of the samples had strongly raised α-fetoprotein concentrations.

Since many of the control samples used in this study were obtained from pregnancies in which there was a risk of rhesus incompatibility, it was important to establish what

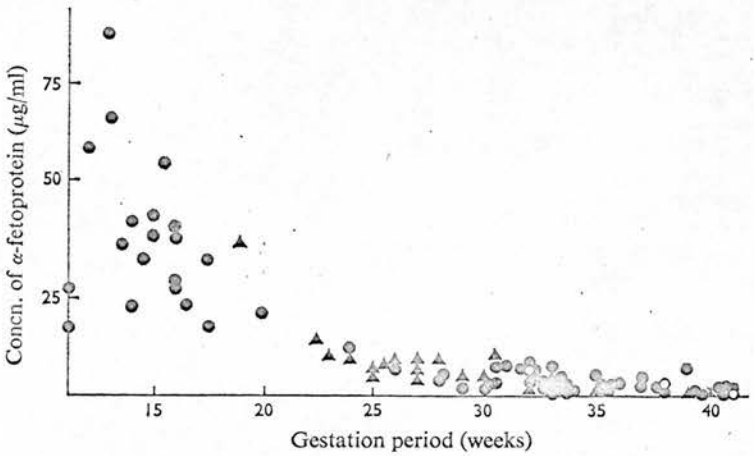

Fig. 1. *α-Fetoprotein concentrations in amniotic fluid at various stages of gestation*
●, Normal samples; ▲, samples obtained during monitoring of pregnancies at risk for rhesus isoimmunization, but where bilirubin concentration was low; ○, unexplained hydramnios; ×, unexplained stillbirth.

Table 1. *α-Fetoprotein concentrations in amniotic fluids from pregnancies leading to various conditions*

Gestation refers to the time at which the fluid was obtained.

Condition	Gestation period (weeks)	Concn. of α-fetoprotein (μg/ml)
Rh isoimmunization necessitating three postnatal exchange transfusions: live-born infant, progressing well	24	1.6
	26	1.2
	28	0.5
	30	1.1
	32	0.4
	33	0
	34	0
Rh isoimmunization necessitating two intrauterine and three postnatal exchange transfusions: live-born infant, progressing well	24	1.1
	25	0.8
	26	1.3
	27	1.2
	28	1.3
Rh isoimmunization necessitating intrauterine exchange transfusion: stillbirth	24	2.2
Rh isoimmunization, hydrops fetalis: stillbirth	27	6.5
Rh isoimmunization: stillbirth	35	1.7
Rh isoimmunization (anti-E): stillbirth	29	0
Duodenal atresia: live birth	36	4.2
Renal hypoplasia: neonatal death	21	5.1
Stunted embryo: termination	12	34.2
Rubella contact: termination	22	4.9
Ectopia vesica: termination	16	23.6
Exomphalos: termination	10	13.7
Diabetic mother, hydramnios: normal infant	35	2.0
Prediabetic mother, hydramnios: mongol infant	39	6.3

effect this had on amniotic-fluid α-fetoprotein concentrations. Two pregnancies were studied where at least four successive amniocenteses had been necessary to monitor foetal status. In both the degree of isoimmunization was sufficient to require postnatal transfusions, and in one intrauterine transfusions had also been necessary. In neither case was there any indication of a raised α-fetoprotein concentration (Table 1), nor was there in four cases of rhesus isoimmunization leading to intrauterine death. A variety of other conditions where there seemed a possibility of elevated amniotic-fluid α fetoprotein concentration were tested, but in none was an abnormal value found (Table 1).

The problem in the prenatal diagnosis of anencephaly is to detect it early enough to allow termination of pregnancy. There are two reasons why this should now be possible. First, even though normal α-fetoprotein concentrations in amniotic fluid are high before 20 weeks (Fig. 1), several of the anencephalic fluids between 27 and 35 weeks' gestation had α-fetoprotein concentrations well above the entire normal range. Secondly, in a case of myelocele spina bifida, fluid was obtained at 13 weeks and had an α-fetoprotein concentration of 350μg/ml (Brock & Sutcliffe, 1972). Since this condition resembles anencephaly in that the neural plate is exposed, one would expect even higher α-fetoprotein concentrations in early amniotic fluids from anencephalic pregnancies.

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Dopamine β-Hydroxylase Activity in Five Cases of Phaeochromocytoma

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Weinschilboum *et al.* (1971) have shown that dopamine (3,4-dihydroxyphenethylamine) β-hydroxylase (EC 1.14.2.1) activity in blood correlates well with the activity in the peripheral sympathetic nervous system and that the adrenal medulla plays no significant part in maintaining the enzymic activity in the blood. We have now measured the dopamine β-hydroxylase activity (by a new and sensitive method) in the plasma and tumour of five patients bearing phaeochromocytoma, as this tumour might be considered as an accessory adrenal medulla.

All the tumors had been confirmed by biological, clinical and anatomo-pathological observations. They were carried in ice from the operating room and frozen at −20°C within 1h. For assay of dopamine β-hydroxylase activity they were thawed and homogenized in 200vol. of tris buffer containing Triton X-100.

Heparinized blood samples were centrifuged and the plasma was stored at −20°C. The dopamine β-hydroxylase activity was stable for months under these conditions.

For the measurement of dopamine β-hydroxylase activity we used a new sensitive radioassay method based on a double enzymic reaction (Bonnay *et al.*, 1971). In the first step dopamine β-hydroxylase hydroxylates the substrate (phenethylamine for tissue and tyramine for blood; Weinschilboum & Axelrod, 1971; Molinoff *et al.*, 1971), and then the reaction products are submitted to the action of the phenylethanolamine *N*-methyltransferase in the presence of *S*-adenosyl[^{14}C]methionine. The labelled *N*-methylated product is extracted and determined by measurement of its radioactivity by liquid-scintillation counting.

Table 1 shows that a significant decrease in blood dopamine β-hydroxylase activity appears 1 week after surgery. The possible exception (TYP) might be due to the unusual character of this malignant phaeochromocytoma (HVA secretant and hepatic metastasis). However, there was no relation between tumour and plasma dopamine β-hydroxylase activities, nor between dopamine β-hydroxylase activities and catecholamine concentrations.

It is now well established that the adrenal medulla does not participate in maintaining dopamine β-hydroxylase activity in the blood. However, after the ablation of a phaeochromocytoma, which might be considered as an accessory adrenal medulla, the plasma enzyme activity falls significantly (by about 50%).

Several hypotheses may be advanced to solve this apparent contradiction. First, a phaeochromocytoma may have the specific character of releasing the enzyme into the

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ENZYMES IN UNCULTURED AMNIOTIC FLUID CELLS

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SUMMARY

The activities of five enzymes, isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, phosphohexose isomerase and lactate dehydrogenase have been measured in amniotic fluid cells at different stages of pregnancy. With the exception of phosphohexose isomerase very low and highly variable values were found and it was necessary to use spectrofluorometry to detect activity. Contamination of samples by red cells was a frequent problem. The usefulness of enzyme measurements in uncultured amniotic fluid cells for the antenatal diagnosis of inborn errors of metabolism is discussed in the light of these experiences with undiluted urine.

The use of amniotic fluid cell studies for the antenatal diagnosis of inherited disease has been increasingly exploited in the past three years[1–4]. Cultured and uncultured cells have been used for a variety of diagnoses using enzymological methods. The use of uncultured cells is attractive since it avoids the delay and uncertainty of culture and also permits an earlier diagnosis[3]. Indeed, a therapeutic abortion for Tay–Sachs disease has apparently been carried out entirely on the basis of a low level of hexosaminidase A in uncultured cells[4].

In this paper we report our experience with the measurement of five enzymes in uncultured amniotic fluid cells. Specimens of amniotic fluid were obtained either by transabdominal amniocentesis (5–10 ml) or at artificial rupture of the membranes using an amnioscope (20–40 ml), or at hysterotomy (60, 150 ml). Specimens were centrifuged at 1000 *g* for 10 min, and the cell-free supernatant was removed. The cell buttons were washed twice in physiological saline and, if necessary, erythrocyte contamination minimized by hypotonic shock. For enzyme measurements the cells were disrupted ultrasonically in 0.1 M potassium phosphate buffer, containing 30 μM NADP and 1 mM mercaptoethanol, with the temperature maintained below 10°.

Glucose-6-phosphate dehydrogenase (G6PDH), isocitrate dehydrogenase (ICDH), 6-phosphogluconate dehydrogenase (6PGDH) and lactate dehydrogenase (LDH) were assayed spectrofluorometrically by adding 20–100 μl of cell extract to 2.3 ml of a solution containing 90 mM potassium phosphate pH 7.95, 4.5 mM $MgCl_2$ and 2 mg bovine serum albumin. 0.4 μmoles of glucose-6-phosphate, 6-phospho-

gluconate, isocitrate and 4 μmoles of lactate were added separately for measurement of the respective enzymes. The reaction was initiated by adding 0.5 μmole NADP or 1 μmole NAD. Protein determinations were made on soluble and insoluble protein by Lowry's method[5].

TABLE I

ENZYMES IN AMNIOTIC FLUID CELLS

Case No.	*Gestation weeks*	*Total protein μg*	*ICDH**	*G6PDH**	*6PGDH**	*PHI**	*LDH***	*Number of hypotonic shocks*	*Source*
1 A	14	200	0.01	0	0	37	0	1	H
1 B	14	560	0.08	0.02	0.01	NM	0.25	2	H
2	18	552	0.23	1.13	0	NM	NM	12	H
3	18	20	1.22	1.15	0	<8	12.2	3	A
4	25	625	0.55	1.88	0	47	6.4	0	A
5	26	1890	<0.01	0.28	0	38	0.29	0	A
6	31	190	0.23	2.95	0	93	7.5	0	A
7	32	350	0.21	11.0	0	99	3.90	0	A
8	34	940	0.16	0.17	0	93	5.17	0	A
9	37	170	0.57	2.75	0	419	0.72	1	A
10	39	1610	0.67	2.0	0.60	136	1.01	0	ARM
11	40	552	NM	0.33	0	NM	5.66	3	ARM

* μmoles TPN converted per min per g total protein at 25°, except for PHI at 37°.
** μmoles DPN converted per min per g total protein at 25°.
H = Hysterotomy, A = Amniocentesis, ARM = Artificial Rupture of Membranes, NM = Not measured.

The levels of enzyme activity found in amniotic fluid cells are shown in Table I. ICDH, G6PDH and LDH were present in the cells in low concentrations and 6PGDH was detectable on two occasions only. However, PHI was present in appreciable quantities and could be confidently measured both by spectrofluorometry and by ultraviolet spectrophotometry, which in our system had a sensitivity of about 10^{-4} μmoles/min. The large variation in activities for each enzyme did not depend on the volume of the amniotic fluid specimen, and was not diminished by expressing in units per g soluble protein (as opposed to per g total protein). In the case of PHI there may be an increase in specific activity with gestational age.

The most obvious feature of the results in Table I is the variability of the values. A comparable range of activities for α-1,4-glucosidase has been recently reported[6]. More important, however, are the extremely low activities of the enzymes tested here. Preliminary experiments in which G6PDH was measured by ultraviolet spectrophotometry indicated that only in a few cases could activity be confidently detected. Subsequent measurements of all the enzymes except PHI was by spectrofluorometry, which increases sensitivity more than 100 fold. Even so, activities for G6PDH, 6PGDH and ICDH were barely detectable. This is particularly true for samples obtained by amniocentesis performed before the 20th week of pregnancy, which rarely yielded more than 100 μg of cell protein. In our experience such samples provide dubious material for precise enzymology, for there is always a chance of significant contamination of amniotic fluid cells by red cells[6], and also by enzymes circulating in the amniotic fluid whose origin is at the moment unknown. These considerations will have to be taken into account before a diagnosis of an inborn error of metabolism is made on the basis of a measured activity.

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OBSERVATIONS ON THE ORIGIN OF AMNIOTIC FLUID ENZYMES

BY

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OBSERVATIONS ON THE ORIGIN OF AMNIOTIC FLUID ENZYMES

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Summary

An enquiry has been made into the origins of a group of amniotic fluid enzymes which have complex specific activity patterns during gestation. Between 13 and 18 weeks of gestation, the specific activities of hexosaminidase, α-1,4-glucosidase, and the placental and heat-labile alkaline phosphatases in amniotic fluid were in excess of those expected on the hypothesis of a serum origin. In contrast, a significant fraction of the acid phosphatase activity during this period could be attributed to its influx from maternal serum. In amniotic fluid at term, hexosaminidase, heat-labile alkaline phosphatase and acid phosphatase appeared to originate from the surrounding tissues, although fetal urine was found to be an additional source of acid phosphatase. The placental alkaline phosphatase in amniotic fluid at term had a mean specific activity which was the closest to that expected on the hypothesis of a serum origin. It is probable that tissue protein is released into the amniotic fluid at term, and possibly also between 13 and 18 weeks of gestation. The methods used to draw these conclusions may be of general use for examining the origins of individual proteins in amniotic fluid.

A NUMBER of studies have suggested that the majority of the protein in amniotic fluid is of serum type. Using paper electrophoresis, McKay *et al.* (1958), Abbas and Tovey (1960), Brzezinski *et al.* (1964), Viergiver *et al.* (1962), and Heron (1966) have all shown the similarity between the amniotic fluid and serum proteins. Derrington and Soothill (1961) immunized rabbits with amniotic fluid obtained at term, and found by immunoelectrophoresis that the antibody reponse was directed solely toward serum protein. Sutcliffe and Brock (1972) immunized rabbits with amniotic fluid obtained at various stages of gestation, and found that throughout gestation most of the immune response was directed towards serum protein.

Although proteins of non-serum origin must constitute a minor fraction of the total amniotic fluid protein, their study is of interest for a number of reasons. Knowledge of the way in which local tissues contribute to the fluid may increase understanding of the formation and maintenance of the amniotic fluid. Secondly, the identification of proteins which come from tissues of fetal origin should be of value in the antenatal diagnosis of some genetic disease (O'Brien *et al.*, 1971; Friedland *et al.*, 1971) and, finally, data collected throughout pregnancy may be useful in the assessment of fetal maturity.

Immunological and enzymological techniques can be used in attempts to find tissue protein in the amniotic fluid. In a previous paper Sutcliffe *et al.* (1972a) reported the activity patterns of a group of amniotic fluid enzymes during gestation, and raised the possibility of these measurements being used for the assessment of fetal maturity. In this paper the biological implications of the results are considered from two stand-points.

First the extent to which the enzymes are of serum origin is discussed, and then their possible tissue origins are considered.

Materials and Methods

The assay systems used have been described by Sutcliffe *et al.* (1972a). Before assay the maternal and fetal sera were diluted by up to 10 and 5 times respectively with physiological saline. Extracts of amniotic fluid cells were made using the methods of Sutcliffe and Brock (1971). In order to follow the release of cellular enzyme small serial samples of the supernatant were collected during the course of sonication. The maximum specific activities of released anzyme were used to calculate the ratio of supernatant to cellular enzyme in samples of amniotic fluid at term.

Results

Amniotic fluid protein concentration

Figure 1 shows the distribution of protein concentrations in amniotic fluid found throughout pregnancy. Although there was a considerable scatter of results, there was a rise in mean protein concentration between 10 and 25 weeks. The quadratic curve of best fit was calculated for this period by multiple regression analysis, and is shown in Figure 1. Between 25 and 32 weeks the protein concentration fell by half and remained at this level until term. Line *a* in Figure 2 shows the rate of accumulation of protein in the amniotic fluid between 10 and 25 weeks of gestation. It was constructed by taking the product of the mean amniotic fluid volume for each weekly period between 10 and 25 weeks of gestation and the weekly protein concentration as calculated from the quadratic curve of best fit. The mean volumes were taken from the combined data of Wagner and Fuchs (1962), Gadd (1966), Rhodes (1966), Gillibrand (1969) and Abramovitch (1970). The difference between the products for consecutive weeks was taken as the rate of protein accumulation per week.

Enzymes in serum and urine

The specific activities of hexosaminidase, acid phosphatase, α-1,4-glucosidase and the heat-labile and placental alkaline phosphatases were measured in maternal and fetal sera, and in fetal urine. The results are shown in Table I. The specific activity of heat-labile alkaline phosphatase could not be measured in maternal serum at term because of the exceedingly high levels of placental alkaline phosphatase.

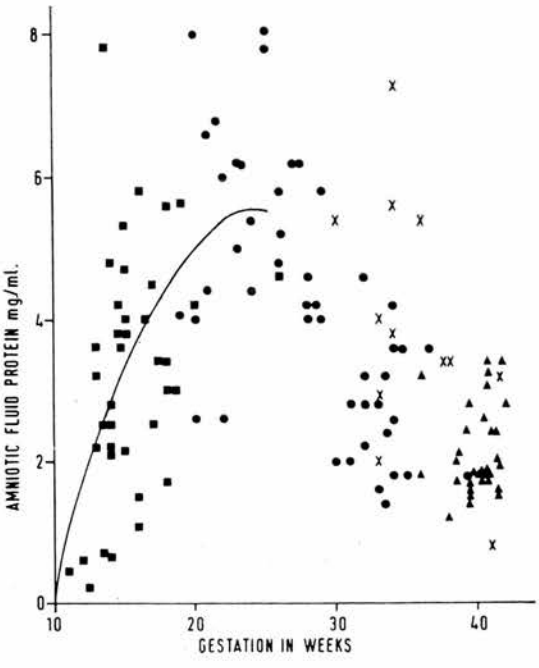

FIG. 1

The protein concentration in amniotic fluid supernatant measured by Lowry's method. The best fitting quadratic curve for the data between 10 and 25 weeks of gestation was plotted. It is of the form: $P = 1{\cdot}266G + 0{\cdot}026G^2 - 9{\cdot}822$ where P = protein concentration, and G = gestation in weeks.

■ = Hysterotomy. ● = Amniocentesis. ▲ = Artificial rupture of membranes, normal pregnancy. x = Artificial rupture of membranes, ancephaly.

Enzymes in amniotic fluid cells

The liberation of enzyme from amniotic fluid cells during the course of sonication is shown in Figure 3. Between 40 and 150 seconds of sonication were required for maximum enzyme liberation. From the results the quantity of *cellular* enzyme was calculated and expressed as the specific activity of enzyme (moles per hour) per precipitate from 1 ml. of amniotic fluid (Table II). In each case the quantity of enzyme present in 1 ml. of amniotic fluid *supernatant* was

TABLE I

Enzymes in maternal and fetal serum and in fetal urine

Gestation (weeks)	No.	Mean (and range) of enzyme specific activities, expressed in μmole per hr. per ml.				
		Hexosaminidase	Acid phosphatase	Alkaline phosphatase		α-glucosidase
				Heat labile	Placental	
Maternal Serum						
9–11	10	2·46 (1·72–3·42)	0·48 (0·35–0·75)	0·54 (0·42–0·81)	0·10 (0·07–0·12)	0·07
13–18	11	3·00 (2·05–4·08)	0·5 (0·34–0·79)	0·65 (0·39–0·81)	0·33 (0·12–0·82)	0·08
39–41	16	8·33 (5·21–13·0)	0·55 (0·41–0·78)	*	11·90 (4·60–22·6)	—
Fetal Serum						
13–18	10	2·20 (1·2–7·23)	0·87 (0·40–1·90)	4·43 (2·65–6·7)	0·26 (0–0·66)	0·26 (0·13–0·42)
30–38	7	1·28 (0·27–1·79)	0·60 (0·44–0·79)	2·33 (1·0–3·54)	0·3 (0–0·45)	— 0·11, 0·15
39–41	11	2·2 (1·70–3·82)	1·64 (0·70–2·51)	2·91 (1·31–5·23)	0·38 (0·21–0·83)	—
Fetal Urine				*Total*		
13–18	11	0·47 (0·15–0·90)	0·56 (0·28–1·10)	0·08 (0–0·18)		0·03 (0–0·07)
22–40	6	0·26 (0·05–0·37)	2·20 (0·45–4·38)	0·14 (0·04–0·13)		0·25 (0·10–0·45)

* Accurate measurement not possible due to high levels of placental alkaline phosphatase.

TABLE II

Ratio between the enzyme specific activities in the supernatant and cellular fractions of amniotic fluid

Enzyme	Case	Amniotic fluid enzyme content		
		Supernatant (μmole/hr./ml.)	Cells (μmole/hr. in precipitate from 1 ml. of amniotic fluid)	Supernatant/cell ratio
Hexosaminidase	D	1·44	0·67	2·15
	E	2·00	0·56	3·57
	T	1·45	0·64	2·27
Acid phosphatase	D	0·21	0·02	10·5
	E	0·24	0·03	8·00
	T	0·13	0·03	4·33
Heat labile alkaline phosphatase*	D	1·11	0·02	56·0
	E	0·87	0·03	29·0
	T	0·75	0·02	37·5

* Measured as heat labile alkaline phosphatase in supernatant, and total alkaline phosphatase in cells.

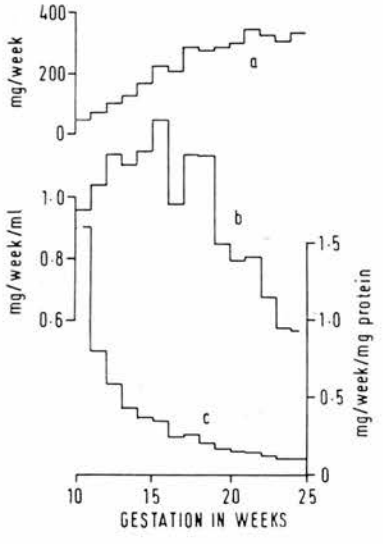

FIG. 2

Histograms of amniotic fluid protein influx calculated from the regression curve in Fig. 1 and the mean amniotic fluid volumes in the literature. The graphs show the rate of protein influx expressed (a) per week (b), per week per ml. of amniotic fluid and (c) per week per mg. of amniotic fluid protein.

measured as moles per hour per ml. of supernatant. The ratio of the supernatant to cellular enzyme was then calculated. In each case the quantity of enzyme in the supernatant exceeded that in the cell fraction (Table II).

DISCUSSION

Immunological methods have shown that most of the protein in amniotic fluid is of serum type (Derrington and Soothill, 1961; Sutcliffe and Brock, 1972). Thus the protein concentration determined by Lowry's method measures the approximate concentration of serum protein in the amniotic fluid. Figure 1 shows that this protein concentration rose from 10 weeks of gestation to reach a maximum between 20 and 30 weeks, and then declined until term. These findings are in good agreement with the work of Queenan *et al.* (1970), who used the biuret method for protein measurement.

In contrast to the protein concentration, the specific activities of some amniotic fluid enzymes studied by Sutcliffe *et al.* (1972a) showed little or no tendency to rise initially and reach a maximum between 20 and 30 weeks. In most cases they found maximal enzyme specific activities at term and/or between 13 and 18 weeks of gestation. These obvious differences lead one to question the extent to which serum can be a major source of these enzymes in amniotic fluid.

For an amniotic fluid enzyme to be of serum origin, three conditions must be fulfilled. Firstly, it must be able to pass into the amniotic sac from the serum. Secondly, its specific activity pattern during gestation should be predictable from the concentration changes of other serum proteins in the amniotic fluid. Finally, the gradient of enzyme specific activity between the serum and the amniotic fluid should be similar to the concentration gradients of proteins which are known to be of serum origin.

Molecular Weight

Previous studies (Usategui-Gomez *et al.*, 1966; Gitlin and Biasucci, 1969; Sutcliffe and Brock, 1972) suggest that serum proteins which have molecular weights of less than about 170 000 can permeate into the amniotic fluid. Four of the enzymes studied here have molecular

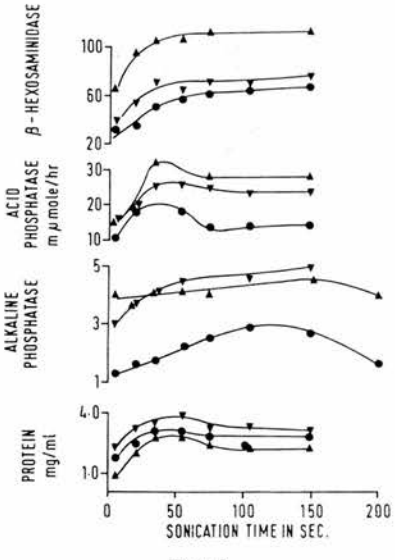

FIG. 3

The liberation of amniotic fluid cell enzymes during sonication. The maximum specific activities were used to calculate the enzyme specific activities in amniotic fluid.

weights under 170 000. Robinson and Stirling (1968) found the molecular weight of hexosaminidase to be 100 000. Placental alkaline phosphatase is dimeric at physiological *p*H, and has a molecular weight of 116 000 (Sussman and Gottlieb, 1969). The three forms of acid phosphatase which have been found in human placenta have molecular weights respectively of 35 000, 105 000 and over 200 000 (Di Pietro and Zengerle, 1967). No data is available for human α-1,4-glucosidase; however, the enzyme purified from bovine and from rat liver have molecular weights of 107 000 and 114 000 respectively (Bruni *et al.*, 1969; Jeffrey *et al.*, 1970). Human heat-labile alkaline phosphatase exists in a number of distinct forms and has not yet been purified.

The effect of lability on expected enzyme specific activity patterns

Enzymatic proteins are more labile than the majority of serum proteins, and most of the enzymes studied here progressively lose activity when incubated *in vitro* at 37 °C. (Fig. 4). This observation makes it important to decide whether the specific activity pattern of a labile enzyme of *serum* origin will differ from that of the amniotic fluid protein concentration. Because of the difficulty of extrapolating from *in vitro* lability data to the *in vivo* situation, a theoretical examination of enzyme lability will be made, and its result will be compared with the pattern expected for a stable enzyme of serum origin.

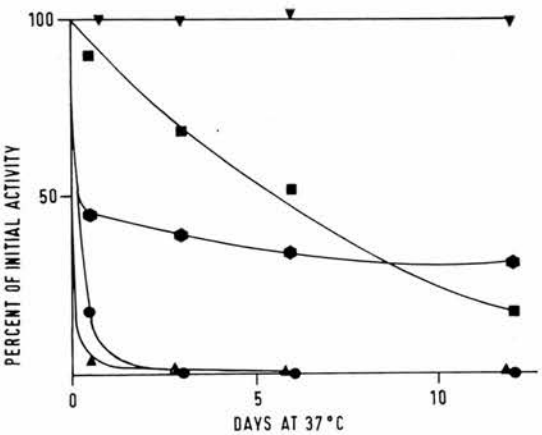

FIG. 4

The decay of enzyme activity in an amniotic fluid obtained at 16 weeks of pregnancy and incubated at 37 °C. The enzymes are placental alkaline phosphatase (▼), ceat-labile alkaline phosphatase (■), acid phosphatase (●), hexosaminidase (●) and α-1,4-glucosidase (▲).

Stable amniotic fluid enzymes of serum origin would have patterns of specific activity per ml. parallel to the protein concentration (provided the enzyme levels in serum remain constant). However, the phenomenon of enzyme lability will reduce the rate of accumulation of measureable enzyme, so that the specific activity of a very labile enzyme is controlled by its rate of influx into the amniotic fluid. The rate of influx of the enzyme will be similar to that of the other serum proteins, in proportions controlled by molecular weight and other characteristics. Before about 25 weeks the rate of influx of protein into the amniotic fluid will be in proportion to its rate of accumulation (see Fig. 2, line a), since removal of protein by fetal swallowing is negligible at this time (Abramovitch, 1970).

Since the total specific activity of a highly labile enzyme will be in proportion to the rate of influx of protein into the amniotic fluid, division of the rate of influx of protein by the mean total fluid volume and the mean total fluid protein content respectively will provide patterns which are in proportion to a labile enzyme's specific activity per ml. of amniotic fluid and per mg. of protein. The results of this calculation are shown as histogram lines *b* and *c* respectively in Figure 2. The influx of total protein on a volume basis is maximum at about 16 weeks whilst expression on a protein basis results in a maximum between 10 and 11 weeks of gestation. This result suggests that in the case of labile enzymes early in gestation, the mere demonstration of a difference between the patterns of enzyme activity and of protein concentration in the amniotic fluid is insufficient to allow the conclusion that the enzyme comes from a source other than serum. This conclusion applies particularly to α-1,4-glucosidase, heat-labile alkaline phosphatase, and hexosaminidase, all of which show considerable increases between 10 and 15 weeks of gestation. The analysis cannot, however, be extended beyond about 25 weeks of pregnancy because the increasing rate of fetal swallowing (Pritchard, 1965) will increase the rate of protein efflux from the fluid and so prevent an accurate assessment of influx.

Analysis of concentration gradients

If an enzyme is primarily of serum origin, the ratio of its specific activities between serum and amniotic fluid should be similar to the concentration ratios of other proteins which are found in amniotic fluid, and which are known to be exclusively of serum origin. The α_2 group-specific component (Gc) in amniotic fluid is known to be of maternal serum origin throughout gestation (Ruoslahti *et al.*, 1966; Sutcliffe *et al.*, 1972b), and so can be used as a measure of the maternal serum: amniotic fluid protein concentration gradient. Similarly, α-fetoprotein may be used as a measure of the fetal serum: amniotic fluid protein concentration gradient, since it is practically absent from the maternal serum (Ruoslahti and Seppala, 1972). The mean concentration gradient for Gc has been found to increase during gestation from 15 at between 13 and 18 weeks of gestation, to 32 at term (Sutcliffe and Brock, 1972). In the case of α-fetoprotein the mean gradient between 13 and 18 weeks has been found to be 206 (Sutcliffe and Brock, 1972), a magnitude which is similar to that found earlier in gestation by Gitlin and Boesman (1966).

Table III shows the mean specific activities observed for the enzymes in amniotic fluid at term and between 13 and 18 weeks of gestation. The expected specific activities have been calculated from the maternal and fetal serum enzyme specific activities (Table I) and the maternal or fetal serum : amniotic fluid protein gradients as outlined above. The term data has not been tested on the hypothesis of a fetal serum origin since the α-fetoprotein gradient at term is very high (Gitlin and Boesman, 1966). The results show that in all cases the specific activities observed in amniotic fluid are in excess of those calculated on the hypothesis of a serum origin for these enzymes. Particularly striking are the low levels which would be expected on the hypothesis of a fetal serum origin.

The validity of the analysis in Table III must be examined, since surface properties and molecular weight are of major importance in determining the rate of diffusion of individual proteins. The molecular weights of most of the enzymes studied are greater than those of Gc and α-fetoprotein. Thus the concentration gradients of the latter proteins may be *underestimates* of the expected specific activity gradients of the enzymes. If this is so, the conclusion that many of the enzymes are of non-serum origin is made more likely. However, an independent line of evidence suggests that the

TABLE III

The mean specific activities observed for the amniotic fluid enzymes, and those calculated from the maternal and fetal serum enzyme activities summarized in Table I

	No.	Mean observed (μmoles of substrate hydrolyzed/ hr./ml. of amniotic fluid)	Means* expected (μmoles of substrate hydrolyzed/ hr./ml. of amniotic fluid)		Ratio between observed and total expected values
			Maternal serum origin	Fetal serum origin	
13–18 weeks gestation					
Hexosaminidase	36	1·06	0·20	0·01	5·0
Acid phosphatase	35	0·06	0·03	<0·01	1·5
Heat labile alkaline phosphatase	30	0·24	0·04	0·02	4·0
Placental alkaline phosphatase	29	0·09	0·02	<0·01	3·0
α-1,4 glucosidase	30	0·54	0·01	<0·01	27·0
Pregnancies at term					
Hexosaminidase	25	1·72	0·26	—	6·6
Acid phosphatase	25	0·23	0·02	—	11·5
Heat labile alkaline* phosphatase	25	0·60	0·02	—	30·0
Placental alkaline phosphatase	25	0·65	0·37	—	1·8

* Using data from maternal serum obtained between 13 and 18 weeks of gestation (see Table I).

analysis is of value, at least in the case of placental alkaline phosphatase at term, for which an expected to observed ratio of 1·8 was calculated. Between 30 weeks of gestation and term, the concentration of major serum proteins in amniotic fluid falls by approximately half (Sutcliffe and Brock, 1972). Therefore, if the amniotic fluid placental alkaline phosphatase is of serum origin, its specific activity would not rise at the same rate as in the maternal serum. Between 30 and 40 weeks of gestation the specific activities of the enzysme in both amniotic fluid (Sutcliffe *et al.*, 1972a) and in maternal serum (Hunter, 1969) increase by 3 to 3·5 fold. This suggests that there is approximately twice the expected enzyme specific activity in amniotic fluid at term, and that the enzyme is therefore not in simple equilibrium with that in maternal mixed venous serum. This bears out the conclusion from Table III.

Alternative Tissue Sources of Enzyme

Having shown that serum is probably neither a major source of α-1,4-glucosidase, hexosaminidase or of placental or heat-labile alkaline phosphatase between 13 and 18 weeks, nor of hexosaminidase, acid phosphatase, or heat-labile alkaline phosphatase towards term, alternative tissue sources must be discussed. Maternal protein of non-serum origin can only enter the amniotic fetal from the uterine decidua and myometrium. Potential fetal sources include fetal urine, skin, respiratory and alimentary secretions, as well as umbilical cord (a potential source of mucopolysaccharide), placenta, amnion, chorion and amniotic fluid cells. Geyer (1970) has suggested that amnion is a major source of some amniotic fluid enzymes at term.

It is possible that amniotic fluid cells contribute to the fluid enzymes. However, it is difficult to understand how they could cause the changes in enzyme activity observed between 10 and 20 weeks of gestation, since Nelson and Emery (1970) have shown that the concentration of amniotic fluid cells is maximal at term. The relationship between the enzyme specific activities in the cells and the supernatants of three samples of amniotic fluid obtained at term was therefore studied. Table II shows that in one ml. of amniotic fluid, there was more enzyme activity in the supernatant fluid than in the cells. In the case of heat-labile alkaline phosphatase there was between 29 and 56 times as much enzyme in the supernatant as in the cells, whereas the ratio for acid phosphatase was 4 to 10, and for hexosaminidase 2 to 4. From these results it is clear that at term the minority of amniotic fluid enzymes is cellular, and that the cells cannot be a major source of alkaline phosphatase or acid phosphatase. It remains to be decided whether the supernatant : cell ratio for hexosaminidase indicates that cells are a major source of this enzyme.

A similar supernatant : cell analysis has not been made on amniotic fluid early in gestation. However, there are reasons to suggest that amniotic fluid cells are an insignificant source of supernatant enzyme between 13 and 18 weeks of gestation. Firstly, the cell concentration is very low at the time of the early specific activity peaks of hexosaminidase, α-1,4-glucosidase, and heat-labile alkaline phosphatase. Further, between 20 and 30 weeks, when the cell concentration is increasing at its maximal rate (Nelson and Emery, 1970) no obvious increase in enzyme specific activity occurs.

The similarity between the early activity peaks of α-1,4-glucosidase and heat-labile alkaline phosphatase suggests that the same tissue is responsible for liberating both enzymes. However, it is not known whether this tissue is maternal or fetal. Genetic studies should provide the clearest answer to this question, yet both successful (Nadler and Messina, 1969) and unsuccessful (Nadler *et al.*, 1970) prenatal diagnoses of Pompe's disease have been reported using the measurement of supernatant α-1,4-glucosidase. However, it is difficult to decide on the biological significance of this observation owing to the lack of extensive control data, and possible complications due to unrecognized fetal heterozygosity. Of considerable interest is the finding of Salafsky and Nadler (1971) that the majority of amniotic fluid α-1,4-glucosidase at 16 weeks is bound to free cellular organelles. These organelles must be of fetal origin which suggests that the enzyme is also of fetal origin. Paradoxically, the same workers (Salafsky and Nadler, 1972) have also claimed that the α-1,4-glucosidase in amniotic fluid is not

kinetically identical to that of fetal tissue or amniotic fluid cells. At present, no firm conclusion can be reached about the origin of the α-1,4-glucosidase of amniotic fluid. However, if the enzyme is of maternal origin, then it must be synthesized within the uterus, since its diffusion pathway into the amniotic sac must be independent of maternal serum.

In contrast to α-1,4-glucosidase, heat-labile alkaline phosphatase is found at high specific activities both early and *late* in gestation. Electrophoresis of various human tissue extracts have shown that at least four distinct forms of the phosphatase exist (see Suzuki *et al.*, 1969), so that it is unwise to regard the amniotic fluid enzyme as a single entity, or to assume that the same entity participates in both the early and late peaks of activity. Indeed, since α-1,4-glucosidase is not elevated at term, it seems that the phosphatase peak at term is not due to the same process of tissue break-down as occurred earlier in gestation.

There is a lack of similarity between the patterns of α-1,4-glucosidase, hexosaminidase and acid phosphatase during the last eight weeks of gestation. Although all three enzymes are of lysosomal origin and are ubiquitously distributed in body tissues, acid phosphatase alone shows a marked increase in specific activity toward term. This suggests that he increased levels of acid phosphatase are not due to local tissue changes, and argues against the suggestion of Seelich and Gomolka (1952) that the placenta is a major source of amniotic fluid acid phosphatase. These workers claimed that the ratio of specific activity of acid phosphatase and placental alkaline phosphatase in the amniotic fluid agreed well with that in the placenta; however, Ahmed and King (1959) were unable to confirm this. The absence of an elevation of acid phosphatase in maternal or fetal serum in the last eight weeks of pregnancy suggests that the placenta is not a major source of acid phosphatase.

Table I shows that the specific activity of acid phosphatase in fetal urine is considerably greater than that of either hexosaminidase or α-1,4-glucosidase. Further, the specific activity of acid phosphatase in fetal urine is considerably greater than in most amniotic fluids, so that fetal urine may be tentatively advanced as a major source of amniotic fluid acid phosphatase during the last eight weeks of gestation.

Conclusion

This paper shows that the majority of the amniotic fluid enzymes studied are probably not of fetal or maternal serum origin. The complex patterns of enzyme activity found between 13 and 18 weeks of gestation, and during the last two months of pregnancy, suggest that significant quantities of tissue protein are released into the amniotic fluid. However, the sources of non-serum protein in the amniotic fluid remain in doubt, and may possibly be identified by immunological studies.

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Origin of Amniotic Fluid Group-specific Component

THE use of amniotic fluid for the prenatal diagnosis of genetic disease has prompted investigations into the origin of amniotic fluid protein. Several observations (refs. 1–5 and R. G. S. and D. J. H. B., manuscript in preparation) have suggested that the foetus contributes protein to the fluid early in pregnancy, and it has also been claimed that during this time amniotic fluid is an extension of the foetal extracellular space[6,7]. Conversely, in term amniotic fluid, the serum proteins transferrin and group-specific component (Gc) have been found to be of maternal origin[8–10]. Serum proteins make up the great majority of amniotic fluid protein at all stages of gestation, but it is not known whether maternal or foetal serum is the source early in gestation. In this study, Gc polymorphism is used to investigate this point.

Polymorphism studies on Gc protein have shown that the protein in foetal serum is synthesized by the foetus from ten weeks of gestation onwards[11]. Thus, in cases where the foetal phenotype is discordant with the mother's, the amniotic fluid Gc would be concordant with the maternal phenotype if it is of maternal origin, but discordant if it is of foetal origin. In the latter case, the expected frequency of discordance between the foetal and maternal phenotypes is equal to the heterozygote frequency in the population[12].

Antibody-antigen crossed electrophoresis (AACE) is known to show Gc as a heterogeneous protein[13], but has not been previously used for Gc phenotyping. We used the method as it allows a detailed examination of the amniotic fluid Gc pattern without previous concentration of the fluid, and is sensitive enough to show small contributions from one or other allele. AACE was carried out using 1% agarose and the buffer systems of Hirschfeld[14]. The first dimension was run at 6 V cm^{-1} for 3 h, and the second dimension at 1.5 V cm^{-1} for more than 15 h. The antiserum was raised in rabbits by subcutaneous injection of $\times 10$ concentrated amniotic fluid, which was dispersed in Freund's complete adjuvant for the first three injections. Amniotic fluid samples before 20 weeks of gestation were obtained at terminations of pregnancy, and thereafter at amniocentesis in cases of suspected rhesus isoimmunization. The Gc precipitate was identified in the gels by its characteristic heterogeneity[13,14], and also by the use of a specific anti-Gc antiserum (Behringwerke).

Table 1 Concordance and Discordant for Maternal Gc Phenotype

Gestation (weeks)	Concordant pairs Maternal Gc phenotype			Discordant pairs All phenotypes
	1–1	2–1	2–2	
10–18	11	8	2	0
19–27	5	5	2	0
28–38	14	8	1	0

Number of pairs studied in which concordance or discordance for Gc phenotype was found between the amniotic fluid and maternal serum, broken down according to gestational age and maternal Gc phenotype. Phenotype frequencies: 0.54; 0.38; 0.09.

Table 1 shows that concordance of phenotype was found in all of the fifty-six maternal serum-amniotic fluid pairs studied. From the frequency of heterozygotes, approximately 38% discordance in at least one of the gestational groups would be expected if there were a major foetal contribution to the Gc of amniotic fluid. Among the cases summarized in Table 1 were eleven in which a sample of foetal serum was available for phenotyping. In four of these cases, discordance was found between the foetal and maternal phenotypes and, in each case, the phenotype of the amniotic fluid Gc was that of the mother.

From these results we conclude that throughout gestation the Gc in amniotic fluid is of maternal origin, and that it enters the fluid by passing through the placental or reflected membranes. This finding sounds a cautionary note for any attempts to carry out prenatal diagnoses of genetic disease either by direct screening of serum proteins or by linkage analysis using serum protein polymorphisms.

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ALPHA-FETOPROTEIN IN THE ANTENATAL DIAGNOSIS OF ANENCEPHALY AND SPINA BIFIDA

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Summary α-fetoprotein (A.F.P.) has been measured in the amniotic fluids of thirty-one pregnancies leading to anencephaly (or anencephaly and spina bifida), three leading to spina bifida, and three leading to hydrocephaly (or hydrocephaly and spina bifida). In the anencephalic cases all A.F.P. concentrations were well above the normal range between 25 and 35 weeks of gestation; after 35 weeks five out of nine cases were clearly above normal. In a single case of spina bifida, where amniotic fluid was obtained at 13 weeks, A.F.P. concentration was four times greater than the highest normal level. After 30 weeks' gestation amniotic fluids from pregnancies leading to spina bifida and hydrocephaly could not be distinguished from normal pregnancies. These results suggest that amniotic-fluid-A.F.P. measurements will be valuable in the early antenatal diagnosis of anencephaly and spina bifida and will enable termination of these pregnancies.

Introduction

ANENCEPHALY and spina bifida cystica are the most common of the congenital malformations of the central nervous system, with a combined incidence of up to 7 in 1000 in the U.K.[1,2] The risk of either disorder is increased to 1 in 20 in a family where there is already an affected sib [3] and to 1 in 10 or above in a family where there are two affected sibs.[4] One of the prerequisites of antenatal diagnosis, the identification of pregnancies at increased risk, is thus satisfied. The other prerequisite, a marker molecule which will indicate an affected fetus early enough to allow termination of pregnancy, has so far not been found. We suggest that α-fetoprotein (A.F.P.) could act as such a marker molecule.

Materials and Methods

Amniotic fluids, obtained at delivery or by trans-abdominal amniocentesis, were centrifuged for 5 minutes at 125 *g* and the clear supernatants removed. These supernatants were stored at −25°C for up to 3 years. Since the effect of protracted storage on A.F.P. is not known, each sample from a pregnancy leading to a congenital malformation was matched with a control sample of the same approximate gestational age, which had been stored for the same time. Congenital malformations were classified as anencephaly (with or without spina bifida), spina bifida, and hydrocephaly (with or without spina bifida). Most of the control samples were obtained from terminations of pregnancy or from amniocenteses carried out during the management of rhesus-incompatible pregnancies.

A.F.P. was measured by one-dimensional antibody-antigen crossed electrophoresis using rabbit antiserum provided by Dr. J. V. Clark and also bought from Behring-werke. The monospecific antiserum was used at a final concentration of 0·25% in a 1% agarose gel. Amniotic fluid (5–15 µl.) was placed in the sample wells and electro-

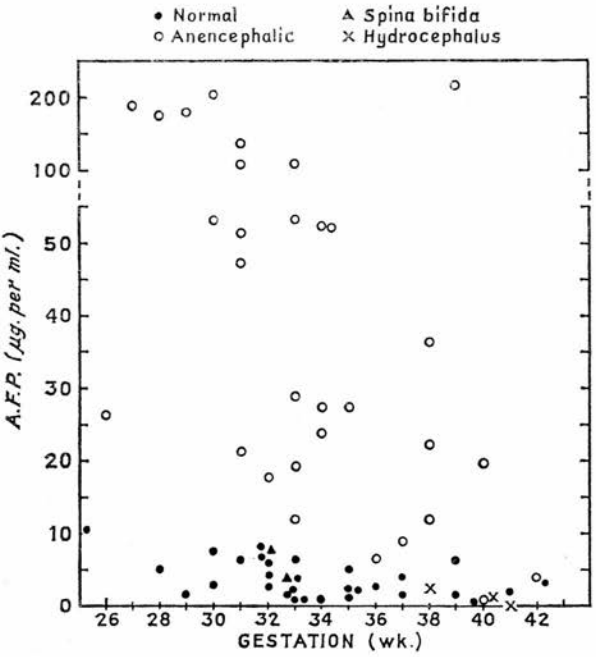

Fig. 1—A.F.P. in amniotic fluid between 25 and 42 weeks of pregnancy.

Note change in scale at 50 µg. per ml.

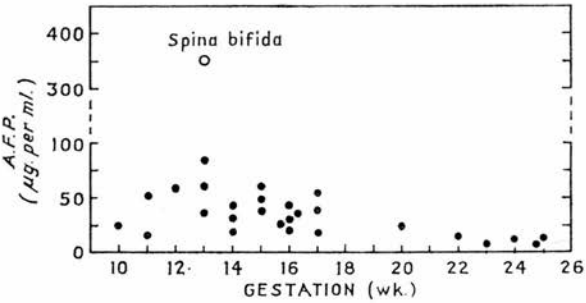

Fig. 2—A.F.P. in amniotic fluid between 10 and 25 weeks of pregnancy.

phoresis carried out overnight. The sensitivity limit of the assay was about 1 μg. per ml., though values under 3 μg. per ml. were somewhat unreliable. Absolute values of A.F.P. were calculated with reference to a standard supplied by Prof. G. Abelev. If a Behringwerke standard is used the values in figs. 1 and 2 must be multiplied by 0·7. Protein was measured by the method of Lowry et al.[5] Norgaard-Pedersen has described a similar method of measuring A.F.P.[6]

Results

Concentrations of A.F.P. in the amniotic fluids of pregnancies leading to anencephaly, spina bifida, and hydrocephaly are shown in figs. 1 and 2. Fig. 1 covers the gestational range from 25 to 42 weeks when most of the abnormal amniotic fluids were obtained. From 25 to 35 weeks' gestation there is a distinction between the anencephalic fluids and the normal controls; even after 35 weeks' gestation most of the anencephalic amniotic fluids have greatly raised A.F.P. concentrations. The mean A.F.P. concentration of the control fluids at 25–42 weeks' gestation was 3·1 μg. per ml. (range 0–10·5 μg. per ml.), while only four of the thirty-one anencephalic amniotic fluids fell below 12 μg. per ml., all of these being from pregnancies of 36 weeks' duration or longer.

In contrast, the concentration of A.F.P. in fluids from cases of spina bifida and hydrocephaly were not distinguishable from the normal controls between 32 and 41 weeks (fig. 1). However, a single amniotic-fluid sample was obtained at 13 weeks' gestation from a hysterotomy in which the fetus had a myelocele spina bifida. In this case the concentration of A.F.P. in the fluid was 350 μg. per ml., which was over four times as great as the highest normal control value (fig.

2). In our experience A.F.P. levels are not very much affected by severe rhesus isoimmunisation, by idiopathic hydramnios, or by stillbirth (unless caused by anencephaly).

Discussion

Attempts have been made to find marker molecules in amniotic fluid which would indicate fetuses with anencephaly and/or spina bifida.[7-9] Several workers have reported that bilirubin or a bilirubin-like molecule with an extinction coefficient at 450 nm. is present in the amniotic fluid surrounding anencephalic fetuses at or near term.[10-12] This suggested to us that enhanced leakage or transudation of fetal-blood components was occurring either directly into the fluid or into the fluid via the cerebrospinal fluid (C.S.F.). If any of these components were to be of diagnostic use it would have to be unique to the fetus, and of sufficiently high molecular weight not to equilibrate rapidly with maternal fluid-spaces—i.e., a specifically fetal protein. A.F.P.[13] was an obvious candidate. It is produced in the fetal liver as early as the 6th week of gestation, reaches its highest concentrations in fetal serum at about 13 weeks, and then falls steadily to term.[14] It may be found in amniotic fluid throughout pregnancy with a highly sensitive radioimmunoassay.[15] However, we have had no difficulty in detecting A.F.P. in amniotic fluid at most stages of pregnancy with a relatively insensitive method; only at term did we sometimes fail to find A.F.P. (indicating a concentration below 1 μg. per ml.). As in fetal serum, amniotic-fluid-A.F.P. concentration is highest at about 13 weeks' gestation and then falls. The concentration gradient between fetal serum and amniotic fluid A.F.P. is about 200/1.[16]

Figs. 1 and 2 suggest that both anencephaly and spina bifida may be detectable in utero early enough to allow termination of pregnancy. In fig. 1 the difference between anencephalic and normal A.F.P. levels is greater in the 25 to 35 week period than it is after 35 weeks. Though the five spina bifida cases (three associated with hydrocephaly) were indistinguishable from controls after 32 weeks' gestation, the single early case had greatly raised A.F.P. at 13 weeks' gestation. There are different types of spina bifida, and myelocele and total myeloschisis are more likely to be associated with extrusion of fetal proteins into the fluid than are meningomyelocele, meningocele, or

spina bifida occulta. The early case was a definite myelocele, the later cases probably all meningomyelocele or meningocele. This may indicate that only unclosed neural-plate malformations can be diagnosed antenatally.

Anencephalics seem to have a choroid plexus, and this is a possible source of both the hydramnios and high amniotic-fluid-A.F.P. levels. In spina bifida, A.F.P. must presumably come from fetal C.S.F., though its concentration there has not previously been measured. We had some difficulty in obtaining fetal C.S.F. entirely free of blood; two moderately clean samples had A.F.P. levels of 0·96 and 0·86 mg. per ml., at 15 and 16 weeks' gestation respectively, though with normal fetal serum levels of A.F.P. reaching 3 mg. per ml. at 15 weeks' gestation [14,16,17] this could be accounted for by contamination.

A.F.P. crosses the placental barrier and may be found in maternal serum with a sensitive radioimmunoassay.[18] In cases of fetal distress maternal serum-A.F.P. levels of up to 9 μg. per ml. have been observed. Possibly the raised amniotic-fluid A.F.P. that we have found in anencephaly and myelocele spina bifida may also find its way into the maternal serum. So far we have not been able to test this, but we would not regard as improbable the eventual antenatal diagnosis of anencephaly and spina bifida through the monitoring of A.F.P. levels in maternal serum.

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ENZYMES IN AMNIOTIC FLUID: A STUDY OF SPECIFIC ACTIVITY PATTERNS DURING PREGNANCY

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Summary

The specific activity patterns of a group of enzymes were studied in the supernatants of amniotic fluid obtained between 10 weeks of gestation and term. A number of different patterns were found. α-1,4-Glucosidase showed a peak in specific activity between 13 and 18 weeks of gestation. Heat-labile alkaline phosphatase had an early peak of specific activity which was similar to that of α-1,4-glucosidase and, in addition, a specific activity peak after 37 weeks. Acid phosphatase showed a peak of specific activity after 32 weeks. Hexosaminidase remained unchanged during most of pregnancy, although the lowest levels were found before 14 weeks. Finally, placental alkaline phosphatase showed a gradual increase in specific activity between 10 and 30 weeks, after which time a more rapid increase occurred. Expression of the results on a protein basis led to an exaggeration of the specific activity peaks in early and late gestation. The significance of the results is discussed in the context of their possible use for the estimation of fetal maturity.

AMNIOTIC fluid analysis has been shown to be of value in the prenatal diagnosis of both genetic and non-genetic conditions. Tay-Sachs disease can be diagnosed between 15 and 28 weeks of gestation by measurement of hexosaminidase A in amniotic fluid (O'Brien *et al.*, 1971; Friedland *et al.*, 1971). Bilirubin measurement has become routine for the diagnosis and assessment of rhesus isoimmunization (see, for example, Robertson, 1969), and the measurement of fluid phospholipids has been claimed to be of value in determining the liability of a fetus to respiratory distress syndrome at birth (Gluck *et al.*, 1971; Bhagwanani *et al.*, 1972).

A greater understanding of the origin and quantitative behaviour of the proteins in amniotic fluid would help in attempting to extend the diagnostic potential of amniocentesis. This paper reports the specific activity patterns of a number of amniotic fluid enzymes during pregnancy, and discusses their potential usefulness in the diagnosis of fetal maturity. The factors which contribute to the changes in enzyme specific activity during gestation are discussed in a subsequent paper (Sutcliffe and Brock, 1972).

MATERIALS AND METHODS

Amniotic fluid was obtained from three sources. Samples obtained early in gestation were taken from hysterotomy sacs, and those from about 20 to 38 weeks were taken by

amniocentesis at the clinic for patients with rhesus iso-immunization. Collections of amniotic fluid at term were made using an amnioscope during artificial rupture of the membranes. In all cases, the duration of pregnancy was measured from the first day of the last menstrual period. All samples which were visibly contaminated by blood or meconium were excluded from the series. In the patients having repeated amniocentesis, only the initial specimen was collected. Specimens with a $\triangle$OD which fell outside the A1, B1, B2, C1 and C2 zones of Robertson (1969) were excluded.

The samples of amniotic fluid were centrifuged for 45 minutes at 27 000 g. at 4 °C., and the clear supernatant was removed. Enzyme assays were carried out within two days of the sample being collected, the material being stored at 4 °C. during this period.

Hexosaminidase was assayed in a system of 0·1 M. citrate buffer *p*H 4·25, 5·3 mM. p-nitrophenyl-2-acetamido-2-deoxy-β-*D*-glucopyranoside and 5 μl. of amniotic fluid in a final volume of 0·15 ml. Acid phosphatase was assayed in a system of 0·1 M. citrate buffer *p*H 4·9, 10·5 mM. "Sigma 104" phosphatase substrate (p-nitrophenyl phosphate), 0·4 per cent formaldehyde and 20 to 40 μl. amniotic fluid in a final volume of 0·15 ml. The formaldehyde inhibited any red cell acid phosphatase present. α-1,4-Glucosidase was assayed in a system of 0·18 M. citrate *p*H 4·9, 12·8 mM. p-nitrophenyl-α-*D*-glucopyranoside and 10 μl. of amniotic fluid, in a final volume of 0·15 ml. Alkaline phosphatase was assayed in a system of 62 mM. sodium carbonate buffer *p*H 10·75, 12·5 mM. magnesium chloride, 32·5 mM. "Sigma 104" phosphatase substrate and 5 to 20 μl. of amniotic fluid in a final volume of 50 μl. To determine the specific activity of the placental (heat-stable) alkaline phosphatase, an aliquot of amniotic fluid was incubated at 65 °C. for 55 minutes after the method of Hunter (1969). The difference in specific activity between the unheated and heated amniotic fluid aliquots represented the heat-labile alkaline phosphatase. In all assays a control without amniotic fluid and a control without substrate were included.

The assay solutions were incubated for 2 hours at 37 °C. under saturated vapour pressure to minimize evaporation. The reactions were then stopped by the addition of 0·4 M. glycine-sodium hydroxide buffer *p*H 10·3, to a final volume of 1·0 ml. The optical density of the solutions was measured spectrophotometrically at 400 mμ. The enzyme specific activities were calculated by subtracting the sum of the controls from the full systems, and using the molar extinction coefficient of p-nitrophenol ($1{\cdot}775 \times 10^4$ under these conditions) to express the results in μmoles substrate hydrolyzed per hour per ml. of amniotic fluid. The protein concentration in amniotic fluid was measured by Lowry's method (Lowry *et al.*, 1951), and it was used to calculate specific activity in μmoles per hour per g. of protein.

Results

1. Enzyme kinetics

Table I shows the *p*H optima and Km. values for the enzymes studied. Routine assays were carried out at optimal *p*H and at substrate concentrations which were essentially saturating. The exception to this was the hexosaminidase assay where the sparing solubility of the substrate precluded saturating conditions.

Table I

*Michaelis constants (Km.) and p*H *optima of the amniotic fluid enzymes studied*

Enzyme	Km.*	*p*H optimum
Hexosaminidase	2·6	4·25
α-1,4-glucosidase	3·3	4·9
Acid phosphatase	0·8†	4·9
Total alkaline phosphatase	6·0	10·75

* expressed as mM substrate.
† substrate inhibition above 9mM substrate.

2. Enzyme specific activity patterns

α-1,4-glucosidase (Figs. 1a and 1b)

Figures 1a and 1b show the patterns of specific activity on volume and protein bases respectively. A peak of activity was found between 13 and 18 weeks of gestation. After this period consistently low specific activities were observed.

Heat-labile alkaline phosphatase
(Figs. 2a and 2b)

Figures 2a and 2b show the patterns of specific activity on volume and protein bases respectively. An initial peak in activity occurred

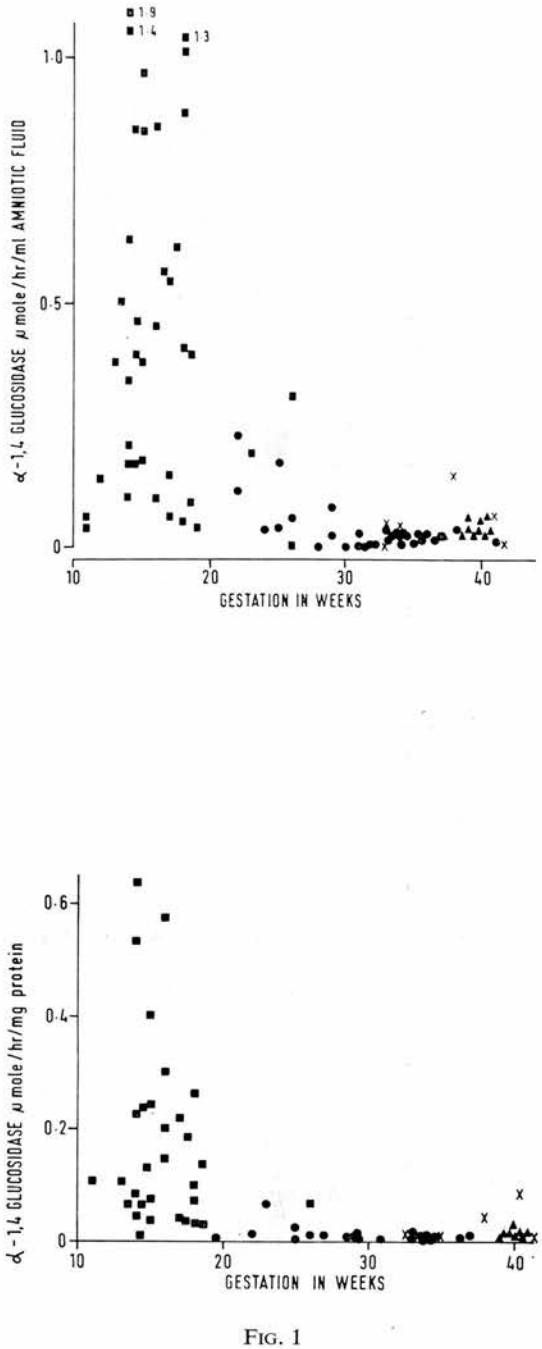


FIG. 1

Amniotic fluid α-1,4-glucosidase, expressed (a) per ml. of amniotic fluid, and (b) per mg. of protein, plotted against gestational age. ■: hysterotomy. ●: amniocentesis. ▲: artificial rupture of membranes, normal pregnancy. ×: artificial rupture of membranes, anencephaly.

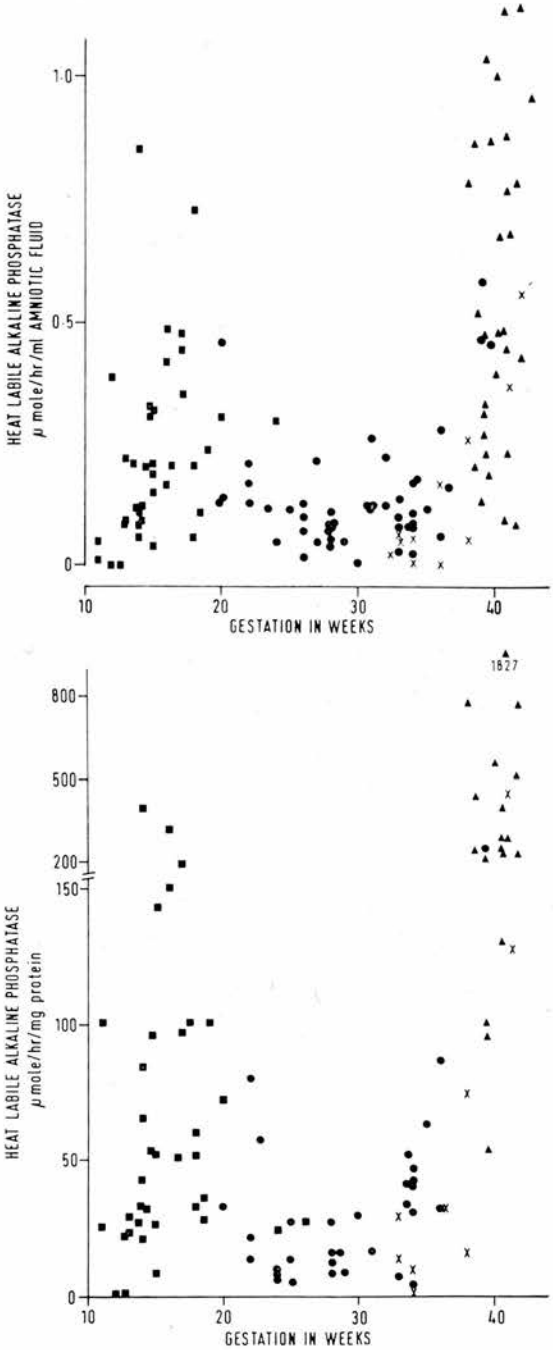


FIG. 2

Amniotic fluid heat-labile alkaline phosphatase, expressed (a) per ml. of amniotic fluid, and (b) per g. of protein, plotted against gestational age. Symbols as in Fig. 1.

between 13 and 18 weeks, and during this time a close relationship was found between the specific activities of heat-labile alkaline phosphatase and α-1,4-glucosidase in individual amniotic fluids (Fig. 3). Heat-labile alkaline phosphatase was found at very low levels between 18 and 36 weeks of gestation, after which there was a considerable rise (accompanied by a wide scatter). When enzyme specific activity is expressed on a protein basis (Fig. 2b) both peaks of activity are exaggerated, and the rise toward term occurred earlier.

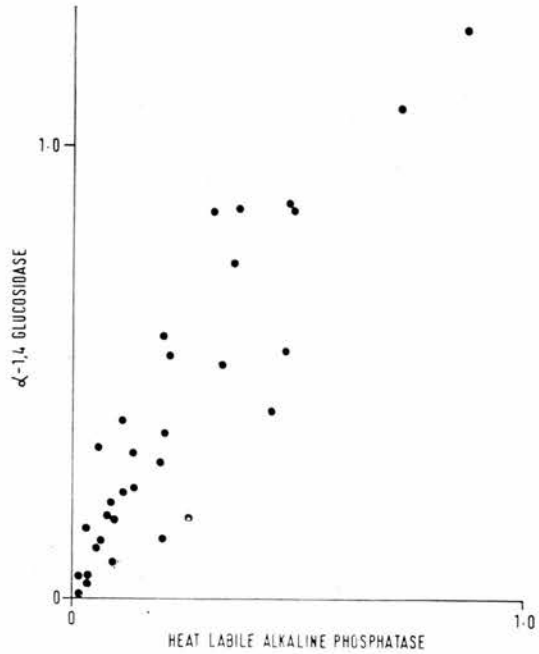


FIG. 3

The relationship between amniotic fluid α-1,4-glucosidase and heat-labile alkaline phosphatase between 10 and 20 weeks of gestation, expressed as μmole/hr./ml. of amniotic fluid.

Acid phosphatase (Figs. 4a and 4b)

Little change in specific activity per ml. of amniotic fluid was observed before about 32 weeks. After this time there was an increase in specific activity, together with considerable variation between individual values. Figure 4b shows that a minor peak in specific activity per g. of protein occurred between 13 and 18 weeks of gestation, as well as the more obvious peak at term.

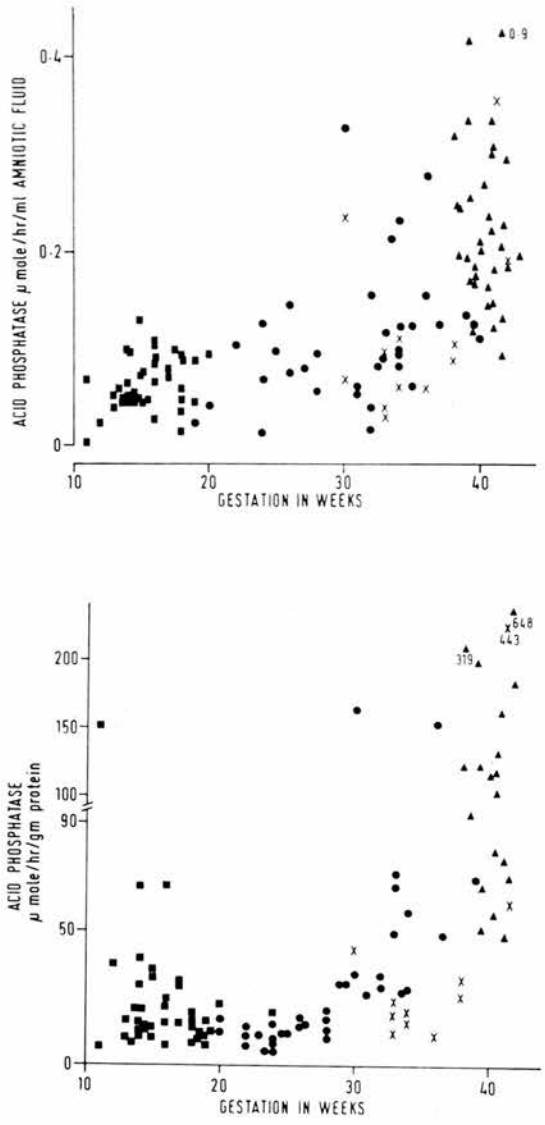
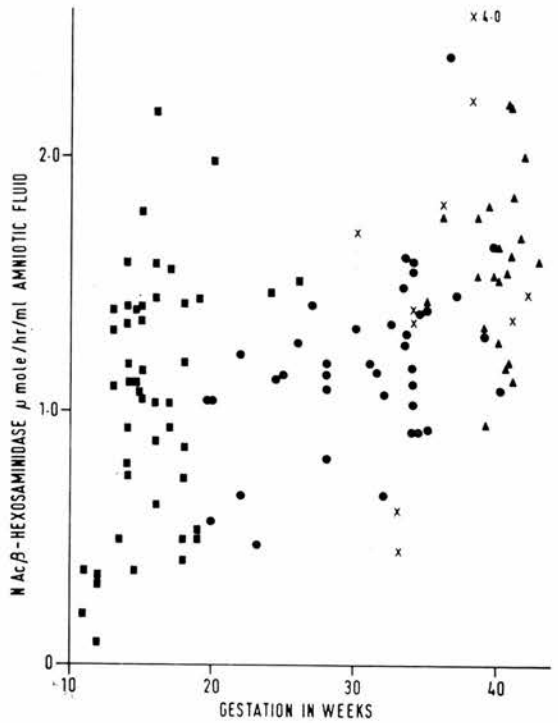


FIG. 4

Amniotic fluid acid phosphatase, expressed (a) per ml. of amniotic fluid, and (b) per g. of protein, plotted against gestational age. Symbols as in Fig. 1.

Hexosaminidase (Figs. 5a and 5b)

No clear pattern emerged from the enzyme specific activity per ml. of amniotic fluid, although the lowest specific activities are found before 14 weeks of gestation. However, Figure 5b shows that the specific activity per mg. of protein is minimal between 20 and 30 weeks.



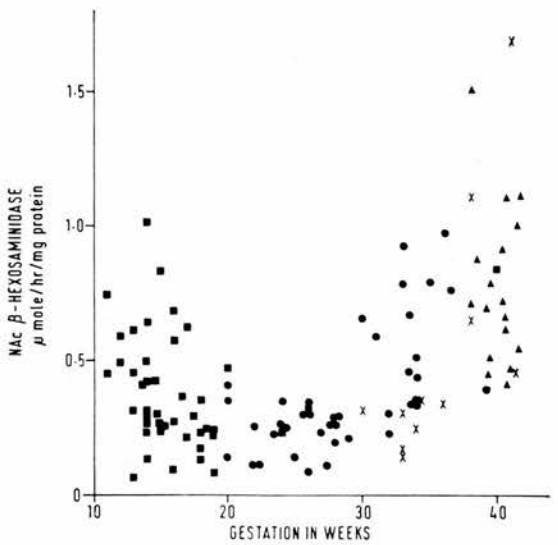
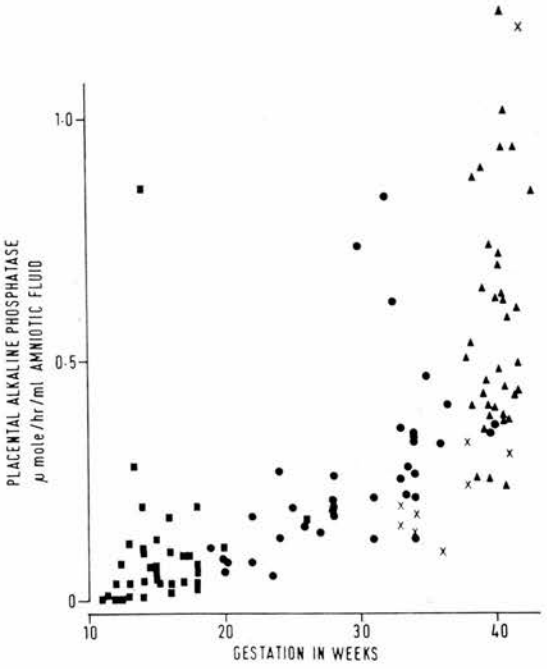


FIG. 5
Amniotic fluid hexosaminidase, expressed (a) per ml. of amniotic fluid, and (b) per mg. of protein, plotted against gestational age. Symbols as in Fig. 1.



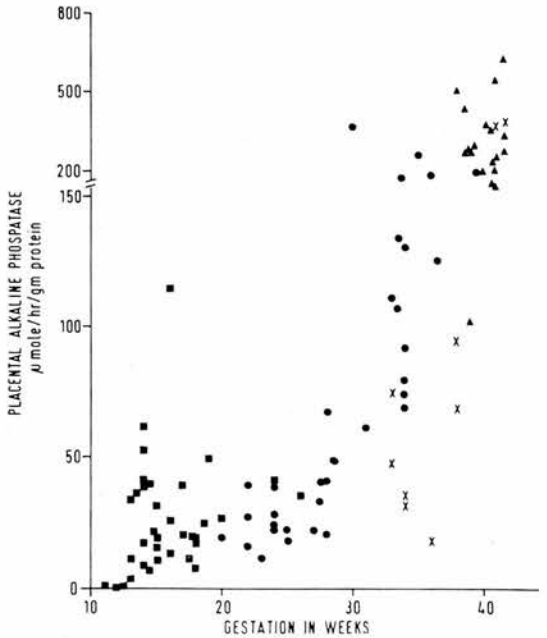


FIG. 6
Amniotic fluid placental alkaline phosphatase, expressed (a) per ml. of amniotic fluid, and (b) per g. of protein, plotted against gestational age. Symbols as in Fig. 1.

Placental alkaline phosphatase
(Figs. 6a and 6b)

A gradual increase in specific activity was observed between 10 and 30 weeks, after which the specific activity rose more rapidly. Between 30 and 40 weeks, the specific activity of placental alkaline phosphatase in amniotic fluid increased by 3 to 3·5-fold. Expression of activity on a protein basis led to an exaggeration of the rise in the latter half of pregnancy.

Discussion

The results show that complex changes of enzyme specific activity occur in the amniotic fluid as gestation proceeds. Generally, hexosaminidase had the highest specific activity in amniotic fluid and, with the exception of α-1,4-glucosidase, all the enzymes were at their highest specific activities at term.

Between 13 and 18 weeks of gestation, there was a peak in the specific activities of α-1,4-glucosidase and heat-labile alkaline phosphatase. The close correlation between the values for these enzymes in individual amniotic fluids (Fig. 3) suggests that their activity peaks are due to the same developmental event. The effects of this event are not limited to these two enzymes, for there is a simultaneous increase in the specific activity of hexosaminidase and also minor activity peaks for the other enzymes.

Between 20 and 30 weeks of gestation, the specific activities of α-1,4-glucosidase and heat-labile alkaline phosphatase fell to their lowest levels, whilst those of the other enzymes remain unchanged. After 30 weeks, the specific activities of hexosaminidase and α-1,4-glucosidase remained unchanged, whilst those of acid phosphatase, placental alkaline phosphatase, and (somewhat later) heat-labile alkaline phosphatase rose to their maximal levels. Roopnarinesingh *et al.* (1972) also found that the specific activity of placental alkaline phosphatase increased during this time, though these workers used 56 °C. as their inactivation temperature and so may also have been measuring a labile fraction of alkaline phosphatse. The considerable scatter of the specific activities of the phosphatases at term may be partially due to errors in estimating gestational age, or to the varying proximity of the onset of labour. Such errors would have their greatest effect upon enzymes undergoing rapid changes in specific activity. The lack of correlation between the levels of the three enzymes in the individual amniotic fluids after 37 weeks does not seem to be due to technical problems. Meconium-stained amniotic fluids were excluded from the series as they contain excessive quantities of heat-labile alkaline phosphatase (Geyer and Schneider, 1970). It was possible that the samples of amniotic fluid obtained at artificial rupture of the membranes were not typical of the rest of the fluid. This point was investigated in three patients at induction of labour. In these patients, ten consecutive 20 ml. samples of amniotic fluid were taken for analysis while it drained from the amnioscope. In no case was significant variation found in the protein concentration or in any of the enzyme specific activities.

In this study no significant correlations were found between the amniotic fluid protein concentration and the enzyme specific activities. However, the specific activities on a protein basis were plotted against gestational age because this caused an exaggeration of the early and late peaks of enzyme specific activity. This occurs because the protein concentration reaches a maximum at approximately 25 weeks of gestation (Queenan *et al.*, 1970; Sutcliffe and Brock, 1972). Although the biological basis of this expression of specific activity is questionable, the exaggeration it brings about may be valuable if the measurements are to be used for maturity testing or other studies during the last trimester of pregnancy.

The patterns of enzyme specific activity reported here show that complex factors control the composition of amniotic fluid, and the possible nature of these factors is dealt with in a subsequent paper (Sutcliffe and Brock, 1972). Yet, whatever the biological implications of these findings, the trends observed during the latter half of pregnancy suggest that the measurement of enzymes in amniotic fluid may be of use in the assessment of fetal maturity. In particular, the changes in phosphatase activity, on both volume and protein bases, appear to merit further investigation.

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