

BIOCHEMICAL STUDIES ON  
NUCLEIC ACIDS AND PROTEINS  
IN HUMAN PLACENTAE, FOETAL  
MEMBRANES AND ORGANS, AND  
CERTAIN BLOOD CELL - CULTURE  
SYSTEMS

By

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"Physico-chemical embryology has, indeed, arrived at the stage immediately prior to birth and all it needs is a skilful obstetrician, for, when once it has reached the light of day and has passed for ever out of the foetal stage, it will be well able to take care of itself."

J. Needham, 1931.

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I N T R O D U C T I O N

The application of chemical methods to the study of embryological problems is relatively recent. Indeed, it may be stated that the penetration of physico-chemical concepts into embryology has not been entirely peaceful. In the past, the opinion has been held that the embryo seems to be resolved to acquire a certain form and structure and that the development of forms of organisms seems to be utterly refractory to explanation by mechanical or physico-chemical principles.

In common with other fields of science, expansion of research follows either the introduction of new promising tools for investigation or a break-through discovery or else it may draw its impetus from the emergence of an important problem that needs a rapid solution. In the field of chemical embryology all such factors played a role in the expansion of recent research, as well as in providing the subject of this thesis. Recent tools of research include reliable chemical methods for the estimation of nucleic acids, fractionation of proteins by electrophoresis and the technique of blood (leucocyte) culture as an in vitro method for the study of growth of human cells. An undoubted break-through discovery has been the realization of the chemical basis of heredity and of the important biological role of DNA and RNA in growth and development. An urgent need for research in the field of chemical embryology followed the recognition of the role of chemical drugs in the interference with

embryonic growth. The thalidomide tragedy is such an outstanding example.

Nucleic acids:

According to Davidson (1965), the foundation of the knowledge of the chemistry of the cell nucleus was laid by Miescher (1844-95). Miescher based his investigations firstly on isolated nuclei from pus cells and then on salmon sperm heads. It was subsequently shown that nucleic acids are normal constituents of all cells and tissues which were examined.

The distinction between the DNA and the RNA was made when studies on the nucleic acid from the thymus gland (which provided one of the best animal sources) and the nucleic acid from yeast showed that the two acids are different. On hydrolysis, the yeast nucleic acid yielded uracil in place of thymine and ribose in place of deoxyribose. Since most nucleic acids from animal sources appeared to resemble thymus nucleic acid and since the nucleic acid from the wheat embryo appeared to be very similar to yeast nucleic acid, this led to the erroneous conclusion that there are two nucleic acids in nature, one obtainable from the nuclei of animal cells and the other from the nuclei of plant cells (Jones, 1920). The validity of this concept was questioned by the subsequent demonstration of pentose nucleic acids in several animal tissues (Jones and Perkins, 1924-5).



The foundation of modern knowledge about nucleic acids was, however, laid only in the early forties, with the introduction of new techniques for cyto-chemistry and cell fractionation. It was soon established that both DNA and RNA are normal constituents of all cells of plants and animals, DNA being confined to the nucleus, while RNA is found mostly in the cytoplasm (Davidson and Waymouth, 1944).

Recent years have witnessed a vast expansion of nucleic acid research. This has been prompted by two main factors, firstly the realization of the controlling role of nucleic acids in the life of the cell and secondly, research facilities afforded by the introduction of several important technical advances. The role of RNA in protein synthesis was first indicated by the histochemical experiments of Caspersson (1950) and Brachet (1950) which demonstrated that RNA is particularly abundant in cells engaged in the synthesis of protein either for growth or for secretion. More attention to the genetic role of DNA was paid when Avery, McLeod and McCarty (1944) showed that DNA extracted from encapsulated smooth strains of pneumococcus type III could, on addition to the culture medium, transform unencapsulated "rough" cells into the fully encapsulated smooth type III. The smooth cells so developed could propagate indefinitely in the same form, producing DNA with the same capabilities. Among the technical advances that have facilitated nucleic acid

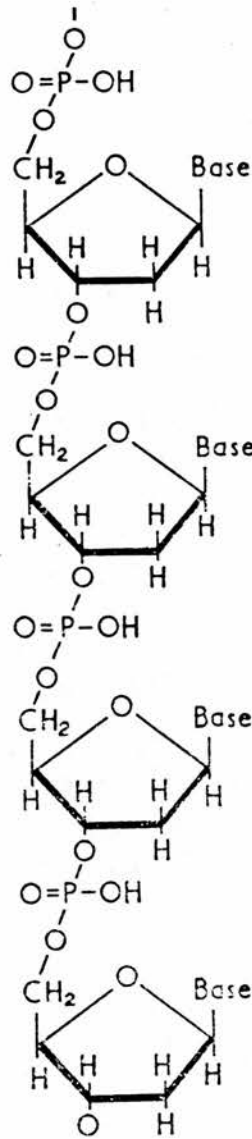


Fig. 1 : Part of the polynucleotide chain in DNA. ( from Davidson, 1965 )

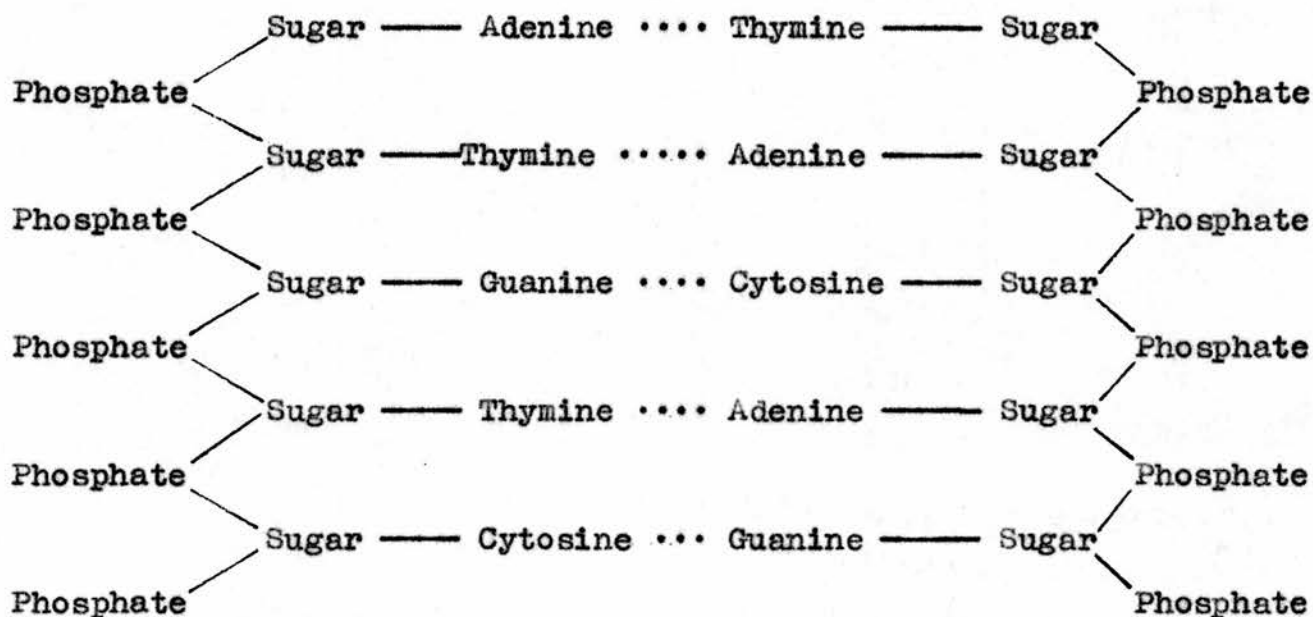


Fig. 2: Complementary sequences of bases in the two strands of the double helix of DNA (after Perutz, 1962)

research was the introduction of new and greatly improved methods of nucleic acid estimation by Schneider (1945) and by Schmidt and Thannhauser (1945).

The structure of the DNA is that of a polymer, the monomer units of which are deoxyribonucleoside monophosphates (deoxyribonucleotides): Fig. 1. One deoxyribonucleotide is composed of one phosphoric acid group, a pentose sugar which is D-2-deoxyribose and a base. The base is a purine or pyrimidine, the purine is a cytosine or thymine and the pyrimidine is adenine or guanine. The DNA molecule has a long unbranched chain structure. The nucleotides in the polynucleotide chain are linked with 3',5' phosphodiester linkages. The molecular configuration of DNA is that of a double right handed helix consisting of two polynucleotide chains winding round the same axis and held together by their bases (Watson and Crick, 1953). The bases are fitted in pairs, one purine and one pyrimidine. In fact, the only pairs which are recognized to fit together are adenine with thymine and guanine with cytosine (Fig. 2). Accordingly, the order in which the bases occur in one chain automatically determines the order in the complementary chain. Apart from this essential condition, there are no restrictions on the sequence of pairs of bases along the chains. There are wide variations in the molar proportions of bases in DNA from different species but the DNA from the different organs and tissues of any species is closely similar. In

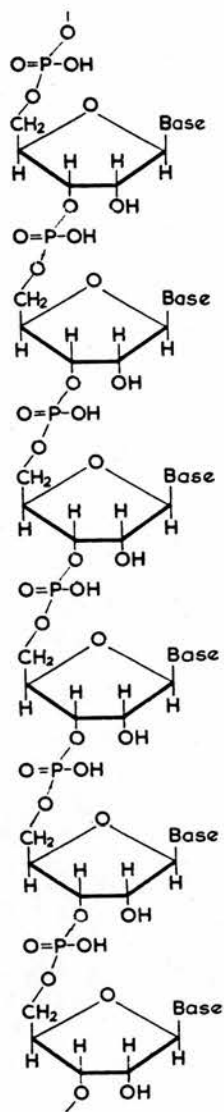


Fig. 3 : A section of the polynucleotide chain in the RNA molecule. ( from Davidson, 1965 )

DNA the sum of the purine bases is equal to the sum of the pyrimidine bases and the sum of the amino bases (adenine and cytosine) is equal to the sum of keto (oxo) bases (guanine and thymidine) (Chargaff, 1955). It follows that adenine (A) and thymine (T) are present in equimolar amounts, and so are also guanine (G) and cytosine (C). This equivalence of A and T and of G and C is of the utmost importance in relation to the formation of the DNA helix. Two main classes of DNA exist: the (A - T) type in which adenine and thymine are in excess and the much rarer (C - G) type in which cytosine and guanine predominate. Most DNA's have high molecular weights, varying from  $10^6$  to  $10^9$  or more. DNA exists in nature in the cell nucleus as deoxyribonucleoprotein. In human germ cells it has been calculated that the DNA contains about  $10^9$  base-pairs and is about one metre in length (Perutz, 1962). It is of course divided among the 23 chromosomes. The amount in the ordinary somatic cells is twice this. The sequence of the nucleotides among the chain is difficult to determine due to the difficulty in obtaining a single molecular species of DNA from most natural sources and partly due to the fact that the DNA molecule is a very large polymer composed of only 4 different monomer types.

The structure of the RNA is that of a polymer, the monomer units of which are ribonucleoside monophosphates (ribonucleotides): Fig. 3. The main internucleotide linkages are phospho-ester groups connecting C-5' in one

nucleotide with C-2' or C-3' in the next nucleotide. One ribonucleotide unit is composed of one phosphoric acid group, a pentose sugar (D-ribose) and a base (purine or pyrimidine). The purine base may be cytosine or uracil. The pyrimidine base may be adenine or guanine. RNA exists in the cell in combination with protein as ribonucleoprotein. The molar proportions of bases vary widely according to the material of origin. The detailed structure of the RNA molecule has not, however, been fully established.

The living cell contains 3 main types of RNA: ribosomal RNA (rRNA); soluble, transfer or acceptor RNA (sRNA); messenger, informational, complementary, translational or transcript RNA (mRNA). Ribosomal RNA forms about 80 per cent of the cellular RNA and is contained in the ribosomes. Soluble RNA constitutes about 15 per cent of cellular RNA and is contained in the soluble cytoplasmic fraction of the cell, hence its name. The messenger RNA forms 5 per cent or less of the cellular RNA and is contained in the nucleus, where it carries information from the DNA, hence its name. The biological function of these 3 types of RNA will be detailed below.

The genetic function of DNA is now well established. The method of replication whereby a parent molecule of DNA gives rise to two identical daughter molecules ensures that each cell, produced as the result of mitotic division, receives exactly the same complement of DNA both

qualitatively and quantitatively as that of the parent cell. The DNA of any species is biologically distinctive. The DNA in different tissues of any one species is not only the same in amount per somatic cell (Boivin and associates, 1948; Vendrely and Vendrely, 1948) but is also the same in quality. The amount of DNA in sperm nuclei, which contain the haploid number of chromosomes, is approximately half that found in the somatic cell nuclei of the same species. Variations in the DNA content fall within limits which are narrow enough to make DNA almost certainly the least variable of all cell components. The stability of the DNA has been utilized in two ways: Firstly, it allows the calculation of the number of cells in a piece of tissue by determination of the total DNA content. Secondly, DNA can be used as a reference substance in terms of which the chemical composition of a tissue may be expressed. For example, the RNA/DNA ratio and the protein/DNA ratio give an indication about the content of RNA or protein per cell.

The function of nucleic acids in protein synthesis has been clarified recently (Davidson, 1965). The sequences of bases in some region of the DNA of the cell determine the sequence of amino acids in any protein. DNA passes the information coded in it to the mRNA. This mRNA carries the information to the ribosomes. It is in the ribosomes that the actual process of protein synthesis begins. As mRNA is present in the cell nucleus,



it has to migrate to the cytoplasm to control the protein synthesis in the ribosomes. The amino acids available for protein synthesis are first activated by a special activating enzyme in the presence of A.T.P. The sRNA accepts the activated amino acid and forms a sRNA-amino acid derivative, and carries it to the ribosomes. The sRNA codes with the mRNA in the ribosomes in such a way as to ensure that the amino acid that it carries will be correctly placed in sequence in the growing polypeptide chain. Under the influence of transferase enzymes, the sRNA is released and can go through the process again. The sRNA, as may be noted, exists in many forms, each form being specific for one amino acid. One of the characteristics of animal cells is that the total task of protein synthesis is subdivided, so that certain cells specialize in the synthesis of certain proteins. In other words, the entire length of DNA is not utilized in all cells so that there are silent as well as active regions. According to Campbell and Sargent (1967), the process may be under control of a gene regulator, probably histones because of their close association with DNA in the cell nucleus.

Apart from the function of RNA in protein synthesis, a considerable amount of evidence has now been accumulated to suggest that RNA metabolism is intimately connected with memory storage and learning (Hydén and Egyhazi, 1964).

### Separation of protein fractions by electrophoresis:

It was not until 1947-1949 that methods of paper electrophoresis were developed for wide use (Block and associates, 1955). It was shown that by applying an electric potential across the ends of strips of filter paper saturated with buffer, mixtures of proteins, peptides or amino acids could be separated into narrowly circumscribed areas. These proteins can be fixed on the paper by heat denaturation and then stained, or each band can be eluted and used for other purposes. The introduction of the method opened a wide field for research. Block and co-authors, reporting in 1955, stated that more than 300 papers have appeared on the subject of paper electrophoresis since 1948.

Certain embryological problems offer a useful field of investigation by electrophoresis. The origin of the amniotic fluid proteins (and of the fluid as a whole) and the origin of the foetal serum proteins are such examples. The importance of such studies in the wider problem of foetal growth and differentiation remains to be seen.

### Human leucocyte culture:

The aim of culturing cells outside the body is to isolate them from the controlling influence of the whole organism and to bring them into a suitable state for examination and experimentation. Although reports dating back to 1915 have shown that in vitro mitosis of peripheral

blood leucocytes does occur (Mellman, 1965), it was not until 1960 when Nowell made the observation of great practical use. Nowell (1960) showed that the addition of phytohaemagglutinin extracted from the red kidney bean, *Phaseolus vulgaris*, produced a high degree of mitotic activity in leucocytes in vitro. Under the influence of phytohaemagglutinin the small lymphocyte in peripheral blood morphologically alters into a blast-like cell that divides in culture.

The usefulness of the peripheral blood cultures for human cytogenetic studies is established. This culture system can also be adopted with advantage for other experiments, such as the study of the effects of physical and chemical agents (Nowell, 1961; Jackson and Lindahl-Kiessling, 1963; Bender and Gooch, 1963). For such purposes, leucocyte cultures offer the advantages of being readily obtainable, of being free of contamination by other tissues, of being of short term and of growing in suspension.

Many different methods have been described in the literature for measuring growth in cultures (Woodliff, 1964). Chemical methods are among the most reliable of these, and among the chemical methods the estimation of DNA is probably the most reliable. Since the constancy of the deoxyribonucleic acid (DNA) content per nucleus - as long as no changes in ploidy occur - is now well established, the determination of DNA seems to be the most reliable

method for following growth in tissue culture (Bonting and Jones, 1957).

Although the results of in vitro experiments do not necessarily reflect the behaviour of tissues in vivo, the application of the method to the study of certain embryological chemical problems may be of help, in providing useful basic information. One such example is to test the effect of teratogenic chemical drugs on the growth pattern of the culture.

The thalidomide tragedy:

In November 1961, Lenz voiced the suspicion that the use of thalidomide as a sedative during early pregnancy might be responsible for the occurrence of congenital malformation in the foetus. In 1962, Lenz was capable of providing conclusive evidence that thalidomide is teratogenic in man. He reported that there was no case in which the mother of a normal infant had taken thalidomide between the third and eighth weeks after conception. Moreover, he found that in every case in which the foetus showed the particular malformation, the mother had taken the drug. The peculiar deformities produced have been described by McBride (1961) and by Willman and Dumoulin (1962) as symmetrical aplasia or hypoplasia of the extremities. During the critical phase of limb development (4th to 6th week), a small quantity of thalidomide is sufficient to cause the malformation (Ferguson, 1962; Stabler, 1962).

The behaviour of thalidomide may be related to some

chemical property of its molecule, although the mechanism of its embryotoxic activity is still not well understood. Smith and co-workers (1965) suggested that thalidomide may be interfering with some mechanism which is specifically involved in embryonic development, since thalidomide exhibits a high but transient toxicity only during the period of morphogenesis. A complete solution of the problem is not obtainable, however, in the fragmentary state of knowledge about chemical embryological processes. The thalidomide tragedy, it may be said, has exposed the wide gaps in our knowledge. Basic studies in chemical embryology are needed and especially on human material.

This thesis presents some basic studies on the chemical aspects of human embryonic and foetal growth. A study in vitro of the effect of thalidomide is also presented.

M A T E R I A L

Foetal organs and membranes:

Foetuses were obtained from cases of therapeutic terminations of pregnancy. The gestational age ranged from 4 to 20 weeks. Since most terminations are done in the first trimester of pregnancy, most of the foetuses were obtained during this period. Therapeutic terminations were preferred for the study because they provide generally healthy normal and intact tissues for analysis. Two spontaneous abortions at 16 and 24 weeks were included in the study. Therapeutic abortions are not readily available after the 20th week of pregnancy. In the 2 cases, the appearance of the two foetuses was healthy. The results in the two cases are however expressed separately and are not included with the rest of the cases. The age of the foetus was determined from its length according to the rule outlined by Potter (1961). One month after conception, the length is slightly less than 1 cm. At the end of 2 months it is about 2 cm. From the third to the 5th month, the age in months is the square root of the length in centimeters. After the 5th month, the length in cms. is approximately the number of months multiplied by 5.

Twenty one therapeutic abortions were examined (though not all the foetal organs were available to this work in all of them). The two spontaneous abortions have been already mentioned. Seven placentae were available for examination from periods of gestation

ranging from 33 to 40 weeks.

The organs examined were the liver, brain, kidney, lung, heart, placenta and amnion. The foetuses and the placentae were obtained immediately after operation or delivery. The dissection was quickly done and the organs blotted, weighed and homogenized. The whole brain was used to avoid possible variations from one region to another (Kissane and Robins, 1958; May and Grenell, 1959). The placental tissue was also washed quickly with distilled water and then with 0.9 per cent saline to eliminate blood contamination.

Material for electrophoresis:

1. Maternal serum
2. Foetal serum
3. Amniotic fluid
4. Placental extract.

Maternal serum:

Venous blood was obtained from pregnant or parturient women. No anti-coagulant was used and time was allowed for clot formation and clot retraction to take place. The blood was centrifuged at 2000 r.p.m. for 15/ and the serum separated. Haemolysis should be avoided since it introduces an artifact of the beta-zone and poor separation of the alpha-2 -beta and gamma globulin zones (Milles and co-workers, 1960). Haemolysed samples were discarded.



Dialysis was carried out by means of Visking dialysis tubing. This tubing requires pre-treatment before use. The tubing was first cut into the lengths likely to be required, washed with distilled water and then kept in  $10^{-3}M$  EDTA buffer. Before use, the tubing was washed well with distilled water, dried and knotted at one end using its own substance for the knot. The serum was pipetted into the dialysis sac which was then knotted from the other end. The dialysis was carried out for 3 hours against a 10 times dilution of the veronal acetate buffer used in the electrophoresis experiment. The total proteins were estimated by the method outlined later (Miller's method).

Foetal serum:

Cord blood was used. From one case of therapeutic abortion at 19 weeks, foetal blood was obtained by heart puncture.

The same treatment outlined for the maternal blood was followed.

Amniotic fluid:

Amniotic fluid was obtained by: (a) abdominal amniocentesis from women with rhesus incompatibility, (b) abdominal hysterotomy from cases after therapeutic termination of pregnancy, (c) high rupture of the membranes in cases of induction of labour.

The amniotic fluid was prepared for electrophoresis

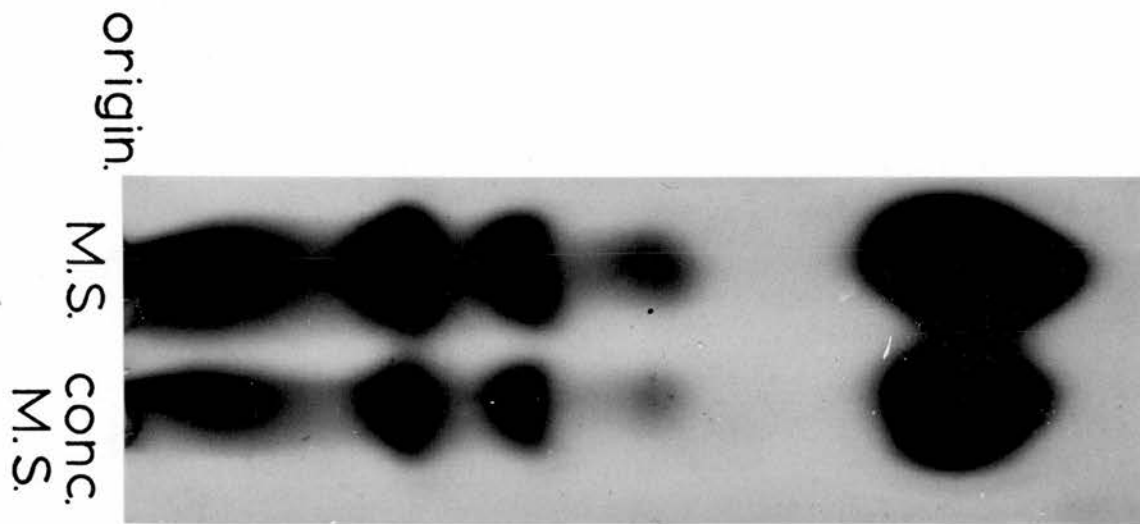


Fig. 4 : The effect of concentration by sucrose on the electrophoretic pattern of the proteins in the maternal serum.

Conc.M.S.: concentrated maternal serum.

M.S. : maternal serum.

in the following way. The fluid was filtered through a Whatman, No.1 filter paper to get rid of lanugo hair, vernix caseosa and a gelatinous material invariably present in the amniotic fluid (Viergiver and co-workers, 1962). The total protein was then estimated by the method described by Miller (1959). The amniotic fluid was then concentrated, by pipetting into a dialysis tubing covered for one hour with dry sucrose (Hsiao and Putnam, 1961). The sugar was then removed by washing the sac with distilled water. The concentrated amniotic fluid was dialysed for 20 minutes against the veronal acetate buffer used for the electrophoresis experiment.

The effect of concentration was tested in the following experiment using maternal serum. A sample of maternal serum was divided into two halves. One half was treated as mentioned for sera while the other half was concentrated using sucrose as mentioned above. The two sera were then run parallel to each other on one strip. The result which is shown in Fig. 4. shows no effect of the concentration on the protein pattern. Concentration is necessary in the case of the amniotic fluid before electrophoresis in view of the small amount of protein normally present.

#### Placental extract:

Placentae were obtained immediately after delivery or operation. A piece was dissected, washed with distilled water, blotted between sheets of filter paper and

weighed. Every 100 gm. of placental tissue was homogenized in 200 ml. of saline using a Waring blender. The homogenate was filtered through Whatman No.1 filter paper. The protein content was estimated by Miller's method. The filtrate was then dialysed for 3 hours against the veronal acetate buffer used for the electrophoresis experiment.

Human leucocyte cultures:

- Blood was obtained (a) from healthy individuals  
(b) from the Blood Transfusion Service.

For the preliminary studies on the normal pattern of leucocyte growth in culture heparinized blood was obtained from volunteering individuals. The dose of heparin used was 10-15 units/ml. of blood.

For the experiment of testing the effect on the culture of chemical agents, blood was obtained from the Blood Transfusion Service. In this way, the blood from a single donor was used for the whole experiment and the results are made more comparable.

METHODS

METHODS FOR THE EXTRACTION AND ESTIMATION OF NUCLEIC  
ACIDS AND PROTEINS

The chemical structure of the nucleic acids provides three main features which can be used for their determination in biological material. The nucleotide units of which the nucleic acids are composed consist of three components: a purine or pyrimidine base, a sugar (ribose in RNA, deoxyribose in DNA) and phosphoric acid. Accordingly, the estimation of nucleic acids can be achieved by means of:

- (a) utilizing the ultra-violet absorption of the bases. This will naturally not differentiate between DNA and RNA.
- (b) estimation of phosphorus. This again will not differentiate between the two nucleic acids.
- (c) using specific reactions for the determination of the ribose content of RNA and the deoxyribose content of DNA.

In order to estimate the two nucleic acids independently, either a method based on the specific sugar reactions is to be used or alternatively the two nucleic acids must be first separated. In this work, the determination of DNA and RNA was based on their specific reactions.

Before applying these methods, the nucleic acids must be first extracted from the biological material. In this work, the method of Schneider (1945) as modified by Lang and co-workers (1965) has been used. In this technique,

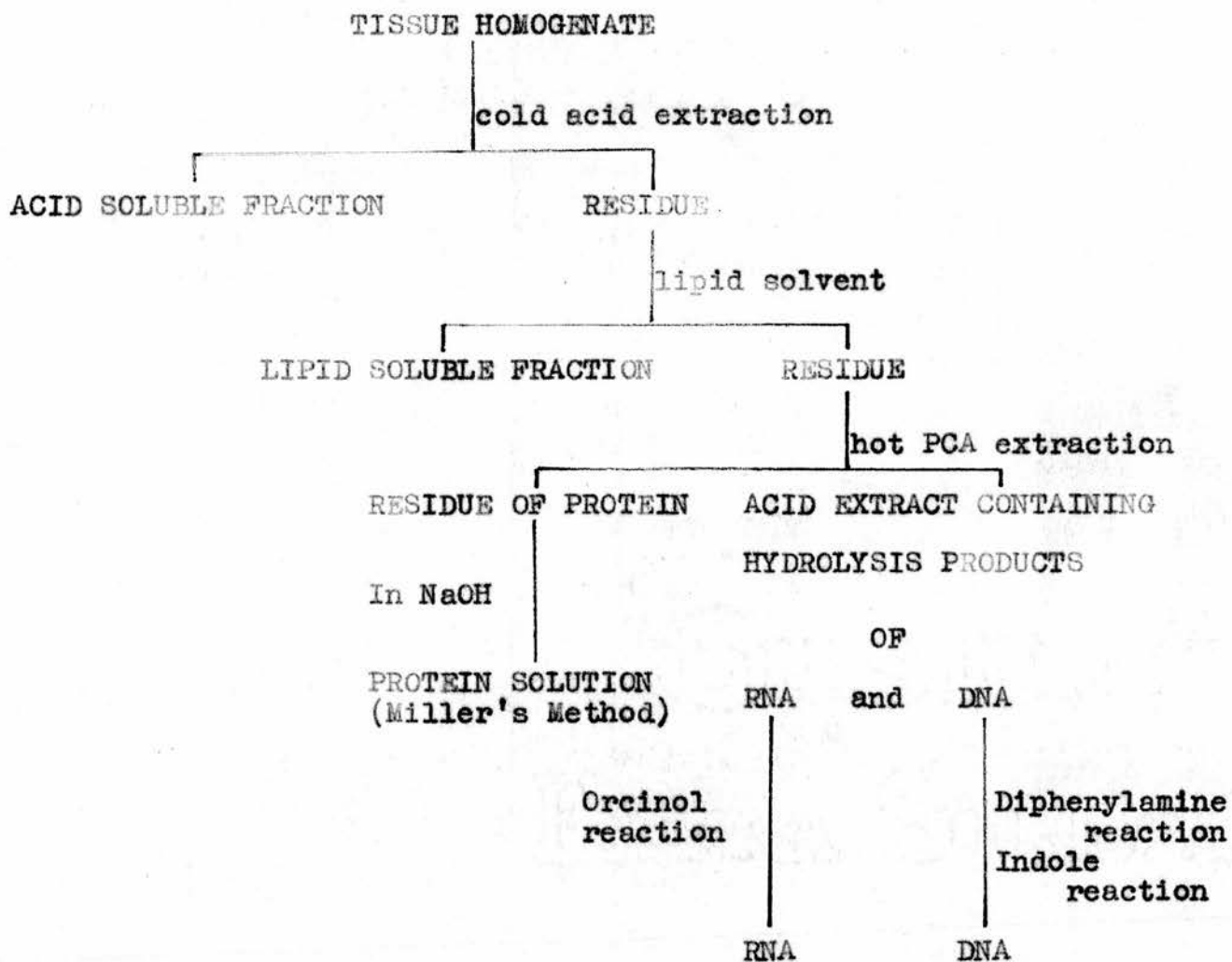


Fig. 5: Schematic representation of the method used for estimation of DNA, RNA and proteins.

both nucleic acids are extracted simultaneously by hot acid. The DNA and RNA concentrations are then determined by means of specific sugar reactions.

Before the extraction of nucleic acids, it is necessary to remove substances that would otherwise interfere with the reactions used subsequently for the estimation of nucleic acids. This commonly involves two steps: the removal of acid soluble compounds, and the removal by organic solvents of lipids and phospholipids.

Finally, it is essential to protect the nucleic acids in the biological material from the action of degrading enzymes, by taking proper precautions during the removal and disintegration of the tissues.

The steps of nucleic acids determination can thus be outlined as follows:

I. The preparation of tissue samples:

- a. Precautions during removal and disintegration of tissues.
- b. Removal of compounds which may interfere with nucleic acids estimation:
  1. acid soluble compounds.
  2. tissue lipids and phospholipids.

II. The extraction of nucleic acids.

III. Estimation of DNA and RNA content in the extract.

A schematic representation of the method used is shown in Fig. 5.



The preparation of tissue samples:

Precautions during removal and disintegration of tissues:

Nuclease enzymes (degrading nucleic acids) occur in most tissues. These can interfere seriously with the estimation of nucleic acids unless precautions are taken. Homogenization with ice cold 0.25 N perchloric acid (PCA) causes rapid inactivation of the nucleases (Munro and Fleck, 1966).

In this work, tissue samples were obtained as soon as possible after delivery or operation. Homogenization was done immediately after. Aliquots of 300 mgm of wet tissue were homogenized in a mortar in 5.0 ml. ice cold 0.25 N perchloric acid.

Removal of compounds which may interfere with nucleic acids estimation:

1. Acid soluble compounds: These are free nucleotides and nucleotide co-enzymes, sugars and inorganic and organic phosphorus compounds.

The homogenate was centrifuged for 5 minutes at 2000 r.p.m., the supernatant discarded, the precipitate washed again in 5.0 ml. ice cold 0.25 N perchloric acid, centrifugation repeated and the supernatant discarded.

0.25 N PCA solution was used since Munro and Fleck (1966) have shown that concentrations above 0.3 N PCA caused progressive losses of RNA from the precipitate. The authors also pointed out the importance of maintaining a low temperature during the extraction process.

2. Tissue lipids: The acid insoluble fraction was washed twice with 5.0 ml. of cold 95 per cent (v/v) ethanol, centrifuged at 2,000 r.p.m. for 5 minutes and then the alcoholic fraction discarded.

Cold lipid solvents were used for hot lipid solvents cause a low recovery of DNA (Marko and Butler, 1951).

### Extraction of nucleic acids

There are three main methods for the extraction of nucleic acids: the Schneider procedure (1945), the Schmidt-Thannhauser procedure (1945) and the Ogur-Rosen procedure (1950). A schematic representation of the three methods is shown in Fig. 6.

The Schmidt-Thannhauser procedure (1945) involves certain hazards of alkaline digestion (Logan, Mannell and Rossiter, 1952; Drasher, 1953; Webb and Levy, 1958; Munro and Fleck, 1966). These hazards affect both the RNA and the DNA fractions. In the Ogur-Rosen procedure (1950), a source of error is that the cold N perchloric acid may not be adequate for complete extraction of the tissue RNA, and that some of the DNA may be extracted with the RNA (Munro and Fleck, 1966).

The Schneider procedure was used in this work. Its only disadvantage, when compared to the other two methods, is that both the RNA and DNA are extracted simultaneously. Accordingly, to obtain absolute values for each nucleic acid separately, only the colour reactions for deoxyribose

and ribose can be used. In the other two methods, on the other hand, the DNA and the RNA are extracted separately, and so the phosphorus reaction and U.V. absorption of bases can be used.

The conditions under which nucleic acids are extracted in the Schneider procedure are critical (Munro and Fleck, 1966). The following factors have been varied by various workers using the method: the acid used (TCA or PCA), the concentration of the acid, the temperature of extraction, the duration of extraction and the number of extractions. Schneider (1945) used a single extraction with 5 per cent TCA at 90°C for 15 minutes. Later, Schneider and co-workers (1950) used 0.6 N perchloric acid instead of TCA, at 90°C for 15 minutes. In this work, two extractions with 1 N perchloric acid at 80°C for 30 minutes each were done, according to the modification of Lang and co-workers (1965). In a preliminary trial of the method, we found that a third extraction was not necessary, as exemplified in the following experiment. Foetal organs (liver, brain, kidney and lung) from a 16-weeks foetus were extracted 3 times with 1N PCA at 80°C for 30 minutes, using equal volumes of PCA for each extraction. The three extracts were not pooled and the RNA and DNA were estimated in each extract. RNA was estimated by the orcinol reaction (Schneider, 1957). DNA was estimated by the indole reaction (Cerioti, 1952). Table 1 shows the results obtained, expressed as

percentages of the total yield. The findings are in agreement with those obtained by Lang and co-workers (1965).

Table 1.

Effect of the number of extractions on the recovery of DNA and RNA in foetal liver, brain, kidney and lung

Organ	Percentage of RNA extracted			Percentage of DNA extracted		
	Extract I	Extract II	Extract III	Extract I	Extract II	Extract III
Liver	91.56	7.14	1.30	95.29	4.42	0.29
Brain	89.47	8.12	2.41	96.82	3.18	0.0
Kidney	88.63	9.01	2.36	93.29	6.12	0.59
Lung	93.83	4.86	1.31	96.85	3.15	0.0
Mean	90.87	7.28	1.85	95.56	4.22	0.22

Extraction method of nucleic acids used in this work

1. To the acid insoluble and lipid free residue, obtained by the previous steps, 3.0 ml. 1 N PCA were added, the mixture thoroughly stirred with a glass rod and then heated in a water bath at 80°C for 30 minutes with occasional stirring.
2. The mixture was then centrifuged for 10 minutes at 2000 r.p.m. The supernatant was then separated and kept.
3. The tissue residue was re-extracted with another 3.0 ml. of 1 N PCA and heated at 80°C for 30 minutes. Centrifugation was then done for 10 minutes at 2000 r.p.m. and the second supernatant added to the first.

4. The pooled supernatant fluids were then made up to 6.0 ml. exactly.
5. The tissue residue contained the proteins, and was dissolved in 3.0 ml. N NaOH at room temperature. The method of protein estimation will be discussed later.

Estimation of DNA and RNA by measurement of deoxyribose and ribose respectively:

In this work, the DNA was estimated by two colorimetric methods: the diphenylamine method (Dische, 1930; Burton, 1956) and the indole method (Cerriotti, 1952). The two methods showed excellent agreement. The RNA was estimated by the orcinol method (Schneider, 1957).

The fluorimetric method for the estimation of DNA, as described by Kissane and Robins (1958) was tried. However, the results in our hands were very low when compared with the two colorimetric methods. The method was therefore dropped.

The diphenylamine method for the estimation of DNA:

The original diphenylamine reaction for measurement of DNA was described by Dische (1930). The method has been widely used since then. In 1956, Burton modified the method in two ways: Firstly, instead of heating at 100°C for 10 minutes, the solution was allowed to stand for about 17 hours at 30°C; Secondly, acetaldehyde was added to the reagent.

The mechanism of the diphenylamine reaction is not

fully clear. The reaction is a colorimetric one and is based on deoxyribose estimation. The links between pyrimidines and deoxyribose are stable while those between purines and deoxyribose are very labile. It appears that diphenylamine reacts with the sugar moiety originally combined with purines in the DNA. The nature of the colour compounds formed in the diphenylamine reaction is unknown. The action of acetaldehyde and some other aldehydes in potentiating the colour development is also obscure.

The effect of various factors on the colour development in the diphenylamine reaction was examined by Burton (1956). The optical density in the absence of added acetaldehyde was only 0.15 to 0.3 of that obtained with the addition of acetaldehyde to the reagent. The optimum concentration of PCA was found to lie between 0.25 and 0.65 N. A concentration of diphenylamine of 1.5 per cent (w/v) was found to give less colour in the absence of DNA, and hence preferably used. Constancy of temperature was not essential and variations between 25 and 35°C did not appreciably affect the colour development. The incubation period could also be between 16 and 20 hours.

The modified method of Burton (1956) is 3.5 times as sensitive as Dische's original procedure and several substances which interfere in the original method do not do so in the modified procedure. It was the method adopted for this work, and will now be described in detail.

Standard:

A highly polymerised calf thymus DNA preparation was used (B.D.H.). A stock standard solution was prepared by dissolving DNA at 0.4 mgm/ml. in 5 mM NaOH. Working standards were prepared every 3 weeks by mixing a measured volume of the stock standard solution with an equal volume of N PCA and heating the mixture at 70°C for 15 minutes. Both the stock and working standard were stored in the refrigerator. No deterioration of the stock standard is expected within 6 months (Burton, 1956).

Reagents:

1. Diphenylamine (Analar)
2. Glacial acetic acid (Analar)
3. Concentrated sulphuric acid (Analar)
4. Aqueous acetaldehyde (B.D.H.): An aqueous solution was prepared at a concentration of 16 mgm/ml. and stored at 4°C.

Preparation of the diphenylamine reagent:

One and a half gram of diphenylamine were dissolved in 100 ml. of glacial acetic acid and then 1.5 ml. of concentrated  $H_2SO_4$  were added. The mixture was stored in the dark (the solution is better freshly prepared just before use). On the day it is to be used, 0.1 ml. of aqueous acetaldehyde (16 mgm/ml.) is added for each 20 ml. of reagent required.

Preparation of a standard DNA curve:

A standard DNA curve was prepared using the working standard in a range of 25 to 250  $\mu\text{g DNA}/2\text{ ml}$ . The average of 6 experiments for each concentration was taken and a curve was drawn by relating the optical density to  $\mu\text{g DNA}$ .

Method:

1. A suitable tissue extract of the nucleic acids in 0.5 N PCA was prepared, as previously outlined.
2. Two ml. of the nucleic acid extract containing between 25 to 250  $\mu\text{g DNA}$  were mixed with 4.0 ml. of the diphenylamine reagent (containing acetaldehyde). A blank containing 2 ml. 0.5 N PCA but no DNA was also prepared to which 4.0 ml. of the diphenylamine reagent were added.
3. The mixture was incubated at  $37^{\circ}\text{C}$  for 20 hours.
4. The optical density of the blue colour developed was measured at 600  $\text{m}\mu$ , against the blank, in a Unicam S.P. 600 spectrophotometer with a light path of 1 cm. (This apparatus was used to read optical densities throughout the whole of this work).
5. The DNA content was calculated by reading the optical density of the solution on the standard DNA curve.

The results of each experiment were the mean of duplicates.



The indole reaction for the estimation of DNA

The method used in this work is that described by Ceriotti (1952) with only one modification, namely that the chloroform was not purified before use. This purification was found to be unnecessary and even disadvantageous by Hutchison and Munro (1961) since it removes the small amount of ethanol added to analytical grade chloroform to prevent decomposition. According to Hutchison and Munro (1961), this was also later confirmed by Ceriotti. Other modifications of the method have been suggested. Bonting and Jones (1957) used a single extraction with a tenfold volume of chloroform to make the method applicable to tissue culture. Keck (1956) used amyl acetate instead of chloroform because it can extract the colour produced by arabinose while chloroform cannot.

The reaction is given by the deoxyribose in the DNA. Probably only the purine-bound deoxyribose reacts, as the pyrimidine-bound sugar molecules are hydrolysed with difficulty. The specificity of the reaction was investigated by Ceriotti (1952). The only substance which could interfere with the reaction was arabinose or substances containing arabinose. The intensity of the colour given by arabinose was, however, very low, compared with the colour produced by DNA (about 8 per cent). This interference by arabinose does not seem to be of much importance when studying animal tissues. In plant tissues, the modification of Keck (1956), already referred

to, may be used to remove arabinose.

Standard DNA solution:

The same standard as that described in the diphenylamine method was used.

Reagents:

1. Indole (Hopkin and Williams Ltd.)
2. Concentrated HCl (specific gravity 1.19) (Analar)

The purity of the hydrochloric acid affects the sensitivity of the indole reaction within a wide range (Deken-Grenson and Deken, 1959)

3. Chloroform (Analar)

Preparation of the indole reagent:

A 0.04 per cent (w/v) solution was prepared. This is done by dissolving the indole in warm distilled water, cooling under running water and then making up to volume with distilled water. The solution is stable for at least two months if kept in a pyrex bottle at refrigerator temperature but should not be stored in a polyethylene bottle (Bonting and Jones, 1957).

Preparation of the standard DNA curve:

The working standard DNA solution was used in a range of 10 - 40  $\mu\text{g}$  DNA. The average of 6 experiments for each concentration was taken. The curve was drawn by plotting the optical density against  $\mu\text{g}$  DNA.

Method:

1. Two ml. of the nucleic acid extract in 0.5 N  $\text{HClO}_4$  were adjusted to contain about 10 to 40  $\mu\text{g}$  DNA. A blank containing 2.0 ml. of 0.5 N PCA but no DNA was also prepared.
2. To this, 1.0 ml. of indole solution and 1.0 ml. of concentrated HCl were added. The mixture was shaken well.
3. The test tubes were placed in a boiling water bath for 10 minutes.
4. The tubes were then cooled under running water.
5. Three extractions, each with 4.0 ml. chloroform, were done. The tubes were then centrifuged at low speed (500 r.p.m.) for 5 minutes to get a completely clear water phase. The colour given by DNA is stable for several hours. On long standing at room temperature, a rose pink colour forms which can interfere with the readings especially at the lowest concentrations. By an additional extraction with chloroform, this colour can be eliminated completely.
6. The upper clear aqueous phase was pipetted in the Unicam 1 cm. cells. The optical density of the yellow colour was read at 490  $\text{m}\mu$  (the wavelength of maximum of absorption) against the blank.
7. The DNA content was calculated by reading the optical density of the solution on the standard DNA curve. The mean of two duplicates for each experiment was taken.

### The Orcinol reaction for the estimation of RNA

The orcinol reaction was first adapted for quantitative determinations and differentiation of various nucleotides by Dische and Schwarz (1937). Several modifications of this reaction were later developed. The method used in this work is that described by Schneider (1957).

The orcinol reaction depends on the hydrolysis of the nucleic acid by acid at 100°C to yield furfural from the ribose. This in turn reacts with orcinol to yield a green pigment. Ferric chloride is used as a catalyst. The purine-bound ribose of RNA reacts much more readily than pyrimidine-bound ribose. Webb and Levy (1958) have shown that a large difference in purine-pyrimidine ratio can sometimes exist between different species of RNA. Consequently, the value to be assigned to the result obtained from the orcinol reaction will depend on the proportion of purine and pyrimidine nucleotides in the molecule. The standard used should, therefore, ideally consist of pure RNA isolated from the tissue on which the determinations are being carried.

The orcinol reaction is very sensitive. An amount as low as 20 µg RNA can be estimated. A number of substances may, however, interfere with the reaction. These include carbohydrate substances (mostly found in plant tissue and yeast) and DNA. A correction for the interference by the DNA should be done. Schneider (1957)

put down an equation for this correction. Steele and co-workers (1949) and Waymouth (1951) subtracted  $0.023 \mu\text{g}$  of ribose for each  $1.0 \mu\text{g}$  DNA in the aliquot. Price (1950) found that DNA gave a 10 per cent colour value with the orcinol reaction. Hutchison and co-workers (1962) observed that DNA reacts with orcinol to give a colour equivalent to 12.5 per cent of the same weight of RNA. In this work, the following method was used for the correction. The orcinol reaction was applied to a standard DNA solution and the curve obtained was compared with a curve obtained with standard RNA. It was found that DNA reacted with orcinol to give a colour equivalent to 14.2 per cent of the same weight of RNA. A correction factor of 14.2 per cent was therefore applied to the orcinol results.

#### Experimental procedure:

##### Standard RNA:

A highly polymerized yeast RNA preparation from the British Drug Houses was used. Stock and working standards were prepared as in the case of DNA.

##### Reagents:

1. Orcinol (B.D.H.)
2.  $\text{FeCl}_3 \cdot 6 \text{H}_2\text{O}$  (Analar)
3. Concentrated HCl (Analar)

##### Preparation of the orcinol reagent:

One gram orcinol was dissolved immediately before use in 100 ml. of conc. HCl containing  $0.5 \text{ g.}$  of  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ .

### Preparation of the standard curve:

A standard curve was prepared using the standard RNA solution in a range of 20-100  $\mu\text{g}$  RNA, relating the optical density to  $\mu\text{g}$  of RNA. The average of 6 experiments for each RNA concentration was taken.

### Method:

1. To 1.5 ml. of 0.5 N PCA containing from 20 to 100  $\mu\text{g}$  RNA, 1.5 ml. of orcinol reagent were added. A blank containing 1.5 ml. of 0.5 N PCA plus 1.5 ml. of orcinol reagent was also prepared.
2. The mixture was then heated for 20 minutes in a boiling water bath.
3. The intensity of the green colour was read spectrophotometrically at 600  $\text{m}\mu$ . The absolute value of the RNA was then obtained from the standard curve.
4. The mean of two duplicates for each experiment was taken.

### Extraction of the residual proteins (Fig. 5)

The method used is that described by Lowry and colleagues (1951). The residue which remained after the nucleic acid extraction with hot perchloric acid contained the proteins. This residue was dissolved in 3.0 ml. 1N NaOH at room temperature.

### Method for the estimation of proteins

The method used is that of Lowry and colleagues (1951) as modified by Miller (1959). The mechanism of the reaction involves two distinct steps which lead to

the final colour development: reaction with copper in alkali and reduction of the Folin reagent. The advantages of the method over other methods for protein estimation have been outlined by Lowry and associates (1951). These advantages include simplicity, specificity, adaptability for small scale analysis and sensitivity. The sensitivity of the reaction is such that the reproducibility of analysis over the range of 40 to 200  $\mu\text{g}$  of protein is on average 2 per cent (Miller, 1959). Few substances can interfere with the reaction and these are not commonly encountered in biological material. These include uric acid, guanine, xanthine as well as most phenols except nitrophenols (Lowry and co-workers, 1951).

The modified method of Miller (1959) has two main advantages over the original method. The first is that it makes it possible to analyse a large number of samples at the same time. This is achieved by using a comparatively smaller volume of more concentrated alkaline copper reagent and a large volume of more diluted Folin phenol reagent, and postponing the final mixing until after the reagent has been added to all samples. The second advantage is that the time of the reaction is reduced to minutes. This is achieved by heating the final mixture of samples and reagents at  $50^{\circ}\text{C}$ , which accelerates the development of colour.

Experimental procedure:Standard protein solution:

The standard solution used was serum 6.4 gm. per cent. The standard solution was diluted with distilled water before use to give a range of 50 to 250  $\mu$ g protein.

Reagents:

1. Copper sulphate ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ) 0.5 per cent (w/v) in 1.0 per cent (w/v) potassium tartrate (dissolved in distilled water). The potassium tartrate solution was not incorporated with the copper sulphate solution except just before use, because of the slow development of a precipitate in the copper tartrate mixture (Oyama and Eagle, 1956). (Solution No.1).
2. Sodium carbonate 10 per cent (w/v) in 0.5 N sodium hydroxide (Solution No.2).
3. Folin and Ciocalteu's reagent (B.D.H.), a 1:11 dilution (v/v) in distilled water was prepared from the stock Folin solution. Both solutions should be kept in refrigerator.

Preparation of the alkaline copper reagent:

This reagent was prepared fresh each time just before the start of the experiment. It was made up of one part of solution No.1 and 10 parts of solution No.2.

Preparation of the standard curve:

A standard curve was prepared using the standard solution in a range of 50 to 250  $\mu$ g of protein. The mean of the results of 6 experiments for each concentration



was taken and a standard curve drawn relating optical density to protein concentration.

Method:

1. The samples were diluted with distilled water so that the protein content falls within the range of the standard curve. The dilution needed varied with the nature of the sample. Thus, for maternal and foetal sera, a 60 fold dilution was generally needed. For amniotic fluids, a 5 fold dilution was adequate. Tissue extracts of the foetal liver were diluted 20 times but other organ extracts were only 10 times diluted. Of these diluted solutions aliquots of 0.1 and 0.2 ml. were used.
2. The protein solution was pipetted in a test tube and then made up to 1.0 ml. exactly with distilled water. A blank containing 1.0 ml. distilled water but no protein was also prepared.
3. One ml. of alkaline copper reagent was added to each test tube.
4. The mixture was left to stand for 10 minutes at room temperature.
5. Three ml. of the diluted folin reagent were added forcibly to ensure adequate preliminary mixing. The final mixing can be postponed until after the reagent has been added to all the samples.
6. The mixtures of samples and reagents were heated for 10 minutes at 50°C in a constant temperature water bath.

A blue colour was formed.

7. After the tubes were cooled at room temperature, the optical density was read at a wavelength of 550 m $\mu$  against the blank.
8. The protein content was then calculated by reading the optical density on the standard curve. The average of duplicates was taken for each solution tested.

## ELECTROPHORESIS ON CELLULOSE ACETATE STRIPS

Three different supporting media can be used in electrophoresis: cellulose acetate membrane, filter paper and starch or agar gel. The advantages of the cellulose acetate membrane over filter paper as a supporting medium for zone electrophoresis were pointed out by Kohn (1960) and can be summarized as follows:

1. Adsorption is minimal and so tailing is eliminated. This improves the accuracy of quantitative determinations.
2. The material is homogenous, microporous and chemically pure.
3. The rapid separation saves time and material.
4. The separation of alpha-1 globulin from the albumin fraction is invariably excellent.
5. Very small quantities of proteins can be successfully separated.
6. Certain proteins which do not separate well on filter paper can be well separated on cellulose acetate.

The advantage of the cellulose acetate over the gel is that the preparation of the material is not needed as it is easily obtainable ready for use.

Apparatus:

EEL horizontal bath with accessory trays for cellulose acetate strips (Evans Electroselenium Ltd., Harlow, Essex, England).

Requirements:

1. Cellulose acetate membrane filter strips (36 x 5 cm.), obtained from Oxoid division of Oxo Ltd., London.

2. Buffer:

Veronal acetate buffer  $\mu$  0.1, pH 8.6. prepared as follows (Bodman, 1960):

Sodium barbitone (Analar) 10.0 gm. in 400 ml. distilled water

Sodium acetate (anhydrous) (Analar) 6.5 gm. in 400 ml. distilled H<sub>2</sub>O

Sulphuric acid 0.1 N (Analar) 64.4 ml.

Make up to one litre with distilled water.

3. Staining solution:

A 0.2 per cent (w/v) solution of Ponceau S (for electrophoresis, G.T. Gurr, Ltd., London) in 3.0 per cent (w/v) aqueous trichloroacetic acid was prepared.

4. Washing solution:

5 per cent (v/v) acetic acid.

- The buffer compartments of the apparatus were filled with the above mentioned buffer.

Procedure: The procedure was carried out according to Kohn, 1960.

1. Preparation of the strip:

The large strips 36 x 5 cm. were cut by a sharp razor to the size of 12 x 2.5 cm. The strip should be thoroughly inspected under a good light for the presence of imperfections such as parallel ridges or spots. Strips showing these faults may produce irregular bands and

imperfect separation, and are therefore discarded.

## 2. Marking of the strip:

Before impregnation with the buffer, the back surface of the strip was marked for the site of application of the sample. Two samples were applied on each strip. A margin of 0.5 cm. was left on each side of the strip. A distance of 0.5 cm. was also left between the two applications. The site of application of the sample was also made at a distance of 2.5 cm. from the cathode end of the strip. This was found to be the best position in this work.

## 3. Preparation of the wicks:

Wicks, 2.5 x 6 cm. were made from Whatman 3 mm. paper. The wicks were soaked in the buffer and then placed in position on the tray with their lower ends dipping into the buffer compartment.

## 4. Impregnation of the strip:

This was performed by first floating the strip on the surface of the buffer in a flat container for two minutes, followed by dipping it into the buffer solution for about 5 minutes. This is essential since quick submersion traps air and creates opaque spots, which take a long time to soak up. The impregnated strip was then removed from the buffer and lightly blotted on both sides between sheets of filter paper so that no excess moisture was seen. It should not, however, dry up and show any white opaque areas. If this happens the strip should be once

more impregnated and blotted.

#### 5. Placing of the strip in position:

The impregnated blotted strip was put across the tray with the face up and each end resting on a damped wick. The tray was then covered with its lid and the strip was made taut by gentle pulling of the buttons of the lid outwards and downwards. The bath was then covered with its glass lid and connected to the power unit. The polarity switch should be placed in the correct position (A or B). The power unit was switched on and the current adjusted to give 0.4 mA/cm width of the strip.

#### 6. Application of the sample:

After leaving the impregnated strip in position and under current for 10 minutes, the lids were removed and the samples were applied by a 10  $\mu$ l pipette as a streak along the pencil mark. A fine straight and narrow application line is essential for satisfactory separation. The lids were placed again and the current re-adjusted since it tends to rise at the beginning of the run. The run lasted for 6 hours.

The dose of the sample applied is important. The protein content should not be less than 200 - 300  $\mu$ g. per strip if the usual protein dyes are used. As a rule the smaller the volume applied, the neater the separation. In the case of sera, 2  $\mu$ l (100-150  $\mu$ g protein) were applied to one half of the strip. Of the concentrated amniotic fluid, about 6 $\mu$ l (100  $\mu$ g of protein) were applied to the half of the strip.

### 7. Drying:

After a run of six hours, the current was switched off and the strips were removed from the apparatus, blotted between sheets of filter paper and then dried in a hot oven at 80°C for 10 minutes.

### 8. Staining:

The dried strips were placed in the stain in a rectangular glass container and allowed first to float on the surface of the Ponceau S staining solution until the stain has entirely penetrated the bands and then submerged completely. The staining lasted for 10 minutes.

### 9. Washing:

The stained strips were washed with a 5 per cent (v/v) acetic acid solution, with frequent changes of the fluid, until the background of the strip became white and the washing fluid completely colourless.

### 10. Drying of the stained strip:

After complete washing, the strips were removed from the bath, thoroughly blotted between sheets of filter paper and allowed to dry at room temperature. The strips were then placed between blotting paper and pressed, e.g. between the sheets of a thick book, usually overnight. This procedure renders the strips absolutely flat.

### Quantitative evaluation of the results:

This can be achieved either by the spectrophotometric method or by scanning. In the spectrophotometric method, the protein bands on the strip are cut, each band

is eluted by a solution, and the optical density of the elute read in a spectrophotometer. The scanning method was used in this work.

The scanner used was: Chromoscan, double beam recording and integrating densitometer manufactured by Joyce, Lobel and Company Ltd., Princesway, Team Valley, Gateshead-on-Tyne, 11, England.

By scanning, the intensity of zones or bands given on electrophoretic separation is determined by measuring the relative quantity of reflected light absorbed by the stained protein. The scanner passes a narrow beam of light through the treated strip on to a photoelectric cell. The fluctuations in current thus produced are amplified and recorded. The integrator count is directly proportional to the pen deflection in the scanner. For the use of this apparatus, the cellulose acetate strip need not be cleared before scanning.

#### Procedure:

A green filter was used. The whole strip was first scanned and the total integrator counts recorded. Then the strip was rescanned to record the integrator counts separately for each protein fraction.

Taking the total integrator counts for all the fractions on a strip as 100 per cent, the percentage of dye uptake by each protein fraction were accordingly calculated.

The absolute value of each protein fraction was calculated by multiplying the total protein concentration



(estimated by the method previously described) by the percentage of dye uptake of this fraction.

The assumption that the quantity of stain taken up by a protein is proportionate to the concentration of that protein may not be absolutely justified (Bodman, 1960). However, provided that the same staining technique is adhered to, the quantitative evaluation is reasonably valid.

## PREPARATION OF HUMAN LEUCOCYTE CULTURES

The method of leucocyte culture used in this work is a modification of that described by Moorhead and co-workers (1960). An essential part of the method is the addition of phytohaemagglutinin (PHA) to the culture. PHA is an extract of the kidney bean *Phaseolus vulgaris* and it stimulates mitosis in the leucocyte culture (Nowell, 1960; Cooper, Barkhan and Hale, 1961; McIntyre and Ebaugh, 1962). In the absence of PHA from the leucocyte culture, no mitosis takes place, and also the cells do not synthesize DNA and RNA (Epstein and Stohlman, 1964). The mechanism by which PHA induces its action is, however, still unknown. One suggestion that has been made is that it acts as an antigenic stimulus (Pearmain and co-workers, 1963; Elves and co-workers, 1963).

Requirements:

All instruments and solutions used must be sterile.

## 1. Culture medium 199 (Glaxo)

For use, the medium was diluted 10 times with sterile distilled water.

## 2. Phytohaemagglutinin (Burroughs, Wellcome and Co. London)

One vial of PHA was dissolved in 5.0 ml. of sterile distilled water just before use.

## 3. Culture bottles.

Screw-top glass bottles, 20 ml. in capacity, were used. White rubber liners of the screw tops should be

used since black or red rubber liners may be toxic to the cells due to some chemicals which have been used in their processing (Paul, 1965).

Preparation of growth medium:

The growth medium was prepared just before use. To 100 ml. of the diluted 199 medium, 5 ml. of PHA solution were added. A bicarbonate solution was also added. Four ml. of the growth medium were pipetted into each culture bottle.

Method:

1. Heparinized blood was allowed to stand for 30 - 60 minutes at room temperature. If no adequate sedimentation was obtained after this time, the blood was centrifuged at 500 r.p.m. for 10 minutes.
2. The supernatant plasma containing leucocytes in suspension was removed by a sterile pasteur pipette.  
N.B. Within limits, the presence of red blood cells does not seem to interfere with the multiplication of the white blood cells in the culture (Mellman, 1965). Excessive red blood cells, on the other hand, may be detrimental to the culture because they metabolize essential nutrients. A grossly haemolyzed supernatant plasma destroys mitotic activity in the culture.
3. One ml. of the plasma leucocyte suspension was placed into each culture bottle containing 4 ml. of the growth medium.

4. The cultures were then incubated at 37°C with the room air as gas phase and the screw-top tightened.

5. The bottles were shaken gently twice daily.

The change in the colour of the phenol red indicator included in the culture medium may indicate the survival of the culture. If the cells are alive, the colour becomes yellow due to pyruvic and lactic acid production from glycolysis (Woodliff, 1964). If the colour of the indicator becomes deeper yellow, the bottle cap is to be loosened by a quarter of a turn for approximately one hour to allow the excess of CO<sub>2</sub> to escape.

6. The culture was terminated after 24 hours in half of the bottles. The other bottles were left till 72 hours after the start of culture.

Preparation of thalidomide to test its effect on leucocyte culture:

Thalidomide: "Distaval" (The Distillers Company-Biochemicals - Ltd. London):

The 170 mgm tablet containing 100 mg. of thalidomide was dissolved in 100 ml. of distilled water to avoid any toxic effect of the other solvents on the cells. The solution was prepared immediately before use since it is unstable in aqueous solution (Williams and co-workers, 1965).

Thalidomide is sparingly soluble in water. The solution was therefore centrifuged at 2000 r.p.m. for 15 minutes, and the clear supernatant was taken. This would contain only 6 mgm of thalidomide (Williams and co-workers, 1965).

The solution was sterilized through a Seitz filter. The solution was then added to the culture to make a concentration of  $3.6 \times 10^{-5}M$  per ml. of the culture.

Preparation of Putrescine to test its effect on leucocyte culture:

Putrescine dihydrochloride: Roche products Ltd., Welwyn Garden City, England:  $H_2N.(CH_2)_4NH_2.2HCl$ .

The amount of putrescine dihydrochloride needed for the experiment was dissolved in distilled water and then neutralised with 0.1 N NaOH to a pH of 7.2-7.4. The neutralised solution was sterilized through a Seitz filter. The solution was then added to the culture to make a concentration of  $10^{-3}M$ ;  $0.5 \times 10^{-2}M$  or  $10^{-2}M$ . per ml. of the culture. (molecular weight of putrescine dihydrochloride is 161).

Method of extraction and estimation of nucleic acids and proteins from leucocyte culture

1. After 24 or 72 hours, the incubation was interrupted and the bottles containing the cultures were centrifuged (2000 r.p.m./10 minutes). The supernatant was then pipetted off and discarded.
2. In order to wash the cells free from any contamination by the growth medium or the plasma, the sediment was washed with 5.0 ml. of saline, centrifuged (2000 r.p.m. for 10 minutes) and the saline pipetted off and discarded. The process was then repeated.

3. To the cell sediment at the bottom of each bottle, about 0.5 ml. of 1N perchloric acid were added and the cells were disrupted by a glass rod. At this stage, the cultures of 3 bottles were pooled to provide an adequate extract in which the nucleic acids can be estimated within the range of the standard curves. The pooled cultures were placed in a centrifuge tube in a final volume of about 2.5 ml. of 1N perchloric acid.

4. The pooled cultures in the centrifuge tube were heated in a water bath at 70°C for 20 minutes, then centrifuged at 2000 r.p.m. for 10 minutes, and the supernatant collected. The process was repeated once more on the residue using about 2.5 ml. of 1N perchloric acid. The supernatants were combined and the volume made up to 5 ml. This solution constituted the nucleic acid fraction and the nucleic acids were estimated by the methods already described. Only the indole method was used for the estimation of DNA, since the indole method is more sensitive than the diphenylamine method. The residue remaining after the extraction of the nucleic acids contained the proteins and was digested in 3 ml. of 1N NaOH at room temperature. The proteins were then estimated by the method already described.

RESULTS

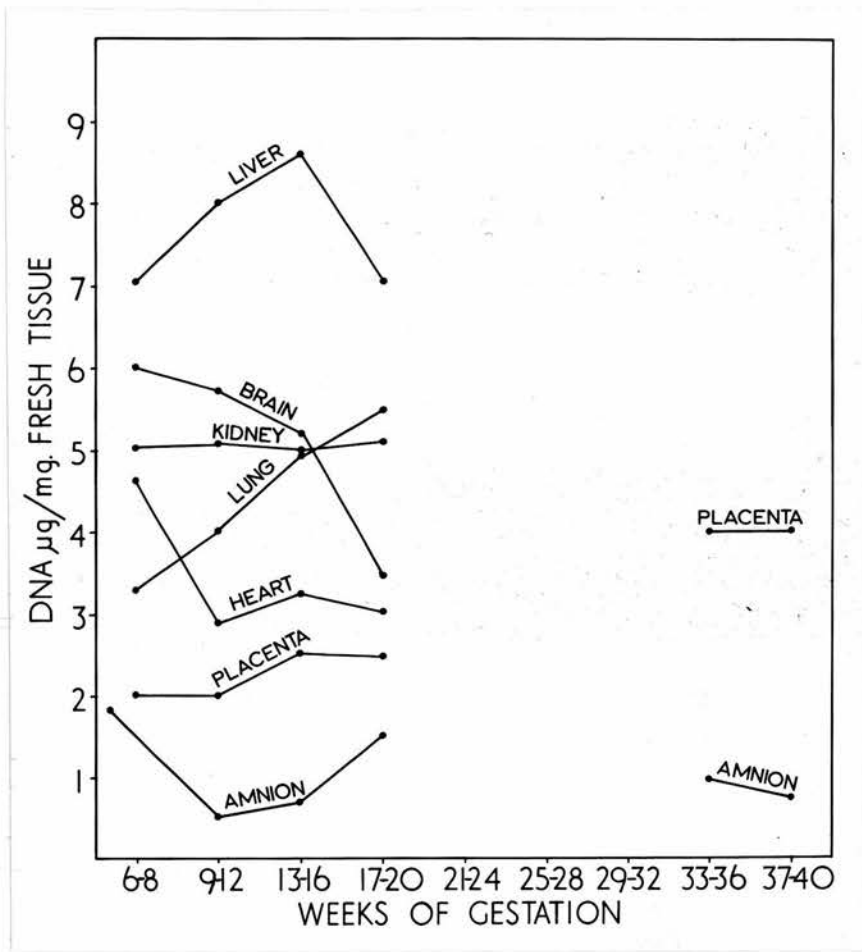


Fig. 7 : The DNA concentrations in human foetal organs and membranes at various periods of gestation.



The concentration of nucleic acids and proteins in the foetal organs and membranes at different periods of gestation

Liver: Table 2 and Fig. 8a.

Table 2.

The mean concentrations of nucleic acids and proteins in the human foetal liver (6 - 24 weeks gestation), expressed in  $\mu\text{g}/\text{mgm}$  of fresh tissue.

Weeks of gestation	Number of cases	RNA	DNA	Proteins	RNA/DNA	Proteins/DNA
6 - 8	4	7.32 $\pm 2.0$	7.08 $\pm 2.13$	56.55 $\pm 15$	1.03 $\pm 0.12$	7.99 $\pm 0.56$
9 - 12	5	9.24 $\pm 1.3$	8.01 $\pm 1.3$	70.38 $\pm 8.5$	1.15 $\pm 0.17$	8.79 $\pm 1.4$
13 - 16	7	9.73 $\pm 0.5$	8.57 $\pm 1.3$	81.81 $\pm 10.3$	1.14 $\pm 0.14$	9.55 $\pm 1.5$
17 - 20	4	7.81 $\pm 1.1$	7.13 $\pm 1.1$	72.50 $\pm 4.1$	1.10 $\pm 0.01$	10.17 $\pm 1.5$
16*	1	8.78	5.74	63.00	1.53	10.98
24*	1	7.85	8.10	88.00	0.97	10.86

\* Spontaneous abortion.

The following points may be noted:

1. The relatively high values of DNA and RNA.
2. The increase in DNA concentration until the 16th week and the decrease thereafter.
3. The correlation between the curves of DNA, RNA and Proteins.
4. The relatively high RNA/DNA ratio and protein/DNA ratio.

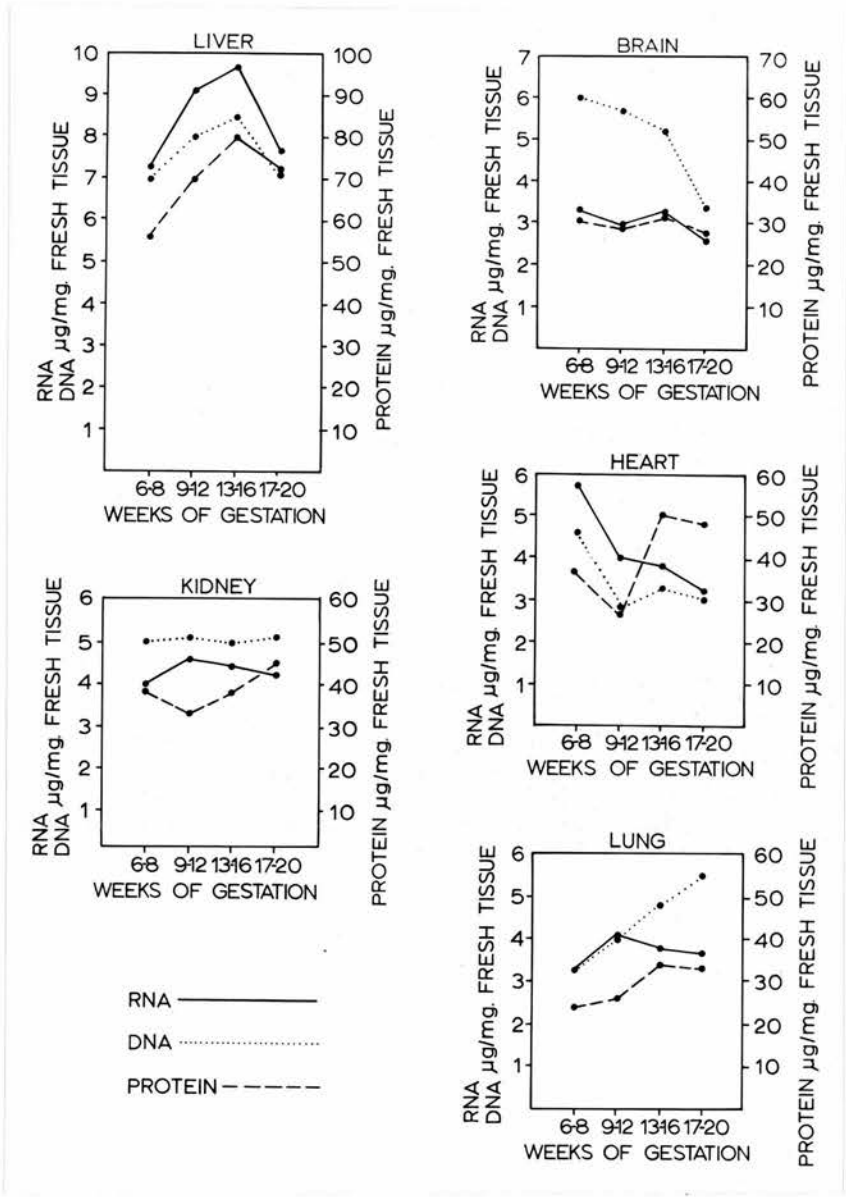


Fig. 8a : The DNA, RNA and protein concentrations in human foetal organs at various periods of gestation.

Brain: Table 3 and Fig. 8a.

Table 3.

The mean concentrations of nucleic acids and proteins in the human foetal brain (6 - 24 weeks gestation), expressed in  $\mu\text{g}/\text{mgm}$  of fresh tissue.

Weeks of gestation	Number of cases	RNA	DNA	Proteins	RNA/DNA	Proteins/DNA
6 - 8	4	3.41 $\pm 0.91$	6.0 $\pm 1.4$	31.63 $\pm 6.8$	0.57 $\pm 0.08$	5.27 $\pm 0.8$
9 - 12	6	2.97 $\pm 0.72$	5.78 $\pm 1.3$	29.79 $\pm 4.9$	0.51 $\pm 0.09$	5.15 $\pm 2.1$
13 - 16	7	3.35 $\pm 0.46$	5.19 $\pm 1.0$	33.49 $\pm 7.1$	0.65 $\pm 0.01$	6.45 $\pm 1.5$
17 - 20	4	2.58 $\pm 0.36$	3.42 $\pm 0.8$	28.69 $\pm 3.9$	0.75 $\pm 0.08$	8.39 $\pm 1.0$
16*	1	1.87	2.31	17.00	0.81	7.36
24*	1	1.56	0.98	21.25	1.59	21.68

\* Spontaneous abortion

The following points may be noted:

1. The relatively low values of RNA and RNA/DNA ratio.
2. The decreasing DNA concentration.
3. The increasing of RNA/DNA ratio and also the protein/DNA ratio.



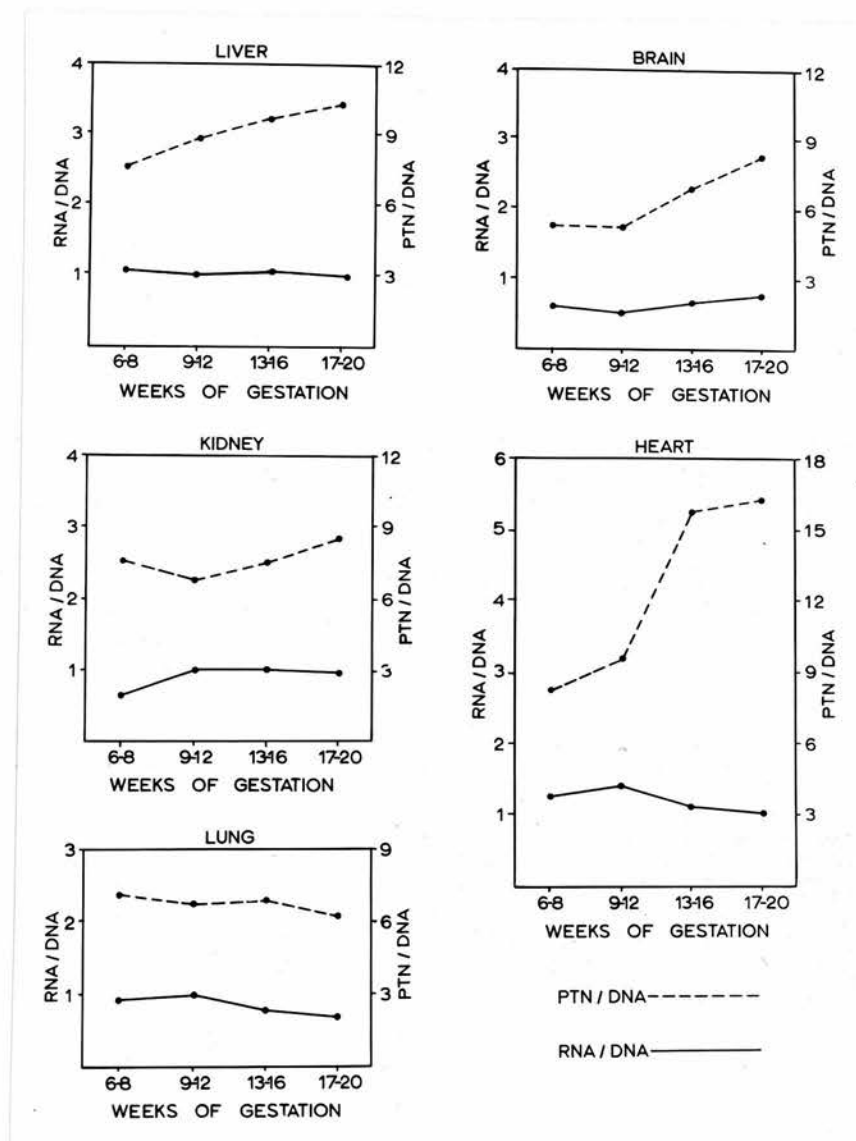


Fig. 9a : The RNA/DNA and the protein/DNA (PTN/DNA ) ratios in human foetal organs at various periods of gestation.

Kidney: Table 4 and Fig. 8a.

Table 4.

The mean concentrations of nucleic acids and proteins in the human foetal kidney (8 - 24 weeks gestation), expressed in  $\mu\text{g}/\text{mgm}$  of fresh tissue.

Weeks of gestation	Number of cases	RNA	DNA	Proteins	RNA/DNA	Proteins/DNA
8	2	4.01 $\pm 0.35$	5.07 $\pm 1.17$	38.6 $\pm 7.6$	0.79 $\pm 0.26$	7.6 $\pm 2.3$
10 - 12	3	4.73 $\pm 1.3$	5.09 $\pm 2.26$	33.77 $\pm 15.3$	0.95 $\pm 0.26$	6.63 $\pm 2.6$
13 - 16	7	4.54 $\pm 1.1$	5.01 $\pm 1.44$	38.73 $\pm 4.7$	0.91 $\pm 0.16$	7.73 $\pm 1.5$
17 - 20	4	4.19 $\pm 0.6$	5.11 $\pm 1.00$	45.13 $\pm 4.6$	0.82 $\pm 0.13$	8.83 $\pm 3.2$
16*	1	3.86	4.58	37.0	0.84	8.08
24*	1	4.33	5.08	53.0	0.85	10.43

\* Spontaneous abortion.

The following points may be noted:

1. The relatively stable DNA concentration.
2. The relatively stable RNA/DNA ratio.

Heart: Table 5 and Fig. 8a.

Table 5.

The mean concentrations of nucleic acids and proteins in the human foetal heart (8 - 24 weeks), expressed in  $\mu\text{g}/\text{mgm}$  of fresh tissue.

Weeks of gestation	Number of cases	RNA	DNA	Proteins	RNA/DNA	Proteins/DNA
8	3	5.77 $\pm 1.9$	4.65 $\pm 1.43$	36.07 $\pm 10$	1.24 $\pm 0.42$	7.8 $\pm 2$
9 - 12	3	4.09 $\pm 0.42$	2.91 $\pm 0.14$	27.88 $\pm 7.6$	1.41 $\pm 0.61$	9.58 $\pm 2.3$
13 - 16	4	3.83 $\pm 0.46$	3.31 $\pm 0.49$	51.15 $\pm 6.9$	1.16 $\pm 0.18$	15.45 $\pm 2.6$
17 - 20	4	3.28 $\pm 0.85$	3.09 $\pm 0.33$	49.25 $\pm 2.6$	1.06 $\pm 0.36$	15.94 $\pm 1.2$
24*	1	2.74	2.54	52.5	1.08	20.67

\* Spontaneous abortion.

The following points may be noted:

1. The decreasing concentration of DNA.
2. The high RNA/DNA ratio.
3. The increasing protein/DNA ratio.

Lung: Table 6 and Fig. 8a.

Table 6.

The mean concentrations of nucleic acids and proteins in the human foetal lung (8 - 24 weeks), expressed in  $\mu\text{g}/\text{mgm}$  of fresh tissue.

Weeks of gestation	Number of cases	RNA	DNA	Proteins	RNA/DNA	Proteins/DNA
8	3	3.32 $\pm 0.82$	3.34 $\pm 0.94$	23.58 $\pm 5.5$	0.99 $\pm 0.15$	7.06 $\pm 0.97$
9 - 12	3	4.13 $\pm 0.4$	4.02 $\pm 0.39$	26.33 $\pm 10$	1.03 $\pm 0.03$	6.55 $\pm 1.76$
13 - 16	2	3.83 $\pm 0.0$	4.93 $\pm 0.71$	34.5 $\pm 4.20$	0.78 $\pm 0.11$	7.00 $\pm 0.17$
17 - 20	2	3.70 $\pm 0.57$	5.50 $\pm 0.16$	33.63 $\pm 3.3$	0.67 $\pm 0.1$	6.11 $\pm 0.42$
16*	1	4.78	6.31	36.0	0.76	5.71
24*	1	3.90	4.94	47.25	0.79	9.56

\* Spontaneous abortion.

The following may be noted:

1. The increasing DNA concentration.
2. The low RNA/DNA ratio.

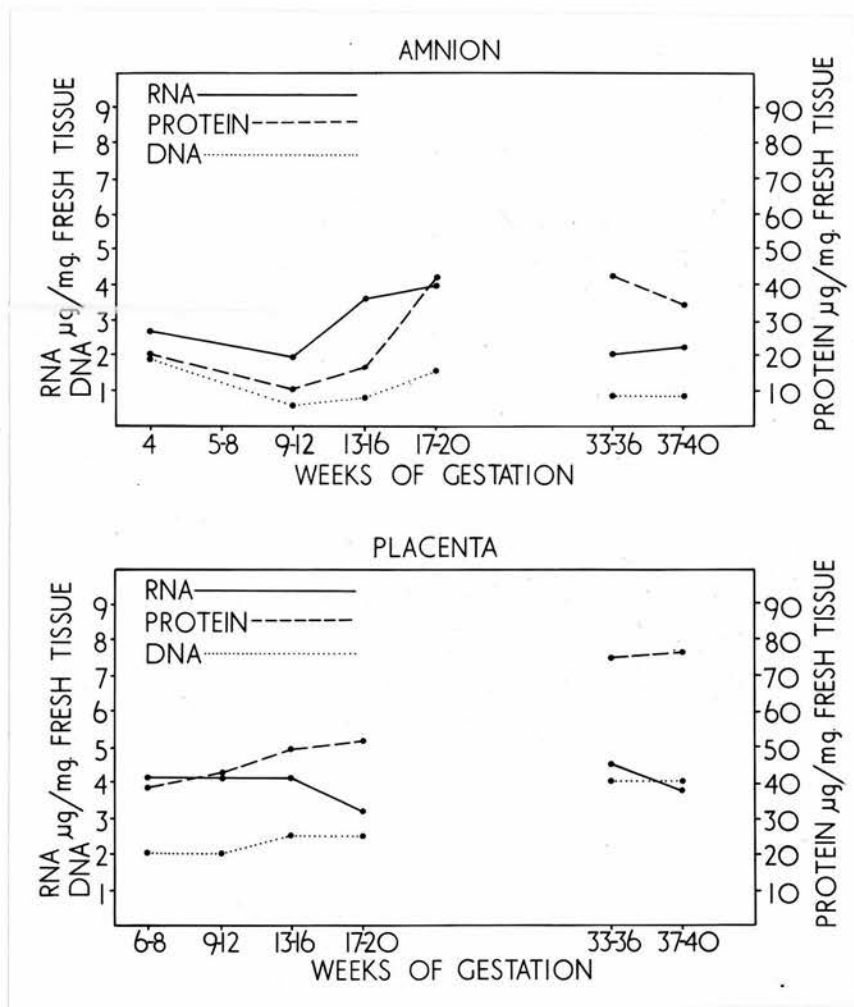


Fig. 8b : The DNA, RNA and protein concentrations in human amniotic membrane and placenta at various periods of gestation.



Placenta: Table 7 and Fig. 8b.

Table 7.

The mean concentrations of nucleic acids and proteins in the human placenta (6 - 40 weeks), expressed in  $\mu\text{g}/\text{mgm}$  of fresh tissue.

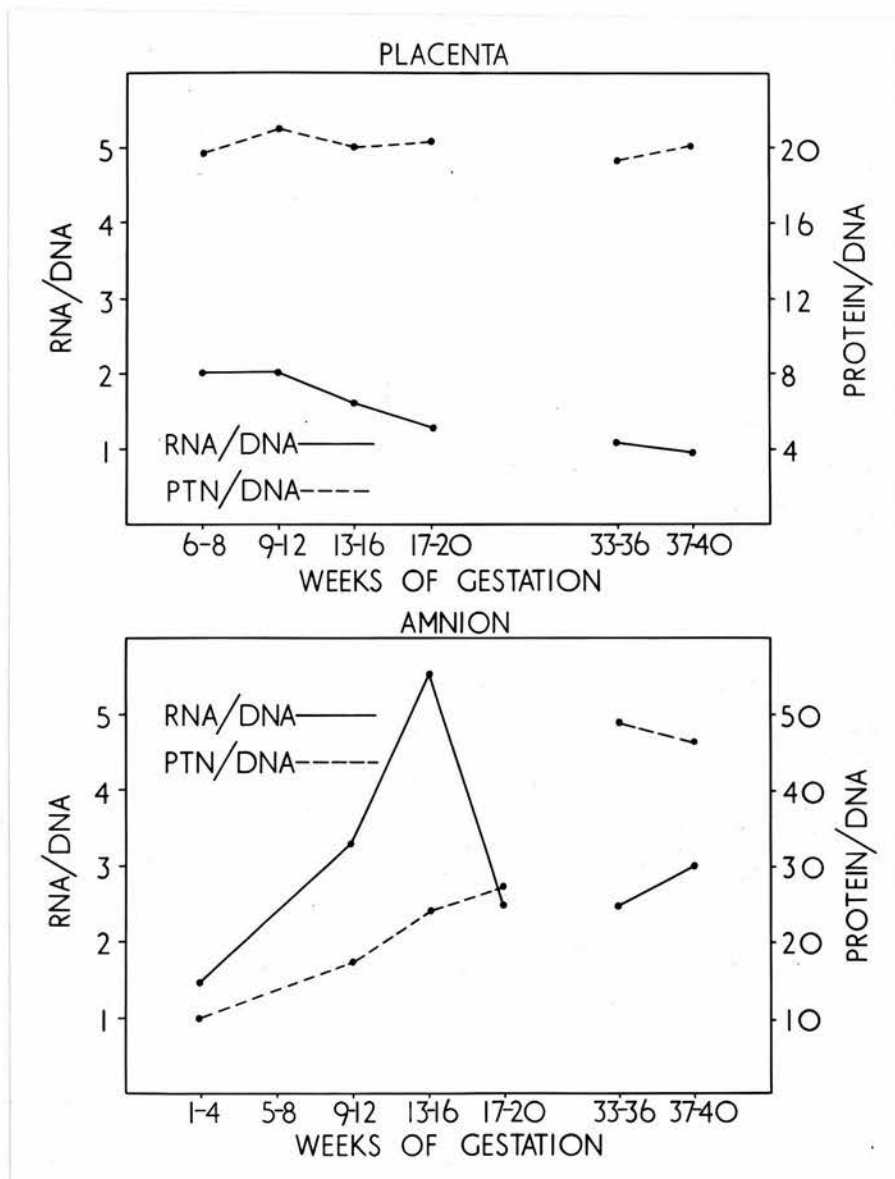
Weeks of gestation	Number of cases	RNA	DNA	Proteins	RNA/DNA	Proteins/DNA
6 - 8	3	4.10 $\pm 0.56$	1.99 $\pm 0.57$	38.71 $\pm 13$	2.06 $\pm 0.26$	19.45 $\pm 3.9$
9 - 12	3	4.17 $\pm 0.05$	1.99 $\pm 0.48$	41.31 $\pm 11$	2.09 $\pm 0.52$	20.76 $\pm 4.9$
13 - 16	5	4.12 $\pm 0.52$	2.49 $\pm 0.75$	49.10 $\pm 8.1$	1.65 $\pm 0.5$	19.72 $\pm 4.9$
17 - 20	4	3.25 $\pm 0.67$	2.45 $\pm 0.2$	51.25 $\pm 6.4$	1.33 $\pm 0.2$	20.92 $\pm 2.6$
33 - 36	1	4.54	3.96	74.75	1.15	18.88
37 - 40	6	3.76 $\pm 0.56$	3.96 $\pm 0.71$	75.70 $\pm 15$	0.95 $\pm 0.16$	19.12 $\pm 1.73$
24*	1	4.16	3.13	45.5	1.33	14.54
18*	1	1.87	0.84	44.69	2.22	55.5
16*	1	1.26	0.57	17.32	2.21	30.4

\* Spontaneous abortion.

\* Vesicular mole.

The following may be noted:

1. The increasing DNA concentration.
2. The high protein/DNA ratio.
3. The relatively high RNA/DNA ratio at early pregnancy. This ratio decreased with progress of pregnancy, mainly due to the increase in the DNA concentration.



**Fig. 9b :** The RNA/DNA and protein/DNA ratios in human placenta and amniotic membrane at various periods of gestation.

Amnion: Table 8 and Fig. 8b.

Table 8.

The mean concentrations of nucleic acids and proteins in the human amnion (4 - 40 weeks), expressed in  $\mu\text{g}/\text{mgm}$  of fresh tissue.

Weeks of gestation	Number of cases	RNA	DNA	Proteins	RNA/DNA	Proteins/DNA
4	1	2.71	1.85	19.25	1.46	10.41
9 - 12	2	1.91 $\pm 0.33$	0.59 $\pm 0.2$	10.17 $\pm 4$	3.24 $\pm 0.4$	17.24 $\pm 0.8$
13 - 16	1	3.61	0.65	15.20	5.55	23.38
17 - 20	1	3.87	1.51	42.25	2.56	27.98
33 - 36	1	2.14	0.87	42.80	2.46	49.20
37 - 40	4	2.22 $\pm 0.7$	0.74 $\pm 0.3$	34.15 $\pm 7.9$	3.0 $\pm 1.2$	46.15 $\pm 4.3$
24*	1	3.22	1.26	40.38	2.56	32.05

\* Spontaneous abortion.

The following points may be noted:

1. The low concentration of DNA.
2. The high RNA/DNA ratio and the high and increasing protein/DNA ratio.

An electrophoretic study of proteins of the maternal serum,  
foetal serum, amniotic fluid and placenta

The protein content of the maternal serum, from 81 cases, at different stages of pregnancy is shown in table 9.

Fractionation of the maternal serum proteins in 24 cases from the 19th week to term is presented in tables 10a and b.

Table 9.

Protein content of the maternal serum throughout pregnancy (as estimated by the method of Miller, 1959).

Weeks of gestation	Number of cases	Total protein in gm. per cent		
		range	mean	standard deviation
18 - 28	26	5.10 - 7.88	6.59	± 0.67
30 - 38	34	5.40 - 7.65	6.86	± 0.74
Full term delivery	21	4.58 - 7.20	5.19	± 0.77

The table shows that there is a trend for the protein content of the maternal serum to be, on average, lower at term. The difference between the mean at term and the means during pregnancy is statistically significant.

Table 10a

Fractionation of maternal serum proteins throughout the 2nd half of pregnancy by electrophoresis.

Percentage of the protein components (as obtained by scanning)

Weeks of gestation	No. of cases		Albumin	Alpha-1 globulin	Alpha-2 globulin	Beta globulin	Gamma globulin	A/G
19-28	8	range	43.44 - 59.22	3.91 - 9.47	8.47 - 17.74	10.79 - 17.88	10.79 - 20.73	0.77 - 1.45
		mean	51.05	5.77	12.27	15.38	15.53	1.04
		S.D.	± 6.1	± 1.95	± 2.42	± 2.1	± 3.57	± 0.26
30-38	9	range	43.45 - 57.69	3.89 - 8.00	8.77 - 14.95	12.36 - 17.55	11.55 - 20.31	0.77 - 1.36
		mean	50.69	6.03	11.56	15.78	15.94	1.03
		S.D.	± 4.99	± 1.52	± 1.84	± 1.33	± 3.04	± 0.20
Full term delivery	7	range	44.76 - 48.10	5.34 - 10.63	12.56 - 16.56	13.29 - 19.10	13.15 - 18.5	0.81 - 0.93
		mean	45.80	7.77	14.79	15.81	15.83	0.85
		S.D.	± 1.21	± 1.85	± 1.40	± 1.96	± 2.05	± 0.03

Table 10b

Fractionation of maternal serum proteins throughout the 2nd half of pregnancy by electrophoresis.

Absolute values for serum protein fractions in gm. per cent in relation to the total protein as estimated by the method of Miller (1959).

Weeks of gestation	No. of cases		Total protein	Albumin	Alpha-1 globulin	Alpha-2 globulin	Beta-globulin	Gamma globulin
19-28	8	range	6.3 - 7.88	3.0 - 4.66	0.27 - 0.65	0.59 - 1.22	0.84 - 1.33	0.85 - 1.47
		mean	7.10	3.63	0.41	0.87	1.09	1.10
		S.D.	± 0.53	± 0.64	± 0.12	± 0.20	± 0.15	± 0.20
30-37	9	range	6.3 - 7.50	2.74 - 4.24	0.28 - 0.55	0.66 - 0.98	0.84 - 1.32	0.66 - 1.35
		mean	6.85	3.48	0.41	0.79	1.08	1.09
		S.D.	± 0.61	± 0.64	± 0.11	± 0.34	± 0.17	± 0.21
Full term delivery	7	range	5.80 - 7.56	2.60 - 3.64	0.37 - 0.66	0.78 - 1.17	0.87 - 1.40	0.80 - 1.24
		mean	6.58	3.01	0.51	0.98	1.04	1.04
		S.D.	± 0.63	± 0.39	± 0.15	± 0.14	± 0.19	± 0.13

The decrease in the mean level of the albumin fraction at full term delivery is statistically significant.

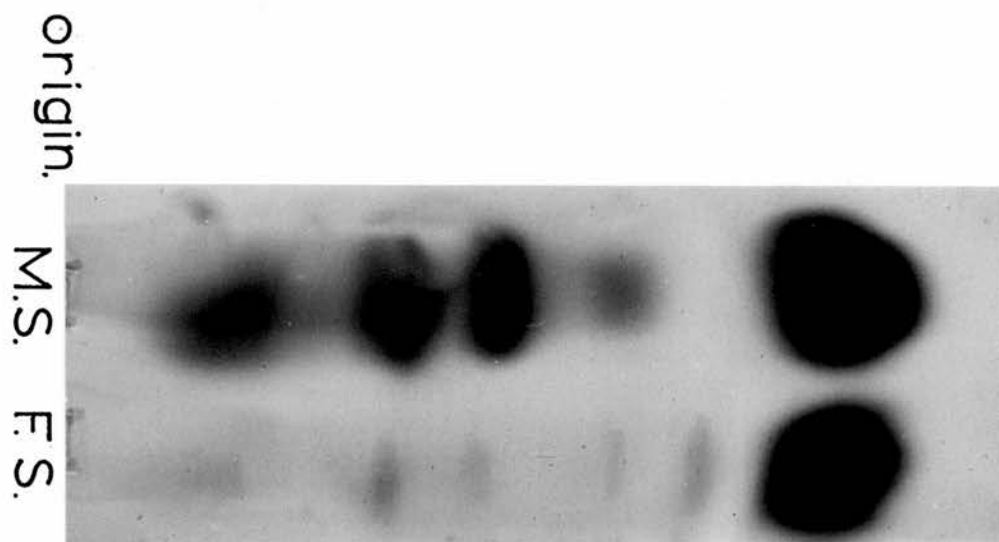


Fig. 10a : Separation pattern of maternal serum and the corresponding foetal serum at 19 weeks, by cellulose acetate electrophoresis. Veronal acetate buffer. pH 8.6.  $\mu$  0.1. A current of 0.4 mA/cm width of the strip for 6 hours. Ponceau S staining.

M.S. : Maternal serum.

F.S. : Foetal serum.

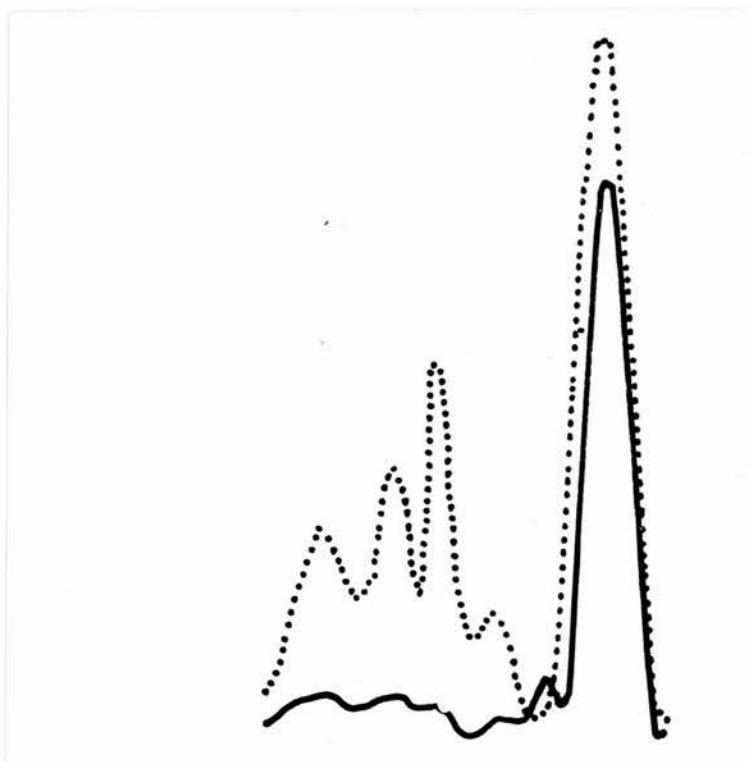


Fig. 10b : Electrophoretic pattern obtained by scanning of the above strip.

..... Maternal serum.

\_\_\_\_\_ Foetal serum.

The protein fractions in the foetal serum in 7 cases: one at 19 weeks and six at term is presented in tables 11a and b.

Table 11a.

Protein fractions by electrophoresis in the foetal serum at 19 weeks (one case) and at term (six cases).

Percentage of the protein fractions as obtained by scanning.

		Albumin	Sub- stance "X"	Alpha-1 globulin	Alpha-2 globulin	Beta globulin	Gamma globulin	A/G
19 weeks		66.05	5.51	5.51	6.42	6.42	10.09	1.95
Term	range	51.34 -61.37	-	2.6 -6.59	7.96 -10.92	7.57 -12.50	17.36 -23.80	1.06 -1.59
	mean	56.16	-	4.59	9.58	9.24	20.43	1.28
	S.D.	3.67	-	± 1.42	± 1.10	± 2.18	± 2.61	± 0.19

Table 11b.

Protein fractions by electrophoresis in the foetal serum at 19 weeks (one case) and at term (six cases).

Absolute values for serum protein fractions in gm. per cent in relation to the total protein as estimated by the method of Miller (1959).

		Total proteins	Albumin	Sub- stance "X"	Alpha-1 globulin	Alpha-2 globulin	Beta globulin	Gamma globulin
19 weeks		3.25	2.14	0.18	0.18	0.21	0.21	0.33
Term	range	5.79 -6.49	3.06 -3.83	-	0.16 -0.39	0.50 -0.71	0.49 -0.72	1.02 -1.50
	mean	6.16	3.46	-	0.28	0.59	0.57	1.26
	S.D.	± 0.32	± 0.30	-	± 0.09	± 0.14	± 0.14	± 0.36

In the 19 weeks foetus, the total proteins are lower, the percentage of albumin is higher, an unidentified substance "X" is



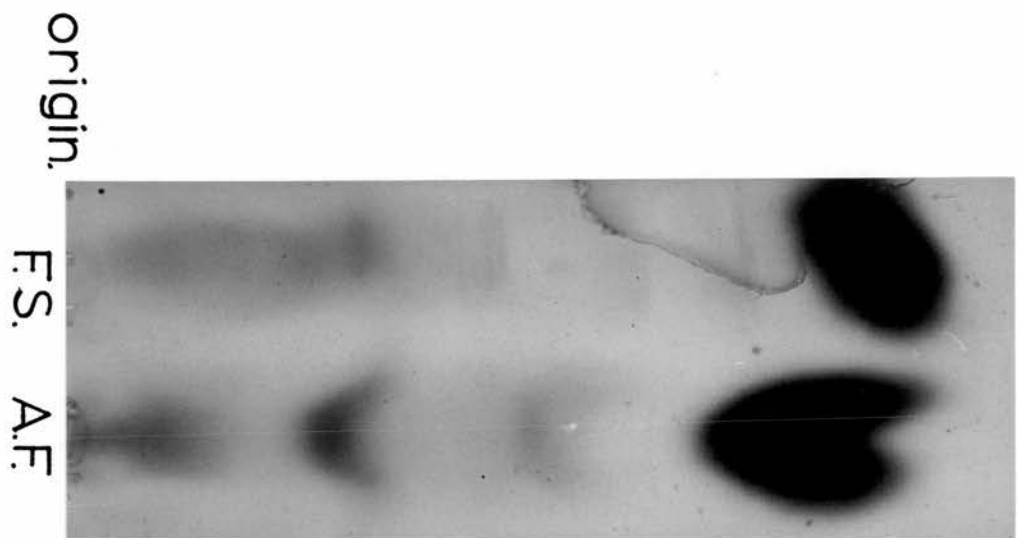


Fig. 11 : Separation pattern of foetal serum and the corresponding amniotic fluid at 19 weeks, by cellulose acetate electrophoresis.

F.S. : Foetal serum.

A.F. : Amniotic fluid.

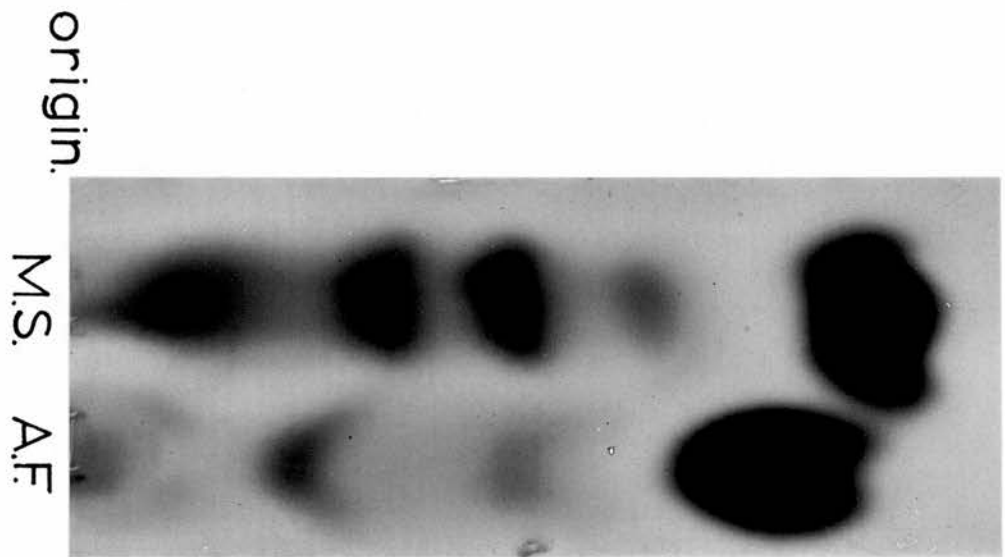


Fig. 12a : Separation pattern of maternal serum and the corresponding amniotic fluid protein at 19 weeks, obtained by cellulose acetate electrophoresis. Method as described in the text.  
M.S. : Maternal serum                      A.F. : Amniotic fluid

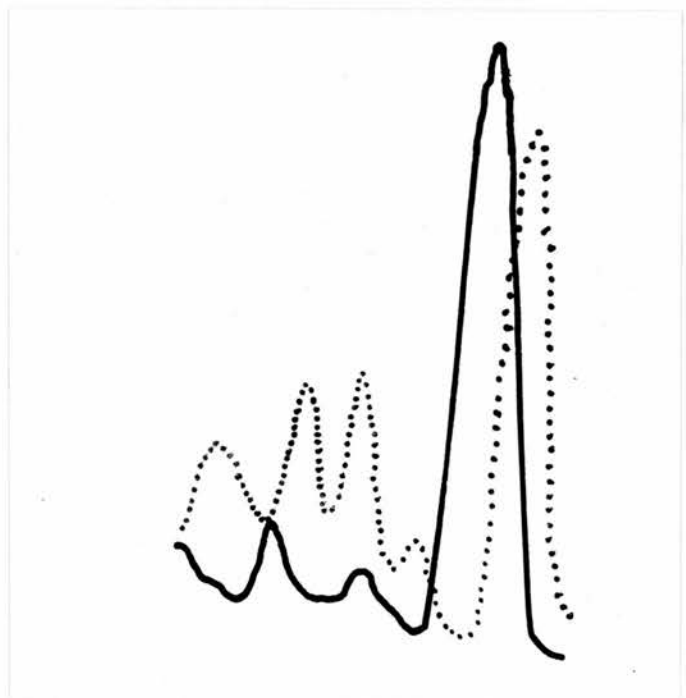


Fig. 12b : Electrophoretic protein pattern obtained by scanning of the above strip.

..... Maternal serum.  
\_\_\_\_\_ Amniotic fluid.

present, the alpha 1-globulin fraction is not much different, the alpha 2 and the Beta-globulin fractions are somewhat lower, and the gamma globulin is much less, almost one half.

A comparison of the protein fractions in the maternal serum, foetal serum and amniotic fluid in a case at 19 weeks gestation is shown in tables 12a and b and Figs. 10a and b, and 11)

Table 12a.

Comparison between the protein fractions by electrophoresis in the maternal serum, foetal serum and amniotic fluid in a case at 19 weeks gestation.

Percentage of the protein fractions as obtained by scanning.

	Albumin	Sub- stance "X"	Alpha-1 globulin	Alpha-2 globulin	Beta globulin	Gamma globulin	A/G
Foetal serum	66.05	5.51	5.51	6.42	6.42	10.09	1.95
Maternal serum	43.44	-	9.47	17.74	15.78	13.57	0.77
Amniotic fluid	60	-		12.5	15	12.50	1.5

Table 12b

Comparison between the protein fractions by electrophoresis in the maternal serum, foetal serum and amniotic fluid in a case at 19 weeks gestation.

Absolute values for serum protein fractions in gm. per cent in relation to the total protein as estimated by the method of Miller(1959)

	Total protein	Albumin	Sub- stance "X"	Alpha-1 globulin	Alpha-2 globulin	Beta globulin	Gamma globulin
Foetal serum	3.25	2.14	0.18	0.18	0.21	0.21	0.33
Maternal serum	6.90	3.00	-	0.65	1.22	1.09	0.94
Amniotic fluid	0.32	0.19	-		0.04	0.05	0.04

In the 19 weeks foetus, substance "X" is present in its serum. This substance is absent in the maternal serum and the amniotic fluid.

Fractionation of the maternal and their corresponding foetal serum proteins in 6 cases at term is shown in tables 13a and b.

Table 13a.

Fractionation by electrophoresis of the maternal and the corresponding foetal serum proteins in 6 cases at term.

Percentage of protein components as obtained by scanning.

		Albumin	Alpha-1 globulin	Alpha-2 globulin	Beta globulin	Gamma globulin	A/G
Maternal serum	range	44.76 -48.6	5.34 -10.04	14.03 -16.57	13.29 -19.10	13.15 -18.50	0.81 -0.93
	mean	45.85	7.29	15.17	16.11	15.58	0.85
	S.D.	± 1.2	± 1.83	± 1.42	± 2.00	± 2.00	± 0.03
Foetal serum	range	51.34 -61.37	2.60 -6.59	7.96 -10.92	7.57 -12.50	17.36 -23.80	1.06 -1.59
	mean	56.16	4.59	9.58	9.24	20.43	1.28
	S.D.	± 3.67	± 1.42	± 1.10	± 2.18	± 2.61	± 0.19
"t" test	p	<0.01	<0.02	<0.01	<0.01	<0.01	<0.01

In the foetal serum, the albumin percentage is higher, and the globulin percentages are lower with the notable exception of gamma globulin. The A/G ratio is also higher.

Table 13b

Fractionation by electrophoresis of the maternal and the corresponding foetal serum proteins in 6 cases at term.

Absolute values for serum protein fractions in gm. per cent in relation to the total protein as estimated by the method of Miller (1959).

		Total protein	Albumin	Alpha-1 globulin	Alpha-2 globulin	Beta globulin	Gamma globulin
Maternal serum	range	6.06 -7.56	2.60 -3.64	0.37 -0.61	0.81 -1.17	0.89 -1.40	0.80 -1.24
	mean	6.64	3.05	0.48	1.00	1.07	1.04
	S.D.	± 0.64	± 0.39	± 0.15	± 0.14	± 0.19	± 0.13
Foetal serum	range	5.79 -6.49	3.06 -3.83	0.16 -0.39	0.50 -0.71	0.49 -0.72	1.02 -1.50
	mean	6.16	3.46	0.28	0.59	0.57	1.26
	S.D.	± 0.32	± 0.30	± 0.09	± 0.14	± 0.14	± 0.36
"t" test	p	=0.1	=0.05	<0.01	<0.01	<0.01	=0.2

The total protein content of the amniotic fluid in 159 samples, from the 12th week of gestation to the 42nd week is presented in table 14 and in Fig. 13.

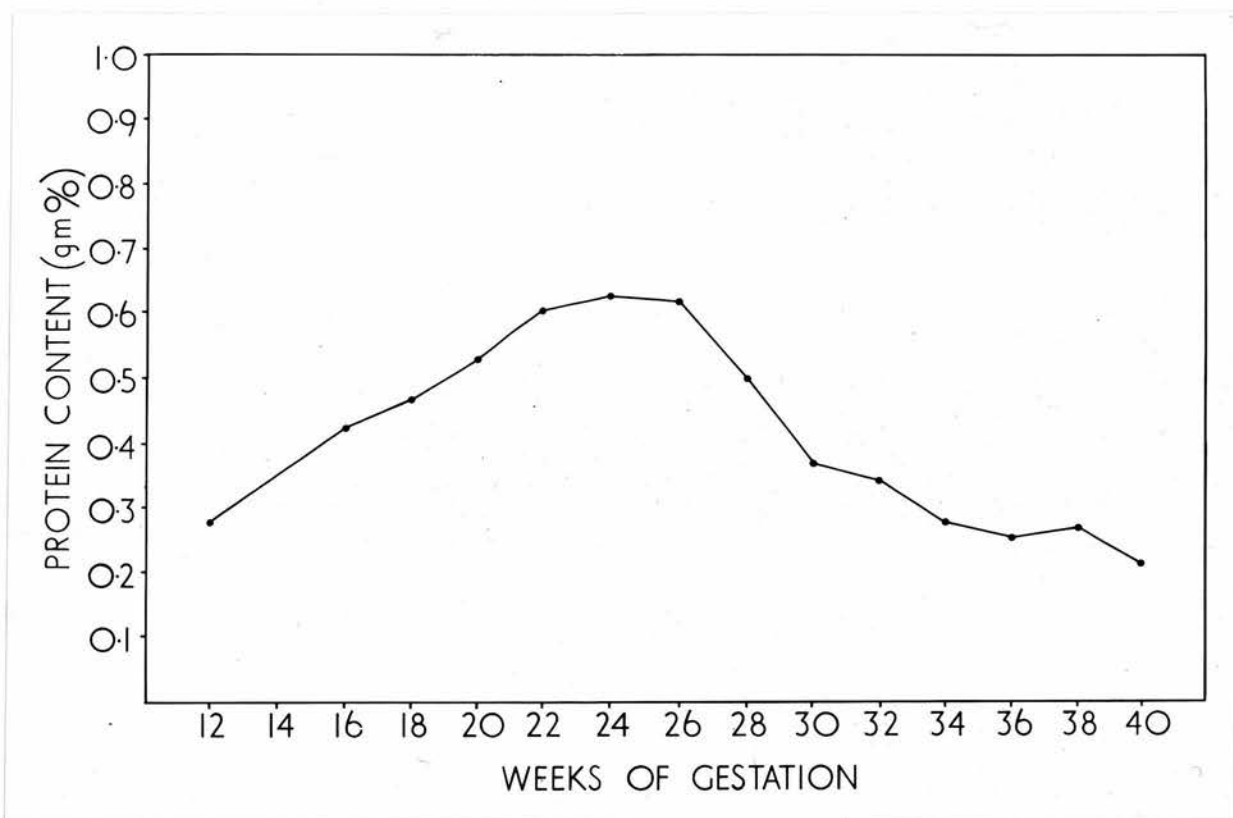


Fig. 13 : The mean protein concentrations in 159 samples of amniotic fluid at various periods of gestation.

Table 14.

Protein content of the amniotic fluid throughout pregnancy, in gm. per cent, by the method of Miller (1959).

Gestation in weeks	Number of cases	range	mean	S.D.
12	3	0.10 - 0.38	0.28	± 0.18
16	2	0.34 - 0.51	0.43	± 0.12
18	6	0.32 - 0.62	0.47	± 0.12
20	3	0.44 - 0.65	0.53	± 0.10
22	10	0.41 - 0.78	0.60	± 0.12
24	8	0.46 - 0.74	0.63	± 0.09
26	10	0.36 - 0.93	0.62	± 0.16
28	17	0.34 - 0.73	0.50	± 0.13
30	12	0.24 - 0.64	0.37	± 0.06
32	21	0.22 - 0.46	0.34	± 0.07
34	20	0.15 - 0.39	0.27	± 0.09
36	19	0.14 - 0.36	0.25	± 0.06
38	4	0.20 - 0.32	0.26	± 0.05
40 +	24	0.11 - 0.33	0.21	± 0.05

The total protein content of the amniotic fluid is relatively low at the 12th week, increases to a maximum between the 22nd and the 26th week, and then declines till term.

Fractionation by electrophoresis of the amniotic fluid proteins in 36 cases, from the 17th week to term is presented in Tables 15a and b.

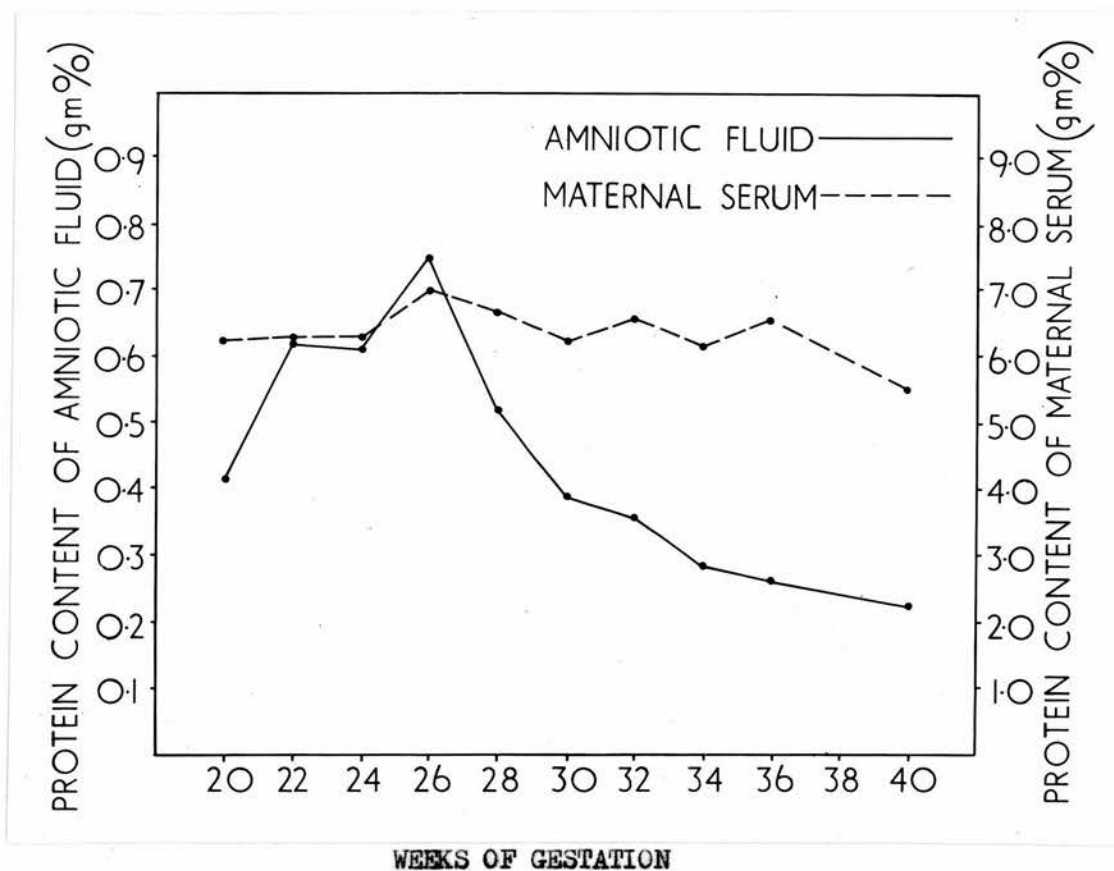


Fig. 14 : The mean protein concentrations in 62 maternal sera and the corresponding amniotic fluids, during the second half of pregnancy.



Table 15a.

Fractionation by electrophoresis of the amniotic fluid proteins throughout pregnancy.

Percentage of the protein fractions as obtained by scanning.

Weeks of gestation	No. of cases		Albumin	Alpha globulin	Beta globulin	Gamma globulin	A/G
17-28	13	range	56.30 -77.63	4.93 -17.04	9.09 -16.78	5.59 -13.31	1.30 -3.47
		mean	66.63	11.97	12.23	9.17	1.996
		S.D.	± 6.23	± 3.41	± 2.59	± 2.78	± 0.76
29-38	16	range	60.70 -76.16	5.30 -14.29	9.27 -16.17	3.70 -11.30	1.55 -3.20
		mean	67.49	10.89	14.02	7.60	2.08
		S.D.	± 4.01	± 2.51	± 2.03	± 2.10	± 0.43
Full term delivery	7	range	59.63 -76.65	5.99 -20.61	8.98 -21.71	4.89 -8.38	1.48 -3.30
		mean	65.48	13.57	13.94	7.01	1.90
		S.D.	± 5.66	± 4.71	± 4.20	± 1.19	± 0.64

No definite significant change can be observed over the period of examination.

Table 15b.

Fractionation by electrophoresis of the amniotic fluid throughout various periods of pregnancy.

Absolute values in gm. per cent in relation to the total proteins as estimated by the method of Miller (1959).

Gest- ation in weeks	No. of cases		Total protein	Albumin	Alpha globulin	Beta globulin	Gamma globulin
17-28	13	range	0.32 -0.93	0.19 -0.58	0.02 -0.14	0.04 -0.09	0.03 -0.12
		mean	0.55	0.36	0.07	0.07	0.05
		S.D.	± 0.19	± 0.13	± 0.03	± 0.02	± 0.02
29-38	16	range	0.2 -0.72	0.14 -0.51	0.01 -0.05	0.03 -0.08	0.01 -0.07
		mean	0.38	0.26	0.04	0.05	0.03
		S.D.	± 0.14	± 0.08	± 0.02	± 0.02	± 0.02
Full term del- ivery	7	range	0.19 -0.28	0.12 -0.21	0.02 -0.05	0.02 -0.05	0.01 -0.02
		mean	0.23	0.15	0.03	0.03	0.02
		S.D.	± 0.03	± 0.03	± 0.01	± 0.01	± 0.0004

All the absolute values in table 15b show a decrease with the progress of pregnancy.

Comparison between the protein fractions by electrophoresis in the maternal serum, foetal serum and amniotic fluid in 6 normal patients delivered at term is shown in table 16.

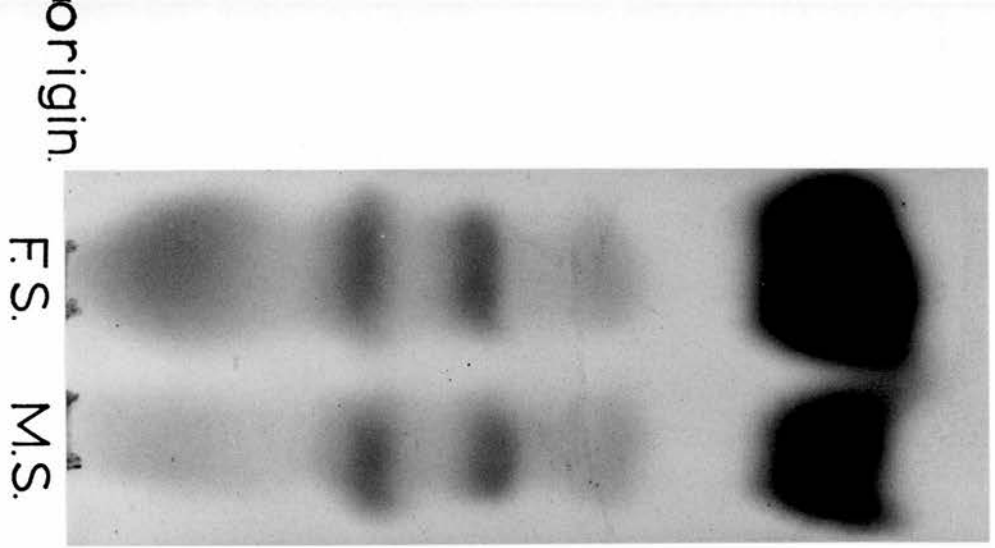


Fig. 15a : Separation pattern of maternal serum and the corresponding foetal serum at term, by cellulose acetate electrophoresis.

F.S. : Foetal serum.

M.S. : Maternal serum.

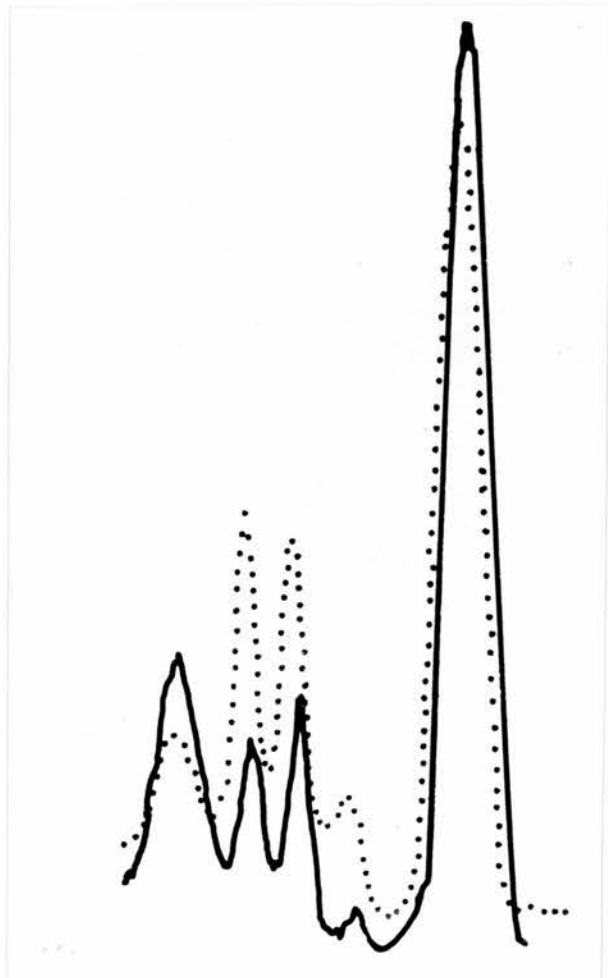


Fig. 15b : Electrophoretic pattern obtained by scanning of the above strip.

..... Maternal serum.

\_\_\_\_\_ Foetal serum.

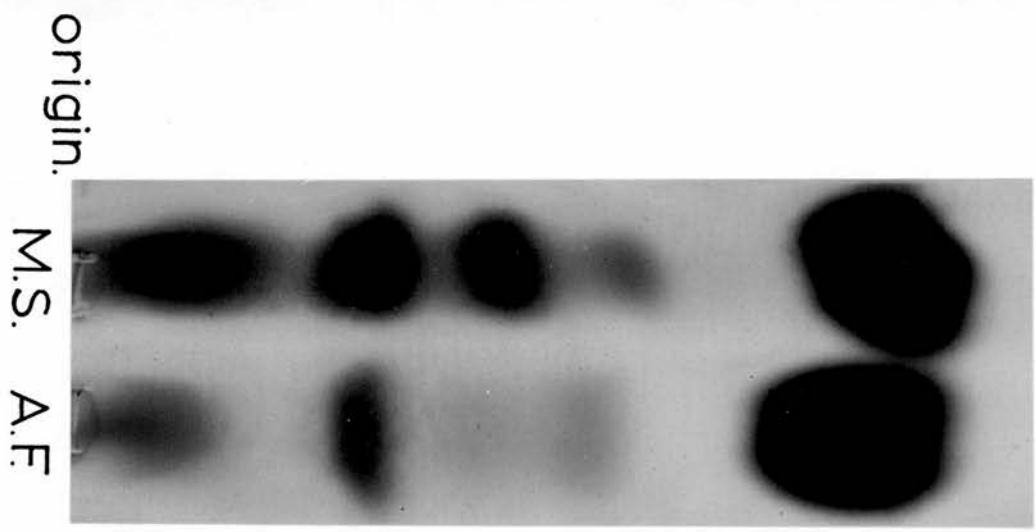


Fig. 16a : Separation pattern of maternal serum and the corresponding amniotic fluid at term, by cellulose acetate electrophoresis.

M.S. : Maternal serum.

A.F. : Amniotic fluid.

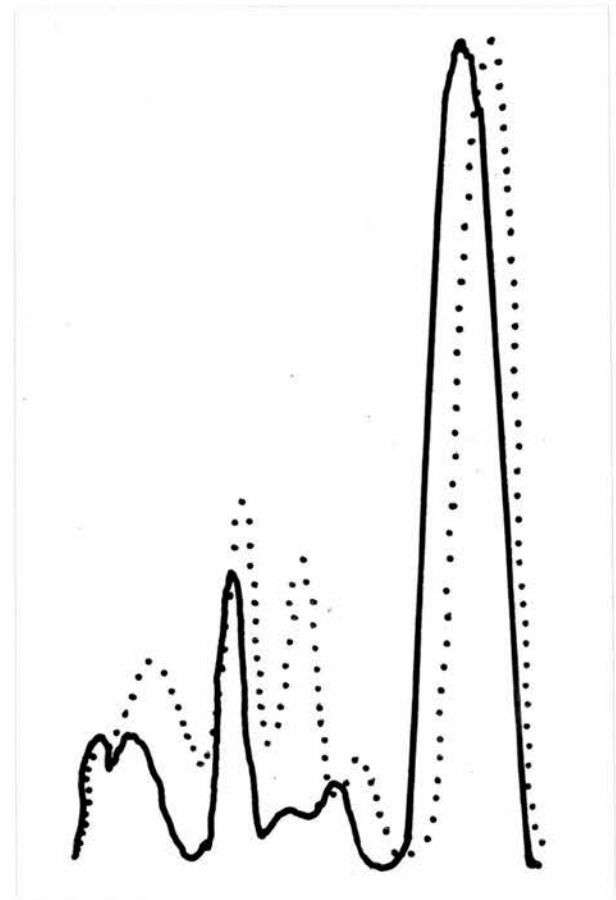


Fig.16b: Electrophoretic pattern obtained by scanning of the above strip.

..... Maternal serum.

\_\_\_\_\_ Amniotic fluid.

origin.  
A.F.  
F.S.

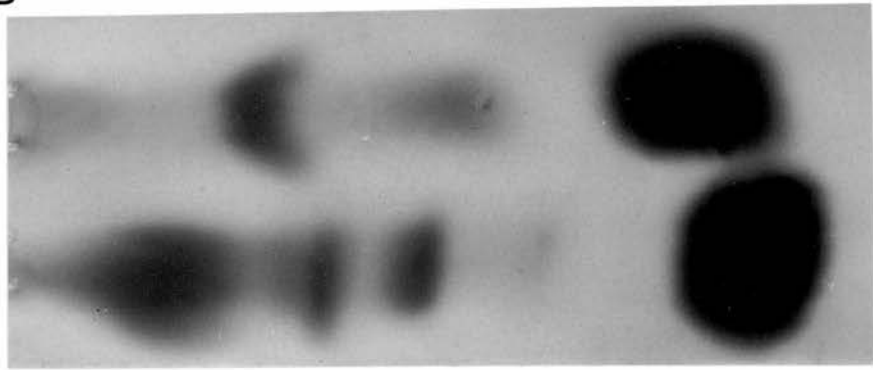


Fig. 17a : Separation pattern of foetal serum and the corresponding amniotic fluid at term, by cellulose acetate electrophoresis.

A.F. : Amniotic fluid.

F.S. : Foetal serum.

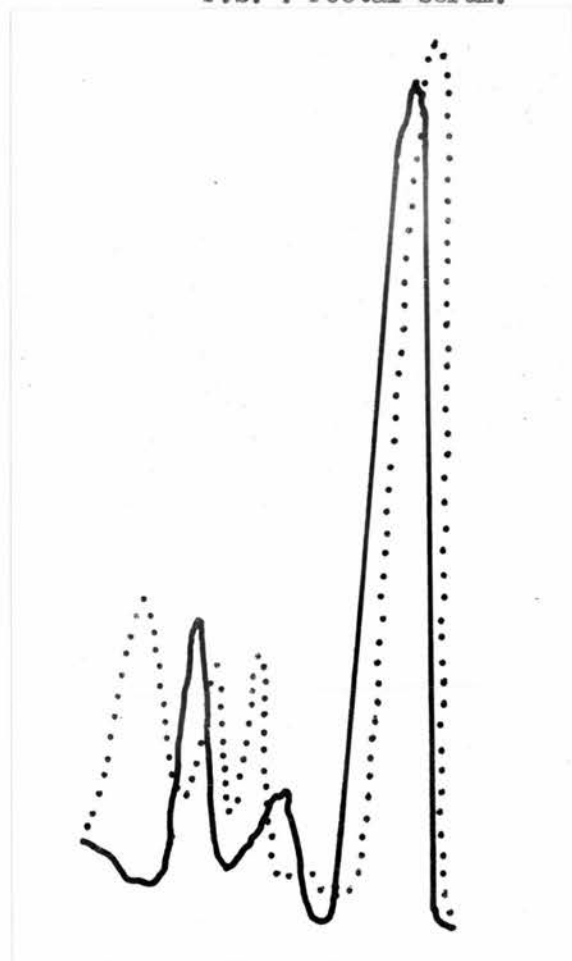


Fig. 17b : Electrophoretic pattern obtained by scanning of the above strip.

..... Foetal serum.

\_\_\_\_\_ Amniotic fluid.

Table 16.

Comparison between the protein fractions by electrophoresis in the maternal serum, foetal serum and amniotic fluid in 6 normal patients delivered at term.

Percentage of the protein fractions as obtained by scanning.

		Albumin	Alpha-1 globulin	Alpha-2 globulin	Beta globulin	Gamma globulin	A/G
Maternal serum	range	44.76 - 48.1	5.34 - 10.04	14.03 - 16.57	13.29 - 19.10	13.16 - 18.50	0.81 - 0.93
	mean	45.85	7.29	15.17	16.11	15.58	0.85
	S.D.	± 1.2	± 1.83	± 1.42	± 2.00	± 2.00	± 0.03
Foetal serum	range	51.34 - 61.37	2.60 - 6.59	7.96 - 10.92	7.57 - 12.50	17.36 - 23.80	1.06 - 1.59
	mean	56.16	4.59	9.58	9.24	20.43	1.28
	S.D.	± 3.67	± 1.42	± 1.10	± 2.18	± 2.61	± 0.19
Amnio- tic fluid	range	59.63 - 69.55	10.94 - 20.61		11.82 - 21.71	4.89 - 7.77	1.48 - 2.24
	mean	63.62	14.83		14.77	6.78	1.75
	S.D.	± 3.71	± 3.63		± 3.92	± 1.13	± 0.28

In the amniotic fluid, the percentage of albumin is highest (consequently also the A/G ratio) and the gamma globulin is lowest. Separation of alpha 1 and 2 globulins is not always complete in the amniotic fluid.

Fractionation by electrophoresis of the placental proteins at 19 weeks of pregnancy (one case) and at term (3 cases) is shown

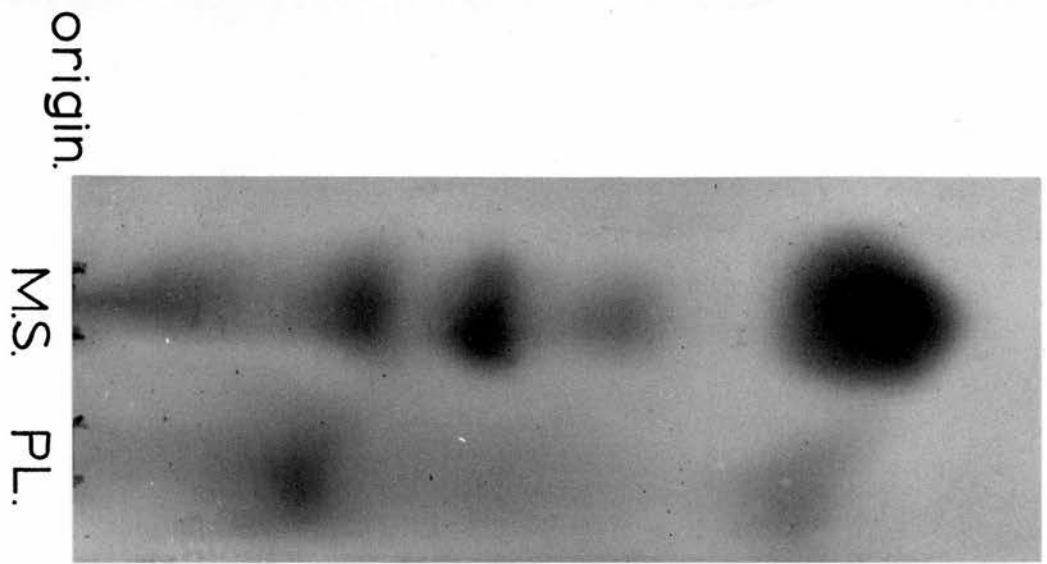


Fig. 18a : Separation pattern of maternal serum and the corresponding placental extract at 19 weeks, by cellulose acetate electrophoresis.  
 M.S. : Maternal serum                      Pl. : Placental extract

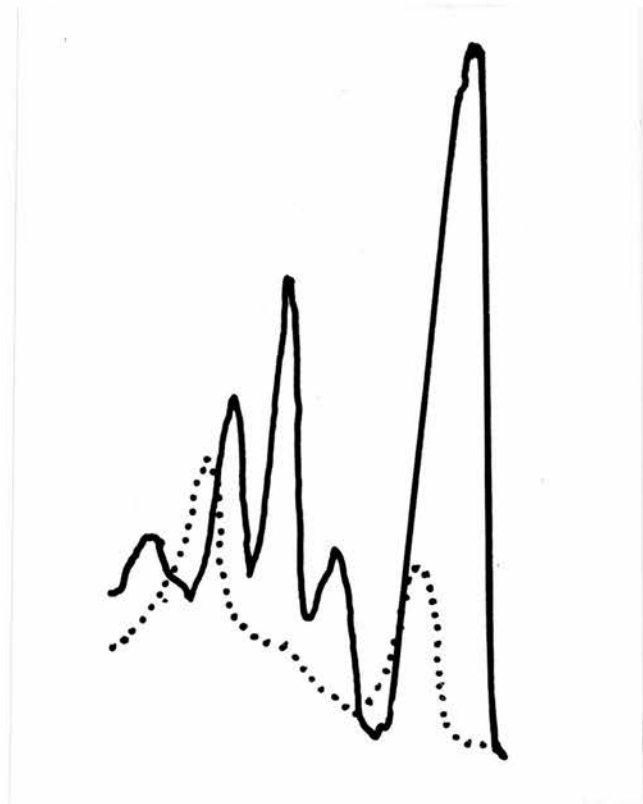


Fig. 18b : Electrophoretic protein pattern obtained by scanning of the above strip.

———— Maternal serum.  
 ..... Placental extract.

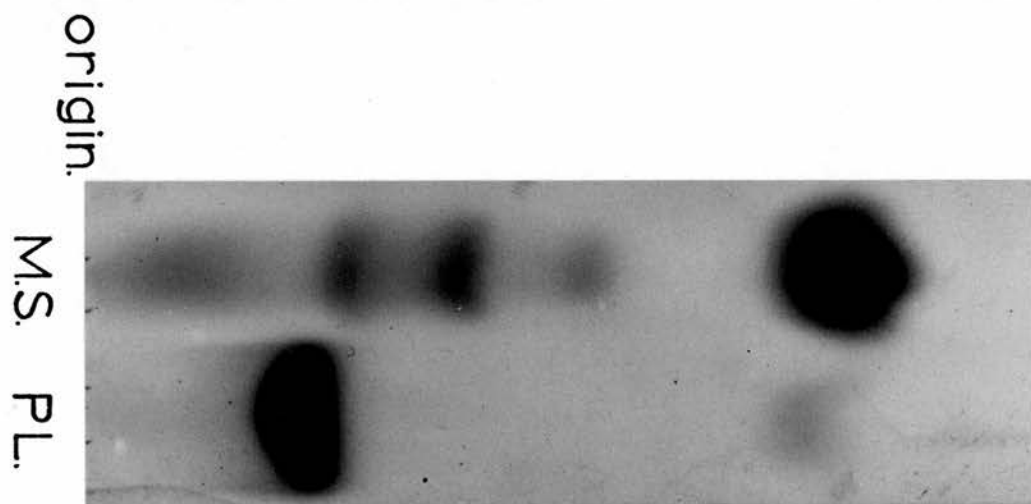


Fig. 19a : Separation pattern of maternal serum and the corresponding placental extract at term, by cellulose acetate electrophoresis.

M.S.: Maternal serum

Pl.: Placental extract

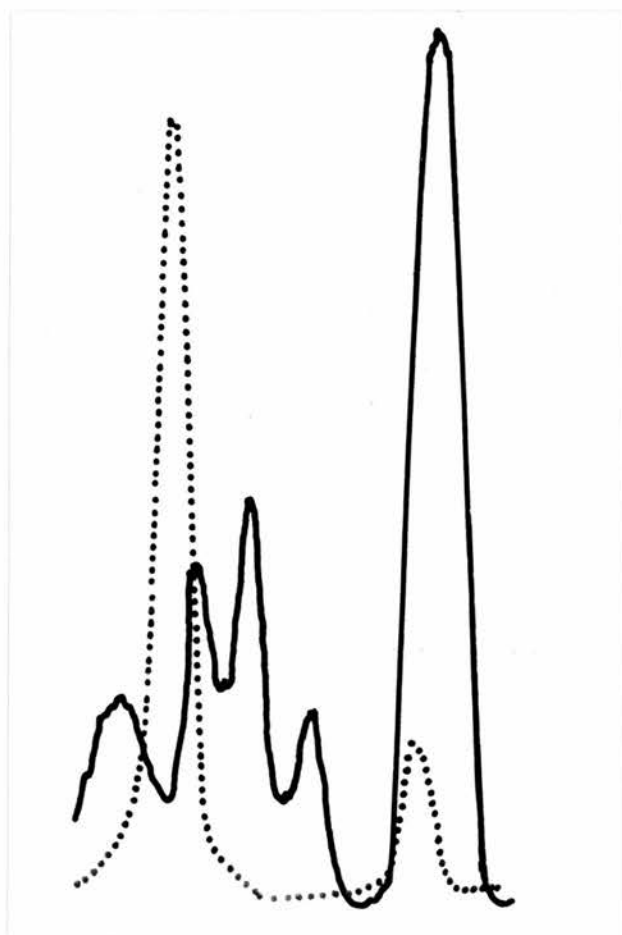


Fig. 19b : Electrophoretic protein pattern obtained by scanning of the above strip.

———— Maternal serum.

..... Placental extract.



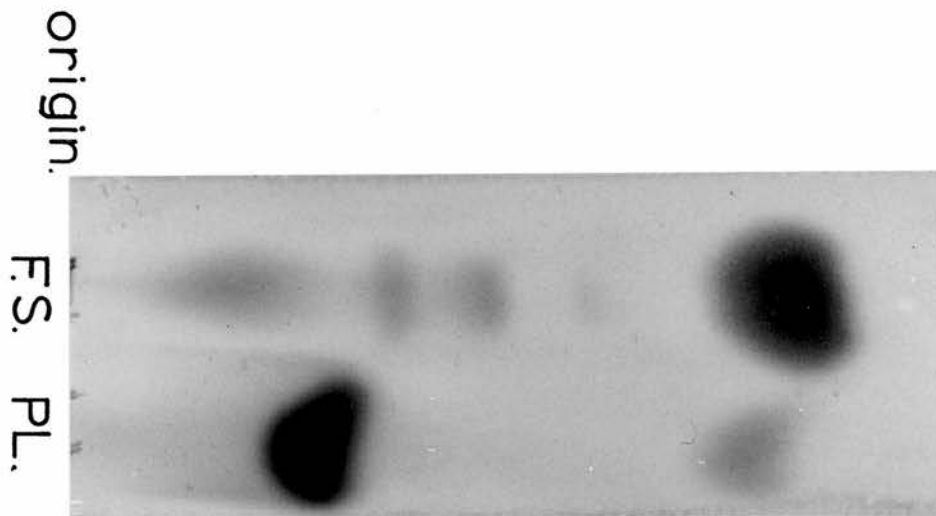


Fig. 20a : Separation pattern of foetal serum and the corresponding placental extract at term, by cellulose acetate electrophoresis.  
 F.S. : Foetal serum. PL. : Placental extract.

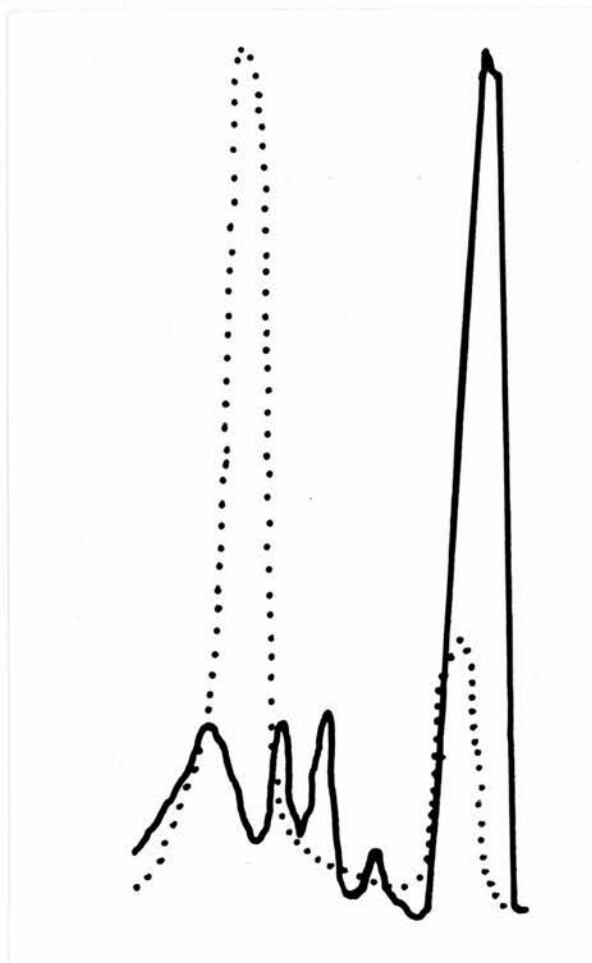


Fig. 20b : Electrophoretic protein pattern obtained by scanning of the above strip.  
 ————— Foetal serum.  
 ..... Placental extract.

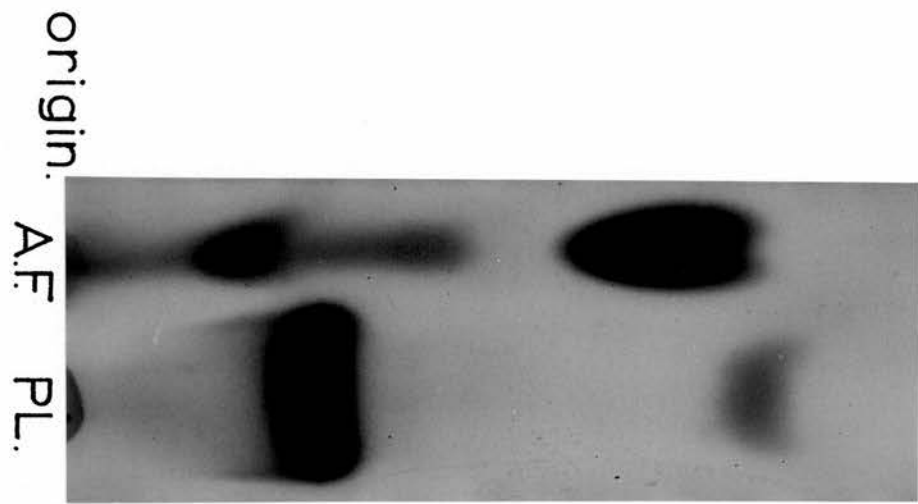


Fig. 21a : Separation pattern of amniotic fluid and the corresponding placental extract at term, by cellulose acetate electrophoresis.  
A.F. : Amniotic fluid. PL. : Placental extract.

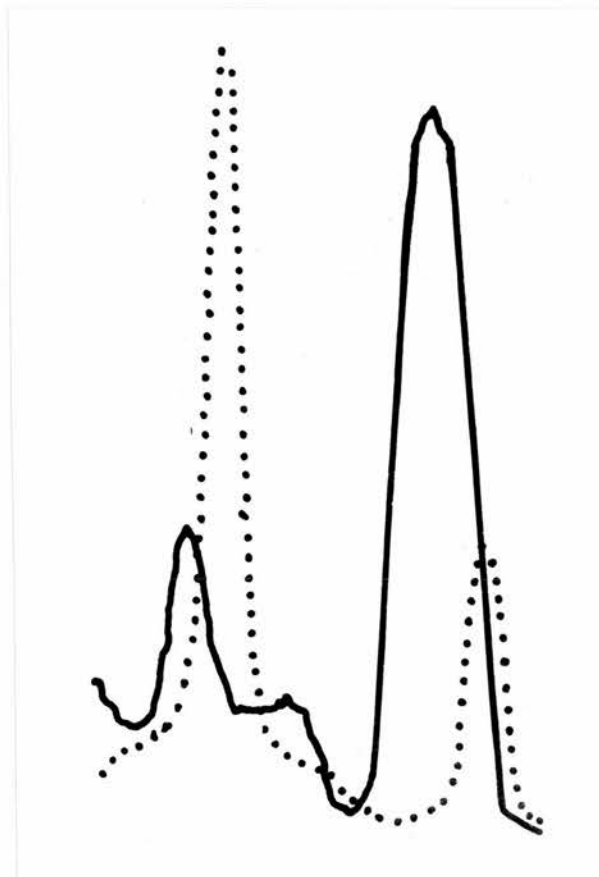


Fig. 21b : Electrophoretic protein pattern obtained by scanning of the above strip.  
—— Amniotic fluid.  
..... Placental extract.

in tables 17a and b and Figs. 18a and b, 19a and b, 20a and b, and 21a and b.

Table 17a.

Fractionation by electrophoresis of the placental proteins at 19 weeks of pregnancy (one case) and at term (mean of 3 cases).

Percentage of protein components as obtained by scanning.

	Fraction I	Fraction II
19 weeks	47.37%	52.63%
Term	31.86 ± 2.5%	68.14 ± 2.5%

Table 17b.

Fractionation by electrophoresis of the placental proteins at 19 weeks of pregnancy (one case) and at term (mean of 3 cases).

Absolute values in mgm/gm fresh tissue, in relation to the total protein as obtained by the method of Miller (1959).

	Fraction I	Fraction II	Total protein in mgm/gm wet tissue
19 weeks	9.60	10.67	20.27
40 weeks	10.56	22.59	33.15

The mobility of fraction "I" was similar to that of serum albumin. The mobility of fraction "II" was between that of the beta and gamma globulin fractions of serum.

A study of the effect of putrescine and thalidomide on leucocyte cultures.

Results are summarized in Tables 18 - 20.

Table 18.

DNA, RNA and protein in leucocyte culture under normal growth conditions.

(The blood was obtained from one individual)

Age of culture	DNA in $\mu\text{g.}$	RNA in $\mu\text{g.}$	Protein in mgm	RNA/DNA	Protein/DNA
24 hours	16.5	69.0	0.9	4.06	54.5
72 hours	42.0	82.0	1.24	1.95	29.5

It is apparent from this table that at 72 hours the increase in the total DNA was much more marked than the increase in either the RNA or the protein, which results in a decrease in the RNA/DNA and the protein/DNA ratios.

Table 19a.

DNA, RNA and protein in leucocyte cultures under the effect of putrescine and thalidomide. Experiment I.

(The blood was obtained from one individual).

Dose per ml. of culture	Age of culture	DNA in $\mu\text{g.}$	RNA in $\mu\text{g.}$	Protein in mgm.	RNA/DNA	Protein/DNA
Control	24 hours	20.0	107.50	2.4	5.35	120
	72 hours	40.5	140.25	3.7	3.46	91
Putrescine $10^{-3}\text{M}$	24 hours	18.75	105.00	2.72	5.61	145
	72 hours	40.50	110.35	3.55	2.72	87.6
Putrescine $0.5 \times 10^{-2}\text{M}$	24 hours	24.75	92.50	2.60	3.74	105
	72 hours	59.0	110.00	3.76	1.84	63.7
Putrescine $10^{-2}\text{M}$	24 hours	21.00	103.75	2.75	4.94	131
	72 hours	40.50	93.50	3.57	2.31	88
Thalidomide $3.6 \times 10^{-5}\text{M}$	24 hours	26.25	105.00	2.60	4.00	99
	72 hours	57.91	119.35	3.04	2.06	52.5
Thalidomide $3.6 \times 10^{-5}\text{M}$ plus Putrescine $0.5 \times 10^{-2}\text{M}$	24 hours	18.75	119.00	2.61	6.35	133
	72 hours	49.15	154.00	3.55	3.13	72

Table 19b.

DNA, RNA and protein in leucocyte cultures under the effect of putrescine and thalidomide. Experiment II.  
(The blood was obtained from one individual.)

Dose per ml. of culture	Age of culture	DNA in $\mu\text{g.}$	RNA in $\mu\text{g.}$	Protein in $\text{mgm.}$	RNA/DNA	Protein/DNA
Control	24 hours	41.5	180.0	4.10	4.34	99
	72 hours	68.0	200.0	4.50	2.94	66
Putrescine $10^{-3}\text{M}$	24 hours	34.0	195.0	3.50	5.74	103
	72 hours	59.0	230.5	5.08	3.91	86
Putrescine $0.5 \times 10^{-2}\text{M}$	24 hours	39.5	187.5	3.20	4.75	81
	72 hours	106.8	273.0	5.40	2.56	50.5
Putrescine $10^{-2}\text{M}$	24 hours	32.5	157.5	2.75	4.85	84.6
	72 hours	60.0	209.0	3.56	3.45	59
Thalidomide $3.6 \times 10^{-5}\text{M}$	24 hours	25.0	157.5	2.53	6.30	101
	72 hours	88.0	211.0	4.68	2.40	53
Thalidomide $3.6 \times 10^{-5}\text{M}$ plus Putrescine $0.5 \times 10^{-2}\text{M}$	24 hours	32.5	187.5	2.35	5.77	72
	72 hours	90.0	212.0	4.31	2.36	48

Table 20.

Comparison of the effect of Putrescine, Thalidomide and a mixture of the two drugs on the DNA content of leucocyte cultures, at 72 hours, expressed as the percentage increase as compared with the control cultures.

Dose per ml. of culture	The percentage DNA increase as compared with the control culture	
	Experiment I	Experiment II
Putrescine $0.5 \times 10^{-2}M$	+ 45.68	+ 57
Thalidomide $3.6 \times 10^{-5}M$	+ 43	+ 29.4
Thalidomide $3.6 \times 10^{-5}M$ plus Putrescine $0.5 \times 10^{-2}M$	+ 21.35	+ 32.4

This table suggests that a mixture of Putrescine and Thalidomide results in an effect less than that produced by Putrescine alone. Although thalidomide, in the same concentration, when used alone, had an effect similar to (but lower than) that of putrescine.

DISCUSSION



THE CONCENTRATIONS OF NUCLEIC ACIDS AND PROTEINS IN THE  
HUMAN FOETAL ORGANS AND MEMBRANES AT DIFFERENT PERIODS OF  
GESTATION

The rapid cellular proliferation and protein synthesis characteristic of embryonic and foetal tissues provide a good target for nucleic acid studies. The role of DNA in cellular multiplication and the role of RNA in protein synthesis have been already discussed in the introduction to this thesis.

Studies on the chemical content of embryonic and foetal organs can be divided into two phases, those before and those after 1945, when convenient and reliable methods for the extraction and estimation of nucleic acids were introduced (Schmidt and Thannhauser; Schneider). The results in the two phases are not comparable and in this discussion only results obtained by the more recent and reliable methods will be mentioned.

Various studies on the nucleic acid content of animal embryonic tissues have been published. In some of these studies, ova and whole embryos were investigated. In other studies individual organ development was followed. As examples of the first approach, the studies of Haggis (1964) on the *Rana pipiens*, Leng et al. (1965) on the mosquito, and Gluck and Kulovich (1964) on very early chick embryos, may be mentioned. The study of Winick and Noble (1965) on the rat and the study of Gluck and Kulovich (1964) on the chick embryo are examples of the

second category.

No values have been so far published relating to the content of nucleic acids (RNA and DNA) in human foetal organs. Hence, no comparison of the results obtained in this work in human foetal organs with similar observations in the literature can be made. It should be emphasized that the studies to be presented here cover only the first half of pregnancy, with the exception of some observations near term on the placenta and amnion. This limitation was imposed by the fact that suitable fresh material (therapeutic abortion) could only be obtained during this period. This, however, is the period of maximal growth and development.

The results obtained in this work, however, appear to be higher when compared to the few figures reported by Davidson and co-workers (1951) and by Slater and co-workers (1964) in human adult organs, and by Umana (1966) in human infant liver. This is understandable in view of the very rapid rate of growth and development in the foetus. Arey (1965) put the amount of the monthly increase of the foetal weight, when the value at the start of each month is taken to be equal to unity as follows:

## Rate of increase of foetal weight.

1st lunar month		40,000
2nd	" "	49
3rd	" "	13
4th	" "	6.5
5th	" "	1.95
6th	" "	1.07
7th	" "	0.69
8th	" "	0.55
9th	" "	0.43
10th	" "	0.38

Concentration of DNA in foetal organs and membranes

As can be seen from Fig. 7, the pattern of the variation in the DNA concentration with the stage of pregnancy appeared to be characteristic for each organ. These differences may be related to differences in the rate of development of these organs at particular stages of pregnancy. Differential growth is a characteristic feature of developmental anatomy. In fact, the development of an organism is characterized by progressive alterations of form and proportions, both externally and internally. Some embryological observations are therefore relevant to the interpretation of the results obtained by the chemical methods.

Brain: As can be seen in Fig. 7 and Table 3, the DNA concentration of the foetal brain was comparatively

high at very early stages of pregnancy (6 - 8 weeks) and then declined to its lowest value, at the end of the period studied, in mid-pregnancy.

The human embryo is characterized by an early and rapid development of the cephalic region. The percentage of the volume of the head and neck to the total body volume is given by Arey (1965) as follows:

2nd foetal month	43%
6th " "	36%
at birth	32%
Maturity (adult)	10%

The central nervous system is relatively huge in young embryos, and its weight in comparison with the whole body weight decreases from about 25 per cent in the second month of pregnancy to about 15 per cent at birth, and 2.5 per cent in the adult (Arey, 1965).

A correlation of the embryological and chemical data seems to suggest that the brain attains its maximum growth by cell division at a very early stage of development, subsequent growth probably occurring largely by cell enlargement.

Heart: As can be seen in Fig. 7 and Table 5, the DNA concentration in the heart was at its highest value at a very early stage of pregnancy (8 weeks), this concentration falling rapidly afterwards.

The heart and the blood vessels are the first of the

embryonic systems to reach a functional state (at about the 12-somite stage: Arey, 1965). Very early in the development, the embryo satisfies its metabolic needs by simple interchanges with the fluid medium in which it is immersed. Soon, however, with increase in size and complexity, a circulatory system becomes necessary in order to make use of the required food and oxygen obtainable from the mother's blood, and this is achieved by the development of the cardiovascular system. According to Jackson (1909), the heart forms more than 5 per cent of the total body volume at 4 weeks of intra-uterine life, and then decreases rapidly in relative size dropping to 0.85 per cent in the third month and to about 0.7 per cent in the newborn.

The decreasing values for the DNA concentration in the heart (Table 5 and Fig. 7) may thus indicate that the maximum growth by cell division occurs in the heart very early, and that subsequent growth occurs more by cell enlargement.

Liver: Fig. 7 and Table 2 show an increase in the DNA concentration in the liver from the 6th to the 16th week, and a decrease thereafter.

The early and rapid development of the liver in the embryo is well known. Its development follows that of the heart. According to Arey (1965), the chief ventral prominence at the early embryonic stages is formed by the

heart, and shortly afterwards also by the rapidly growing liver. The two organs determine the shape of the ventral body until the 8th week, when the gut dominates the belly cavity. According to Jackson (1909), the liver reaches its maximum relative size (7.5 per cent of the total body) during the second and third months of intra-uterine life, and decreases in the fourth month.

With the DNA concentration as indicator of cell division, it may be suggested therefore that the maximum growth of the liver by cell division occurs in the period in which the DNA concentration is highest, and that further growth of the liver probably takes place mainly by cell enlargement.

Lung: Fig. 7 and Table 6 show that the DNA concentration of the lung increases progressively in the first half of pregnancy. This may be correlated with the data reported by Jackson (1909), who found that the lungs are relatively small at early pregnancy and that they increase steadily in size, reaching a maximum of about 3.29 per cent relative to the total body weight during the fourth month of pregnancy.

Kidney: From Fig. 7 and Table 4, it can be seen that the DNA concentration of the kidney showed no noticeable change during the period examined.

Amniotic membrane: The DNA content of the amniotic membrane showed a decrease with the advance of pregnancy

(Table 8 and Fig.7) which may suggest that the amnion was not actively growing by cell division during this period.

Among the organs examined, the amniotic membrane showed the lowest DNA concentration whereas the liver showed the highest DNA concentration. Such variations are in accord with what may be expected from the histological picture and the cellularity of the different organs.

Placenta: Table 7 and Fig. 7, show that the DNA concentration in the placenta increased towards term. These results may be compared with those reported by Brody (1953) and Mori (1965). Brody (1953) used the cysteine reaction in hot trichloroacetic acid extracts to determine the nucleic acids in 16 human placentae of different ages ranging from the 10th week to full term. The author found a small increase in DNA with advance of pregnancy. Mori (1965) reported an increase of DNA concentration in the human placenta with ageing. The DNA concentration of the placenta at term was found by Mori (1965) to be nearly twice the value obtained for the 3-months chorion.

It is interesting to discuss the significance of the variations in the rate of development of the various organs in foetal life. It may be suggested that organs, the function of which is needed early in foetal life, reach their maximum development earlier than other organs, the function of which is not needed. This may be illustrated by the finding of a progressive rise of DNA

concentration in the lung in the first half of pregnancy, in contrast to the decreasing concentration of DNA in the heart over the same period (Fig. 7). It is possible that the foetus utilizes the growth energy available to it to the best advantage by giving priorities to certain organs rather than others. The foetus relies at a very early stage of development on the function of the heart, whereas a functioning lung is only required after birth. A functioning liver is probably essential early in foetal life. Thus Mori and Iso (1965) have shown in pregnant rabbits that the relative amount of  $S^{35}$ -methionine incorporated into protein and the efficiency of protein synthesis were high in the foetal liver. The function of the foetal liver in the synthesis of foetal serum proteins will be discussed in another part, in relation to our electrophoresis results.

The present results on the human foetal organs seem to be in agreement with the results of Gluck and Kulovich (1964) in the chick embryo. These authors stressed the finding that the developing organs were unique and that each organ showed its own characteristic changes and that no two embryonic organs were identical in their biochemical development.

#### Concentration of RNA and Proteins in the foetal organs and membranes

Other parameters studied in this work were the estimation of RNA and protein content of the foetal organs



and membranes. A study of the curves of these two components in relation to the curves of DNA concentration (Fig. 8a and b) showed no correlation except in the liver, where the RNA, DNA and proteins showed the same pattern. Some useful information may, however, be gained by studying the RNA/DNA ratio and the protein/DNA ratio, as these ratios describe more or less the amounts of RNA and protein per tissue unit, rather than per tissue weight.

Generally, the RNA/DNA ratio for any particular organ did not vary much with the stage of pregnancy (Fig. 9a and b). An exception is the placenta in which this ratio decreased with advancing pregnancy, due to the rise in the DNA concentration. It may be noted that Brody (1953) and Mori (1965) recorded a similar finding in the placentae which they examined. Winick and Noble (1965), in their study of the quantitative changes in DNA, RNA and protein during prenatal and postnatal growth of the rat, recorded that the RNA in most organs remained proportional to DNA during the period studied, resulting in a constant figure for RNA per nucleus. When the RNA/DNA ratio is compared in the various organs (Fig. 9a and b) it will be seen that higher values were obtained for the amniotic membrane, placenta and heart and to a less extent in the liver. These organs also showed the highest protein/DNA ratio. There is thus some correlation between the RNA/DNA and the protein/DNA ratios, in other

words between the RNA and protein content of the cell. This finding adds support to the evidence of the role of RNA in protein synthesis. The finding may again be correlated with embryological data. The function of those organs with the high RNA/DNA and protein/DNA is probably needed at an early stage of embryonic development. Hence, they are enriched with RNA with a resultant larger capacity for protein synthesis.

Studying the changes in the protein/DNA ratio with the progress of pregnancy shows a trend towards a progressive rise, especially in the liver, brain, heart and amnion (Fig. 9a and b). The study of Winick and Noble (1965) on the biochemical development of the rat has shown that growth by cell enlargement (i.e. by accumulation of protein) follows the stage of growth by cell division. It is interesting to note that the lung and kidney showed a minimal rise in the protein/DNA ratio during the period examined (Fig. 9a and Tables 4 and 6). This could be related to the embryological observation that these organs are late to achieve their maximum development and that the function of the two organs is probably not essential to the early foetus. Although the protein/DNA ratio was relatively high in the placenta, it showed no rise with the progress of gestation. It is possible that maximal protein accumulation occurs in the placenta at an early stage of pregnancy. Mori (1965) found that the efficiency of protein synthesis was

highest in the chorion at 3 months gestation.

The biochemistry of development in the human foetus is a branch in its infancy. No similar studies have been previously reported. The small number of recorded observations makes the conclusions and the suggested explanations in this study only tentative until more data are accumulated about this rarely obtainable biological material.

#### AN ELECTROPHORETIC STUDY OF PROTEINS OF THE MATERNAL SERUM, FOETAL SERUM, AMNIOTIC FLUID AND PLACENTA.

##### Maternal serum proteins

The protein content of the maternal serum, has been estimated in 81 cases mainly in the second half of pregnancy. The results have been already presented in table 9. In 24 of these cases, an electrophoretic fractionation of the proteins was also performed (Tables 10a and b). Three points are worthy of discussion: the tendency to lower levels of the total serum proteins, the change in the albumin/globulin ratio and the change in the globulin fractions.

The level of the maternal serum proteins throughout pregnancy has been studied by various workers, but the results are not in complete agreement. That the concentration of the total proteins tends to be lower in the pregnant than in the non-pregnant patient is, however,

uniformly accepted (Mack, 1955). This decrease is manifested as early as the first trimester of pregnancy (Mack, 1955). Slight increases in the total proteins during the last two or three months of pregnancy have been reported by Hoch and Marrack (1948) and by MacGillivray and Tovey (1957). Other investigators did not find such an increase (Cantarow and co-workers, 1933; Leyssac, 1960). Our own estimations show that the levels of the serum proteins in pregnant patients at term are on average significantly lower than in patients before term. It must, however, be realized that our observations are not "longitudinal" observations on the same subjects and that even such longitudinal observations do show wide individual fluctuations (Mack, 1955). Although it is possible that the decrease in the levels of the serum proteins is partly a manifestation of the pregnancy hydraemia, the selective change in the protein fractions suggests the operation of other factors. It is interesting to note that Singh and co-workers (1967) found that the protein and caloric intake and the socio-economic status did not affect the maternal serum proteins at term quantitatively or qualitatively.

The decrease in the albumin/globulin ratio may be partly explained on the basis that the albumin molecules, due to their comparatively small size, diffuse more freely into the tissues and cross the placental membrane more readily than the larger globulin molecules (Mack, 1955).

Thus, by a sieve-like mechanism the globulin molecules may be retained in the maternal circulation. However, it is also possible that the relative decrease in albumin may be due to albumin synthesis not keeping pace with albumin utilization. The decrease in albumin/globulin ratio is also partly due to an increase in the total globulin concentration, which is more difficult to explain, though it may have a protective role by helping to compensate osmotically for the diminished number of albumin molecules and thus preventing additional fluid losses to the tissues.

The change in the relative concentration of the different globulin fractions is also worthy of discussion. While the concentration of the alpha and beta globulins is increased, the concentration of gamma globulin is declining. Selective filtration on the part of the placenta has been suggested (Mack, 1955), and is supported by the relatively high level of gamma globulin in the foetal serum as will be discussed later.

#### Foetal serum proteins

The protein fractions and the total proteins in the foetal serum have been studied in 7 cases: one at 19 weeks pregnancy and six at term. The results have already been presented in Tables 11a and 11b. The maternal serum proteins have also been studied in these cases and the results, compared with those of the foetal serum

proteins, have been shown in tables 12a and 12b and 13a and 13b.

The protein concentration of foetal serum at term was lower than that of the corresponding mother (table 13b). The difference was, however, not statistically significant. Moore et al. (1949), Mack (1955) and Oberman et al. (1956) observed a lower concentration in the foetal serum than in the maternal serum, whereas Brown et al. (1959) and Orlandini et al. (1955) observed no difference.

It is also apparent that the overall pattern of the foetal serum proteins at term was different from that of the maternal serum (Tables 13a and b and Figs. 15a and b): the albumin/globulin ratio was higher, the albumin was more, the alpha and beta globulins were less and the gamma globulin was more. The relatively higher concentration of albumin is in agreement with the results of Longsworth et al. (1945), Moore et al. (1949), Mack (1955), Oberman et al. (1956), Brown et al. (1959) and Derrington and Soothill (1961). Viergiver, Stroup, Sheff and Westphal (1962) considered that the overall pattern of the foetal serum at term corresponded more closely to the serum of healthy non-pregnant women than that of normal mothers at delivery, and suggested that the physiologic alterations of the maternal blood during pregnancy may be due to an effort on the part of the mother to provide the infant at birth with a normal blood pattern. The high gamma globulin in the serum of newborn babies may offer a

degree of protection against ordinary bacterial and viral infections.

Considering the foetal serum proteins before term, no dogmatic conclusion can be drawn from our single case at 19 weeks pregnancy. The result, however, is in agreement with the few previously reported investigations on the subject. The differences in protein pattern between our early case and the cases at term lie in the lower level of the total proteins, the presence of an unidentified substance "X" and variations in the percentages of the different protein fractions. The low level of proteins in the foetal serum at early and mid-pregnancy has also been reported by Bergstrand and Czar (1967), Westin and co-workers (1959), Leyssac (1960) and Thalme (1967). The presence of substance "X" in the electrophoretic pattern of the foetal serum proteins in early and mid-pregnancy provides an interesting feature. This additional band is situated between the albumin fraction and alpha 1-globulin fraction. Bergstrand and Czar (1956, 1957) noted the presence of this band and named it substance "X" because of its unknown nature. They found it in foetal sera between the 9th and the 19th week of gestation but not in the sera of premature babies or in maternal sera. They also observed that this substance constituted a considerable part of the foetal serum proteins in early pregnancy (on average, 10 per cent) and that it decreased significantly as pregnancy

advanced, both relatively and absolutely. The presence of this substance "X" was also demonstrated by Bang and Páby (1956), Talavera (1957) and Westin and co-workers (1959). Variations in the percentages of the different protein fractions in the foetal serum from those at term can also be observed (Tables 11a and b). In our case at 19 weeks gestation, the albumin is much higher, the alpha-1 globulin fraction is not much different, the alpha-2 and the beta globulins are somewhat lower, and the gamma globulin is much less, almost one half. Moore and co-workers (1949) observed higher percentages of albumin, alpha globulin and beta globulin, and a lower percentage of gamma globulin in the earlier part of pregnancy. Mack (1955) investigated the serum proteins of a five months foetus and found that the alpha and beta globulin fractions were somewhat higher than those of the cord blood of healthy newborn infants at term, while the gamma globulin fraction was about one third less. Bergstrand and Czar (1957) analysed the serum protein patterns of foetuses, 9 to 19 weeks old and noted that, with the progress of pregnancy, the relative amounts of albumin, alpha-1 globulin and probably also of gamma globulin increased whereas the amount of beta globulin decreased and the percentage of alpha-2 globulin did not change.

The bearing of these results on the problem of the origin of the foetal proteins will now be discussed.



### Origin of the foetal serum proteins

The following three sources have been suggested for the foetal serum proteins: from the maternal serum across the placenta, synthesis by the foetal liver and synthesis by the placenta.

#### 1. From the maternal serum across the placenta:

Because of the presence of maternal antibodies in the foetal serum, Moore and co-workers (1949) suggested that the foetal serum proteins may be of maternal origin. That proteins from the mother may cross the placenta to the foetus has been shown both in animals and in the human. Schechtman and Abraham (1958) injected pregnant rabbits intravenously with human and bovine albumin. The injected albumins were demonstrated in the foetal blood and were found to retain their original electrophoretic mobilities despite passage through several layers of maternal and foetal tissues. Bangham and associates (1958) also reported that plasma albumin and gamma globulin traverse the placenta in the rhesus monkey. The transfer from mother to foetus of several labelled proteins was studied in human pregnancies near term by Gitlin and associates (1964). All the plasma proteins studied appeared in at least some of the foetuses after injection into the mothers, but in greatly different amounts. Several bits of evidence suggest that the gamma globulin fraction in the foetal serum is mainly or completely a transplacental transfer from the mother. Thus, Slater

(1954) found that the high gamma globulin level in the serum of women suffering from liver cirrhosis was reflected in an equally high serum gamma globulin in the newborn. More convincing evidence has been provided by Good (1955) in his study of a mother with the condition of agammaglobinaemia. The cord blood of this mother's baby contained only traces of gamma globulin. A rise in the level of gamma globulin in the infant's serum was not observed until the third month of life. Dancis, Braverman and Lind (1957) found that the foetal liver cannot synthesise gamma globulin, nor does the placenta. If the source of the gamma globulin in the foetus is the maternal serum, a selective transmission by the placenta must be present, because the level of gamma globulin is higher in the foetal than in the maternal serum (Tables 13a and b). This has in fact been shown by Bangham and associates (1958) in the rhesus monkey, where the transmission of the gamma globulin was found to be 15 - 20 times more rapid than that of the albumin.

## 2. Synthesis by the foetal liver:

The demonstration of the unknown protein component, substance "X", in the foetal serum in early and mid pregnancy and its absence in the maternal serum (Figs. 10a and b), is not in a very good agreement with the hypothesis of a passive placental transfer of maternal serum proteins to the foetal circulation. The foetal liver may be

suggested as a possible site for plasma protein synthesis (Mack, 1955). Dancis, Braverman and Lind (1957) showed that the liver of human fetuses of 3-4 months gestation is already capable of synthesizing plasma proteins with the exception of gamma-globulin. Protein synthesis in the foetal organs of the rabbit was studied by Mori and Iso (1965) by injecting 25 days pregnant rabbits with  $S^{35}$ -methionine into the ear vein. The animals were sacrificed 20 minutes after the injection. The maternal liver, decidua, chorion and foetal liver were removed. The authors found that the relative amount of  $S^{35}$ -methionine incorporated into protein and the efficiency of protein synthesis were highest in the foetal liver, followed by the chorion, maternal liver, and least in the decidua. Mori and Iso (1965) concluded that the direct precursors of foetal proteins are free amino acids of maternal origin which are transferred across the placental barrier to the foetal circulation. They emphasized that the placenta does not play any role in the synthesis of these amino acids. Dancis and Shafran (1958) studied protein synthesis by the guinea pig foetus and concluded that the foetus near term can synthesize all plasma proteins recognizable electrophoretically with the exception of gamma globulin. The authors also stated that the foetus utilizes amino acids transferred from the maternal circulation for the synthesis of plasma proteins.

### 3. Synthesis by the placenta:

Dancis and co-workers (1957) studied the protein synthesis by the human placenta and concluded that both early and term placentae were capable of synthesizing proteins with the electrophoretic mobilities of alpha and beta globulins but not proteins that were electrophoretically identifiable as albumin or gamma globulin.

In this study, the proteins of 4 placentae (one at 19 weeks and 3 at term) were investigated (Tables 17a and b). The total protein content was higher in the term placentae. By electrophoresis, the proteins could be separated into two fractions (Figs. 18a and b) and (Figs. 19a and b). The nature of fraction "I" which is the band with the albumin mobility is not known, but it may possibly represent a contamination of the placental extract with maternal blood as suggested by Dancis and co-workers (1957). Fraction II, on the other hand, could not be entirely due to blood contamination because of its situation and amount. This fraction II may include the placental enzyme proteins. As can be seen in Table 17b, the increase in the total protein content of the placenta at term was due to an increase in the amount of fraction II.

#### Amniotic fluid proteins

The total protein content in the amniotic fluid throughout pregnancy:

The protein content of the amniotic fluid has been

analysed throughout pregnancy and the results of 159 estimations are presented in Table 14 and Fig. 13. In 15 cases, repeated estimations were performed on the same case: twice in 5 cases, 3 times in 7 cases, 5 times in 2 cases and 6 times in one case. Two other points about this material need to be mentioned. The first is that, apart from the cases at term, the amniotic fluid was obtained from Rh negative patients only. There is no evidence, however, that the amniotic fluid proteins are different from other normal cases (Abbas and Tovey, 1960; Heron, 1966). The second point is that the series of patients at term includes 3 postmature cases (40+, 41 and 42 weeks). The results in these 3 cases are not different from the rest of the cases at term.

As shown in Table 14 and Fig. 13, the protein content of the amniotic fluid is comparatively low at the 12th week of pregnancy, increases to a peak between the 22nd and the 26th week of gestation, then declines towards term. The results of serial estimations in individual cases also show this trend. Because of a relatively wide range in the protein values, this estimation alone does not appear to be of value in the individual case in assessing the maturity of the foetus.

In 62 cases in the 2nd half of pregnancy, the total proteins in the maternal serum were also estimated. There was no correlation between the level of the proteins in the maternal serum and that in the amniotic fluid

(Fig. 14).

Comparison of these results with those reported by previous workers on isolated periods of gestation shows that they are in agreement with the results reported at term by Makepeace and co-workers (1931), Imaz and Gascon (1950), Hanon and co-workers (1955), Abbas and Tovey (1960) and Derrington and Soothill (1961). The results also appear to be in agreement with those reported by Heron (1966) between the 36th and the 43rd weeks of pregnancy, and by Bevis (1953), between the 22nd and the 40th week, and by McKay and co-workers (1955) in the first trimester. Other authors reported higher values for the proteins in the amniotic fluid at the stages of pregnancy which they examined: Cantarow and co-workers (1933) between the 7th and the 9th month of pregnancy; Westin and co-workers (1960) between the 12th and the 26th weeks; and Schreiner (1964) between the 10th and 16th week and between the 35th and 40th week. Mentasti (1959) observed no variation in the total protein content of the amniotic fluid after the 6th month of pregnancy.

The electrophoretic pattern of the amniotic fluid proteins:

The proteins of the amniotic fluid were fractionated by electrophoresis in 36 cases (Tables 15a and b; Figs. 12a and b, and Figs. 16a and b). The amniotic fluid in the period examined (17 weeks to term) is shown to

contain all the protein fractions found in the maternal serum and the foetal serum (with the exception of substance "X" found in the young foetuses) (Tables 12a and b; Figs. 17a and b and Fig. 11). One point, however, may be worthy of note. A small spot at the point of application was observed in some electropherograms of the amniotic fluid. A similar observation was made by Brzezinski and associates (1961). Viergiver and co-workers (1962) also observed the same spot in most of their strips and called it the static component "S", the nature of which being unknown. On the other hand, Heron (1966) did not notice the presence of this non-moved component in his electropherograms.

As is shown in Table 15a, the percentage of the different protein fractions in the amniotic fluid did not show a significant change during the examination period (17 - 42 weeks). The albumin/globulin ratio also did not change appreciably. This result is in agreement with Westin and co-workers (1960) who stated that the albumin/globulin ratio between the 14th and the 26th weeks of pregnancy was not different from that to be expected at term. The result, however, does not confirm the claim of Heron (1966) that a progressive increase in the albumin/globulin ratio occurs as pregnancy advances, and that this may help in the assessment of maturity. The result is also different from that found by Wirtschafter and Williams (1957) in the rat. The latter authors

reported that the A/G ratio in the amniotic fluid of the rat was subject to a characteristic change as pregnancy advanced, with a dramatic reversal on the 21st day of gestation just prior to parturition.

Comparison between the protein fractions by electrophoresis in the maternal serum, foetal serum and amniotic fluid:

In 6 normal patients delivered at term, electrophoresis was performed on the maternal serum proteins, the foetal serum proteins and the amniotic fluid proteins (Table 16, Figs. 15a and b; 16a and b and 17a and b). In 16 other patients, the serum proteins and the amniotic fluid proteins were studied, the results being the same as in the 6 cases. Two features are worthy of note in this comparison. The first is that the percentage of the albumin fraction is higher in the amniotic fluid than in the maternal or even the foetal serum. McKay and co-workers (1958), Abbas and Tovey (1960), Derrington and Soothill (1961), Brzezinski and co-workers (1961) and Viergiver and associates (1962) also reported a higher percentage for the albumin fraction in the amniotic fluid proteins than in the maternal serum proteins. The second interesting feature is the markedly lower level of the gamma globulin in the amniotic fluid than in the corresponding maternal and foetal sera. Brzezinski and co-workers (1961) and Viergiver and co-workers (1962) reported similar findings. Westin, Lind and Nilsson



(1960) failed to demonstrate the presence of gamma globulin fraction in the amniotic fluids during their examination period from 14 to 26 weeks of pregnancy.

In most of the amniotic fluid electropherograms, the beta globulin fraction is noticed to be slightly shifted towards the cathode end as compared with the corresponding maternal serum (Figs. 12a and b). This shift is also noticeable when the amniotic fluid is run in parallel with the corresponding foetal serum (Figs. 17a and b).

The origin of the amniotic fluid proteins:

The bearing of the results on the problem of the origin of the amniotic fluid proteins will now be discussed.

The relative shift in the beta globulin fraction of the amniotic fluid towards the cathode, as compared to the maternal or foetal serum (Figs. 12a and b and 17a and b) may suggest a qualitative difference in the protein, and hence a contribution by the amniotic epithelium. A further support for such a contribution is the relatively high RNA/DNA ratio and Protein/DNA ratio in the amniotic membrane, as already shown in the results (Table 8). These findings indicate active protein synthesis and since this marked protein synthesis is not apparently all needed for growth of the membrane, it may indicate active secretion. Earlier histological studies have also suggested that the amniotic epithelium may have a secretory function (Taussig, 1927; Palliez and co-workers, 1956).

The relatively higher albumin/globulin ratio in the amniotic fluid than in the maternal serum may support an origin of the amniotic fluid proteins by dialysis from the maternal serum, as suggested by Barbanti (1956), McKay and associates (1958) and Abbas and Tovey (1960). The albumin, on account of its low molecular weight (69,000. Abbas and Tovey, 1960) appears in relatively larger amounts in the amniotic fluid. The composition in this respect resembles other interstitial fluids in the adult such as the synovial, oedema, lymph and ascitic fluids which are presumably dilute dialysates of the serum (McKay and associates, 1958). The ultrastructure of the amniotic membrane, on the other hand, suggests that at least in the latter part of pregnancy, the movement of material from the serum to the amniotic fluid is highly selective and specialized (Bourne, 1962).

The contribution by the foetal serum to the amniotic fluid proteins has been suggested by Mentasti (1959) who found a statistically significant correlation between the concentration of the protein in the amniotic fluid and those of the foetal serum, and by Brzezinski and co-workers (1961) who found that the protein pattern of the amniotic fluid bore more resemblance to that of the foetal serum than to that of the maternal serum. Although this contribution to the amniotic fluid proteins is possible, the results in this study do not provide a positive evidence in its favour.

A STUDY OF THE EFFECT OF THALIDOMIDE AND OF  
PUTRESCINE ON GROWTH IN LEUCOCYTE CULTURES

The thalidomide tragedy has stimulated scientific and even public interest in the embryopathic activity of drugs. Although it is now several years after the discovery of the teratogenic properties of thalidomide, the mechanism of the action of the drug is still poorly understood. The fact that the drug exerts its effect during the stages of active embryonic growth and differentiation may suggest the possibility that the drug interferes with some mechanism which is especially involved in embryonic development. Knowledge of the biochemical processes in embryonic development is still lacking. However, a biochemical approach to the problem of the teratogenic activity of the drug may, in spite of the handicaps produced by the gaps in our knowledge, provide some useful information. But, before such an approach can be made, two basic questions must be satisfactorily answered: The first question is whether the teratogenic effect is produced by the drug itself or by one of its numerous metabolites. The second question is what are those structural features of the thalidomide molecule that are important for its teratogenic activity. These two questions have been investigated by other workers.

Experimental data on the first question, namely, whether one of the many thalidomide metabolites is responsible for the teratogenic effect, were provided by

Fabro, Schumacher, Smith, Stagg and Williams (1964), and by Keberle, Faigle, Fritz, Knusel, Loustalot and Schmid (1965). Under the experimental conditions employed by those authors, none of the metabolites led to any malformations in the fetuses, whereas thalidomide itself was clearly teratogenic. The nature of the compound transferred across the placenta to the embryo was investigated by Williams and co-workers (1965). An oral dose of C<sup>14</sup>-thalidomide was administered to a 10 days pregnant rabbit. When the embryo was examined 4 hours later, thalidomide was the major component found. The authors concluded that thalidomide itself is the compound which crosses the placental barrier and penetrates the tissues of the embryo, but once it has reached the embryo, it can be converted into all its metabolites by spontaneous hydrolysis. The authors also stated that the hydrolysis products do not pass readily the placental barrier. The significance of this latter observation is twofold: firstly, it implies that these metabolites accumulate in the embryo to a high concentration. Secondly, it shows that, although these metabolites are not teratogenic, when administered to the mother, this does not exclude their teratogenic effect, since they are unable to cross the placental barrier.

The second question, namely the structural characteristics of the thalidomide molecule relevant to its teratogenic effect, have been studied by Smith and

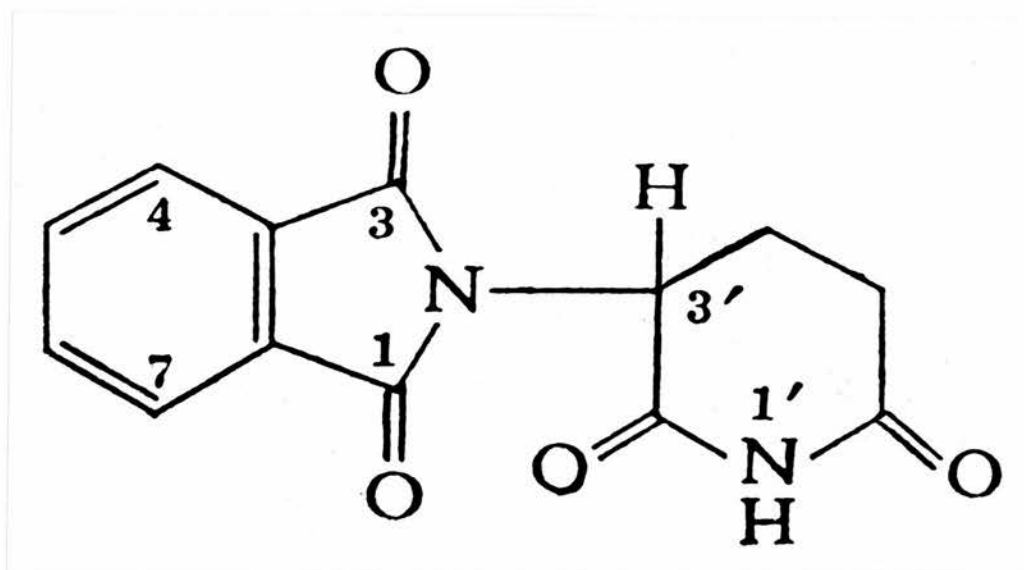


Fig. 22 : The structure of thalidomide molecule ( Williams and associates, 1965 ).

The thalidomide molecule is described chemically as  $\alpha$ -phthalimidoglutarimide. The compound is made up of two parts, the phthalimide ring structure on the left and the glutarimide structure on the right.

associates (1965). The authors investigated the embryopathic properties in the rabbit of a number of compounds chemically related to thalidomide. Their results suggested that only the phthalimide group (Fig. 22) is important for the embryopathic activity of the thalidomide molecule. The relevance of this observation to the mechanism of the action of the drug will be referred to later in this discussion.

Several mechanisms have been suggested for the possible mode of interference of thalidomide in the chemical processes of development. Roath and associates (1963) suggested that thalidomide may act as an antagonist against glutamic acid or glutamine. This hypothesis was proposed on the basis that thalidomide gives rise in the body to a number of derivatives of glutamic acid, and that these derivatives or thalidomide itself may interfere with the biochemical and physiological functions of glutamic acid. The available evidence seems, however, to be against this hypothesis. For Keberle and associates (1965) found that the thalidomide metabolites did not interfere with the growth-promoting properties of glutamic acid in the micro-organisms tested. McColl, Globus and Robinson (1965) likewise believe that the teratogenicity of thalidomide in rats is not due to an inhibition of glutamic acid metabolism because the teratogenic effect of the drug was not modified following the administration of glutamic acid and glutamine. Finally, thalidomide-like

compounds which do not contain the glutarimide ring have been shown also to exert teratogenic effects (Bignami, Bovet, Bovet-Nitti and Rosnati, 1962).

Another suggestion was that thalidomide may act as a folic acid antagonist (Kempner, 1962) or as an antagonist of other members of the B-group of vitamins e.g. riboflavine (Leck and Millar, 1962). Microbiological tests, however, showed no antagonism between thalidomide metabolites and aneurine, riboflavine, pantothenic acid, nicotinic acid, biotin, vitamin B<sub>12</sub>, p-aminobenzoic acid, inositol, and vitamin B<sub>6</sub> (Keberle and associates, 1965).

Kapeller-Adler (1965) offered another suggestion, based on the ability of the thalidomide to combine with polyamines and diamines to form stable products. It was suggested that thalidomide, by combining with putrescine, cadaverine or other polyamines, may deprive the human embryo of these biogenic amines at an early critical stage of foetal development. This hypothesis was tested in this study on human leucocyte culture.

#### Growth in leucocyte cultures

Leucocyte cultures are suitable for the study of normal and abnormal growth under the effect of various agents. The human blood cells can be obtained readily and repeatedly, and are relatively free of contamination by other tissues. Other advantages are the short term of the culture, and the presence of the growing cells in

suspension so that they do not need to be brought in suspension (as in monolayer cultures) by using chemical or physical agents which may damage the cells.

The growth in the leucocyte cultures was estimated by measurement of DNA, RNA and protein in the cultures. Some preliminary observations on the growth under normal conditions were made. An example of one experiment was shown in Table 18. The results showed that at 72 hours, the increase in the DNA was much more marked than the increase in either the RNA or the proteins. This observation is in line with other investigations on the subject. Thus, McIntyre and Ebaugh (1962) found that RNA synthesis precedes DNA synthesis, but after the first 24 hours, DNA synthesis is higher in rate than RNA synthesis. According to Jackson and Killander (1964), mitosis starts at the second day of the culture and reaches its maximum at the third and fourth days of culture, after which it declines.

#### Effect of thalidomide on leucocyte cultures

The effect of thalidomide was first tested in a concentration of  $1.2 \times 10^{-5}M$ . No difference was, however, detected in the values obtained between the treated and the control cultures. The concentration of thalidomide was therefore increased to  $3.6 \times 10^{-5}M$ . Tables 19a and 19b represent the results obtained, from which it can be seen that thalidomide ( $3.6 \times 10^{-5}M$ ) seemed to have enhanced



the DNA synthesis, where no apparent effect on the RNA and the protein synthesis could be detected.

These results seem to be in agreement with Fabro (1965) who studied the effect of thalidomide on the DNA and RNA synthesis in regenerating rat liver, following partial hepatectomy. His results suggested that treatment with thalidomide did not appear to interfere with the synthesis of DNA and RNA in the regenerating rat liver. Roath and associates (1963) used 3  $\mu$ g of thalidomide per ml. of leucocyte culture, and on measuring blast transformation in these cultures obtained an almost 50 per cent growth inhibition. The discrepancy between their results and those obtained in the present work may be due to the fact that a three fold amount of thalidomide was applied in this study.

Care should be taken in the interpretation of these results. Although no inhibition of DNA synthesis could be demonstrated, this does not exclude the presence of more subtle and undetected changes. It may be mentioned in this connection that Jensen (1965) reported the presence of chromosome aberrations in cultures of human leucocytes when thalidomide was added to the medium in a dose of 1 microgm/ml. The aberrations were reported as chromatid and iso-chromatid gaps and breaks with formation of small acentric fragments, as well as few deletions and dislocations.

Effect of putrescine on leucocyte culture

The effect of putrescine on leucocyte culture was studied in 3 concentrations:  $10^{-3}M$ ,  $0.5 \times 10^{-2}M$  and  $10^{-2}M$ . The results in two experiments are included in Tables 19a and 19b. A stimulant effect on the DNA synthesis in the culture at 72 hours was observed with a concentration of  $0.5 \times 10^{-2}M$ , but not with the lower or higher concentration.

Recently, interest has been aroused in the important biological role of polyamines and related amines (Tabor, Tabor and Rosenthal, 1961; Tabor and Tabor, 1964). A role as growth factors for certain micro-organisms has been shown by Herbst and Snell (1949) who found that *H. Para influenza* attained maximum growth when putrescine was added to the growth medium at a concentration of 0.1 microgm/ml. In the chick embryo, Caldarera and associates (1965) showed the possible relationship between polyamines and nucleic acids. The authors showed that during development of the chick embryo, the polyamines (spermidine, spermine, putrescine and cadaverine) are biosynthesized in considerable quantities in embryonic cells from the earliest stages of development and that their concentration reached a maximum between the 5th and 10th day of incubation. Moreover, they found that the concentrations of DNA and RNA also showed a parallel behaviour. The authors, therefore, suggested that polyamines may be involved in the process of morphogenesis and growth. In the human, Kapeller-Adler (1965)

suggested that cadaverine and putrescine may play an important role during gestation, since the enzyme histaminase (diamine oxidase), present in human placenta, has been found to display throughout normal human pregnancy a significant activity towards cadaverine and putrescine.

Effect of a mixture of putrescine and thalidomide on leucocyte cultures

Table 20 summarizes the findings obtained in two experiments, in which the effect of a mixture of the two drugs on the DNA content of leucocyte cultures was studied at 72 hours. Each experiment was performed using the blood from a single donor. The results show that in both experiments, putrescine ( $0.5 \times 10^{-2}M$ ) increased the DNA content of the culture at 72 hours by almost 50 per cent. Thalidomide ( $3.6 \times 10^{-5}M$ ) also increased the DNA formation but to a somewhat lesser extent. A mixture of both drugs did not have, however, an additive effect since lower DNA figures than those with putrescine alone were obtained.

The explanation of the latter results may lie in a competitive interaction between thalidomide and putrescine. It may also be mentioned that Williams and associates (1965) incubated thalidomide ( $4 \times 10^{-5}M$ ) at  $37^{\circ}C$  separately with each of the following amines - spermidine, spermine, cadaverine and putrescine, and

noted the disappearance of the thalidomide, measured spectroscopically, after two hours of incubation.

According to the same authors, the reaction of thalidomide with these polyamines appears to be an acylation reaction involving the phthalimide group of thalidomide molecule.

It is interesting to note that Smith and associates (1965) suggested that the embryopathic activity of the thalidomide probably stems from its phthalimide group.

Whether the interaction between thalidomide and such biogenic amines as putrescine is the basis of its embryotoxic effect remains to be assessed. The results of the present experiments on leucocyte culture can be explained on this basis, but more work is needed to provide the final answer.

S U M M A R Y

THE CONCENTRATIONS OF NUCLEIC ACIDS AND PROTEINS IN THE  
HUMAN FOETAL ORGANS AND MEMBRANES AT DIFFERENT PERIODS OF  
GESTATION.

Although nucleic acids concentrations in some animal embryonic organs have been studied by various workers, no such studies have been performed on human material. In this work, chemical estimations were carried out on organs of foetuses obtained by therapeutic abortion. The duration of gestation ranged from 4 to 20 weeks. Two abortuses expelled spontaneously at 20 and 24 weeks were also studied. The organs investigated include the liver, heart, kidney, brain and lung. Placentae and amniotic membranes during the first half of pregnancy and in some cases near term were also studied.

The pattern of variations in nucleic acid and protein concentrations, with the stage of gestation, was different for each organ. This may be related to the differential rates of development in individual organs.

The role of DNA in cellular multiplication is well-known. If the DNA concentrations are taken as indicative of the state of active growth by cell division, an interesting picture emerges in comparing the different organs. In the heart and brain, the concentration of DNA was highest during the early part of the period studied and fell gradually afterwards. This correlates

with the embryological fact that the development of these two organs occurs very early in intra-uterine life. In the lung, on the other hand, the concentration of DNA rose during the period studied, probably also in correlation with the later stage of development of the organ the function of which is not needed during intra-uterine life. In the liver, the concentration of DNA increased to a peak at 16 weeks, and then decreased. This is also in correlation with embryological data. In the kidney, there was no noticeable variation in the concentration of DNA over the period studied. In the placenta, the DNA concentration increased especially later in gestation. In the amnion, the concentration did not show a significant change.

The highest concentration of DNA was in the liver and the lowest in the amnion. This is in accord with histological data.

There was no general correlation between the curves of DNA concentration and those for RNA or proteins for each organ, with the exception of the liver. If the RNA and protein concentrations are parameters of growth by cell enlargement, then this lack of correlation may be explained by suggesting that growth by cell division and growth by cell enlargement do not always go hand in hand.

Generally, the RNA/DNA ratio for any particular organ did not vary much with the stage of pregnancy, except in the case of the placenta where the ratio increased.

Comparison of the RNA/DNA ratio in the various organs, however, shows that higher values are seen in the amniotic membrane, placenta and heart, and to a less extent in the liver. These organs also showed the highest protein/DNA ratio, a finding in support of the role of RNA in protein synthesis.

A trend towards an increase in the protein/DNA ratio with the progress of pregnancy was noted, especially in the liver, brain, heart and amnion. This may indicate an increasing role for growth by cell enlargement as pregnancy advances.

Attention is drawn to the limitation in drawing definite conclusions from such studies, in view of the scarce availability of human material.

Electrophoretic study of proteins of the maternal serum, foetal serum, placenta and amniotic fluid.

The concentration of proteins in the maternal serum was estimated in 81 cases, mainly in the second half of pregnancy. In 24 of the cases, electrophoretic fractionation of the proteins was also performed. The protein fractions and the total proteins in the foetal serum were studied in 7 cases: one at 19 weeks pregnancy and six at term. The maternal serum proteins and the amniotic fluid proteins have also been studied in these 7 cases. The proteins of the placenta were studied by electrophoresis in 4 cases: one at 19 weeks and 3 at



term. The protein concentration in the amniotic fluid was analysed in 159 samples between the 12th and the 42nd week. In 15 cases, two to six serial estimations were performed on the same case at different stages of pregnancy. In 62 of the cases, the total proteins were also estimated in the maternal serum. The proteins of the amniotic fluid were fractionated by electrophoresis in 36 cases. In 7 of these, the maternal and foetal sera were also investigated. In 16 other cases, the serum proteins in the mother only were investigated.

Comparison of the maternal serum proteins, foetal serum proteins and amniotic fluid proteins show that, apart from quantitative variations in the total proteins, qualitative variations are also detectable. The albumin fraction and hence the albumin/globulin ratio is relatively highest in the amniotic fluid, followed by the foetal serum and then the maternal serum. The gamma globulin, on the other hand, is highest in the foetal serum and lowest in the amniotic fluid. Another qualitative change which may be of significance is that the beta globulin fraction in most of the amniotic fluid electropherograms was slightly shifted towards the cathode end, when compared with the corresponding maternal or foetal serum.

Changes in the protein pattern with the progress of gestation were also observed. In the maternal serum, the total protein and the albumin percentage were, on average, lower at term than during pregnancy. In the serum of the

foetus at 19 weeks pregnancy, the total proteins were lower, the albumin percentage was higher, and the gamma globulin was much less than in the sera of mature foetuses. Moreover, an unidentified band, substance "X", was present. This band was absent in the sera of mature foetuses, maternal sera, and in amniotic fluids. In the amniotic fluid, the total protein content was comparatively low at the 12th week of pregnancy, increased to a peak between the 22nd and 26th week, and then declined towards term. There was no significant change, however, in the electrophoretic pattern.

These observations may be of significance when the problem of the origin of the foetal serum proteins and the proteins of the amniotic fluid is considered. The presence of substance "X" in the foetal serum early in pregnancy is not in good agreement with the hypothesis of passive placental transfer of maternal serum proteins to the foetal circulation and may suggest protein synthesis by the foetal liver. The high percentage of the gamma globulin fraction in the foetal serum suggests a selective transmission through the placenta from the maternal serum. As regards the origin of amniotic fluid proteins, a possible contribution by the amniotic epithelium may be suggested by the qualitative change in the beta-globulin fraction. This is also supported by the relatively high RNA concentration in the amniotic membrane, as shown in this work. The relatively high

level of albumin in the foetal serum and in the amniotic fluid can be easily explained on the basis of a passive transfer from the maternal serum, the relatively smaller albumin molecules being transferred more rapidly.

One conclusion of this study is that in individual cases the total protein concentration in the amniotic fluid and the albumin/globulin ratio do not provide reliable guides to the estimation of maturity. This is contrary to a claim by some authors.

The protein concentration in placental extracts at term was higher than at 19 weeks. On electrophoresis, the placental proteins were fractionated into two bands, one with a mobility similar to that of the maternal serum albumin and the other with a mobility between that of the beta-globulin and gamma-globulin fractions of the maternal serum. The latter band constituted the major part of placental proteins at term.

#### A study of the effect of putrescine and thalidomide in human leucocyte culture

The pattern of growth in human leucocyte culture, as measured by the estimation of the total DNA, RNA, protein, RNA/DNA ratio and protein/DNA ratio, was investigated under normal growth conditions, and in two controlled experiments under the effect of putrescine, thalidomide and a combination of the two drugs.

The addition of thalidomide to the culture medium

did not inhibit the growth of the culture, as indicated by the continuation of DNA synthesis. On the other hand, the presence of thalidomide in the culture treated with putrescine appeared to interfere with the growth stimulating effect of putrescine.

A possible explanation of this result may lie in the interaction between thalidomide and putrescine, an interaction which also occurs with other biogenic amines. Ample evidence points to the important biological role of these amines in early development. Whether, however, this interaction between the thalidomide and natural amines forms the basis of its embryotoxic activity remains to be assessed.

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