

Quantitative Studies on the Nucleic Acids in Human Placenta

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Human placental tissue offers a favourable material for the study of quantitative changes of the nucleic acids during development.¹

During an investigation concerning these problems it was found necessary to make some comparative studies on different procedures for the determination of the nucleic acids. This was considered important from two points of view. Up to the present, the human placenta has not been investigated for nucleic acids and might present some peculiarities. Some earlier comparisons of the procedures of Schneider² and Schmidt and Thannhauser³ have revealed discrepancies between the methods. As these procedures have never been run together with a method that yields pure nucleic acid fractions, it was thought that by comparing them with the method of Hammarsten⁴, more precise information concerning the source of error in the respective method would be obtained.

EXPERIMENTAL

Placentas from different stages of pregnancy were obtained at abortions, provoked on social-medical indications according to Swedish law, and at parturitions*. All the patients were in good health. When possible, the age of the placentas was estimated from the over-all (crown-heel) length of the foetus, applying Haase's (quoted from Wylie and Amidon⁵) rule. In some instances, however, the foetus was destroyed at operation, and in these cases the age of the placenta was calculated from the time of the last menstrual period.

Immediately after removal the placentas were freed from the foetal membranes and the decidua. The remaining foetal placenta was cut into small sections, wiped free of

* The material was kindly placed at my disposal through the generosity of Docent S. Karlson, M.D., Head of the General Maternity Hospital, Stockholm, and Dr. E. Westberg, M.D., Head of the Department for Gynecology and Obstetrics at St. Erik's Hospital, Stockholm.

blood and placed in a large volume of absolute alcohol. The dissection of the foetal portion of the placenta from the maternal elements was in some cases rather difficult in the fresh state, especially as it had to be done very quickly. It was much easier carried out after the specimens had been fixed in alcohol for some time.

The placentas were homogenized in alcohol in a Turmix blender. The homogenates were filtered with the aid of a Buechner funnel, repeatedly washed with ethyl ether and allowed to dry at room temperature.

Extraction of trichloroacetic acid- (T.C.A.) soluble phosphorus. About 100 mg of the dry powder is suspended in 15 ml of ice-cold 10 % T.C.A. The suspension is allowed to stand at 0° C for 60 minutes. It is then spun in a refrigerated centrifuge. The tissue is resuspended three times in the same amount of T.C.A., the centrifugate always being discarded.

Extraction of lipid-soluble phosphorus. To remove the T.C.A. the tissue is suspended twice in 80 % alcohol and then washed three times in absolute alcohol. It is then again suspended in a large volume (50 to 80 ml) of an ethyl ether-alcohol mixture (1 : 3). The suspension is boiled for two hours under reflux and then washed twice in alcohol.

Vibration. The tissue residue is then thoroughly homogenized in a Turmix blender and placed in a thick-walled glass tube with a capacity of about 100 ml. This is filled with glass beads, and alcohol is poured into the interstices. The specimen is vibrated for 96–120 hours in the manner prescribed by Hammarsten⁴. The tissue is then washed twice with ethyl ether and allowed to dry at room temperature.

Extraction of nucleic acids according to the method of Hammarsten. The extraction procedure outlined by Hammarsten was followed. Some details, however, may be pointed out. The tissue is extracted thirteen times. The last extraction is done over-night. This comprehensive extraction procedure was found to be necessary particularly to obtain maximum yield of pentose nucleic acid (P.N.A.). After extraction and centrifugation the polynucleotides are precipitated as copper salts. The collected supernates are allowed to stand for 30 to 32 hours.

After alkaline hydrolysis the desoxy-pentose nucleic acid (D.N.A.) is precipitated with 0.1 M lanthanum nitrate in 0.01 N H₂SO₄⁶. The precipitate is finally dissolved in a small volume of 1 M K₂CO₃.

The tissue residue after the extractions with the salt solutions, and the undissolved precipitate after the potassium-urea extractions, are repeatedly washed with distilled water and combined.

Nucleic acid extraction according to the method of Schneider. Between 30 and 60 mg of dry tissue is suspended in 2.5 ml of 5 % T.C.A. at room temperature. After centrifuging the tissue is resuspended in 4.5 ml of T.C.A. and allowed to stand at 90° C for 20 minutes. The residue is washed with 2.5 ml of T.C.A. at room temperature. The supernatant solutions are collected and diluted with distilled water to a given volume.

Separation of nucleic acids by the Schmidt and Thannhauser procedure. 75 to 150 mg of dry tissue is suspended in a given volume of 1 N KOH, and hydrolysed in the water bath at 37° C for 24 hours. To 2 ml of this solution is added 2.5 ml of an ice-cold mixture of 5 % T.C.A. and 6 N HCl (5 : 1). The mixture is spun in the refrigerated centrifuge. The precipitate is washed twice with 5 % T.C.A. The supernatant solutions, containing also P.N.A., are diluted with distilled water to a given volume. The precipitate, containing among other substances, D.N.A. and protein, is dissolved in a given volume of 1 N KOH.

Quantitative determination of nucleic acids. Nucleic acids, extracted and separated according to Hammarsten's technique and separated by the Schmidt and Thannhauser method, are estimated as phosphorus. The phosphorus is determined according to Teorell's ⁷ modification of Fiske and SubbaRow's ⁸ method.

The nucleic acids in the T.C.A. extract are estimated colorimetrically as sugars. For the determination of D.N.A. the present writer has worked out a modification of Dische's ⁹ cysteine reaction. A detailed account of this method is given elsewhere ¹⁰. The regression line for the relation between concentration and absorption is satisfied by the equation $y = -0.056 + 0.1054 x$, y indicating the extinction. The amount of D.N.A. phosphorus present is obtained by using this formula. The assay of P.N.A. is made according to Dische's ¹¹ basic cysteine reaction. Its adoption to T.C.A. extracts has been described elsewhere ¹². The extinction of an amount of P.N.A., equivalent to 1 μ g phosphorus, is 0.062, under the experimental conditions described.

All colorimetric determinations were carried out in a Beckman quartz spectrophotometer, using a 1 cm cell.

RESULTS

The analytical results are summarized in Table 1. Every figure is the mean of duplicates. It will be seen that there is good agreement between the values obtained by the method of Hammarsten and by the application of the cysteine reactions to the hot T. C. A. extract. The Schmidt and Thannhauser values, on the other hand, show considerable divergencies.

The amount of nucleic acids per unit dry tissue powder decreases with the aging of the placenta. This decrease is brought about by the reduced P.N.A.

Table 1. Comparison between results obtained with different methods.

Method		Hammarsten							T. C. A. extraction							Schmidt-Thannhauser			
Weeks of pregn.	P _{tot}	NAP	PNAP	DNAP	P _{res}	P _{tot} - P _{est}	PNAP/DNAP	NAP	PNAP*	DNAP*	P _{extr}	P _{extr} - P _{NAP}	P _{res}	PNAP/DNAP	NAP	PNAP	DNAP	PNAP/DNAP	
																			9
12	6 580	4 105	2 315	1 790	495	1 980	1.30	4 260	2 495	1 765	6 125	1 865	455	1.41	6 580	4 000	2 580	1.55	
24	5 248	4 075	1 805	2 270	327	846	0.80	4 005	1 760	2 245	4 750	745	498	0.78	5 248	2 878	2 280	1.20	
40	4 533	3 250	1 210	2 040	374	909	0.59	3 295	1 235	2 060	4 165	870	368	0.60	4 533	1 943	2 590	0.75	

P_{tot}: phosphorus left after lipid and cold T.C.A. extraction.
 NAP: nucleic acid phosphorus.
 PNAP: pentose nucleic acid phosphorus.
 DNAP: desoxypentose nucleic acid phosphorus.
 P_{res}: residual phosphorus in tissue after extraction.
 P_{est}: NAP + P_{res} according to Hammarsten.
 P_{extr}: phosphorus extracted by Schneider's procedure.
 The figures are given as μ g phosphorus/gram dry tissue powder.

* Calculated from sugar determination.

content. The D.N.A. increases slightly. The P.N.A./D.N.A. ratio will thus show a marked change during the course of pregnancy.

The hot T.C.A. extract contains a considerable amount of phosphorus, which is probably not nucleic acid phosphorus as calculated from the sugar determinations. An equal amount of phosphorus is extracted by the method of Hammarsten, but is not precipitable with copper.

There is an appreciable quantity of phosphorus left in the tissue residue after both extraction procedures.

DISCUSSION

The question of the ability of T.C.A. to remove acid-soluble phosphorus has been discussed by several authors. Barrenscheen and Peham¹³ consider that extraction with 10 % T.C.A. at 0° C removes quantitatively the nucleosides and nucleotides. There are, however, also other fractions which have to be removed, and Davidson, Frazer and Hutchison¹⁴ have made a comprehensive investigation of this question. They compared the results following three, six and twenty extractions with 10 % T.C.A. There was little difference after three and six times. Twenty extractions gave a larger yield, but the authors conclude from turnover rate determinations that this phosphorus is not only acid-soluble phosphorus, which has not been removed by earlier washings, but also phosphorus split off from other fractions by T.C.A. hydrolysis. On the other hand, these authors hold that, however numerous the washings, the contamination of protein-bound phosphorus with traces of inorganic phosphate cannot be ruled out. This is of course of utmost importance in tracer work but has no bearing on the results in the present investigation. Four extractions with 10 % T.C.A. at 0° C were supposed to be sufficient.

To find out whether the lipid extraction was complete, two of the placentas were further twice extracted with a CHCl₃-alcohol (1 : 3) mixture, each time for 90 minutes, in the water bath at 60° C. The amount of phosphorus in the tissue after this treatment was exactly the same as before.

The values obtained by the extraction procedure of Schneider, together with the colorimetric methods used here for the determination of the sugar components of nucleic acids, are in good agreement with the results obtained by Hammarsten's method. The possible effect of several substances on the P.N.A. determinations in the hot T.C.A. extract has been discussed earlier.¹² Direct evidence of the possibility of determining the nucleic acids by the colorimetric sugar reactions has been produced^{10,12}. The agreement between the results obtained by Hammarsten's method and the T.C.A. extraction procedure affords strong indirect evidence that the nucleic acid determinations, based

upon estimation of nucleic acid-bound sugar as performed in this investigation, may be considered reliable. When separation of the nucleic acids is unnecessary, the T.C.A. extraction procedure offers an accurate and convenient means for quantitative determination of nucleic acids.

Contrary to the findings of Schneider^{2,15} all the phosphorus in the hot T. C. A. extract of placental tissue could not be accounted for by nucleic acids as calculated from sugar determinations.

The method of Schmidt and Thannhauser has been repeatedly criticized and Schneider¹⁵ made a comparison of his method and that of Schmidt and Thannhauser. He investigated the different Schmidt and Thannhauser fractions for phosphorus, pentose and desoxy-pentose. The values obtained by the sugar determinations showed good agreement with those obtained by his own method. On the other hand, he states that "results based upon phosphorus determinations were less consistent and reliable". From his tabulated values it will be seen that the amount of phosphorus in the tissue residue after extraction with hot T. C. A. is fairly high, especially in liver, kidney and brain tissue, in the brain tissue amounting to about 63 per cent of the nucleic acid phosphorus. Schneider¹⁵ does not make any comment on this fact. Davidson, Frazer and Hutchison¹⁴ made a comprehensive investigation on the protein-bound phosphorus fractions in rat and rabbit liver. They too found considerable quantities of phosphorus in the residue after hot T. C. A. extraction. They demonstrated that the bulk of this phosphorus cannot be regarded as phospho-protein and assumed that the hot T. C. A. extraction is incomplete. These authors made no determinations of the amount of the different nucleic acids in the hot T. C. A. extract. From their results, however, it is clear that the amount of phosphorus in this extract is less than the combined amounts of phosphorus in the different Schmidt and Thannhauser nucleic acid fractions. They also state, "It would appear probable, therefore, that fraction A₂S (corresponding to the P. N. A. P. fraction in this investigation) contains phosphorus compounds other than the acid-soluble ribonucleotides and the inorganic phosphate derived from phospho-protein".

With both the Hammarsten and the T.C.A. extraction procedure I obtained a large phosphorus fraction in the present material, which probably is not nucleic acid phosphorus. In the Schmidt and Thannhauser procedure, however, this fraction will be determined as nucleic acid phosphorus. In an early placenta the fraction amounts to 44 per cent and in a late case to 26 per cent of the nucleic acid phosphorus.

It is probable that the residues after the salt as well as the T.C.A. extraction procedure contain small amounts of nucleic acid. In two cases the residues after Schneider extraction were further extracted with T. C. A.

Table 2. Prolonged T.C.A. extraction of Schneider residue.

Weeks of pregnancy	Tissue residue	T.C.A. extract		
	P	P	PNAP	DNAP
40	121	170	83.0	17.5
24	182	332	86.0	19.3

P: μg phosphorus/gram dry tissue powder.

PNAP: μg pentose nucleic acid phosphorus, calculated from sugar determination.

DNAP: μg desoxypentose nucleic acid phosphorus, calculated from sugar determination. For further explanations, see text.

at 90° C for 60 minutes. Phosphorus in residue and extract and nucleic acid-bound sugars in extract were determined. The results are summarized in Table 2. These nucleic acids amount to 3.3 per cent of the total nucleic acids in one case, and to 2.6 per cent in the other. Assuming that the same amount is still left in the residue, though this is not probable, the nucleic acids non-extracted by the Schneider procedure would amount to between 5 and 6 per cent.

SUMMARY

The nucleic acid content of the human placenta during development has been determined using different methods.

1. There was good agreement between the results obtained by Hammarsten's method and by the application of the cysteine reactions to the hot T. C. A. extract. The Schmidt and Thannhauser values showed considerable divergencies.

2. A large, probably non-nucleic acid fraction, in a young placenta amounting to 44 per cent of the nucleic acid phosphorus and in an old placenta to about 26 per cent, is extracted by the methods of Hammarsten and Schneider. This fraction will not be determined as nucleic acids when using the Hammarsten procedure or the cysteine reactions on the hot T. C. A. extract, but introduces a large error when the nucleic acids are determined according to the Schmidt and Thannhauser procedure.

3. After the extraction procedures of Hammarsten and Schneider the tissue residue contains a considerable quantity of phosphorus, constituting about 12 per cent of the nucleic acid phosphorus. It has been demonstrated that the Schneider residue probably contains 5 to 6 per cent of the total amount of nucleic acids.

4. A decrease of P. N. A. per unit of dry tissue powder was obtained with all the methods, and when applying salt or T. C. A. extraction a small increase of D. N. A. was obtained. The P. N. A./D. N. A. ratios decrease considerably with the aging of the placenta.

For valuable discussion and advice I desire to acknowledge my great indebtedness to Professor E. Hammarsten and to Doctor Bo Thorell.

For this investigation the author received a doctoral bursary and a grant from the Reserve Funds of Karolinska Institutet.

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Received November 18, 1952.