A STUDY OF THE PREPARATION OF THYMUS NUCLEIC ACID ACCORDING TO GULLAND *ET AL.*

by

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One of the most used methods for the preparation of thymus nucleic acid is that described by GULLAND and co-workers[1] founded on the original work of SEVAG *et al.*[2].

The method consists of repeated treatments of freshly prepared thymus nucleoprotein in 10% NaCl with amyl alcohol and chloroform. The chloroform serves, according to SEVAG, as a liquid adsorbent. After each treatment the denatured protein is centrifuged away. When protein precipitate is no longer formed, methyl alcohol is added, giving a threadlike precipitate of DNA.

The GULLAND method has never been closely investigated or discussed. It must, however, be of great interest to know the yield of DNA and to follow the gradual removal of protein. A closer investigation of the preparation methods would for instance give a picture of the treatment necessary for a complete separation of protein and acid.

The method is based upon the implicit assumption that nucleic acid is freed from the protein, each treatment with amyl alcohol and chloroform giving a solution containing less protein and relatively more nucleic acid.

A quite different picture, however, is given by results from analyses made on samples from each treatment. In the following this picture is drawn, leading up to a preliminary discussion on the protein separation mechanism.

EXPERIMENTAL

The method of GULLAND *et al.* was used with small modifications, all of which were dictated by the fact that GULLAND and his co-workers started with rather large quantities of thymus, whereas I used only a few hundred grams in each preparation. For details see an earlier article[3]. All analyses were made on fresh material. The solutions were used after only a few days storage in the cold and none of the intermediate products were dried.

After each treatment with amyl alcohol and chloroform, the chloroform was blown off, keeping the salt concentration as constant as possible, and the remaining mixture was centrifuged, samples were taken from the supernatant liquid and the precipitate of denatured protein was collected, washed with ethyl alcohol, and redissolved in fairly strong NaOH.

The nitrogen content of all solutions was determined by the Kjeldahl method.

The absorption in UV-light of the different samples, all diluted to pH 12, was measured in a Beckman spectrophotometer against NaOH solutions of the same pH. The amyl alcohol used was also measured as a control.

The extinction was determined for wavelengths between 235 and 400 mμ. The maximal absorption in all measurements fell at 258–260 mμ.

In Table I the quotients between the maximum extinction coefficients and the nitrogen content in mg/ml are given for the original NP, for the solutions and redissolved

References p. 47.

precipitates after the first five alcohol-chloroform treatments, for the redissolved end products of DNA and for the mother solution from which DNA was precipitated with methyl alcohol. As the absorbing effect is associated with the nucleic acid part and the nitrogen content is approximately the same in the nucleic acid and the protein, the quotients will directly reflect the change in nucleic acid content of the samples.

The values for the redissolved denaturation products (given in brackets in Table I), and for the solution after only one treatment are less precise than all the other values in the table, owing to opalescence giving a relatively high unspecific absorption which had to be corrected for [4] or, in the case of the precipitate after treatment No. 3 (Table I), when only rather small amounts of material were available. In all other cases the extinction at 350–400 mμ was less than 1% of the extinction at 260 mμ and no correction was made.

The values for original NP, measured on rather concentrated solutions, is somewhat high compared with earlier determinations[3,5] which gave quotient values of 72–74.

After treatment No. 6 no samples were taken in order to conserve material for the precipitation of DNA.

TABLE I

QUOTIENT BETWEEN THE MAXIMUM EXTINCTION COEFFICIENT (pH > 12) AND NITROGEN CONTENT IN mg/ml FOR NP SOLUTIONS AFTER REPEATED TREATMENT WITH AMYL ALCOHOL—CHLOROFORM (pH 6.3)
The values in brackets are for the redissolved protein precipitates.

Original NP	*After treatment*						*DNA precipitate*	*DNA mother liquid*
	1	*2*	*3*	*4*	*5*	*6*		
78	89 (62)	91 (88)	91 (70)	88 (—)	85 (—)		140*	—
76	— (67)	83 (66)	86 (67)	87 (—)	87 (—)	—	144	66
76	92 (67)	88 (70)	90 (54)	89 (—)	86 (—)	—	150	65

* after only 5 treatments.

The three preparations in Table I were all performed buffered with phosphate to pH 6.3. Preparations were also made at higher pH values (see Table II). The sensitivity to the denaturing agent increased, however, with higher pH and especially at pH 12–12.5 the amount of material in solution decreased rapidly.

TABLE II

QUOTIENT BETWEEN MAXIMUM EXTINCTION COEFFICIENTS (pH 12) AND NITROGEN CONTENT IN mg/ml FOR NP SOLUTIONS AFTER REPEATED TREATMENT WITH AMYL ALCOHOL—CHLOROFORM AT DIFFERENT pH VALUES. COMPARE ALSO TABLE I.

pH	*Original NP*	*After treatment*				
		1	*2*	*3*	*4*	*5*
8.5– 8.7	78	98	90	91	90	96
12.0–12.5	78	93	90	90	—	—

The quotients were measured for different salt concentrations, whence of less than about 15% a very small increase was found. Above this value, the increase was greater. If the solutions were allowed to stand for several weeks between the last treatments with amyl alcohol and chloroform, there was a marked increase in the quotients.

References p. 47.

The yield of DNA with the normal treatment (Table I) was 5–10% of that theoretically possible in terms of the DNA content of the starting nucleoprotein.

At pH 6.3 the yield increased with increasing quotients.

Two preparations of NP which had been treated six times were investigated in the Tiselius' electrophoresis apparatus after dialysis overnight against 1 *M* NaCl buffered to pH 9.5 with borate-NaOH buffer. Two peaks were seen in the electrophoresis diagram. One was very small and moved very slowly. The other was found to have a mobility towards the anode of $9.3 \cdot 10^{-5}$ and $9.6 \cdot 10^{-5}$ cm²/volt for the two different runs. The mobilities are in good agreement with those found for ordinary nucleoprotein (the faster being the less viscous) as can be seen from the values given by FLEMING AND JORDAN[6]. The large peak was very sharp and thin owing to the high viscosity and therefore it was not possible to measure its area.

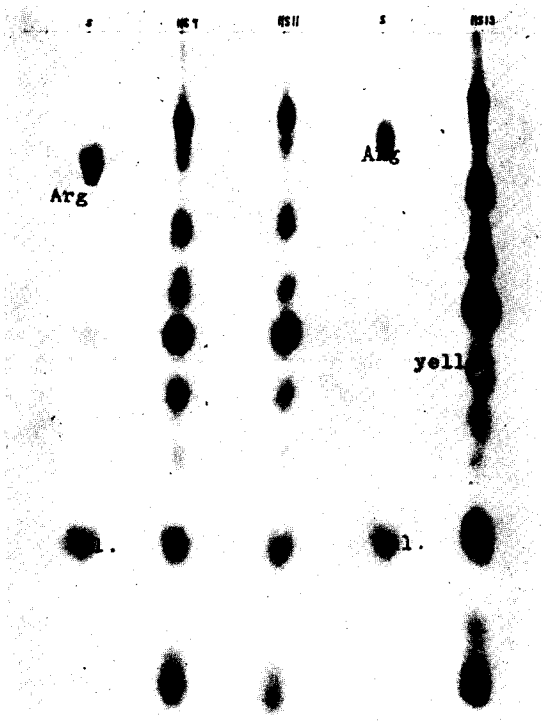

Fig. 1. Three differently treated preparations of nucleoprotein. The preparation giving the chromatogram to the right was treated fewer times with amyl alcohol — chloroform than the other two. This is the only one showing the yellow spot of proline.

DISCUSSION

From Tables I and II it is obvious that there is no large change in the proportion between nucleic acid and protein after each treatment, except for the increase after the very first one. Obviously, too, one is also throwing away a large amount of nucleic acid with the "protein" precipitate. It is also striking that, when one adds methyl alcohol, only a portion of the absorbing material is precipitated as is seen from the quotient of the remaining mother liquid. This quotient is, however, lower than the one for the original nucleoprotein. In all, the DNA yield is only about one tenth of that theoretically possible.

FLEMING AND JORDAN[6] have shown, in an electrophoretical study, that NP dissociates into a nucleoprotein part and a smaller protein part, as represented by the following equation

$$(np)' \rightleftharpoons p + (np)''$$

The dissociation increases with increasing pH and at pH 12 it has been shown that there is no (np)′ left.

As is seen from Tables I and II, the quotient for the nucleoprotein solution increases

References p. 47.

from 77 to a constant value of about 89. This may be described as a result of the dissociation and a selective effect of the chloroform as described by SEVAG *et al.*[5]. The precipitate being richer in the protein part (p) than the supernatant.

The electrophoresis diagram for six times-treated NP showed, as told above, one large peak, moving with about the same speed as nucleoprotein and one small peak moving very slowly. Compared with FLEMING AND JORDAN's diagram the component giving the small peak ought to be protein (p), the peak for treated NP being smaller than for the untreated preparation.

When nucleic acid is precipitated from the solution the quotient decreases to 66.

As we know that there may be free protein (p) in the solution, we can calculate the composition of this mixture on the assumption that it contains (p) and a nucleoprotein with an absorption—nitrogen quotient of 74. Compared with the quotient 66 for the mixture, this will give the amount of free protein as 11% of the total.

We can now also compare the original quotient (77) with the quotient of the treated nucleoprotein solution (89). This would give the following picture of the dissociation. 100 grams of (np)′ with an DNA content (as determined earlier) of 44% would give 86 grams of a mixture of (np)″ and (p) with a DNA content of 51%. 14 grams (p) is thrown away in the precipitate (together with nucleoprotein not counted within the 100 grams of starting material). The mixture of (np)″ and (p) contains 9 grams of (p) (see the above assumption), total (p) then being 23 grams and 77 grams of (np)″ with a DNA content of 57%. If, instead, we use the value of the DNA content of the original nucleoprotein of 50% given by FLEMING AND JORDAN in their experiment at pH 12 the amounts of (p) and (np)″ will naturally be the same, but (np)″ will have a DNA content of 65%. This calculated DNA content is the same as that experimentally found by FLEMING AND JORDAN by analysis of the contents in their electrophoresis cells after separation. It is not possible, however, to compare the calculated values of the amounts of the (np)″ and (p) with values found by FLEMING AND JORDAN, because, under the assumption that they do not lose any DNA in their analysis, they get a distribution of the protein part which is not in accordance with the values given for the nucleic acid content of the different components found in the same material. The sum of the components is, moreover, about 10% higher than the amount of starting material.

In the above calculations, the nitrogen percentage is assumed to be the same for nucleic acid and for the protein part. The same assumption was made by FLEMING AND JORDAN.

It may be stressed that the homogeneity and other similar characteristics of the substances represented by the symbols (np)′, (np)″ and (p) are not taken up for discussion.

SEVAG *et al.*[5] who first introduced this method of separation with amyl alcohol and chloroform, used it on nucleoprotein which had been treated at 50–55° in a solution containing sodium carbonate in order to separate nucleic acid from the protein before the treatment with the amyl alcohol-chloroform mixture. They worked on streptococcal nucleoprotein. MCCARTY AND AVERY[7] apply the amyl alcohol-chloroform treatment directly after the lysis of their pneumococcus cells. They state also that "A considerable amount of active material is entrained in the chloroform-protein gel". This "active material", found to be DNA, is later recovered. No analysis of the mother liquor from the precipitation with alcohol is made.

GULLAND *et al.* treated their material more vigorously than I, and obtained an output of slightly more than half the theoretically possible.

References p. 47.

In other publications this difficulty to separate and prepare nucleic acid from mildly treated nucleoprotein does not seem to have been encountered and may, especially in the extension of the work on the transforming factor of DNA-type, be a possible and dangerous pitfall.

From the comparison with the work of FLEMING AND JORDAN it can be assumed that only the already dissociated part of the protein will be removed. Nothing is yet known as to whether the small output of DNA received yields an acid which is representative of the whole starting material. To increase the output the equilibrium might be shifted or some sort of mild hydrolysis applied.

The purity of the preparations

An investigation was carried out on the amino acid contamination of DNA prepared according to GULLAND *et al.* and as described above.

GULLAND *et al.* worked, as already mentioned, with large amounts of starting material and tested the resulting end product of DNA for protein with relatively simple tests (the biuret test and the test according to SAKAGUCHI). They found that 9 treatments with amyl alcohol-chloroform was enough to give a pure product. Starting with a few hundred grams and centrifuging at higher speeds, seven treatments sufficed in this investigation to yield a nucleate which was negative in the above-mentioned tests as well as with ninhydrin. If this nucleate, however, is hydrolyzed in 9 *N* HCl at 110° for 24 hours in a sealed tube, a strong colour is then given by the ninhydrin.

Paper chromatography was carried out on hydrolyzed and on non-hydrolyzed DNA. More than 15 preparations were made and in no cases were spots given by the non-hydrolyzed material, whereas DNA which had been hydrolyzed gave spots for all the amino acids found in the original nucleoprotein, except proline.

From the picture of the three preparations (Fig. 1), all containing proline at the start but treated differently, proline can be seen to disappear in the NP solutions which have been treated the greatest number of times.

HAMER[8] has given the following list of amino acids contained in thymus nucleoprotein: alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, *iso*leucine, leucine, lysine, phenylalanine, serine, threonine, tyrosine, valine and proline. All these amino acids were also found in the nucleoprotein preparation which was used as starting material for DNA in this investigation.

DALY *et al.*[9] and ALLGÉN[10] add methionine to the list. This amino acid was also found in my nucleoprotein with microbiological tests. No attempt has been made to use this test on the DNA preparation. No cysteine, nor tryptophan was found. Recently HARPER AND MORRIS[11] have investigated the amino acid content of chick erythrocyte nucleohistone. The composition found was the same as for my nucleoprotein preparations.

The paper chromatography was carried out in butyl alcohol-acetic acid and phenol. The amount of non-hydrolyzed nucleate put on the paper was at least five times that of the hydrolyzed. All samples were desalted according to CONSDEN *et al.*[12] in a desalting apparatus of the Shandon type.

As a control the number of treatments with amyl alcohol-chloroform were increased to fifteen. No effect on the composition of the paper chromatogram was observed.

To determine the absolute amount of the "impurity", samples were hydrolyzed as above and the colour given by ninhydrin was measured according to MOORE AND STEIN[13] at a wavelength of 570 mμ in a Beckman spectrophotometer. As a comparative solution,

References p. 47.

a mixture of three parts of arginine and one part of each of the other amino acids found in the DNA was used. This mixture was diluted so as to give an absorption very close to that of the DNA solution.

Two determinations were made in this way on two different preparations. The amino acid content was found to be between 0.20 and 0.25% of the dry DNA, taken as acid. When the sample was hydrolyzed it became a little coloured. This does not seem, however, to disturb the measurements at the given wavelength to any significant degree. The amount of DNA was measured both by absorption measurements at 260 mμ and by weighing (the sodium content was determined as sulfate and weighed).

Great difficulty is introduced by the fact that adenine when hydrolyzed can give glycine[14,15]. It is obvious, however, that when adenine is bound to desoxyribose it does so only to a very small extent, if at all. ALLGÉN[10], who also points out this difficulty, has come to the same conclusion. In any case, the value 0.25% for the amino acid content must only be looked upon as an upper limit. A lower limit can be given by an estimation of the number of visible spots in the paper chromatogram produced by a given amount of DNA. This value is found to be 0.1%, which, however, must be somewhat low because of a small loss of material during desalting.

Most preparations were made at pH 6.3 but some were also performed at pH 7.0 and 8.7 without any difference. In one preparation, the preparation of the nucleoprotein itself was done at a higher pH, 7.2. This had no influence on the composition.

The investigation shows that methyl alcohol gives a precipitate of DNA which after washing is shown to contain only very small amounts of amino acids, although the mother liquid contains protein. The necessity of vigorous hydrolysis to obtain a positive ninhydrin reaction indicates that the amino acids are firmly bound to the DNA.

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SUMMARY

The method of GULLAND *et al.* of preparing DNA has been investigated. It is found that, when using as mild methods of preparation as possible, the output is 5–10% of the DNA in the nucleoprotein used as starting material. The amount of liberated nucleic acid corresponds to the amount of protein which dissociates according to FLEMING AND JORDAN. The prepared DNA contains between 0.1–0.25% of amino acids. A vigorous hydrolysis is necessary to get a positive ninhydrin reaction. All the amino acids of the nucleoprotein are found in the hydrolysate with the exception of proline.

RÉSUMÉ

La méthode de GULLAND *et al.* pour la préparation du DNA a été étudiée. En utilisant des méthodes de préparation aussi douces que possible, le rendement est de 5 à 10% du matériel de départ. La quantité d'acide nucléique libéré correspond à la quantité de protéine qui se dissocie selon FLEMING ET JORDAN. Le DNA préparé contient entre 0.1 et 0.25% d'aminoacides. Une hydrolyse poussée est nécessaire pour obtenir une réaction positive à la ninhydrine. On retrouve dans l'hydrolysat tous les aminoacides de la nucléoprotéine à l'exception de la proline.

References p. 47.

ZUSAMMENFASSUNG

Die Methode von GULLAND und Mitarbeitern zur Darstellung von DNA wurde untersucht. Es wurde gefunden, dass bei der Verwendung von so mild als möglichen Darstellungsmethoden die Ausbeute 5–10% des Ausgangsmaterials beträgt. Die Menge der in Freiheit gesetzten Nucleinsäure entspricht der Menge des nach FLEMING UND JORDAN dissozierten Proteins. Die dargestellte DNA enthält zwischen 0.1–0.25% Aminosäuren. Eine kräftige Hydrolyse ist nötig um eine positive Ninhydrinreaktion zu erhalten. Alle Aminosäuren des Nucleoproteins mit Ausnahme des Prolins werden im Hydrolysat gefunden.

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