A CRITICAL STUDY OF SOME COMMONLY USED METHODS OF PREPARING DESOXYRIBONUCLEATE

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In the preparation of biochemical substances from animal or plant tissues it is usually necessary to make a choice between a high yield and a high quality of the product.

Poor quality may result because of change in the natural structure of the material caused by too drastic treatment during the preparation. If, on the other hand, a mild method is used and a large amount of substance is obtained, this may contain a high percentage of impurity.

In the following the preparative methods for desoxyribonucleate of SEVAG, LACKMAN AND SMOLENS[1], GULLAND, JORDAN AND THRELFALL[2], HAMMARSTEN[3], SCHWANDER AND SIGNER[4] and KAY, SIMMONS AND DOUNCE[5] are compared.

In all these methods substances rich in nucleic acid are first prepared. KAY *et al.* start with a preparation containing chromatin threads. The other methods start with nucleoprotein which has been prepared by extracting the tissue—usually thymus glands—with distilled water or 1 *M* NaCl and then precipitating the nucleoprotein from the extract.

A closer investigation of the treatment necessary to free the nucleic acid from the protein moiety might also give some information about the bonds between the two components, as they exist *in vitro*.

Preparation according to SEVAG *et al. and according to* GULLAND *et al.*

In an earlier investigation[6] the preparative method of GULLAND *et al.* has been discussed. The nucleoprotein is first prepared according to MIRSKY AND POLLISTER[7], *i.e.* extracting thymus with 1 *M* NaCl and then precipitating by diluting to 0.14 *M* NaCl. The resulting nucleoprotein is repeatedly treated with a mixture of amyl alcohol and chloroform and the denaturated protein is sedimented by centrifugation.

It was found that if the work was carried out in the cold a very low yield of DNA (5–10% of the nucleic acid in the nucleoprotein) was obtained. In order to receive a higher yield SEVAG *et al.*, who originally introduced the amyl alcohol-chloroform method, have used a mild hydrolysis with sodium carbonate before starting the chloroform treatment.

If no form of hydrolysis is applied the yield of DNA that it is eventually possible to precipitate corresponds to the amount of protein that according to FLEMING AND JORDAN[8] is in dissociation equilibrium with the nucleoprotein.

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Since hydrolysis is necessary to obtain high yields, the association between protein and nucleic acid in salt solutions is apparently stronger than is generally considered.

The resulting nucleate prepared according to GULLAND *et al.* contained between 0.1–0.25% of amino acids.

Preparation according to HAMMARSTEN *and according to* SCHWANDER AND SIGNER

In HAMMARSTEN's method the nucleoprotein is dissolved in a saturated NaCl solution. The protein precipitates and after three days the solution is filtered and the sodium nucleate is precipitated from the filtrate by addition of alcohol.

The nucleoprotein used as starting material is obtained from the tissue by extracting three times with distilled water and is then precipitated from the extract with calcium chloride.

In two experiments of the present investigation each of the three aqueous extractions was followed separately. The tissue had been ground together with solid carbon dioxide and the first portion of distilled water was added to the mixture when it still contained a considerable amount of solid carbon dioxide. Sixteen hours later, when the temperature had risen to 0° C and there were still a few ice crystals in the liquid, the suspension was centrifuged in the cold. The supernatant solution was called solution A.

The two following extractions (called B and C) were performed at + 4° C for 24 hours before centrifugation. HAMMARSTEN points out that very little nucleoprotein dissolves in the first portion of distilled water. It is during the two following extractions of the tissue that a large amount of nucleoprotein goes into solution. This is quite in accordance with the dependence of nucleoprotein solubility on salt concentration[9]. The first addition of water does not decrease the salt concentration from about 0.14 *M* to a sufficiently low value about 0.03 *M* unless large volumes are used. It may be pointed out that this difficulty has not always been observed[10].

In the present investigation the protein precipitates obtained upon addition of calcium chloride were centrifuged and weighed. The wet substance from extract A weighed 7 grams, from B 110 grams and from C 40 grams. The starting material was 350 grams of thymus gland.

The nitrogen content and the UV absorption (at pH 12.5) of the resulting nucleates from extracts A, B, and C were determined—the nitrogen content according to Kjeldahl and the UV absorption in a Beckman spectrophotometer. Maximal absorption was found at 260 mμ. The absorption between 370 mμ and 400 mμ was less than 2% for solutions of the substances from extract A, and less than 1% for B and C, where the value at 260 mμ is taken to be 100%.

Since the UV absorption at 260 mμ is associated with the DNA part and the nitrogen contents of the DNA and the protein parts are of the same order of magnitude, the quotient between absorption and nitrogen will give an indication of the extent to which the nucleate preparation has been freed from protein.

In Table I the values for two DNA preparations are given. The extinction at 260 mμ (1.00 cm cuvette) has been divided by the nitrogen content in mg/ml. Between precipitation 1 and 2 the substance, following the preparation scheme, has been redissolved in distilled water.

The quotient between the extinction and the nitrogen content for pure DNA is about 160 (pH > 12), that for undialysed thymus nucleoprotein is about 74 and for dialysed about 88.

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TABLE I

THE QUOTIENT BETWEEN THE EXTINCTION AT 260 mμ (pH 12.5) AND THE NITROGEN CONTENT IN mg/ml FOR TWO PREPARATIONS OF DNA

Solution A: DNA from a distilled water extraction at 0° C and lower for 16 hours (see text).
Solution B and C: DNA obtained by two subsequent extractions of the same tissue at + 4° C for 24 hours.

	Solution A		*Solution B*		*Solution C*	
	Prep. 1	*Prep. 2*	*Prep. 1*	*Prep. 2*	*Prep. 1*	*Prep. 2*
1st precipitation (redissolving)	82	95	116	120	134	130
2nd precipitation	82	92	138	140	155	152

Thus, the end product from extract A shows only a small relative increase in DNA over an undialysed preparation. If the part of the protein which was in dissociation equilibrium with the nucleoprotein had been precipitated and lost by filtration that would account for the increase of the quotient. Preparations from B, on the other hand, show a large relative increase of DNA, while the end product from extraction C is a fairly pure nucleate.

The possibility to separate protein and nucleic acid by saturated NaCl thus seems to be dependent upon autolysis which has already been carried out by cellular enzymes. At a temperature between — 79° and 0° C the enzymes have little effect. If, however, the ground tissue is allowed to stand in contact with the extraction liquid for a longer time and at higher temperature + 4°, the protein and the nucleic acid can later be separated. This effect may also be influenced by the decrease in salt concentration.

The difference in pH between the three extracts is small. The pH of extract A is 6.5–6.6, of B and C one tenth of a unit lower.

The dependence of the DNA isolation procedure upon the step leading up to the nucleoprotein precipitation is further evidenced if one compares the results of extractions according to HAMMARSTEN and according to MIRSKY AND POLLISTER[7]. In the latter case the extractions are carried out in 1 *M* NaCl and the nucleoprotein extracts are then treated according to HAMMARSTEN, *i.e.* dissolved in a saturated NaCl solution, filtered and the sodium nucleate precipitated with alcohol.

(The MIRSKY-POLLISTER nucleoprotein is as usual precipitated by dilution to 0.14 *M* NaCl and then redissolved and reprecipitated in the same manner 6 to 8 times.)

The whole procedure is illustrated by diagram 1. From the diagram it can be seen that for the two preparations (II and III) where the initial steps follow the method of MIRSKY AND POLLISTER the saturated NaCl filtrate is poorer in UV absorbing material than the precipitate that is retained by the filter. The end products after redissolving twice and reprecipitating with alcohol give the quotients 90 and 89. These values are close to the quotient value for dialysed nucleoprotein (see above).

If instead the tissue is treated according to HAMMARSTEN from the start (preparation I), the precipitate in the filter contains only traces of absorbing material. The end product on the other hand gives the quotient 161 which is the same as for pure DNA.

In these preparations the nucleoprotein was allowed to stand in saturated NaCl

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for a longer period than the usual three days. Preparation I and II stood five days and preparation III eight days.

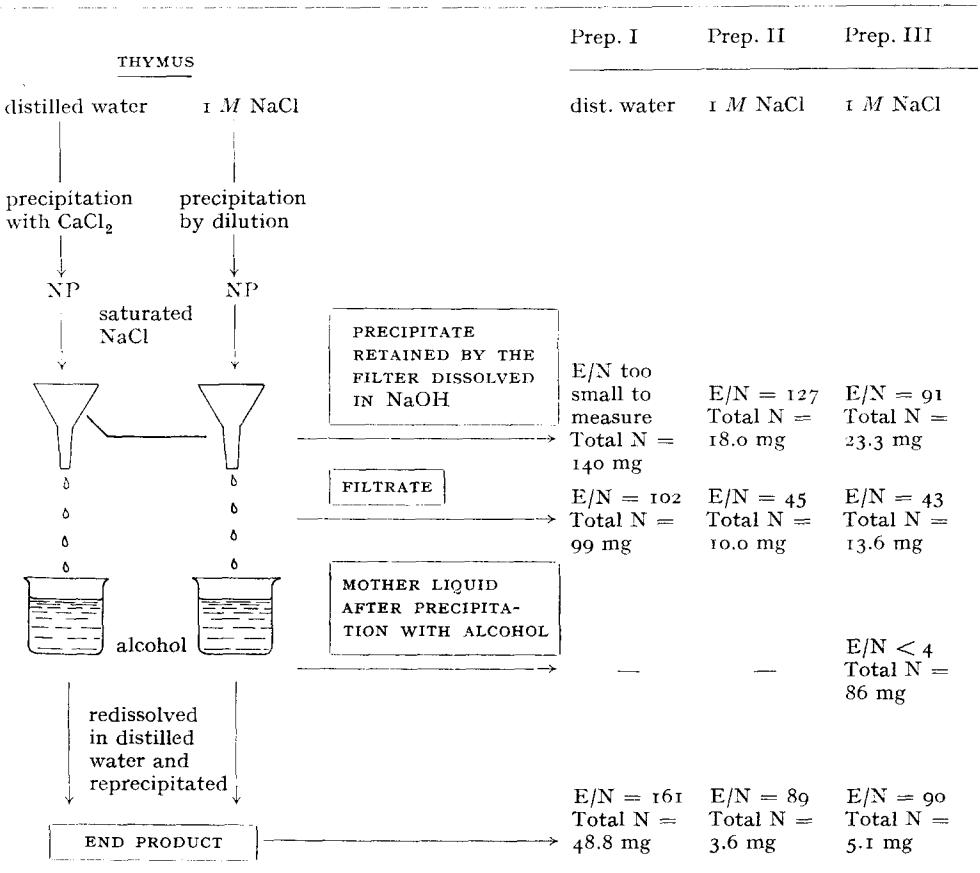

Diagram 1. The diagram shows the difference between the DNA prepared using HAMMARSTEN's method alone (preparation I) and the substance by first isolating nucleoprotein by the method of MIRSKY AND POLLISTER and subsequently using HAMMARSTEN's method (preparation II and III).
E = extinction at 260 mμ (1.00 cm cuvette, pH = 12.5)
N = mg nitrogen per ml

SIGNER AND SCHWANDER[11] have used nucleoprotein prepared according to MIRSKY AND POLLISTER to prepare DNA by the HAMMARSTEN method.

Their nucleoprotein was allowed to stand in saturated NaCl for 14 days. The aqueous solution of once precipitated DNA had to be centrifuged with the Sharples centrifuge in order to free it from insoluble material. The yield was relatively low, 5 grams sodium nucleate having been obtained from 700 grams of thymus, whereas HAMMARSTEN obtained 140 grams from 7000 grams of tissue. Since no analytical data are given a discussion of the quality of the end product is not possible.

SCHWANDER AND SIGNER[4] later substituted filtration with celite for the centrifugation in the Sharples centrifuge. The modified procedure takes one month. The yield is good, 1.8 grams of sodium nucleate from 100 grams of thymus, and impurity with

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protein amounts to only 0.2%. It must be pointed out, however, that the extraction of the tissue with 1 *M* NaCl was allowed to take "mehrere Tage". Sodium citrate was added to stop any enzyme activity. Since, according to WEBB[12], sodium citrate has no effect for instance on the activity of desoxyribonuclease from mammalian tissue the effectiveness of the procedure is questionable.

The DNA prepared completely according to HAMMARSTEN showed the normal increase in absorption[13] in ultraviolet light if alkali was added. The amino acid contamination was determined colorimetrically according to MOORE AND STEIN[11] after hydrolysis for 24 hours in boiling 7 *N* HCl. The standard consisted of a mixture of three parts of arginine, and one part of aspartic acid, glutamic acid, glycine, histidine, *iso*leucine, lysine, phenylalanine, serine, threonine, tyrosine and valine. The amino acid contamination varied from one preparation to another, the lowest values being about 1%. To diminish the amino acid content to 0.2–0.1% the lanthanum salt method of CHARGAFF *et al.*[15] was used.

The yields of DNA using HAMMARSTEN's method range between 1.5–2.0 g from 100 g thymus. From diagram 1 it is seen that nearly all the absorbing material in the nucleoprotein of preparation I is recovered in the end product, whereas only about one fifth of the nitrogen is recovered. The reason for this is obviously that the calcium chloride precipitates a mixture of nucleoprotein and other water-soluble proteins with a much lower content of DNA than is generally accepted for nucleoprotein (40–50%).

Preparation according to KAY *et al.*

A very good yield of DNA is obtained by the method of KAY *et al.*[5]. The DNA-rich substance prepared from thymus gland is treated with the detergent Duponol E. M. which contains dodecyl sulphate and its homologues. The protein precipitates and is centrifuged away. The nucleate is then precipitated fron the solution by alcohol. In this way 3 grams of nucleate are easily obtained from 100 g thymus. The content of amino acids is between 0.3–0.4%.

Detergents of the Duponol type form strong complexes with protein through their polar ends, mostly stoichiometrically[16]. These complexes precipitate. The dodecyl sulphate takes the place of the nucleic acid in the nucleoprotein complex. When the nucleoprotein is suspended in saline solution and treated with detergent, a large amount of the DNA is forced into solution (the solubility may be increased by a non-polar association between DNA and detergent). Afterwards, when NaCl is added to make the solution 1 *M*, the part of the nucleoprotein that has not been affected by the detergent also goes into solution. The insoluble protein-detergent complex can then be removed by centrifugation.

The DNA and the remaining nucleoprotein in the supernatant is precipitated by addition of alcohol. Thereafter the treatment with detergent can be repeated.

Thus by this method loss of DNA due to coprecipitation of nucleoprotein with protein is to a large extent avoided.

The nucleate is washed with alcohol and acetone. If the washing is repeated many times and done very carefully the preparation will only contain traces of detergent.

The contamination with detergent was calculated from the sulphur content, determined colorimetrically after burning with vanadium pentoxide according to KIRSTEN[17]. The sulphur content of the nucleate was less than 0.05%.

After the washing with alcohol and acetone the substance is dried in the air at

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room temperature. This treatment probably is the cause of some denaturation of the acid.

Thus non-dried DNA prepared according to HAMMARSTEN or GULLAND *et al.* gives an increase in UV absorption from 100 to 133 at 260 mμ if the pH is raised to 12, whereas DNA prepared according to KAY *et al.* gives an increase of only 18 units.

The small hump at 0.9% NaCl in the relative viscosity-NaCl concentration curve which is given by KAY's preparations was not found for my nucleates prepared by the same method. This may, perhaps, be due to the fact that higher speeds were used on the centrifuges in the latter case and that the relative viscosity was somewhat lower (4.5–5.5) than the viscosity found by KAY *et al.* for their preparations.

The preparative method using detergent can not only be used on material from thymus glands treated according to KAY *et al.* but also on nucleoprotein prepared with extraction with 1 *M* NaCl according to MIRSKY AND POLLISTER. This accords well with the theory of the detergent effect. Thus the affinity between the DNA and the protein in the nucleoprotein complex *in vitro* must be less than the affinity between the detergent and the protein.

ACKNOWLEDGEMENTS

The author wishes to thank Prof. A. TISELIUS for his kind advise and helpful discussions.

The sulphur analyses were kindly carried out by Dr. W. KIRSTEN, Institute of Medical Chemistry, Upsala.

This investigation has been financed by grants to the Institute of Biochemistry from the Rockefeller and Wallenberg Foundations.

SUMMARY

At least three different mild methods of desoxyribonucleate isolation are available at present.

1. The method of SEVAG *et al.* which gives a good yield but involves hydrolysis or (following GULLAND's modification) without hydrolysis gives a low yield.

2. The method of HAMMARSTEN, which gives a good yield but seems to depend on enzymic activity in the starting material. While the method is very mild protein contamination of the product may be relatively great.

3. The method of KAY *et al.*, which gives the highest yield, but some denaturation as judged by the UV absorption is caused by the washing and drying procedure.

RÉSUMÉ

Il existe actuellement au moins trois méthodes douces différentes d'isolement du désoxyribonucléate.

1. La méthode de SEVAG *et coll.* qui donne un bon rendement mais qui suppose une hydrolyse ou qui (selon la modification de GULLAND), sans hydrolyse, donne un rendement faible.

2. La méthode d'HAMMARSTEN, qui donne un bon rendement mais qui dépend, semble-t-il, de l'activité enzymatique du matériel de départ. Quoique cette méthode soit très douce, la contamination du produit par des protéines peut être relativement élevée.

3. La méthode de KAY *et coll.*, qui donne le meilleur rendement, mais dans laquelle les procédés de lavage et de séchage entraînent une dénaturation partielle, si l'on en juge par l'absorption en U.V.

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ZUSAMMENFASSUNG

Augenblicklich stehen zur Isolierung von Desoxyribonucleaten zu mindest drei verschiedene milde Methoden zur Verfügung.

1. Die Methode von SEVAG *et al.*, die bei einer guten Ausbeute aber gleichzeitig Hydrolyse verursacht, oder, nach der Modifikation von GULLAND, ohne Hydrolyse eine niedrige Ausbeute ergibt.

2. Die Methode von HAMMARSTEN, die eine gute Ausbeute ergibt, aber von der enzymatischen Aktivität des Ausgangsmaterials abhängig zu sein scheint. Da die Methode sehr mild ist, kann die Verunreinigung des Produktes mit Eiweiss relativ gross sein.

3. Die Methode von KAY *et al.*, die die höchsten Ausbeuten liefert, aber bei dem Wasch- und Trockenverfahren nach der UV-Absorption zu urteilen geringe Denaturierung verursacht.

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Received October 22nd, 1953