cubated with radioactive iodide, were found to convert the inorganic iodine into protein bound form[6].

SUMMARY

The construction and operation of an hydraulically-driven tissue homogenizer are described.

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A RAPID METHOD FOR PREPARING POLYMERIZED DEOXYRIBONUCLEIC ACID FROM TISSUES, BASED ON SEPARATION OF NUCLEI*

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Present methods for isolating deoxyribonucleic acid (DNA) suffer from one or more of the following disadvantages: (1) they are time-consuming and cumbersome; (2) they can be used only for tissues like thymus and sperm, in which the nuclear/cytoplasmic ratio is quite high; or (3) they require use of either strong acids and bases or high temperatures. Recently we reported a method for the isolation of DNA from fish sperm which, because of the mild conditions employed, yielded a highly polymerized product[1]. But this method, as well as those so far devised for thymus, cannot readily be applied to most other tissues because of their relatively high content of non-nuclear materials. It seemed to us that the isolation of DNA from ordinary tissues could be accomplished provided those tissues could be homogenized in such a way as to release intact nuclei, and provided the nuclei could be separated from the large mass of non-nuclear materials. A rapid method for preparing an homogenate with a high yield of intact nuclei was presented in the preceding paper[2].

The present report describes a method for the quick separation of these nuclei from extraneous cellular elements. This method makes use of the strong affinity of nuclei for a negatively-charged, inert material, and thereby avoids the usual lengthy centrifugation procedures. Since the adsorbing material appears to have no affinity

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for the non-nuclear elements of the homogenate, a concentrate of nuclei can be prepared from most mammalian tissues in a matter of 15 minutes. Then, after the nuclei have been dispersed with a strong salt solution, their DNA can be separated from their basic protein[3] which adheres tenaciously to the adsorbing material. Since cytoplasmic ribonucleic acid (RNA) is removed during isolation of the intact nuclei, the DNA sample can be freed of the small amounts of RNA of nuclear origin by precipitation techniques. In this way, DNA can be isolated, in a form quite free of protein and RNA, in about an hour.

EXPERIMENTAL

Material

Potassium arsenate stock solution. A 0.2 *M* solution of KH_2AsO_4 was prepared, and its pH was adjusted to 7.0 by titration with a concentrated KOH solution. This neutral solution was then diluted with water until its molarity was 0.014.

Homogenization medium. 23.9 g of solid KCl were dissolved in 2 liters of the 0.014 *M* potassium arsenate solution, and the mixture was diluted to 4 liters. This yielded a medium which was 0.007 *M* in arsenate ion and 0.038 *M* in chloride ion.

After a series of trials with buffers of varying composition, the 0.007 *M* arsenate solution proved best for maintaining normal nuclear dimensions and for preventing granulation of the liver nucleus—a common occurrence in sucrose and phosphate buffers. Arsenate was also selected because it is a strong nucleodepolymerase inhibitor[4].

The homogenizer, the Büchner funnels, the filter flasks, and the beakers were cooled with brine at —5°. The brine was circulated through jackets surrrounding the homogenizer and vessels, by means of a centrifugal pump. A mechanical mixer equipped with a glass, or preferably a Teflon, paddle was used for all mixing operations. The abrasive nature of diatomaceous earth used in the procedure described below precludes the use of a stainless-steel mixing paddle.

The amounts of tissues used for homogenization were varied according to their DNA contents. The following amounts were found adequate: 10 g of liver; 10 g of kidney; 5 g of hepatocarcinoma; 5 g of most glandular tissues; and 3 g of spleen. These amounts of tissue were mixed with 40 ml of the buffer, and homogenized.

Separation of nuclei from homogenate

The homogenate obtained from the hydraulic homogenizer under the conditions previously described[2] was quickly filtered through a single layer of 9XX nylon cloth. The retained connective tissue was washed with 20 ml of homogenization medium, and the filtrates were collected in a vessel immersed in an ice bath. 4.2 g of dry Hyflo Super Cel (diatomaceous earth) were added to the filtrate, and the mixture was stirred at high speed, for about 10 seconds, with a Teflon paddle. The mixture was poured into an 111-mm cooled Büchner funnel containing a filter paper (11 cm) overlaid with 2.9 g of Hyflo Super Cel which had been suspended in water and sucked very dry. Provided the conditions described above were maintained, filtration with the aid of suction was rapid. The pad was washed with chilled homogenization solution until the filtrate was colorless; then another 100 ml of this solution were sucked through the pad. As a rule, a total of 300 ml of solution suffices to remove non-nuclear materials. When sucked dry following this treatment, the pad will be white, and indistinguishable in color from the Super Cel itself. A yellowish pad indicates incomplete homogenization, which prolongs the filtration time because of the entrapment of whole cells and mitochondria on the filter pad.

Isolation of DNA from the nucleus preparation

The Super Cel pad with its adsorbed nuclei was lifted out of the funnel, and sufficient saturated solution of NaBr (15 to 20 ml) was added to it, with stirring, to give a free-

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flowing slurry. The addition of the highly concentrated salt solution burst the nuclei, dissociating the nucleoprotein and leading to a metathetical exchange reaction[3]. During stirring, a small amount of solid NaBr was added to insure saturation of the mixture with the salt. The saturation with NaBr also aided the removal, by precipitation, of some of the protein, but the bulk of the nuclear proteins (histones) was firmly adsorbed by the diatomaceous earth. The suspension was next filtered through a 5.5-cm, jacketed Büchner funnel, and the filtrate was collected in a jacketed, 125-ml suction flask. Two sheets of Whatman No. 1 filter paper overlaid with 2 mm of Hyflo Super Cel were used for the filtration. To remove residual nucleic acids, the pad was washed three times with 4-ml portions of a chilled, saturated NaBr solution.

The very viscous, clear filtrate containing all the NaDNA was diluted with 1 part water to 5 of filtrate. While the solution was being swirled by hand, 2 to 3 volumes of alcohol were added to it, and the precipitated, fibrous mass of NaDNA was removed with a glass rod. (The fibers should not be compressed on the rod since this prolongs the time required for their re-solution.) The fibrous mass was removed from the rod with a sharp scalpel, and placed in a small, jacketed beaker containing 20 ml of homogenization medium. The mixture was stirred with the Teflon paddle until all the nucleic acid was dissolved. This required about 10 minutes. To this solution were added 2 g of Hyflo Super Cel, followed by solid NaBr to saturation. This mixture was then filtered through a chilled, 5.5-cm Büchner funnel fitted with a filter pad as described above. The viscous, completely colorless filtrate was diluted with water (1 part for each 5 parts of filtrate), and the DNA was again precipitated with 2 to 3 volumes of alcohol. For purposes of subsequent chemical analysis, it was convenient to store the white, fibrous NaDNA in 70% alcohol until it was eventually completely desalted in a smaller version of a continuous-extraction apparatus described earlier[1]. The nucleic acid sample was then dried over NaOH and $Mg(ClO_4)_2$ at room temperature, *in vacuo* (0.4 mm Hg), for 12 to 18 hours. The salt-free preparation of DNA obtained was fibrous and, in this solid state, contained 9 to 13% water. It was readily soluble in water. If the nucleic acid were wanted for physical studies, we stored the freshly prepared samples in solutions of either sodium arsenate buffer or sodium citrate[17] at low temperature.

TABLE I

SOME CHEMICAL AND PHYSICAL PROPERTIES OF DNA ISOLATED BY PROCEDURE DESCRIBED HERE

DNA source	*N* %	*P* %	*N/P*	*Absorbancy (1%, 1 cm, 258 mμ max, 25° ± 0.4°)*				
				μ = 1.0 (as KCl)	*pH*	*μ = 0*	*pH*	$\frac{A_S\ (258\ m\mu),\ \mu = 0}{A_S\ (258\ m\mu),\ \mu = 1}$
Mouse liver (C57 leaden)	14.47	8.05	1.79	161	6.7	196	7.1	1.21
Mouse liver (C57 leaden)	14.64	8.13	1.80	149	6.3	182	6.9	1.22
Mouse hepatocarcinoma (C_{954})*	13.70	7.90	1.73	173	6.4	208	6.9	1.20
Mouse hepatocarcinoma (C_{954})*	13.89	7.94	1.75	156	7.7	—	—	—
Mouse kidney	14.57	7.87	1.85					
Mouse spleen	13.89	7.74	1.79					

* See first footnote p. 265.

The phosphorus content of the nucleic acid samples was determined colorimetrically by the Fiske-Subbarow procedure[6], modified by the use of ferrous sulfate

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as a reducing agent (precision*, 100 ± 0.6 %). Nitrogen analyses were carried out with a Jenden-Taylor micro-Kjeldahl apparatus[7] on nucleic acid hydrolysates prepared with sulfuric acid, potassium sulfate, and mercury catalyst (precision, 100 ± 0.5%).

Purity of isolated DNA

Tests for salts and protein. Our procedure gave nucleic acid samples free of occluded salt and contaminating protein. It was not possible to demonstrate the slightest trace of NaBr by use of a microchemical test in which bromide ion is liberated as HBr by concentrated sulfuric acid[10]. A nucleic acid hydrolysate was obtained by hydrolysing the DNA in a sealed tube with 4 *N* HCl, for 4 hours at 100°. This hydrolysate failed to give a positive test for amino acids (except glycine, which arose from purine destruction[11]) when it was chromatographed on filter paper and sprayed with ninhydrin in the usual manner. Both the Sakaguchi test for arginine (which is very sensitive but somewhat erratic) and the biuret test were negative.

TABLE II

SPECTROGRAPHIC ANALYSIS OF NUCLEIC ACID ASH

(Expressed as percent of total ash)

Element	*Mouse liver NaDNA*	*C_{954} hepatocarcinoma NaDNA*
Mg	0.01	0.01
Ca	0.1	0.05
Al	0.006	0.002
Pb	0.29	0.14
Cr	0.001	0.001
Fe	0.03	0.009
Ag	0.0005	0.0002
Sn	0.16	0.08
Cu	0.005	0.005

Test for ribonucleic acids. A sample of DNA was hydrolysed with 72% perchloric acid, and the hydrolysate, when chromatographed, revealed no trace of uracil when irradiated with ultraviolet light. It was, however, possible to demonstrate the presence of RNA in very small amounts in the following manner: 25 mg of DNA were hydrolysed with 9 *N* HCl in a sealed tube, at 110°, for 24 hours. This hydrolysate was chromatographed on a wide strip (40 cm) of Schleicher and Schüll No. 598 paper, in the *iso*propanol solvent of WYATT[12]. The area on the chromatogram just below thymin, where uracil is expected to be found, was cut out and extracted three times with boiling water. The extracts were concentrated to 0.2 ml, and rechromatographed on a narrow strip of the same type of paper. Under ultraviolet light it was then possible to see small, fluorescence-quenching areas corresponding to uracil. A rough estimate of the uracil content of the hydrolysate, based on the area occupied by the uracil on the chromatogram, indicated that DNA isolated by this procedure contained less than 1% RNA.

Spectrographic analysis for heavy metals. As a further test of contamination by foreign materials, we subjected the DNA isolated by the procedure described here to

* The term "precision" is defined in the usual sense as the maximum deviation (in per cent) from the mean.

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spectrographic analysis. DNA samples isolated from normal mouse liver and from a hepatocarcinoma* were ashed in platinum crucibles for 4 hours at 450°. The ash was weighed in graphite crucibles, and subjected to D.C. excitation**. The semiquantitative results shown in Table II were calculated from spectra. The bulk of the ash from these nucleic acid samples was undoubtedly primary sodium phosphate (or sodium metaphosphate), and these heavy metals and alkaline earths constituted no more than 0.5% of the nucleic acid ash. The significance of these metals in nucleic acid structure is not understood at the present time, but interesting speculations have already been made[13,14]. The generally larger amounts of the heavy metals in nucleic acid from the normal liver may reflect its greater age (several months) as compared with the carcinoma nucleic acid (7 to 12 days).

DISCUSSION

Recently, MARKHAM AND SMITH stated that nucleoproteins prepared from whole tissue without any attempt at separation of its nuclear and cytoplasmic components must be mixtures of ribo- and deoxyribonucleic acids originating from both parts of the cell[15]. Indeed, it has long been recognized that the ideal way to obtain DNA uncontaminated by cytoplasmic material is to make use of a nuclear preparation as starting material. Unfortunately, not only is the large-scale isolation of uncontaminated nuclei by centrifugation procedures a burdensome task requiring a day or more, but in addition, the yields from such procedures are often low[5]. A method for circumventing these difficulties is described here. The controlled hydraulic homogenization of tissues, combined with the rapid separation of nuclei from the homogenate by an adsorption technique, made available to us a ready source of nuclei from which DNA was easily isolated. This procedure has wide applicability, and we were able to obtain fibrous DNA from several mammalian (including brain, lung, and colon) and many invertebrate tissues.

The yields of nuclei obtained by our method are dependent upon the extent of tissue homogenization. Incomplete homogenization prolongs the time required for isolation of nuclei because the diatomaceous earth is obstructed by the cellular material and the filtration rate is thereby slowed. A contaminated nuclear preparation yields DNA samples that are difficult to purify, and subsequent purification results in large losses of DNA. Homogenization carried out with an annular orifice equal to or less than the diameter of the nuclei disrupts the nuclei. Since the nucleoproteins released from the nuclei are not retained by the diatomaceous earth, their DNA is lost.

It seems unlikely that the retention of the nuclei on the diatomaceous earth is due to physical obstruction of their passage through this material, since the channels and voids between its particles are larger than the dimensions of an average nucleus. We observed that cytoplasmic constituents, red blood cells, and intact cells readily passed between the siliceous particles while the nuclei were retained. Microscopic examination of such trapped nuclei revealed that their surfaces were completely covered with the smallest particles present in diatomaceous earth. Since diatomaceous

* C_{954} hepatocarcinoma (obtained from Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine) was carried by serial transplantation in C57 leaden mice.

** These analyses were carried out by Mr. G. M. GORDON of the Division of Mineral Technology, University of California, Berkeley.

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earth is negatively charged[16], we have assumed that the nuclear retention is an adsorptive effect. These considerations suggest that the nuclear membrane is strongly positive, conceivably as a result of the charge carried by the strongly basic histones at neutral pH. So far, we have not succeeded in removing these intact nuclei from the adsorbent.

The DNA isolated by this procedure appears to be of acceptable purity as judged by lack of contamination with protein, RNA, and inorganic salt. The nitrogen : phosphorus ratios shown in Table I are in fair agreement with the theoretical value of 1.7 calculated for a statistical tetranucleotide composed of 1 mole of each of the following nucleotides: adenylic, guanylic, cytidylic, and thymidylic acids.

The absorbancy data shown in Table I bear on the macromolecular state of the DNA samples[18]. The more degraded a nucleic acid sample, the higher its absorbancy values will become[19], but the less will be the depressing effect of salt upon the absorption. This will result in a ratio, $A_s\,(\mu = 0)/A_s\,(\mu = 1)$, approaching unity. Our samples have given values of about 1.21 for this ratio. Such values are in good agreement with the figure given by REICHMANN, RICE, THOMAS AND DOTY for the samples they isolated from calf thymus[20] and used for molecular weight studies.

SUMMARY

A method for the isolation of fibrous DNA from tissues with low nuclear/cytoplasmic ratios is described. This method is based on the actual removal of nuclei from ordinary tissues by controlled homogenization, and subsequent separation of the nuclei from their homogenates by adsorption. The DNA samples obtained appear to be free of significant contamination by protein, RNA, and inorganic salts, and their absorptive response to ultraviolet illumination under conditions of changing ionic strength indicates that they are highly polymerized. Complete isolation of DNA samples can be carried out in about an hour.

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