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SOME PROPERTIES OF NUCLEIC ACIDS EXTRACTED WITH PHENOL

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SUMMARY

Preparations of nucleic acids obtained by extraction of mouse liver, HeLa cells and cell fractions with phenol and deoxycholate have been characterized with regard to the differential solubility of ribonucleic acid and deoxyribonucleic acid in ethanol, density-gradient centrifugation and the presence of high-molecular-weight contaminants. Ribonucleic acid obtained by this method is less soluble than deoxyribonucleic acid. It was precipitable with 20 % ethanol, nearly free of deoxyribonucleic acid, but containing 4–5 times its weight of polysaccharide which is not removed by repeated fractional precipitation nor entirely by α-amylase (EC 3.2.1.1) digestion, but is removed by density-gradient centrifugation. Deoxyribonucleic acid could be subsequently precipitated with 50 % ethanol free of ribonucleic acid but contaminated with polysaccharide. The buoyant density of the latter is identical with deoxyribonucleic acid and they are not separated by density-gradient centrifugation. The contaminating polysaccharide appears to be a single entity, the β-subunit of glycogen granules. Its isolation and some of its properties are described. Its effect upon the properties of the nucleic acids is discussed.

INTRODUCTION

By phenol and detergent extraction, biologically active preparations of viral nucleic acids are obtained. The biologic activity is attributed to the native state of the nucleic acid, but usually such preparations have not been throughly characterized. The infectivity might be affected in part by contaminants which stabilize or potentiate, particularly, since addition of various supplements increase the observed level of infectivity. Preparations obtained by a method frequently used in this laboratory have been partially characterized. Certain observations pertinent to the presence and nature of significant contaminants are recorded.

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METHODS AND MATERIALS

Cultivation of HeLa cells

A continuous line of cells, HeLa, were grown in serial passage in monolayers in a medium described by EAGLE[1] containing 10 % calf serum. These were grown on the walls of stationary Roux bottles. All cultures were supplemented with penicillin (100 units per ml) and streptomycin (0.1 mg/ml) and periodically tested for bacteria and pleuropneumonia like organisms.

Fractionation of HeLa cells

HeLa cells ($50 \cdot 10^6$) were scraped from the walls of the culture vessel into 0.25 M sucrose, containing 0.018 M $CaCl_2$, disrupted in a Potter–Elvehjem homogenizer, fractionated by centrifugation through a layer of high-density sucrose (0.34 M). The supernatant, containing the cytoplasmic fraction was extensively dialysed against phosphate-buffered saline, until the dialysate contained no more than 2 μg of hexose. The nuclear pellet was resuspended in phosphate-buffered saline and washed once.

Extraction of mouse liver with phenol and sodium deoxycholate

Livers (7.2 g) from five adult mice were excised, washed with phosphate-buffered saline, and frozen at −40°. Later, these were homogenized 4 min in a blender at 4° with a fluid composed of 87 ml of water-saturated phenol, 3.6 ml of 10 % sodium deoxycholate (in distilled water), 11 ml of 2 M NaCl, and 72 ml of 1 M NaCl containing 0.02 M phosphate buffer. The aqueous phase was separated after centrifugation at 2000 rev./min (15 min), added to an equal volume of phenol and extracted again by shaking for 8 min at 20°. After a third extraction with phenol, the aqueous phase was freed of phenol by 4 extractions with ether. The latter was removed with a stream of N_2. This is essentially the method of COLTER[2].

Extraction of HeLa cells and cell fractions with phenol and sodium deoxycholate

Suspensions of HeLa cells in phosphate-buffered saline or cytoplasmic fractions prepared as above (55 ml) were adjusted to 1 M with solid NaCl, then 5 ml of 10 % sodium deoxycholate and 5 ml of 2 M NaCl were added. The preparation was extracted with phenol as described above. Attempts to extract nuclei were performed in the same manner. Consistently the nuclear extract contained no DNA. However, extraction of reconstituted mixtures of dialysed cytoplasm (from $2 \cdot 10^8$ cells) and nuclei (from $2.5 \cdot 10^8$ cells) yielded preparations containing DNA (225 μg) in the same manner as whole-cell homogenates. Further, phenol extraction of mixtures of nuclei (from $2.5 \cdot 10^8$ cells) and the phenol extract of cytoplasm (from $2 \cdot 10^8$ cells) yielded DNA (171 μg). The observation is that the extractability of the nuclei is

influenced by cytoplasm components. Thus far the observation has not been further delineated and experiments dealing with isolated nuclei are not reported in the following.

Analyses

DNA was determined by the method of DISCHE[3] using diphenylamine. RNA was determined by the orcinol method[3]. Hexose was determined by a modified diphenylamine reagent in which nucleic acids do not interfere. The method has been described elsewhere and consists of heating at 100° (40 min) 1 volume of sample with 3 volumes of reagent (1.33 g of diphenylamine in 100 ml of glacial acetic acid plus 50 ml of concentrated HCl) and determining the absorbancy at 635 mμ (ref. 4).

Enzymes

RNAase (EC 2.7.7.16), 3 times crystallized and α-amylase were obtained from Nutritional Biochemical Corporation, Cleveland, Ohio; DNAase (EC 3.1.4.5), 3 times crystallized was obtained from General Biochemicals, Chagrin Falls, Ohio.

Phosphate-buffered saline

This is isotonic saline (0.14 M NaCl) containing 0.01 M phosphate buffer (pH 7.4).

EXPERIMENTAL AND RESULTS

Precipitability by ethanol of nucleic acids prepared by phenol extraction

Upon addition of 2 volumes of ethanol and standing 1 h at 4°, the nucleic acids precipitated from the aqueous phase of the phenol extract of mouse liver (described in the section on Methods). The precipitate was dissolved in 5 ml of half-concentrated phosphate-buffered saline, analyzed, and found to contain 38.7 mg DNA, 68.7 mg RNA, and 21.0 mg hexose; ratios 1:1.77:0.54. The material was twice reprecipitated with 1 volume of ethanol from half-concentrated, phosphate-buffered saline then treated with RNAase and reprecipitated. The precipitates at each stage were analyzed and the results recorded in Table I. It will be noted that the carbohydrate is not removed by repeated ethanol precipitation, in fact, it persists in a nearly constant ratio to the DNA. Further, from this mixture RNA is precipitated like DNA by 1 volume of ethanol. Removal of the RNA by enzyme digestion and addition of ethanol does not affect the precipitability of the DNA and carbohydrate whose ratio remains constant.

An extract of HeLa cells prepared as described under Methods, was shown by analysis also to contain RNA, DNA, and carbohydrate. This was precipitated with 2 volumes of ethanol. The precipitate was dissolved in phosphate-buffered saline

TABLE I

COMPOSITION OF THE PRECIPITATES FROM REPEATED PRECIPITATION OF A CRUDE PHENOL EXTRACT OF MOUSE LIVER WITH 1 VOLUME OF ETHANOL

The concentrations of DNA, RNA and polysaccharide in the redissolved precipitates are expressed as μg/ml. Precipitation No. 3: before precipitation, the preparation was treated at 20°. (30 min) with 25 μg RNAase per ml.

Succession precipitations	*DNA*	*RNA*	*Polysaccharide*	*DNA : RNA : polysaccharide weight ratio*
1	1250	1476	619	1 : 1.18 : 495
2	413	471	211	1 : 1.14 : 512
3	105	42	57.6	1 : 0.40 : 547

and reprecipitated with one-fourth volume of ethanol whereupon 60–80 % of the RNA and nearly all the carbohydrate were found in the pellet while the DNA and some RNA and carbohydrate remained with the supernatant (Table II, Expts. 2a, 3a). Thus, under these conditions a major portion of the RNA is more insoluble in 20 % ethanol than the DNA; this is in contrast to that described for other preparations[5].

TABLE II

PRECIPITATION OF MIXTURES OF NUCLEIC ACIDS (DNA, RNA) AND POLYSACCHARIDE WITH 20 % ETHANOL WITH AND WITHOUT α-AMYLASE TREATMENT

α-Amylase treatment was for 3 h at 37°; ratio of amylase to polysaccharide was 1 : 15 in Expt. 1b and 1 : 10 in Expts. 2b and 3b. DNA, RNA and polysaccharide are expressed as total μg in the precipitate (Ppt.) or supernatant (Sup.) after addition of one-quarter volume ethanol. Expts. 1 and 2 represent successive quarter-volume-ethanol precipitation without enzyme treatment.

Expt. No.	*DNA*		*RNA*		*Polysaccharide*		*Polysaccharide/RNA*
	Ppt.	*Sup.*	*Ppt.*	*Sup.*	*Ppt.*	*Sup.*	*Ppt.*
1	—	—	28.7	87.0	146	72.0	5.10
2	—	—	14.1	5.47	60.5	22.6	4.28
1a	—	—	15.6	37.4	63.5	87.5	4.18
1b	—	—	4.7	39.3	8.3	152	—
2a	0	114	122	24.0	870	0	7.13
2b	28.2	85.8	78.8	74.2	339	545	—
3a	0	197	314	193	1570	127	5.00
3b	0	201	218	244	309	1430	—

Phenol extracts of the cytoplasm of HeLa cells containing only two components, carbohydrate and RNA, were first precipitated with 2 volumes of ethanol and then twice precipitated with 20 % ethanol. The carbohydrate extracted with the RNA originally, upon repeated fractional precipitation produces a pellet of relatively constant proportions (Table II, Expts. 1, 2).

Thus beginning with a fresh crude phenol extract of whole cells in 1 M saline one can anticipate the precipitation of one fraction of RNA (94.2 %) by quarter volume ethanol which is nearly free of DNA (3.6 %) and containing 4–5 times its weight of polysaccharide (94.5 %); further addition, to give 1 volume of ethanol, precipitates most of the DNA (91.3 %) with some polysaccharide (5.5 %); while 2 volumes of ethanol yield only a small residue of DNA (4.9 %) and RNA (5.8 %).

The percentages are of the total precipitable material. The biological significance (messenger RNA, transfer RNA, viral infectious RNA, etc.) of these fractions will be determined later.

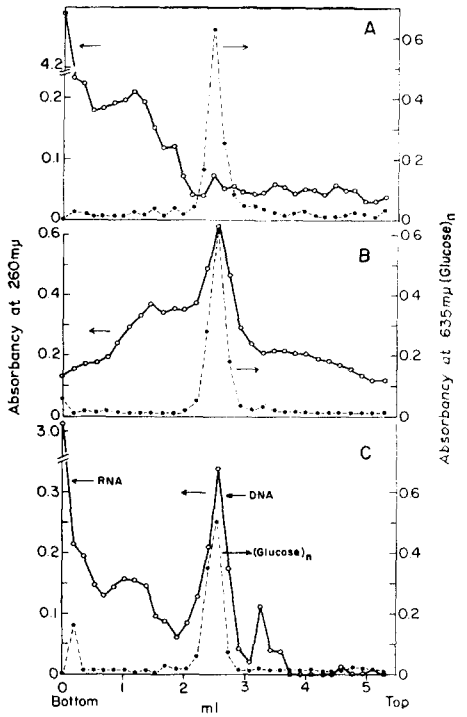
Density-gradient centrifugation in CsCl

Since the principal components of the phenol extract (DNA, RNA, polysaccharide) are not separated by ethanol fractionation, the extract was subjected to density-gradient centrifugation in CsCl which is known to separate the DNA and RNA. In addition, samples of the extract were treated with RNAase or DNAase before centrifugation. Material prepared as above from mouse liver precipitated once with 2 volumes of ethanol, and once with 1 volume was dissolved in half-concentrated phosphate-buffered saline. To 0.1 ml of phosphate-buffered saline was added 0.3 ml of this preparation, it was incubated 30 min at 20°, precipitated with 2 volumes of ethanol, centrifuged at 2000 rev./min (10 min), and the precipitate dissolved in 0.5 ml of half-concentrated phosphate-buffered saline after washing with 67 % ethanol. This preparation was layered over 4.8 ml of 60 % (w/w) CsCl and centrifuged at 40 000 rev./min at 4° for 64 h and 35 min. Two similar preparations were made with the exception that the 0.1 ml of phosphate-buffered saline above was replaced by 0.1 ml of RNAase (100 μg/ml) or 0.1 ml of DNAase (100 μg/ml). Fractions of 5 drops each were removed from the tubes from the bottom. The drops were diluted to 3 ml in water. 1 ml was used for hexose determination after absorption was measured optically at 260 mμ.

The resulting data are plotted in Fig. 1. It is concluded from the RNAase effect (Curve B) and the DNAase effect (Curve A) that in Curve C, the absorbancy measured at 260 mμ at the bottom of the tube represents RNA and that in the center DNA. To confirm this, the ultraviolet spectrum of Fractions 14 and 15 of the RNAase-treated sample (Curve B) and 13 through 15 of the untreated sample (Curve C) were pooled and upon assay found to contain by ultraviolet adsorption 115 μg nucleic acid per ml, and by the DISCHE test 107 μg DNA per ml, *i.e.*, the ultraviolet-absorbing material of the central peak can be accounted for as DNA.

At the point in the density-gradient tube where the DNA is found, a sharp clearly defined band of turbidity was always observed. This effect is produced by the polysaccharide which is present at this point rather than the DNA, since it remains after enzymatic digestion with DNAase (Curve A, Fig. 1). The carbohydrate has no absorption at 260 mμ (though in some preparations it scatters light), its buoyant density is seen to be identical with the DNA; however, it is clearly not an integral part of the DNA molecule but rather is itself a high polymer with properties remarkably similar to DNA. A further characterization of this material and justification for these conclusions are presented in the next section of the paper.

Bottom fractions obtained from the untreated and DNAase-treated samples (Curves A and C, Fig. 1) were pooled. After dilution, these were precipitated with 2 volumes of alcohol, the precipitate was dissolved in 1.5 ml of water and upon analysis found to contain 19 μg of nucleic acid per ml (ultraviolet absorption) and 22 μg of RNA per ml (orcinol method). The ultraviolet absorption of the bottom fraction can be accounted for by the RNA content.



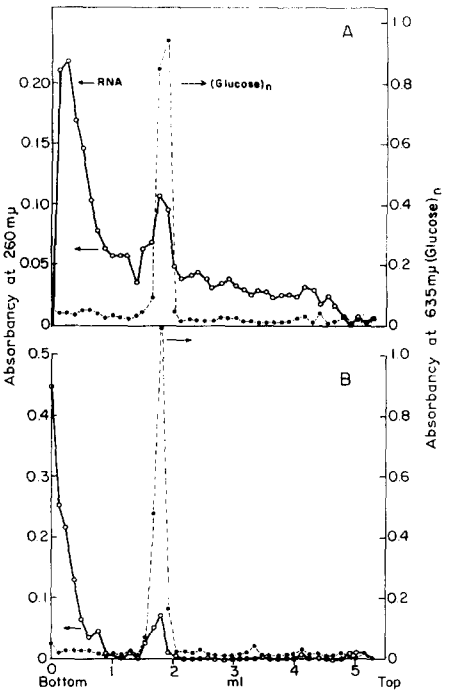


Fig. 1. The nucleic acid and polysaccharide contents of individual fractions taken from CsCl density-gradient centrifugations of nucleic acid preparations are plotted. The preparation in 1A was pretreated with DNAase, that in 1B pretreated with RNAase and 1C was untreated. The chromogenic activity of the polysaccharide is plotted with the broken line against the right ordinate; the nucleic acid is plotted with the solid line against the left ordinate.

Fig. 2. The nucleic acid and polysaccharide contents of individual fractions taken from CsCl density-gradient centrifugations of a nucleic acid extract of HeLa cell cytoplasm are plotted. The preparation in 2A was pretreated with RNAase, that in 2B was untreated. The chromogenic activity of the polysaccharide is plotted with the broken line against the right ordinate; the nucleic acid is plotted with the solid line against the left ordinate.

While the carbohydrate can be separated from the nucleic acids by acid precipitation of the latter, more gentle methods yielding products which tend to the native state do not separate the DNA and carbohydrate. The carbohydrate can be obtained in pure form from a density gradient by starting with phenol extracts of HeLa-cell cytoplasm which contain no DNA.

To 4.3 ml of 57.5 % CsCl, 1.05 ml of sample were added, centrifuged at 40 000 rev./min for 66 h. Fractions (5 drops) collected through the bottom of the tube were diluted to 3 ml, and assayed for RNA (260 mμ) and carbohydrate. In a duplicate experiment the sample was pretreated with RNAase. The results are plotted in Fig. 2. At the point of maximum hexose content, there was a very sharp visible band of turbidity but no DNA. The slight absorption at 260 mμ at this point is probably due to light scattering. The RNA is at the bottom of the tube. Again the position of the polysaccharide is independent of the presence of DNA but occupies the same position as the latter. By dilution of the CsCl and precipitation with ethanol the carbohydrate can be obtained in a nearly pure state.

Properties of the carbohydrate component

The third component obtained by density-gradient centrifugation of a cytoplasmic phenol extract of HeLa cells was partially characterized. It was found to be non-dialysable, to contain no protein by the Lowry method and no absorption at 260 or 280 mμ. It has a very uniform buyoant density, identical with DNA. It reacted with the modified diphenylamine reagent in a manner typical of hexose, to yield a blue-purple reaction product whose absorption spectrum in the visible region was identical with that of hexose (Fig. 3). The specific absorptivity of oyster glycogen in this test

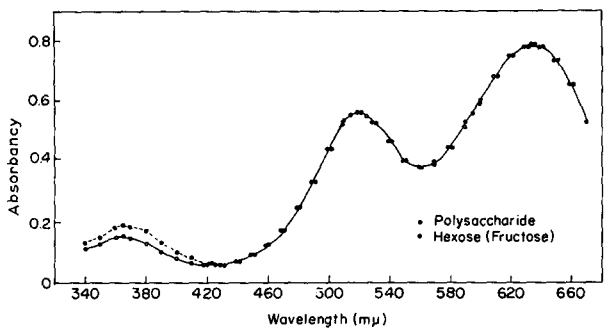


Fig. 3. The absorption curve in the range 340–660 mμ is represented for the colored product produced by purified polysaccharide from HeLa cells and by a typical hexose, fructose, using the modified diphenylamine reaction.

is equivalent to an equal weight of glucose while that of fructose is different but equivalent to an equal weight of inulin. The colored product resulting in each case is identical for hexoses and fructose while other sugars, either higher or lower homologous, are distinctly different. From a known weight of material the specific absorptivity was determined (6.66) and found to correspond to that of glucose (6.25) rather than other hexoses. The various hexoses and their polymers react with diphenylamine at different rates. Appropriate concentrations of glucose, fructose, oyster glycogen and the unknown polysaccharide were chosen so as to yield the same amount of colored product at the end of 40 min heating. At intervals from 5 to 40 min the development of color was followed. The data are recorded in Table III from which it can be seen that the unknown polymer reacts at a rate closely approximating glucose or oyster glycogen and distinct from fructose or inulin. Further the material is partially reduced by α-amylase (EC 3.2.1.1) treatment to a state no longer precipitable by ethanol (Table II, Expts. 1b, 2b, 3b). Thus the material appears as a high-molecular-weight polymer of glucose, a type of animal glycogen.

Electron micrographs of the material confirmed the impression of a highly homogeneous, uniform structure. Preparations, negatively stained or shadowed revealed one type of particle, approx. 30 mμ in diameter, irregular in shape with some degree of substructure (Fig. 4).

TABLE III

RATE OF COLOR (635 mμ) DEVELOPMENT CONSEQUENT TO THE REACTION OF HEXOSES AND THEIR POLYMERS WITH DIPHENYLAMINE

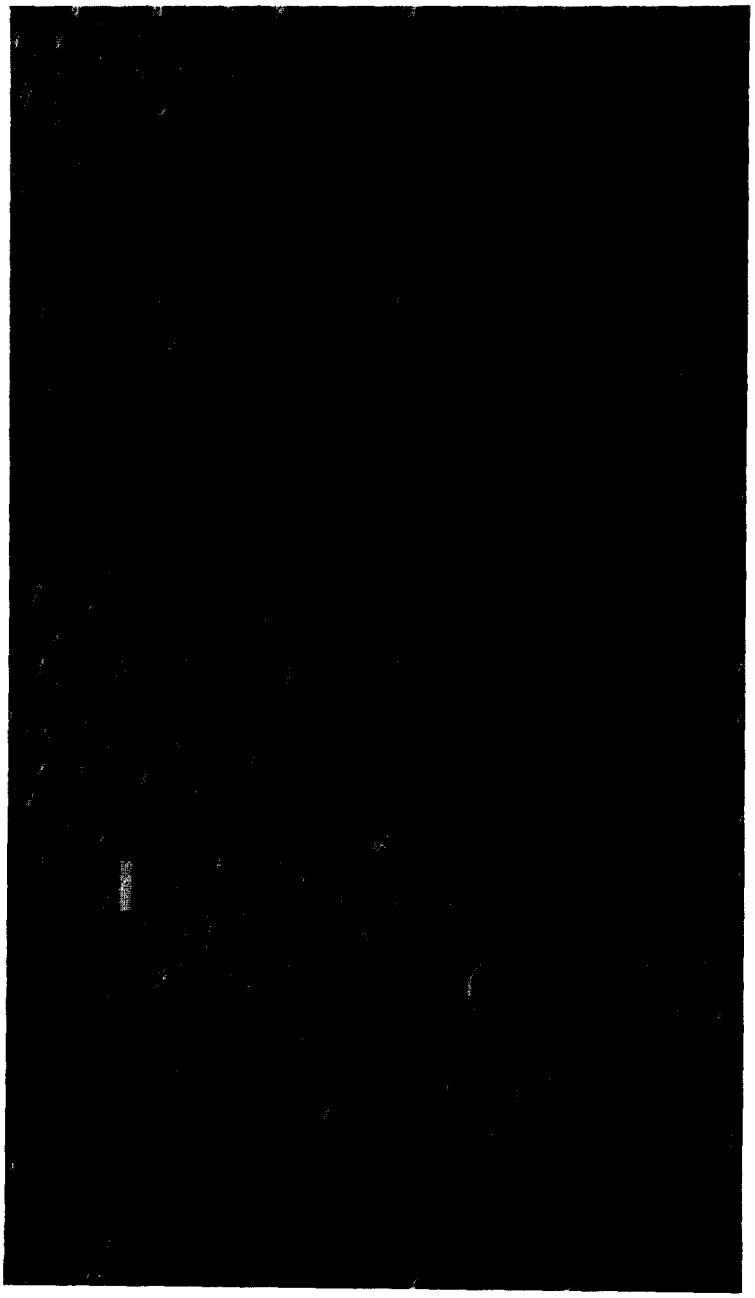
Polysaccharide (HeLa) is the purified polysaccharide isolated from HeLa cell cytoplasm. Glycogen is a commercial preparation from oysters. The concentrations of glucose, polysaccharide (HeLa) and oyster glycogen were 48 μg/ml; that of inulin and fructose 10 μg/ml. Heating with the diphenylamine reagent was as described for the analytical procedure for hexose (under METHODS) for the various intervals indicated.

Time (min)	*Absorbancy at 635 mμ*				
	Glucose	*Polysaccharide (HeLa)*	*Glycogen*	*Inulin*	*Fructose*
5	0.000	0.002	0.000	0.045	0.049
10	0.022	0.021	0.022	0.114	0.130
15	0.055	0.058	0.058	0.168	0.190
20	0.098	0.108	0.108	0.203	0.226
25	0.153	0.158	0.168	0.234	0.263
30	0.207	0.223	0.219	0.255	0.284
40	0.300	0.335	0.328	0.279	0.311

Effect of α-amylase on the solubility of nucleic acids in ethanol

It was considered that the anomalous solubility behavior of the RNA (less soluble than DNA) as compared with other preparations of it[5] might result from its interaction with the carbohydrate present. Hence, the effect of α-amylase treatment upon the solubility of preparations of RNA and DNA in ethanol–water mixtures was next determined. An ethanol-precipitated fraction of a phenol extract of HeLa-cell cytoplasm containing RNA and carbohydrate was dissolved in 0.133 ml of phosphate-buffered saline and divided into two parts. To one part (0.133 ml containing 50.0 μg RNA and 156 μg carbohydrate), 0.05 ml of phosphate-buffered saline was added, to the other 0.05 ml of a preparation of α-amylase (10 μg). After incubation for 3 h at 37°, the preparations were diluted 4-fold with phosphate-buffered saline and one-fourth volume of ethanol added and allowed to stand at 4° over night. The precipitates and supernatants were analysed for carbohydrate and RNA. It will be noted in Table II (Expts. 1a, 1b) that the insoluble RNA fraction was reduced from 15.6 μg to 4.7 μg by the treatment and that a major portion of the carbohydrate was no longer precipitable. While a portion of the RNA was still precipitable with one-fourth volume of ethanol, a portion of the carbohydrate likewise was insoluble.

In a further experiment, a preparation containing RNA, DNA and carbohydrate was treated in a similar manner, the data are recorded in Table II (Expts. 2a, 2b). As expected under conditions of one-quarter volume ethanol, the DNA is not precipitated, while 80 % of the RNA is insoluble. The fraction of RNA which was originally precipitable was reduced by 35 % after amylase treatment. This correlates with the reduction in precipitable carbohydrate. Similar results were obtained in Expts. 3a, 3b (Table II). The solubility in ethanol of RNA is enhanced by the removal of a portion of the polysaccharide by α-amylase treatment. However, exhaustive treatment with α-amylase (4 h) as established by a time study results in a residue of carbohydrate which remains precipitable with ethanol. There is an indication that this enzyme-resistant residue in the three-component preparation is



4a

4b

Fig. 4. 4a is an electron micrograph of polysaccharide purified by density-gradient centrifugation. It was air-dried on a collodion membrane and shadowed with palladium at an angle arctan 0.2. The bar represents 0.1 μ. 4b is an electron micrograph of purified polysaccharide on a carbon-coated grid, negatively stained with potassium phosphotungstate (pH 7.4). The bar represents 0.1 μ.

larger than that obtained with pure preparations of polysaccharide. This residue may be responsible for the fraction of RNA which remains insoluble after α-amylase treatment.

DISCUSSION

Since, earlier, phenol was used successfully for carbohydrate extraction, it was to be anticipated that nucleic acids extracted with phenol would contain polysaccharides. The relatively large amount of polysaccharide present in the preparation, its uniformity and homogeneity, the persistence with which it follows the nucleic acids through further purification, the identity of its buoyant density with that of DNA and its apparent interaction with RNA are observations not wholly expected. It is not entirely clear, but it appears that the insolubility of RNA of these preparations in 20 % ethanol is not an intrinsic property of the RNA; rather it is effected by the polysaccharide. In contrast the solubility of the DNA in 20 % ethanol and its precipitation by 50 % ethanol is in accord with other observations.

The glucose content, susceptibility to the action of α-amylase, ethanol precipitability and non-dialysable nature all indicate that the polysaccharide is a type of glycogen. The electron micrographs of it bear a striking resemblence to the β-particles of glycogen described by REVEL[6]. The glycogen rosettes which are light visible as cytoplasmic granules are viewed by some as aggregates of β-particles held together by some foreign material[6]. A recent note describes ribosome-like particles passing through a phenol-extraction procedure to precipitate with a RNA fraction which is rapidly labeled by short pulses of radioactive phosphorus (messenger RNA)[7]. Because of the procedure employed in those studies, the ribosome-like particles are more likely carbohydrate of the type here described than protein. The older literature describes a strict temporal relation between the visualization of glycogen granules and mitotic activity[8]. The causal relationship assumed to be one of energetics may more correctly entail some transport or structural function of glycogen if these associations *in vitro* reflect a situation *in situ*.

From a methodological viewpoint, the carbohydrate contaminant may adversely affect the interpretation of experiments designed to characterize high-molecular-weight nucleic acid by light-scattering, solubility or possibly centrifugation, *i.e.*, in situations where rigorous methods of purification cannot be employed. Further, fractions taken from CsCl density-gradient centrifugation, will give an intensely false DISCHE reaction for DNA if they contain Cl^- as well as polysaccharide[4]. This assumes importance since in fresh preparations the buoyant density of the polysaccharide and the DNA are identical and cannot be separated by the method. The particulate nature of the polysaccharide causes significant light scattering which can effect absorbancy measured at 260 mμ. 8 μg of polysaccharide will produce an orcinol reaction equivalent to 1 μg of RNA. Since the former is often present in 4–5 times the weight of the latter, the orcinol method is applied only with care to these preparations.

If the carbohydrate affects the physical properties of the nucleic acid, it may also alter its biologic reactivity when tested *in vitro*, *e.g.*, the infection of cells with nucleic acid from viruses. The latter process is reportedly sensitive to histones, chelat-

ing agents and various ions[9]. In some instances infectious nucleic acid has been demonstrated in phenol extracts of infected cells (which contain the carbohydrate) more readily than in extracts of purified virus[10]. In this connection, the greater ease with which we have been able to extract DNA from whole cells, as well as from a mixture of nuclei and cytoplasm, than from the nuclei alone (section on Methods) suggests some effect of contaminants upon the extractability of the nucleic acid, as possibly upon reactivity.

COLTER[2] using this method of nucleic acid extraction has reported the nucleic acid obtained to be free of carbohydrate. This conclusion was based upon a negative test for glucosamine which was observed with his preparations. The polysaccharide described in the present experiments is a polyglucose and probably contains no hexosamine and hence was undetected though present as a major component in his preparation.

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REFERENCES

1 H. EAGLE, *J. Exptl. Med.*, 102 (1955) 37.
2 J. S. COLTER, R. A. BRAN AND K. A. O. ELLEN, *Biochim. Biophys. Acta*, 55 (1962) 31.
3 A. DISCHE, in E. CHARGAFF AND J. N. DAVIDSON, *The Nucleic Acids*, Vol. 1, Academic Press, New York, 1955, p. 285.
4 W. W. ACKERMANN, F. SOKOL AND A. J. BRANDAU, *Anal. Biochem.*, in the press.
5 A. R. MATHIESON AND M. R. PORTER, *J. Chem. Soc. London*, (1958) 1301.
6 J. P. REVEL, *Histochem. Cytochem.*, 12 (1964) 104.
7 J. LASZLO AND K. S. MCCARTY, *Proc. Am. Assoc. Cancer Res. Abstr.*, 5 (1964) 39.
8 R. R. KUDO, *Protozoology*, Charles C. Thomas, 1946.
9 C. E. SMULL, M. F. MALLETTE AND E. H. LUDWIG, *Biochem. Biophys. Res. Commun.*, 5 (1961) 247.
10 H. F. MAASSAB, *J. Immunol.*, 90 (1963) 265.