Extraction, Separation and Labeling with ^{14}C-Glucose of Hela Cell Polyglucose, RNA and DNA and Comparison of the Molecular Weights and Buoyant Densities of Polyglucose from Poliovirus-Infected and Noninfected Cultures

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Synopsis

The aqueous phase of phenol extracts of HeLa cells contains polyglucose $(CHO)_n$, RNA, and DNA. These macromolecules were precipitated together and removed from 50% (v/v) ethanol solutions with a stirring rod. The viscous precipitate had the classical white appearance of DNA, but contained an average of 439, 670, and 220 μg (from 3×10^7 cells) of $(CHO)_n$, RNA, and DNA, respectively. The $(CHO)_n$ was separated from the RNA, either by CsCl density gradient centrifugation or by precipitating the RNA with trichloroacetic acid (TCA). Both methods of separation resulted in preparations of $(CHO)_n$ with similar specific activities (radioactive counts/μg/min). However, electron micrographs showed that the $(CHO)_n$ separated by using TCA had a greater variation in particle size when compared with $(CHO)_n$ separated by CsCl centrifugation. With the CsCl methods, the number-average molecular weights, as determined by electron microscope particle-counting, and the buoyant densities of $(CHO)_n$ whose synthesis was stimulated by poliovirus infection and $(CHO)_n$ from noninfected cultures, were found to be similar. When the $(CHO)_n$ was extracted from HeLa cells with TCA, rather than phenol, the yield was 1.68-fold greater and its specific activity was an average of twice that of the $(CHO)_n$ extracted with phenol. The time at which cells were pulse-labeled with ^{14}C-glucose, after reducing the glucose in the culture medium to 0.01 of normal, was found to be important, in that the specific activity of the $(CHO)_n$ increased 23.4-fold over a 4-hr period and the amount extracted decreased 8.2-fold. The increase in the specific activities of RNA and DNA was not as large as that of the $(CHO)_n$ and the amounts extracted were not significantly changed. The sedimentation coefficients of RNA and $(CHO)_n$ which were separated from each other with TCA were 6.4 and 116 S, respectively, whereas, without separation, two peaks were seen, with values of 25.4 and 31.4 S. Chloride ions reduce the sensitivity of the Burton test for DNA. However, the Burton reagent will detect $(CHO)_n$ even in the presence of DNA if the assay mixture is heated. Chloride ions increase the sensitivity of the Burton reagent to detect melizitose and, at concentrations above $1.5M$, synthetic polyglucose by increasing the absorption of the colored $(CHO)_n$ reaction product(s).

Introduction

A polysaccharide $(CHO)_n$, the β subunit of glycogen granules, and considered to be composed of polyglucose, has been extracted from HeLa cells

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by using phenol. Ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) are also extracted by the same methods. In CsCl, the buoyant density of the $(CHO)_n$ is similar to HeLa cell DNA[1] and sea urchin egg $(CHO)_n$ is similar to mitochondrial DNA.[2] These similarities may cause errors in ultraviolet and chromogenic assays for DNA.[1-7]

In 50% ethanol, HeLa cell $(CHO)_n$,[8] as well as synthetic and other natural $(CHO)_n$,[9] interact with and precipitate heat-denatured DNA when the $(CHO)_n$/DNA weight ratio approaches 2.5. A requirement for this interaction is a $(CHO)_n$ with a molecular weight of 10,000 or greater. Glucose does not reduce the solubility of heat-denatured DNA, nor will it inhibit the interaction of the larger $(CHO)_n$ with heat-denatured DNA.[9]

RNA also interacts with $(CHO)_n$ in 20% ethanol. The RNA has the capacity to precipitate up to six times its weight of $(CHO)_n$. The $(CHO)_n$ alone is soluble in 20% ethanol. Denatured RNA, in contrast to native RNA, is soluble in 20% ethanol and is rendered precipitable by the presence of $(CHO)_n$.[10] In addition, the copolymer polyadenylic-cytidylic acid, as well as the homopolymer, poly(adenylic acid), interacts with $(CHO)_n$. The latter interaction was inhibited in $2.4M$ guanidine. This inhibition of the interaction is considered to be due to a preferential interaction of guanidine with the poly(adenylic acid)[11] as well as with the $(CHO)_n$.[12]

These associations of polynucleotides with neutral polysaccharides initiated speculations regarding an *in vivo* function of $(CHO)_n$ (other than the storage of energy-rich sugars) involving, perhaps, the conformation, protection, and the regulation of expression of nucleic acid.[8,10,11]

Accumulations or increased synthesis of $(CHO)_n$ have been detected in a variety of pathological processes. For instance, this host response has been observed in irradiation,[13-15] brain stab wounds,[16] regeneration of human skin,[17] human skin grafts,[18] herpes,[19] poliovirus,[20,21] and psittacosis[22] infections. The $(CHO)_n$ may then be involved in some way in host inflammatory and repair reactions. The findings above and the older observation that the appearance of $(CHO)_n$ is related to the mitotic cycle[23] seem important and warrant studies which attempt to detect alterations in the $(CHO)_n$ whose synthesis was stimulated during infection with poliovirus. In addition, it is hoped that the following observations may facilitate *in vivo* and *in vitro* investigations of $(CHO)_n$ and the possible association of it with nucleic acids. These experiments were initiated to study and describe: (1) the proportions of $(CHO)_n$, RNA, and DNA which precipitated together in 50% ethanol; (2) the techniques which will separate the RNA from the $(CHO)_n$; (3) differences in the $(CHO)_n$ which is extracted with phenol or trichloroacetic acid; (4) the labeling with ^{14}C from ^{14}C-glucose of HeLa cell $(CHO)_n$, RNA, and DNA; and (5) a modification of the Burton test for DNA which will detect DNA as well as $(CHO)_n$. By using some of these methods, the number-average molecular weight and buoyant density of $(CHO)_n$, whose synthesis was stimulated as a result of poliovirus infection[20] may be compared with those of $(CHO)_n$ extracted from noninfected cultures. Studies of metabolic changes in virus infected

cells[20,24,25] and (CHO)$_n$–polynucleotide interactions[8–10] have utilized the techniques for phenol extracting, separating, and purifying (CHO)$_n$, RNA, and DNA. However, the experiments involving the separation of (CHO)$_n$ from RNA have not been described.

Materials and Methods

HeLa cells were cultured in Roux bottles in Eagle's medium and labeled, as indicated in the specific experiments, for 1 hr with 3 μCi of uniformly labeled ^{14}C-d-glucose (specific activity, 22.3 μCi/mmole, Calbiochem Corporation). These methods have been previously described.[20,24,25] The (CHO)$_n$, RNA, and DNA were extracted from the cells with phenol by using the method described by Colter et al.[26] The method of extraction was as follows: 10 ml of 1M NaCl containing 10% (w/v) sodium desoxycholate was added to the Roux bottles containing HeLa cell monolayers. This effectively removed the cells from the glass surface, and stopped the incorporation of the ^{14}C from ^{14}C-glucose into the macromolecules. This suspension was removed from the Roux bottles and each bottle was rinsed with 10 ml of water-saturated phenol. The phenol was then added to the saline–cell suspension. The combined solutions were shaken for 8 min at room temperature, centrifuged (1100g/10 min), and the aqueous phase was removed and extracted again with 10 ml of water-saturated phenol. The remaining phenol was removed with ether and the ether was removed with nitrogen.

The macromolecules were precipitated from the aqueous phase by addition of ethanol. The concentrations of ethanol varied with the type of experiment and are indicated in each of the following experiments. Of importance is the observation that a precipitate containing (CHO)$_n$, RNA, and DNA may be quantitatively removed from the 50% (v/v) solution without centrifugation. That 20% (v/v) ethanol will selectively remove (CHO)$_n$ and RNA from saline solutions containing the three types of macromolecules has been previously described.[1]

The RNA, DNA, and (CHO)$_n$ were quantitatively determined by using, respectively, the orcinol,[5] Burton,[27] and the diphenylamine reagents.[3,4] The standards were prepared from yeast RNA, calf thymus DNA (Worthington Biochemicals, Freehold, New Jersey), and glucose. Synthetic polyglucose (L524023-0) and melezitose were purchased from Merck and Difco Laboratories, respectively.

The following procedure was used to extract the (CHO)$_n$ from HeLa cell cultures with trichloroacetic acid (TCA).[28] The monolayers containing approximately 3 $\times$ 10^7 cells were rinsed twice with phosphate (0.01M) buffered (pH 7.2) saline (0.14M) (PBS). A 3-ml portion of 10% (w/v) TCA was added to the Roux bottles, and the cells were removed from the glass surface with a rubber policeman. The cell suspension was treated with ultrasonic waves (10 kcp's, 10 min, 5°C), centrifuged (1100g, 10 min), and 5 volumes of absolute ethanol were added to the supernatant fluid. The solution was held at 5°C for 16 hr and the precipitate was removed by

centrifugation. The supernatant fluid (10 ml total volume) contained an average of 10 radioactive counts/ml. The precipitate was dissolved in PBS and reprecipitated with 5 volumes of ethanol. The $(CHO)_n$ precipitate was then dissolved in 2 ml PBS, and 0.3 ml was assayed for radioactivity by scintillation counting[11,12,24] and 0.1 ml was used for $(CHO)_n$ analysis.

The $(CHO)_n$, which is separated from RNA by CsCl density centrifugation, has a rather uniform particle size,[1,29] This property permits a comparison of the number-average molecular weights of $(CHO)_n$ from infected and noninfected HeLa cells. The methods used to culture[24,25] and infect[20] HeLa cells with poliovirus have been described. The following experiments use $(CHO)_n$ extracted from cultures which were infected for 2 hr and pulse-labeled with ^{14}C-glucose during the first to the second hour after infection.

The method for determining the molecular weight of $(CHO)_n$, by using an electron microscope particle counting technique[29,30] is as follows. A portion of a standard suspension of latex particles, single sphere weight 0.382×10^{-15} g (as specified by Dow Chemical, Midland, Michigan) which contained 1.37×10^{12} spheres/ml as determined from dry weight measurements, was mixed with 0.5% bovine serum albumin. This mixture was sonically treated (3 min, 10 kcps, 5°C) and a portion of it was added to water containing a known weight of $(CHO)_n$, as determined by chromogenic assay. A drop of the mixture was placed on a collodion membrane floating on water. A block of sintered glass was then submerged in the water and copper grids were placed upon the block. The grids were brought up under the $(CHO)_n$-latex mixture and the drop was lifted out of the water. The block was then placed on a moist towel and the fluid in the drop was pulled through the sintered glass by capillary action. This method was developed to minimize the aggregating effects caused by drying. The grids were then shadow-cast with palladium at an angle of 7:1 and electron micrographs were taken of random fields. The reported weight-average molecular weight of the $(CHO)_n$ was determined by light scattering to be 4.6×10^6, whereas the number-average molecular weight, as determined by particle counting, was 1.35×10^6. The range in the number-average values was $2.24–0.823 \times 10^6$.[29] The electron micrograph of the $(CHO)_n$ separated from RNA with TCA was taken using grids prepared in manner described above.

The sedimentation coefficients $s_{w,20}$ of the macromolecules involved in the following experiments are 116 and 11.1 S for $(CHO)_n$ and DNA, respectively.[8] The RNA which was separated from the $(CHO)_n$ by TCA precipitation had one peak with a value of 6.4 S. Of interest is the finding that when a preparation containing RNA and $(CHO)_n$ (which had not been exposed to TCA) was examined in a Beckman model E analytical centrifuge using schlieren optics, two components were seen, but the $s_{w,20}$ values were 25.4 and 31.4 S. In contrast, the $s_{w,20}$ of $(CHO)_n$ and DNA were not significantly different when the two macromolecules were centrifuged together.[8]

Results

Cultures of HeLa cells (3×10^7 cells) were extracted with phenol. Ethanol (50% v/v) was added to the aqueous phase and the resultant precipitate (which had the viscous, white appearance of DNA) was removed from the fluid by winding it on a stirring rod. The precipitate was dissolved in $1M$ NaCl, and absolute ethanol was slowly added while the solution was being mixed with a Super mixer until a final concentration of 20% (v/v) was reached. This solution was held at 5°C for 15 hr. A white turbidity formed which, with time, precipitated. The precipitate, containing RNA and $(CHO)_n$, was removed by centrifugation ($1100g$, 10 min, 5°C) and the concentration of ethanol in the supernatant fluid was increased to 50% (v/v). The resultant, a viscous precipitate containing DNA, was removed with a stirring rod. Both precipitates were dissolved in PBS, and 40% (w/v) TCA was added until a concentration of 5% was reached. The supernatant fluids were removed and the precipitates were washed in 5% TCA, then hydrolyzed in hot (100°C, 10 min) 5% TCA. The amount of RNA and DNA in the preparations obtained by precipitation with 20% and 50% ethanol, respectively, and the amount of $(CHO)_n$ in the 5% TCA supernatant fluid were quantitatively determined. The DNA precipitate contained approximately 1 μg $(CHO)_n$ per 100 μg DNA, as determined by the amount of $(CHO)_n$ in the TCA supernatant fluid. The majority of the $(CHO)_n$ was found in the TCA supernatant fluid from the RNA preparation.

The total quantity of $(CHO)_n$, RNA, and DNA present in these various fractions from nine HeLa cell cultures are listed on Table I. The proportions of the three components in the original precipitate (50% ethanol) are also listed, i.e., the amounts of RNA relative to $(CHO)_n$, DNA relative to $(CHO)_n$, and DNA relative to RNA.

TABLE I

Quantitative and Qualitative Analysis of Precipitates Containing DNA, RNA and Polyglucose and their Relative Proportions[a]

Expt.	DNA, μg	RNA, μg	$(CHO)_n$, μg	RNA/DNA	$(CHO)_n$/DNA	$(CHO)_n$/RNA
1	173	624	430	3.61	2.49	0.691
2	279	684	463	2.46	1.68	0.677
3	238	712	400	3.00	1.68	0.569
4	195	540	462	2.77	2.36	0.855
5	177	618	488	3.48	2.76	0.789
6	200	710	364	3.55	1.82	0.512
7	200	620	389	3.10	1.94	0.627
8	244	706	470	2.89	1.93	0.666
9	270	816	483	3.02	1.79	0.592
Average	220	670	439	3.10	2.05	0.663

[a] DNA, RNA and polyglucose $(CHO)_n$ extracted from cultures of HeLa cells.

In these experiments (Table I), TCA was used to separate the RNA from the $(CHO)_n$. This method is both rapid and convenient, and the TCA does not interfere with the diphenylamine assay for $(CHO)_n$. In addition, the $(CHO)_n$ is recoverable from the TCA by precipitation with ethanol.[10] One disadvantage in this method of separation is that the $(CHO)_n$ has a greater range in particle size than the $(CHO)_n$ which is separated from the RNA by CsCl density gradient centrifugation. Figure 1 is an electron micrograph which illustrates the variation in the $(CHO)_n$ particle sizes when TCA precipitation of the RNA was used to purify the $(CHO)_n$.

To test whether this separation from RNA with TCA yielded $(CHO)_n$ with a specific activity (radioactive counts/μg/min) similar to that of $(CHO)_n$ purified by CsCl centrifugation, the products of both methods were compared. In addition, answers to two other questions were sought: (1) Is the buoyant density of the $(CHO)_n$ extracted from poliovirus infected cultures the same as that of control cultures? (2) Is there a difference in the molecular weights of the two $(CHO)_n$ preparations?

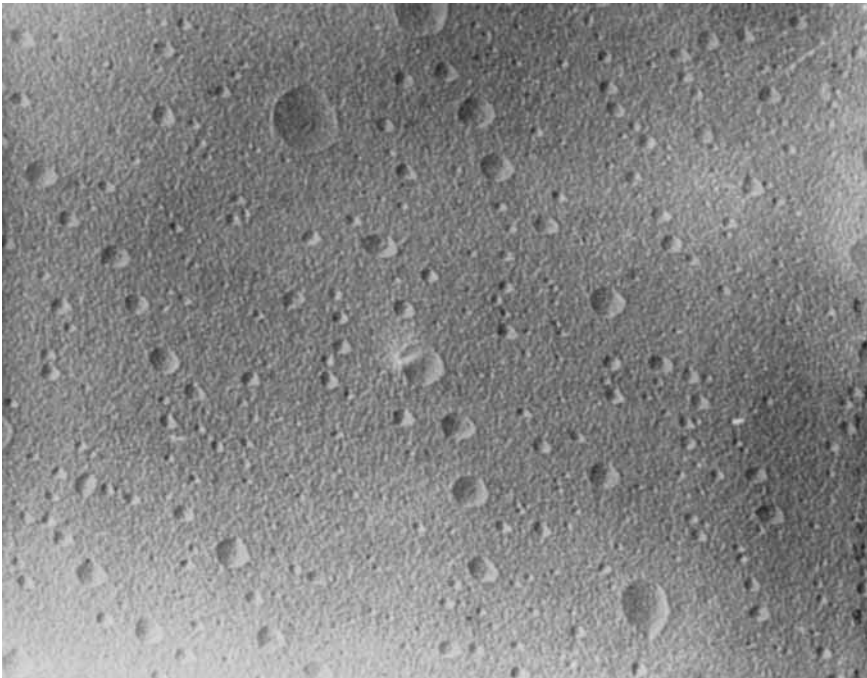Fig. 1. Electron micrograph of $(CHO)_n$ which was extracted from HeLa cells with phenol and purified by precipitating the RNA with TCA. The $(CHO)_n$ was precipitated from the TCA with 50% ethanol and then dissolved in water. Grids were prepared using the method described for the molecular weight determinations (Table III). The magnification was 20,000$\times$. Note the variation in the size of the $(CHO)_n$ particles. The larger particles are considered to be aggregates of β-subunits. Greater uniformity in the size of the $(CHO)_n$ particles was seen when the $(CHO)_n$ was purified by CsCl centrifugation.[1,29]

Control, noninfected cultures and poliovirus-infected cultures were pulse labeled with 3 μCi of ^{14}C-glucose during the first to the second hour after infection. During the time of labeling, the glucose in the culture media was reduced to 0.01 of normal. The cultures were extracted with phenol and the RNA and $(CHO)_n$ were precipitated in 20% ethanol. A portion of each of the precipitates was layered over 60% (w/v) CsCl. Following the centrifugation (66 hr, 125,000g), 0.3-ml fractions were collected and diluted with water. The fractions were assayed for absorption at 260 mμ, as well as for $(CHO)_n$ and radioactive count. Figure 2 illustrates the results. For reasons of clarity, the 260 mμ absorption profiles for the RNA (which descends to the bottom of the gradient[1]) are not illustrated. It is apparent that poliovirus infection did not change the buoyant densities of the $(CHO)_n$. The specific activities (radioactive counts/μg/min) of both $(CHO)_n$ preparations in fractions 9, 10, and 11 are listed on Table II. These fractions did not absorb ultraviolet light at 260 mμ.

The RNA in the second portion of the RNA–CHO preparations was precipitated with 5% TCA; the specific activities of the $(CHO)_n$ were determined and are also listed on Table II. The average specific activity of the $(CHO)_n$ in the fractions (7.9 and 3.4) and the specific activities of the $(CHO)_n$ purified with TCA (8.2 and 3.4) seem sufficiently close to conclude that both methods of purification yield $(CHO)_n$ with similar specific activities.

The $(CHO)_n$ in the number 10 fractions (Fig. 2 and Table II) was used for the molecular weight determinations. It was precipitated with and washed in 50% ethanol and dissolved in water. An appropriate volume of water containing a known weight of $(CHO)_n$ was added to a portion of the standard latex suspension previously described. Electron microscope
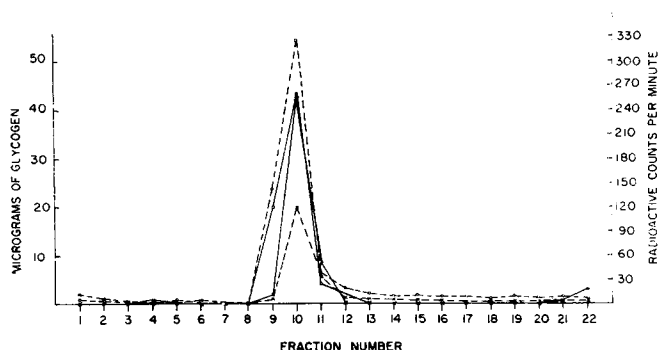

Fig. 2. CsCl density gradient centrifugation of radioactive $(CHO)_n$ extracted from (O) poliovirus-infected and (X) noninfected HeLa cells: (——) weight of $(CHO)_n$ extracted; (- -) radioactivity. The fractions were diluted with water, assayed for radioactivity, $(CHO)_n$ content, and absorption at 260 mμ. The specific activities of the $(CHO)_n$ in fractions 9, 10 and 11 are listed on Table II. Although virus infection stimulated $(CHO)_n$ synthesis, the buoyant density of $(CHO)_n$ extracted from infected cells was similar to that of $(CHO)_n$ extracted from noninfected cells.

TABLE II

Comparison of the Specific Activities of Polyglucose Extracted from Control and Polio-
virus Infected HeLa Cells Using Phenol, and Purified With CsCl Centrifugation or
Trichloroacetic Acid Precipitation of the RNA

Sample	No. of fraction (from CsCl gradient) [a]	Specific activity, counts/μg/min		
		Of each fraction	Avg. of fractions	TCA method
Infected	9	7.1 ⎫		
	10	7.6 ⎬	7.9	8.2
	11	9.0 ⎭		
Control	9	3.3 ⎫		
	10	2.8 ⎬	3.4	3.4
	11	4.2 ⎭		

[a] The CsCl gradient is illustrated on Fig. 2.

grids were prepared with this mixture and the ratios of $(CHO)_n$ particles
to latex spheres were determined. Table III presents these data. From
these ratios and the known concentration of spheres, the number of $(CHO)_n$
particles in the mixture was determined. From the dilution factor, the
number of the $(CHO)_n$ particles in the original sample was calculated. The
average weight of a $(CHO)_n$ particle was calculated by dividing the total
weight of the $(CHO)_n$ by the number of particles in the original sample.
The number-average molecular weights of the $(CHO)_n$ (1.85×10^6 and
2.0×10^6) are the product of Avogadro's number times the weight in grams
of a single particle.

From the previous experiments (Table II), one may not conclude that the
$(CHO)_n$ extracted from HeLa cells by using TCA will have a specific
activity comparable to that of the $(CHO)_n$ extracted by using phenol.
This was investigated by reducing the level of glucose to 0.01 of normal and
exposing the HeLa cell cultures for 1 hr to 3 μCi ^{14}C-glucose and by de-
termining the specific activity of the $(CHO)_n$ which was extracted with
phenol and purified by precipitating the RNA with TCA. The specific
activity of this $(CHO)_n$ was compared with $(CHO)_n$ extracted from dupli-
cate cultures using TCA. The method used for extracting the $(CHO)_n$
with TCA is described in the Materials and Methods Section. The specific
activities of these $(CHO)_n$ preparations and the total amount of $(CHO)_n$
extracted in three experiments are listed on Table IV.

In addition to the methods of purification and extraction (Tables II and
IV), another important factor in accurately determining the rate of syn-
thesis of $(CHO)_n$ and the amount of incorporation of ^{14}C from ^{14}C-glucose
into RNA and DNA is the time at which the cultures are pulse-labeled with
^{14}C-glucose following the lowering of the glucose level in the culture medium.
To examine this factor, the glucose in the Eagle's culture medium was
reduced to 0.01 of normal, and HeLa cell cultures were pulse-labeled at

TABLE III

Molecular Weight of Polyglucose Extracted from Poliovirus-Infected and Noninfected HeLa Cells

$(CHO)_n$ sample	Latex: $(CHO)_n$ [a]	Counts[b]		Avg. ratio $(CHO)_n$/latex	Particle concn [c]	Particle weight, g	Molecular weight [d]
		Latex	$(CHO)_n$				
Noninfected	6:1	398	1819	5.0	4.13×10^{13}	3.44×10^{-18}	2.00×10^{6}
Infected	6:1	727	1972	3.48	2.87×10^{13}	3.07×10^{-18}	1.85×10^{6}

[a] The sample counted was prepared by adding 6 parts of latex containing 1.37×10^{12} spheres/ml to one part of $(CHO)_n$ containing 142 μg/ml isolated from noninfected cultures and to one part of $(CHO)_n$ containing 88 μg/ml isolated from infected cultures.

[b] The total number of latex and $(CHO)_n$ particles in the 24 fields examined.

[c] The number of particles in the original solution as calculated from the ratio of $(CHO)_n$/latex and the dilution factor.

[d] The number-average molecular weight is the product of Avogadro's number times the particle weight in grams.

TABLE IV

Specific Activities and the Total Amounts of Polyglucose Extracted with Phenol
as Compared with Trichloroacetic Acid

Expt.	Specific activity of $(CHO)_n$, counts/min/μg		Total $(CHO)_n$ extracted, μg	
	TCA	Phenol	TCA	Phenol
1	1.40	0.742	1174	718
2	1.53	0.908	1194	630
3	1.53	0.588	1204	788
Average	1.49	0.746	1191	712

hourly intervals with 3 μCi of ^{14}C-glucose. The cultures were extracted
with phenol, the RNA and $(CHO)_n$ were precipitated from the saline phase
with 20% (v/v) ethanol and separated from each other by precipitating the
RNA with TCA. The DNA was then precipitated by increasing the
ethanol to 50% (v/v). After washing the RNA and DNA with 5% (w/v)
TCA, the specific activity of each type of macromolecule was determined.
The specific activity and the total quantity of $(CHO)_n$, RNA and DNA
extracted from the cultures in a representative experiment are listed on
Table V. When compared with the nucleic acids, the specific activity of
the $(CHO)_n$ increased more with time while the amount of extractable
$(CHO)_n$ decreased.

If the chloride ions and $(CHO)_n$ are present in preparations of DNA, the
amount of color produced in the Dische test for DNA may be altered.[4]
What is needed then, is a test for DNA which is not influenced by $(CHO)_n$
but which will detect $(CHO)_n$ if $(CHO)_n$ is present in the assay mixture.
The following experiments describe how the Burton reagent may be used
to meet this twofold need.

TABLE V

Specific Activities and the Amounts of Polyglucose, RNA, and DNA Extracted
from HeLa Cells Which Were Pulse-Labeled with ^{14}C-Glucose [a]

Labeling interval, hr	Specific activity, counts/μg/min			Amount extracted, μg		
	$(CHO)_n$	RNA	DNA	$(CHO)_n$	RNA	DNA
0–1	3.2	3.2	1.1	132	1166	1218
1–2	8.3	4.4	1.6	76	996	1120
2–3	28.8	4.3	1.5	36	1330	1088
3–4	74.8	5.0	1.8	16	1264	1144

[a] The glucose in the culture medium was reduced to 0.01 of normal and the cultures
were then pulse-labeled with 3 μCi of ^{14}C-glucose at each hourly interval thereafter, as
indicated. Note that the specific activity of the polyglucose $(CHO)_n$ increased at each
successive time interval while the amount of $(CHO)_n$ extracted decreased.

Since it is known that chloride ions reduce the sensitivity of the Dische test and play some role in the development of the color produced in the reaction between diphenylamine and $(CHO)_n$,[4] the effect of chloride ions in the Burton test for DNA, as well as in the reaction between $(CHO)_n$ and the Burton reagent was examined. Melezitose and synthetic polyglucose were selected for this study because their composition is known and it is also known that fructose is more reactive than glucose in the diphenylamine test for $(CHO)_n$.[4]

Heating (100°C) the $(CHO)_n$–Burton reagent reaction mixture is essential for development of color and chloride ions affect the development of the colored reaction product(s). Figure 3 illustrates these effects, as well as the inhibitory effect of NaCl on amount of color produced in the Burton test for DNA. On a molar basis, CsCl is as effective as NaCl in affecting the color produced in the reaction between $(CHO)_n$ and the Burton reagent. Therefore, the anion, rather than the cation, is considered to be the active constituent.

Using the data in Figure 3, one could obtain presumptive evidence as to whether a preparation of DNA contained $(CHO)_n$ by heating the reaction mixture and noting an increase in the optical density at 600 mμ. The factors to be considered are the concentration of NaCl, the period of time the reagent mixture is heated and the type and concentration of $(CHO)_n$ present. Greater sensitivity may be obtained by recording the spectra (350–900 mμ) of the colored reaction products after heating. If no $(CHO)_n$ is present, the adsorption spectra of DNA will not be altered by heating. If $(CHO)_n$ is present in a DNA preparation which is not heated, the adsorp-
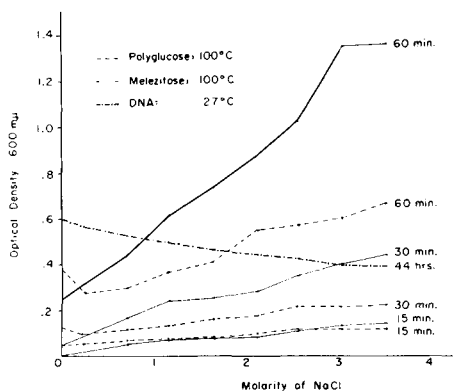

Fig. 3. Effects of chloride ions on the Burton test for DNA and the reactions between the Burton reagent and melezitose or synthetic polyglucose. A 2-ml portion of the Burton reagent was added to 100 μg of DNA or 1250 μg of $(CHO)_n$ in 1 ml of water containing NaCl in the concentrations indicated above. The DNA solutions were held for 44 hr at 27°C. The $(CHO)_n$ solutions were heated at 100°C for 15, 30, and 60 min and the optical density of all the solutions was recorded at 600 mμ. Note that chloride ions reduced the amount of color produced in the Burton test for DNA while increasing the amount of color produced by melezitose and, above 1.5 molar, polyglucose. Without heating, no color was produced in the $(CHO)_n$ solutions.
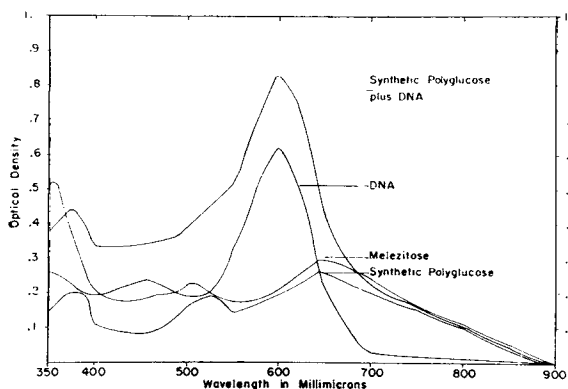
Fig. 4. Absorption spectra of the products in the reactions between the Burton reagent and melezitose, synthetic polyglucose, DNA, and DNA plus synthetic polyglucose. A 2-ml portion of the Burton reagent was added to 1 ml of $1.65M$ NaCl containing either 1250 μg of melezitose, synthetic polyglucose, 1250 μg of synthetic polyglucose plus 100 μg DNA, or 100 μg of DNA alone. The solutions were held for 48 hr at 27°C. No color developed in the solutions containing only the $(CHO)_n$, whereas the spectrum of the DNA and DNA plus polyglucose solutions was essentially that of DNA illustrated above. The solutions were heated (100°C, 30 min) and the spectra were recorded with a Beckman DK-2B spectrophotometer using 1-cm silica cells. Note that the $(CHO)_n$ has three absorption maxima, none corresponding to the maximum of DNA (600 mμ), and that the spectra of polyglucose is additive to that of DNA.

tion spectra will be that of DNA. If $(CHO)_n$ and DNA are present and the reagent mixture is heated, new adsorption peaks referable to the reaction product(s) of the Burton reagent plus $(CHO)_n$ appear. Figure 4 illustrates these findings.

Discussion

That the $(CHO)_n$, RNA, and DNA precipitate together in 50% ethanol–saline solutions and that the precipitate has the white, viscous appearance of DNA may lead to errors in the quantitative and qualitative estimates of each macromolecule present in the precipitate. Another source of confusion and possible error is that in CsCl, the buoyant density of DNA and $(CHO)_n$ is similar. (This is considered not to be a result of an interaction between native DNA and $(CHO)_n$ but rather an individual characteristic of the $(CHO)_n$ and DNA.[8]) Some of the CsCl fractions, then, may contain DNA as well as $(CHO)_n$. The error in estimating the quantity of DNA in such fractions stems from the fact that when heated, the chloride ion (contributed by CsCl) plus $(CHO)_n$ plus the diphenylamine in the Dische reagent contribute color, which may result in an erroneously high value for DNA.[4] In addition, the $(CHO)_n$ scatters ultraviolet light.[1,2,9] Here, the DNA was separated from the $(CHO)_n$ by precipitating with 20% ethanol the RNA–$(CHO)_n$ from $1M$ saline containing RNA, $(CHO)_n$, and DNA prior to assaying for DNA (Table I). Also, the chloride ions may be eliminated by precipitating the macromolecule(s) in the CsCl fractions with

50% ethanol. Ethanol (50% v/v) will precipitate 42% (w/v) but not 36% (w/v) CsCl (5°C, 1 hr). This solubility characteristic was used to advantage in removing the $(CHO)_n$ from diluted CsCl fractions (Table II, Fig. 2).

Another way to eliminate the interference of $(CHO)_n$ in chromogenic assays for DNA is by using the Burton test. The Burton reagent is not heated during the assay for DNA.[27] The $(CHO)_n$,even in the presence of chloride ions, will not produce color if the reagent mixture is not heated (Fig. 3). The obvious disadvantage is that if $(CHO)_n$ is present in the DNA preparations, the Burton reagent will not detect it if the reagent is used as directed. However, simply by heating the reagent mixture, the $(CHO)_n$ may be detected. If the DNA preparations contain $(CHO)_n$, not only will the $(CHO)_n$ increase the optical density at 600 mμ following heating, but absorption peaks attributable to $(CHO)_n$ will appear at other wave lengths. For example, synthetic polyglucose has absorption maxima at 640, 525, and 370 mμ, and its spectra will be additive to the spectra of DNA (Fig. 4). At NaCl concentrations above 1.5M the intensity of the color produced by all $(CHO)_n$ examined is increased. Therefore, to increase the potential of the Burton reagent to detect $(CHO)_n$, NaCl may be added to the assay mixture. Addition of chloride will not reduce the optical density of DNA, nor will heating (30 min, 100°C) increase the optical density of solutions containing only DNA, once the maximum color attributable to DNA has been reached. Solutions saturated with NaCl may be used (in 2 ml of the Burton reagent and 1 ml of sample, 0.86M is the solubility limit of NaCl). However, if chloride ions are present during the development of color produced by DNA, the sensitivity of the Burton test is reduced (Fig. 3). Reference blanks containing the Burton reagent and concentrations of aqueous NaCl identical to that in the test sample will not compensate for the error. If the DNA standards contain concentrations of chloride ions equivalent to the test sample, the two may be related with no error.

In practice, after the maximum color produced by the DNA is attained, the reagent mixture is heated (100°C). If an increase in the optical density at 600 mμ is detected, then this is presumptive evidence for the presence of $(CHO)_n$. Adding NaCl prior to heating and noting the change in the spectra (350–900 mμ) following heating, increases the sensitivity of the reagent to detect $(CHO)_n$. An increase in the ratio of the optical density prior to heating to that following heating at wavelengths of approximately 650–875 mμ and 575–350 mμ is a more conclusive indication for the presence of $(CHO)_n$ than an increase at 600 mμ (Fig. 4).

Further experiments, similar to those illustrated in Figures 3 and 4, indicate that the Burton reagent used in the manner described here will give quantitative and qualitative estimates of $(CHO)_n$ which may be present in preparations of DNA. These findings will be presented in a following communication.

Girard and Baltimore[31] have observed that the rates of sedimentation of poliovirus RNA, messenger, and HeLa cell ribosomal RNA through sucrose gradients were increased by a factor of 1.5–2.0 by an unidentified HeLa cell

cytoplasmic component(s) with a molecular weight(s) of 10,000 or greater. Here, the $s_{w,20}$ of 6.4 S for RNA, which was separated from $(CHO)_n$ by TCA precipitation of the RNA, was increased to 25.4 S when $(CHO)_n$ was present in the preparation.   In addition, the RNA reduced the $s_{w,20}$ of the $(CHO)_n$ from 116 to 31.4 S.   An assumption implicit in these interpretations is that when RNA and $(CHO)_n$ were centrifuged together, the slow- and fast-moving components were RNA and $(CHO)_n$, respectively, or complexes of both macromolecules.

One interpretation of these findings is that the $(CHO)_n$ and RNA interact and the sizes and/or shapes of the RNA and $(CHO)_n$ are altered.   If the interaction between RNA and $(CHO)_n$ results in complexes with greater sizes and molecular weights, one expectation might be that the $s_{w,20}$ value for the RNA–$(CHO)_n$ complexes would be increased.   Another interpretation is that when the RNA was separated from the $(CHO)_n$ by precipitation with TCA, the RNA was degraded.   It is known that repeated TCA precipitations of RNA increase the solubility of the RNA in 20% ethanol.[10] If such treatment reduced the size of the RNA, one expectation might be that its solubility would increase and the $s_{w,20}$ value would be decreased. Regarding the $(CHO)_n$, if TCA treatment aggregates the particles as the electron micrographs suggest, then, the $s_{w,20}$ value could be expected to be increased.   However, as with DNA,[32] the morphology of the $(CHO)_n$ may be altered by the type of receiving surface used in the preparation of the electron microscope grids.   For example, light-scattering measurements suggest that the $(CHO)_n$ from HeLa cells is a linear molecule, 2700 Å in length and with a thickness of 50 Å,[29] whereas electron micrographs do not show this linearity.   Therefore, caution is required in interpreting the sedimentation characteristics of the $(CHO)_n$ on the basis of its appearance in shadow-cast collodion membrane preparations (Fig. 2).   At this time, definitive interpretation of these $s_{w,20}$ alterations cannot be made.   However, since it is not generally known that $(CHO)_n$ accompanies and interacts with nucleic acids, this presentation of the experimental findings seems warranted.

RNA governs the solubility of $(CHO)_n$ in 20% ethanol–saline solutions by interacting with and precipitating the $(CHO)_n$.[10]   The present experiments (Table I) demonstrate that DNA, RNA, and $(CHO)_n$ have the capacity to interact with each other in 50% ethanol.   When the resultant precipitate was removed from the supernatant fluids and the components were separated and analyzed, it was found that for each microgram of DNA. approximately 2 and 3 μg of $(CHO)_n$ and RNA, respectively, were present, The limits of the combining capacities of the macromolecules cannot be determined from these data.   However, the combining capacity of RNA with $(CHO)_n$ is below the previously described 1:6 ratio.[10]   Since, the solubility in ethanol of RNA is less than that of $(CHO)_n$[10] and that of $(CHO)_n$ is less than that of DNA,[8] it may be likely that the order in which the three macromolecules combined with each other was RNA, $(CHO)_n$, and then DNA.

The type(s) of bonding that occur between these macromolecules is not known. Considered to be a main force involved in these interactions is hydrogen bonding between RNA and $(CHO)_n$, heat-denatured HeLa cell DNA and $(CHO)_n$, and poly(adenylic acid) and dextran.[8-11] In a similar manner, all three macromolecules examined here could be interacting via hydrogen bonds.

The relative amounts of $(CHO)_n$, RNA, and DNA in HeLa cells which are maintained in Eagle's medium, containing normal amounts of glucose, will vary with respect to the age of the culture. In addition, we have observed differences in the amounts of $(CHO)_n$ extractable from S-3 HeLa cells which originated from different clones. The quantitative differences in the amounts of $(CHO)_n$ relative to RNA or DNA in experiments presented in Tables I and V are examples of the variation which may be encountered between experiments using the same cell line. This variation emphasizes that the proportions listed in Table I will not be constant for all HeLa cells.

Once the RNA and $(CHO)_n$ were separated from DNA by precipitation with 20% ethanol, the RNA and $(CHO)_n$ in the RNA–CHO complex may be separated from each other, either by CsCl density gradient centrifugation or by precipitatoin of the RNA with TCA (Table II). Both methods yield $(CHO)_n$ with similar specific activities, indicating that the separation was complete. Additional evidence of purity of the $(CHO)_n$ is that the $(CHO)_n$ in the CsCl fractions did not absorb ultraviolet light at 260 mμ, which indicates that RNA or DNA was not present in the $(CHO)_n$ preparations. Therefore, the specific activities of the $(CHO)_n$ separated either by TCA or CsCl centrifugation is similar and the more rapid TCA method yields $(CHO)_n$ suitable for metabolic studies.[20,25]

A difference noted in the $(CHO)_n$ separated by TCA rather than CsCl centrifugation was that the TCA method yielded $(CHO)_n$ with a greater range in the particle size (Fig. 1). Cold TCA yields $(CHO)_n$ whose particles are prone to aggregation and appear larger than those purified by CsCl. Particles smaller than those purified by CsCl centrifugation are also seen. Apparently, TCA has the capacity to aggregate as well as degrade the $(CHO)_n$ particles. This range in particle size renders the $(CHO)_n$, which was separated from RNA using TCA, unsuitable for accurate number-average molecular weight determinations. However, $(CHO)_n$ purified by CsCl centrifugation is quite uniform and permits a reliable molecular weight determination. Such $(CHO)_n$ was used here to determine whether there was a change in the buoyant density in CsCl or in the molecular weight of the $(CHO)_n$, whose synthesis was stimulated by poliovirus infection.

The molecular weight of $(CHO)_n$, extracted from infected cells (1.85×10^6) is not considered to be significantly different from that of noninfected cells (2.0×10^6). Although poliovirus infection induces a stimulation in the rate of $(CHO)_n$ synthesis,[20] the $(CHO)_n$ containing the newly added glucose residues has a buoyant density and a molecular weight similar to

that of the $(CHO)_n$ extracted from noninfected cells. These results indicate that poliovirus infection does not change the size or number of $(CHO)_n$ particles per unit weight of the extracted $(CHO)_n$. One explanation is that in HeLa cells the stimulated synthesis of $(CHO)_n$ is the result of an increased turnover of $(CHO)_n$. This stimulation of $(CHO)_n$ synthesis is not reflected in any detectable change or shift in the glycolytic and Krebs cycle or hexosemonophosphate shunt pathways. Interestingly, at the same time post-infection, the incorporation of ^{14}C from glucose into HeLa cell RNA, DNA, and protein is inhibited by virus infection.[24] To the author's knowledge, attempts to describe any change in the properties of $(CHO)_n$ whose synthesis was stimulated in other pathological processes has not been reported. Since $(CHO)_n$ may have a function other than as a source of energy during differentiation,[33] the importance of $(CHO)_n$ would seem to warrant such investigations in diseased, as well as normal cells.

Phenol extraction of HeLa cells yields $(CHO)_n$ with a specific activity which is 50% that of $(CHO)_n$ extracted with TCA (Table IV). One explanation for the greater specific activity may be that of the phenol method is more selective for a specific population of $(CHO)_n$, whereas the TCA method yields a greater quantity of $(CHO)_n$, having a more heterogenous nature. Various authors have commented on some of these considerations.[34-38]

Another explanation is that TCA extraction yields $(CHO)_n$ contaminated with other molecules which were also labeled with ^{14}C, originating from glucose. If so, when the total radioactive count is related only to the weight of the $(CHO)_n$, the specific activity of the $(CHO)_n$ would be erroneously high. Protein–$(CHO)_n$ complexes, which are soluble in TCA, have been described,[39] and complexes of a like nature could account for the greater specific activity of the $(CHO)_n$ extracted with TCA. The chromogenicity of a known weight of $(CHO)_n$ which was extracted from HeLa cells with phenol was equivalent to that of an equal weight of glucose. This suggests that the species of $(CHO)_n$ examined here is polyglucose and is free of contaminants.[1] Since phenol tends to eliminate proteins, it seems likely that $(CHO)_n$ extracted with phenol will reflect a more accurate estimate of the amount of incorporation of ^{14}C than $(CHO)_n$ which is extracted with TCA. The phenol method of extraction has been used to determine the rate of synthesis of $(CHO)_n$ in poliovirus[20] and in vaccina virus-infected[25] HeLa cells. When TCA was used to extract $(CHO)_n$ under similar circumstances, erratic results were obtained.

If radioactively labeled tracer studies involve the incorporation of ^{14}C from glucose into $(CHO)_n$, RNA, and DNA, either the glucose in the culture media must be reduced to allow sufficient incorporation of ^{14}C into the macromolecules or large quantities of ^{14}C-glucose must be added to the media.[25] For reasons of toxicity, as well as economy, reducing the glucose levels is the method of choice. However, the time when cultures are pulse labeled after lowering the glucose level in the culture media is an important consideration. The specific activities of RNA and DNA both increase to a

similar extent over the 4-hr period (an increase of 1.56- and 1.64-fold, respectively), whereas, the amount extracted is not significantly altered. In comparison, the specific activity of the $(CHO)_n$ increased 23.4-fold and the amount extracted decreased 8.2-fold over the 4-hr period (Table V). Clearly, the $(CHO)_n$ is more responsive to the decreased glucose level.

Alpers has also described a decrease in the amount of glycogen in HeLa cells cultured in "low-glucose" media. Phosphorylase, glycogen synthetase, glucose-6-phosphate, UDPG, ATP, and total adenine nucleotides were also decreased in these low-glucose cells.[40] Conceivably, the increased specific activity of the nucleic acids observed here could be a result of a reduced intercellular pool size of adenine nucleotide. This would allow more ^{14}C from the distal precursor, glucose, to be incorporated into RNA and DNA. Regarding the increased specific activity of $(CHO)_n$, the decreased glucose pool size may account for the increased incorporation of ^{14}C into the extractable $(CHO)_n$. However, since Alpers has shown that the activities of the enzymes involved in glycogen synthesis were low, the increased specific activity and the decreased quantity of extractable $(CHO)_n$ may be the net result of the decreased glucose pool size, decreased enzyme activity and the degradation of preexisting, nonlabeled $(CHO)_n$. Stetten et al. have shown that glycogen is metabolically inhomogenous,[41] and in this instance, increased glycogenolysis would tend to increase the specific activity of the $(CHO)_n$, as well as decrease the amount of extractable $(CHO)_n$.

An important consideration is that $(CHO)_n$, RNA, and DNA all respond, with time, to the decreased glucose level in the culture media. This factor should be considered in experiments concerned with the incorporation of ^{14}C from ^{14}C-glucose into macromolecules.

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