The Isolation and Characterization of a Novel Microsome Fraction from Washed and Detergent-Treated Nuclei of HeLa Cells by the Use of Solutions Containing Deoxyribonucleic Acid*

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ABSTRACT: A membrane fraction was isolated from washed and detergent-treated HeLa cell nuclei by extraction with a buffer containing deoxyribonucleic acid (DNA). The nuclei were recovered quantitatively following this extraction and did not differ in their appearance under the phase-contrast microscope from that of unextracted nuclei. The extract was characterized as a membrane fraction by its chemical composition and its content of the microsomal marker enzyme, reduced nicotinamide–adenine dinucleotide–cytochrome *c* reductase. This fraction was rich in ribosomal aggregates which were identified by their characteristic sedimentation in zonal centrifugation, extreme sensitivity to degradation with ribonuclease (RNase), and content of rapidly labeled RNA. The kinetics of the appearance of newly synthesized RNA and DNA in this fraction, following exposure of intact cells to labeled precursors, helped to differentiate this fraction both from the cytoplasmic microsomes and from the RNA and DNA of the residual nuclei which remained after extraction. The extract fraction incorporated amino acids into acid-insoluble material *in vitro*. The products of the incorporation resembled the products obtained using cytoplasmic microsomes as the enzyme source more than the products obtained when nuclear microsomes were used even though the fraction was derived from washed nuclei. Other polyanionic materials such as sodium poly(ethenesulfonate) also caused the release of similar fractions from washed nuclei.

The possibility that this fraction had its origin in the nuclear membrane is discussed.

One major difference in subcellular organization between bacteria and cells derived from higher organisms is the existence in cells from higher organisms of a compartmentalization which separates the bulk of the cell's protein-synthesizing machinery (*i.e.*, the cytoplasm) from the genetic material in the nucleus. Up to this time most biochemical studies have assumed or implied a clear distinction between these compartments and only a very few papers have attempted to deal with certain "cytoplasmlike" characteristics which are found associated with carefully purified nuclei (Penman, 1966; Holtzman *et al.*, 1966).

In the course of our studies on the subcellular localization of a number of macromolecular events in HeLa cells (Bach, 1962; Bach and Johnson, 1964, 1966a,b) we have developed a method for isolating a unique membrane fraction from washed nuclei which has both cytoplasmic and nuclear properties. It is the purpose of this paper to describe the isolation and characterization of this fraction as well as some of the dynamic properties of the macromolecules which are associated with it.

Methods and Materials

Culture, Exposure to Isotope, and Homogenization. HeLa cells were grown attached to glass in Eagle's basal medium supplemented with 33 units/ml of penicillin, 0.17 mg/ml of streptomycin sulfate, and 10% (v/v) calf serum. Cultures (2 to 3 days old) were harvested by scraping and suspended in culture medium at a concentration of 2×10^7 cells/ml. [^{3}H]Urd[1] (0.9 μM, sp act. 3.7 mc/μmole) or [^{3}H]dThd (1.5 μM, sp act. 7 mc/μmole) was added for varying exposure times after the cells had been stirred gently for 30 min at 37°. To establish the rate of incorporation of [^{3}H]-dThd as a function of the DNA content of the various subcellular fractions in the presence of the large amounts of exogenous DNA which were added during isolation, HeLa cells were grown in a medium which was supplemented with 0.2 μM [^{14}C]dThd (0.94 mc/mmole). This medium was changed twice and replenished with fresh [^{14}C]dThd over the last 36 hr of the growth period. Cells were then harvested, resuspended in medium, and exposed to [^{3}H]dThd while in suspension as described above.

After incubation with the labeled precursors, the

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[1] Abbreviations used: [^{3}H]Urd, tritiated uridine; [^{3}H]dThd, tritiated thymidine; [^{14}C]dThd, ^{14}C-labeled thymidine; PEP, potassium phosphoenolpyruvate; PK, pyruvate kinase; SDOC, sodium deoxycholate; SDS, sodium dodecyl sulfate; OD_{260}, absorption at 260 mμ; OD_{565}, absorption at 565 mμ; NADH, reduced nicotinamide-adenine dinucleotide; ATP, GTP, and UTP, adenosine, guanosine, and uridine triphosphates.

cells were washed twice in Puck's salts solution and allowed to swell in distilled water at 0° for 30 min before adjustment to 0.25% with respect to Triton X-100 (Hymer and Kuff, 1964) and homogenized in a tight-fitting Dounce homogenizer (Penman *et al.*, 1963). Immediately following homogenization the suspension was adjusted to contain the following: 0.25 M sucrose, 5 mM magnesium acetate, 0.1 M KCl, and 0.05 M Tris-HCl buffer (pH 7.8). A medium of this composition but without the detergent will be referred to as solution A.

Particle Fractionations. The nuclei were harvested by centrifugation at 600*g* for 15 min. They were washed twice in solution A plus detergent by centrifugation at the same speed. Where indicated, they were further purified by sedimentation through a layer in solution A which was 2.2 M with respect to sucrose at 37,000 rpm for 90 min in the SW 39 rotor of the Spinco Model L ultracentrifuge (Widnell and Tata, 1964).

Mitochondria were obtained from the supernatant fractions following removal of the nuclei by centrifugation at 12,000*g* for 15 min. In the experiments where mitochondria were isolated no Triton X-100 was used during homogenization. The mitochondria were washed once by fractional centrifugation of the resuspended pellet between 600*g* and 12,000*g*.

Large microsomes were obtained by sedimentation at 30,000*g* for 30 min and purified by centrifugation through a layer of solution A which was 0.9 M with respect to sucrose at 37,000 rpm for 90 min (Wettstein *et al.*, 1963). Small microsomes were similarly purified from the supernatant of this centrifugation, or from pellets which were obtained by centrifugation at 157,000*g* for 90 min. Ribosomes were obtained by treatment of microsomes with 0.5% SDOC prior to centrifugation. The final supernatant is referred to as the soluble fraction.

The DNA extract of the nuclei was obtained by resuspending the washed nuclei in solution A which was supplemented with 5 mg/ml of salmon sperm DNA (3 mg/ml initial volume of packed cells). The DNA had been shear degraded by sonication in the Raytheon 10-kcycle sonic disintegrator for a total of 20 min. The sonication was interrupted periodically to prevent overheating. Such DNA solutions had a markedly reduced viscosity and could be pipetted readily. After standing at 0° for 30 min two volumes of solution A were added to the suspended nuclei per volume of DNA solution used and the suspension was centrifuged at 12,000*g* for 15 min. (Even centrifugation at 1000–5000*g* was sufficient to completely sediment the nuclei.) The pellet is referred to as extracted nuclei and the supernatant as the DNA extract of nuclei. This extract was fractionated further into microsomes and ribosomes as described above.

Enzymatic Assays. NADH–CYTOCHROME *c* REDUCTASE. This enzyme activity was assayed as described by Hudack and Brummond (1961) in a final incubation volume of 0.5 ml using the Cary Model 11 recording spectrophotometer to follow the absorption changes.

SUCCINIC DEHYDROGENASE. Activity was measured by following the reduction of ferricyanide (Bonner, 1955) in a Gilford multiple sample absorption recorder which was equipped with an auxiliary dwell timer.

PROTEIN SYNTHESIS. The priming activity of isolated RNA fractions for amino acid incorporation *in vitro* was determined with an *Escherichia coli* S-30 fraction as described by Nirenberg (1963). The RNA used for these studies was isolated by a combination of the methods of Georgiev *et al.* (1963) and Ralph and Bellamy (1964). The bulk of the RNA was first isolated by extraction with phenol at 0°. This was followed by release of the rapidly labeled RNA from the interface pellets which were obtained in the first extraction by heating to 65°. The composition of the aqueous and phenol layers in these extractions and the further processing of the aqueous layers were precisely as described by Ralph and Bellamy (1964).

The ability of various particulate fractions from HeLa cells to incorporate amino acids into protein was assayed by a modification of the methods of Ochoa and Weinstein (1964). For these experiments cell homogenates were prepared in a buffer which was 0.01 M with respect to Tris-HCl (pH 7.8), 3 mM magnesium acetate, 25 mM KCl, 0.25 mM sucrose, and 6 mM β-mercaptoethanol (solution B). The use of Triton X-100 stimulated incorporation. A pH 5 fraction was prepared from the supernatant fraction of rat liver homogenates following centrifugation at 105,000*g* for 90 min by precipitation with dilute acetic acid at pH 5.0. The fraction was redissolved in solution B and the pH was adjusted to 7.8 with dilute KOH. This fraction was stored over liquid nitrogen. Incubation tubes contained 25 mM Tris-HCl (pH 7.8), 6.0 mM magnesium acetate, 75 mM KCl, 1.5 mM ATP (sodium salt), 50 μM GTP, 4 mM PEP, 6 mM β-mercaptoethanol, 40 μM each of 20 L-amino acids including one tritiated amino acid (sp act. 200–300 mc/mmole), 2.4 μg of PK, 60 μg of liver tRNA, rat liver pH 5 fraction, and HeLa particulate enzyme preparation in a total volume of 0.10 ml. The pH 5 fraction was added at three times the protein concentration of the particulate fraction. After incubation for 20 min at 37°, samples were precipitated with trichloroacetic acid and processed for analysis of content of radioactivity as described by Wettstein *et al.* (1963).

When the simultaneous incorporation of two radioactive amino acids was studied the radioactive amino acids were first diluted with unlabeled amino acids and chromatographed singly on thin layers of silica gel using the following solvents: 1-butanol–acetic acid–water (4:1:5, v/v) for phenylalanine, and 2-butanone–propionic acid–water (75:25:30, v/v) for leucine. The bands corresponding to the ninhydrin-positive marker spots were eluted with 0.01 N HCl, concentrated to dryness *in vacuo*, and reconstituted in a small volume of 0.01 N HCl. They were then standardized with respect to concentration and specific activity by an ultramicroadaptation of the ninhydrin reaction and radioactive counting. For this procedure 7.5 μl containing 1.0–30 mμmoles of amino acid, along with 150 μl of ninhydrin reagent (Moore and Stein, 1954),

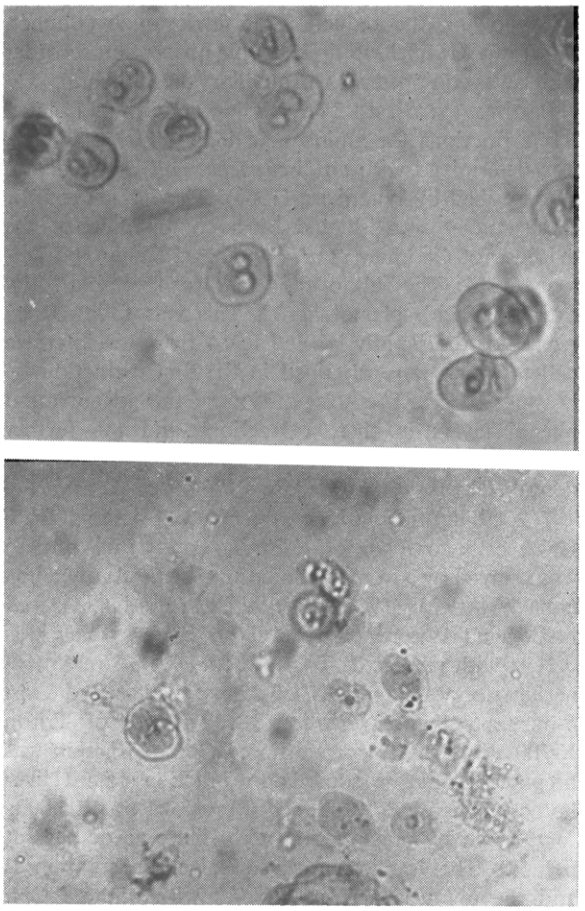

PLATE 1: Appearance of washed nuclei before and after extraction with solutions containing DNA. The extracted nuclei were stabilized with 0.5 mM $CaCl_2$ and 5% dextran and washed repeatedly to remove the added DNA. Magnification 915×.

in a final volume of 160 μl were placed in a boiling water bath for 20 min. Samples were diluted to 0.65 ml with 50% aqueous isopropyl alcohol and OD_{565} was converted to millimicromoles of amino acid by use of a standard curve for the same amino acid.

For the characterization of radioactive protein products by electrophoresis on polyacrylamide gels, uniformly ^{14}C-labeled algal protein hydrolysate served as the source of radioactive amino acids. Following a 40-min incubation, the mixtures were solubilized as described by Summers *et al.* (1965) by the addition of one-tenth volume each of 5 M urea, 10% SDS, and glacial acetic acid. After standing at 37° for 30 min, the samples were dialyzed overnight against 4000 volumes of 0.01 M phosphate buffer (pH 7.2) which was 0.5 M with respect to urea, 0.1 M with respect to β-mercaptoethanol, and 0.1% with respect to SDS. This step also served to remove the free amino acids. The samples were then concentrated 10–20-fold by dialysis against a saturated sucrose solution in the same buffer. Electrophoresis was conducted in a gel which was only 7.5% with respect to acrylamide but was otherwise identical with that described by Summers *et al.* (1965). Separations using 50 μl of each protein concentrate were run on a vertical sheet of gel in an apparatus which was similar to that described by Raymond (1962) at 160 v (10 v/cm) for 3.5 hr in a tap water cooled system. The gels were dehydrated, and fixed in 60% aqueous acetone for 1 hr, and then stained in Amido Black stain (Raymond and Wang, 1960) for at least 36 hr. After electrolytic destaining in 5% acetic acid, the stained bands were scanned using a Joyce Chromoscan. The gels were then cut into 2.5 mm-long fractions which were solubilized by brief heating to 90° with 0.25 ml of Superoxol (W. Benjamin, personal communication). Care was taken to minimize the duration of the heating as extended heating led to losses of radioactivity. After cooling, the addition of 0.5 ml of methanol, followed by 0.4 ml of Hyamine and heating to 50–60°, completely removed any residual peroxide from the samples. The counting efficiency of such samples was identical with that for other Hyamine-containing samples provided that all bubbling had stopped before scintillator solution was added.

Analytical. Radioactivity was determined by plating perchloric acid hydrolysates of radioactive nucleic acid containing samples and Hyamine dispersions of protein samples using diotol scintillator fluid (Herberg, 1960) and counting on a Packard Tri-Carb liquid scintillation spectrometer.

For double isotope counting (^{3}H and ^{14}C) the external standard feature of the Packard Model 3000 liquid scintillation spectrometer was used. The volume of the samples was strictly controlled at 12 ml to avoid the variation in the external standard counts with sample volume. The ratio of the external standard counts in the high-energy (blue) and tritium (red) channel was related to counting efficiency of tritium and carbon in the two channels by empirical curve fitting. In most cases the relationship was linear in a log–log plot. The derived equations were then programmed on the IBM 1620 computer and used to estimate the disintegrations per minute of ^{3}H and ^{14}C in the various samples.

Cell fractions for determination of RNA, protein, and phospholipid content were prepared by the method of Schmidt and Thannhauser (1945) modified by the use of chloroform–methanol (Bligh and Dyer, 1959) for the extraction of lipids. Protein determinations were made by the method of Oyama and Eagle (1956), RNA by the orcinol method (Dische, 1955), DNA by the Burton modification of the diphenylamine method (Burton, 1956), and phospholipid by multiplying the P_i content (Fiske and Subbarow, 1925) of the wet-ashed chloroform–methanol extracts by 25 (Ernster *et al.*, 1962).

Salmon sperm DNA, poly U, and PK were purchased from the California Corp. for Biochemical Research. PEP was prepared by converting the tricyclohexylammonium salt which was purchased from the same source. Liver tRNA was purchased from

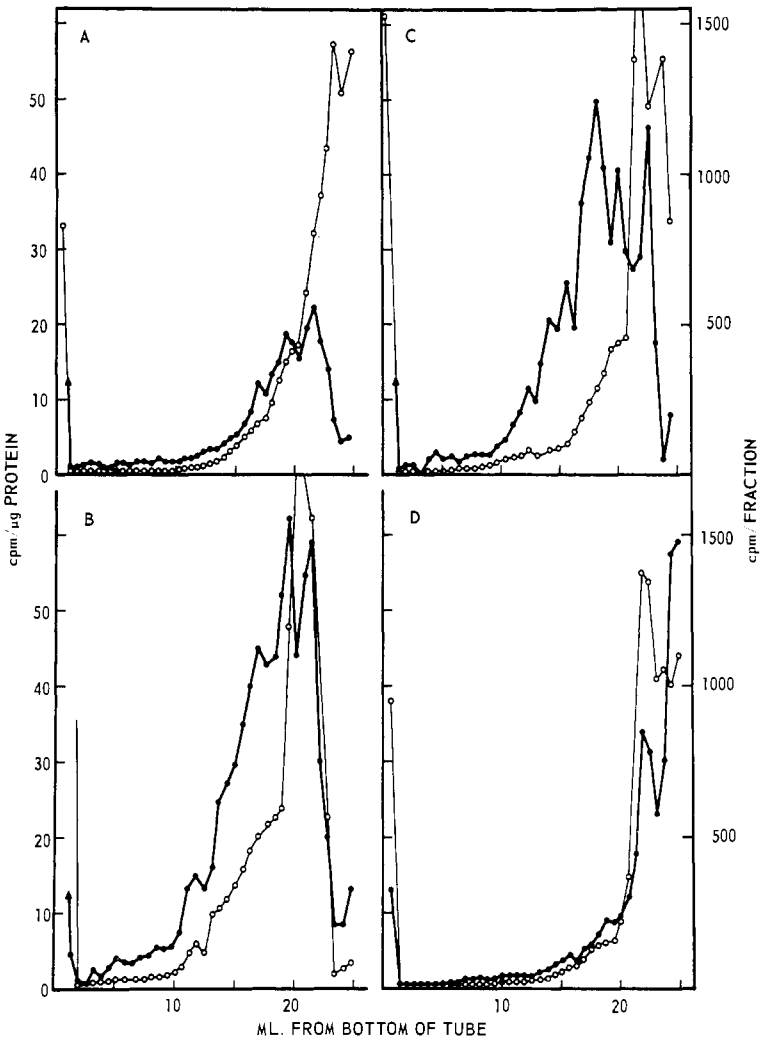

FIGURE 1: Sucrose gradient centrifugation of extracts from cells labeled with [^{3}H]Urd for 5 min. Isotopic labeling and cell fractionation as described in Methods. Sucrose gradients were linear (15–30% sucrose in solution A) and contained 15 μg/ml of sodium poly(ethenesulfonate), mol wt 12,900 (Bach, 1964). Centrifugation was at 25,000 rpm for 2 hr in the SW 25 rotor of the Spinco Model L ultracentrifuge. Results represent the ratio of acid-insoluble radioactivity to protein (closed circles) and total acid-insoluble radioactivity per fraction (open circles). (A) Cytoplasmic fraction. (B) DNA extract of nuclei. (C) DNA extract of nuclei after treatment with 2.5 μg/ml of DNase at 37° fo 5 min. (D) DNA extract of nuclei after treatment with 0.062 mμg/ml of RNase at 37° for 5 min. Tube A was charged with 1.3 mg of protein. Tubes B–D were charged with 0.5 mg of protein each.

Nutritional Biochemicals Corp., and *E. coli* cells for the preparation of the S-30 fraction from General Biochemicals Corp. The tritiated nucleosides and amino acids were obtained from the New England Nuclear Corp. Calf thymus DNA, pancreatic DNase (EC 3.1.4.5), and yeast RNA were obtained from Worthington Biochemical Corp.; basal medium, Eagle, from Microbiological Associates, and carboxymethylcellulose, from the Dow Chemical Co. The sample of sodium poly(ethenesulfonate) was prepared by the Farbwerke Hoechst, Germany, and heparin was an Upjohn Co. product having an activity of 138 units/mg.

Results

Preservation of Intact Nuclei during Extraction with DNA. Extraction of washed nuclei with a DNA-containing buffer had no demonstrable effect on their appearance under the phase-contrast microscope (Plate I). Furthermore, the recovery of nuclei following such extractions, as determined by hemocytometer counts, was essentially quantitative.

The Presence of Membrane-Bound Ribosomal Aggregates in the DNA Extract Fraction. Figure 1 demonstrates that the DNA extracts of the nuclei are a much better source of polysomelike aggregates than the cyto-

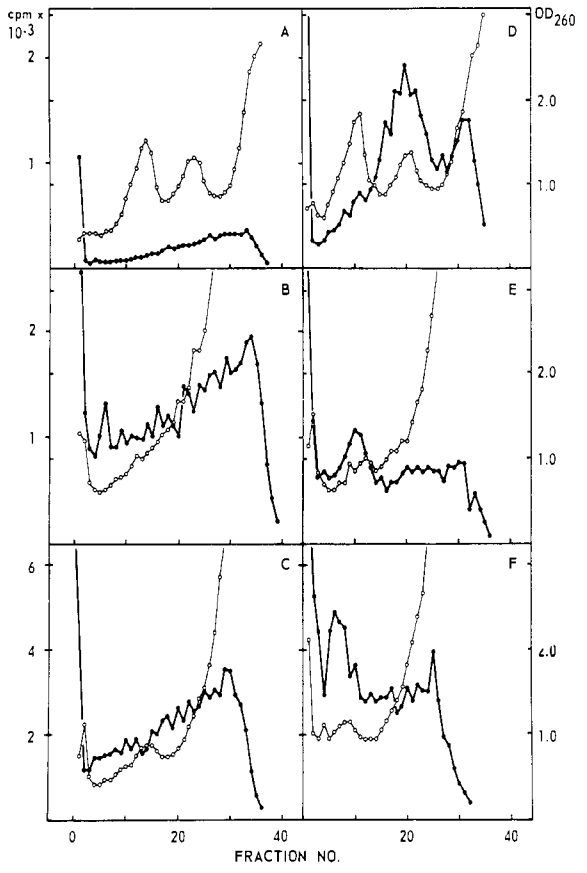

FIGURE 2: Sucrose gradient analysis of the RNA from cell fractions which were obtained as described in Methods. The isolated fractions were adjusted to 0.5% with respect to SDS and layered over linear sucrose gradients (15–30%) which contained 0.5% SDS (Gilbert, 1963). Centrifugation was at 20° and 25,000 rpm for 11 hr in the SW 25 rotor. Total acid-insoluble radioactivity (closed circles). Absorption at 260 mμ (open circles). Note that because of addition of DNA to fractions B, C, E, and F OD_{260} does not represent RNA. (A) Cytoplasmic particulate fraction, 5-min labeling. (B) DNA extract of nuclei, 5-min labeling. (C) Nuclear residue from same fractionation. (D, E, and F) Same as A–C, but after 90-min labeling.

plasm. Thus, in fractions from the same homogenate the ratio of the radioactivity from [^{3}H]Urd to protein in the polysome region (tubes 30–36) of gradient separations of the cytoplasmic fraction (Figure 1A) is strikingly smaller than that from the DNA extract of the nuclei (Figure 1B). Because of the presence of a large amount of added DNA in the nuclear extract, measurement of OD_{260} would be meaningless. The aggregates survived treatment with relatively high concentrations of DNase (Figure 1C) but were degraded by minute amounts of RNase (Figure 1D). This supports the conclusion that they were held together by RNA rather than by the DNA which was added during isolation.

TABLE 1: Chemical Composition and Content of NADH–Cytochrome *c* Reductase and Succinic Dehydrogenase of Subcellular Fractions from HeLa Cells.[a]

Fraction	Protein (mg)/Fraction	RNA (mg)/Fraction	Phospholipid (mg)/Fraction	RNA: Protein	Phospholipid: Protein	NADH–Cytochrome *c* Reductase[c]	Succinic Dehydrogenase[d]
Total homogenate	47 ± 2[b]	7.0 ± 0.6	4.7 ± 1.0	0.15	0.10	0.95 ± 0.05	0.137 ± 0.008
Washed nuclei	3.6 ± 0.2	1.47 ± 0.08	1.22 ± 0.06	0.41	0.34	...	...
Extracted nuclei	3.2 ± 0.3	0.42 ± 0.09	0.43 ± 0.01	0.13	0.13	0.62 ± 0.08	0.127 ± 0.000
DNA extract of nuclei: Total	...	...	...	...	...	1.50 ± 0.3	0.148 ± 0.000
DNA extract: Microsomes	0.448 ± 0.007	0.14 ± 0.01	0.058 ± 0.010	0.3	0.13	...	...
SDOC ribosomes	0.089 ± 0.005	0.15 ± 0.02	0.39 ± 0.01	1.7	4.4	...	...
Mitochondria	...	...	...	...	...	0.025 ± 0.004	0.27 ± 0.02
Cytoplasmic: Microsomes	1.91 ± 0.07	0.96 ± 0.02	1.8 ± 0.02	0.50	0.95	0.72 ± 0.01	0.033 ± 0.005
SDOC ribosomes	1.00 ± 0.007	0.83 ± 0.05	...	0.82	...	...	...
Soluble	6.0 ± 0.2	0.96 ± 0.04	0.068 ± 0.011	0.16	0.011	0.003	0.021 ± 0.005

[a] Chemical analyses were carried out on homogenates prepared using Triton X-100. Enzyme analyses were a separate experiment in which Triton X-100 was omitted to preserve mitochondria and NADH–cytochrome *c* reductase activity. "Dense nuclei" which sedimented through 2.2 M sucrose were used in both these experiments. [b] Standard deviation of the mean. Triplicate samples for proteins based on linear enzyme concentration–activity plots (five to six points) for the two enzyme activities. RNA and phospholipid values are average of duplicates. [c] Units/mg of protein. [d] OD_{440}/min mg of protein.

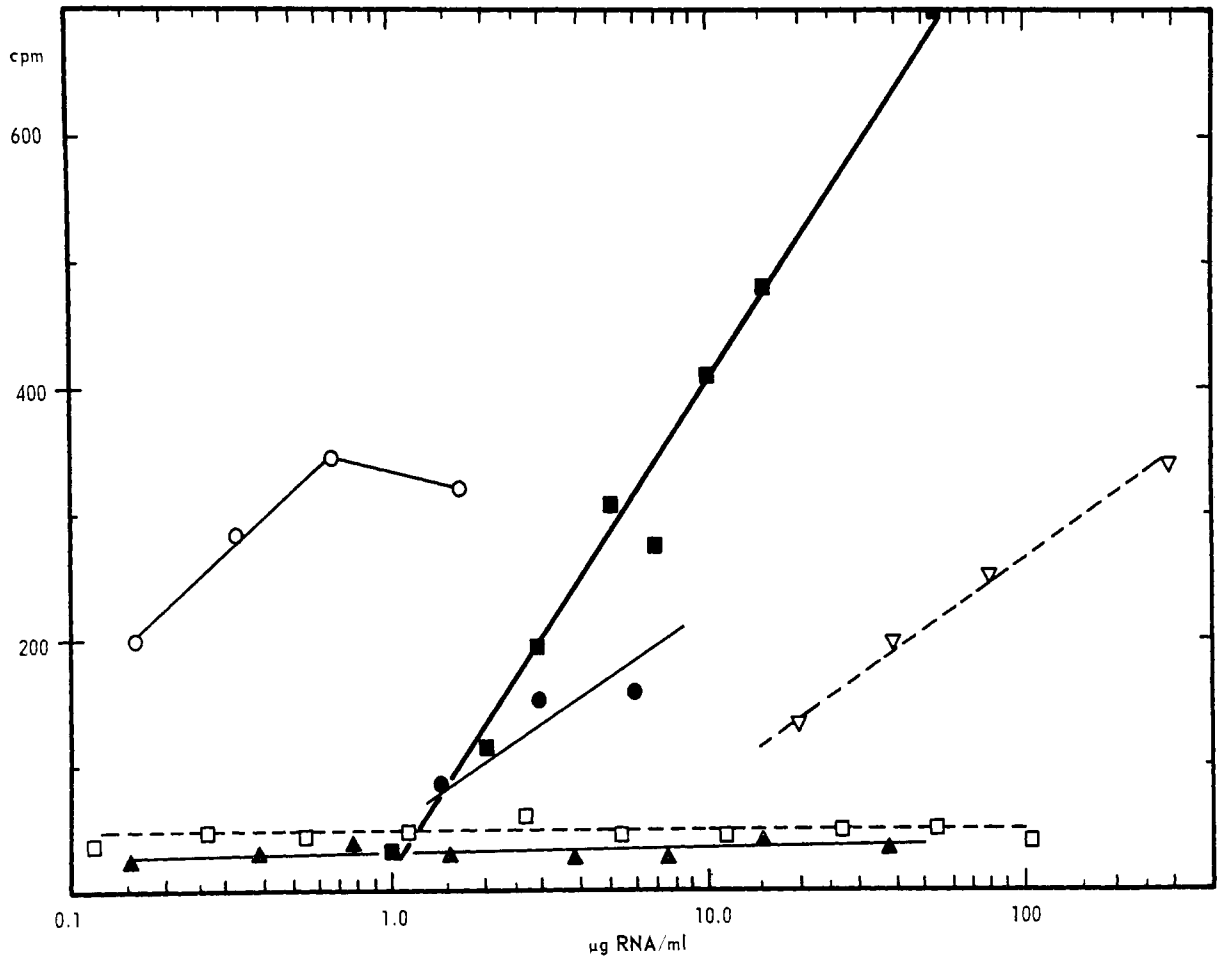

FIGURE 3: Stimulation of protein synthesis by RNA fractions isolated from HeLa cells. The final millimolar concentrations of the components of the incubation mixture were as follows: Tris-HCl (pH 7.8, 100), magnesium acetate (14), KCl (50), ATP (1.0), GTP (0.02), PEP (75), β-mercaptoethanol (6.0), 20 L-amino acids (0.20 each) including L-[^{3}H]phenylalanine (sp act. 600 mc/μmole). In addition each 0.25-ml incubation contained 4 μg of PK, 1 mg of *E. coli* S-30 fraction (protein basis), and RNA additions as indicated. Incubations were for 20 min at 37°. Results are the averages of triplicates and are corrected for incorporation in the absence of added mRNA. (●) Nuclear RNA isolated at 0°. (▲) RNA isolated from DNA extract of nuclei at 0°. (○) RNA isolated from DNA extract of nuclei at 65°. (□) RNA isolated from cytoplasmic microsomes at 0°. (▽) RNA isolated from cytoplasmic microsomes at 65°. (■) Poly U × $^1/_{20}$.

By far, most of the ribosomes and all the polysome-like aggregates of the HeLa cells which were used in these experiments were bound to membrane fractions. Thus, in the absence of SDOC all the radioactivity in sucrose density gradients of the cytoplasmic fraction and of the DNA extract of the nuclei was found in the pellets at the bottom of the tubes. The addition of SDOC was essential for the demonstration of polysomelike patterns such as those in Figure 1. Furthermore, as demonstrated in Table I, the phospholipid: protein and RNA:protein ratios of the microsome fractions (both from the cytoplasm and from the DNA extract of the nuclei) are characteristic of those described for endoplasmic reticulum by other investigators (Ernster *et al.*, 1962; Dallner *et al.*, 1963; Rees and Rowland, 1961), and the ribosomes which were obtained from these by treatment with SDOC had high RNA:protein ratios. These fractions had the highest specific activity for NADH–cytochrome *c* reductase, a marker enzyme for membrane fractions. In these cells the mitochondria were almost devoid of this enzymatic activity, and thus no corrections for mitochondrial contamination of the microsome fractions (Hudack and Brummond, 1961) were applied.

Characterization of the RNA and Protein Synthetic Capacity of the DNA Extract Fraction. In addition to their behavior in gradient density centrifugation (Figure 1), the polysomelike aggregates in the DNA extract of the nuclei were characterized by several other criteria.

SIZE DISTRIBUTION OF RNA. Figure 2 shows that after exposure of intact cells to [^{3}H]Urd for 5 min the radio-

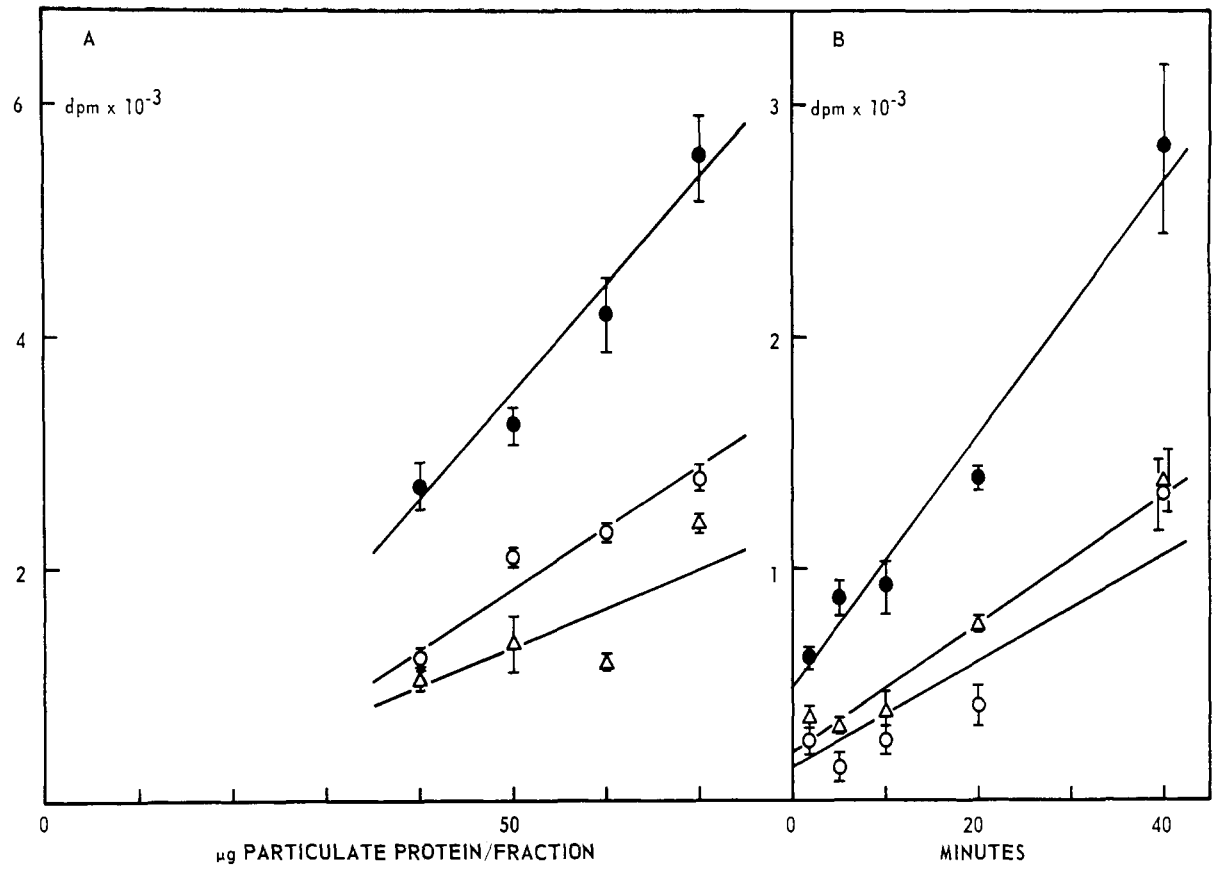

FIGURE 4: *In vitro* incorporation of L-[^{3}H]leucine by subfractions of HeLa homogenates as a function of enzyme concentration and incubation time. See Methods for details. (A) Effect of particulate enzyme concentration; incubation time = 20 min. (B) Effect of incubation time. All tubes contained 100 μg of particulate enzyme. (—●—) Microsomes. (—○—) DNA extract. (Δ) Residual nuclei.

active material in the DNA extract of the nuclei had the same distribution in SDS-containing gradients as the radioactive RNA from the nuclei and the cytoplasmic fraction. This distribution was highly heterogeneous with a peak around 10S, and corresponded to the size distribution reported from other laboratories for nuclear RNA or mRNA (Otsuka and Terayama, 1964; Lang and Sekeris, 1964). On prolonged exposure to [^{3}H]Urd the distribution of radioactivity shifted toward that for rRNA and ribosomal precursor RNA (parts D–F, Figure 2) with a notable absence of a 16S peak in the nuclear and the DNA extract fractions. This is in agreement with the reported kinetics of synthesis of these components (Scherrer and Darnell, 1962; Rake and Graham, 1964; Girard *et al.*, 1965) and with the reported absence of 16S RNA from nuclei (Penman, 1966). The RNA in the DNA extract fraction resembled nRNA in this respect.

MESSENGER ACTIVITY OF ISOLATED RNA. The RNA which was extracted from these fractions was active in stimulating the incorporation of phenylalanine into acid-insoluble material in the *E. coli* S-30 fraction of Nirenberg (1963), as is shown in Figure 3. The RNA which was extracted at 0° (largely r- and tRNA) was inactive, while the RNA which was obtained from the DNA extract of the nuclei at 65° was by far the most active, suggesting the enrichment in mRNA in this fraction. Sekeris and Lang (1964) and McNamara (1964) have also reported enrichment in mRNA activity in RNA which had been obtained by extraction with hot phenol. The availability of a radioactive marker in the RNA fractions which were used in the experiment described in Figure 3 permitted the examination of the specific activities of the RNA fractions after short pulse labeling. These are summarized in Table II. The fractions which were the most active in stimulating protein synthesis also had the highest over-all specific activity. Analysis of density gradient centrifugal distributions of the same fractions on sucrose–SDS gradients demonstrated the expected enrichment in heterogeneous RNA in the 65° extracts and in tRNA and rRNA in the 0° extracts.

PROTEIN-SYNTHESIZING ABILITY OF ISOLATED MICROSOMES *in Vitro*. Microsomes in the DNA extract of the nuclei as well as the cytoplasmic microsomes from HeLa cells were able to effect the incorporation of amino acids into protein in the presence of added

TABLE II: Purity and Specific Activity of RNA Isolated from Subfractions of HeLa Cells.

Fraction[a]	Temp (°C)	Total Yield (mg)	RNA Content (%)[b]	Sp Act. (cpm/mg of RNA)
Nuclear	0	0.27	6	603
	65	None	...	...
DNA extract of nuclei	0	21.0	14	248
	65	0.080	50[c]	680[c]
Cytoplasmic microsomes	0	13.5	85	80.3
	65	0.32	98	98.9

[a] RNA was isolated by the phenol method as described in Methods. The 65° isolate was obtained by the reextraction at 65° of the interphase pellet which formed following extraction at 0°. [b] RNA content by orcinol method compared to OD_{260}. [c] No pentose color found in small amount available. RNA content is maximum based on amount of DNA found and maximum level of RNA which would not have been detected. Specific activity is a minimum figure.

TABLE III: Effect of Various Additions to or Omissions from the Incubation Mixture on the Incorporation of L-[^{3}H]Leucine by Particulate Preparations from HeLa Cells.[a]

Expt	Conditions	Net Cpm Incorporated: Nuclear	DNA extract	Microsomes
I	Complete medium	316	367	382
	Minus PK	148	357	134
	Minus PEP and PK	67.2	175	117
	Minus ATP, GTP, PEP, and PK	71.4	28.7	17.9
	Minus GTP	115	139	280
	Plus 50 μM each UTP and GTP	280	550	448
II	Control	75.8	75.7	93.4
	Minus 19 amino acids	76.5	61.0	27.3
III	Control	798	223	274
	Plus 10^{-4} M puromycin	49.1	53.2	23.3
	Plus 20 μg/ml of DNase	705	213	230
	Plus 20 μg/ml of RNase	53.4	153	28.5

[a] The complete medium consisted of 25 mM Tris-HCl buffer (pH 7.8), 75 mM KCl, 6 mM $MgCl_2$, 6 mM β-mercaptoethanol, 1.5 mM ATP (sodium salt), 50 μM GTP, 4 mM PEP, 40 μM each of 20 L-amino acids including L-[^{3}H]leucine (sp act. 100 mc/mmole), 2.4 μg of PK, 30 μg of liver tRNA, and 100μg each of liver pH 5 fraction and HeLa particulate enzyme (on a protein basis) in a final volume of 0.10 ml. Incubations were for 20 min, and results are the averages of triplicates corrected for zero-time incorporation.

pH 5 fraction and tRNA (Figure 4). In this experiment the incorporation was optimal, linearly related to enzyme concentration, and directly dependent on incubation time. When activity was expressed on the basis of RNA content rather than protein, incorporation by all these fractions was almost equivalent. In the process of testing for similar responses with other amino acids, it was found that conditions had to be varied somewhat to obtain optimal incorporation in each case. Thus the dependence of incorporation on ATP and an energy-generating system, the effect of addition of the 18 or 19 unlabeled amino acids, as well as the effect of varying the nature and concentration of tRNA and monovalent cations were examined in detail. Incorporation was linearly dependent on the final concentration of the radioactive amino acid at least up to a concentration of 0.4 mM in the assay medium.

The effect of omission of various parts of the energy-generating system, the unlabeled amino acids, or GTP is shown in Table III. Clearly omission of any part of the energy-generating system is strongly inhibitory. By the same token (not shown) addition of higher concentrations of these compounds was without effect short of inhibition at excessive concentrations. A requirement for unlabeled amino acids could only be shown in the microsome fraction. On the other hand, analysis of the free amino acids in the enzyme preparations accounted for at most 10% of the total amino acids supplied so that the correction for dilution of added radioactive amino acids was small. Table III also shows results which demonstrate that the reaction measured was in fact protein synthesis since it was strongly inhibited by puromycin (Yarmolinsky and de la Haba, 1959) and by RNase but not by DNase. The failure to observe inhibition by DNase also eliminated the possibility that protein synthesis depended on attachment of ribosomes to the DNA used during isolation of the nuclear membrane fraction (McCarthy and Holland, 1965; Bladen *et al.*, 1965) or the presence of "natural" DNA-containing aggregates in the DNA extract fraction (Naora, 1966).

The nature of mRNA in any preparation ultimately defines the structure of the proteins for which it serves as a template. Thus an examination of the proteins which were produced in *in vitro* incubations under optimal conditions is potentially the best method for characterizing the mRNA fraction. This was done by a comparison of the relative incorporations of two amino acids which were labeled with two different

TABLE IV: Ratio of *in Vitro* Incorporation of L-Leucine and L-Phenylalanine by Particulate Preparations from HeLa Cell Homogenates.[a]

Source of Radioactivity		Ratio in Substrate Mix	Ratio in Incorpd Protein ± Std Dev			*P*	
^3H	^{14}C	^3H:^{14}C	Microsomes[b]	DNA Extract[c]	Nuclei[c]	DNA Extract	Nuclei
Leu	Leu	7.3	7.23 ± 0.13	7.65 ± 0.55	8.09 ± 0.34		Not significant
Leu	Phe	2.75	23.5 ± 0.9	15.5 ± 3.7	11.2 ± 4.3	Not significant	<0.01
Phe	Leu	23.2	0.97 ± 0.25	3.1 ± 2.5	20.2 ± 12.9	Not significant	<0.01
Phe	Phe	8.94	8.9 ± 1.1	11.0 ± 2.1	*d*		Not significant

[a] Enzyme preparations were prepared as described in Methods. Incubation conditions as in Table I except tRNA concentration was 150 μg/ml, and 200 μg of pH 5 fraction was used for 100 μg of particulate enzyme, two radioactive amino acids were used (as indicated), and incubations were carried out for 10, 20, 30, 40, and 60 min. Isotope ratio averages for quadruplicate samples at each time point were used to compute the averages shown, and were corrected, where necessary, by the unequal dilution of the radioactive amino acids because of different pool sizes in the enzyme preparations. *P* values are for probability of sample being identical with the microsome fraction, based on Student's t test. Results show corrected disintegrations per minute of ^3H to disintegrations per minute of ^{14}C. [b] Enzyme (particulate and pH 5) contained 0.9 mμmole of leucine and 0.42 mμmole of phenylalanine, compared to 4.0 mμmoles supplied by substrate mix. [c] Enzyme contained 0.21 mμmole each of leucine and phenylalanine. [d] Uptake of [^{14}C]-phenylalanine was so low that results were not significant.

isotopes under incubation conditions where the incorporation of both amino acids was strictly dependent on the amount of particulate enzyme preparation (and therefore mRNA) present, the kinetics of the uptake of both amino acids were comparable, and the dilution of the two amino acids by free amino acids in the various enzyme preparations was known and corrected for. It was found necessary to rechromatograph all radioactive amino acids immediately before use in order to remove radiodecomposition products. Only under these conditions were the isotope ratios constant over varying enzyme concentrations or incubation times. The results shown in Table IV indicate that the ratio of incorporation of leucine to phenylalanine was different in the three enzyme fractions while the controls, where the same amino acid contained both isotopic markers, were consistent. The incorporation into the DNA extract fraction was not significantly different from the incorporation into the nuclear fraction. Similar results were obtained on three separate occasions, using enzyme preparations derived from three different cell fractionations and varying either enzyme concentration or incubation time. The incorporation of four other amino acids (lysine, tryptophan, proline, and glutamic acid) was also examined, but in no case was there a reproducible difference in incorporation ratios between the three enzyme fractions.

Another approach to the comparison of the nature of the products from *in vitro* protein synthesis was obtained by fractionating the products by gel electrophoresis (Summers *et al.*, 1965). Typical separations are shown in Figure 5. The band patterns were reproducible from run to run and from preparation to preparation and closely resemble those obtained by Warner (1966) from ribosomal proteins of HeLa cells which were exposed to labeled amino acids before homogenization. Furthermore, the addition of radioactive amino acids to a protein solution before application to the gels failed to result in the detection of any

TABLE V: Relative Distribution of Radioactive Proteins in Various Fractions Following Electrophoresis of Products from *in Vitro* Incubations on Polyacrylamide Gels.[a]

Fraction	Microsomes	DNA Extract	Nuclei
1	4.1	6.8	8.4
2	5.5	5.0	7.4
3	10.7	9.9	11.5
4	9.3	8.6	8.9
5	8.1	7.7	8.0
6	17.7	14.5	15.4
7	9.9	8.3	6.9
8	10.5	7.3	6.5
9	10.9	9.9	7.6
10	10.1	13.1	11.2
11	2.0	3.6	5.7
12	1.0	5.2[b]	2.4

[a] See Methods for experimental details. Results are presented as per cent of total radioactivity recovered from the gel. [b] This value is largely based on a single test tube which contained an abnormally high amount of radioactivity.

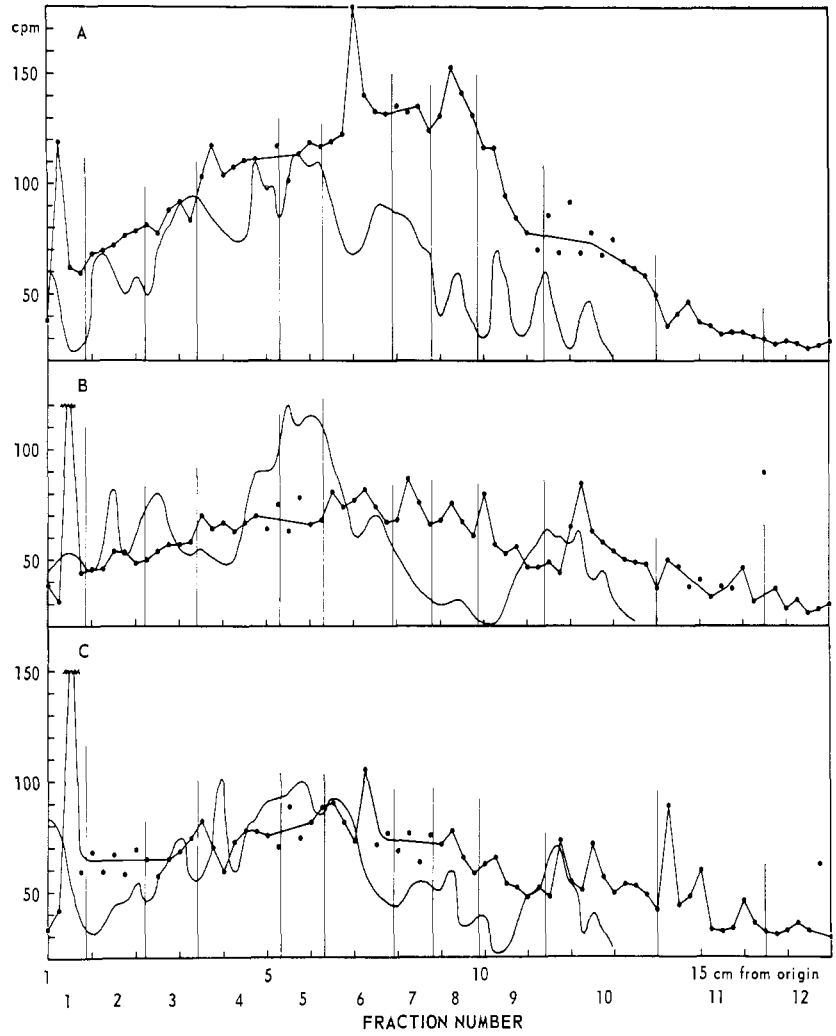

FIGURE 5: Electrophoretic separation of the solubilized products from *in vitro* amino acid incorporating systems derived from HeLa cells. See Methods for experimental details. Continuous line tracing absorption due to staining with Amido Black on an arbitrary scale. (—●—) Counts per minute in respective fractions. Distance is measured from the (−) end of the gel. (A) Cytoplasmic microsomes. (B) DNA extract of nuclei. (C) Residual nuclei.

radioactivity in the fractions after processing. Thus the radioactivities which were observed truly represent incorporation into protein. The patterns for the three enzyme preparations were quite similar in general, while differing in some details. The patterns were divided into 12 regions and the relative amount of radioactivity in these was compared. Table V presents the results of such a comparison. There was no significant difference in the relative abundance of some bands (3, 4, 5, and 10) accounting for 40% of the total radioactivity. Several bands showed relatively small differences (2, 6, 7, and 9) while a few (especially bands 1, 8, and 11) showed pronounced differences. It is noteworthy that with the exception of band 12 the relative amount of radioactivity in any band in the DNA extract fraction was intermediate in magnitude between the amount for the same band in the nuclear and the cytoplasmic fraction (*e.g.*, 1, 7, 8, 9, and 11) or differed from one of these to an insignificant extent. These results indicated in a more quantitative sense that although the DNA extract fraction originated from washed nuclei, the mRNA which it contained had a more cytoplasmic character than the remaining nuclear mRNA.

Labeling Kinetics for RNA and DNA in the DNA Extract Fraction. RIBONUCLEIC ACID. In agreement with the reports of Darnell *et al.* (1963) and Girard *et al.* (1965), our studies on the incorporation of radioactivity into RNA of various subcellular fractions (Figure 2) show that during exposures of less than 30 min the radioactive RNA in all subcellular fractions was heterogeneous in size and gave no indication of incorporation of radioactivity into ribosomal RNA. The distribution of the radioactive RNA in various subcellular fractions (as a per cent of total radioactivity) at various times after the addition of the isotopically tagged precursor is shown in Figure 6. As expected, the nuclei accounted

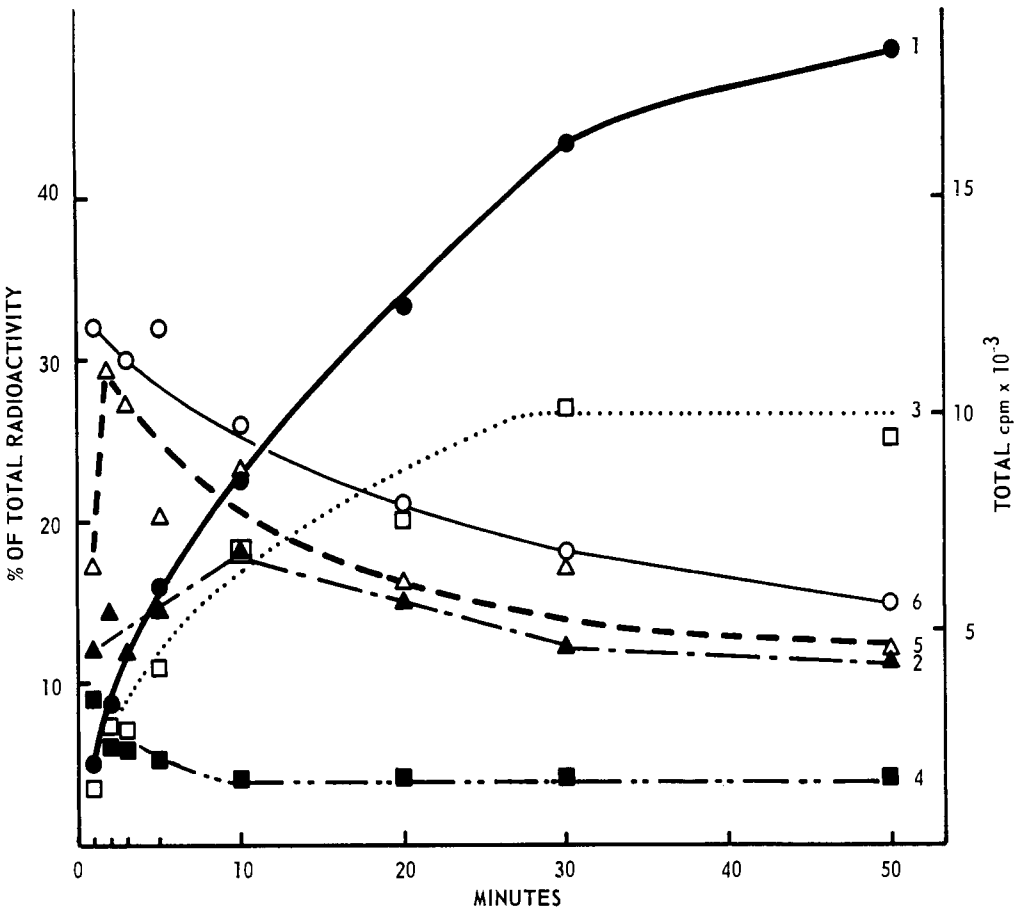

FIGURE 6: Time course of incorporation of [^{3}H]cytidine into various subcellular fractions from HeLa cells. Washed cells were suspended in Eagle's basal medium (2×10^7/ml) and exposed to 4 μc/ml (3.7 mc/μmole) of [^{3}H]cytidine. At times shown aliquots were pipetted into crushed, frozen buffer. Cells were washed and homogenized as described (Bach and Johnson, 1966a). Results represent per cent of total radioactivity where total recovery was nearly quantitative. Curve 1: uptake of radioactivity into the total aliquot. Curves 2–6: the per cent of the total radioactivity which was found in the following. Curve 2: the cytoplasmic fraction sedimenting at 12,000g for 30 min. Curve 3: the cytoplasmic fraction sedimenting between 12,000 and 105,000g. Curve 4: the cytoplasmic-soluble fraction. Curve 5: the DNA extract of the nuclei. Curve 6: the residual nuclear fraction after extraction with DNA solution.

for most of the radioactivity in the earliest time points, again demonstrating that these organelles are the major if not the only site of RNA synthesis in these cells (Harris, 1963; Moldave, 1965; Smellie, 1963).

Similarly the slow incorporation of radioactivity into the cytoplasmic soluble fraction and the free cytoplasmic ribosomes was expected. Of particular interest here is the labeling of the membrane-bound cytoplasmic fraction (rough endoplasmic reticulum), which preceded the appearance of label in the free ribosomes by some 10 min, and the very early appearance of radioactivity in the DNA extract of the nuclei. This pattern is consistent with the transport of RNA from the nuclei *via* the DNA extract fraction and the rough endoplasmic reticulum to the free ribosomes. To gather further evidence for such a transport of RNA an attempt was made to examine the rate at which the RNA left the DNA extract fraction by studying the inhibition of further RNA synthesis by means of actinomycin D. Latham and Darnell (1965a,b) had shown that mRNA transport to the cytoplasm continued for some 7 min following inhibition of new synthesis by this drug. The results of such an experiment were complicated by the increase in the per cent of total radioactivity in the nuclear fraction upon addition of actinomycin D. This may be explained by the very rapid decrease in total radioactivity in the cells after addition of actinomycin D. Contrary to previous reports for mammalian systems (Pitot *et al.*, 1965; Bloom *et al.*, 1965) the half-life of newly synthesized RNA in HeLa cells under the conditions of our experiments was of the order of only 6 min. As the more labile RNA was degraded the more stable rRNA precursors accounted for an increasing fraction of the total radioactive RNA which remained in the cells, and much of this was presumably nuclear. Nonetheless the results were consistent with

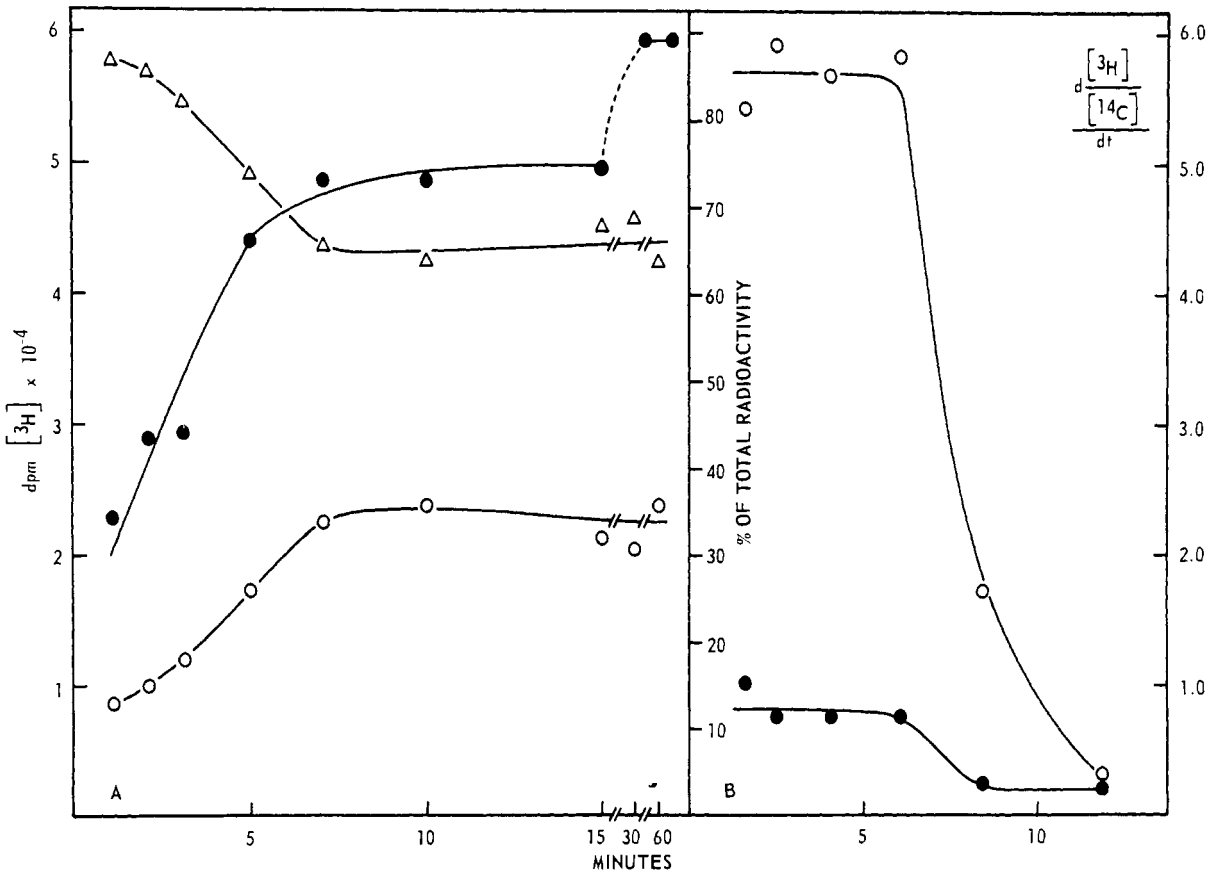

FIGURE 7: Kinetics of incorporation on [^{3}H]dThd into subcellular fractions from HeLa cells. Cells were prelabeled with [^{14}C]dThd during growth and then pulse labeled with [^{3}H]dThd while in suspension. See Methods for details. (A) (—●—) Incorporation of [^{3}H]dThd into total homogenate. (—○—) Per cent of total radioactivity found in DNA extract of nuclei. (—▲—) Per cent of total radioactivity found in residual nuclei. (B) Rate of change of specific activity with time. Results represent change in the ratio of disintegrations per minute of ^{3}H:disintegrations per minute of ^{14}C per minute of uptake time and are derived from the difference in specific activity at any two subsequent time points. (—●—) Residual nuclei. (—○—) DNA extract of nuclei.

a rapid displacement of the [^{3}H]RNA from the DNA extract fraction and the cytoplasmic microsomes into the "free ribosomes."

DEOXYRIBONUCLEIC ACID. Earlier results from relatively crude experiments (Bach, 1962) had suggested that during very short exposures more [^{3}H]dThd was incorporated into the microsomes than into the nuclei. We subsequently found (Bach and Johnson, 1964) that this incorporation was limited to the DNA extract fraction. It was of interest to establish whether the radioactive DNA in the microsomes of the DNA extract fraction was in kinetic equilibrium with the total DNA of the cells. The results of a double-label experiment designed to answer this question are shown in Figure 7. In this experiment incorporated radioactivity from [^{14}C]dThd is equated to total DNA. The amount of this radioactivity did not change during the entire experimental period. The radioactivity from [^{3}H]dThd reflects "newly" synthesized DNA. Although the residual nuclear fraction contained the bulk of ^{3}H and ^{14}C, the specific activity and the rate of change of the specific activity (part B, Figure 7) of the DNA extract fraction were several times greater than that of the residual nuclei. Furthermore, there was no evidence of transfer of any ^{3}H-labeled DNA from one fraction to the other once incorporation had stopped. (The per cent of total radioactivity in both fractions did not change once incorporation of new [^{3}H]dThd had stopped.)

The uptake of [^{3}H]dThd in this experiment was limited to 7 min by the rapid conversion of dThd to free thymine in the medium in the presence of the high concentration of cells which was being used. In another experiment (not shown) addition of a second aliquot of [^{3}H]dThd after uptake had stopped resulted in a second spurt of incorporation, thus confirming that the cessation of incorporation which was seen was not due to any damage to the cells and that, presumably, DNA synthesis proceeded throughout the experimental period by utilizing endogenous dThd.

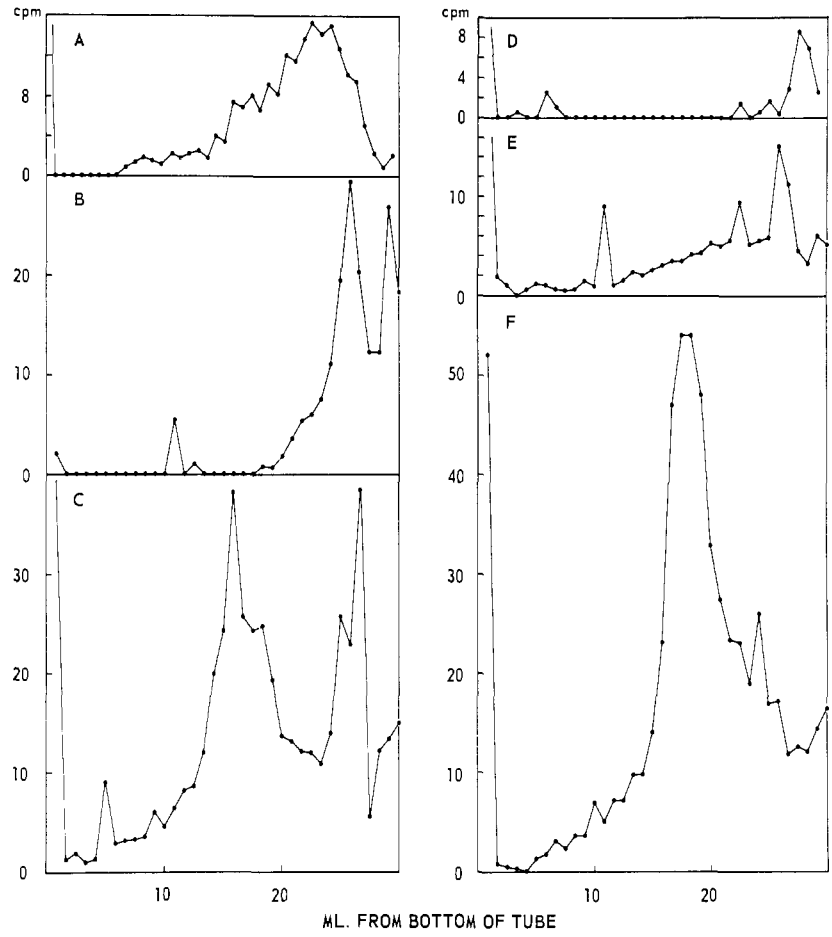

FIGURE 8: Effect of various polymers in extracting polysomes from washed nuclei. Cells were labeled with [^{3}H]Urd for 5 min. Washed nuclei extracted as described in Methods using the following polymers: (A) 5 mg/ml of shear-degraded salmon sperm DNA, (B) 15 mg/ml of yeast RNA, (C) 15 mg/ml of sodium poly(ethenesulfonate) (mol wt 12,000), (D) 15 mg/ml of sodium carboxymethylcellulose, (E) 15 mg/ml of dextran sulfate (mol wt 2×10^6), and (F) 15 mg/ml of heparin (U.S.P.). Results represent counts per minute per microgram of protein.

It is therefore possible to consider the incorporation of [^{3}H]dThd during this period as a pulse-chase experiment under "steady-state" conditions.

Can Other Polyanionic Compounds Replace DNA in the Preparation of the "DNA Extract" Fraction? Several polyanionic materials, in addition to DNA, were found to be active in extracting ribosomal aggregates from washed nuclei (Figure 8). When polysulfates such as sodium poly(ethenesulfonate), sodium dextran sulfate, and heparin were used, aggregates of ribosomes were isolated in very viscous fractions which were high both in radioactivity and in protein content. These fractions were found considerably further down the gradient tubes than the polysomelike aggregates in the DNA extract. Electron microscopy and sucrose gradient centrifugation after treatment of the pelleted aggregates with RNase suggested that these aggregates were also held together by mRNA. The extraction of the nuclei with DNA was only quantitatively dependent on the condition of the DNA. Thus the use of "native" calf thymus or salmon sperm DNA in place of the shear-degraded preparation which was normally used gave similar results. Since the DNA used in these preparations was frequently dissolved in distilled water, and was therefore denatured, it is likely that double strandedness is not a criterion for activity. Use of commercial tRNA failed to yield a polysomelike fraction, however.

Table VI describes the results which were obtained when other polymers were used in place of DNA as an extractant for cells which had been prelabeled with [^{3}H]dThd. While some of these reagents caused the extraction of a considerable amount of radioactive material and of protein from the nuclear fraction, in no case was the specific activity of the residual nuclei as low as when DNA had been used for extraction. Both yeast RNA and sodium poly(ethenesulfonate) extracted a higher percentage of the radioactivity, but

TABLE VI: Comparison of the Efficacy of Other Polymers for the Extraction of Microsomes Labeled with [^{3}H]dThd from Isolated Nuclei.[a]

	Polymer Used							
	DNA (5 mg/ml)		Yeast RNA (5 mg/ml)		Sodium Poly-(ethenesulfonate) (mol wt 12,900) (10 mg/ml)		Sodium Carboxymethyl-cellulose (10 mg/ml)	
Fraction	%[b]	Rel Sp Act.[c]	%[b]	Rel Sp Act.[c]	%[b]	Rel Sp Act.[c]	%[b]	Rel Sp Act.[c]
Cytoplasmic microsome[d]	5.5	0.17	7.7	0.17	5.1	0.17	14.2	0.17
Nuclear extract	18.5	3.0	38.3	3.1	39.7	7.7	6.0	7.5
Residual nuclei	76.0	1.3	54	6.6	55	16.9	80.0	5.0

[a] Results of this experiment were obtained with KB cells which had been prelabeled for 2 hr while still attached to glass, and homogenized as described by Bach (1962). [b] Per cent of total radioactivity recovered. [c] Relative specific activity is based on the total homogenate (680 cpm/mg of protein) equal to 1.00. [d] This fraction was common to all the extractions and equaled 1837 cpm.

a greater portion of the protein must also have been extracted to explain the specific activities which were obtained.

Discussion

This paper characterizes a membrane fraction derived from HeLa cells which appears to be unique in its origin and in the combination of nuclear and cytoplasmic biochemical functions in which it appears to be involved. By this time the observation of the occurrence of polysomes in mammalian cells, and especially in HeLa cells, is not novel (Zimmerman, 1963; Darnell *et al.*, 1963; Goodman and Rich, 1963). However, the results presented here differ in two respects from the earlier work. (1) Ribosomal aggregates were isolated from a membrane fraction which was derived from washed nuclei. (2) The use of SDOC to dissociate the ribosomal aggregates from membranes was essential for their demonstration in any of the fractions.

The Characterization of the Ribosomal Aggregates. Brief labeling with amino acids could not be utilized to demonstrate the existence of polysomelike aggregates in the HeLa cells which were used in this study. Thus the characterization of the particulate fraction which contained rapidly labeled RNA depends on the characterization of the RNA it contained, the enzymatic properties of the aggregates, the size of the aggregates, and the effect of incubation with nucleases on the size of the aggregates. The results presented here demonstrate that aggregates of polysome size range were present in the DNA extract fraction, that their size was profoundly decreased following brief treatment with minute concentrations of RNase, and that the preparations in which they were found effectively incorporated amino acids into acid-insoluble material without the addition of mRNA or synthetic oligonucleotide templates. Moreover, the radioactive RNA which was isolated from these aggregates had a heterogeneous size distribution which is one of the characteristics which has become associated with mRNA. In agreement with previous work, these preparations contained little if any radioactive material which corresponded to rRNA or ribosomal precursor RNA after a 5-min labeling period. Finally, the RNA which was obtained from the DNA extract fraction by extraction with phenol at 65° had the most messenger activity (Georgiev *et al.*, 1963), when it was compared to the messenger activity of other RNA fractions from the same cells and to that of poly U.

It is unlikely that the relatively large amounts of contaminating DNA which were present in some of the preparations (Figure 3, Table II) contributed to the amino acid incorporation, either directly (Holland and McCarthy, 1964; McCarthy and Holland, 1965; Naora, 1966) or indirectly, for the following reasons. (1) Treatment of the reaction mixture with 1 mg/ml of DNase was without effect. (2) Even though the 0° phenol extract of the DNA extract fraction contained over 80% DNA it did not stimulate amino acid incorporation. (3) The addition of pyrimidine nucleoside triphosphates, which would be required for RNA synthesis in the dialyzed S-30 fraction, did not affect incorporation. Similarly, the failure to demonstrate stimulatory activity with the 0° extracts and the 20-fold larger incorporation in the presence of poly U confirm that the incubation system contained sufficient amounts of t-RNA to support an even much larger incorporation of a single amino acid than that induced by the addition of the 65° phenol extract fractions.

The Characterization of the mRNA in the DNA Extract Fraction as Cytoplasmlike. There is no *a priori* reason to believe that mRMA in nuclei codes

for different proteins from the mRNA in the cytoplasm. The biosynthesis of truly nuclear proteins, such as histones and residual nuclear proteins, is not completely understood and has not, to our knowledge, been achieved under conditions which are appropriate for general protein synthesis. Nevertheless, an attempt to compare the fine structure of the mRNA in the various microsome preparations seemed desirable. For a number of technical reasons a comparison of the proteins which were synthesized by these microsomes *in vitro* was chosen for this purpose. There have been numerous reports of successful synthesis of functional proteins *in vitro* when the synthesis depended on the mRNA which was originally present in the microsome preparations (for a few examples, see von der Decken and Campbell, 1962; Manner *et al.*, 1965; Nisman and Pelmont, 1964; Clark *et al.*, 1965).

The criteria for the characterization of the protein products which were applied in this study are not nearly as critical as those of immunological or functional integrity which were used by the workers cited above. However, there was good reason to believe that the differences in the protein fractions which were observed (Tables IV and V, Figure 5) are meaningful and that the mRNA in the DNA extract fraction is truly a cytoplasmic mRNA. Although it is not of critical importance in the studies reported here, the striking resemblance in the electrophoretic patterns which we obtained (Figure 5) with those reported by Warner (1966) for *in vivo* labeled ribosomal proteins is worthy of note, and strengthens the case for a real meaning to the patterns which were obtained. The rapidly labeled RNA in the DNA extract fraction was very similar to nuclear RNA in its distribution in sucrose gradient density centrifugations and in its specific activity (Figure 2). At the same time, this study indicates that the mRNA of this fraction was cytoplasmic in nature, results which might appear contradictory at first glance. It is possible that the bulk of the radioactive RNA in the nuclei as well as in the DNA extract fraction was not mRNA. It must be borne in mind that these experiments cannot distinguish conclusively between mRNA and the as yet poorly understood rapidly labeled nuclear RNA which has been described recently (Houssais and Attardi, 1966; Attardi *et al.*, 1966; Warner *et al.*, 1966; Shearer and McCarthy, 1967).

The Membrane-Bound Nature of Polysomes. Our results clearly demonstrate that by far most of the ribosomal aggregates containing newly synthesized RNA in the HeLa cells which were used in this study were bound to membranes in the cells. By contrast, Penman *et al.* (1964) using suspension-grown cultures derived from the S-3 clone of HeLa cells reported that the polysomes in their cells were free in the cytoplasm and that no SDOC was required for their release. While it is possible that the discrepancy is due to the fact that their polysomes were identified as active centers of protein synthesis by use of amino acid tracers and our polysomes were identified as containing newly synthesized RNA by use of uridine tracer, this interpretation is not likely. It is more likely that the reason for the discrepancy lies in the differences in the nature and cultural characteristics of the cells involved. (1) Whereas the cells which were derived from the S-3 clone were specifically selected for their failure to attach to glass and for their ease of culture in suspension (S. Penman, personal communication) all efforts to grow the cells which were used in this study in suspension were unsuccessful. (2) While Penman *et al.* (1964) as well as a number of further papers in which results from experiments with the suspension-grown cells were described all were able to demonstrate and to characterize the polysomes in the cells following brief exposures to radioactive amino acids, the pool sizes for amino acids in our cells were so large that such experiments were uniformly unsuccessful. (3) The calculated generation times for the suspension-grown cells are approximately half those for our cells. All these differences are consistent with the interpretation that the suspension-grown cells are in a state of rapid, logarithmic growth during which pool sizes would be expected to be small and the endoplasmic reticulum to be poorly developed. The cells used here, by contrast, had a slower growth rate which might well be more representative of the cells which are normally found in intact animals. The important role of membrane-bound ribosomal aggregates for functional protein synthesis in a variety of tissues has been reported repeatedly (Hendler, 1965; Campbell *et al.*, 1964; Ganoza *et al.*, 1965; Pitot, 1964; Henshaw *et al.*, 1964).

Kinetic Evidence for the Role of the DNA Extract Fraction in DNA Synthesis and RNA Transport. The labeling kinetics of the RNA in the DNA extract fraction (Figure 6) are certainly characteristic of a "typical" kinetic intermediate. This fraction contained a small percentage of the total radioactivity initially, then rapidly increased in relative abundance, and declined again. The actinomycin D "chase" experiment is also consistent with this interpretation. The extreme rapidity with which this fraction became labeled suggests that it must be a relatively early intermediate in the transport process. It is interesting to note that the time at which the DNA extract fraction contained the highest per cent of total radioactivity (3 min, Figure 6) is almost the same as the time for which Latham and Darnell (1965b) and Joklik and Becker (1965a,b) reported the primary existence of mRNA bound in soluble form to 40S rRNA precursor in HeLa cell cytoplasm. No such complexes were found in these studies, and newly synthesized RNA, when dissociated from the polysome-sized aggregates in which it seemed to be bound, always behaved as a heterogeneous material with an approximate mean sedimentation constant of 10 S. While it is, of course, possible that a mRNA form which was bound to 40S RNA could have been demonstrated in the cytoplasm of our cells at yet earlier labeling times, this is highly unlikely. First, as already stressed, the growth rate of our cells was only about one-half that of the suspension-grown cells. Second, and more significant, it is not logical to presume the existence of an intermediate which first appears in

free form in the cytoplasm, then migrates back to a membrane near the nucleus only to be transported back into the cytoplasm while it is attached to membranes. Thus under the conditions of these experiments, transport of RNA from the nucleus to the cytoplasm in membrane-bound complexes seems to be the more likely path.

The kinetics of the incorporation of [^{3}H]dThd into the DNA extract (Figure 7) suggest that DNA synthesis in this fraction is somehow different from the bulk of cellular DNA synthesis. The only way in which the absence of transfer of newly synthesized DNA from one fraction to the other can be explained is by proposing the existence of two sites at which DNA synthesis took place simultaneously. The discrepancy between the large initial abundance of newly synthesized DNA in the residual nuclei and the lower rate of synthesis in this fraction can be explained if the rate of activation of dThd to the triphosphate level was faster in the nuclei than in the DNA extract fraction, thus giving the slower polymerization reaction a head start. On the other hand, if the pool size of the intermediates was comparable and at equilibrium, polymerization would cease equally promptly in both fractions upon exhaustion of the [^{3}H]dThd supply.

While the involvement of membranous systems in functional protein synthesis in mammalian and bacterial systems is well known, their role in functional DNA synthesis is more speculative at this time. Thus Ganesan and Lederberg (1965) reported evidence for a very active DNA polymerase and "newly synthesized" DNA in a membrane fraction from *Bacillus subtilis*. It is tempting to speculate that this may represent the "reading head" for *in vivo* DNA replication which has been proposed by Jacob *et al.* (1963) although it is clearly too early to draw such a conclusion (A. T. Ganesan, personal communication). Despite the added complexities which are inherent in the use of mammalian cells, our results seem to offer a close parallel to those of Ganesan and Lederberg (1965) and may offer a method for the isolation of derepressed DNA from these cells.

Origin of the DNA Extract Fraction. There are several explanations for the results reported here which can be ruled out. The DNA extract fraction was not an artifact caused by a viral, bacterial, or PPLO contamination of the cell cultures. All efforts to demonstrate such contaminations failed even though PPLO contaminants in other cell lines were readily detected (H. H. Buskirk, personal communication). Similarly, the "creation" of the DNA extract fraction by the disaggregation of large clumps of cell debris is ruled out by the specificity with which this fraction became labeled when [^{3}H]dThd or [^{3}H]Urd were added to the cultures, and because the fraction was obtained from nuclei which have been sedimented through 2.2 M sucrose. Finally, the fact that other, less viscous anionic polymers also were able to effect the isolation of a fraction similar to the DNA extract fraction (Figure 8, Table VI) is argument against a nonspecific "cleansing action" whereby nuclei would be "scrubbed" of contaminating cytoplasmic debris while passing through the viscous DNA solution. Again, the labeling kinetics and the amino acid incorporation data also argue against this.

Our results indicate that the gross morphology of the nuclei was unaffected by their extraction with DNA (Plate I). Similarly, the relatively large amount of RNA which was obtained in the DNA extract fraction, along with the observed high yield of nuclei following extraction, render the intranuclear origin of the DNA extract fraction unlikely. On the other hand, the RNA in this fraction differed from particulate cytoplasmic RNA in several respects. (1) After long-term exposure to [^{3}H]Urd the radioactive RNA in the DNA extract fraction distinctly resembled nuclear RNA in the absence of a 16S peak (Figure 2D,E) (Penman, 1966). (2) The specific activity of the extracted RNA (Table II) was at least six times that of the cytoplasmic RNA fractions, and was similar to the specific activity of nuclear RNA. (3) The kinetics of incorporation of [^{3}H]Urd into this fraction was clearly different from the kinetics of incorporation into cytoplasmic fractions. Similarly the demonstration of the presence of DNA polymerase activity in this fraction (Bach, 1962) and particularly of unique kinetics for the incorporation of [^{3}H]dThd into it (Figure 7) suggest that the fraction must originate very near the nucleus. In fact, for these results to agree with autoradiographic data on [^{3}H]dThd incorporation (Sheek and Magee, 1961; Kit *et al.*, 1963) the components of this fraction must not be resolvable from the cell nuclei by visible light microscopy. These considerations lead to the conclusion that the DNA extract fraction has its origin in the nuclear membrane. Direct confirmation of this proposal might come from high-resolution electron microradioautography following very short periods of incorporation of [^{3}H]dThd. Unfortunately, published results from such studies are contradicatory on this point (Revel and Hay, 1961; Caro, 1962; Caro *et al.*, 1962; Meek and Moses, 1963; Pelc, 1963; and Silk *et al.*, 1961). Part of the difficulty is in the resolution which is possible by this method as well as the amount of isotope incorporation which is required for meaningful detection. Efforts to demonstrate the identity of the DNA extract fraction with portions of the nuclear membrane by a direct examination of nuclear preparations for possible loss of the membrane following extraction with DNA also failed because of the marked decrease in the stability of the nuclei to washing following the extraction step. Nor would a demonstration of such a loss necessarily prove that the membrane fraction which was "lost" was really the one which was present at this site in the intact cell.

A possible explanation for the mode of action of these polymers in promoting the release of the "DNA extract" fraction from washed nuclei might be a competition for binding between the sites that hold the nuclear membrane to the nucleus and sites which can be bound to anionic polymers. Polycations, and especially histones, might have such a binding specificity.

This interpretation is strengthened by our observation (unpublished data) that extraction by DNA was strongly dependent on the magnesium concentration and when spermidine replaced magnesium essentially no extraction of a microsome fraction in DNA-containing buffers occurred.

Whatever the mechanism involved, and whatever the origin of the DNA extract, it is clear that the methods described here permit the isolation of a unique subcellular fraction, having both nuclear and cytoplasm-like properties which may have considerable use in the further exploration of macromolecule synthesis and transport in mammalian cells.

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