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Nuclear Envelope of Chinese Hamster Ovary Cells. Re-formation of the Nuclear Envelope following Mitosis†

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ABSTRACT: We have developed a technique for isolating nuclei and nuclear envelope(s) (NE) from Chinese hamster ovary (CHO) cells which does not depend on the use of detergents to solubilize contaminating cytoplasm. In our procedure NE are prepared from purified nuclei by nuclease digestion and subsequent high salt–sucrose gradient centrifugation. The nuclei and NE fractions are free of significant contamination by other subcellular organelles as judged by electron microscopy and enzyme analysis. Examination of the peptide and glycopeptide composition of the NE fraction by sodium dodecyl sulfate–polyacrylamide gel electrophoresis reveals a very complex coomassie blue staining profile with prominent bands in the 55 000–75 000 molecular weight range. Using this NE isolation technique, we have examined the breakdown and re-formation of the NE during a limited stage (late G_2, M, and early G_1) of the replicative cycle in synchronized populations of CHO cells. Our data demonstrate that a minimum of 60% of the early G_1 NE protein and a minimum of 50% of the early G_1 NE phospholipid were present in the cell during the preceding G_2 phase of the cell cycle and were reutilized in the re-formation of the NE occurring during late M and early G_1. Our evidence suggests that the vast majority of the newly synthesized peptides and glycopeptides of the NE which appear in the daughter NE are synthesized during the early G_1 phase of the replicative cycle. Examination of the NE peptides by one-dimensional gel electrophoresis suggests that no reproducible changes in NE peptide composition can be correlated with specific phases of the cell cycle.

The NE has been shown by a variety of microscopic techniques to break down and re-form during cell division in most species of plants and animals [for a review, see Franke & Scheer (1974)]. Unfortunately, very little is known about the fate of the NE during mitotic breakdown nor is there good experimental data concerning the origin of the components which reform the NE in late M and early G_1. Similarly, the mechanism(s) whereby the NE is disassembled at the initiation of M and re-formed in late M has not been elucidated.

It has been suggested by a number of workers (Porter & Macado, 1960; Moses, 1964; Robbins & Gonatas, 1964; Murray et al., 1965; Brinkley et al., 1967) that, following NE breakdown, fragments of NE mingle with and become indistinguishable from the endoplasmic reticulum (ER). Furthermore, it has been argued that components of the ER are utilized in the re-formation of the NE which occurs during late M–early G_1 (Porter & Macado, 1960; Moses, 1964; Flickinger, 1974). Other investigators have suggested that the NE or components of the NE persist through mitosis as distinct cytoplasmic entities which are biochemically different from the ER. It has been suggested that these hypothetical cytoplasmic components are specifically reutilized to re-form the NE at the completion of M (Erlandson & DeHarven, 1971; Scott et al., 1971; Maruta & Goldstein, 1975; Maul, 1977). Finally, some workers in the field have suggested that the NE which reappears at the end of M is a product of de novo synthesis of all the NE components (Jones, 1960).

To study the composition and biosynthesis of the intact NE and to examine biochemical changes which might occur in the NE during the cell cycle, it was necessary to develop a technique which would permit the isolation, from cultured cells, of NE containing the inner and outer membranes of the NE as well as the pore–lamina complex (Aaronson & Blobel, 1974; Riley et al., 1975; Dwyer & Blobel, 1976; Hodge et al., 1977).

Using a NE isolation technique dependent on nuclease digestion of purified nuclei followed by high salt–sucrose gradient centrifugation, we have investigated the breakdown and re-formation of the NE during the G_2–M–G_1 transition in CHO cells. Our data strongly suggest that the majority of NE peptides and phospholipids which are found in the early G_1 NE were present as cell components prior to the initiation

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of mitosis. Furthermore, our data suggest that a burst of NE-specific protein and phospholipid synthesis occurs in early G_1.

Materials and Methods

Materials. All tissue culture media were purchased from Grand Island Biological Co. (Grand Island, NY). Tissue culture plastic ware was obtained from Corning Glass Works (Corning, NY). Ultrapure sucrose, [*methyl*-^{3}H]thymidine, [5-^{3}H]uridine, and L-[4,5-$^3H_2$]leucine were obtained from Schwarz/Mann Division, Becton, Dickinson and Co. (Orangeburg, NY). Deoxyribonuclease I (DNase I) and ribonuclease A (RNase A) were purchased from Worthington Biochemical Corp. (Freehold, NJ) and were shown to be protease-free via the assay of Tomarelli et al. (1949). [*methyl*-^{3}H]Choline chloride and [^{32}P]orthophosphate were obtained from Amersham/Searle Corp. (Arlington Heights, IL). All reagents for electrophoresis were purchased from Bio-Rad Laboratories (Rockville Centre, NY). All other reagents were obtained from Scientific Products (Ocala, FL).

Cell Culture and Cell Synchrony. CHO cells (originally obtained from Dr. Kenneth Ley, Sandia Laboratories, Albuquerque, NM) were maintained at 37 °C in suspension culture in Ham's F-10 nutrient medium, supplemented with 10% (v/v) calf serum and 5% (v/v) fetal calf serum. Cell density was monitored daily and maintained between 1.2×10^5 and 4×10^5 cells/mL.

Cells were synchronized via a modification of the isoleucine deprivation technique first introduced by Tobey & Crissman (1972). In the procedure utilized, suspension cultures (usually 6 L) were allowed to grow to stationary phase [$(6–8) \times 10^5$ cells/mL] in complete medium. The cells were left for 12–24 h at stationary phase and then harvested and resuspended in fresh, complete medium containing 1 mM hydroxyurea (HU). Ten hours after the addition of the medium supplemented with HU (when the majority of the cells were arrested at the G_1/S boundary), the cultures were reharvested and again resuspended in fresh medium. Immediately following resuspension, the cells began to traverse the cell cycle.

In order to make meaningful comparisons among the many experiments performed, we have plotted synchronization curves as the fraction of cells divided $[(N - N_0)/N_0]$ vs. time, N_0 being the number of cells before division and N being the number of cells at any given time during division.

Isolation of Nuclei and NE. A flow chart of this procedure is presented in Figure 1. All procedures were carried out at 0 °C unless otherwise noted. For each NE preparation $(3–4) \times 10^9$ cells were harvested, washed twice with phosphate-buffered saline (PBS, pH 7.2), and resuspended at $(2–4) \times 10^7$ cells/mL in a homogenization buffer containing 10 mM Tris (pH 7.6), 10 mM KCl, and 10 mM EDTA. The cells were allowed to swell for 15 min and then were lysed (in 15-mL batches) by five to seven strokes in a Dounce homogenizer fitted with a tight pestle. Homogenization was continually monitored by phase-contrast microscopy to assure maximum cell lysis with minimum nuclear breakage.

Immediately following lysis, the homogenate obtained from each batch of cells was diluted with 2 volumes of 65% (w/w) sucrose, 50 mM Tris (pH 7.6), and 7.5 mM $MgCl_2$ and kept on ice until homogenization of the entire cell sample was completed. The diluted homogenate was centrifuged in a Sorvall HB-4 rotor for 30 min at $4936g_{max}$. This initial centrifugation concentrated the nuclei in the pellet and removed most of the contaminating organelles. The crude nuclear pellet was resuspended in 24 mL of 65% (w/w) sucrose, 50 mM Tris (pH 7.6), and 2.5 mM $MgCl_2$ (65% sucrose–TM). Aliquots of the resuspended crude nuclear pellet were overlaid with 6 mL of 60% sucrose–TM, 12 mL of 55% sucrose–TM, 6 mL of 50% sucrose–TM, and 7 mL of 30% sucrose–TM to form a discontinuous sucrose gradient. The gradient(s) was subsequently spun for 1 h at $72100g_{max}$ in a Beckman SW27 rotor. Nuclei banding at the 65–60% and 60–55% sucrose interfaces were removed, combined, and diluted to 50% sucrose with TM buffer. By the use of an SW27 rotor ($72100g_{max}$ for 1 h), these nuclei were sedimented through another discontinuous gradient containing 12 mL of 55% sucrose–TM and 3 mL of 60% sucrose–TM and collected onto a cushion of 3 mL of 65% sucrose–TM. Both gradients were necessary to ensure virtually complete removal of minor contaminants. Material caught on the 65% sucrose–TM cushion was removed, diluted to 10% sucrose with distilled water, and pelleted in an HB-4 rotor at $1020g_{max}$ for 5 min. This pellet was designated the purified nuclear fraction. Catching the nuclei on a 65% sucrose–TM cushion decreased the difficulty encountered in resuspending the nuclei and therefore significantly reduced premature nuclear lysis.

The nuclei were lysed by being resuspended in 1 mL of 10 mM Tris (pH 8.5), containing 0.1 mM $MgCl_2$. The inclusion of 0.1 mM $MgCl_2$ facilitated chromatin swelling and subsequent nuclear lysis (Kay et al., 1972). After 20 min in 10 mM Tris and 0.1 mM $MgCl_2$, DNase I and RNase A were added to a final concentration of 100 μg/mL and the incubation was continued for 20 min at 22 °C. Following lysis (which was monitored with phase optics), 4 volumes of 60% (w/w) sucrose, 50 mM Tris (pH 7.6), and 500 mM $MgCl_2$ was added to the lysed nuclei. The inclusion of 500 mM $MgCl_2$ significantly reduced the DNA and histone contamination of the NE. It should be noted, however, that 500 mM $MgCl_2$ might be expected to strip some extrinsic proteins from the NE but it would not be expected to remove the major intrinsic proteins responsible for NE structure. The solutions were thoroughly mixed and the suspensions placed in the bottom of a Beckman SW41 centrifuge tube. A linear 20–45% (w/w) sucrose gradient containing 50 mM Tris (pH 7.6) and 500 mM $MgCl_2$ was formed on top of the sample, and the gradients were spun for 2 h at $153244g_{max}$ in a Beckman SW41 rotor. NE banded at 1.19 g/cm^3 (35% sucrose in 500 mM $MgCl_2$). The purified NE were diluted with 50 mM Tris (pH 7.6) and 500 mM $MgCl_2$ and then pelleted in a Beckman fixed-angle 40 rotor for 30 min at $130766g_{max}$. The NE were subsequently resuspended in 10% sodium dodecyl sulfate ($NaDodSO_4$), 10 mM Tris (pH 8.5), and 1% β-mercaptoethanol if they were to be analyzed by electrophoresis or in 10% sucrose, 10 mM Tris (pH 7.6), and 0.5 mM $MgCl_2$ if chemical or enzymatic analysis was to be performed on the sample.

Isolation of Plasma Membrane. Plasma membranes were isolated by the aqueous two-phase polymer technique of Brunette & Till (1971).

Chemical Assays. All protein determinations were performed according to the procedure of Lowry et al. (1951). Phospholipid phosphorus (Rouser et al., 1966) was measured in chloroform–methanol extracts (Rouser & Fleischer, 1967) of various subcellular fractions. Phospholipid content of the various fractions was extrapolated from the phosphorus determination by multiplying the phosphorus value by a factor of 25. Cholesterol was determined according to Glick et al. (1964). In order to determine the DNA or RNA content of the NE fraction, we grew cells for five generations in the presence of 0.5 μCi/mL [^{3}H]thymidine or 0.4 μCi/mL [^{3}H]uridine, respectively, prior to preparation of NE. The specific radioactivity of DNA or RNA (counts per minute per

microgram of nucleic acid) was determined from the cell homogenate by liquid scintillation counting, and the spectrophotometric assay DNA content was determined by the diphenylamine assay (Burton, 1956) while RNA content was determined by the orcinol (I-Sanlin & Schjeide, 1969) assay of extracts prepared according to Fleck & Munro (1962). The specific radioactivity obtained for DNA and RNA was then used to determine the relative nucleic acid content of nuclei and NE.

Enzyme Assays. 5′-Nucleotidase was assayed via the technique of Widnell & Unkless (1969) with inorganic phosphate release being measured by a modification of the technique described by Chen et al. (1956). Succinate dehydrogenase was assayed according to Veeger et al. (1969).

Electron Microscopy. Nuclei and NE were fixed (0 °C, 2 h) in 1.9% glutaraldehyde, 8% sucrose, 50 mM phosphate (pH 7.6), and 2.5 mM $MgCl_2$. Both the nuclei and NE fractions were postfixed in the same buffer containing 1% osmium tetroxide (22 °C, 1 h). Samples were sequentially dehydrated in increasing concentrations of ethanol and finally embedded in Spurr's low-viscosity medium (Spurr, 1969). Thin sections were stained with uranyl acetate (Watson, 1958) and lead citrate (Venable & Coggeshall, 1965) and then examined in a Hitachi HS8 electron microscope at 50 kV.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. All electrophoretic analyses were performed in 1.5-mm thick slab gels according to the procedure of Laemmli (1970). The gels used were composed of a 5.6% acrylamide stacking gel overlaying a linear 7.5–12.5% acrylamide gradient separation gel. The acrylamide/bis(acrylamide) ratio was maintained at 37.5:1 in both the stacking and running gels. Purified NE were solubilized by boiling at 100 °C for 5 min in 10% $NaDodSO_4$, 10 mM Tris (pH 8.5), and 1% β-mercaptoethanol (βME). Plasma membrane was solubilized by resuspension and boiling in 2% $NaDodSO_4$, 62.5 mM Tris, 10% glycerol, and 0.1% βME ["sample buffer" (Laemmli, 1970)]. Lysed nuclei, sampled after DNase I and RNase A digestion, and whole-cell homogenates were solubilized by boiling in 1 volume of 2× sample buffer. Each sample was then exhaustively dialyzed against sample buffer containing 0.1 M phenylmethanesulfonyl fluoride (PMSF). Inclusion of PMSF in the sample buffer was essential since we found that NE solubilized in sample buffer lacking PMSF were subject to extensive proteolysis, possibly as the result of the copurification of an intrinsic protease with the purified envelopes (Carter et al., 1976).

Following electrophoresis, the slabs were fixed for 30 min in 10% trichloroacetic acid and then allowed to equilibrate overnight in 25% ethanol and 8% acetic acid. The following day the gels were stained with coomassie brilliant blue according to Weber & Osborn (1969) and finally destained in 25% ethanol and 8% acetic acid.

Labeling of Cell Cultures. In our "isotope dilution studies", cells were maintained for five generations prior to synchronization in a particular radioactive precursor (at the specific activity indicated) and then the cultures were synchronized in the presence of either 0.2 μCi/mL [^{3}H]leucine, 0.1 μCi/mL [^{3}H]choline, or 0.4 μCi/mL[^{32}P]orthophosphate. In order to determine the dilution of radiolabel which occurred during the mitotic phase of the cell cycle, we harvested the labeled cell cultures immediately prior to entrance into M (i.e., in very late G_2) and washed them twice in phosphate-buffered saline, pH 7.2 (PBS). One aliquot of cells was used to prepare G_2 NE while the remaining cells were returned to culture in fresh medium containing no radioactive precursor. After the cells had completed M, they were used to prepare the G_1 NE.

Pulse-label experiments with [^{3}H]leucine were performed by growing and synchronizing cultures in the absence of radiolabel. Following release of the cells from HU, the cultures were resuspended in medium containing 3.3 mg/L leucine (25% of the leucine normally present in F-10 medium). One hour before each sequential NE isolation, an aliquot of cells was removed from the synchronized stock culture and [^{3}H]-leucine was added to a final concentration of 0.2 μCi/mL [^{3}H]leucine. Following maintenance for 1 h in medium supplemented with [^{3}H]leucine, the NE were isolated.

Pulse labeling of cultures with [^{32}P]orthophosphate was performed as in [^{3}H]leucine pulses except that medium added after the release of the cells from the HU block was complete F-10. [^{32}P]Orthophosphate was added to a final concentration of 0.8 μCi/mL.

Determination of Specific Activities. The specific activity of [^{3}H]leucine-labeled cell components was determined by liquid scintillation counting (LSC) and protein assay either after solubilization of the cell component in 10% sodium dodecyl sulfate, 10 mM Tris (pH 8.5), and 1% β-mercaptoethanol and exhaustive dialysis against sample buffer containing 0.1 mM phenylmethanesulfonyl fluoride or following precipitation of the isolated component with 10% trichloroacetic acid (Cl_3AcOH). The specific activity of ^{32}P-labeled phospholipid was determined from phospholipid phosphorus assays (Rouser et al., 1966) of chloroform–methanol extracts (Rouser & Fleischer, 1967) of the specific cell component.

Determination of Precursor Pool Equilibration Rates. Cell cultures were grown for five generations in either 0.2 μCi/mL [^{3}H]leucine or 0.4 μCi/mL [^{32}P]orthophosphate. The cells were then synchronized in the presence of 0.2 μCi/mL [^{3}H]leucine or 0.4 μCi/mL [^{32}P]orthophosphate. Five to six hours after release from HU, an aliquot of cells was taken from the culture and the initial precursor pool specific activity was determined as described below. Immediately after removal of this initial aliquot, the remaining cells were washed twice with PBS, sampled again, and returned to unlabeled medium. Each hour over the next 4 h a sample of cells was removed from the unlabeled medium and the precursor pool specific activity determined.

For a determination of the size of the [^{3}H]leucine pool, cells were collected on a glass fiber filter, washed quickly with 10 volumes of ice-cold PBS, and solubilized in 0.2 N NaOH for 5 min at 70 °C. The solubilized material was chilled and precipitated with 10% trichloroacetic acid overnight at 4 °C, and the supernatant was collected by centrifugation. [^{3}H]-Leucine in the supernatant (i.e., the acid-soluble pool) was determined by LSC, and total cell protein was assayed according to Lowry et al. (1951). Amino acid analysis of the acid-soluble material demonstrated that >90% of the radiolabel was associated with a peak which was superimposable on a nonradiolabeled leucine carrier.

Phospholipid precursor pool size was based on the specific activity of labeled phosphatidic acid. [^{32}P]Phosphatidic acid specific activity was determined by harvesting cells via centrifugation and immediately extracting the harvested cells with chloroform–methanol (Rouser & Fleischer, 1967). After the Folch backwashes of the chloroform–methanol extract, phosphatidic acid was found in the lower organic phase. Phosphatidic acid was purified by two sequential separations on silica gel thin-layer chromatography using solvent system I developed by Skipski & Barclay (1969) for acidic phospholipids. The purified phosphatidic acid was assayed for phosphorus (Rouser et al., 1966), and the ^{32}P content was determined by LSC.

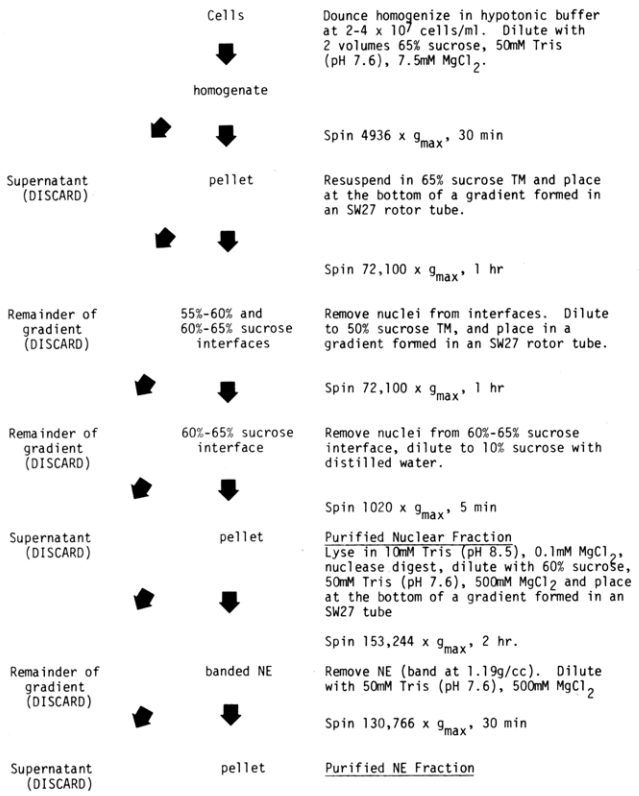

FIGURE 1: Flow diagram of the nuclei and NE isolation procedure.

Measurement of Nuclear Surface Areas. Nuclear surface areas were measured by point and intersection planimetry of photographs of thick sections of embedded whole cells. Samples of cells were taken from a synchronized culture 5 (late G_2) and 8 h (early G_1) after release from HU. Samples were fixed in 2% glutaraldehyde, 2% formaldehyde, and 50 mM phosphate (pH 7.4) for 30 min at 22 °C. Postfixation was performed in 1% OsO_4 and 100 mM phosphate (pH 7.4) for 30 min at 22 °C. Samples were then dehydrated and embedded as described previously. Thick sections were cut on a Sorvall microtome and stained with 1% toluidine blue in 1% sodium borate for 15 s at ~200 °C. Sections were photographed by using a Wild MII microscope. The resulting photographs were subsequently subjected to planimetry according to Weibel (1969).

Results

Evidence That the Organelle Isolated Is NE. Electron micrographs of the purified nuclear fraction (Figure 1) show no obvious contamination of the nuclei with mitochondria, rough endoplasmic reticulum (RER), vesicles, or large sheets of plasma membrane (Figure 2A). A higher magnification micrograph (Figure 2B) of nuclei isolated in the purified nuclear pellet clearly shows an outer (single arrow) nuclear membrane and suggests the presence of an inner nuclear membrane. However, in this micrograph (Figure 2B) the inner nuclear membrane is not easily distinguishable from the heterochromatin and probably lamina complex (Dwyer & Blobel, 1976).

The NE isolation procedure described under Materials and Methods (and Figure 1) is a combination and modification of the procedures published by Kay et al. (1972) and Monneron et al. (1972) for the isolation of NE from rat liver nuclei. Electron micrographs of sections through the purified NE fraction (Figure 3) demonstrate that most, if not all, of the isolated membranes retain the double membrane (single arrow) characteristic of the NE. Unfortunately, the morphology of

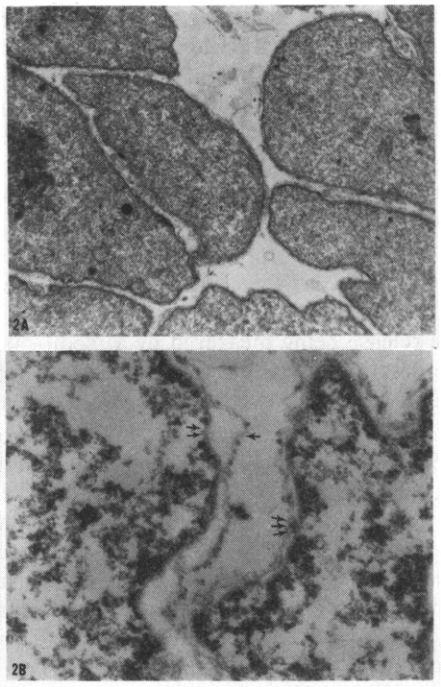

FIGURE 2: Electron micrograph of the purified nuclear fraction. Purified nuclei were fixed, embedded, and stained as described under Materials and Methods. (A) Representative section through purified nuclear fraction (×3000). (B) Higher magnification electron micrograph of chosen nuclei with the attention being directed to the nuclear membranes (×28 000): (↑) outer membrane; (↑↑) inner membrane; (↑↑↑) pores.

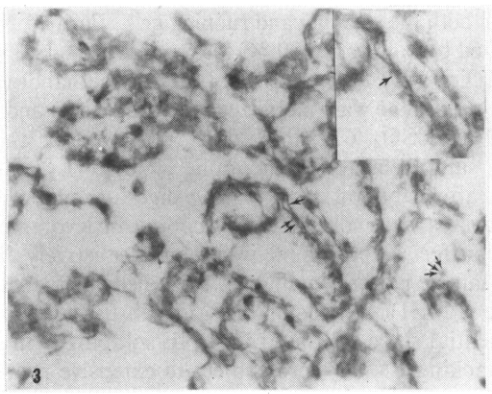

FIGURE 3: Electron micrograph of the purified NE fraction. The NE fraction was fixed, embedded, and stained as described under Materials and Methods (×15 925). Region of double membranes (↑) can be seen as well as pores in transverse (↑↑) and tangential (↑↑↑) sections. Inset: higher magnification of a portion of the NE fraction showing a pore structure (↑) in transverse section (×31 850).

the pore structures is not well-preserved by this isolation technique. Nevertheless, potential pore structures are identified in the micrograph (double arrow, Figure 3). Clearly, the presence of porelike structures and double membranes in this purified fraction supports its identity as an NE fraction. Furthermore, few, if any, smooth vesicles are apparent in this NE preparation (Figure 3), suggesting limited microsomal contamination. Similarly, no mitochondria or unbroken nuclei are observed in this purified NE fraction.

Typical recoveries of protein, phospholipid, and DNA obtained during preparation of the NE are presented in Table I. Approximately 14 and 30% of the homogenate protein and DNA, respectively, are recovered in the nuclear fraction. On the basis of the recovery of DNA, it appears that ~30% of the starting nuclei are isolated in the purified nuclear fraction (Figure 1). On the basis of the recovery of phospholipid in

Table I: Recovery of Protein, DNA, and Phospholipid in Subcellular Fractions[a]

fraction	% of total cellular DNA	% of total cellular protein	% of total nuclear DNA	% of total nuclear protein	% of total nuclear phospholipid
homogenate	100	100			
nuclei	30	14	100	100	100
NE	0.1	1	0.3	8	50

[a] Recoveries were determined by assay of individual fractions sampled during the isolation procedure.

Table II: Enzyme Activities of Subcellular Fractions[a]

fraction	5′-nucleotidase (nmol of PO_4 per mg of protein per h)	succinate dehydrogenase (nmol of succinate oxidized per mg of protein per h)
homogenate	49 (6)	470 (2)
nuclei	ND (2)	ND (2)
NE	ND (2)	ND (2)
plasma membrane	255 (2)	ND (2)

[a] Each fraction was assayed as described under Materials and Methods immediately following isolation of NE fractions. ND = no detectable activity. The minimum detectable activity in succinate dehydrogenase assays was 50 nmol of succinate oxidized per mg of protein per h and the minimum detectable activity in 5′-nucleotidase assays was 3 nmol/(mg of protein·h). Numbers in parentheses are the number of assays performed on separate isolates. It should be noted that homogenates and plasma membrane fractions were treated with 500 mM $MgCl_2$, nuclease digestion and pH 8.5 Tris-HCl as in the NE preparation (Figure 1). We found, as did Monneron & d'Alayer (1978), that such treatment does not markedly affect the enzyme activities of the isolated fractions.

the NE fraction, it can be estimated that ~50% of the NE present on the starting nuclei are recovered in the purified NE fraction. Virtually the same data are obtained if the yield is determined on nuclei isolated from cells at the G_2/M or M/G_1 boundary, suggesting that nuclei immediately prior to dissolution or just after re-formation are as stable as nuclei isolated from an asynchronous population of cells.

Characterization and Purity of Nuclear and NE Fractions. In order to further confirm the relative purity of the isolated nuclear and NE fractions, as well as to determine the composition of the nuclei and NE, we have examined the chemical content and a number of the enzymatic activities displayed by both fractions.

Enzyme Activity. The activity of 5′-nucleotidase was examined in homogenate, nuclei, and NE fractions in order to determine possible plasma membrane contamination of the purified NE fraction. Table II clearly shows that no detectable 5′-nucleotidase activity is found associated with either the nuclei or the NE. Similarly, assays for succinate dehydrogenase activity (as a marker for mitochondrial contamination) indicated that neither the nuclear nor the NE fraction had significant quantities of this enzyme associated with them (Table II).

Chemical Composition. An analysis of the relative chemical composition of the homogenate, nuclei, and NE fractions is presented in Table III. Among the data presented in Table III which should be stressed is the fact that the purified NE fraction does contain small amounts of residual DNA and RNA. Similar findings with regard to nucleic acid composition of isolated NE have been reported in previously published

Table III: Chemical Content of Subcellular Fractions[a]

fraction	μg of DNA per mg of protein	μg of RNA per mg of protein	μg of phospholipid per mg of protein	μg of cholesterol per mg of protein
homogenate	90	220	90	ND
nuclei	200	90	30	ND
NE	10	20	200	20
plasma membrane	ND	ND	ND	117

[a] Individual fractions were sampled during the isolation procedure and assayed for protein, DNA, RNA, phospholipid, or cholesterol. A plasma membrane enriched fraction was prepared from CHO cells via the two-phase aqueous polymer technique of Brunette & Till (1971). ND = not determined.

characterizations of NE from liver (Kasper, 1974) and may, in fact, represent physiologically significant components of the NE (Franke, 1974).

To confirm the membranous nature of the isolated NE fraction, we determined the phospholipid content in the homogenate, nuclei, and NE fraction. As can be seen from the data presented in Table III, the NE fraction contained 200 μg of phospholipid per mg of protein, indicating that the fraction isolated was relatively protein rich perhaps due to the proteinaceous lamina which is probably associated with the inner nuclear membrane. In order to demonstrate that this relatively low phospholipid/protein ratio was not due to incomplete extraction of phospholipid, we prepared NE from cells which had been maintained for five generations in medium containing 0.1 μCi/mL [^{3}H]choline. Ninety-seven percent of the [^{3}H]choline present in the NE fraction was extracted by chloroform–methanol and could be accounted for in subsequent phosphorus determinations (data not shown).

The cholesterol content of NE and plasma membrane was compared in order to determine if there were any clear differences in cholesterol composition between these two fractions taken from CHO cells. As can be seen in Table III, fivefold less cholesterol was found in the NE as compared to the plasma membrane. These data are in agreement with previously published work concerning the cholesterol content of NE isolated from a variety of cell types (Keenan et al., 1970; Franke, 1974; Franke & Scheer, 1974; Kasper, 1974).

Peptide Composition. When the purified NE fraction was examined by $NaDodSO_4$ gel electrophoresis (Laemmli, 1970), a complex coomassie blue staining profile was obtained which was clearly distinguishable from the stained profiles obtained from whole-cell homogenate, purified nuclei, and plasma membrane derived from CHO cells (Figure 4).

The major coomassie brilliant blue staining components of the NE fraction range in molecular weight from 55 000 to 75 000. The majority of the remaining NE peptides and glycopeptides are of a molecular weight higher than 75 000. It should be noted (compare nuclear with NE fraction, Figure 4) that few, if any, peptides are found in the NE fraction which comigrates with the histones (arrows) found in the nuclear fraction, thereby again arguing against substantial contamination of the NE with DNA.

Cell Synchrony. The relative synchrony obtained by the technique outlined under Materials and Methods is presented in Figure 5. The [^{3}H]thymidine incorporation into DNA indicates that following release from HU DNA synthesis was initiated immediately and that the DNA synthetic phase (S phase of the cell cycle) lasted ~5 h. A 5-h S phase is in good agreement with the value previously obtained by Tobey & Crissman (1972) using these same cells. Concurrent with cessation of DNA synthesis, mitotic cells appeared (Figure

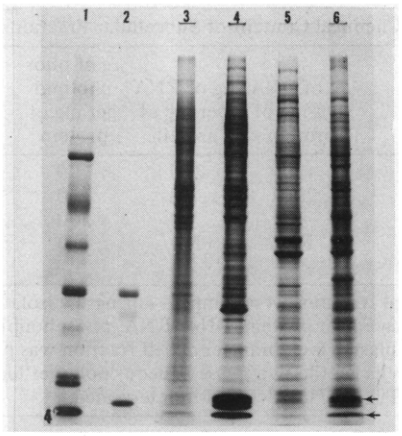

FIGURE 4: Coomassie blue stained profiles of subcellular fractions. Fractions were isolated and electrophoresed as described under Materials and Methods. 75 μg of protein was applied to each slot. From left to right: lane 1, standards (phosphorylase A, M_r 100 000; bovine serum albumin, M_r 69 000; ovalbumin, M_r 43 000; DNase I, M_r 31 500; soybean trypsin inhibitor, M_r 23 000; cytochrome *c*, M_r 13 500); lane 2, DNase I and RNase A; lane 3, NE; lane 4, nuclei; lane 5, plasma membrane; lane 6, whole-cell homogenate. Arrows indicate the region in which histones migrate in these gels.

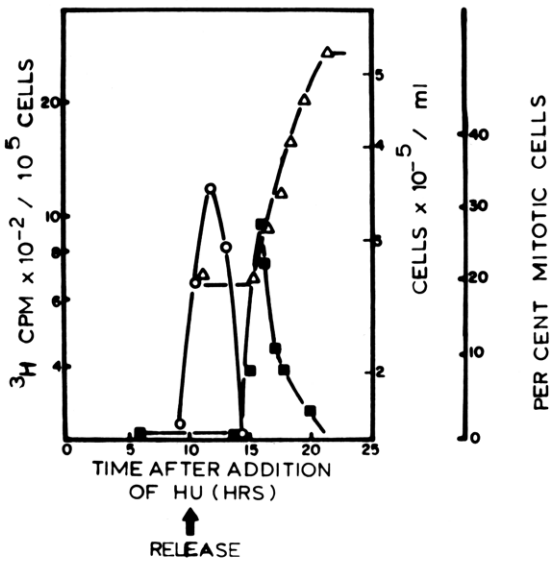

FIGURE 5: Cell cycle parameters in synchronized CHO cells. A suspension culture was synchronized as described under Materials and Methods. DNA synthesis was determined by pulsing duplicate 2-mL aliquots of cells for 10 min at 37 °C with 2.6 μCi/mL [^{3}H]-thymidine. Labeled cells were then washed 3 times with ice-cold 10% Cl_3AcOH. Radioactivity in the precipitate was determined by LSC. Cell number was determined from four replicate counts by using a Levy–Hausser counting chamber. Mitotic index is expressed as the percent of cells in mitosis as reflected by phase microscopy. [^{3}H]-Thymidine incorporation into Cl_3AcOH-insoluble material (○); cell number (△); mitotic index (■).

5). The mitotic phase of the cell cycle was complete in ~4–5 h (i.e., occurred during a time from 5–10 h after release from HU or 15–20 h after addition of HU, Figure 5). During this time period the cell population increased by ~80% (Figure 5). The fact that mitotic figures begin appearing as soon as DNA synthesis is terminated (Figure 5) makes it impossible to accurately measure the G_2 phase of the cell cycle under the conditions we have used to optimize cell yield. However, previous work by Tobey & Crissman (1972) suggests that the G_2 phase in these cells is relatively short. It should also be noted that the maximum mitotic index at any time after release from HU is 25–30% of the total cell population. Thus, during the 4–5-h mitotic phase described in Figure 5 a proportion of

Table IV: Dilution of [^{3}H]Leucine-Labeled NE Protein[a]

sp act. of NE protein (cpm/mg of protein)		
G_2/M	M/G_1	M/G_1:G_2/M ratio
5.3 × 10^5	3.9 × 10^5	0.74
9.3 × 10^5	6.7 × 10^5	0.72
6.3 × 10^5	4.2 × 10^5	0.67
2.7 × 10^5	2.1 × 10^5	0.78
1.4 × 10^5	1.1 × 10^5	0.79
		$\bar{x}$ = 0.74
		SD = ±0.05

[a] Cells were grown and synchronized in media containing 0.2 μCi/mL [^{3}H]leucine, and NE were isolated from half of the cell population immediately before synchronous division (G_2/M NE). Four hours later (when ~80% of the cells had completed M) NE were isolated from the other half of the cell population which had proceeded through M in the absence of [^{3}H]leucine (M/G_1 NE). Specific activities were determined as described under Materials and Methods. $\bar{x}$ = average percent of M/G_1 peptide which pre-exists in G_2/M (precursor pool equilibration not considered). SD = standard deviation. Each set of points is a separate experiment and represents the average of duplicates within each experiment.

the cells either is in the preceding G_2 or has progressed into the next G_1 phase of the cell cycle and is coexisting with cells in mitosis. Due to this limitation in reaching maximal synchrony along with adequate cell numbers, we are forced to refer to the 4–5-h time period during which the cell population divides as the G_2–M–G_1 transition. It is during this transitional period of the cell cycle that the NE breaks down and re-forms.

Peptide Composition of the NE during Different Phases of the Cell Cycle. In an effort to determine whether any changes occur in CHO NE during the cell cycle which could be detected as changes in the coomassie blue staining profile of NE peptides and glycopeptides separated on NaDod-SO_4–polyacrylamide gel electrophoresis, we have isolated NE from cells at various stages of the cell cycle. In this study NE were isolated from (a) cells which were in the logarithmic phase of growth, (b) cells which had been grown to stationary phase, allowed to reinitiate the cells cycle in fresh HU containing media, and then collected 10 h after reinitiation of the cell cycle (i.e., cells at G_1/S boundary, Figure 5), (c) cells 5 h after release from HU (i.e., cells at the G_2/M bounadry), and (d) cells 10 h after release from HU (i.e., cells in the following G_1). In agreement with the previously published work of Sieber-Blum & Burger (1977), we cannot find any consistent differences in NE peptide composition which can be related to the different phases of the cell cycle which we have analyzed (data not shown).

Dilution of [^{3}H]Leucine-Labeled NE. Using synchronized and uniformly labeled CHO cells, we have examined the dilution of labeled NE peptides during the G_2–M–G_1 transition. Such label dilution experiments were directed at determining whether peptides present in NE isolated from early G_1 cells (10–12 h after release from HU) were synthesized de novo during the G_2–M–G_1 transition or whether the peptides present in the early G_1 NE preexisted in the cell prior to mitosis.

CHO cells were grown for five generations and synchronized in the presence of 0.2 μCi/mL [^{3}H]leucine. In late G_2 (5 h after HU removal, Figure 5), the cell culture was harvested and washed twice with PBS and then the cells were divided into two equal aliquots. From one aliquot NE were immediately isolated (described in Table IV as the G_2/M NE population). The remaining cells were returned to fresh medium free of [^{3}H]leucine but containing 13.2 mg/L cold leucine. Four hours later (after 80% of the cells had completed M, Figure 5), these cells were harvested and NE immediately

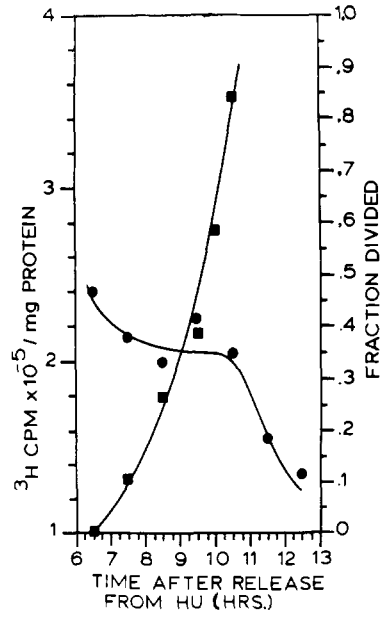

FIGURE 6: Dilution of [^{3}H]leucine-labeled NE protein. A suspension culture was grown to stationary phase in the presence of 0.2 μCi/mL [^{3}H]leucine and synchronized in medium containing 0.2 μCi/mL [^{3}H]leucine. 6.5 h after release from HU, NE were prepared from an aliquot of cells. The remaining cells were washed twice with PBS and returned to culture in complete medium lacking [^{3}H]leucine. NE were isolated each hour after removal of [^{3}H]leucine, and the specific activity of each fraction was determined as described under Materials and Methods. Fraction of cells divided was determined from four replicate countings in a Levy–Hausser counting chamber. NE specific activity (●); fraction divided (■).

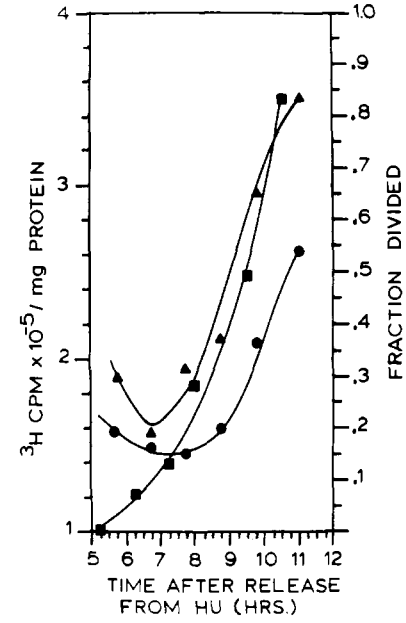

FIGURE 7: Incorporation of [^{3}H]leucine into NE protein. A suspension culture was grown to stationary phase and synchronized. The culture was released from HU blockade into media containing 25% of the normal F-10 leucine concentration. 1 h before synchronous division (i.e., 5.25 h after removal from HU), an aliquot of cells was removed, pulsed with 0.2 μCi/mL [^{3}H]leucine for 1 h, and harvested for NE isolation. Each hour thereafter, an additional aliquot of cells was pulsed for 1 h with 0.2 μCi/mL [^{3}H]leucine, NE were isolated, and the specific activity was determined on each NE and homogenate fraction. Fraction of cells divided was determined from four replicate countings in a Levy–Hausser counting chamber. NE specific activity (●); homogenate specific activity (▲); fraction divided (■).

isolated (described as the M/G_1 NE population in Table IV). The specific activity of these two NE fractions was then determined and compared. As can be seen in Table IV, the average specific activity of the M/G_1 NE is ~25% less than the specific activity of the NE isolated from the G_2/M cell population. These data suggest that although some de novo synthesis of NE protein occurred over the 4-h time span between the isolation of the G_2/M and M/G_1 NE, the majority of the peptides present in the M/G_1 NE preexisted in the G_2/M cells. The data presented in Table IV do not, of course, take the precursor pool into account. The effect of the labeled precursor pool on the apparent reutilization of NE peptides will be discussed below.

The reduction in specific activity broadly described in Table IV was further investigated by isolating NE at a time point (6.5 h after release from HU) when fewer than 5% of the cells had completed M and every hour thereafter until greater than 80% of the cells had completed M (Figure 6). Cells were grown and synchronized in the presence of [^{3}H]leucine as described for the experiments presented in Table IV. Six and one-half hours after release from HU (Figure 6) cells were harvested and washed. One aliquot of cells was used immediately for NE isolation (G_2/M NE), while the remaining cells were returned to culture in complete medium lacking [^{3}H]-leucine. Each hour thereafter, for the next 6 h, an aliquot of cells was harvested, NE were isolated, and the specific activity of the NE protein was determined. The specific activity of the NE at each time point is displayed in Figure 6 along with the fraction of cells divided at each time point. Figure 6 suggests that NE which are in the late G_2 phase of the cycle (7.5 h after release from HU) have lost ~10% of the label initially associated with the envelopes, suggesting that during very late G_2 some dilution of the labeled components of the NE may occur. As the NE enter M there appears to be a decrease in the rate of dilution, and then as the NE enter the following G_1 a marked resumption of label dilution occurs (Figure 6).

In order to determine whether a particular peptide or group of NE peptides was preferentially lost during the G_2–M–G_1 transition, we solubilized [^{3}H]leucine-labeled NE (isolated during the experiments described in Table IV) in $NaDodSO_4$, separated labeled peptides and glycopeptides on a discontinuous $NaDodSO_4$–polyacrylamide gel electrophoresis, and examined the profile by fluorography. Although the data are not shown, it is apparent that, within the detection limits of the fluorographic process and one-dimensional gel electrophoresis, no individual NE peptide or glycopeptide is preferentially lost during the G_2–M–G_1 transition.

Incorporation of the [^{3}H]Leucine into NE Protein. One likely, if not the most likely, explanation for the reduced specific activity found in M/G_1 NE relative to G_2/M NE (Table IV) is dilution of the preexisting (i.e., G_2/M) label with unlabeled NE components synthesized and inserted into the NE during either the G_2, M, or G_1 phase of the cell cycle. The data presented in Figure 6 suggest that some dilution of label may occur in G_2 immediately after removal of [^{3}H]leucine from the culture medium while the majority of the dilution occurs in G_1. In order to determine whether this dilution results from phase-specific biosynthesis of NE peptides, we synchronized cells as described above and then 5.25 h after release from HU an aliquot of cells was taken and pulsed for 1 h in medium containing 0.2 μCi/mL [^{3}H]leucine but only 25% of the standard F-10 leucine concentration. Cells were pulsed with 0.2 μCi/mL [^{3}H]leucine, and NE were isolated every hour from 5.25 to 11.5 h after release of cells from an HU blockade (Figure 7). After 1 h in labeled precursor, the cells were homogenized, an aliquot was taken (the "homogenate" in Figure 7), and the NE were isolated from the remaining material.

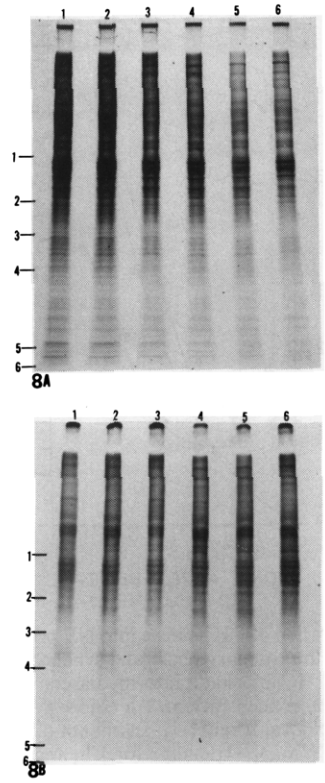

FIGURE 8: $NaDodSO_4$ gel electrophoresis of [^{3}H]leucine pulse-labeled NE peptides. NE isolated during the experiment described in Figure 7 were solubilized and subjected to electrophoresis and fluorography. (A) Coomassie blue stained profile corresponding to the fluorogram in (B). Lane 1, 72 μg of protein; lane 2, 74 μg of protein; lane 3, 76 μg of protein; lane 4, 71 μg of protein; lane 5, 56 μg of protein; lane 6, 42 μg of protein. (B) Fluorogram depicting [^{3}H]leucine-labeled NE isolated at the six time points indicated in Figure 7. Lane 1, 5.75 h after HU removal; lane 2, 6.75 h after HU removal; lane 3, 7.75 h after HU removal; lane 4, 8.75 h after HU removal; lane 5, 9.75 h after HU removal; lane 6, 11 h after HU removal. Equal numbers of counts (11 000 cpm) were applied to each lane. Left side: 1–6 denote molecular weight markers as in Figure 4.

The specific activity of each NE and homogenate fraction was determined, and the specific activities were plotted as a function of time after release from the HU block. As can be seen in Figure 7, the specific activity of the NE fractions remains relatively constant (±5%) from 6 to 9 h after release from HU (by which time ~40% of the cells in the population have divided). However, between 9 and 10 h after release from HU (when the majority of the cell population is in the early portion of the G_1 phase of the cell cycle, Figure 7) incorporation of [^{3}H]leucine into the NE increased 1.25 times that seen in M and by 11 h after release incorporation of [^{3}H]-leucine into the NE was ~1.56 times that seen in M. Taken together, the data in Figures 6 and 7 suggest that the majority of NE synthesis (and consequently dilution of preexisting label) occurs in early to mid G_1, with NE-specific synthesis in M being low relative to G_1. Some NE-specific synthesis may occur late in G_2; however, it is, at best, difficult to detect by using this large-scale synchronization technique.

Determination of acid-soluble [^{3}H]leucine counts (counts per minute per milligram of protein) in the whole-cell homogenate at each time point clearly demonstrates that the increase in specific activity of NE and homogenate protein seen in G_1 is not due to increased transport of labeled precursor but rather is due to enhanced de novo biosynthesis (data not shown).

For a determination of whether specific peptides of the NE might be preferentially synthesized during the G_2–M–G_1 segment of the cell cycle, NE fractions, obtained at the time points taken in Figure 7, were solubilized in $NaDodSO_4$ and the peptide composition was displayed by coomassie blue staining and fluorography on a 7.5–12.5% discontinuous $NaDodSO_4$–polyacrylamide gel electrophoresis. The fluorogram presented in Figure 8B indicates that, at the limits of detection of one-dimensional $NaDodSO_4$ gel electrophoresis and fluorography, all NE peptides are synthesized at some point in the G_2–M–G_1 transition. Comparison of the coomassie blue stained profile (Figure 8A) with the intensities of the individual bands in the exposed fluorogram (Figure 8B) suggests that the NE peptides are labeled approximately in proportion to their relative staining with coomassie blue.

Table V: Dilution of Labeled NE Phospholipids[a]

radiolabel	NE phospholipid sp act. (cpm/μg of lipid phosphorus) G_2/M	NE phospholipid sp act. (cpm/μg of lipid phosphorus) M/G_1	M/G_1:G_2/M ratio
[^{3}H]choline	2.0×10^4	1.4×10^4	0.70
[^{32}P]orthophosphate[b]	0.8×10^4	0.5×10^4	0.63
			$\bar{x} = 0.67$

[a] Cells were grown and synchronized in media containing 0.1 μCi/mL [^{3}H]choline or 0.4 μCi/mL [^{32}P]orthophosphate, and NE were isolated from half of the cell population immediately before synchronous division (G_2/M NE). Four hours later (when ~80% of the cells had completed M) NE were isolated from the other half of the cell population which had proceeded through M in the absence of label (M/G_1 NE). Specific activities were determined as described under Materials and Methods. $\bar{x}$ = average percent of M/G_1 phospholipid which preexists in G_2/M (precursor pool equilibration not considered). [b] Data were calculated from the experiment shown in Figure 11.

Dilution of [^{3}H]Choline-Labeled NE. Using [^{3}H]choline as a precursor of phosphatidylcholine, we have examined the relative reutilization of NE phospholipids during the G_2–M–G_1 transition. As in our work with [^{3}H]leucine-labeled NE, we initially followed the dilution of label at two time points within the cell cycle, the so-called G_2/M and M/G_1 stages of the cell cycle (Table V).

CHO cell cultures were grown for five generations in medium supplemented with 0.1 μCi/mL [^{3}H]choline and then synchronized in complete medium containing 0.1 μCi/mL [^{3}H]choline. Five hours after release from HU (i.e., at the G_2/M transition) the labeled culture was harvested, washed twice with PBS, and divided into equal aliquots. G_2/M NE were prepared from one aliquot while the other aliquot was returned to culture in complete F-10 medium without [^{3}H]-choline but containing 0.7 mg/L cold choline. Following division (i.e., 9 h after release from HU, see Figure 5), M/G_1 NE were isolated and chloroform–methanol extracts were prepared from both preparations. The specific activities of the G_2/M NE and the M/G_1 NE phospholipids (counts per minute per microgram of lipid phosphorus) were determined and compared (Table V).

The simplest interpretation of the data in Table V would suggest that approximately 65% of the M/G_1 NE phospholipid was present in the cell prior to mitosis while 35% of the M/G_1 phospholipid was synthesized during the G_2–M–G_1 transition. Reutilization of label is not considered in these data but will be discussed below.

Dilution of [^{32}P]Orthophosphate-Labeled NE Phospholipid. Since choline labels a specific phospholipid which could conceivably behave differently than the remainder of the NE phospholipid, we felt it important to determine the dilution of total NE phospholipid. To do this, we chose to label cells to a constant specific activity with [^{32}P]orthophosphate. In

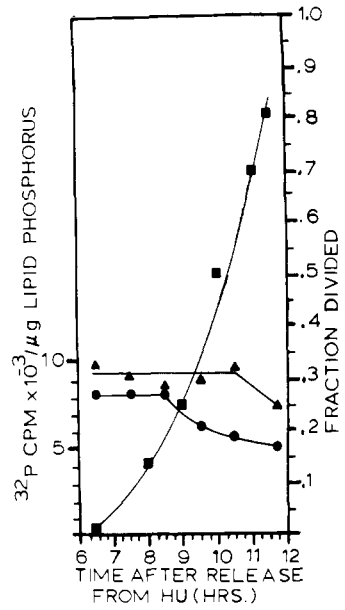

FIGURE 9: Dilution of ^{32}P-labeled NE phospholipid. A suspension culture was grown to stationary phase in the presence of 0.4 μCi/mL [^{32}P]orthophosphate and synchronized in medium containing 0.4 μCi/mL [^{32}P]orthophosphate. 6.5 h after release from HU NE were prepared from an aliquot of cells. The remaining cells were washed twice with PBS and returned to culture in complete medium lacking ^{32}P. NE and homogenate were isolated each hour after removal of ^{32}P, and phospholipid specific activities were determined on each fraction. Fraction of cells divided was determined from four replicate countings on a Levy–Hausser counting chamber. NE specific activity (●); homogenate specific activity (▲); fraction divided (■).

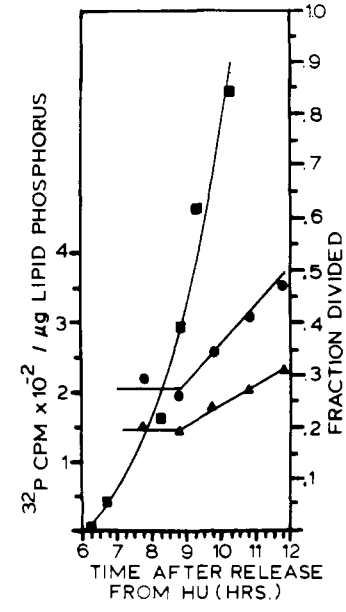

FIGURE 10: Incorporation of ^{32}P into NE phospholipid. A suspension culture was grown to stationary phase and synchronized. 7 h after removal of HU an aliquot of cells was removed and pulsed with 0.8 μCi/mL [^{32}P]orthophosphate for 1 h and NE were isolated. Each hour thereafter (for 6 h) another aliquot of cells was pulsed with 0.8 μCi/mL ^{32}P for 1 h and NE were isolated. Phospholipid specific activity was determined on the NE and homogenate isolated after each pulse. Fraction of cells divided was determined from four replicate countings in a Levy–Hausser counting chamber. NE specific activity (●); homogenate specific activity (▲); fraction divided (■).

these experiments CHO cell cultures were maintained and synchronized in complete F-10 medium supplemented with 0.4 μCi/mL [^{32}P]orthophosphate. As in the previous radiolabel dilution experiments (Tables IV and V, Figure 6), the culture, labeled to a constant specific activity with ^{32}P, was harvested ~6.5 h after release from HU (at the G_2/M transition) and washed twice with PBS. One aliquot of the washed cells was immediately used for preparation of NE while the remaining cells were returned to complete medium lacking ^{32}P and allowed to proceed through M. Each hour after the cells were returned to unlabeled medium, a percentage of the cells was harvested, the homogenate was prepared, and NE were isolated. After the last isolation was completed (11.5 h after release from HU, Figure 9), phospholipid was extracted and the NE phospholipid specific activity (counts per minute per microgram of lipid phosphorus) determined. Figure 9 describes the time course of the change in phospholipid specific activity in cell homogenate and the NE fraction. Clearly the specific activity of both the NE and homogenate remains relatively constant while the majority of the cells are in late G_2 and M and then begins to drop as the cells enter G_1. After 80% of the cells have completed division (Figure 9, 11.5 h after release from HU), the specific activity of the NE is ~65% of the NE phospholipid specific activity before M (Figure 9). The simplest explanation for these data is that 65% of the phospholipid present in NE 12 h after release from HU (i.e., early G_1; see Figure 9) was present in the cell prior to M.

Incorporation of [^{32}P]Orthophosphate into NE Phospholipid. In order to confirm that the dilution of NE phospholipid specific activity, seen in Table V and Figure 9, was due to synthesis of new phospholipid, which was in turn incorporated into NE, cell cultures were grown to stationary phase and then synchronized in complete medium containing no label. Seven hours after release from HU (i.e., at the G_2/M transition, Figure 10), an aliquot of cells was removed from the stock culture and pulsed for 1 h with 0.8 μCi/mL [^{32}P]orthophosphate. After 1 h in ^{32}P, the labeled culture was harvested and both NE and homogenate were prepared. Similarly treated aliquots of cells were pulsed for 1 h, with ^{32}P over the next 5 h. The specific activity of phospholipid was then determined on each homogenate and NE fraction. The time course of ^{32}P incorporation into NE and homogenate phospholipid can be seen in Figure 10. As would have been predicted from Figure 9, after 30–40% of the cells have completed division the homogenate and NE phospholipid specific activity begins to rise. This increase in specific activity of the two fractions remains linear over the remainder of the time course studied. These data (Figure 10) clearly suggest that a major fraction of the phospholipids which are destined to become part of the NE are synthesized and incorporated into the organelle during early G_1.

Rate of Precursor Pool Equilibration. One of the major problems encountered by most investigators interested in determining the rate of turnover or reutilization of radiolabeled cell components has been the contribution of the intracellular radioactive pool to the data obtained after removal of the exogenous label. The size of this intracellular radioactive precursor pool has been shown to be directly related to the rate of internal pool depletion via protein synthesis and the contribution of radiolabeled component breakdown to the pool. That the radiolabeled pool is not immediately depleted by the addition of excess quantities of unlabeled precursors to the external medium has been rigorously demonstrated by Eagle et al. (1959) and more recently by Righetti et al. (1971). Therefore, in order for us to rigorously interpret the label dilution data described throughout the previous experiments, we examined the kinetics of precursor pool equilibration in synchronized cultures which were manipulated in a fashion identical with that already described in Tables IV and V and Figures 6 and 10. Such data are, of course, essential if we are to make more precise estimates of the amount of NE

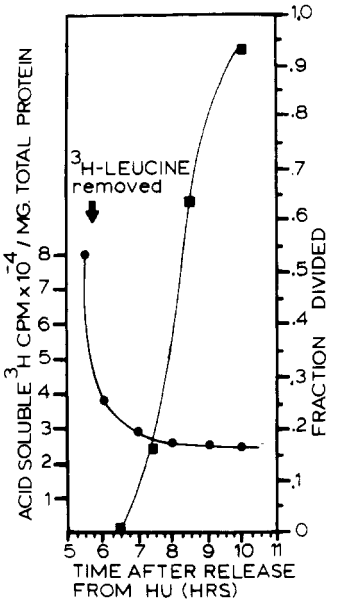

FIGURE 11: [^{3}H]Leucine pool dilution. A suspension culture was grown for five generations in 0.2 μCi/mL [^{3}H]leucine and synchronized in medium containing 0.2 μCi/mL [^{3}H]leucine. 5.5 h after HU removal a sample was taken and acid-soluble [^{3}H]leucine was determined as described under Materials and Methods. The remaining cells were washed twice with PBS, and another sample was taken for determination of Cl_3AcOH-soluble [^{3}H]leucine counts. The washed cells were then returned to unlabeled media, and samples were taken each hour for determination of Cl_3AcOH-soluble [^{3}H]leucine counts. Fraction of cells divided was determined from four replicate countings in a Levy–Hausser counting chamber. Acid-soluble ^{3}H counts per minute per milligram of total protein (●); fraction divided (■).

peptide and phospholipid synthesis (and subsequently dilution of preexisting label) which occurs during the G_2–M–G_1 transition in our synchronized cultures.

In the experiments presented in Figure 11, CHO cells were grown for five generations and synchronized in medium supplemented with 0.2 μCi/mL [^{3}H]leucine. Five hours after release from HU an aliquot of cells was removed from the culture and the soluble [^{3}H]leucine pool size was determined (see Materials and Methods). The remaining cells were then harvested and washed twice with PBS (as was done in all our previously presented dilution experiments). Immediately after the PBS washes (6 h after HU removal) another aliquot of cells was collected and the soluble [^{3}H]leucine pool size was determined. The culture was then returned to complete medium without [^{3}H]leucine. Each hour after returning the cells to unlabeled medium an aliquot of cells was collected for pool determination. The specific activity of the Cl_3AcOH-soluble [^{3}H]leucine counts was subsequently determined and plotted as a function of time (Figure 11). As can be seen in Figure 11, ~55% of the [^{3}H]leucine, which was in the cytoplasmic precursor pool before the cells were harvested, was removed during the initial two PBS washes. Subsequent culturing of the cells in [^{3}H]leucine-free medium reduced the soluble [^{3}H]leucine pool to a specific activity which was ~30% of the specific activity at the initial time point (Figure 11).

By setting the initial, pre-PBS wash, soluble [^{3}H]leucine pool at 100% (initial time point, Figure 11) and then averaging the fractional value of this initial pool (which was measured during the G_2–M–G_1 transition in the absence of label), we have calculated a mean soluble [^{3}H]leucine pool specific activity during the G_2–M–G_1 transition. This calculation yields a mean intracellular leucine pool specific activity equal to 36% (average of two experiments) of the [^{3}H]leucine found in the soluble intracellular pool prior to removal of label from the medium. This mean value represents the percentage of [^{3}H]leucine in the soluble pool of cells which divided in the absence of label, relative to those cells labeled to constant specific activity (i.e., prior to the washes). Since the leucine pool size has been shown to remain constant in HeLa cells during the G_2–M–G_1 phase of the cell cycle (Robbins & Scharff, 1966), we have concluded that the protein synthesized following removal of [^{3}H]leucine from the medium is synthesized with a specific activity that is, on the average, 36% of the specific activity of proteins synthesized when [^{3}H]leucine was maintained in the culture.

Using the formula below (which is derived under Appendix), we have estimated the amount of early G_1 NE protein synthesized during the G_2–M–G_1 transition. We have based these calculations on the ratios of specific activities of G_2/M NE to M/G_1 NE presented in Table IV

$$p = (SA_{G_1} - SA_{G_2})/(SA' - SA_{G_2})$$

where p = proportion of G_1 NE synthesized during G_2–M–G_1, SA_{G_1} = specific activity of M/G_1 NE, SA_{G_2} = specific activity of G_2/M NE, and SA′ = relative specific activity of pool after label removal. Setting SA_{G_2} equal to unity gives SA_{G_1} a value of 0.74 (Table IV). Substituting these values into the equation, we have

$$p = (0.74 - 1.0)/(0.36 - 1.0) = 0.41$$

Thus, when one takes into account the fact that the soluble pool is not completely depleted of [^{3}H]leucine after removal of [^{3}H]leucine from the media, it becomes clear that 40% of the M/G_1 NE protein was synthesized during the G_2–M–G_1 transition. By simple subtraction then, ~60% of the M/G_1 NE protein must have preexisted M.

We have used the same experimental design and logic to determine the dilution of the ^{32}P-labeled phosphatidic acid pool in order to more precisely estimate NE phospholipid biosynthesis during the G_2–M–G_1 transition. Phosphatidic acid was chosen since it is a precursor of virtually all phospholipids and is not shunted into other pathways (Howard & Howard, 1974; Spector, 1972).

In these experiments (Figure 12) CHO cells were grown for five generations in medium supplemented with 0.4 μCi/mL [^{32}P]orthophosphate and synchronized. Six hours after release from HU (Figure 12) an aliquot of cells was harvested and immediately extracted with chloroform–methanol to give an initial value for phosphatidic acid specific activity. The remaining cells were also harvested and washed twice in PBS, and another aliquot of the cell population was extracted with chloroform–methanol (Figure 12, 6.5 h after HU removal). The remaining cells were returned to complete media free of ^{32}P. As shown in Figure 12, aliquots were harvested from the cell population each hour through the G_2–M–G_1 transition and extracted with chloroform–methanol. The specific activities of phosphatidic acid at each time were determined on the eluted material as described under Materials and Methods.

The rate of decrease in the specific activity of ^{32}P-labeled phosphatidic acid is presented in Figure 12. The arithmetic mean intracellular specific activity during the G_2–M–G_1 transition was calculated to be 38% (average of two experiments) of that found in cells prior to removal of ^{32}P from the media. Calculating from the observed dilution of ^{32}P-labeled NE phospholipid (Table V), we find the proportion of M/G_1 NE phospholipid synthesized during the G_2–M–G_1 transition to be ~0.53.

$$p = (0.67 - 1.0)/(0.38 - 1.0) = 0.53$$

Again by subtraction, we estimate that ~50% of the M/G_1

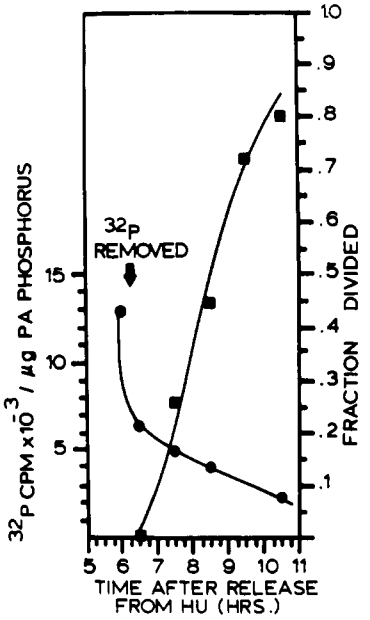

FIGURE 12: [^{32}P]Phosphatidic acid pool dilution. A suspension culture was grown for five generations in 0.4 µCi/mL [^{32}P]orthophosphate and synchronized in medium containing 0.4 µCi/mL [^{32}P]orthophosphate. 5 h after HU removal an aliquot of cells was harvested by centrifugation and phosphatidic acid specific activity was determined. The remaining cells were washed twice with PBS, another aliquot was removed for phosphatidic acid specific activity determination, and the bulk of the cells was returned to unlabeled medium. Aliquots were removed for phosphatidic acid specific activity determination each hour thereafter until 90% of the cells had completed division. Fraction of cells divided was determined from four replicate countings in a Levy–Hausser counting chamber. Phosphatidic acid specific activity (●); fraction divided (■).

NE phospholipid preexisted M.

Changes in Nuclear Surface Area. Since a change in total nuclear surface area might reflect synthesis of NE and at the same time affect the interpretation of our data, we have determined mean nuclear surface areas in a population of cells before and after the G_2–M–G_1 transition (i.e., at 15 and 18 h in Figure 5). Cells were synchronized as described under Materials and Methods, and 5 h after release from HU an aliquot of cells was removed and prepared for sectioning as described under Materials and Methods. After 75% of the cells had completed division (8 h after HU removal), another aliquot of cells was removed and prepared for sectioning. Thick sections were taken from both cell samples, stained, and photographed. Measurements of the surface area of individual nuclei were made by planimetry (Weibel, 1969). The nuclei in both cell populations were found to have a mean nuclear surface area of ~100 µm^2. If we assume that the mean nuclear surface area after the cells have completed mitosis represents the mean surface area of "daughter" nuclei only, then the difference in the G_1 (8 h after release from HU) nuclear surface area relative to the G_2 nuclear surface area will reflect what increase, if any, has occurred during the G_2–M–G_1 transition. If no increase in NE surface area occurred during the G_2–M–G_1 transition, this would be reflected by a mean nuclear surface area in G_1 which is exactly one-half of the G_2 mean nuclear surface area. Our measurements suggest that the total nuclear surface area has doubled during the G_2–M–G_1 transition (G_1 and G_2 mean nuclear surface areas being approximately equal). If such a doubling in nuclear surface area was due entirely to NE synthesis (i.e., no stretching or expansion of available NE), we would expect a 50% dilution of NE protein during the G_2–M–G_1 transition. Our label dilution experiments indicate that the G_1 NE peptides have a specific activity which is 60% of the G_2 NE while the G_1 NE phospholipids have a specific activity which is 50% that of the G_2 NE. Therefore, in our opinion, both our labeling data and our surface area data are, within the limits of resolution, complementary.

Discussion

In this manuscript we have examined the question of whether or not cellular proteins and phospholipids which preexisted the mitotic phase of the CHO cell cycle are used by the CHO cell in the re-formation of the NE which occurs immediately after telophase.

$NaDodSO_4$ gel electrophoresis of NE isolated at various stages of the CHO cell cycle showed no *consistent* differences in peptide composition which could be assigned to any phase of the cell cycle. It is of interest to note that we do not see any cell cycle dependent changes in the peptides migrating between M_r 55 000 and 75 000, as have been reported by Hodge et al. (1977) to occur in the HeLa cell nuclear matrix. In this regard, our data agree with those of Sieber-Blum & Burger (1977), who also did not see cycle-specific changes in CHO cell NE.

In this manuscript, we have presented evidence, obtained primarily from what we have dubbed "label dilution" studies, which strongly suggests that at least 60% of the early G_1 NE protein and at least 50% of the early G_1 NE phospholipid existed in the cell prior to mitotic breakdown and re-formation (i.e., in late G_2). Our data further suggest that the remaining 40% of the early G_1 NE protein and the remaining 50% of the early G_1 NE phospholipid come from de novo synthesis of NE components. Our pulse-label experiments suggest that relatively little NE protein or phospholipid synthesis occurs in M and that the majority of NE synthesis occurs in early G_1. Unfortunately, the degree of synchrony obtainable with such a large number of cells does not allow us to determine whether some NE components are specifically synthesized in the G_2, M, or G_1 phases of the cell cycle. However, our data do indicate that at some point in the G_2–M–G_1 transition all of the NE peptides separable on one-dimensional $NaDodSO_4$–polyacrylamide gel electrophoresis are labeled with [^{3}H]-leucine.

Measurement of nuclear surface area in populations of cells synchronized in G_2 and G_1 indicates that there is no significant difference in the average surface area of the two nuclear populations. These data suggest that the *total* nuclear surface increases twofold between G_2 and early G_1. If we assume that increases in surface area are directly correlated with NE biosynthesis, then these data predict that our label dilution experiments would detect a 50% dilution of NE protein during the time period examined. This is in good agreement with our calculated results of 40% dilution and 60% reutilization of preexisting peptides.

The 60% of the early G_1 NE protein which our data suggest preexisted M, when taken together with our data suggesting a burst of early G_1 NE synthesis, strongly implies that a majority of very early G_1 NE proteins come from preexisting cellular components. Such a finding would be in agreement with earlier studies demonstrating that NE can re-form at the G_1 stage of the cell cycle even if cycloheximide is added to the cells during mitosis in order to block protein synthesis (Sieber-Blum & Burger, 1977). Thus, in our opinion, our data, taken together with the data of others, unequivocally rule out "complete" de novo synthesis of NE protein as being responsible for mitotic reassembly. Unfortunately, we cannot state unequivocally that the early G_1 NE protein which preexisted M resided solely in the G_2 NE. The possibility

clearly exists that G_1 NE components are made prior to M and then stored in some cellular compartment for use in restructuring the NE after division. The most likely organelle for such a "storage function" would be the endoplasmic reticulum. Testing such a possibility must await the availability of NE-specific probes which can be applied to the cells at various phases of the cell cycle to determine whether "NE-specific" components exist in other cellular organelles prior to or during M.

The same problems and interpretation just discussed with regard to NE peptides apply to those experiments which indicate that 50% of the early G_1 NE phospholipid preexisted M. The slightly higher rate of NE phospholipid biosynthesis relative to NE protein biosynthesis over the same G_2–M–G_1 transition may reflect a high turnover rate for phospholipids. Such a higher rate of phospholipid turnover, relative to protein turnover, has been reported in other tissue culture cells (Cunningham, 1972).

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Appendix

Derivation of the Equation for Calculating the Proportion of M/G_1 NE Synthesized de Novo during the G_2–M–G_1 Transition. p = proportion of M/G_1 NE synthesized during the G_2–M–G_1 transition; SA_{G_1} = specific activity of M/G_1 NE; SA_{G_2} = specific activity of G_2/M NE; SA′ = mean intracellular pool specific activity during the G_2–M–G_1 transition, assuming an initial pool specific activity of 1.0.

The NE specific activity at M/G_1 (SA_{G_1}) is equal to some proportion (p) which was synthesized at a diluted pool specific activity (SA′) plus some proportion ($1 - p$) synthesized before label removal at the G_2/M NE specific activity (SA_{G_2}). This can be expressed by

$$SA_{G_1} = p(SA') + (1 - p)SA_{G_2} \quad (1)$$

Rearranging eq 1 gives

$$SA_{G_1} = p(SA') + SA_{G_2} - p(SA_{G_2}) \quad (2)$$

$$SA_{G_1} - SA_{G_2} = p(SA') - p(SA_{G_2}) \quad (3)$$

$$SA_{G_1} - SA_{G_2} = p(SA' - SA_{G_2}) \quad (4)$$

$$(SA_{G_1} - SA_{G_2})/(SA' - SA_{G_2}) = p \quad (5)$$

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Defective Poly(adenosine diphosphoribose) Synthesis in Xeroderma Pigmentosum†

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ABSTRACT: The response of poly(adenosine diphosphoribose) [poly(ADPR)] polymerase to treatment of cells with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine or ultraviolet (UV) irradiation was evaluated in long-term lymphocyte lines derived from three normal donors and from five patients with xeroderma pigmentosum representing complementation groups A-E. Measurements of poly(ADPR) synthesis were conducted in cells that were made permeable to exogenously supplied nucleotides and then incubated with [^{3}H]NAD$^+$ and optimal concentrations of other components required for poly(ADPR) synthesis. All of the lymphocyte cell lines obtained from normal donors showed an increase in poly(ADPR) synthesis in response to treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine or UV irradiation. While the xeroderma pigmentosum cell lines showed increased poly(ADPR) synthesis in response to treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, they were all defective in their poly(ADPR) synthesis response to UV irradiation. In cells from xeroderma pigmentosum complementation group A, the increase in poly(ADPR) synthesis was similar to that seen in normal cells but it occurred at a prolonged interval after UV irradiation. Cells from xeroderma pigmentosum complementation groups B and C showed no increase, and cells from complementation groups D and E showed only slight increases in poly(ADPR) synthesis in response to UV irradiation. When xeroderma pigmentosum cells were UV irradiated and then permeabilized and treated with deoxyribonuclease I, they showed a normal increase in poly(ADPR) synthesis, demonstrating that their ability to synthesize poly(ADPR) in response to deoxyribonucleic acid (DNA) damage was not destroyed by treatment with UV irradiation. The failure of xeroderma pigmentosum cells to demonstrate an increase in poly(ADPR) synthesis in response to UV irradiation is consistent with a role for poly(ADPR) in the DNA repair process.

Poly(ADPR)[1] polymerase is a chromosomal enzyme which uses the ADPR moiety of NAD$^+$ to synthesize the nucleic acid homopolymer poly(adenosine diphosphoribose) (Hayaishi & Ueda, 1977). The activity of this enzyme increases when cells are subjected to a variety of treatments which cause DNA damage (Berger et al., 1979b; Davies et al., 1977; Jacobson & Jacobson, 1978; Miller, 1975). For example, normal human lymphocytes treated with UV irradiation, *N*-acetoxy-*N*-acetyl-2-aminofluorene, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), or bleomycin all showed an abrupt increase in poly(ADPR) synthesis along with an increase in DNA repair synthesis (Berger et al., 1979b). While these studies showed that poly(ADPR) synthesis was stimulated by DNA damage and also that the increase in activity occurred in temporal association with DNA repair (Berger et al., 1979b), they did not demonstrate whether one process was dependent on the other.

To further investigate the relation between poly(ADPR) synthesis and DNA repair, we examined poly(ADPR) synthesis in cells obtained from patients with xeroderma pigmentosum (XP). Such cells are defective in their ability to repair UV-induced DNA damage (Cleaver, 1969; Setlow et al., 1969). They do repair some types of DNA damage such as that caused by MNNG (Cleaver, 1973). We reasoned that if poly(ADPR) synthesis is a component of the DNA repair process, then cells from patients with xeroderma pigmentosum might show a defective poly(ADPR) synthesis response to UV irradiation but a normal response to treatment with MNNG. These studies were conducted with long-term lymphocyte cell lines derived from three normal donors and from five patients with xeroderma pigmentosum representing each of the complementation groups from A to E. Cells from each of the different complementation groups are presumably defective in a different aspect of the repair of UV-induced DNA damage since the defect in DNA repair is corrected in heterocaryons obtained by fusing cells from any two of the different complementation groups (Kraemer et al., 1975). The long-term lymphocyte cell lines were used in order to provide sufficient cells for the studies described. These cell lines from patients with xeroderma pigmentosum have previously been shown to be abnormal in their response to UV irradiation (Andrews et

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[1] Abbreviations used: ADPR, adenosine diphospohoribose; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; NAD, nicotinamide adenine dinucleotide; UV, ultraviolet; XP, xeroderma pigmentosum.

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