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Cadmium Modification of Nucleolar Function and Structure in *Physarum polycephalum*

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Cadmium Modification of Nucleolar Function and Structure in *Physarum polycephalum*. SINA, J. F., AND CHIN, B. (1979). *Toxicol. Appl. Pharmacol.* **50**, 253–259. Exposure of *Physarum* to $CdSO_4$, 1.5×10^{-3} M for 2–3 hr in early S phase (the onset of DNA synthesis and nucleolar reconstruction) resulted in the appearance of ring-shaped nucleoli. We have investigated the effects of cadmium on nucleolar (ribosomal) RNA synthesis during this interval with acrylamide–agarose gel electrophoresis. Cadmium significantly depressed [^{3}H]uridine incorporation into rRNA, with the extent of inhibition increasing with time. The effect of cadmium on RNA synthesis was quantitative, not qualitative: Cadmium depressed the amplitude of individual RNA peaks but not their distribution in RNA profiles. Cadmium exposure did not result in the appearance of new (or altered) rRNA peaks, induce detectable degradation/turnover of RNA, or alter rRNA processing. Surprisingly, no significant difference in rRNA content and distribution was observed in cells treated with cadmium which induced rings and shorter exposures which did not, suggesting that the inhibition of rRNA synthesis and the formation of ring-shaped nucleoli are coincident manifestations of cadmium toxicity at the cellular level but are not causally related.

Cadmium and its compounds are extremely toxic (Fulkerson and Goeller, 1973), and have been shown to be teratogenic (Ferm, 1971; Barr, 1973; Chernoff, 1973), mutagenic (Röhr and Bauchinger, 1969; Murray and Flessel, 1976), and carcinogenic (Gunn *et al.*, 1963; Haddow *et al.*, 1964; Lemen *et al.*, 1976). The literature is replete with epidemiological and clinical investigations of cadmium toxicity, as well as with studies of whole bodies, organs, and tissues. Such work allows us to attribute distinct symptomatology and aberrations to cadmium exposure but does not elucidate the bases of toxicity. This laboratory has therefore focused attention on the smallest integrated unit in biology—the cell—to identify mechanisms of cadmium toxicity.

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The slime mold, *Physarum polycephalum*, is a good model system for toxicological studies at the cellular level. *Physarum* can be cultured as a multinucleate, naturally synchronous cell, from which a number of identical explants can be easily excised (Chin *et al.*, 1972) for studies with toxic substances.

This laboratory has established the nucleus to be an intracellular target for cadmium toxicity. Exposure of *Physarum* to $CdSO_4$ affects a number of basic biological, biochemical, and morphological nuclear events. Mitosis is delayed, and DNA and RNA synthesis are inhibited (B. Chin, unpublished data). Ultrastructural aberrations resulting from cadmium exposure are manifest first in the nucleolus and lead to the formation of ring-shaped nucleoli (Sina and Chin, 1978).

Since an intimate relationship between RNA and the nucleolus has been demon-

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strated in the literature (for reviews, see Busch and Smetana, 1970; Ghosh, 1976), and since many agents which cause aberrations in nucleolar structure also disrupt rRNA synthesis (Busch and Smetana, 1970), it occurred to us that this model provides an opportunity to study the toxicity of cadmium on structure *and* function at the organelle level. Herein we report that cadmium has a specific, quantitative effect on ribosomal (nucleolar) RNA synthesis, and that this biochemical lesion occurs conomitant with the ultrastructural lesion but is not causally related to the development of ring-shaped nucleoli.

METHODS

P. polycephalum, strain M_3CV, was maintained in microplasmodial form in submerged culture in an axenic medium (Chin and Bernstein, 1968) at 23°C. For experiments, the organism was cultured as single-cell plasmodia on the surface of liquid medium (Chin *et al.*, 1972). Incorporation of radioisotopic precursor, preparation of macromolecular fractions, and electron microscopy were performed as previously described (Sina and Chin, 1978).

The RNA extraction technique of Zellweger and Braun (1971) was modified for this study. *Step 1.* Half a plasmodium (~7 cm diameter) was homogenized in 5.7 ml ice-cold buffer [0.5 M NaCl, 0.02 M Na-citrate, 0.01 M Tris, pH 7.4], plus 0.3 ml 20% SDS (w/v), and 0.2 ml diethyl pyrocarbonate (Solymosy *et al.*, 1968) using a Dounce homogenizer with B pestle. The homogenate was shaken with a Vortex agitator for 3 min; 6 ml of ice-cold, buffer-saturated phenol was added; and shaking was continued for 5 min. Phenol, interphase, and aqueous layers were separated by centrifugation in a Sorvall centrifuge (HS-4 swinging bucket rotor) at 10,000*g* × 6 min. The phenol layer containing protein and the interphase containing DNA and RNA were collected together; the aqueous phase containing RNA was collected separately.

Step 2. The aqueous phase from step 1 was extracted twice with 3 ml of cold phenol. In each extraction, the three phases were collected separately. The phenol layer was discarded, and the interphase was saved for further extraction (step 3). An equal volume of 95% ethanol was added to the aqueous phase and the mixture (RNA–ethanol I) was stored at −20°C for step 4.

Step 3. The initial interphase and phenol layers, collected together in step 1, were reextracted with 4 ml of buffer and 2 ml of phenol. After centrifugation, the aqueous phase from this and subsequent extractions were pooled for final extraction of RNA (step 4). The interphase from this extraction was combined with pooled interphases from step 2; 2 ml of buffer and 3 ml of phenol were added, and the mixture was shaken in a water bath at 60°C for 7 min to release RNA in the interphase to the aqueous phase. The mixture was immediately chilled in an ice slurry. After centrifugation, the aqueous phase was pooled for step 4. The interphase was collected separately, 2 ml of buffer and 3 ml of phenol were added, and the mixture was reextracted at 60°C; the aqueous phase from this extraction was also pooled for step 4.

Step 4. The pooled aqueous phases (step 3) were extracted once more with phenol at 4°C. The aqueous phase was collected and an equal volume of 95% ethanol at −20°C was added (RNA–ethanol II). RNA–ethanol I and II were combined; DNA was collected from the ethanol with a glass rod (Marmur, 1961). Less than 1% of RNA label was lost with the DNA. RNA precipitated during overnight storage at −20°C.

Step 5. RNA was collected by centrifugation at 10,000*g* × 30 min, resuspended in 1.0 ml of buffer, and reprecipitated overnight in 6 ml ethanol at −20°C. The efficiency and uniformity of the extraction procedure was monitored by measuring tritium in isolated RNA as a percentage of counts in the initial homogenate; yields from control and cadmium-treated cells were always in the order of 80%.

To maximize the resolution of isolated rRNA, electrophoretic separation was carried out on 2.0% polyacrylamide gels, strengthened with 0.5% agarose. These gels, when poured 18 cm in length, allowed the successful examination of RNA molecules ranging from 40 to 4 S. Agarose–acrylamide gels were made by the method of Loening (1967), as modified by Peacock and Dingman (1968). Stock solutions were: 3× buffer (0.12 M Tris, pH 7.8, 0.06 M Na-acetate, 6.0 mM Na-EDTA); 15% recrystallized acrylamide/0.75% bisacrylamide (w/v); and 6.0% ammonium persulfate (w/v).

Agarose[2] (0.15 g) in 15.8 ml of distilled water was refluxed at 100°C for 15 min and cooled to 40°C. Four milliliters of acrylamide/*N,N'*-methylenebis-acrylamide (Bis) was mixed with 10.0 ml of 3× buffer, the solution was degassed for 30 sec, and 0.01 ml of TEMED (*N,N,N',N'*-tetramethylethylene diamine) was added. The agarose and acrylamide solutions were mixed, and 0.175 ml of freshly prepared 6% ammonium persulfate was added. The mixture was poured immediately into 18 × 0.5 cm (i.d.) glass tubes and allowed to polymerize overnight.

Physarum RNA, (50 μl), suspended in RNA isolation buffer, was mixed with 50 μl 40% sucrose

[2] Sigma, St. Louis, Mo.

(containing bromophenyl blue), and the entire 100-μl sample was layered onto a gel. As an external standard, 10 μl of *Escherichia coli* 16 and 23 S RNA (125 A_{260} units/ml)[3] was mixed with 10 μl of 40% sucrose, layered onto a separate gel, and electrophoresis conducted with gels containing *Physarum* RNA. Electrophoresis of the gels were performed at 5 mA/gel for 3 or 5 hr. For electrophoresis, the same running buffer (0.04 M Tris, pH 7.8, 0.02 M Na-acetate, 2.0 mM Na-EDTA, 0.1% SDS) was used in upper and lower reservoirs. Gels were prerun for 30 min at 5 mA/gel before use. Sample gels were run at 5 mA/gel for 3 hr (to examine molecules ranging from 40 to 4 S) or 5 hr (for greater separation of ribosomal peaks from 40 to ~12 S).

The gel containing *E. coli* standard was removed from the tube, placed in a quartz cuvette, and scanned at 260 nm in a Gilford 240 spectrophotometer equipped with linear transport. Ultraviolet absorption was recorded automatically on a Gilford 6040 recorder. Gels containing *Physarum* RNA were sliced into 2-mm sections, and each slice was incubated overnight in 0.5 ml of NCS tissue solubilizer.[4] Five milliliters of toluene scintillator containing 5.0 g of PPO and 0.5 g of Me_2POPOP per liter was added to each sample; and samples were counted in a Packard liquid scintillation counter to determined counts per minute per slice. Sedimentation coefficients were determined graphically by the method of Richards *et al.* (1965), based on the positions of external (*E. coli*) and internal (*Physarum*) standards.

RESULTS

We have previously reported that a 2- to 3-hr exposure of *P. polycephalum* to 1.5×10^{-3} M $CdSO_4$, initiated with the onset of DNA synthesis and nucleolar reconstruction, resulted in the formation of ring-shaped nucleoli; and that a 4-hr exposure, initiated at the same time, depressed RNA content by 60%, as measured by orcinol determination (Sina and Chin, 1978). The absence of positive uranyl acetate staining in ring nucleoli is indicative of an RNA deficiency in the central zone, suggesting that cadmium interferes with the synthesis, organization, and/or stability of nucleolar RNA. An implied relationship between the effects of cadmium on nucleolar rRNA and the formation of ring nucleoli was studied by examining rRNA profiles from control and cadmium-treated cells.

In these experiments, the label from radioisotopic uridine was restricted to nucleolar RNA by limiting labeling periods to 30 or 60 min: The primary rRNA transcript of *Physarum* (40 S) requires 60 min to be processed to the large ribosomal component (26 S) and transported to the cytoplasm; the small ribosomal component (19 S) requires 20–30 min (Braun *et al.*, 1966; Melera and Rusch, 1973).

RNA profiles developed by gel electrophoresis provide both qualitative and quantitative data. Cadmium, however, inhibits RNA synthesis (Sina and Chin, 1978). In order to accurately compare RNA profiles from control and cadmium-treated cells quantitatively, an internal standard was developed: A plasmodium was prelabeled with [U,^{14}C]uridine[5] for 2 cell cycles and briefly washed to remove adhering isotope. Turnover of ribosomal RNA in the cytoplasm is relatively slow (Rusch, 1970), and the bulk of cellular RNA contained ^{14}C label at the end of the prelabeling period. The experimental design called for cutting the prelabeled plasmodium in half, maintaining one-half as a control, and exposing the other half to cadmium. When [5-^{3}H]uridine[6] was used to label RNA during exposure periods, nucleolar RNA from control and cadmium-treated cells could be compared by adjusting samples to contain equivalent amounts of ^{14}C prelabel.

The validity of the internal standard, however, depends on the stability of cytoplasmic RNA during expsoure to cadmium. To address this point, a plasmodium was prelabeled with [^{14}C]uridine, washed, and cut into replicate disks. Six disks were harvested immediately and macromolecular fractions prepared (zero time). Six disks were incubated on control medium, and six on medium

[3] Miles Laboratories; Elkhart, Ind.

[4] Amersham/Searle, Arlington Heights, Ill.

[5] 0.2 μCi/ml, sp. act. 462 mCi/mmol; New England Nuclear, Boston, Mass.

[6] 30 μCi/ml, sp. act. 30 Ci/mmol; Nuclear Dynamics, Inc., El Monte, Calif.

containing 1.5×10^{-3} M cadmium for 4 hr. At the end of the exposure, the disks were harvested and macromolecular fractions prepared. Degradation of bulk RNA was not observed in cadmium-treated disks: counts per minute per milligram of protein at zero time = 5078 ± 157 (100%); control at 4 hr = 4198 ± 126 (83%); cadmium at 4 hr = 4303 ± 210 (85%). In addition, exposing cells to cadmium did not result in the degradation of individual RNA species. ^{14}C RNA profiles obtained after electrophoresis of RNA from prelabeled cells exposed to control and cadmium-containing medium at early prophase for 1 hr were identical: RNA peaks did not rise, fall, nor shift, nor did background levels increase in cadmium-treated samples, as compared with controls.

The effect of cadmium on ribosomal RNA synthesis was studied directly by examining gel profiles of rRNA under three conditions: cadmium and [^{3}H]uridine from early prophase (0 min) to 30 min; cadmium and [^{3}H]uridine from 0 to 60 min; and cadmium from 0 to 120 min, but [^{3}H]uridine from 60 to 120 min only. Three major rRNA peaks were clearly and reproducibly identified in the profiles: 40 S, the primary transcript; 26 S, the large ribosomal component; and 19 S, the small ribosomal product. In addition, dependent on timing of exposure to label, we observed a small 34 S peak [possibly corresponding to the large rRNA intermediate reported by Davies and Walker (1977)], a 21.5–22.5 S peak [which could correspond to the pre-19 S RNA species (Braun *et al.*, 1966)], and a shoulder on the 26 S peak at ~28 S [corresponding to pre-26 S rRNA (Jacobson and Holt, 1973; Melera and Rusch, 1973)]. In gel profiles within an exposure interval, the same peaks were observed whether the profile was from control or cadmium-treated cells. Electrophoretic profiles of RNA from control and cadmium-treated samples, from 0- to 30-min to 0- to 60-min intervals, were virtually identical. However, a significant quantitative difference was observed over the 60- to 120-min interval (Fig. 1).

Two methods were employed to define this quantitative effect of cadmium on RNA synthesis. First, total counts under the major

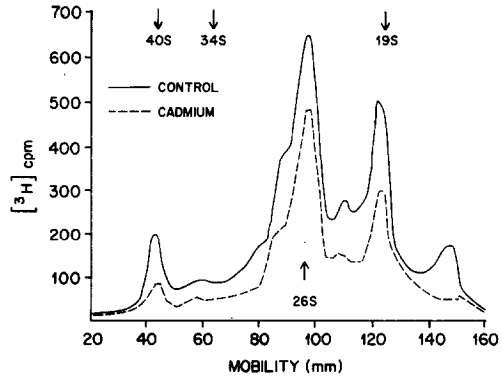

FIG. 1. Representative electrophoretic profiles of RNA separated in 2.0% polyacrylamide gels for 5 hr: RNA from plasmodial halves incubated with and without cadmium, from early prophase to 120 min; [^{3}H]uridine was added to the medium from 60 to 120 min. Profiles corrected to equivalent total ^{14}C cpm (internal standard).

TABLE 1

DISTRIBUTION OF [^{1}H]URIDINE AMONG MAJOR *Physarum* rRNA PEAKS[a]

Labeling interval (min)		^{3}H cpm/peak (% total cpm) 40 S	26+28 S	19 S
0–30	C[b]	114 (7)	997 (61)	515 (32)
	P[b]	55 (4)	792 (62)	416 (33)
0–60	C	70 (4)	1170 (66)	519 (30)
	P	67 (4)	1143 (71)	440 (25)
60–120	C	260 (6)	2244 (58)	1382 (36)
	P	39 (2)	1150 (66)	563 (41)

[a] Tritiated counts under each major rRNA peak were tabulated from gel electrophoretic profiles. These are expressed as actual counts/peak and as a percentage (in parentheses) of the total counts within each sample for three representative experiments. Note that no significant shift in distribution of label among the peaks is apparent; the approximate proportionality of label among RNA peaks in eight separate experiments (both control and experimental) was 40 S, 4 ± 2%; 26+28 S, 65+5%; 19 S, 30 ± 4%.

[b] C: RNA from control cells: P: RNA from cadmium-treated cells.

ribosomal peaks of cadmium-treated samples were tallied and compared to counts/peak in corresponding control profiles. Representative data for each experimental interval are shown in Table 1. (Each experiment was reproduced two or three times with similar results.) Ribosomal RNA synthesis was inhibited significantly in the second hour of S, but only slightly in the first; a 10–20% decrease in total counts in rRNA profiles was seen from 0 to 60 min, while a 50% decrease was observed from 60 to 120 min.

Second, we examined incorporation of isotope into total RNA (i.e., tRNA, mRNA, and rRNA). In each experiment, before RNA was extracted, small aliquots of control and cadmium homogenates were removed, acid-precipitable fractions were prepared from these, and specific activities for control and cadmium-exposed samples were measured as [^{3}H]uridine counts per minute per milligram of protein. A progressive depression of incorporation into total RNA was observed in cadmium-treated cells, decreasing 6% from 0 to 30 min, 18% from 0 to 60 min, and 35% from 60 to 120 min. [A decrease in total RNA was previously reported (Sina and Chin, 1978)].

In addition, these data provide insight into the effect of cadmium on processing of the 40 S transcript. In electrophoretic profiles, aberrations in rRNA processing are manifest as new (or shifted) intermediate peaks, or in the accumulation of specific intermediates or products in excess of expected (i.e., proportionate) levels (Granwick, 1975; Wilkinson and Pitot, 1973; Schlessinger *et al.*, 1974; Craig and Perry, 1970). In our RNA profiles, we found the same peaks in experimental and control profiles; nor did we observe any shifts in the position of peaks. Counts per peak for 40, 26+28, and 19 S peaks were calculated as a percentage of the total counts in profiles. (Again, Table 1 provides representative data.) Analysis of data from eight experiments comparing cadmium-treated and control rRNA samples over different time intervals showed no significant differences in distribution of counts, the approximate mean proportionality (±range) in all samples being 40 S : 4±2%; (26+28 S) : 65±5%; 19 S : 30±4%. The uniform distribution in the labeling pattern, with and without exposure to cadmium, suggests that cadmium does not significantly alter the route of RNA processing once the 40 S molecule is transcribed. A reasonable interpretation is that a key disruptive action of cadmium on RNA occurs in the synthesis of ribosomal (as well as total) RNA, rather than on post-transcriptional events.

With regard to the nucleolar lesion, we have shown (Sina and Chin, 1978) that exposure for 1 hr to cadmium is not sufficient to produce ring nucleoli, while a 2-hr exposure is necessary. Therefore, if inhibition of rRNA synthesis is causal for ring nucleoli, we would expect to observe differences between RNA profiles after 1 and 2 hr of cadmium exposures.

Two halves of a plasmodium were exposed to 1.5×10^{-3} M cadmium from MII to MII+1 hr. At MII+1 hr, one-half was exposed to [^{3}H]uridine plus cadmium the

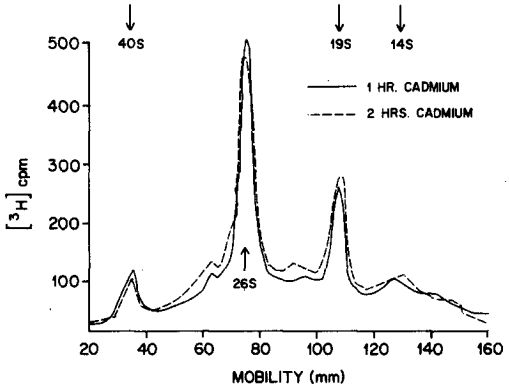

FIG. 2. Electrophoretic separation of RNA in 2.0% polyacrylamide gels for 5 hr: RNA from plasmoidal halves incubated on 1.5×10^{-3} M cadmium, from early prophase to 60 min; one-half was then incubated on [^{3}H]uridine (1 hr cadmium) and the other half was incubated on [^{3}H]uridine plus cadmium (2 hr cadmium) from 60 to 120 min. Profiles corrected to equivalent total ^{14}C cpm (internal standard).

other to label alone. At MII+2 hr, explants were excised from both halves and incubated on fresh medium until the completion of nucleolar reconstruction (MII+3 hr) when the explants were examined by phase microscopy. RNA was extracted from the rest of the material at MII+2 hr. Although the explant which had been exposed to cadmium for 1 hr only contained few, if any, nucleolar rings, while the explant which had been exposed to cadmium for 2 hr contained 70–90% ring nucleoli, RNA profiles from both plasmoidal halves were essentially identical (Fig. 2). Ribosomal RNA synthesis in both 1- and 2-hr cadmium-exposed cells was quantitatively equivalent. Acid-precipitable counts were determined for 1- and 2-hr samples in a separate experiment, and both showed depression of [^{3}H]uridine incorporation to ~30% of control values after the second hour. Furthermore, the distribution of label among the peaks was essentially the same in the two exposures: 1 hr, 40 S = 10%, 26 S = 66%, 19 S = 24%; 2 hr, 40 S = 5%, 26 S = 65%, 19 S = 30%.

DISCUSSION

In this study, we have used the model system, *P. polycephalum*, to advantage both in examining effects of cadmium on nucleolar RNA synthesis in detail, and in correlating this biochemical effect with an ultrastructural lesion reported earlier, ring-shaped nucleoli (Sina and Chin, 1978). Cadmium exposure in our system results in a quantitative decrease in newly synthesized RNA, reflected in a reduction of the primary 40 S transcript (and succeeding rRNA peaks) in electrophoretic profiles and in the depression of [^{3}H]uridine incorporation into acid-precipitable material. RNA synthesis is impaired more markedly during the second hour of exposure than during the first. Furthermore, even when cadmium exposure is terminated, RNA synthesis continues to decline.

The data in the literature imply that a cause–effect relationship exists between changes in RNA synthesis and the formation of ring-shaped nucleoli (Busch and Smetana, 1970; Smetana *et al.*, 1970, 1976; DeBarsy *et al.*, 1974). In contrast, we have demonstrated with cadmium that, although inhibition of RNA synthesis and formation of ring-shaped nucleoli occur concomitantly (Sina and Chin, 1978), they are not causally related. Were they causal, one to the other, the lesions should be inseparable by experimental manipulation. Cadmium exposure for 2 hr from early prophase results in 70–90% ring nucleoli upon completion of reconstruction at 3 hr. Exposure during the first hour of S is necessary for complete expression of the lesion, but not sufficient. If exposure is terminated at 1 hr, ring nucleoli are not formed. If cells are exposed to cadmium for the first hour and incubated in the absence of cadmium for the second hour, 26 and 19 S RNA levels decline to the same level as continuously exposed, ring-forming cells, suggesting a separability of the two lesions. More directly, the fact that no qualitative or quantitative difference is observed between rRNA gel profiles from 2- and 1-hr exposed cells (Fig. 2), even though the former results in formation of ring nucleoli and the latter does not, indicates that inhibition of RNA synthesis is not the critical event in ring formation.

In summary, the inhibition of RNA synthesis by cadmium occurs concomitant to the nucleolar lesion, but is not directly causal to ring formation. These cadmium-induced structural and functional lesions result from impairment of two separate nuclear events.

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