ARGININE DEPRIVATION IN KB CELLS

II. Characterization of the DNA Synthesized during Starvation

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ABSTRACT

DNA synthesis in cells deprived of arginine was examined. Three lines of evidence indicated that tritiated thymidine ([^{3}H]TdR) incorporation in arginine-starved cells was due to replicative rather than repair DNA synthesis. (*a*) When made in the presence of bromodeoxyuridine, the [^{3}H]TdR-labeled DNA sedimented at hybrid density in isopycnic gradients. (*b*) As determined by the diphenylamine reaction, there was a 15% increase in the chemical amount of DNA per culture 30 h after arginine deprivation. (*c*) [^{3}H]TdR incorporation was hydroxyurea-sensitive.

Alkaline velocity sedimentation of the total DNA made during starvation revealed the existence of two distinct size classes: most of the DNA sedimented at a position analogous to that of control DNA, but 40% migrated one-third the distance of the bulk. After arginine restoration, these shorter pieces appeared to be chased into DNA of normal length; thus, one lesion in deprived cultures may cause an arrest in completion of DNA stretches to mature size. These findings, together with results of morphological studies of starved cells, suggest that changes induced by arginine deficiency effect the organization of nucleoproteins. These changes are reversible upon arginine restoration.

In a preceding paper (22) we presented evidence that when the essential amino acid arginine (arg) was withdrawn from an exponentially growing culture of KB cells, these cells ceased to divide but continued to engage in DNA synthesis for at least 36 h (1 1/2 normal cell doubling times) after starvation was instituted. While the rate of tritiated thymidine ([^{3}H]TdR) incorporation into macromolecular products gradually declined to 5% of the initial rate, the fraction of cells incorporating thymidine in autoradiographic preparations remained constant (~40%) throughout the starvation period. Further, cells both initiated and terminated DNA synthesis, because when [^{3}H]TdR was present throughout the starvation period, 80% of the cells accumulated autoradiographic evidence of incorporation by the end of the deprivation period.

Various reports in the literature have shown that in the absence of arg, morphological disorganization of nuclei and fragmentation of chromosomes take place (1, 8, 9, 16). These findings posed the question of whether the observed [^{3}H]TdR incorporation was the result of continued replicative (scheduled) DNA synthesis or was the result, in part or exclusively, of repair of lesions in the nucleoprotein. Here we present data which show that the bulk of incorporation in the absence

of arg is replicative. Degradation of DNA and gross alterations in chromosome structure are also investigated.

MATERIALS AND METHODS

Cells and Experimental Procedure

The established human cell line, KB, was used. Source, stock maintenance, and details of the experimental protocol are described in an accompanying paper (22). Briefly, sparse, exponentially growing KB monolayers were overlaid with minimum essential medium enriched with 5% dialyzed calf serum and lacking arg (arg^-) or containing 0.5 mM arg (arg^+, control). Subsequently, parallel cultures were subjected to experimental manipulation at various times between 0 and 30 h. In some experiments, starvation was reversed by the readdition of arg to give a final concentration of 0.5 mM after 30 h under the starved condition and incubation was continued.

Measurement of DNA Content

The diphenylamine reaction described by Burton (4) for the colorimetric estimation of DNA was used.

Isopycnic Gradient Analysis

DNA was isolated by the method of Marmur (13). 2 ml of 1 × standard saline citrate (0.15 M NaCl and 0.015 M $C_6H_5Na_3O_7$, pH 7.4) containing a 50-μg sample of DNA was adjusted to a density of 1.70 gm/cm^3 with CsCl. Gradients were centrifuged for 64 h at 33,000 rpm in an SW56 rotor. The gradients were collected in 0.1-ml samples by piercing the bottom of the tube. Absorbance at 260 nm was measured with a Gilford spectrophotometer, and fractions were precipitated with trichloroacetic acid (TCA), filtered onto nitrocellulose filters, and assayed for radioactivity in a Nuclear Chicago liquid scintillation spectrometer (Nuclear-Chicago Corp., Des Plaines, Ill.).

Alkaline Sucrose Gradient Analysis

Zonal sedimentation of DNA in alkaline sucrose gradients was performed according to a modification of the method of Doerfler (6). Samples containing 5 × 10^4–1 × 10^5 cells were layered on gradients of 5–20% sucrose and allowed to lyse in 0.5 N NaOH for 2 h at room temperature. The gradients were centrifuged for 135 min at 25,000 rpm in a Spinco SW41 rotor. Approximately 0.25-ml fractions were collected by pumping from the bottom of the tubes directly onto numbered Whatman number 3 MM filter paper circles. The samples were then processed by the method of Champe et al. (5) and counted in a liquid scintillation spectrometer.

Chromosome Preparations

Chromosomes were prepared from KB monolayers by standard karyotyping procedures (14). Slides were stained with Giesma stain after hydrolysis in 1 N HCl at 60°C for 10 min and examined under the microscope at a magnification of × 1,250.

Chemicals and Radioisotopes

[Methyl-^{3}H]thymidine (sp act = Ci/mM) was purchased from New England Nuclear, Boston, Mass. The other reagents used in these experiments were obtained from the following sources: diphenylamine (J. T. Baker Chemical Co., Phillipsburg, N. J.), CsCl (Gallard Schlesinger, Carle Place, N. Y.), bromodeoxyuridine (BrdUrd; Sigma Chemical Co., St. Louis, Mo.), colchicine (K & K Laboratories Inc., Plainview, N. Y.), and hydroxyurea (Nutritional Biochemical Corp., Cleveland, Ohio).

RESULTS

Replicative Nature of the DNA Synthesis

During 30 h of incubation in the absence of the essential amino acid, arg, KB cell division is arrested, but the majority (80%) of the cells engage in detectable DNA synthesis as determined by autoradiography of [^{3}H]TdR incorporation (22). To deduce the effect of arg starvation on cell cycle functions, it was necessary to determine whether this activity represented true replicative (i.e. scheduled) synthesis or enhanced repair incorporation, possibly secondary to nuclear lesion(s) induced by arg deficiency. The thymidine analogue, BrdUrd, if incorporated on a nascent single replicating strand, will create hybrid duplexes of DNA with heavier than normal buoyant density. These can be isolated on isopycnic gradients. Repair incorporation in very small regions throughout the DNA will not generate two distinct density classes.

KB cultures that had been arg-starved for 12 h, in parallel with control cultures, were incubated in medium containing BrdUrd, 20 μg/ml, for 5 h. During the last hour, [^{3}H]TdR, 0.5 μCi/ml was added as a more sensitive marker of newly synthesized DNA. Cultures were harvested and the DNA was extracted and subjected to analysis on isopycnic neutral CsCl gradients. Gradient patterns of control and arg-starved DNA are presented in Fig. 1. In both gradients, the bulk of the DNA sedimented at a buoyant density, η = 1.70 g/cc, characteristic of normal KB cell DNA. In addition, both cell samples contained small, well-separated, heavier peaks of material at $\eta \simeq 1.74$, the reported density of the hybrid product of semiconservative replication (7). The ^{3}H label was largely associated with the more dense frac-

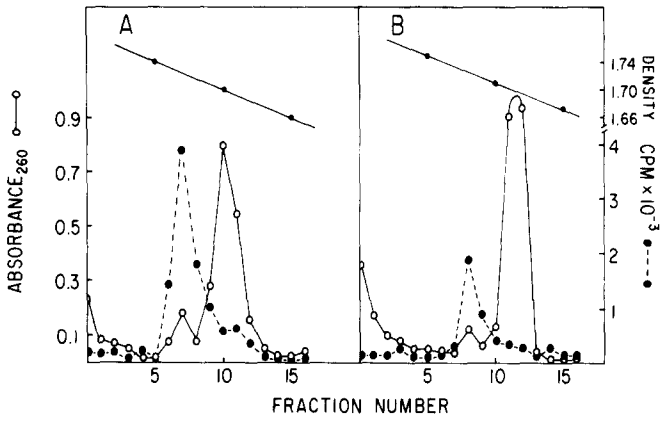

FIGURE 1 Synthesis of hybrid density DNA in the presence of BrdUrd by arg$^-$ cells. Cultures deprived of arg for 12 h (B) and control cultures (A) were incubated with BrdUrd, 20 μg/ml for 5 h. [^{3}H]TdR, 0.5 μCi/ml was added during the final hour (t = 16–17 h). DNA from both sets of cultures was then extracted, layered on CsCl gradients, and centrifuged to equilibrium (see text). Fractions were collected and assayed for absorbance at λ = 260 nm (——) and for acid-insoluble radioactivity (----).

tions: 89 and 85% of the total radioactivity in arg$^+$ and arg$^-$ cells, respectivley, coincided with nascent hybrid material rather than being distributed throughout the DNA, as would be expected in repair processes. It was concluded that the [^{3}H]TdR incorporation observed in arg-starved cells represented replicative, or S phase, DNA synthesis.

In view of the fact that replication continues in the absence of arg, it should be possible to demonstrate net increase in the amount of DNA during the starvation period. Total DNA was quantitated by the diphenylamine method in sample populations of arg$^-$ and fully nourished cultures. These results are presented in Table I. There was a little more than a 100% increase in the DNA content of fully nourished cells between 0 and 30 h (1 1/4 doubling times). In the arg$^-$ cultures the amount of DNA as determined chemically increased by 15–17%. This amount agreed well with a net increase approximated from the decreased rate of [^{3}H]TdR incorporation during arg deprivation (see Fig. 2 in reference 22). The result provides additional evidence for the replicative nature of the [^{3}H]TdR incorporation under arg$^-$ conditions.

Sensitivity to the inhibitor hydroxyurea (HU) was also used to distinguish between replicative and repair synthesis. HU selectively suppresses normal replication but does not inhibit repair synthesis (3). As shown in Table II, [^{3}H]TdR incorporation was reduced by more than 90% in both arg-starved and fully nourished cultures at early (6 h) and late (24 and 30 h) times in the presence of 5 mM HU. Actual HU-resistant incorporation in starved cells was less than in controls. By this criterion as well, the bulk of DNA synthesis throughout starvation would appear to be replicative.

TABLE I

*Chemical Amount of DNA in Complete or Arginine-Deprived KB Cell Cultures**

Experiment	Medium	Hours after starvation	DNA/culture	Increase from 0 h
			μg	%
I	Complete	0	67	
	Complete	30	138	106
	Arg$^-$	30	77	15
II	Complete	0	71	
	Complete	30	149	110
	Arg$^-$	30	83	17

* Determined by the diphenylamine reaction; see Materials and Methods for details. Experiments were initiated with ~5 × 10^6 cells/culture in 90-mm Petri dishes.

Stability of the DNA Synthesized

The observation that replicative synthesis continues in the absence of arg did not preclude the possibility that abnormal moieties were generated under arg$^-$ conditions. One approach to studying this possibility was to denature the DNA and to examine the size of single strands by centrifugation through alkaline sucrose velocity gradients. Be-

cause it was possible that strand breakage might not become evident until after arg restoration, the DNA made during starvation was also examined after reversal. A series of exponentially growing cultures were fed with complete and arg$^-$ media and were labeled with [^{3}H]TdR for 28 h, so that all stable DNA synthesized during the starvation period would be radioactively marked. In those samples to be incubated further, 10^{-3} M unlabeled thymidine was added at t = 28 h to stop radioactive incorporation. At t = 30 h, arg, or an equivalent volume of buffered saline, was added to sets of starved cultures which were then reincubated. Samples were harvested for gradient analysis at t = 28 and 42 h. The partition of counts between the "light" and "heavy" portions of the gradients in two experiments are shown in Table III, and typical gradient patterns are shown in Fig. 2.

TABLE II

*Inhibition of DNA Synthesis by 5 mM Hydroxyurea (HU) as a Function of Time after Arginine Deprivation of KB Cells**

Medium	Hours	[^{3}H]TdR incorporated/culture		Inhibition
		Untreated	HU-treated	
		cpm		*%*
Arg$^-$	6	11,695	932	92
	24	3,020	165	95
	30	2,846	75	97
Complete	6	44,509	1,329	97
	24	27,657	671	99
	30	51,614	608	99

* Cultures in arg$^-$ and complete medium were treated with HU, 5 mM, for 1 h beginning at the indicated times after initiation of the experiment. [^{3}H]TdR, 0.5 μCi/ml, was then added to these and untreated cultures for 1 h. Cultures were harvested, processed, and acid-insoluble ^{3}H-incorporation was determined.

In control cultures, fully nourished and labeled with [^{3}H]TdR for 30 h (panel B), most of the DNA (83%) sedimented in a broad peak in the "heavy" region of the gradient, as is characteristic of mammalian cell DNA after relatively long labeling times. At the top of the gradient there was a small amount of DNA heterogeneous in length. 30 h after transfer of exponential cultures to arg$^-$ medium (panel A), the greater portion of the DNA (68%) sedimented at a "heavy" position, representing DNA molecules of normal chain length. However, roughly 32% of the DNA was distinctly smaller with a modal sedimentation rate one-third that of the bulk. As estimated from the relative sedimentation distances (19), the "light" material would average about $^1/_{16}$ the average heavy or normal DNA size. The pattern for mock-restored cultures, i.e. those in which arg starvation continued up to 42 h (panel C), revealed a further accumulation of smaller-sized pieces amounting to 45% of the total. However, 12 h after arg was restored to cells at t = 30 h (panel D), some smaller material appeared either to have moved again into the larger-sized peak or to have been degraded: the "light" peak now comprised 27% of the total. It appeared that

TABLE III

*Size-Partition of DNA Made during Arginine Deprivation of KB Cells: Distribution of [^{3}H]TdR in Alkaline Sucrose Gradients**

Experiment	Medium	Hours after starvation	Heavy (H)		Light (L)	
			cpm	Proportion H/total	cpm	Proportion L/total
I	Complete	28	22,400	0.83	4,675	0.17
	Arg$^-$	28	9,210	0.68	4,320	0.32
	Arg$^-$	42‡	6,040	0.55	4,910	0.45
	Arg$^-$, restored	30 + 12‡ §	9,510	0.73	3,460	0.27
II	Complete	28	20,918	0.85	3,138	0.15
	Arg$^-$	28	7,949	0.67	2,623	0.33
	Arg$^-$	42‡	5,648	0.55	2,542	0.45
	Arg$^-$, restored	30 + 12‡ §	9,085	0.75	2,271	0.25

* The experimental details are the same as for Fig. 2.

‡ A chase with excess cold thymidine (10^{-3} M) was initiated at t = 28 h and continued until the cultures were harvested.

§ Starvation was terminated at 30 h by the addition of 0.5 mM arg.

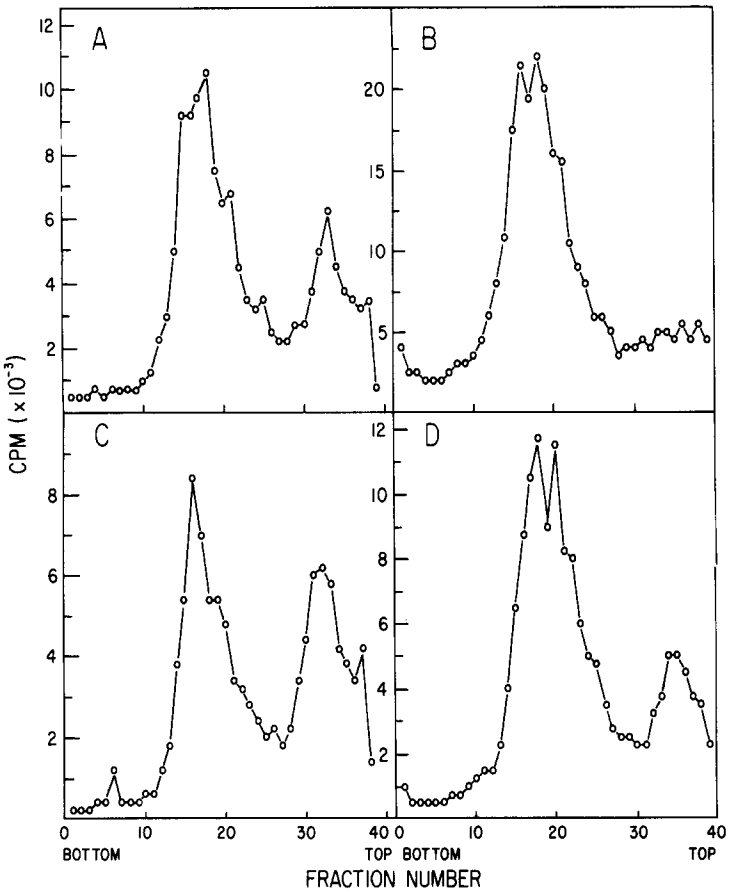

FIGURE 2 Alkaline sucrose rate zonal sedimentation patterns of DNA made during arg deprivation of KB cells. At t = 0 h exponentially growing cells were fed with arg-deprived or complete medium and labeled with [^{3}H]TdR (0.5 μCi/ml). At t = 30 h cells were either (*a*) harvested or (*b*) supplemented with arg or phosphate-buffered saline (PBS) after a 2-h chase with cold thymidine (10^{-3} M). 5×10^4–1×10^5 cell samples were layered on gradients of 5–20% alkaline sucrose. The gradients were centrifuged in a Spinco SW41 rotor at 25,000 rpm for 135 min and harvested as described in Materials and Methods. (panel A) Arg$^-$ medium for 30 h. (panel B) Complete medium for 30 h. (panel C) Arg$^-$ medium for 42 h (mock-restored). (panel D) Arg$^-$ medium for 30 h, restored medium for 12 h.

smaller lengths of DNA were progressively generated during arg starvation. The gradient patterns further suggest, but do not prove, that these fragments may become integrated in DNA molecules of normal size after recovery.

Although velocity gradient patterns show the chain-length distribution of DNA synthesized in the absence of arg, this does not address the possibility that a portion of the DNA is further degraded to acid-soluble pieces, possibly single nucleotides. Comparison of the total acid-precipitable radioactivity in cultures at the end of the labeling period (i.e. at 28 h), and 14 h later, after continued starvation (42 h cultures) or rescue (30- + 12-h cultures), might define the stability of the DNA made during deficiency. The values shown in Table IV represent averages of six replicate determinations on 10^5 cells each. Samples were collected in conjunctions with Experiment I, Table III, and a one-way analysis of variance on the data confirmed an interpretation as follows: approximately 7,600 cpm were incorporated during 28 h of starvation. Some further incorporation occurred between 28 and 42 h; this indicates that the addition of 10^{-3} M unlabeled thymidine did not completely abolish synthesis of

radioactive moieties. (Particularly difficulty in arresting radioactive incorporation seems to be characteristic of arg-starved cultures and must be attributed to complex changes in uptake, metabolism, and pool size in deficient cells.) Nevertheless, there was no difference between the arg- or mock-restored samples (9,175 and 9,345 cpm, respectively). Therefore, although the data are ambiguous with respect to turnover because continued incorporation may mask some degradation, it is clear that restoration of arg does not influence this process and that extensive degradation of previously labeled material does not occur.

TABLE IV

*Stability of the DNA Made during Arginine Deprivation of KB Cells**

Medium	Hours after starvation	[^{3}H]TdR incorporated/10^5 cells
		cpm
Complete	28	178,895
Arg$^-$	28	7,613
Arg$^-$	42‡	9,175
Arg$^-$, restored	30 + 12‡ §	9,345

* Exponentially growing cells were fed with arg-deprived or complete medium at t = 0 h and were labeled with [^{3}H]TdR (2 μCi/ml). Arg was added to two sets of starved cultures at t = 30 h. Cultures were harvested at the times indicated, solubilized in sodium dodecyl sulfate, and prepared for liquid scintillation counting.

‡ A chase with excess cold thymidine (10^{-3} M) was initiated at t = 28 h and continued until the cultures were harvested.

§ Starvation was terminated at 30 h by the addition of 0.5 mM arg.

Chromosome Aberrations

The discovery of smaller pieces of DNA generated during arg deprivation raised the possibility that damage to the DNA might be reflected in chromosome morphology. The types of chromosomes found in two separate experiments are tabulated in Table V. Normal metaphase figures (illustrated in Fig. 3*A*) contained between 70 and 79 chromosomes and showed no evidence of abnormalities such as breaks or condensation of chromosomes. By these criteria, most metaphases in the control, fully nourished preparations were scored as normal. Polyploid cells, occasionally seen in KB stocks and containing between 140 and 150 chromosomes per figure, but not representing true endoreduplicated chromosomes, were simply scored as "not normal." Two types of aberrations were observed: during arg deprivation, strikingly condensed, and therefore

TABLE V

*Abnormalities Observed in Metaphase Figures of KB Cells during Arginine Deprivation or after Arginine Restoration**

Experiment	Medium	Hours after starvation	Metaphases counted	Normal metaphases‡	Aberrant metaphases	
					Condensed	Endoreduplicated
I	Complete	27	50	48	0	0
	Arg$^-$	27	25	6	19	0
	Arg$^-$§	27	25	2	23	0
	Arg$^-$, restored	30 + 3‖	25	20	5	0
	Arg$^-$, restored	30 + 8‖	50	49	0	0
	Arg$^-$, restored	30 + 30‖	50	38	0	12
II	Complete	27	50	49	0	0
	Arg$^-$	27	25	2	23	0
	Arg$^-$§	27	25	3	22	0
	Arg$^-$, restored	30 + 3‖	25	19	6	0
	Arg$^-$, restored	30 + 8‖	50	49	0	0
	Arg$^-$, restored	30 + 30‖	50	35	0	15

* Chromosomes were prepared by standard karyotyping procedure, stained with Giemsa, and examined under a mganification of × 1250; see Materials and Methods for details.

‡ Normal metaphases were not condensed and had a chromosome number between 70–79 chromosomes/figure.

§ Without colchicine.

‖ Starvation was terminated at 30 h by the addition of 0.5 mM arg.

shortened, chromosomes (pictured in Fig. 3*B*); and, after readdition of arg, endoreduplicated figures (shown in Fig. 3*C*). Contrary to previous reports (9), however, no frank chromosome breaks, i.e., actual separations in one or more chromatids, were seen.

Essentially all of the very rare mitotic figures seen 27 h after transfer to arg medium were condensed. To be sure that this aberration was a result of growth in arg-deprived medium and was not simply an effect of colchicine treatment in the nutritionally deficient medium, starved cultures were harvested at t = 27 h without the usual colchicine treatment (see data associated with footnote § in Table V). The results were the same, i.e. the chromosomes were markedly condensed in the absence of arg, whether colchicine was present or not. Proof that this condensation is related to availability of arg can be seen if one examines the chromosomes after arg restoration; the morphology of the chromosome returned to normal as early as 3 h after restoration. Thus, as might be expected, these data suggest that limitation of arg does affect the structure of chromosomes.

Finally, 30 h after restoration there was a dramatic appearance of endoreduplicated figures; approximately one-quarter of the metaphase figures examined at this time, when the mitotic index was ~4%, exhibited this abnormality.

DISCUSSION

These studies have shown that the DNA synthesis seen after arg deprivation of KB cells is largely replicative in nature. Other workers have assumed that only repair synthesis can occur in the absence of arg (18), although, to our knowledge, this has never been directly demonstrated. [^{3}H]TdR incorporated during arg starvation is incorporated along with the heavy TdR analogue, BrdUrd, into hybrid molecules of heavier than normal density, and is sensitive to an inhibitor (HU) specific for replicative synthesis. Further, the net amount of DNA in starved cultures increases.

Extensive breakdown of DNA was not observed, but a significant fraction (32–45%) appeared as shorter than average single strands on denaturing velocity gradients. Such pieces could be generated by an abnormal nuclease activity in deficient cells or by enhanced susceptibility to shear during centrifugation. Either might be a consequence of faulty histone metabolism and, hence, abnormal chromatin configuration.

However, the size of this short material (estimated to be ~25–30 S) is similar to that of the intermediate pieces (11) detected after brief periods of [^{3}H]TdR incorporation during normal mammalian DNA replication (12, 15, 17). Those fragments migrate rather quickly (10) into the larger bulk DNA. Accumulation of intermediate-sized DNA under the arg-starved condition may reflect inhibition of processing, e.g., gap filling or ligating such nascent strands. Similar moieties have been observed to accumulate after inhibition of mammalian cells with HU (21). The cultures in that case are blocked in early S (20). In the experiments reported here, the amount of total culture DNA increased only about 16% during a 30-h starvation, although 80% of the cells (22) incorporated [^{3}H]TdR and there was no evidence of depolymerization. This suggests that the majority of arg-starved cells may not advance far into S.

The enhancement during starvation in intermediate-sized single strands of DNA was not reflected in discontinuities at the morphological level. The increased frequency of chromosome or chromatid breaks and exchanges and severely fragmented chromosomes observed by Freed and Schatz (9) were not seen in this study. However, these aberrations may be unique to their system as other workers have explicitly failed to notice an increase in any major chromosome damage after arg deprivation of Syrian hamster (18) and mouse embryo (2) cells. It was obvious in our experiments, however, that availability of arg does effect the structure of chromosomes because of the marked but reversible condensation observed. It is tempting to propose that this is the result of some change in the packaging of nucleoproteins which is rapidly returned to normal when arg becomes available.

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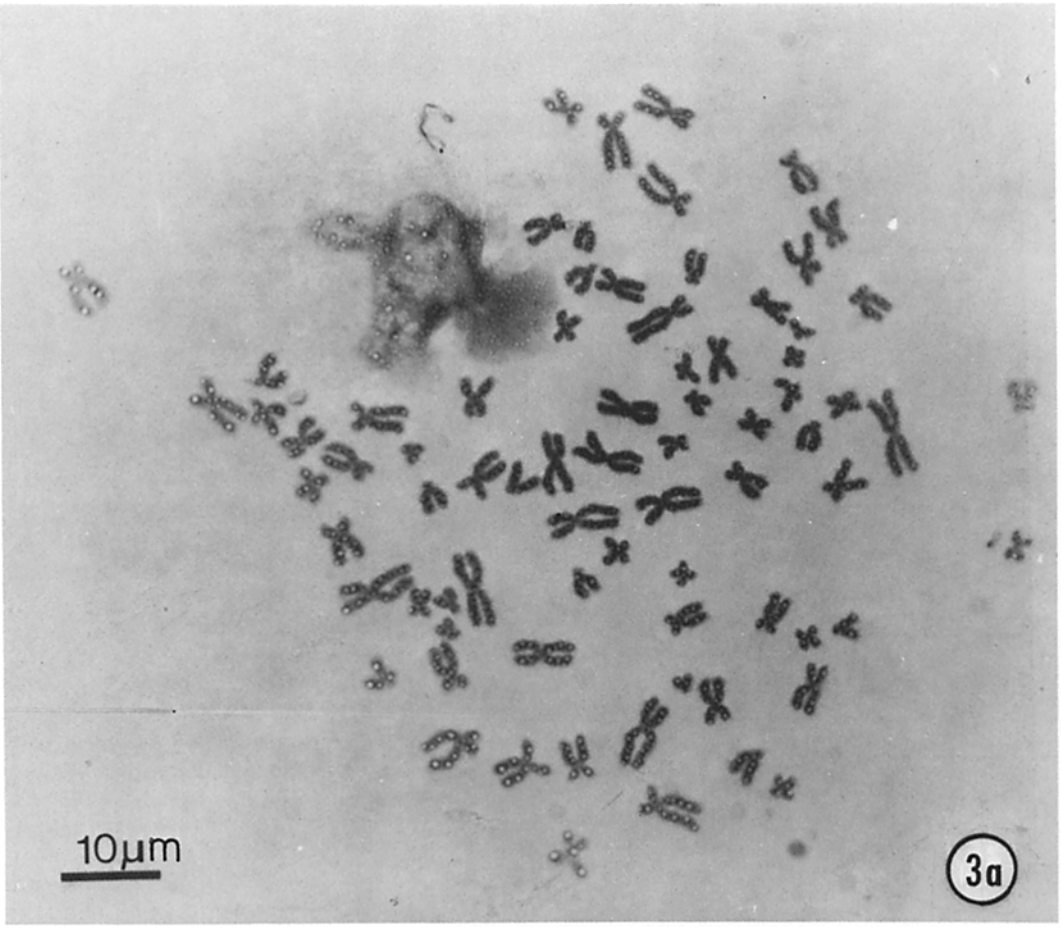
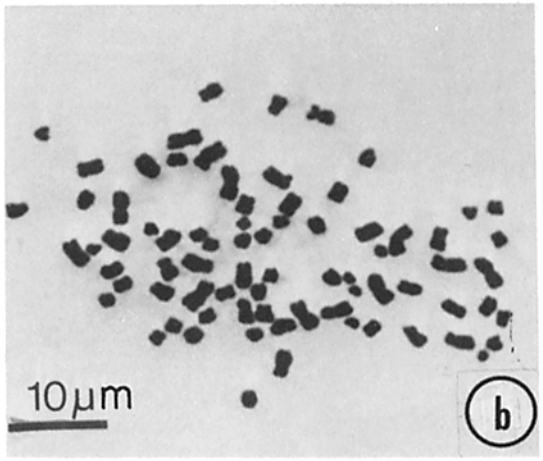


FIGURE 3 Photomicrographs of typical metaphase figures tabulated in Table V. The experimental details are the same as for Table V. (panel *a*) Normal metaphase figure. (panel *b*) Condensed, aberrant metaphase figure (typical appearance in starved cells). (panel *c*) Endoreduplicated, aberrant metaphase figure (anomaly observed after restoration). Note that panels *a* and *b* are reproduced at the same magnification.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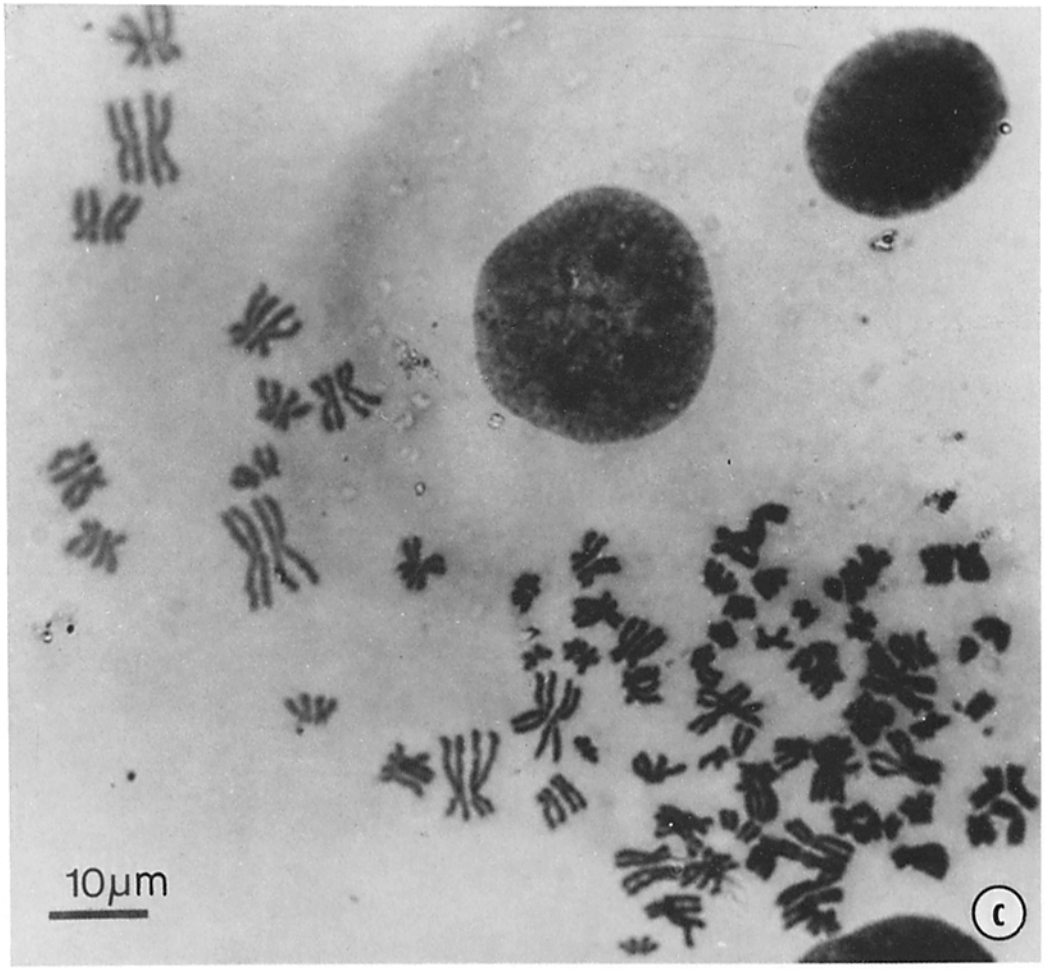
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