AN ELECTRON MICROSCOPE STUDY OF CALF THYMUS NUCLEAR PREPARATIONS ISOLATED IN SUCROSE SOLUTIONS

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ABSTRACT

This work was carried out with the intent of developing a method capable of routinely evaluating calf thymus nuclear preparations with the electron microscope. Examination of small random samples, pre-embedded in agar after fixation with permanganate, were found to give results comparable to those obtained with much larger samples withdrawn randomly from pellets and embedded and sectioned conventionally. Results obtained by this pre-embedding technique with acrolein, osmium tetroxide, or permanganate fixations were equivalent. Calf thymus nuclear preparations isolated in sucrose by the method prevalently used (see 1) are contaminated only slightly with intact cells, to a degree which varies with each preparation. However, intact cells, damaged cells, or nuclei with some cytoplasm constitute together about 30 per cent of the preparation. Particles other than intact cells are not readily distinguishable from one another by light or phase microscope techniques. These preparations can be purified further by centrifuging through a dense sucrose layer. In our hands, however, contamination with some cytoplasm still remains in approximately 10 per cent of the particles. Incubation of the particles prepared without purification procedures, under conditions frequently used, results in the extensive breakdown of particles. Under at least one set of conditions, nuclei are selectively disrupted, leaving primarily damaged cells in the preparation.

INTRODUCTION

Nuclei can be obtained from calf thymus after mild homogenization of the tissue in 0.25 M sucrose solutions with a Waring Blendor, followed by centrifugal sedimentation (see 1). This procedure has proved to be one of the most useful for the bulk isolation of nuclei. For this reason, this preparation has been extensively used in a number of biochemical studies. These works have reported incorporation of precursors into the the nuclear proteins (2, 4, 9), RNA (3, 5, 6, 9, 14), and DNA (7, 9, 14). In addition, evidence has accumulated for the presence of reactions responsible for ATP synthesis (2) and the transport of amino acids (8).

The evidence accumulated in these studies favors the view that the reactions are actually taking place in the nuclei, since the properties of these systems do not seem consistent with a cytoplasmic localization (see 12, 13). For example, the various biochemical systems are dependent on the presence of DNA (*e.g.*, 10, 15, 3, 7) and sodium ion (16), and they have a characteristic sensitivity to metabolic inhibitors (11). Confirmation of these results with preparations purified by centrifugation through sucrose or Ficoll layers (*e.g.*, 8) gives additional weight to this argument. In addition, a number of studies with other preparations have

shown similar results with other tissues where a cytoplasmic contamination should not be of major proportions (*e.g.*, 17–21).

Despite this evidence, the cytological purity of the calf thymus preparation is still in doubt. For example, there is little information about the distribution of impurities in relation to the particles present. This question may be answered at least in part with the electron microscope. Electron microscope studies of this preparation have been carried out previously (3, 22). Unfortunately, these studies have not been reported in full, and the material published is difficult to evaluate. The electron microscope has been known to demonstrate impurities in nuclear preparations unsuspected from light microscope examinations (23). In addition, we were interested in developing a method which would permit a routine evaluation of preparations, at the time at which biochemical or biophysical data are collected under a variety of conditions (*e.g.*, after incubation). It is for these reasons that this study was carried out.

METHODS

The rationale for the use of some of these techniques is discussed below together with the results. The experimental details follow.

Isolation of Nuclei

The method of Stern and Mirsky (26) with subsequent modifications (see 8) has been used as closely as possible. The thymuses of 1- to 6-week-old calves were used. The thymus of a freshly killed calf (stored at 0°C a few minutes after slaughter and used within 1 hour of slaughter) was stripped of capsular tissue and 50 gm were homogenized for 4 minutes in 400 cc of cold sucrose (0.25 M with 0.003 M $CaCl_2$) and 50 cc cold 0.50 M sucrose. The Waring Blendor was used at 1300 rpm (35 v); the material was kept between 0 and 4°C. The homogenate was strained through a double layer of 44 by 36 mesh gauze (Johnson and Johnson) and through no. 15, style 170 flannelette. The filtrate was centrifuged at 600 *g* at 0°C for 10 minutes. The resulting pellet was suspended in 200 cc of 0.25 M sucrose with 0.003 M $CaCl_2$, with a glass rod. The large clumped particles were allowed to settle for 10 minutes and the supernatant was carefully decanted through a double layer of gauze. The preparation was again centrifuged at 600 *g* for 10 minutes. After resuspension of the pellet in a convenient volume, the final preparation was immediately processed for electron microscopy or observed and photographed by the procedure of Barer *et al.* (28). Occasionally, the preparation was also treated in other ways, as described in subsequent sections.

Purification of Nuclei

Results with preparations purified by centrifuging through a sucrose or Ficoll dense layer have been reported (*e.g.*, 8). However, to our knowledge, the experimental details of this purification remain unpublished. For this reason, in these experiments, a purification procedure was used which did not attempt to duplicate those used in other laboratories.[1] Approximately 10 cc of nuclei isolated by the procedure described above and suspended in 0.25 M sucrose with 0.003 M $CaCl_2$ were layered on 30 cc of 1.9 M sucrose. The nuclei were resedimented by centrifuging in a LRA-1 Lourdes refrigerated centrifuge at 36,000 *g* for 60 minutes. The temperature of the chamber was maintained at 0°C.

Incubation

In order to test the possible effect of prolonged incubation and shaking, the preparations isolated without purification were incubated in a medium containing 0.021 M glucose, 0.013 M sodium chloride, 0.026 M sodium phosphate, 0.0015 M calcium chloride, and 0.198 M sucrose at 37°C. The preparations were incubated either for 30 minutes without shaking at pH 7.4, or with shaking for 30 minutes at 110 cycles per minute at pH 7.4 or for 60 minutes at 140 cycles per minute at pH 6.3. These conditions were used in previous studies (see 3, 37). A portion of the incubated preparations invariably clumped. The clump was removed by filtering the preparations through a loose nylon mesh, and the free particles or the clump itself were examined with the electron microscope.

Phase Contrast Microscopy

The preparations were observed and photographed with the phase microscope with positive contrast. In this procedure, 3 drops of 30 per cent bovine albumin (California Corporation for Biochemical Research, C Grade) in 0.6 sodium chloride (at pH 7.4) were added to ½ drop of the preparation (see 28). After 2 to 12 minutes at room temperature the preparation was photographed in a Petroff-Hausser bacterial counter. Since whole cells and nuclei differed in density, it was necessary to have two exposures for each field, one at the top and one at the bottom of the preparation. The relative concentrations of the nuclei and cells were subsequently estimated from photographs at a final magnification of 880 times, where the light particles were considered intact cells and the dark particles, nuclei.

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[1] The method of purification published by McEwen *et al.* (33), which appeared after this paper was submitted for publication, differs significantly from this procedure.

Electron Microscopy

GENERAL TECHNIQUES: A number of techniques were used for the fixation and staining of the preparation. After fixation and most frequently preembedding in agar, the material was dehydrated, embedded in Epon 812 (29), and sectioned with a glass knife in a Porter-Blum microtome to a thickness of about 500 A. The observations were carried out with an RCA EMU-2 electron microscope. Buffered 0.75 per cent sodium permanganate (see 30), buffered 0.5 per cent osmium tetroxide (32), and 10 per cent acrolein in potassium phosphate buffer (27) were used as fixatives. The preparations were fixed in sodium permanganate or osmium tetroxide in the presence of a concentration of 0.125 M sucrose with 0.014 M sodium veronal, 0.014 M sodium acetate, and 0.058 M sodium chloride, brought to pH 7.4 by the addition of hydrochloric acid. The acrolein-fixed preparations were in the presence of 0.1 M potassium phosphate buffer at pH 7.5 and 0.15 sucrose.

For additional contrast, the acrolein-fixed preparations at times were stained for 15 minutes with 1 per cent phosphotungstic acid during dehydration in absolute alcohol (41). In other cases, sections from acrolein-fixed material were stained with 1 per cent osmium tetroxide (see 34), lead acetate (36), and phosphotungstic acid (see 24). Usually, treatment with uranyl acetate (saturated aqueous solution) for 1 hour before dehydration (see 38) preceded the staining of the acrolein-fixed sections. In the osmium tetroxide–fixed material, the sections were stained with lead hydroxide (35). The permanganate-fixed material showed good contrast and required no staining.

HANDLING OF THE PREPARATION: The suspensions were diluted so that 3 ml would yield about 0.1 ml of wet pellet. The suspensions were fixed with chilled media (about 5°C) and left at room temperature for 20 minutes with intermittent shaking. In the case of acrolein fixation, the preparation was treated in this fashion for 10 minutes.

After sedimentation at 600 *g* for 10 minutes, the material was washed with ion-free water. The resedimented specimen was suspended in ion-free water, then warmed to 45°C, and small aliquots were rapidly mixed with a few drops of 2 per cent agar at 45°C. The agar was allowed to harden by rapid cooling. The agar-embedded material was sliced into small, 1-mm cubes and carried through dehydration and embedding procedures (29).

For comparison, occasionally, the preparations were not embedded in agar. In these cases, randomly selected portions of the fixed pellet were embedded independently and without selection of orientation (5 samples).

RESULTS AND DISCUSSION

Rationale for the Choice of Techniques

The determination of the purity of subcellular fractions has always been the preoccupation of the cytochemist involved in the isolation of subcellular particles. A number of procedures have been utilized successfully, depending on the fractions under study.

In the case of nuclei, cytochemical procedures and the electron microscope have frequently been used. In addition, in order to distinguish between nuclei and intact cells, a method has been developed by Barer *et al.* (28). In this method, the particles are suspended in concentrated protein solutions. When viewed with positive phase contrast, the dark particles are considered nuclei, and the light particles whole cells.

In view of the thinness of the cytoplasmic layer in sections of whole thymus (Fig. 1), the cytochemical procedures seem the least promising. The distances between the edges of the cell membrane and the nuclear membrane, measured at randomly selected points in randomly selected portions of the tissue, are tabulated in Table I. The mean distance is 0.29 μ. Although these measurements represent only 6 random samples of the tissue, they do serve as an indicator of these distances. The values recorded vary widely from the mean, as shown by the extremely high standard deviations. It is clear that the ring of cytoplasm is thin. In fact, it is possible that, in a large proportion of the preparation, it might not be resolvable with light microscopy. It is also possible that most intact cells might be indistinguishable from nuclei if the disruptive isolation procedures or the cytochemical methods were to shrink the cytoplasm even minimally. This is a problem of more than academic importance. For example, in our experiments the acrolein-fixed particles were found considerably shrunken, whereas the permanganate-fixed nuclei were found considerably swollen.

The method of Barer *et al.* (28) has been used to evaluate a number of preparations (*e.g.*, 28, 37). Unfortunately, this procedure might be based on an entirely arbitrary principle. Whether the assumption of a difference between the appearance of cells and that of nuclei when suspended in concentrated protein solutions is justified remains largely untested. Some of our results may support this criterion in detecting intact cells (see Table V). Particles which appear as intact cells with the

electron microscope, and which most likely correspond to damaged cells, may at times be indistinguishable from nuclei when viewed with the phase contrast procedure. At any rate, the procedure is not likely to distinguish effectively between nuclei and cells in various degrees of disruption. Disrupted cells might constitute a large fraction of the preparation. In fact, this difficulty is illustrated by our failure to detect with this technique the presence of cytoplasm in damaged cells in the experiments outlined in Table V. However, the method is one of the few available alternatives and, in addition, it seems effective for detecting truly intact cells.

Evaluation of subcellular fractions by means of electron microscopy is beset with a number of difficulties. The procedure must enable one to distinguish clearly between the different materials in the preparation. In addition, the material observed must be a meaningful sample of the preparation.

In most works, osmium tetroxide treatment has been preferred as a general fixative. However, for optimal preservation of nuclear material, it has generally become necessary to use special procedures (see 38). In fact, the fixatives used in this study do not always show an optimal preservation of nuclear contents. At first sight, osmium tetroxide would seem not to be an attractive choice, since it has been known to produce a number of disruptive artifacts in several membrane systems (39, 40). This was found not to be the case in our prepara-

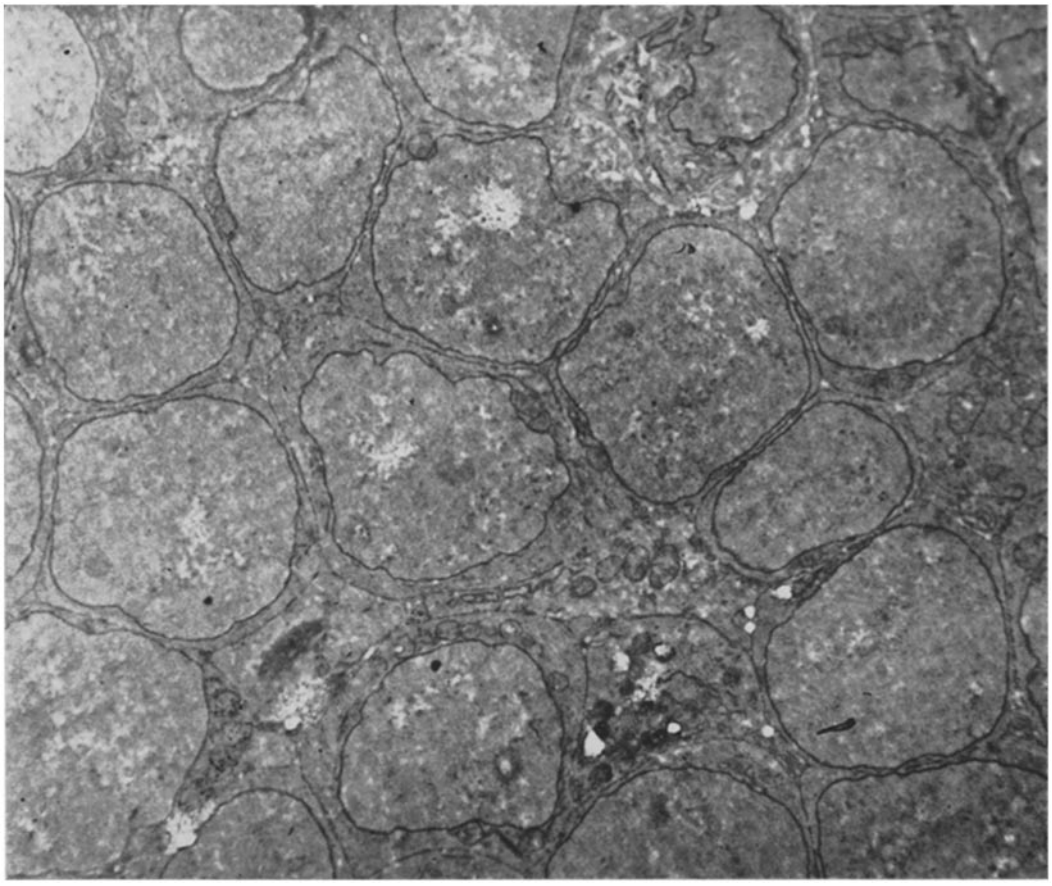


FIGURE 1 Section of whole thymus, permanganate fixation. The section selected is typical of the ones used for the measurements of thickness of the cytoplasmic ring. There is evidence of damage from the sectioning procedure. × 6700.

tions, where it produced less gross disruption than the other two fixatives (see Table III). However, the contrast afforded by osmium tetroxide was frequently found not to be sufficient for most routine counts, even where improved by staining. We have seen that the cytoplasmic ring in sections of the tissue is rather thin. Therefore, it would seem for this reason it has been avoided in similar studies with isolated preparations (see 39). However, in this study, sucrose has been found not to interfere significantly, so long as the concentration is not too high or the time of fixation too prolonged.

TABLE I

Thickness of Cytoplasmic Ring in Sections of Whole Tissue

Specimen	No. of measurements	Mean thickness (μ)	Standard deviation
1	137	0.34	0.27
2	55	0.26	0.26
3	56	0.24	0.13
4	60	0.26	0.15
5	66	0.29	0.24
6	74	0.29	0.21

An electron microscope study with a single fixative is very difficult to evaluate. Therefore, a number of additional procedures were tried. Osmium tetroxide and acrolein were also used (Table II). Lead hydroxide staining of sections was used in conjunction with osmium tetroxide fixation. With acrolein fixation, uranyl acetate treatment usually preceded other stainings where phosphotungstic acid, osmium tetroxide, and lead acetate were used. No significant differences were found in comparisons of the results of the various stains used after acrolein fixation.

A tabulation of the results of osmium tetroxide, permanganate, and acrolein preparations (Table II) shows them in close agreement. The criteria

TABLE II

Comparison of Nuclear Preparations Fixed with Different Fixatives

Experiment	Fixative	% intact cells	% ruptured cells	% nuclei with tabs of cytoplasm	% nuclei with traces of cytoplasm	% intact nuclei	% ruptured nuclei	Total No. of particles
1	OsO_4 (lead hydroxide stain)	20.2 ±1.8	12.2 ±3.0	17.5 ±4.0	10.6 ±2.0	34.7 ±3.6	4.5 ±1.6	245
	$NaMnO_4$	13.7 ±2.9	12.0 ±2.4	16.0 ±1.1	12.0 ±2.0	32.0 ±4.9	14.3 ±2.0	175
2	Acrolein (stained sections)	4.3 ±1.8	12.4 ±1.2	9.4 ±1.9	15.7 ±1.8	50.2 ±11.2	8.0 ±2.6	1073
	$NaMnO_4$	7.8 ±1.6	14.2 ±3.4	15.1 ±4.3	19.8 ±3.6	34.5 ±3.2	8.6 ±1.4	232
3	Acrolein (PTA stained)	1.9 ±0.7	0.0	15.2 ±1.4	30.6 ±1.8	38.3 ±1.7	14.1 ±1.5	376
	$NaMnO_4$	2.4 ±0.7	2.9 ±1.1	19.8 ±2.4	23.6 ±2.9	38.3 ±4.9	13.0 ±2.7	416

most probable that distinguishing between the different kinds of particles (for example, disrupted cells and nuclei with tabs of cytoplasm) would require optimal preservation of the nuclear and cell membranes. It is known that permanganate gives excellent fixation and contrast of membrane systems (*e.g.*, 42). Accordingly, it was the method chosen for this study. Permanganate is known to react with sucrose, the isolation medium used, and used for the classification are discussed in a separate section (see below). The use of small samples was found to be valid where a pre-embedding technique is used (see Table IV).

Since a selective breakdown may alter the proportion of the different particles, it was deemed desirable to check the magnitude of the breakdown of the particles exposed to each fixation procedure. The preparations, after fixing and washing (see

Methods), were sampled in a Petroff-Hausser bacterial chamber and photographed with the light microscope. These results were compared with those obtained with unfixed, fresh nuclei (Table III, control). Counts from the photographs show that in contrast to osmium tetroxide fixation, both permanganate and acrolein fixation disrupt a significant proportion of the particles (Table III). However, the results obtained with the three fixatives (Table II) appear comparable. Since osmium tetroxide did not disrupt a significant proportion of the particles, it is unlikely that the disruption encountered with acrolein or permanganate significantly altered the estimates.

TABLE III

Effect of Fixative on the Stability of the Particles

Typically, 7 or 8 samples of a total of 1500 to 3000 particles were used for each determination. The deviations recorded are standard errors.

Fixative	% control	
	Preparation 1	Preparation 2
Control (fresh, unfixed nuclei)	100.0 ± 7.8	100.0 ± 4.5
$NaMnO_4$	81.0 ± 7.4	66.5 ± 2.0
Acrolein	68.7 ± 5.7	72.8 ± 4.5
OsO_4	—	97.3 ± 5.6

TABLE IV

Comparison of Conventional and Agar–Pre-embedded Preparations

Pre-embedding	Intact whole cells	Ruptured whole cells	Nuclei with tabs of cytoplasm	Nuclei with traces of cytoplasm	Nuclei free of all cytoplasm	Ruptured nuclei	Total No. of particles
	%	%	%	%	%	%	
Agar	1.4	4.2	15.4	20.3	35.3	23.4	286
	±0.6	±0.6	±1.9	±2.1	±1.8	±4.4	
None	2.7	6.1	19.6	19.0	39.0	13.0	673
	±1.0	±1.9	±1.7	±1.5	±4.1	±3.4	

The problem of sampling the preparation for the electron microscope is not without its difficulties. The pellet may be sampled at random (3, 22), or the sample may include all layers of a thin pellet, where each slice will represent a sample of all layers in the pellet (43, 45). Alternatively, a valid aliquot of a preparation may be sampled (see 44). Since nuclear pellets are not firmly packed, it is difficult to withdraw a longitudinal sample representing all layers. A reasonable estimate of the distribution of particles from randomly withdrawn samples or by sectioning a small pellet *in toto* requires either that a very large number of samples be carried through the preparatory procedures or that a very large number of particles be counted. It would be more convenient to proceed with a small but valid sample of the suspension where the particles are randomly distributed in the embedding medium. Any one section, therefore, would contain a random sample of the preparation. If consecutive sections are avoided, it is possible to get from relatively few slices a valid sample of the preparation. For this reason, after fixation of the suspensions the preparations were suspended rapidly in agar at 45°C, after which the agar was immediately allowed to harden. Procedures carried out in this fashion give a reliable estimate, as shown by comparing results obtained by this method with counts from five randomly selected samples without pre-embedding (Table IV). There is some indication that the agar treatment may damage some of the nuclei. The criteria used for these classifications are discussed in the following section.

Classification of the Particles

The counts were carried out after dividing the population of particles into arbitrary morphological groups: intact cells, ruptured cells, nuclei with tabs of cytoplasm, nuclei with traces of cytoplasm, nuclei free of cytoplasm, and fragmented nuclei. The criteria used are obviously subjective and largely self-explanatory. Figs. 2 to 8 illustrate typical particles. Fig. 8 illustrates the survey picture commonly used in the actual counts. However, a total magnification of 8500 times was usually employed. The particles in each morphological group were spot-checked at a higher magnification (about 30,000 times), but no serious discrepancies were encountered.

Usually, duplicate counts of the same preparation are in good agreement. There is some difficulty in distinguishing between closely related groups. For example, counts of intact cells together with ruptured cells are in excellent agreement. However, the counts of either classification alone may differ significantly from those of a duplicate count. This difficulty reflects in part, no doubt, the introduction of a subjective criterion. However, there is also an additional problem. Though a section may not reveal damage in a presumably intact cell, this damage may actually be present in some other area. The technique of Barer *et al.* (28) may throw light on this difficulty. Estimates carried out with this technique reveal a much lower proportion of cells than those shown by the electron microscope (Table V). This discrepancy is particularly serious where a disruptive procedure such as prolonged incubation is carried out (Table V, experiment 4). The most likely explanation of this observation is that the phase contrast technique fails to permit distinguishing between damaged cells and nuclei. This could occur, for example, if a refractive index differential between the particles and the medium could not be maintained by a damaged cell membrane. Accordingly, an estimate of intact cells with the phase contrast technique would appear more realistic, whereas the electron microscope would seem to fail to detect damage in some cells. It is likely, therefore, that the estimates of intact cells obtained with the electron microscope include partially damaged cells.

TABLE V

Comparison of Percentages of Whole Cells Obtained with Procedure of Barer et al. (28) and with Electron Microscopy

Experiment	Phase microscopy		Electron microscopy	
	Whole cells	Total no. of particles	Whole cells	Total no. of particles
	%		%	
1	9.0 ± 1.5	1030	17.5 ± 2.3	420
2	0.7 ± 0.1	1667	1.8 ± 1.0	119
3	2.0 ± 0.6	342	4.9	41
4 Unincubated	4.1 ± 1.3	727	10.3 ± 2.1	407
4 Incubated	7.4 ± 2.0	146	48.0 ± 4.6	102

Evaluation of the Nuclear Preparations

A tabulation of the results obtained from several different preparations shows that they are relatively free of apparently intact cells[2] (Table VI). This is in agreement with previous works where 3 per cent (22, 37) and 4.5 to 7.7 per cent (22) contamination with intact cells was reported. This amount seems to vary considerably from preparation to preparation. If we were to tabulate intact and damaged cells together (intact, ruptured, and with tabs of cytoplasm), they would account for a relatively large portion of the particles in the suspension (29 per cent). By the same token, completely pure nuclei in good morphological state account for a somewhat larger proportion (31 per cent). Except for the tabulations of intact cells, the tabulations under any classification seem

[2] The amount reported is likely to be an overestimate (see Table V and discussion above).

TABLE VI

Summary of All Preparations

Seven experiments. Permanganate-fixed and agar-pre-embedded.
The deviations recorded are standard deviations.

Fraction	Per cent
Apparently intact cells	6.0 ± 4.7
Intact and disrupted cells (intact, ruptured, and with tabs of cytoplasm)	29.4 ± 7.4
All nuclei (with traces of cytoplasm, free of contaminants, or ruptured)	73.3 ± 9.6
Nuclei, apparently intact and free of contaminants	31.5 ± 7.7

to be fairly reproducible from experiment to experiment.

The estimate of the intact and damaged cells is considerably higher than that obtained by Ficq and Errera with the light microscope (37). On the basis of Unna staining, their estimate of the two classifications together is 13 per cent. This discrepancy is not surprising, considering the thinness of the cytoplasmic ring; the electron microscope would be expected to reveal impurities unsuspected with the light microscope.

Purification of the Preparation

Results have been reported for preparations centrifuged through a dense layer of sucrose or Ficoll (*e.g.*, 8). To our knowledge, the details of the method have not been published.[3] For this reason, the method used in these experiments may differ significantly from those used in other laboratories. No effort was made to duplicate the procedures of other workers. In this work, the isolated nuclei were layered on 1.9 M sucrose and subsequently sedimented at 36,000 *g* for 1 hour. The results, shown in Table VII, show that the procedure does bring about a considerable separation. In fact, it is questionable whether there are any intact cells left in the preparation. However, nuclei with some cytoplasm are still present (about 10 per cent). It is conceivable that modifications of this centrifugation procedure may permit a cleaner separation. It should be noted, however, that damaged cells present after the incubation seem to sediment together with the nuclei in the counting chamber (see discussion below) and therefore might not significantly differ in sedimentation properties from pure nuclei. However, our results do not provide an unequivocal answer to this question.

The possibility of purification by centrifugation through a dense layer appears very promising. Our results, unfortunately, reveal a serious morphological deterioration of the nuclei (70 per cent appear ruptured). This breakdown could perhaps be avoided by the use of media other than sucrose. The use of Ficoll or other substances of high molecular weight may provide a suitable alternative. We have not examined such preparations.

[3] See footnote 1.

FIGURE 2 Unusually large intact cell. Permanganate fixation, agar pre-embedding. × 5300.

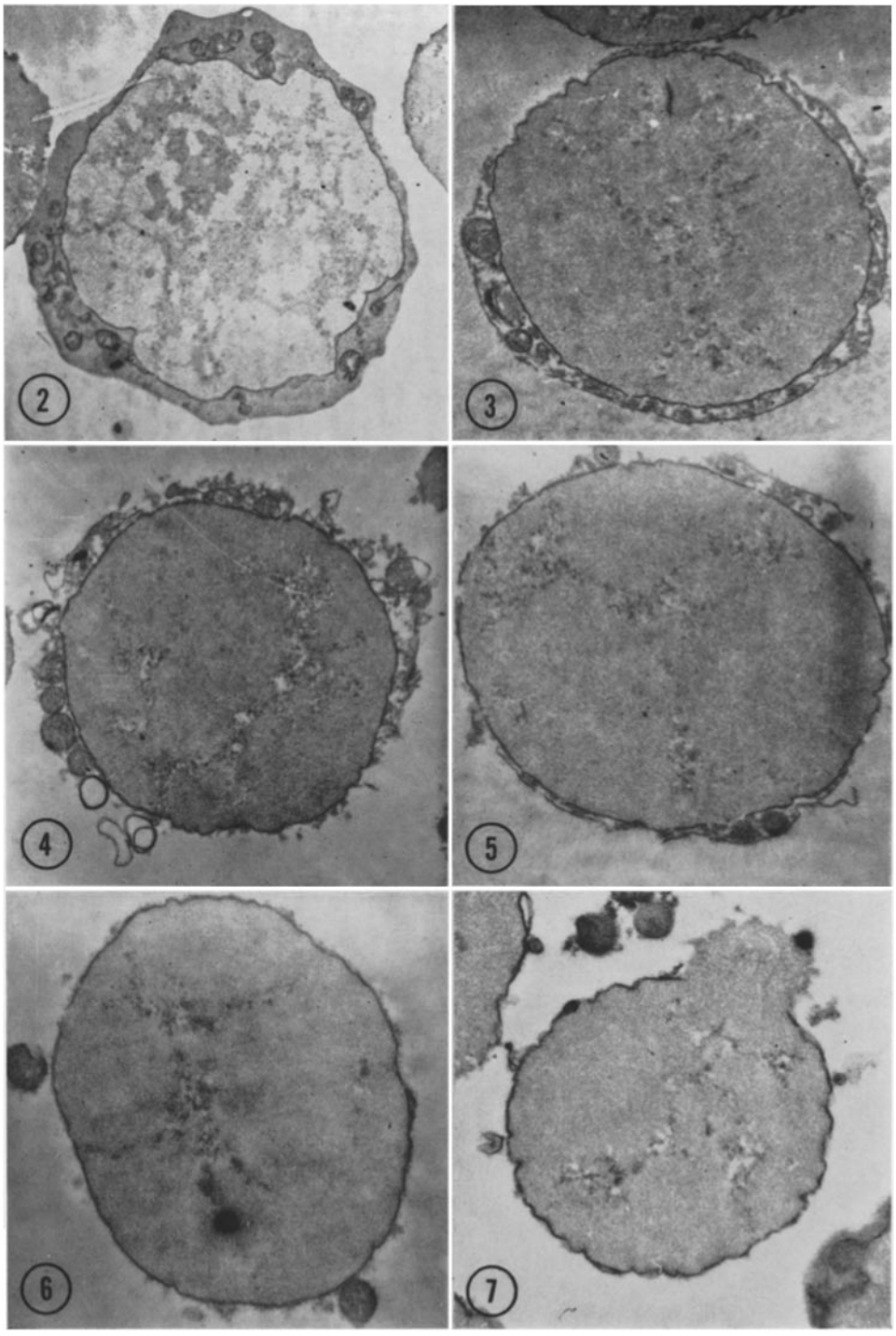
FIGURE 3 Ruptured cell. Permanganate fixation, agar pre-embedding. Although the particle is damaged, a good deal of organization remains, including large portions of the cell membrane. × 7900.

FIGURE 4 Nucleus with tabs of cytoplasm. Permanganate fixation, agar pre-embedding. The particle has little or no cell membrane. A number of cytoplasmic components are present. × 8400.

FIGURE 5 Nucleus with some cytoplasm. Permanganate fixation, agar pre-embedding. Some cytoplasm is still present. Although in this case a good deal of the cell membrane still remains, this specimen was classified as a nucleus with traces of cytoplasm. This is not a completely typical example and it was selected partly to illustrate the difficulties encountered in the classification. × 8700

FIGURE 6 Intact nucleus. Permanganate fixation, agar pre-embedding. Traces of cytoplasm or cytoplasmic components occasionally remain, as in this specimen. × 8800.

FIGURE 7 Ruptured nucleus. Permanganate fixation, agar pre-embedding. The nuclear membrane is usually clearly ruptured. Occasionally, damage is more extensive. × 8300.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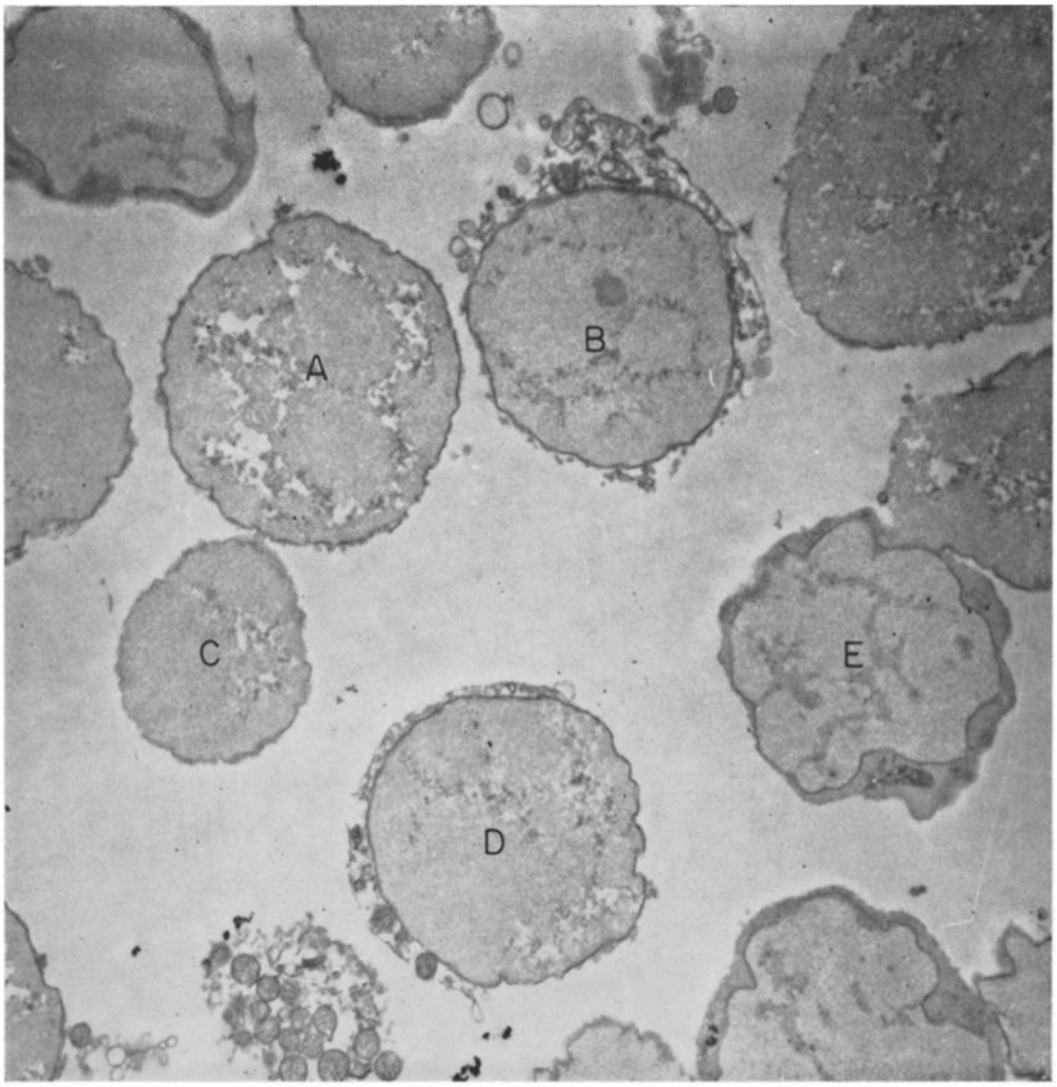

FIGURE 8 Portion of a field at approximately the magnification used for the counts. Permanganate fixation, agar pre-embedding. *A*, intact nucleus. *B*, nucleus with tabs of cytoplasm. *C*, intact nucleus. *D*, nucleus with tabs of cytoplasm. *E*, ruptured whole cell; although little damage is evident, the membrane is not clearly visible and there is some distortion at the cell surface. × 6700.

Effect of Incubation on the Population of Particles

It is possible that the proportion of the different particles in suspension may be altered by the experimental conditions, which may selectively rupture some of the particles. In order to test this point, samples of the particles, isolated without further purification, were incubated under the various conditions used in other works (*e.g.*, see 3, 37). At the end of the incubation, the preparations were sampled (Table VIII) and compared with the unincubated fresh preparation (the control). Under all incubation conditions, extensive breakdown took place (see Table IX). A large clump of material was invariably present. Although the particles remaining in suspension were usually sampled, this population where tested

TABLE VII

Purification of the Preparation by Centrifugation through a Dense Sucrose Solution

	Intact whole cells	Ruptured whole cells	Nuclei with tabs of cytoplasm	Nuclei with traces of cytoplasm	Nuclei free of cytoplasm	Ruptured nuclei	Total No. of particles
	%	%	%	%	%	%	
Experimental (purified preparation)	0.2 ±0.1	0.0	1.6 ±0.6	8.2 ±1.7	19.9 ±2.4	70.0 ±5.9	437
Control (original preparation)	4.5 ±1.7	4.5 ±1.0	8.6 ±1.8	16.2 ±3.7	45.9 ±5.5	20.3 ±3.0	266

TABLE VIII

Comparison of Preparation before and after Incubation

Experiment	Incubation	Intact whole cells	Ruptured whole cells	Nuclei with tabs of cytoplasm	Nuclei with traces of cytoplasm	Nuclei free of all cytoplasm	Ruptured nuclei	Total No. of particles
		%	%	%	%	%	%	
1	Before	7.8 ±1.6	14.2 ±3.4	15.1 ±4.3	19.8 ±3.6	34.4 ±3.2	8.6 ±1.4	232
	After:							
	Clump	46.3 ±5.43	18.2 ±4.16	0.8 ±.8	3.3 ±3.3	28.9 ±4.9	2.5 ±1.6	121
	Particles	40.4 ±2.2	29.7 ±3.8	2.7 ±.8	4.3 ±.8	18.3 ±3.2	4.7 ±1.8	300
2	Before	2.4 ±0.6	2.9 ±1.1	19.7 ±2.4	23.5 ±2.9	38.4 ±5.0	13.0 ±2.7	415
	After	45.5 ±6.2	26.8 ±2.4	3.0 ±1.2	2.2 ±.76	16.4 ±1.9	6.0 ±1.2	134
3	Before	10.3 ±2.1	7.1 ±3.2	16.7 ±1.4	20.1 ±2.6	28.5 ±2.3	17.2 ±2.0	407
	After	48.0 ±4.6	30.4 ±3.0	3.9 ±2.6	7.8 ±1.9	7.8 ±2.6	2.0 ±1.0	102

(Table VIII, experiment 1) did not differ significantly from that in the clump. When samples were incubated at pH 7.4 with or without agitation, the particles were not recognizable with the electron microscope. At pH 6.3 with agitation (140 cycles per minute), a meaningful examination with the electron microscope was possible (Table VIII). In this case, intact and partially ruptured cells were predominant (about 70 per cent). This is in sharp contrast with the original non-incubated preparations, which were relatively pure. With the electron microscope, a large proportion of the particles had the appearance of intact cells. However, it is most likely that they were actually damaged. This is attested by examination of the preparation by the method of Barer *et al.* Under these conditions, most of the particles were indistinguishable from nuclei and sedimented to the bottom of the chamber (Table V, experiment 4). As discussed above, it is not likely that the method of Barer *et al.* can effectively discriminate between nuclei and damaged cells.

It would be interesting to ascertain whether this drastic alteration of the proportion of the particles can be accounted for by the breakdown. To test this point, the particles were counted in a Petroff-Hausser bacterial chamber after incubation under equivalent conditions. Since the breakdown

accounts for the disappearance of 79 per cent of the particles (Table IX, experiment 1), it would seem likely that we are indeed dealing with a breakdown which favors the rupture of nuclei.

It is clear from these results that, whenever the nature of the intact particles is critical to the interpretation, extreme caution should be exercised in analyzing the data without information on the final state of the preparation.

The presence of such a high concentration of disrupted cells raises the question whether the incorporation of radioactive labels into the constituents of the preparation, reported in other works, represents a true nuclear incorporation. There are a number of arguments against the conclusion that the incorporation is not truly nuclear. These stem from the properties of the system; its biochemical behavior is such as to make this interpretation highly unlikely. These arguments have been presented in a number of reviews (see 12, 13), and they are summarized in the introductory section of this paper.

However, it should be recognized that there is little direct evidence that this incorporation is nuclear. For example, three radioautographic studies have been carried out on the incorporation or uptake of amino acids into nuclei (see 3, 8, 37). Incorporation by "all the components of the suspension" has been reported in one study (3), and by many of the particles in another (8). Only one study reports the data in detail (37). In this latter work, the nuclei were isolated by the same procedure. From these results, it would appear that a maximum of about 60 per cent of the particles (usually after 3 hours of incubation) are labeled and that the preparation is very heterogeneous in relation to its capacity to incorporate. In our preparations incubated under similar conditions, we have found (Table VIII) that the proportion of cells (whether intact or damaged) exceeds this percentage. Therefore, it would seem likely that the direct demonstration of the incorporation of amino acids into thymus nuclei awaits new experiments.

It should be noted that the cytoplasm may have an indirect role in this incorporation. It may,

TABLE IX

Particle Breakdown during Incubation

The deviations recorded are standard errors.

Experiment	Procedure	Concentration of particles (particles/cc)
1	Control	$7.37 \pm 0.91 \times 10^8$
	Incubated at pH 6.3; shaking at 140 cycles/min.	$1.53 \pm 0.02 \times 10^8$
	% breakdown, 79	
2	Control	$19.73 \pm 0.77 \times 10^8$
	Incubated at pH 7.4; shaking at 110 cycles/min.	$1.76 \pm 0.22 \times 10^8$
	% breakdown, 91	
	Incubated at pH 7.4; no shaking	$0.55 \pm 0.20 \times 10^8$
	% breakdown, 97	
3	Control	$18.67 \pm 1.12 \times 10^8$
	Incubated at pH 7 4; shaking at 110 cycles/min.	$1.78 \pm 0.07 \times 10^8$
	% breakdown, 91	
	Incubated at pH 7.4; no shaking	$1.74 \pm 0.07 \times 10^8$
	% breakdown, 91	

for example, preserve the integrity of the nucleus rather than actually contribute to its synthetic machinery. A role of cytoplasmic components in the preservation of the viability of nuclei is well recognized from experiments involving transplantation of nuclei (*e.g.*, 25, 31).

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