STUDIES ON THE PERMEABILITY OF CALF THYMUS NUCLEI ISOLATED IN SUCROSE

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ABSTRACT

A study of the permeability of calf thymus nuclei isolated in sucrose was carried out with sucrose-^{14}C, glycerol-^{14}C, and carboxydextran-^{14}C (molecular weight, 60,000–90,000). The results indicate that the nuclei are very permeable to both sucrose and glycerol but they exclude the carboxydextran. Results obtained with other low molecular weight nonelectrolytes (malonamide-^{14}C, erythritol-^{14}C, D-arabinose-^{14}C, and D-mannitol-^{14}C) are in agreement with the view that the nuclei are freely permeable to these molecular species. A sucrose-impermeable space is also present in these preparations and it has been attributed to the presence of intact cells. The high permeability of nuclei to sucrose was confirmed with Ficoll-separated preparations. The possibility of the presence of a substantial particulate space that allows the penetration of dextran cannot be excluded by these experiments, and this space may correspond to damaged nuclei.

INTRODUCTION

The literature on the permeability of nuclei contains a good deal of contradictory information. This information may reflect, in part, differences in the material used or in the experimental manipulations. The presence of apparent pores (2, 14, 17, 27, 33, 53, 54, 58, 69) as seen with the electron microscope argues for a high permeability of nuclei. However, some doubts have been introduced in experiments which indicate that the pores may not be actual patent holes (2, 53, 54). Among these studies, an electron microscope study of rat-thymus nuclei (54) may be of particular relevance for this discussion.

The impermeability of nuclei to at least some cytoplasmic components is suggested by their apparent osmotic behavior in intact cells (19, 64). In fact, in amphibian oocytes, the nuclei shrink in response to the injection into the cytoplasm of polyvinyl pyrrolidinone and bovine serum albumin (34). Direct evidence on the permeability of nuclei can be obtained by the use of molecular or colloidal probes. Electron microscope observations in ameba injected with colloidal gold particles coated with polyvinyl pyrrolidinone and subsequently fixed indicate that, when small enough, these particles penetrate the nuclei (27, 28). The distribution of the particles within the cell suggests that particles above a critical size (120–145 A) are almost completely excluded from the nuclei whereas smaller particles are admitted in varying degrees (28). The appearance of particles in the annuli has been suggested to reflect possibly a passage through these channels (27, 28). In addition to these observations, γ-globulin (molecular weight, 165,000) labeled with fluorescein (see reference 63) has been found to be excluded from the nuclei of *Cecropia* moth oocytes when it is injected into the cytoplasm (29).

Experiments carried out with isolated amphibian oocytes generally agree with the idea that isolated nuclei are impermeable to macromolecules of a molecular weight of the order of about 50,000 or above (13, 32, 37, 49). However, this is not always the case since a permeability to beef hemoglobin has been claimed for the nuclei of frog oocytes (35). The distribution of hemoglobin in nucleated erythrocytes (25) at rest and during hemolysis supports the concept of a high nuclear permeability. The susceptibility of isolated nuclei to the action of enzymes such as RNase and DNase or to the activity of nucleic acids, histones, and protamines (7, 8, 10) also argues for a high permeability to at least some macromolecules. In addition, techniques making use of fluorescein-labeled antibodies demonstrated the appearance of egg and bovine serum albumin as well as γ-globulin inside and at the periphery of nuclei when these proteins were injected into mice (23). Experiments with isolated *Chaetopterus* oocyte nuclei (52) in which the nuclear diameters or the optical path were followed with the interference microscope indicate that, in this system, bovine serum albumin (molecular weight, 56,000) penetrates nuclei readily.

Most works dealing with nuclei isolated from amphibian oocytes show that nuclei have an apparent high permeability to low molecular weight substances such as inorganic salts and sucrose, a view supported by the absence of osmotic behavior in isolated oocyte (13, 32, 37, 49) or rat-liver nuclei (11) in media of low molecular weight solutes. In accordance with this view, equilibration of the nucleus with radioactive sodium seems to occur rapidly in either frog eggs (1) or rat liver (43) in vivo. However, low permeability of isolated mammalian somatic nuclei to substances of low molecular weight, such as Krebs cycle intermediates (51) and amino acids (5), and the presence of active transport mechanisms have been reported (45, 46), i.e. concentration against a gradient which implies the necessity of a semipermeable membrane to avoid an energetically prohibitive backflow. In addition, the accumulation of ions, particularly Na^+, has been shown in several systems (1, 43, 55). Whether this constitutes a passive distribution (brought about by a Donnan effect or binding) or the consequence of active transport, still remains unresolved (see reference 66).

A completely different approach is based on the electrical properties of nuclear membrane measured *in situ* with microelectrodes. It reveals a significant resistance (1–10 ohms cm^2) in the salivary glands of *Drosophila*, chironomids, and sciarids (see reference 47). Although this resistance is much lower than that of most cell membranes, it is much higher than that of the cytoplasm or the nucleoplasm. In the case of the nuclear membrane of frog oocytes and of various marine oocytes (39) the resistance is negligible. In insect nuclei, a potential difference of 2–15 mv between the cytoplasm and the nucleoplasm was also measured.

It is rather difficult to resolve most of these questions from the existing data. It is possible that the nuclear membranes may be labile or that the permeability is a variable function of physiological state, age, stage of development of the cell, or the nature of the tissue from which the nucleus is derived. Experiments carried out with isolated nuclei are particularly open to criticism in relation to the possible lability of the nuclear membrane. Although it may correspond to a simple colloid-osmotic function, the role of the cytoplasm in maintaining the viability of nuclei is well recognized (15, 20). Since the role of the nuclear membrane may be fundamental to the physiology of the cell (note for example the lability of the cell when the nuclear membrane is punctured; references 18, 42, 59), this question deserves a critical reexamination in the laboratory; this reexamination may nevertheless not be definitive. The present report is a modest attempt in this direction.

In this work, calf thymus nuclear preparations were examined with several probe molecules. Evidence is presented that indicates that glycerol-^{14}C and sucrose-^{14}C penetrate readily calf thymus nuclei isolated in sucrose, whereas carboxydextran-^{14}C (molecular weight, 60,000–90,000) does not. In addition, the results are consistent with the conclusion that calf thymus nuclei isolated in sucrose without further purification can be heavily contaminated with cells to an extent hitherto unsuspected from direct electron microscopical observations.

MATERIALS AND METHODS

Isolation of Calf Thymus Nuclei

The methods used for the isolation have been previously described (67). The details of the procedure used in our experiments have been described in our previous paper (41). In this work, thymus from

approximately 8-wk-old calves was used. The tissues were taken from animals within an hour of slaughter. Unless specified, the particles were not purified on a gradient. This method was used rather than more recent procedures, e.g. those of reference 4, as a way of avoiding the use of hypotonic solutions or detergents which could conceivably interfere with the properties of the nuclear membrane. Ficoll gradients were not used as a routine procedure since it has been reported that Ficoll interferes with nuclear metabolism (51). The final pellets were washed with 200 cc of the incubation medium used throughout this study, solution 1. Solution 1 was comprised of 0.40 osmolal sucrose, 0.02 M tris buffer, 5 mM $CaCl_2$, and 5 mM $MgCl_2$, pH adjusted to 7.4 with HCl. After another 10 min, 600 *g* centrifugation the pellets were resuspended in 100 cc of solution 1 which gave a concentration of about 10^8–10^9 particles per cubic centimeter. This was the final thymus suspension used.

Measurement of Spaces

As a general method, ^{14}C-labeled substances were used to measure the particle spaces (New England Nuclear Corp., Boston, Mass.). On the basis of osmotic measurements and Sephadex separation at room temperature, the supplier claims a molecular weight of 60,000–90,000 for the carboxydextran-^{14}C. A determination of molecular weight, at 0°C and in the sucrose medium used in these experiments, was carried out with Sephadex 200 (see reference 70). The results demonstrated a molecular weight within the limits of 75,000 and 100,000.

In each experiment equal aliquots (2 or 3 cc) of the thymus suspension in solution 1 were dispensed into 12-cc cellulose-nitrate centrifuge tubes. All the samples were then incubated at 0°C on ice and shaken on a Dubnoff shaker (130 cycles/min) to insure complete mixing during the permeability process. When not specified, the glycerol-^{14}C distribution was measured after 5 hr in the presence of the isotope, and the sucrose-^{14}C distribution was measured after 10 min (see Fig. 1). At the appropriate times 50 μl of the isotope solution was added to the proper tube. Generally, a 50–200-fold excess of nonradioactive penetrant was added to the suspension to saturate possible binding sites which may have existed in or on the particles. Since no unlabeled carboxydextran was available, it was not added to the ^{14}C form. The incubation time of the different aliquots was exactly the same. The time during which the aliquots were exposed to the radioactive compound was varied. This was accomplished by incubating all aliquots simultaneously but adding the label at zero time or at the appropriate time thereafter. In this way, all the tubes were shaken for equal lengths of time and the possibility of having greater particle damage in the tubes shaken for longer periods of time was avoided.

At the end of the incubation period all the tubes were centrifuged together at 12,800 *g* for 5 min in a Lourdes LRA centrifuge (Lourdes Instrument Corp., Brooklyn, N. Y.) so that the suspended particles could be packed into a compact pellet. Immediately after the centrifugation the supernatant was poured off and saved for counting. The portion of the cellulose nitrate centrifuge tube not adjacent to the pellet was cut off and discarded. The bottom of the remaining portion of the tube was wiped dry, and the excess supernatant on the surface of the pellet was removed by capillarity with tissue paper. The pellet with the remaining portion of the tube was placed in another 12 cc cellulose nitrate centrifuge tube to which was added exactly 1.00 cc of 15% (w/w) trichloroacetic acid or 2 N hydrochloric acid to extract the radioactive substances. The material was then resuspended with vigorous shaking on a test tube mixer (Cyclo-Mixer, Clay-Adams, Inc., New York). After overnight extraction, the extract was centrifuged, and the supernatant was counted for radioactivity. Within experimental error, the radioactivity remaining in this last pellet was accounted for quantitatively by the trapped water.

Counting of Radioactivity

In the earlier experiments the radioactivities of the pellet acid extract and supernatant were counted with a Nuclear-Chicago Corporation (Des Plaines, Ill.) gas-flow counter, model D-47 attached to an Ultrascaler. Later, the counts were done with a Packard Tri-Carb Instrument Company (Downer's Grove, Ill.) liquid scintillation spectrometer, model 3003. No significant difference was found between the two sets of results.

With the gas-flow counter a 2.1 cm diameter filter paper disc on a flat aluminum planchet was impregnated with 50 μl of the radioactive solution to be counted. The disc was counted within 30 sec of impregnation as a way of avoiding differences in counts due to drying. Each sample was counted for 5 min.

For the samples which were counted on the liquid scintillation spectrometer, the 50 μl sample was dispensed into 10 cc of scintillation counting fluid in a screw-cap liquid scintillation counting vial. The scintillation counting fluid was made up of 1,200 cc of toluene, 1,200 cc of methyl cellosolve (2-methoxyethanol), and 100 cc of Liquifluor (New England Nuclear Corp.). The Liquifluor contained 100 g of 2,5-diphenyloxazole (POP), 1.25 g of *p*-bis [2-(5-phenyloxazlyl)]-benzene (POPOP) in 1 liter of toluene. The samples were counted for 2 min on two channels simultaneously to check for the quenching error by the method of ratios. This error was found

to be insignificantly small, so no correction was necessary.

Calculation of Isotope Space

The glycerol spaces were calculated by using the dilution principle: $V_i \times C_i = V_i' \times C_i'$. V_i and C_i were the isotope space and concentration in the pellet. $V_i' = V_i + 1$. Assuming equilibration, C_i equals C_o where C_o is the supernatant concentration.

$$\text{Therefore, } V_i = \frac{C_i' (V_i + 1)}{C_o}.$$

In this study, the ratio (R) of the isotope concentration of the extract (counts per milliliter) to the isotope concentration in the supernatant (counts per milliliter) is frequently used in the discussion of the results. It corresponds to the relative proportion of the pellet occupied by the isotope. As discussed later, the glycerol space corresponds to the total fluid volume. The ratio of the R value of a substance to the R value for glycerol yields directly the proportion of the pellet penetrated.

Electron Microscopy

The thymus suspensions analyzed with the electron microscope were treated in a manner similar to that of Kodama and Tedeschi (41). The suspension was first diluted with solution 1 to a concentration in which 3 cc of suspension contained about 0.1 cc of wet pellet. To 3 cc of the suspension were added 3 cc of fixative, both chilled to 0–4°C. The two fixatives used were osmium tetroxide and sodium permanganate. Both were made at 4% strength in Palade's medium (57) with the pH adjusted to 7.4. The final fixative strength was 2%. The fixation was allowed to proceed in ice for 45 min with occasional shaking. The suspension was then centrifuged for 10 min at 600 *g*. The pellet was washed with 5 cc of deionized water (25°C) for removal of the excess fixative and then centrifuged for 10 min at 600 *g*. This pellet was thoroughly resuspended in two to three drops of deionized water to randomize the sample. The suspension was then warmed to 45°C, two to three drops of 2% agar at 45°C were added to it with mixing, and the agar was allowed to harden (see reference 41). Epon-812 embedding was carried out conventionally (48) and PbO staining (40) was used for OsO_4-fixed preparations.

RESULTS AND DISCUSSION

Particle Composition of Thymus Suspension

An electron microscope analysis of the thymus suspension showed it to be composed almost exclusively of intact and ruptured cells and intact and ruptured nuclei. No electron micrographs are shown since they are indistinguishable from those previously published (41). The contamination due to smaller subcellular particles such as mitochondria, lysosomes, and microsomes was insignificantly small when examined under the electron and light microscopes.

In three separate preparations, air-dried smears of the suspension were stained with Wright's stain, a standard hematological differential cell counting stain (21). 2000–3000 particles in 14–22 different microscope fields were counted in each experiment, and the per cent erythrocyte contamination was calculated. The percentages were 0.10, 0.44, and 0.47, with the mean being only 0.32%. It would, therefore, seem that the suspension contained only thymus cells and nuclei, both intact and ruptured.

Spaces Present in the Suspension

The permeability behavior of the preparation was probed with three substances differing significantly in molecular weights: glycerol-^{14}C, sucrose-^{14}C, and carboxy-dextran-^{14}C (molecular weight, 60,000–90,000). The three spaces are shown in Fig. 1 and are expressed as R values (see Materials and Methods).[1]

From Fig. 1 it is clear that the three markers distributed in three distinct and stable volumes. These distribution volumes did not change with time, except for a small increase in the carboxydextran distribution volume within the first hour. In the experiments of Fig. 1 the glycerol volume was measured at 5 hr only. However, in nine different experiments, a comparison of the R values for glycerol at 5 min and 5 hr shows that the per cent increase was not significantly large. The same can be said about the comparison of the 5-min and 5-hr R values for sucrose (Table I).

These results indicate that the probe molecules equilibrated very rapidly, were stable with time, and separated the pellet into three stable compartments. The per cent distribution of the different fractions is shown in Fig. 2 and expressed as percentages of the largest space, the glycerol space. The sucrose space percentage of the total glycerol space was the mean of 18 experiments whereas the dextran percentage was derived from five experiments. The simplest hypothesis to explain these results is that these distributions reflected actual particle compartments in the pellet. The glycerol

[1] The deviations indicated are standard deviations.

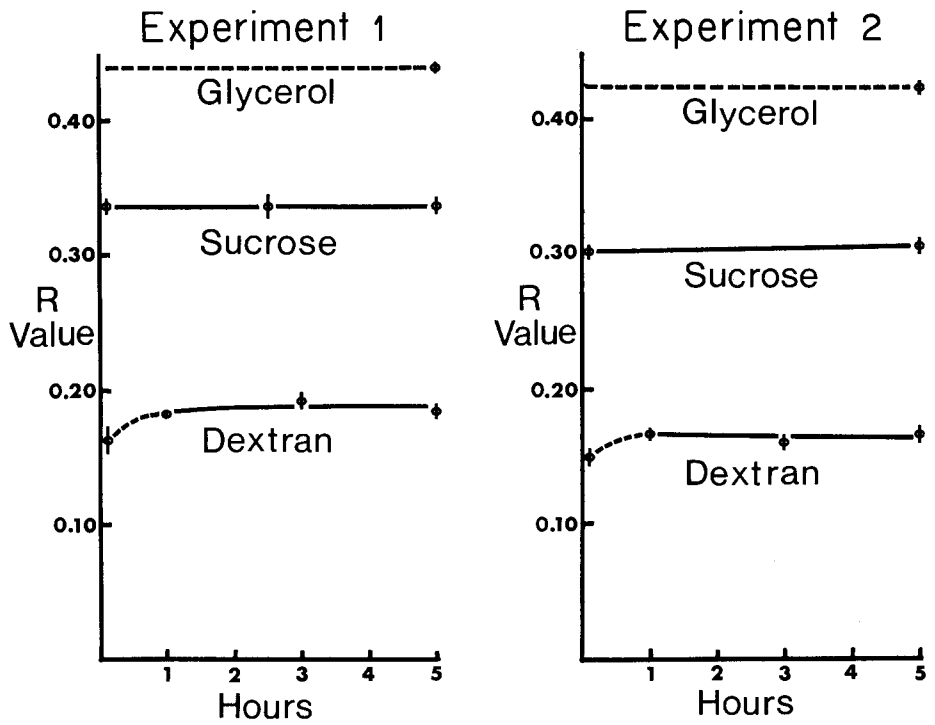

FIGURE 1 Probe molecule space versus time.

TABLE I

Per Cent Increase of 5 Hr R Value over 5 Min R Value for Glycerol and Sucrose

Experiment	Increase	
	Glycerol	Sucrose
	%	%
1	+0.5	−2.4
2	+7.3	+2.4
3	+1.9	+1.6
4	+4.8	−2.2
5	+0.9	−5.6
6	+2.0	0.0
7	−2.5	+2.5
8	+3.0	+3.6
9	+0.2	−0.3
10	—	+2.3
Mean	+2.0% ± 2.8	+0.2% ± 2.8

space minus the sucrose space (33%) would represent the least permeable fraction, whereas the sucrose-permeable, dextran-impermeable space (27%) would represent the slightly more permeable particles. The dextran space (40%) would represent the damaged particles and the fluid trapped between the particles. The glycerol space would then represent the total pellet fluid volume. Data will be presented which indicate that the compartment impermeable to sucrose corresponds to intact cells, that the sucrose-permeable, dextran-impermeable compartment, corresponds to ruptured cells and intact nuclei, and that the dextran space corresponds to ruptured nuclei and extraparticle space. Since it has been shown that the suspension was composed only of cells and nuclei, this reasoning is in line with the expectation that only two possible membrane permeability barriers exist: the cell membrane and the nuclear membrane.

Other low molecular weight solutes (malonamide-^{14}C, erythritol-^{14}C, D-arabinose-^{14}C, and D-mannitol-^{14}C) equilibrate with the sucrose-permeable space within the shortest manipulation time (these results are not presented here).

Total Pellet Fluid Volume

Whether the glycerol space corresponded to the total fluid volume of the pellet, was tested by com-

paring the total fluid volume of the pellet with glycerol-^{14}C space. This was done in two experiments where 5-hr glycerol isotope spaces in one set of pellets were compared with the total water volumes of an identical set. The total water volumes were measured by wet and dry weight differences, with an analytical balance. In the first experiment the pellets were dessicated in a vacuum oven at 60°C for 3 wk, and in the second they were frozen and sublimed under a vacuum in a freeze-dry apparatus at −25°C for 1 wk. The total water volume as measured by wet and dry pellet-weight differences did not represent the solution volume of the pellet, because of the partial molal volume of the sucrose and electrolytes in the suspension medium. Therefore, the total water volume was multiplied by an empirically determined factor of 1.08 for the virtual molal volume of the solutes which was not accounted for when the water was evaporated out of solution. The glycerol space, as it was measured, actually represented the total solution volume of the pellet, not just the total water space. The results are given in Table II.

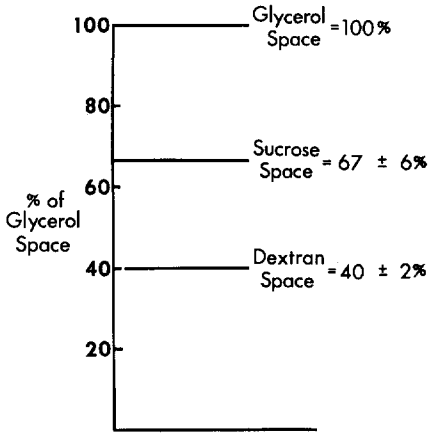

FIGURE 2 Per cent distribution of probe molecules. See text for further explanation.

From the results in Table II it can be seen that the glycerol isotope equilibrated with about 96% of the entire fluid volume of the pellet. This meant that the glycerol space was most probably a measure of the total fluid volume of the pellet.

Volume of Pellet Occupied by Particle Solids

The solid volume of the pellet may be of some interest. It was determined by two general methods, both of which utilized the glycerol space as a measure of the total fluid volume of the pellet. In the first method the solid volume was obtained by measuring the total volume of the pellet and subtracting the glycerol space from it. This was done in two experiments. In one experiment the volume of the pellet was determined by carefully measuring the volume of supernatant from a centrifuged (12,800 *g* for five 5 min) 3.00 cc of thymus suspension with a pipette graduated in 0.01 cc. This volume, subtracted from 3.00 cc, gave the total pellet volume. A more accurate measurement of the total pellet volume was obtained gravimetrically. Exactly 2.00 cc of suspension medium (solution 1) were added to the pellet from a 4.00 cc suspension (eight samples). This resultant suspension was weighed on an analytical balance. A 1.00 cc aliquot of this suspension was then weighed, and the density of the suspension was obtained. Since the weight and density of the suspension were then known, the volume was easily calculated. The 2.00 cc volume of suspension medium which was added to the pellet originally was then subtracted from the calculated suspension volume to give the original total volume of the pellet. The glycerol spaces in both experiments were also measured and subtracted from the re-

TABLE II

Comparison of Glycerol Space and Total Pellet Solution Volume

Experiment	Total pellet H_2O volume*	Total pellet solution volume‡	Glycerol space
	cc	*cc*	*cc*
1 (8 samples)	0.501 ± 0.003	0.541 ± 0.003	0.522 ± 0.019
2 (8 samples)	0.787 ± 0.004	0.850 ± 0.004	0.821 ± 0.011

* From wet and dry weight pellet differences.

‡ Total pellet H_2O volume corrected for molal volume of solutes.

spective total pellet volumes to give the solid volumes of the pellets (see Table III).

The solid volume percentage of the pellet was measured by another independent method by using the dilution principle and the fact that glycerol equilibrated with the total pellet fluid volume. The glycerol isotope concentration was measured in the supernatant of a thymus suspension of exactly 3.00 cc, which was centrifuged in the usual way. The glycerol space of the pellet was also measured. The glycerol isotope concentration was also measured in exactly 3.00 cc of solution 1, and was compared with the glycerol isotope concentration in the supernatant of the suspension. The higher glycerol isotope concentration of the supernatant was then due to the presence of the solids in the suspension. From the dilution equation, (isotope concentration in solution 1) × (3.00 cc) = (isotope concentration in suspension supernatant) × (x cc), the solid volume, which was equal to (3.00 − x) cc, was obtained. These results are given in Table IV.

The mean solid volume percentage of the total pellet volume of all the values (not including experiment 1 of Table III) was 21 ± 2%. Any small error in any of these determinations could have resulted in relatively large errors in the solid volume percentage since the volume being measured was small, but the fact that two independent methods gave comparable results added support to these estimates.

Interpretation of the Sucrose-Impermeable Compartment

According to previous electron microscope data on this thymus suspension, the intact cells represented about 3–8% of the particles (6, 41). Since the sucrose-impermeable compartment was 33% of the total pellet fluid volume, it did not seem likely that the sucrose was separating only the intact cells from the rest of the pellet, but the ruptured cells and intact nuclei as well. However, the possibility remains that the electron microscope data may have misrepresented the true composition of the thymus suspension because some of the intact cells were damaged during the procedures for electron microscopy. If so, the intact cell proportion would actually have been higher before the treatment for electron microscopy. It was shown that the pellet was divided into three definite, stable compartments by sucrose and dextran. The most likely explanation for the presence of three compartments in the pellet was that the sucrose-impermeable compartment represented the intact cells, that the sucrose-permeable, dex-

TABLE III

Pellet Solid Volume Percentage

Pellet volumes measured by pipette and gravimetrically

	Experiment 1*	Experiment 2‡
Total pellet volume, *cc*	0.55 ± 0.008	1.021 ± 0.065
Glycerol space, *cc*	0.423 ± 0.005	0.821 ± 0.011
Pellet solid volume, *cc*	0.13	0.200
Solid volume of total pellet volume, %	24	20

* Total pellet volume obtained by pipette.

‡ Total pellet volume obtained gravimetrically.

TABLE IV

Pellet Solid Volume Percentage Measured by Glycerol Isotope Dilution

Experiment	Final suspension volume	Glycerol isotope concentration in suspension	Glycerol isotope concentration in solution 1	Glycerol space	Solid volume	Solid volume
	cc	*counts/0.05 cc*	*counts/0.05 cc*	*cc*	*cc*	%
1	4.05	51,311 ± 438	48,750 ± 364	0.821	0.202	20
2	3.05	99,577 ± 1,248	96,805 ± 864	0.328	0.085	21
3	3.05	100,861 ± 851	97,114 ± 125	0.388	0.113	23

tran-impermeable compartment represented the particles with intact nuclear membranes (partially damaged cells and intact nuclei), and that the dextran space represented the ruptured nuclei and extraparticle space. If the sucrose-impermeable compartment represented both cells and intact nuclei, there would not have been any other accountable membrane permeability barrier left in the suspension to prevent the dextran space from being equal to the sucrose space.

It has been pointed out in a previous study (41) that a selective breakdown of nuclei upon incubation may alter the proportion of the different particles in the preparation. This occurs under the condition of the experiments which were previously reported by others (7, 9, 30), and duplicated in our work (41). Even under optimal conditions of pH and divalent ion concentration,[2] a nuclear breakdown as high as 50% takes place, e.g., 52 ± 13 (calculated from Table III in reference 4). Such breakdown could increase the proportion of intact cells upon incubation; this would explain the discrepancy between the electron microscope data and the space distribution data. This possibility was avoided in these experiments by keeping the preparation at 0°C where no breakdown was evident. A typical experiment carried out under conditions identical to those of the present experiments show that before incubation 1.37 ± 0.03 × 10^9 particles per cubic centimeter were present. After 5 hr of incubation, the number counted was the same. This experiment was carried out with a Coulter counter (30 μ window) at a dilution of 1/2,406 of the concentration reported. Although no incubation experiments were carried out with an alternative technique, direct counts from photographs of particles in a hemocytometer chamber correspond within 20% to those obtained with the Coulter counter.

STUDIES WITH THE ELECTRON MICROSCOPE AND WITH FRACTIONATION PROCEDURES: In order to help resolve the question whether the sucrose-impermeable space represented only intact cells or intact cells, ruptured cells, and intact nuclei, we carried out a separation procedure described by Allfrey et al. (4). This involved the use of a discontinuous Ficoll (Pharmacia, Fine Chemicals Inc., New Market, N.J. molecular weight, 400,000) density gradient centrifugation. The thymus suspension was layered over a dense Ficoll solution and was centrifuged at 700 *g* for 5 min. The Ficoll solution was made up by adding 20 cc of cold, 4°C, Ficoll stock solution to 55 cc of 0.25 M sucrose with 3 mM $CaCl_2$. The Ficoll stock solution was made by adding 100 *g* of dialyzed, lyophilized Ficoll to 100 cc of 0.25 M sucrose with 3 mM $CaCl_2$. An electron microscope examination of the bottom pellet by others (4) has shown it to be 92–98% nuclei, whereas the interphase was found very rich in intact cells. McEwen et al. (50), using the same procedure, reported that the interphase contained 55–80% whole cells. The spaces present in the different Ficoll fractions together with an electron microscope examination of these fractions should help clarify the meaning of the penetration spaces in the original preparation.

The study was carried out on three suspensions: Ficoll-separated interphase and bottom pellet fractions and the original unseparated thymus suspension. All three of these came from the same original suspension. Sucrose-impermeable spaces were also measured in these three suspensions for comparison of the intact cell proportions. The results are given in Tables V and VI. In the electron microscope analysis the particles were divided into six arbitrary categories: nuclei free of cytoplasm; ruptured nuclei (nuclei with definite tears in the nuclear envelope); nuclei with traces of cytoplasm; nuclei with tabs of cytoplasm (nuclei with about 50% of the cytoplasm still adherent); ruptured cells (cells with noticeable tears in the plasma membrane, but with the cytoplasm largely intact); and intact cells (cells with no visible damage to the plasma membrane). Electron micrographs and a discussion of these classifications have been presented previously (41).

In Table V the intact cell percentages in the interphase fractions are much higher than in the bottom Ficoll fractions with both permanganate and osmium tetroxide fixatives. At the same time, the unseparated suspension percentages were intermediate. This result, qualitatively at least, confirmed the claim (4) that the Ficoll separation procedure did trap intact cells at the Ficoll interphase and concentrate nuclei in the bottom pellet.

The second finding, that permanganate selectively destroyed more intact cells than nuclei, suggests a reason for Kodama and Tedeschi's having obtained such a low intact cell percentage in their

[2] In our previous work (41) the breakdown was much more extensive primarily because of the lower divalent ion concentration. The composition of the incubation medium reported by others was used (8).

TABLE V

Electron Microscope Analysis of Ficoll Interphase, Ficoll Bottom, and Unseparated Suspensions

Fixative	Suspension	Intact cells	Ruptured cells	Nuclei with tabs of cytoplasm	Nuclei with traces of cytoplasm	Nuclei free of cytoplasm	Ruptured nuclei	Total No. particles counted	Total No. sets of photographs counted*
		%	%	%	%	%	%		
$NaMnO_4$	Ficoll interphase	6 ± 3	15 ± 7	32 ± 4	26 ± 8	17 ± 7	4 ± 4	342	10
	Ficoll bottom	0 ± —	2 ± 1	4 ± 1	10 ± 4	66 ± 10	17 ± 6	395	6
	Unseparated	2 ± 1	6 ± 4	19 ± 6	22 ± 10	40 ± 11	10 ± 4	232	9
OsO_4	Ficoll interphase	16 ± 5	36 ± 8	23 ± 7	6 ± 3	12 ± 5	7 ± 4	251	9
	Ficoll bottom	0 ± —	0 ± —	1 ± —	3 ± 7	41 ± 9	54 ± 9	333	9
	Unseparated	8 ± 4	16 ± 4	28 ± 3	6 ± 4	20 ± 8	22 ± 7	197	7

* The standard deviations were calculated from the percentages obtained in the individual sets of photographs each representing one randomly selected section. Each set of photographs was comprised of five photographs, each photograph containing about 5–10 particles.

TABLE VI

Sucrose-Impermeable Compartment Percentage of Glycerol Spece in Ficoll Interphase, Ficoll Bottom, and Unseparated Suspensions

Suspension	Substance-^{14}C	Space R value	Sucrose-^{14}C-impermeable compartment percentage
			%
Ficoll interphase	Glycerol	0.2534 ± 0.0021	71
	Sucrose	0.0727 ± 0.0008	
Ficoll bottom	Glycerol	0.2177 ± 0.0012	16
	Sucrose	0.1819 ± 0.0015	
Unseparated	Glycerol	0.2480 ± 0.0140	52
	Sucrose	0.1187 ± 0.0002	

analyses with a permanganate fixative. With permanganate fixation the intact cell percentages in the unseparated suspension and the Ficoll interphase fraction were 2 and 6%, respectively, whereas with osmium tetroxide these percentages were four and three times greater, respectively, in these two suspensions. Even though osmium tetroxide did preserve more intact cells than permanganate, the intact cell percentages alone could not wholly account for the sucrose-impermeable space in the same suspensions. For example, the intact cell percentage of the unseparated suspension was 8%, but the sucrose-impermeable space was 52%. For the Ficoll interphase fraction, the electron microscope intact cell percentage was 16% and the sucrose-impermeable space percentage was 71%. Although exact correspondence of percentages determined by number and volume cannot be expected, it is likely that this approximation is useful since the particles were of similar sizes. The agreement was at least qualitative, in that, according to both types of measurements, the intact cell proportions were highest in the Ficoll interphase fraction, intermediate in the unseparated suspension, and lowest in the Ficoll bottom fraction. This result suggested that the sucrose-impermeable space was measuring the intact cell volume.

TABLE VII

Electron Microscope Data of Table V, with Intact Cells, Ruptured Cells, and Nuclei with Tabs of Cytoplasm Taken Collectively to Represent Intact Cells

Fixative	Suspension	Intact cells, ruptured cells, and nuclei with tabs of cytoplasm	Nuclei with traces of cytoplasm, nuclei free of cytoplasm, and ruptured nuclei	Total No. particles counted
		%	%	
$NaMnO_4$	Ficoll interphase	53	47	342
	Ficoll bottom	7	93	395
	Unseparated	27	73	232
OsO_4	Ficoll interphase	75	25	251
	Ficoll bottom	1	99	333
	Unseparated	52	48	197

TABLE VIII

Sucrose-Impermeable Compartment Proportions in Ficoll Interphase and Ficoll Bottom Fractions

Experiment	Sucrose-impermeable compartment proportion	
	Ficoll interphase fraction	Ficoll bottom fraction
	%	%
1*	71	16
2	30	13
3	28	10
4‡	52	11
5‡	66	8

* This experiment represents the one presented in Table VI.

‡ The percentages of the Ficoll interphase and Ficoll bottom fractions were actually those of the top and bottom fractions, respectively, of a modified Ficoll separation (see Schrek test). These two experiments are those of Table X.

The data left open the possibility of an artifactual conversion of intact cells into nuclei during the procedures preparatory to the electron microscopy. Destruction and extraction of membrane systems by osmium tetroxide fixation has been documented repeatedly by others (24, 26, 44). If the three cell categories were taken collectively to represent intact cells before the electron microscope treatment, the intact cell proportion would be 53% for the unseparated suspension (Table VII). This value compared well with the 52% measured by the sucrose-impermeable space. In the Ficoll interphase fraction, the electron microscope intact cell percentage was 75%, whereas the sucrose-impermeable proportion was 71%. In this respect, in the study of Kodama and Tedeschi this collective classification produced 30% intact cells even with permanganate fixation. This value compared favorably with the mean sucrose-impermeable compartment proportion of 33% generally found in the present study (Figs. 1 and 2). The results of several experiments detailing the sucrose impermeable space are summarized in Table VIII.

The possibility that Ficoll increased the permeability of these particles to sucrose, in the bottom Ficoll fraction, is ruled out by the following experiment. A thymus suspension was divided into two portions. Ficoll stock solution was added to one portion to give a final Ficoll concentration equal to that used in the separation procedure. Solution 1 was added to the other portion. Both portions were allowed to stand for 15 min, then were centrifuged and washed twice with solution 1. The sucrose-impermeable proportions were measured in both portions. Table IX shows that the sucrose-impermeable space proportion in the solution 1-exposed portion was 30%, whereas in the Ficoll-exposed portion it was 28%.

The excellent agreement between the sucrose-impermeable spaces of the Ficoll fractions in this work and the electron microscope data on the same fractions in the work of Allfrey et al. (4) supports the view that the sucrose-impermeable space corresponds to intact cells. This conclusion implies that, in contrast to the results in our work, in that work (4) no breakdown of cells occurs as a consequence of the procedures preparatory to the electron microscopy.

SCHREK TEST: To further support the idea

that the sucrose-impermeable compartment was the intact cell space, we used the Schrek test. The Schrek test and other similar vital dye tests are used to distinguish intact cells from damaged cells and nuclei (4, 36, 61, 65). Allfrey et al. (4) reported the Schrek test to be valid for evaluating thymus suspensions. Roof and Aub (61) used this test for distinguishing nuclei of lymphocytes. The Schrek test is based on the premise that the nuclear membrane is more permeable than the cell membrane. Hoskins et al. (36) used it to test the number of viable tumor cells in preparations. In the present study, three drops of the suspension to be tested were added to 2 cc of a 0.5% eosin Y solution made up in solution 1, and a drop of this suspension was counted under the light microscope. Supposedly, the nuclei, being permeable to the reasonably small molecule of eosin Y (molecular weight, 692), took up the dye and their entire contents appeared dark red, whereas the intact cells, being impermeable to the dye, were only stained superficially and appeared a very light pink. The difference in appearance of these two particles was very distinctive.

TABLE IX

Effect of Exposure of Sucrose-Impermeable Particles to Ficoll

	R value	Sucrose-impermeable proportion
		%
Ficoll, exposed glycerol space	0.2859 ± 0.0008	28
Ficoll, exposed sucrose space	0.2058 ± 0.0012	
Solution 1, exposed glycerol space	0.3102 ± 0.0037	30
Solution 2, exposed sucrose space	0.2173 ± 0.0024	

The Schrek test was applied to a thymus suspension separated into three fractions by a modified Ficoll separation procedure. The original thymus suspension was layered over the Ficoll solution and centrifuged at a low speed of 300 *g* for 5 min. The top layer of the Ficoll gradient and the particles at the interphase were then withdrawn, resuspended, and relayered over another tube of fresh Ficoll solution. The original Ficoll layer was centrifuged at 1,000 *g* for 10 min for sedimentation of the particles into a pellet. This fraction is the bottom fraction in Table X which was very rich in nuclei. The second Ficoll tube with the relayered suspensions was then centrifuged at 800 *g* for 5 min. The top layer of this tube and the interphase particles were withdrawn (the top fraction in Table X). This fraction was rich in cells. The Ficoll layer of this tube was centrifuged at 1,000 *g* for 10 min for sedimentation of the particles. This fraction was the middle fraction, supposedly intermediate in composition between the top and bottom fractions. The intact cell percentages in the three fractions were counted by the Schrek test and compared with the sucrose-impermeable proportions measured in these same fractions. The results are given in Table X.

In both experiments there was very good correlation between the percentages of intact cells as determined by the sucrose-impermeable space and by the Schrek test. In another experiment a similar comparison was made in an unseparated thymus suspension. The intact cell proportion determined by the Schrek Test was 31% and the sucrose-impermeable proportion was 35%. These results further supported the contention that the sucrose-impermeable space measured the intact cell volume in the thymus suspension.

The high per cent of intact cells in this prepara-

TABLE X

Comparison of Intact Cell Percentages Measured by the Schrek Test and Sucrose-Impermeable Compartment in Modified Ficoll-Separated Suspensions

Experiment	Test	Top Ficoll fraction	Middle Ficoll fraction	Bottom Ficoll fraction
		%	%	%
1	Schrek test	62 ± 2	21 ± 2	10 ± 2
	Sucrose-impermeable compartment	52	18	11
2	Schrek test	73 ± 4	7 ± 1	3 ± 1
	Sucrose-impermeable compartment	66	9	8

tion may seem surprising. Many estimates of intact cells with several techniques give low estimates, frequently well below 10% (3, 6, 8, 28, 41). Some of these studies made use of the electron microscope (6, 41), and some the techniques of Barer et al. (12). On the other hand, some reports have questioned the low yield of intact cells in preparations made with the older methods (16). Although no actual percentages were reported, a heavy contamination with intact cells has been claimed. In this work, the sensitivity to low calcium levels and the lack of osmotic behavior were used as a test for the presence of nuclei. Nuclei supposedly lyse in low calcium (see references 4, 11, 31, 60, 68) and do not exhibit osmotic behavior in sucrose (11). However, more recent reports do not support such heavy contaminations, although higher proportions of cells have been reported than in the earlier papers. In their more current reports on the composition of the suspension, Allfrey et al. (4, 50, 51) reported that the intact cell percentage could be, in some cases, as high as 17–25% when the preparations were examined by electron microscopy after osmium tetroxide fixation. They further reported (4) that by increasing the Waring Blendor homogenization from 4 min at 1,000 rpm to 7 min at 1,600 rpm the intact cell percentage was increased from 8.4 to 60% (4). This meant that Waring Blendor homogenization is potentially capable of producing a calf-thymus suspension very rich in intact cells. Our previous work with the electron microscope substantiates the finding of a contamination close to 30%, only on the assumption that cells, which appear broken in our work with the electron microscope, are actually intact. The present results support this value.

It should be noted that a fairly high proportion of cells can occur in an initially relatively pure preparation by selective rupture of the nuclei during incubation (see reference 41). Even with short incubation times and optimal conditions of pH and divalent ion concentration, the nuclear breakdown can be as high as 50%, e.g. it can be calculated to be 52 ± 13, (Table III in reference 4). This fact may explain some of the discrepancies between the results presented by workers from various laboratories.

The interpretation that the sucrose-impermeable space corresponds to intact cells is supported by the permeability properties of this compartment. The permeability of this space to malonamide is identical with the permeability of thymocytes separated from each other by being pressed through a wire screen. These results will be discussed in detail elsewhere.

Extraparticle Space

One more pellet compartment remained to be defined, i.e., the extraparticle compartment. So far, in the considerations of the pellet compartments, the extraparticle space has been tacitly assumed to be zero, as an approximation. This implied that all of the dextran space was considered to be the ruptured, dextran-permeable nuclear compartment. Unfortunately, the ruptured nuclei and extraparticle space could not be distinguished by using the isotope dilution method, since there was no permeability barrier to separate them. The extraparticle space could not have been higher than the dextran space (40% of the total pellet water), even if there were no ruptured nuclei in the preparation. If there had been no ruptured nuclei present and the entire dextran space were extraparticle volume, the rather close agreement obtained between data on the sucrose-impermeable compartment and data from the electron microscope, Ficoll separation, and Schrek test would not have been so good. For example, the sucrose-impermeable space in terms of the proportion of intact particles would have been 56% instead of 33%. However, in spite of this lack of exact quantitation, the same general conclusion that the sucrose-impermeable compartment represents the intact cells is still justified.

It does not seem plausible that the homogenization procedure was so selective as to rupture cells and not nuclei. Several observations support the idea that ruptured nuclei did exist in the preparation and did not disintegrate completely so long as calcium was present. If the thymus suspension was sonicated at 70–80 kc/min for up to 7 min, gross physical damage was incurred in the particles as evidenced by their ragged outlines when seen under the light microscope. However, the particle concentration was not lowered. The electron microscope also showed that ruptured nuclei did exist in the original preparation (41). This point is somewhat ambiguous since it could be argued that the nuclear rupture was an electron microscope artifact. Nevertheless, these two sets of data suggest that ruptured nuclei may exist in the suspension and that they occupy a portion of the dextran space.

Another alternative that cannot be discounted is the possibility that penetrating macromolecules do not freely distribute in the nucleoprotein meshwork. Data are not yet available to permit examining this alternative critically. It is also possible that some of the dextran is bound to the nuclei and thus increases the apparent extranuclear space.

The erythrocyte may be an atypical case because of its biconcave disc shape which may theoretically allow closer packing than a spherical shape. However, in this case the results of other workers indicate that little extraparticle space is present. Savitz et al. (62), measuring the extraparticle volume in human erythrocyte pellets packed for 25 min at 6,700 *g* and using albumin-^{131}I space, found the extraparticle volume percentage to be 0.8–1.6%. Kahn (38), using a 15 min 1,500 *g* centrifugation found this value to be 7%. Omachi et al. (56), centrifuging for 10 min at 1,500 *g*, obtained a value of 11%. Conway and Downey (22) calculated the theoretical minimum interspace volume between maximally packed rigid spheres to be 26%. Using yeast cells with rigid cellulose cell walls, they obtained values between 22 and 24%. This theoretical value could be reduced considerably if the particles were plastic and conformed to a cubic configuration, a shape which allows maximal packing under the centrifugal force. The centrifugal force used in this study was quite high, 12,800 *g* for 5 min, and sufficient to pack the particles rather tightly and possibly reduce the extraparticle space to a very low value.

Although our experiments are not entirely conclusive, we tentatively propose that damaged nuclei are included in this fraction. In addition, the possibility that a portion of the nuclear pellet space may actually be carboxydextran permeable cannot be excluded.

CONCLUSIONS

The data presented in this paper support the thesis that isolated calf-thymus nuclei are highly permeable to low molecular weight nonelectrolytes, such as glycerol or sucrose, but that they are relatively impermeable to macromolecules (such as carboxydextran: molecular weight, 60,000–90,000). However, caution is necessary in the extrapolation of the properties of this preparation to the in vivo system. The preparatory manipulations may have damaged the particles; the temperature of incubation (0°C) and the nature of the isolation or incubation medium may have led to alteration of their properties.

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REFERENCES

1. Abelson, A. P., and W. R. Duryee. 1949. *Biol. Bull.* **96**:205.
2. Afzelius, B. A. 1955. *Exptl. Cell Res.* **8**:147.
3. Allfrey, V. G. 1954. *Proc. Natl. Acad. Sci. U. S.* **40**:881.
4. Allfrey, V. G., V. C. Littau, and A. E. Mirsky. 1964. *J. Cell Biol.* **21**:213.
5. Allfrey, V. G., R. Meudt, J. W. Hopkins, and A. E. Mirsky. 1961. *Proc. Natl. Acad. Sci. U. S.* **47**:907.
6. Allfrey, V. G., and A. E. Mirsky. 1955. *Science.* **121**:879.
7. Allfrey, V. G., and A. E. Mirsky. 1959. *In* Subcellular Particles. T. Hayashi, editor. The Ronald Press Co., New York. 186.
8. Allfrey, V. G., A. E. Mirsky, and S. Osawa. 1957. *J. Gen. Physiol.* **40**:451.
9. Allfrey, V. G., A. E. Mirsky, and S. Osawa. 1955. *Nature.* **176**:1042.
10. Anderson, N. G. 1953. *Exptl. Cell Res.* **5**:361.
11. Anderson, N. G., and K. M. Wilbur. 1951. *J. Gen. Physiol.* **35**:781.
12. Barer, R., S. Joseph, and M. P. Esnouf. 1956. *Science.* **123**:24.
13. Battin, W. T. 1953. *Exptl. Cell Res.* **17**:59.
14. Beams, H. W., T. N. Tahmisian, R. Devine, and E. Anderson. 1957. *Exptl. Cell Res.* **13**:200.
15. Briggs, R. and T. J. King. 1953. *J. Exptl. Zool.* **122**:485.
16. Brown, J. R. C. 1955. *Science.* **121**:511.
17. Callan, H. G., and S. G. Tomlin. 1950. *Proc. Roy Soc. (London) Ser. B.* **137**:367.
18. Chambers, R. 1921. *J. Gen. Physiol.* **4**:41.
19. Churney, L. 1942. *Biol. Bull.* **82**:52.
20. Comandon, J., and P. de Fonbrune. 1959. *Compt Rend.* **130**:744.
21. Conn, H. J., M. H. Darrow, and V. M. Emmel, editors. 1960. Chapter 6. *In* Staining Procedures. Williams and Wilkins Co. Baltimore, Maryland. 133.

22. CONWAY, E. J., and M. DOWNEY. 1950. *Biochem. J.* **47**:347.
23. COONS, A. H., E. H. LEDUC, and M. H. KAPLAN. 1961. *J. Exptl. Med.* **93**:173.
24. DALLAM, P. D. 1957. *J. Histochem. Cytochem.* **5**: 178.
25. DAVIES, H. G. 1961. *J. Biophys. Biochem. Cytol.* **9**:671.
26. Elbers, P. F. 1961. *Nature.* **191**:1022.
27. FELDHERR, C. M. 1962. *J. Cell Biol.* **14**:65.
28. FELDHERR, C. M. 1965. *J. Cell Biol.* **25**:43.
29. FELDHERR, C. M., and A. B. FELDHERR. 1960. *Nature.* **185**:250.
30. FICQ, A., and M. ERRERA, 1958. *Exptl. Cell Res.* **14**:182.
31. GILL, D. M. 1965. *J. Cell Biol.* **24**:157.
32. GOLDSTEIN, L., and C. V. HARDING. 1950. *Federation Proc.* **9**:48.
33. GREIDER, M. H., W. J. KOS, and W. J. FRAJOLA. 1956. *J. Biophys. Biochem. Cytol.* **2** (Suppl.):445.
34. HARDING, C. V., and C. M. FELDHERR. 1959. *J. Gen. Physiol.* **42**:1155.
35. HOLTFRETER, J. 1954. *Exptl. Cell Res.* **7**:95.
36. HOSKINS, J. M., G. G. MAYNELL, and F. K. A. SANDERS. 1956. *Exptl. Cell Res.* **11**:297.
37. HUNTER, A. S., and F. R. HUNTER. 1961. *Exptl. Cell Res.* **22**:609.
38. KAHN, J. B., JR. 1958. *J. Pharmacol. Exptl. Therap.* **123**:263.
39. KANNO, Y., R. F. ASHMAN, and W. LOEWENSTEIN. 1965. *Exptl. Cell Res.* **39**:184.
40. KARNOVSKY, M. J. 1961. *J. Biophys. Biochem. Cytol.* **11**:729.
41. KODAMA, R. M., and H. TEDESCHI. 1963. *J. Cell Biol.* **18**:541.
42. KOPAC, M. J. 1948. *Ann. N. Y. Acad. Sci.* **50**: 870.
43. LANGENDORF, H., G. SIEBERT, and D. NITZ-LITZOW. 1964. *Nature.* **204**:888.
44. LEE, S. 1960. *Exptl. Cell Res.* **21**:252.
45. LEE, H. J., and D. J. HOLBROOK, JR. 1964. *Arch. Biochem. Biophys.* **108**:275.
46. LEE, H. J., and D. J. HOLBROOK. 1965. *Arch. Biochem. Biophys.* **112**:98.
47. LOEWENSTEIN, W., Y. KANNO, and S. ITO. 1966. *Ann. N. Y. Acad. Sci.* **137**:708.
48. LUFT, J. H. 1956. *J. Biophys. Biochem. Cytol.* **2**: 799.
49. MCGREGOR, M. C. 1962. *Exptl. Cell Res.* **26**: 520.
50. MCEWEN, B. S., V. G. ALLFREY, and A. E. MIRSKY. 1963. *J. Biol. Chem.* **238**:758.
51. MCEWEN, B. S., V. G. ALLFREY, and A. E. MIRSKY. 1963. *J. Biol. Chem.* **238**:2579.
52. MERRIAM R. W. 1959. *J. Biophys. Biochem. Cytol.* **6**:353.
53. MERRIAM R. W. 1961. *J. Biophys. Biochem. Cytol.* **11**:559.
54. MURRAY, R. G., A. MURRAY, and A. PIZZO. 1965. *Anat. Record.* **151**:17.
55. NAORA, H., H. NAORA, M. IZAWA, V. G. ALLFREY, and A. E. MIRSKY. 1962. *Proc. Natl. Acad. Sci. U. S.* **48**:853.
56. OMACHI, A., R. P. MARKEL, and H. HEGARTY. 1961. *J. Cellular Comp. Physiol.* **57**:95.
57. PALADE, G. E. 1952. *J. Exptl. Med.* **95**:285.
58. PAPPAS, G. D. 1956. *J. Biophys. Biochem. Cytol.* **2**(Suppl.):431.
59. PETERFI, T., and A. NEVILLE. 1931. *Protoplasma.* **12**:524.
60. PHILPOTT, J., and J. E. STANIER. 1956. *Biochem. J.* **63**:214.
61. ROOF, B. S., and J. C. AUB. 1960. *Cancer Res.* **20**:1426.
62. SAVITZ, D., V. W. SIDEL, and A. K. SOLOMON. 1964. *J. Gen. Physiol.* **43**:79.
63. SCHILLER, A., R. W. SCHAYER, and E. L. HESS. 1952. *J. Gen. Physiol.* **36**:489.
64. SHINKE, N. 1937. *Cytologia (Tokyo).* (Fujii jubilee volume.) 449.
65. SCHREK, R. A. 1946. *Am. J. Cancer.* **28**:389.
66. SIEBERT, G., and G. B. HUMPHREY, 1965. *Advan. Enzymol.* **27**:239.
67. STERN, H., and A. E. MIRSKY. 1953. *J. Gen. Physiol.* **37**:177.
68. SWINGLE, K. F., and L. J. COLE. 1964. *J. Histochem. Cytochem.* **12**:442.
69. WATSON, M. L. 1955. *J. Biophys. Biochem. Cytol.* **1**:257.
70. WHITAKER, J. R. 1963. *Anal. Chem.* **35**:1950.