METHODS FOR THE PURIFICATION OF THYMUS NUCLEI AND THEIR APPLICATION TO STUDIES OF NUCLEAR PROTEIN SYNTHESIS

V. G. ALLFREY, Ph.D., V. C. LITTAU, Ph.D., and A. E. MIRSKY, Ph.D.

From The Rockefeller Institute

ABSTRACT

Procedures are described for the purification of calf thymus nuclei using mild hypotonic shock to break intact cells, and layering techniques to remove cytoplasmic debris. Ficolc (a high polymer of sucrose) was dissolved in isotonic sucrose to give dense solutions suitable for gradient centrifugation. The method yields nuclei which can incorporate amino acids *in vitro*. Thymus nuclei isolated under isotonic conditions were incubated with C^{14}-amino acids and later purified by centrifugation through dense sucrose solutions. The distribution of radioactivity in different nuclear proteins was measured and it was found that isotopic amino acids are actively incorporated into characteristically chromosomal proteins, such as the arginine-rich and lysine-rich histones. Protein synthesis in the nucleus is markedly inhibited by puromycin and by agents, such as 2,4-dinitrophenol, which inhibit ATP synthesis. The synthesis of histones is also inhibited by puromycin, but the uptake of several amino acids into the lysine-rich histone fraction seems less sensitive to puromycin inhibition than is uptake into the arginine-rich histones or other proteins of the nucleus. High resolution autoradiography using tritiated leucine and observing grain distribution over thin sections of isolated nuclei and whole cells shows that amino acid incorporation occurs *within* the nucleus and is not due to cytoplasmic contamination.

INTRODUCTION

The experiments to be described are concerned with methods of nuclear isolation and with tests for the ability of nuclei, once isolated, to synthesize proteins, including some characteristically nuclear proteins—the arginine-rich and lysine-rich histones.

It will be shown, using high resolution autoradiography, that the cell nucleus of the thymus lymphocyte is actively engaged in amino acid incorporation both *in situ* and *in vitro*, and that amino acid uptake occurs *within* the isolated nucleus and not just on its periphery.

Some related observations deal with the effects of puromycin on the synthesis of histones and other nuclear proteins.

The incorporation of amino acids by thymus nuclear suspensions was reported in 1954 (1), and it was subsequently found that such nuclei retain a capacity to carry out many biosynthetic reactions, including the synthesis of ribonucleic acids (2–5) as well as nuclear proteins (1, 2, 6, 7). Of special interest was the observation that nuclear protein synthesis appears to require a prior synthesis of RNA (2). One advantage of isolated nuclei is their susceptibility to attack by deoxyribonuclease (2) and other enzymes (8). This made it

possible to remove the DNA and to demonstrate the DNA-dependence of nuclear RNA synthesis (9) and to show also that the selective removal of histones (by tryptic digestion) led to a stimulation of this nuclear activity (8).

Calf thymus nuclei, because of their relative ease of isolation and their retention of function, are often selected for biochemical studies of nuclear activity. In such studies of function and intracellular enzyme localization it is important to know the state of purity of the nuclear fraction being investigated, to rule out or correct for errors due to cytoplasmic contamination, and to demonstrate by biochemical or cytochemical means that the isotope incorporations observed did, in fact, occur in the nuclei.

Tests for Purity of Thymus Nuclear Fractions

Most of the isotope incorporation studies made with calf thymus nuclei have employed nuclear fractions prepared in isotonic sucrose solution. In outline, the procedure is as follows (10, 1, 2); minced calf thymus tissue is suspended in 0.25 M sucrose containing small amounts of divalent cations, and the cells are broken by shearing forces, generally in a blendor running at low speed. Subsequent centrifugation sediments the nuclear fraction which can then be washed to remove loosely adhering cytoplasmic debris.

The nuclear sediments obtained in this way have been found to satisfy a number of analytic criteria of purity; for if one judges them by chemical, immunological, or enzymatic tests for cytoplasmic contamination, the nuclear fractions are, on the average, better than 90 per cent pure (2, 10). (A good preparation, for example, will contain less than 5 per cent of the total cytochrome c oxidase or alkaline phosphatase content of the tissue (10, 21–23).)

However, we have known for some time that some cytoplasmic contamination does exist in these preparations, since this was evident in electron micrographs published in 1955 (11) and it can be seen readily in stained preparations under the light microscope.

The contamination consists mainly of small cytoplasmic tabs or strands attached to some of the nuclei, plus a small proportion of intact cells (red cells plus small lymphocytes). The whole cell contamination has been reported to range from 29 cells per 1000 nuclei (11) to 77 cells per 1000 nuclei (2, 12), as judged by counts made on thin sections of the nuclear sediment under the electron microscope. These figures are in accord with cell counts carried out after staining nuclear suspensions with Unna's stain (13). They also agree with other estimates based on the observation that intact cells resist attack by deoxyribonuclease under conditions in which the free nuclei lose nearly all of their DNA (2, 14); the cells can then be easily seen and counted after Feulgen staining of the DNase-treated suspension (15). Intact thymocytes also resist penetration by basic dyes under conditions in which the nuclei stain immediately and this, too, can be used in estimating cell frequencies in nuclear sediments.

For other types of contamination, electron microscopy seems to be a most sensitive indicator. Cytoplasmic tabs are readily observed (11) and estimates of their frequency indicate that 20 to 30 per cent of the nuclei have small tabs of attached cytoplasm (17, 16). A few mitochondria are present, but they are unable to synthesize ATP because of the calcium ions in the medium (21). On a dry weight basis, this visible contamination can amount to only a few per cent of the nuclear mass, but it is difficult to estimate quantitatively. Judging by nucleic acid analyses of the nuclei and of the tissue, and by tests for cytoplasmic enzymes in the nuclear sediment, cytoplasmic contamination probably amounts to less than 10 per cent of the weight of nuclei. We have now elected to remove most of this contamination, taking advantage of the fact that the presence of cytoplasm lowers the density of the nucleus to which it is attached.

Centrifugation in dense media has been employed to float cells and nuclei bearing cytoplasmic tabs away from the heavier and cleaner nuclei in the suspension. Some of the techniques employed to break cells and to minimize cellular and cytoplasmic contamination of thymus nuclear fractions will now be described. Several of these can be employed either before or after amino acid incorporation experiments to yield nuclei of high purity.

METHODS

Preparation of the Nuclear Fraction

The original procedure employed in the isolation of thymus nuclei for use in amino acid incorporation experiments (1, 2) was a modification of a method described by R. Schneider and M. L. Petermann

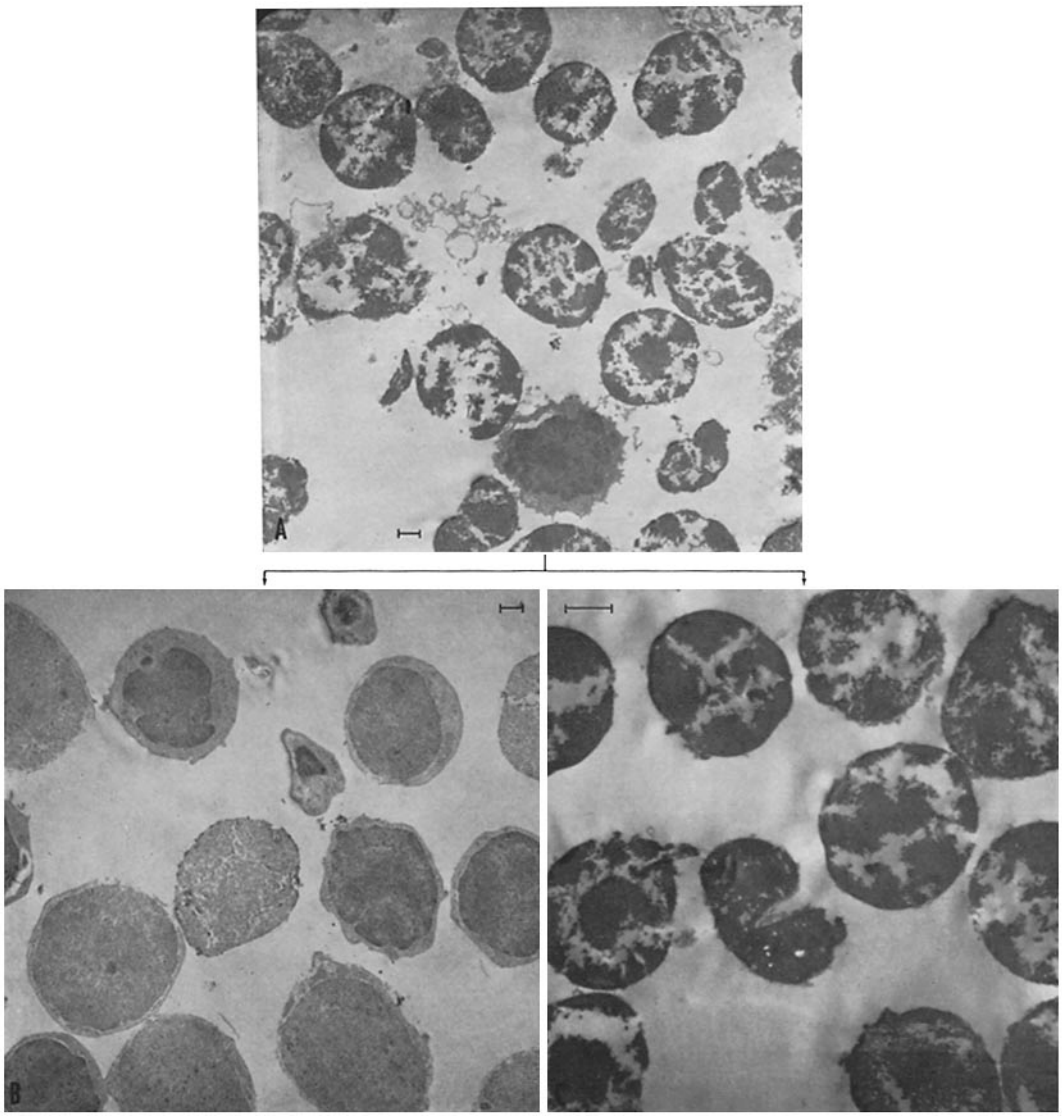

FIGURE 1 Electron micrographs showing stages in the fractionation of calf thymus nuclear suspensions by the Ficoll layering procedure. *A*, Unpurified nuclear sediment. Section through the nuclear pellet obtained after homogenization of the tissue in 0.21 M sucrose and centrifugation in 0.25 M sucrose. Note the presence of an intact cell, cytoplasmic fragments, swollen mitochondria, and cytoplasmic tabs adhering to the nuclei. *B*, Cellular fraction from Ficoll gradient. Cell-rich fraction trapped at the interface between the isotonic sucrose and the dense Ficoll layer. *C*, Nuclear fraction from Ficoll gradient. Nuclear sediment after centrifugation through the Ficoll-isotonic sucrose density barrier. This is a selected field which does not show any cells. Cell counts made on many other fields usually range between 2 and 4 per cent of the number of nuclei. *A* and *B*, × 3,000; *C*, × 6,000.

(18) and applied to calf thymus tissue. The cells were broken by low speed blending in a conventional Waring blendor vessel mounted on a high-torque motor. The medium used was isotonic (0.25 M) sucrose containing 0.002 to 0.003 M $CaCl_2$. (The calcium prevents formation of a nuclear gel and at the same time inactivates the mitochondria.) The nuclei were then collected by differential centrifugation.

The success of the homogenization method de-

pends on the difference in fragility between the intact thymocyte and its nucleus, the shearing forces in the blendor, and other conditions being selected to break the plasma membrane in nearly all of the cells, while leaving most of the nuclei intact.

Using thymus glands from 12- to 20-week-old calves (average wet weight of the glands is about 300 gm), it is often possible to reduce the number of intact cells to about 7 to 8 per cent of the number of nuclei by this simple procedure.

However, the fragility of calf thymocytes is a physiological variable not subject to control and, in some preparations, too many cells remain, sometimes comprising as much as 17 to 25 per cent of the number of nuclei. It should be stressed that, because of their small size, the cells are not easily detected under the ordinary light or phase contrast microscopes. A thin crescent or ring of cytoplasm does become evident in the electron microscope (Fig. 1, *B*), and cell counts are readily made on thin sections through the nuclear pellet.

TABLE I

Effects of Varying Conditions of Blending on Cell Content of Thymus Nuclear Fractions

Conditions of homogenization		Number of cells in nuclear sediment
Time	Speed of blendor	
min.	RPM	*per cent of total nuclei*
4	700	8.4
4	1600	17.
5	1600	35.
7	1600	60.

Some procedures for minimizing the contamination of thymus nuclear fractions by whole cells will now be described. Among the variables considered are the conditions of blending, the effects of detergents, and the tonicity of the medium.

EFFECTS OF VARYING CONDITIONS OF BLENDING: The type of homogenization employed is the first important variable in the isolation procedure. For handling large amounts of tissue, blending is preferred but, when blending is used to mince the tissue and break cells, it requires strict control because high shearing forces must be avoided. Thus, when many thymocytes from a given sample of tissue resist breakage during low speed blending, it is usually not advantageous to increase the speed of the blendor or to prolong blending time, because the nuclei already released cannot withstand very rigorous treatment. The effects of prolonged homogenization and high speed mixing are illustrated in Table I, which records the cell counts in the nuclear sediments under four conditions. It is evident that extended periods of blending and higher speeds lead to cell-rich, rather than nuclear, fractions. (It has been noted by others that the low shearing forces which suffice to break most calf thymocytes leave rat thymocytes intact (19).)

Fortunately, the resistance of the cells to breakage during blending can be reduced. We have employed treatment with detergents and hypotonic shock, and both of these methods significantly lower whole cell contamination of the nuclear fraction.

INCREASING CELL FRAGILITY BY EXPOSURE TO DETERGENTS: The resistance of thymocytes to breakage can be markedly reduced by the addition of detergents at early stages of the isolation. For example, the presence of 0.02 to 0.05 per cent sodium deoxycholate (DOC) during blending reduces cell counts to below 2 per cent. Deoxycholate treatment, however, is not recommended if the nuclei are to be used subsequently for isotope incorporation experiments, because DOC seriously impairs amino acid incorporation in both nuclei and intact cells.

The non-ionic detergent, Tween 80, was also tested after Fisher and Harris described its use in the isolation of nuclei from HeLa cells (20). We have added it to nuclear suspensions containing 20 to 30 per cent intact thymocytes, stirring for 10 to 30 minutes at 0° and counting the residual cells. A concentration of 0.4 volume per cent Tween 80 was found to reduce the cell count to below 8 per cent after stirring for 10 minutes. The nuclei were then washed extensively to remove the detergent and tested for their ability to incorporate C^{14}-alanine into protein. Although some activity remained, nuclei after Tween treatment were much less active in protein synthesis than other nuclear suspensions of comparable or higher purity which were prepared by the hypotonic shock method or by gradient centrifugation (see below.).

Another surfactant tested was Cemulsol NPT 6 (Société des Produits chimiques de Synthèse, Bezons, Seine-et-Oise), introduced by Hubert *et al.* (59) for the isolation of rat liver nuclei. When Cemulsol NPT 6 was added to calf thymus nuclear suspensions to give a final concentration of 0.2 per cent, brief stirring reduced cell counts from 12 to 17 per cent to below 5 per cent. Thymus nuclei treated in this way and washed briefly with isotonic sucrose were capable of amino acid incorporation, as are rat liver nuclei (54, 59), but continued presence of the detergent is harmful to their function.

CELL BREAKAGE IN HYPOTONIC MEDIA: An alternative procedure, now often used, is to increase cell fragility by exposing the minced tissue to a hypotonic environment during blending, thus providing an internal stress on the plasma membrane to supplement the shearing stresses of the blendor.

The effectiveness of this procedure is shown in Table II, which records the number of cells re-

maining in the nuclear fraction after blending in sucrose solutions of different concentrations. In all cases, 50 gm of fresh, minced tissue was added to 450 ml of the indicated sucrose solution containing 0.0024 M $CaCl_2$, and blended for 5 minutes at 1000 RPM (at 2°C). After homogenization, 0.5 M sucrose was added to bring the suspension to isotonicity (0.25 M) and the nuclei were sedimented by centrifugation at 200 *g* avg for 10 minutes. Small portions of the nuclear pellets were fixed, sectioned, and stained as described below.

Cell counts were made in the electron microscope, counting 5 to 10 random fields, each with 40 to 50 nuclei.

The results show that decreasing the sucrose concentration during blending favors cell breakage and gives nuclear fractions with fewer intact thymocytes. Thus, when necessary, the number of intact cells can be reduced to a very small proportion of the total number of nuclei, simply by lowering the sucrose concentration at early stages of the isolation. It should be stressed, however, that blending under hypotonic conditions has its drawbacks, because, as we have shown previously, deviations from isotonicity impair the capacity of the isolated nuclei to incorporate alanine-1-C^{14} into protein. This impairment of function is evident both in nuclei isolated under isotonic conditions and then incubated at lower sucrose concentrations (2), and in nuclei first exposed to hypotonic sucrose and then incubated under isotonic conditions. The longer the exposure to low sucrose concentrations, the greater the loss of function. Even relatively brief exposures to 0.18 M or 0.20 M sucrose lead to a significant reduction in the capacity of the isolated nuclei to incorporate alanine-1-C^{14} into protein.

TABLE II

Effects of Varying Sucrose Concentration on Cell Fragility and on Nuclear Function

Sucrose concentration during blending	Number of cells remaining	Rate of alanine-1-C^{14} uptake
Moles/liter	*per cent of total nuclei*	CPM/*mg protein/hr.*
0.18	1.6	80
0.20	4.4	166
0.25	10.2	468
0.28	25.0	359

Some indication of the effects of exposure to hypotonic media during blending is given in the last column of Table II, which compares the rates of alanine-C^{14} uptake in the different nuclear preparations. It is clear from the data that optimal amino acid uptake occurs in nuclei isolated under isotonic conditions. Note also that the observed uptakes do not correlate simply with cell counts, because fractions containing 25 per cent cells (last line in the Table) are less active than nuclear suspensions containing 10 per cent cells (third line) but maintained at isotonicity throughout the isolation. Of course, some of the incorporation is due to whole cells present in the nuclear suspensions, but autoradiographic data to be presented below shows that much of the protein synthetic activity of the cells also resides in their nuclei.

In studies of the metabolic activities of thymus nuclei, the presence of cytoplasmic tabs or a few intact cells may or may not introduce special biochemical problems. Special precautions must be taken, for example, in studies of nuclear ATP synthesis, where mitochondrial contamination must either be avoided, or the mitochondria selectively inhibited by carbon monoxide (21). On the other hand, for tracer studies of RNA synthesis, virtually all of which occurs in the nucleus (9), the cytoplasmic contamination can usually be safely ignored. In isotopic labeling experiments using amino acids, the number of whole lymphocytes must be minimized because intact lymphocytes are so much more active in amino acid transport and incorporation than are free, isolated nuclei. (Contamination by red cells is not a problem because of their relative inactivity in protein synthesis.) For this reason we frequently resort to blending under hypotonic conditions to reduce the number of intact lymphocytes, despite the resulting impairment in nuclear function.

The following procedure is useful when more than 10 per cent of the cells from a given thymus gland resist breakage during the usual blending in 0.25 M sucrose (2).

METHOD FOR CELL DISRUPTION IN HYPOTONIC SUCROSE SOLUTION: This method employs a brief exposure to hypotonic sucrose during blending and a rapid readjustment to isotonicity before isolating the nuclei. All operations are carried out in the cold (0–2°C). Fifty gm of fresh calf thymus tissue (20 to 40 minutes postmortem at 0°) is finely minced with scissors and transferred to a Waring blendor vessel of 1 liter capacity containing 450 ml of 0.20 M sucrose–3 mM $CaCl_2$. The cells are broken by blending at 700 RPM for 3 minutes (using a high-torque motor). At that time, sufficient 0.5 M sucrose–3 mM $CaCl_2$ is added to bring the sucrose concentration to 0.25 M, and the mixture is filtered through two layers of gauze (Johnson & Johnson Type I) and through two layers of pre-washed, double-napped, white flannelette. The filtrate is blended for an additional 2 minutes at 700 RPM and filtered again through a double thickness of flannelette.

SEDIMENTATION OF THE NUCLEAR FRACTION The filtrate is centrifuged at 400 *g* avg for 7 minutes and the supernatant phase is discarded.

The nuclear sediment is washed twice more with 120 ml portions of 0.25 M sucrose–3 mM $CaCl_2$. At this stage in the isolation the nuclei are better than 90 per cent pure, as judged by the absence of cytoplasmic enzymes such as succinoxidase or cytochrome *c* oxidase (21–23).

Some red cell contamination can be seen overlaying the nuclear pellet. In most incorporation experiments the red cells are so inert that their contribution to C^{14}-uptakes can be ignored, but they can be removed, if necessary, by (*a*) centrifugation at low speeds (*e.g.*, 140 *g* for 5 minutes) which sediments most nuclei and leaves erythrocytes in suspension, or (*b*) by centrifuging the nuclei through a Ficoll or sucrose density barrier (as described below) which leaves the red cells trapped at the interface. The latter procedure is very effective and has yielded nuclear fractions containing less than 0.0001 per cent heme.

Following centrifugation, the nuclear sediment is resuspended in 0.25 M sucrose containing 3 mM $CaCl_2$. A convenient concentration for amino acid incorporation experiments is 40 mg (dry weight) per ml. Approximately this concentration is obtained when the nuclei from fifty gm of tissue are finally resuspended in 40 ml of isotonic sucrose solution. The over-all yield is about 1.6 gm of nuclei (dry weight). Based on DNA analyses, this is a recovery of about 25 per cent of the total nuclei of the tissue.

TESTS FOR PURITY OF THE NUCLEAR SEDIMENT: Nuclear fractions prepared by the hypotonic isolation procedure have been examined five times in the electron microscope to check for whole cells and contamination by cytoplasmic debris.

The cell counts were low: 2.6, 3.7, 4.4, 4.4, and 6.4 per cent, respectively, in the different preparations. These results are in very good agreement with other estimates based on staining the nuclear suspension with 0.2 per cent crystal violet in 0.25 M sucrose; in such tests 2 to 6 per cent of the suspended particles resisted immediate penetration by the dye and were presumed to be intact cells. The remaining 94 to 98 per cent were nuclei and stained darkly within a few seconds.

Some of the nuclei, possibly 20 to 25 per cent of the total, had small tabs of cytoplasm attached to the nuclear envelope. Mitochondria were occasionally seen in close contact with the outer nuclear membrane, but this represents a very minor contamination. Moreover, these mitochondria are inactive; they cannot synthesize ATP because oxidative phosphorylation has been uncoupled by Ca^{++} ions (21). The over-all level of cytoplasmic contamination is estimated as below 5 per cent, a figure in agreement with the virtual absence of typically cytoplasmic enzymes such as cytochrome *c* oxidase and succinoxidase. With respect to the amount of adhering cytoplasm, the nuclei prepared in hypotonic media do not seem very different from the more active nuclear preparations isolated in isotonic sucrose solutions, but cell counts are definitely lower in the former.

Purification of Nuclei by Centrifugation through Dense Media

A critical examination of thymus nuclear fractions prepared under either isotonic or hypotonic conditions shows that some cytoplasmic contamination is present and is readily detectable in the electron microscope (Fig. 1 *A*).

To remove this contamination, either before or after amino acid incorporation experiments, we have employed two procedures which involve sedimentation through media of high density, rejecting intact cells as well as nuclei with large cytoplasmic tabs. Both methods depend on the fact that free nuclei, because of their high nucleic acid content, have a higher average density than do whole cells or nuclei contaminated by cytoplasm.

As density barriers we have employed concentrated solutions of sucrose, or solutions of the sucrose polymer, Ficoll, dissolved in 0.25 M sucrose to maintain isotonicity.

SUCROSE LAYERING PROCEDURES. METHOD I: The sucrose layering procedures now to be described are similar in principle to the method of Chauveau *et al.* (24) which utilizes 2.2 M sucrose to float whole cells and cytoplasmic debris away from the denser nuclei of rat liver cells.

Ten ml aliquots of the thymus nuclear suspension (usually labeled by a prior incubation with C^{14}-amino acids) are carefully layered over 20 ml portions of 2.0 M sucrose–3 mM $CaCl_2$ (in 1 x 3 inch Lusteroid tubes of 30 ml capacity). The tubes are carefully transferred to the cups of the SPINCO swinging-bucket rotor, SW 25, and centrifuged at 20,000 RPM for 30 minutes. The upper, clear layer is removed by aspiration, and the interphase zone, which contains light nuclei and intact cells, is removed with a syringe. The dense sucrose solution is then decanted, leaving the purified nuclear pellet.

Nuclear sediments obtained in this way show less than 2 per cent contamination by intact thymocytes, as judged by electron microscopy and staining tests. Yields, however, are usually low because about half of the nuclei become trapped in the narrow interphase zone between the 2 M sucrose and the upper isotonic medium. (The method is included here because a few of the histone-labeling experiments described below employed nuclear fractions purified in this way.) A double-layering technique has been devised to minimize the entrapment of clean nuclei in the interphase layer.

SUCROSE METHOD II: Ten ml of 2.0 M sucrose –3 mM $CaCl_2$ is placed in the bottom of each cen-

trifuge tube and carefully layered over with 10 ml of 1.6 M sucrose–3 mM $CaCl_2$. The nuclear suspension in isotonic sucrose is applied next, adding 10 ml to each tube, and the tubes are centrifuged as described above.

Two distinct interphase layers are produced in addition to the nuclear pellet at the bottom of the tube. The upper zone of turbidity lies between the isotonic sucrose layer and the 1.6 M sucrose layer. It contains red cells and cytoplasmic debris. A second zone of turbidity occurs between the 1.6 M and 2.0 M sucrose layers; this is made up of intact thymocytes, light nuclei, and nuclei bearing cytoplasmic tabs. (The radioactivity present in this fraction after labeling experiments is sometimes taken as an indication of the synthetic activity of the cells. It is usually about three times higher than that of the nuclear sediment. This is in accord with autoradiographs after leucine-H^3 incorporation, which show that intact cells are far more active in amino acid uptake than are the free nuclei (see below).)

Nuclear sediments obtained by the double-layering technique are as pure as those obtained by Method I; very few cells are present and nuclear yields are higher; about 60 to 70 per cent of the nuclei of the original isotonic suspension are recovered in the sediment. It is of interest that such nuclei do not contain more than trace amounts of heme pigments (less than 0.0001 per cent heme). Nuclei purified in this way were used for most of the studies of amino acid uptake into arginine-rich and lysine-rich histones, as described below.

PURIFICATION OF NUCLEAR FRACTIONS USING FICOLL: Layering techniques using concentrated sucrose solutions are often useful in purifying nuclei *after* isotopic labeling experiments but not *before* them, because exposure of the nuclei to hypertonic media destroys their capacity for subsequent amino acid incorporation (2, 14). Fortunately, other methods exist for increasing the density of a suspending medium without greatly disturbing its isotonicity; for example, the use of high-molecular-weight solutes, such as dextran, glycogen, or polysucrose, allows the preparation of media of high specific gravity and low molar concentration suitable for cell fractionation studies.

The synthetic sucrose polymer, Ficoll, was introduced for this purpose by Holter and Møller in 1958 (25). This material is exceedingly soluble in water and its solutions are not so viscous as those produced by less spherical polymers of comparable molecular weights (mol wt avg about 400,000).

The use of Ficoll in isotonic sucrose solutions allows a purification of thymus nuclei in an "active" state, *i.e.*, still capable of amino acid uptake into protein and active in RNA synthesis. Using layering procedures similar to those just described, free nuclei can be sedimented though a Ficoll density barrier which blocks the passage of whole cells and most of the tabbed nuclei. Although Ficoll itself is somewhat toxic, the purified nuclei are active enough to permit many direct tests of their function, and, when higher levels of uptake are desired, the nuclei can be purified in Ficoll gradients *after* labeling of the original nuclear suspension.

FICOLL-LAYERING TECHNIQUES: Several procedures for purifying nuclei by centrifugation through a Ficoll density barrier have been tested; as expected, the results obtained depend largely on the concentration and density of the Ficoll solution used.

A simple procedure for the purification of thymus nuclear fractions in bulk is described briefly below. It can be scaled down for working with smaller volumes of nuclear suspension.

PREPARATION OF FICOLL: Ficoll, a synthetic polysucrose, is available from Pharmacia, Ltd. (Vanløse, Copenhagen, Denmark). As delivered, it contains unknown substances harmful to nuclear function. Most of the toxicity can be removed by prolonged dialysis against distilled water. (For example, a comparison was made of amino acid uptakes by aliquots of a nuclear suspension after purification in dialyzed and undialyzed Ficoll. The specific activity of the proteins of nuclei prepared with undialyzed (but neutralized) Ficoll was only 105 CPM/mg after 60 minutes' incubation, as compared with 192 CPM/mg in nuclei isolated using the dialyzed Ficoll.) Following dialysis, the material is lyophilized and stored in the dry state.

A stock Ficoll solution is made by dissolving 100 gm of the dialyzed, lyophilized material in 100 ml of cold 0.25 M sucrose–3 mM $CaCl_2$.

LAYERING AND CENTRIFUGATION: Twenty ml of the stock Ficoll solution is added with stirring to 55 ml 0.25 M sucrose–3 mM $CaCl_2$ in a glass centrifuge bottle of 250 ml capacity. One hundred ml of nuclear suspension (prepared according to earlier references (1, 2) or as described above) is carefully layered over the dense Ficoll-sucrose mixture. The tubes are centrifuged in the horizontal rotor of the IEC refrigerated centrifuge for 5 minutes at 1800 RPM (700 *g* avg).

COLLECTION OF FRACTIONS: At that time, there is a clear separation of the particulates into an interphase zone (at the Ficoll density barrier) and a sediment. The upper clear layer is removed by aspiration and discarded. The interphase zone, which contains small intact thymocytes together with some light nuclei (Fig. 1 *B*), is collected with a syringe. Cell counts in this layer are high enough to warrant its use as a good source of functional thymus lymphocytes. (The autoradiographs showing H^3-leucine uptake into whole cells (Fig. 2) were obtained using this cell-rich interphase layer.)

Examination of the sediment at the bottom of the tube shows that it consists almost entirely of purified nuclei. A representative view is shown in Fig. 1 *C*. When this is compared with Fig. 1 *A*, it is clear that the nuclei collected through dense Ficoll solutions have much less adhering cytoplasm than is the rule in the original nuclear suspension. Traces of cytoplasm are still evident attached to nuclear membranes and within invaginations, and the sediment still contains a few whole cells. Several counts made in the electron microscope gave cell frequencies for different preparations ranging from 2 to 4 per cent of the number of nuclei. Two preparations showed 6 to 8 per cent whole cell contamination; these fractions could be further purified by increasing the density of the Ficoll-sucrose barrier or, alternatively, the nuclei could be separated from the cells by centrifugation in a Ficoll density gradient (*e.g.*, 10 to 40 per cent Ficoll in 0.25 M sucrose–3 mM $CaCl_2$.) (A Ficoll gradient was used recently by Birnstiel and Hyde for the isolation of pea nucleoli (26) which were capable of amino acid incorporation.)

Nuclei isolated by the Ficoll layering or gradient techniques retain some of their original capacity to incorporate isotopic amino acids into protein, and this activity is discussed below in connection with autoradiography and with measurements of over-all isotopic amino acid incorporation into the proteins of the nuclear sediment.

NUCLEAR PURIFICATION USING DEXTRAN:

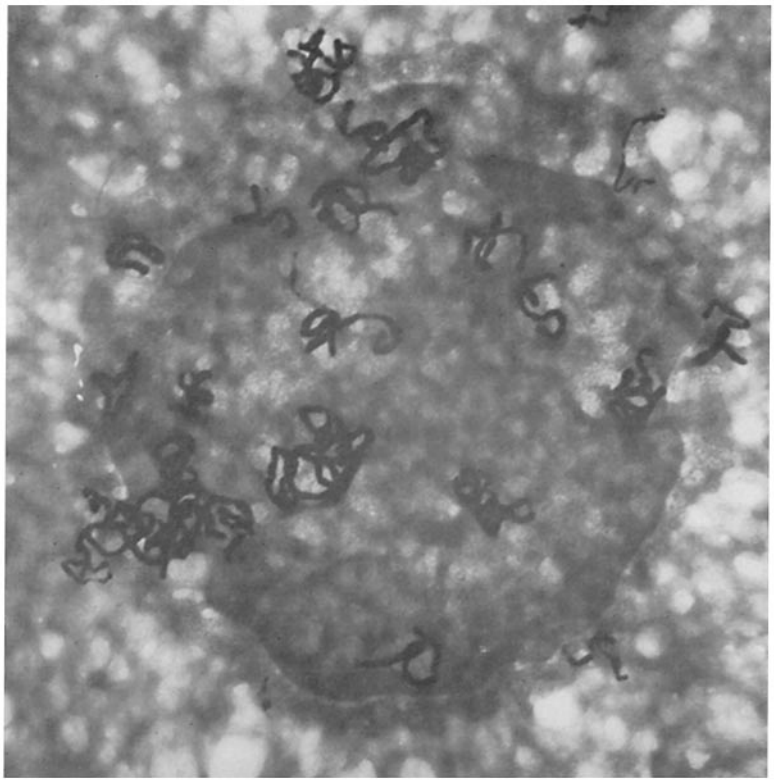

FIGURE 2 Electron microscope autoradiograph showing grain distributions over the nucleus and cytoplasm in a thin section of an intact cell, following incubation in the presence of leucine-4,5-H^3 for 30 minutes at 37°. Note the nuclear localization of many of the grains. × 21,500.

Calf thymus nuclear fractions have also been purified by layering over dense solutions of Dextran 500 (Pharmacia, Ltd.), under conditions similar to those described under *Ficoll-Layering Procedures*. Because dextran solutions of the required density are more viscous, centrifugation takes more time. Nuclear ATP levels usually fall as a result of prolonged anaerobiosis during centrifugation and this results in a lowered capacity to incorporate amino acids into protein (27).

However, dextran itself does not appear to be toxic to nuclei, and the use of zonal centrifugation (28) in 30 per cent dextran–0.25 M sucrose has recently been found to yield purified nuclear frac-

tions capable of incorporating leucine, glycine, and lysine (29).

Incubation Procedure

Nuclear fractions prepared by the hypotonic isolation procedure, and the sediments purified by the Ficoll layering technique were resuspended in 0.25 M sucrose–3 mM $CaCl_2$ to give a final concentration of approximately 40 mg (dry weight) per ml of suspension.

In amino acid incorporation experiments the nuclei were incubated at 37° in the presence of isotopically labeled amino acid, buffer, salts, and added metabolites, as follows: each incubation vessel contained (1) 1.0 ml of nuclear suspension, (2) 0.5 ml of 0.1 M sodium phosphate–0.25 M sucrose buffer, pH 6.75, (3) 0.4 ml of 0.1 M glucose solution containing 3.75 mg NaCl and 4.19 mg $MgCl_2 \cdot 4H_2O$ per ml, and (4) 0.1 ml of water containing the isotopic amino acid. The amino acids used were DL-alanine-1-C^{14} (4.42 mc/mmole), DL-lysine-1-C^{14} (HCl) (9.0 mc/mmole), DL-arginine-G-C^{14} (HCl) (4.9 mc/mmole), and DL-tryptophan-2-C^{14} (0.5 mc/mmole). Usually about 2×10^6 counts were added for each ml of nuclear suspension.

It should be stressed that, at this pH and in the presence of sufficient divalent cations (Mg^{++} and Ca^{++}), most nuclei remain intact throughout a 30-minute incubation (see below). NaCl is added to permit maximum amino acid transport (30).

In some experiments the incubation mixture contained beef pancreatic ribonuclease (Worthington Biochemical Corp., Freehold, New Jersey) at a final concentration of 500 to 1000 μg/ml. This high concentration of RNase was added to inhibit amino acid incorporation by cytoplasmic ribosomes and by ribosomes attached to the outer nuclear membrane. (It was shown previously that amino acid uptake by intranuclear ribosomes is not inhibited by RNase under these conditions (2, 31), although DNase does inhibit protein and RNA synthesis in nuclei (2, 9, 32).)

When puromycin was added to inhibit nuclear protein synthesis, it was used as the hydrochloride and dissolved in the buffered sucrose medium to give a final concentration of 100 μg/ml of incubation mixture.

On Nuclear Stability during Incubation

In a recent report (17) Kodama and Tedeschi have described an extensive breakdown of isolated calf thymus nuclei during incubation at 37°. They reported that "a large clump of material was invariably present," but went on to observe in the electron microscope that there had been a selective destruction of nuclei with concomitant increases in the proportion of remaining cells.

It should be pointed out that the incubation medium used in their experiments differs in two important respects from media used in this laboratory since 1957 (see reference 2, p. 485, (30–31, 38)). First, they omitted the divalent cation, Mg^{++}, and they worked at pH values (pH 6.3 and 7.4) which we have employed only rarely. Under the circumstances, it is not surprising that clumping and dissolution of the nuclei occurred. We pointed out this risk in 1957, in the statement that ". . . at the higher pH (7.3) . . . nuclei formed a heavy gel which was difficult to centrifuge and which precluded a clean separation of nuclei from the surrounding medium" (2). Naturally, since in tracer experiments the reliability of the results depends on being able to wash out the radioactive precursor, flasks containing clumps had to be discarded. For this reason, we introduced a buffer at pH 6.75 and increased the concentration of divalent cations, both of which stabilize the nuclei.

In our experience, clumping is an extreme manifestation of nuclear breakdown and must be avoided. In the absence of sufficient divalent cations and especially at higher pH values, the nucleohistone gel within the nuclei tends to expand; on swelling, it ruptures the nuclei and envelops surrounding particles, including whole cells. The more extreme the clumping, the greater the nuclear breakdown. In some experiments of Kodama and Tedeschi, clumping was so severe that less than 10 per cent of the original number of particles were left in suspension (17). It is not surprising that the ratio of cells to nuclei became much higher after incubation under these conditions.

In our experiments, using sufficient Mg^{++} ions in the incubation medium, the nuclei are far more stable. They are stable in phosphate buffers at pH 6.75 and in "tris" buffers at pH 7.1. This is shown by the data in Table III, which summarizes electron microscope counts of the number of cells and broken cells present in nuclear suspensions before and after 30 to 40 minutes' incubation. It is clear that, even at the end of the incubations, nuclei comprised 87 to 94 per cent of the particles in suspension.

On more prolonged incubations, nuclear breakdown becomes more pronounced. DNA and its breakdown products appear in the medium; this can be followed easily by measuring the E_{260} of the supernatant fluid after centrifuging down the nuclei. Based on DNA recoveries in the sedimented nuclei, breakdown does not exceed 10 to 15 per cent in 30 minutes' incubation.

Preparation and Counting of Nuclear Proteins

Following incubation, the nuclei were purified by the sucrose-layering or Ficoll-layering techniques and fractionated to yield the arginine-rich and lysine-rich histones and residual non-histone proteins. The

lysine-rich fraction (f1 histone) was prepared by extraction of the purified nuclei in 5 per cent perchloric acid, according to the method of Johns and Butler (33). The arginine-rich (f3) fraction was isolated from nuclei previously washed with 80 per cent ethanol, and then extracted with 80 per cent ethanol-20 per cent 1.25 N HCl (34).

In some cases these histone fractions were further purified by chromatography on columns of carboxymethylcellulose, using NaCl gradients at pH 4.2 to elute the lysine-rich histones and 0.1 N HCl to elute the arginine-rich components (35). The isolated histones were washed with acetone and plated for counting as described previously (2).

TABLE III

Cell Counts on Calf Thymus Nuclear Suspensions Before and After Incubation

Preparation*	Number of cells + Broken cells per 100 nuclei		Number of particles counted in incubated samples
	Before incubation	After incubation‡	
1	4.1	11.0	99
2	6.3	13.0	116
3	4.4	6.4	249
4	2.5	5.8	38
5	4.4	11.0	190

* All preparations made by the hypotonic blending procedure described under Methods.

‡ Incubation for 30 to 40 minutes at 38°, 100 shaking cycles/min., in medium described under Methods.

After extraction of the histones, the nuclear residues were treated with 16 per cent trichloroacetic acid (TCA) for 15 minutes at 90° to remove nucleic acids and polysaccharides. The residues were washed repeatedly with 16 per cent TCA, with a 2:2:1 ethanol-ether-chloroform mixture (to remove lipids) and with ether. The residual protein was dried. resuspended in acetone, and plated for counting (2).

Radioactivity measurements were made using a Nuclear-Chicago thin-window, gas-flow counter with coincidence correction for reducing background to 1.4 CPM or lower. The observed counts were corrected for self-absorption according to the method of Schweitzer and Stein (36).

Preparation of Samples for Electron Microscopy and Autoradiography

In general, all preparations were fixed by adding to the final suspension of nuclei (or cells) a volume of 5 per cent aqueous osmium tetroxide sufficient to give an approximate final concentration of 0.5 per cent. Fixation was carried out at 0° for 20 to 30 minutes, during which time the preparations were centrifuged in a clinical centrifuge. The sedimented nuclear pellet was broken into small pieces, dehydrated in an ethanol series and propylene oxide, and embedded in Epon 812.

In most experiments, samples of the pellet were selected at random for sectioning. In some experiments, comparisons were made between the upper and lower layers of the nuclear sediment and failed to reveal a difference between them. (An agar-embedding technique which allows a uniform sampling of thymus nuclear suspensions has recently been described by Kodama and Tedeschi (17) and should prove very useful.)

Sections were cut on a Cambridge Huxley-Model microtome, stained for 30 minutes in 1 or 2 per cent uranyl acetate, and examined in either an RCA EMU-3F or a Siemens Elmiskop I.

Electron microscope autoradiographs were prepared according to the method of Caro and van Tubergen (37). After incorporation of the tritiated leucine, the nuclei were washed to remove free leucine, then fixed, embedded, and sectioned as described above. The sections were covered with liquid photographic emulsion (Ilford L-4) and exposed for 1½ and 2 months. They were developed in Microdol-X and stained with uranyl acetate. Grain counts were made over nuclei, over whole cells, and over the nuclei in whole cells. An average of 6.1 grains per field was counted over the particles. A background of only 1.2 grains per field was observed over empty spaces in the section.

RESULTS

Evidence for Nuclear Protein Synthesis in vitro

In previous work on amino acid uptake by isolated thymus nuclei *in vitro*, many different types of evidence were presented to show that nuclei can, in fact, synthesize protein autonomously, and that they utilize a biosynthetic pathway involving amino acid activation by ATP (38, 39), amino acid transfer to low-molecular-weight RNAs (38, 39), and peptide bond formation on ribonucleoprotein particles ("nuclear ribosomes") (31, 7). Much of this evidence is assembled and summarized in the Discussion.

New evidence for autonomous nuclear protein synthesis, now to be described, includes these demonstrations: (*a*) that nuclei purified by layering over dense Ficoll solutions can incorporate amino acids into their proteins *in vitro*; (*b*) that

nuclei purified by sucrose-layering techniques after labeling experiments contain radioactive basic proteins (arginine-rich and lysine-rich histones) known to be localized in the chromosomes; (*c*) that the addition of mixed cytoplasmic fractions to the nuclei does not augment amino acid uptake; and (*d*) that amino acid uptake, as observed in autoradiographs under the electron microscope, specific activities of the proteins and the number of cells present in the fraction tested.

It is clear that the purified nuclei continue to incorporate radioactive amino acids into their proteins. As expected, the intact cell fractions are much more active in protein synthesis than are the isolated nuclei. This is due in part to the greater intrinsic activity of the intact cell (see below),

TABLE IV

Alanine-1-C^{14} Incorporation by Thymus Nuclei and Cell-Rich Fractions Purified by the Ficoll-Layering Procedure

Conditions of experiment			Specific activity of protein in		Cell count in	
	Sucrose conc'n. during blending	Incubation time	Nuclei	Cell-rich fraction	Nuclear fraction	Cell-rich fraction
	M	*min.*	CPM/*mg*	CPM/*mg*	*per cent*	*per cent*
Nuclei purified in Ficoll *before* incubation*						
A	0.25	30	65.8	508.0	3.7	87.8
B	0.25	30	133.0	415.0	6.1	81.0
C	0.25	30	95.5		4.4	
	0.20	30	42.1		4.4	
Nuclei purified in Ficoll *after* incubation‡						
D	0.21	30	190.0	532.0	4.4	81.0
	0.21	60	593.0	—	4.4	81.0

* Nuclei were isolated in isotonic (0.25 M) sucrose and layered over dense Ficoll in isotonic sucrose (as described under Methods) to yield a nuclear sediment and a cell-rich fraction. These were suspended in the incubation medium described and incubated with alanine-1-C^{14} for 30 minutes.

‡ Nuclei isolated after hypotonic blending (0.21 M sucrose) and isotonic centrifugation (0.25 M sucrose) were incubated with alanine-1-C^{14} and then purified by layering over Ficoll. In this experiment, the nuclear fraction before purification showed protein specific activities of 235 CPM/mg at 30 minutes, and 749 CPM/mg at 60 minutes.

takes place *within* the nucleus and not just on its periphery.

Amino Acid Incorporation by Nuclei Purified in Ficoll

Nuclear fractions prepared in isotonic sucrose (Fig. 1 *A*) were layered over the dense Ficoll-sucrose mixture and centrifuged to yield a cell-rich fraction (Fig. 1 *B*) and a nuclear sediment (Fig. 1 *C*). These subfractions were resuspended in 0.25 M sucrose-3 mM $CaCl_2$ and incubated with alanine-1-C^{14} as described under Methods. After 30 and 60 minutes' incubation, the total mixed protein of the nuclei (or cells) was prepared for counting. The results of several such experiments are summarized in Table IV. The table lists the but it also reflects some nuclear damage caused by exposure to Ficoll. (Evidence for Ficoll toxicity is presented under Methods.)

The use of Ficoll gradients to remove cells has made possible a direct comparison of the activities of nuclei prepared under isotonic conditions throughout (*i.e.* always using 0.25 M sucrose) and of nuclei isolated after blending in hypotonic (0.20 M) sucrose solution. In both cases, the nuclear fraction was purified in Ficoll and then tested for its ability to incorporate alanine-1-C^{14} into protein. The results are presented in Table IV *C*; they show that nuclei exposed to 0.20 M sucrose for only 3 minutes before readjusting to isotonicity are only about half as active as nuclei maintained in an isotonic environment throughout the isolation.

Table IV also lists some related observations on the radioactive protein contents of nuclei which were purified in Ficoll *after* incubating the original nuclear suspension with C^{14}-alanine. It can be calculated that about 80 per cent of the total counts were recovered in the nuclear sediment after centrifugation. Less than 20 per cent of the counts appeared in the cell-rich fraction, which in this case contained 81 per cent intact thymocytes. However, the cell fraction is small, and, on a specific activity basis, cells are about 3 times more active than are the free nuclei in amino acid uptake. The higher activity of intact cells is in accord with autoradiographic evidence to be presented below.

Histone Synthesis in Isolated Nuclei

As an alternative proof of nuclear localization of radioactive proteins after *in vitro* labeling experiments, we have measured amino acid uptake into the histones, selecting in particular the lysine-rich (f 1) and the arginine-rich (f 3) fractions described by Johns and Butler (33) and by Johns *et al.* (34).

In these experiments, the cells were broken by blending in hypotonic sucrose and the nuclei isolated as described under Methods. The nuclei were then incubated in the presence of different C^{14}-labeled amino acids: lysine, arginine, valine, alanine, or tryptophan. (The latter was used to test for adsorption or contamination by other proteins, since the histones do not contain tryptophan (58).)

After incubation, the suspension was layered over dense sucrose solutions (usually by Sucrose Method II) and centrifuged to remove cells and tabbed nuclei. The histones were then prepared from the purified nuclear sediment, using 5 per cent perchloric acid for the extraction of the f 1 fraction (33) and 80 per cent ethanol −20 per cent 1.25 N HCl for the extraction of the f 3 fraction.

The results are presented in Table V, which lists the specific activity of the different histones and, for purposes of comparison, the activities of the total mixed proteins of the purified nuclei, and of the proteins remaining after extraction of the histones. It is evident that appreciable histone labeling has occurred in the isolated nuclei, but it is also clear that histone synthesis does not proceed as rapidly as does the synthesis of other proteins in the thymocyte nucleus. This finding is in accord with earlier experiments on protein synthesis in thymus nuclei (2) and it agrees with studies of histone turnover in the nuclei of nondividing cells (46). (It should be pointed out, however, that histone labeling is very much accelerated in dividing cells, when large quantities of the histones must be made to keep pace with chromosome replication (47–50).)

The ability of nuclei to incorporate amino acids *in vitro* into characteristic chromosomal proteins, such as histones, is especially convincing evidence for *intranuclear* protein synthesis. Localization of the synthetic machinery *within* the nuclei is also indicated by experiments in which ribonuclease added to the medium failed to stop amino acid uptake. In one such experiment, RNase was added to give a final concentration of 1000 μg/ml, and the nuclei were incubated for 10 minutes at 37°. At that time C^{14}-alanine was added and the nuclei were incubated for an additional 30 minutes, still in the presence of ribonuclease. The nuclei were purified by the sucrose double-layering technique (Method II) and the protein was prepared for counting. The specific activity of the protein in the RNase treated nuclei was 101.4 CPM/mg; in appropriate control nuclei (which had been preincubated, but without added RNase) the protein contained 106.8 CPM/mg. Thus less than 5 per cent inhibition had occurred even at this high enzyme concentration. (The concentration of RNase used was 50 to 100 times that required to block amino acid uptake into "microsome" fractions from liver (40, 41).) The results agree with earlier findings that RNase under these conditions does not degrade intranuclear ribosomes (31).

Effects of Puromycin on Nuclear Protein Synthesis

We observed earlier that the antibiotic, puromycin, inhibits nuclear protein synthesis almost completely (39). Yarmolinsky and de la Haba had previously shown its effect on cytoplasmic incorporating systems and pointed out its close structural resemblance to the terminal end of amino acid–transfer RNAs (51). More recent studies by Allen and Zamecnik indicate that Puromycin displaces the growing peptide chain from its site of formation on the ribosomes (52). Thus, its effect in the nucleus can be taken as evidence that nuclear protein synthesis also employs a mechanism involving transfer RNAs and ribosomal sites of peptide bond formation.

TABLE V

Effects of Puromycin and Dinitrophenol on Amino Acid Uptake into Histones and Other Proteins of Thymus Nuclei

	Conditions of experiment				Specific activity of nuclear proteins			
	Isolation method*	Incubation time	C^{14}-labeled amino acid added	Inhibitor added	Total protein	Lysine-rich histones	Arginine-rich histones	Residue proteins‡
		min.			CPM/mg	CPM/mg	CPM/mg	CPM/mg
A	SL-II	15	Arginine-G-C^{14}	None			11.0	27.2
		30	Arginine-G-C^{14}	None			34.0	85.5
		45	Arginine-G-C^{14}	None			61.8	156.8
	SL-I	15	Tryptophan-2-C^{14}	None			0.0	3.2
	SL-II	30	Tryptophan-2-C^{14}	None			0.7§	7.4
B	SL-II	60	Arginine-G-C^{14}	None	319.0	19.5		46.4
		60	Arginine-G-C^{14}	Puromycin (100 μg/ml)	71.5	14.3		12.0
C	HS	60	Lysine-1-C^{14}	None	277.7	142.2		302.8
		60	Lysine-1-C^{14}	Puromycin	32.0	75.5		32.5
D	HS	60	Lysine-1-C^{14}	None	286.6	135.8		274.4
		60	Lysine-1-C^{14}	Puromycin	33.5	69.1		30.1
		60	Lysine-1-C^{14}	DNP (2×10^{-4} M)	42.6	22.1		43.2
E	HS	60	Lysine-1-C^{14}	None	261.6		156.3	426.1
		60	Lysine-1-C^{14}	Puromycin	18.9		27.4	22.9
F	HS	60	Alanine-1-C^{14}	None	157.6	63.9		188.4
		60	Alanine-1-C^{14}	Puromycin	21.2	41.3		18.6

* *Isolation Methods—SL-I, II:* nuclei isolated under isotonic conditions, incubated, and then purified by layering over one or two zones of dense sucrose solution (See Methods). HS: cells broken in hypotonic sucrose before centrifuging down nuclei in 0.25 M sucrose.

‡ Protein residue remaining after extracting the lysine rich histones in 5 per cent PCA (33), or after extracting arginine-rich histones in ethanolic-HCl (34).

§ These counts were removed when the histone was subsequently purified by chromatography (34).

When added to thymus nuclei, puromycin inhibits the synthesis of histones as well as that of other nuclear proteins. The magnitude of the effect is shown in Table V. At puromycin concentrations of 100 μg/ml, about 90 per cent inhibition of total protein synthesis is observed; a correspondingly high level of inhibition occurs in the synthesis of the arginine-rich (f 3) histone fraction.

Puromycin also inhibits amino acid uptake into the lysine-rich (f 1) histone fraction, but less effectively. The data in Table V indicate some of the differences in the degree of inhibition of the synthesis of these different histones. It is generally observed that C^{14}-amino acid uptake into the f 1 fraction is far less sensitive to puromycin than is uptake into the f 3 histones. The reason for the difference is not yet clear, but it has been confirmed many times, using different C^{14}-amino acids. We considered the possibility that the perchloric acid extraction procedure used to prepare the lysine-rich histones might have introduced an artifact by fixing radioactive amino acids on the f 1 histones by some process which does not require the formation of peptide bonds. This is unlikely because the incorporations ob-

served are clearly energy-dependent. The presence of 2×10^{-4} M dinitrophenol blocks nuclear ATP synthesis and inhibits amino acid uptake into all the histones by 89 per cent.

It may be suggested that the lysine-rich histones contain peptide bonds which are not synthesized by the usual biosynthetic route involving transfer-RNAs and ribosomes. Among the possible alternative bonds are those linking amino acid carboxyl groups to the epsilon amino groups of lysine. This possibility is subject to direct test using end-group reagents (such as cyanate (53)) to determine the nature and number of the amino ends in the protein. Such experiments are now in progress. An alternative view, that part of the lysine-rich histone represents a complex of small peptides linked to protein chains, is also under consideration.

In any case, the fact that puromycin does inhibit the synthesis of the arginine-rich histones and other histones in isolated nuclei indicates that these basic proteins are probably synthesized on ribosomal sites, as are most of the other proteins of the nucleus.

Attempts to Augment Nuclear Amino Acid Uptake by Adding Cytoplasm

It has sometimes been suggested that cytoplasmic contamination may have an indirect role in promoting nuclear amino acid incorporation, for example, by supplying necessary co-factors, or helping to preserve the integrity of the nuclei (*e.g.*, reference 17). Some direct tests of this hypothesis are possible; so far, the results have proved negative.

For example, in one experiment, the cytoplasmic fraction remaining after low speed centrifugation of the nuclei was added back to aliquots of the final nuclear suspension. Six mg (dry weight) of cytoplasm in 2.0 ml of isotonic sucrose was added to 30 mg portions of nuclei. Leucine-1-C^{14} uptake in nuclei receiving this total cytoplasmic supplement was compared with that of control nuclei receiving an equal volume of isotonic sucrose. The results, after 30 minutes' incubation, showed 11,160 counts in the control nuclei and 7600 counts in the nuclei receiving the cytoplasmic supplement. Thus, there was no indication that the cytoplasmic fraction is able to augment protein synthesis in the isolated nuclei.

High resolution autoradiographs also support the view that the presence of a cytoplasmic tab is not a necessary prerequisite for amino acid uptake in the isolated nucleus (see below).

Autoradiographic Evidence for Intranuclear Protein Synthesis

High resolution autoradiography using the electron microscope has allowed a closer inspection of the sites of amino acid incorporation in the isolated nuclei and in whole cells. In these experiments, the nuclear fraction was prepared by homogenizing the tissue briefly in hypotonic (0.21 M) sucrose and then centrifuging under isotonic conditions (see Methods). The nuclear sediment was resuspended in 0.25 M sucrose–3 mM $CaCl_2$ and added to the buffered incubation medium containing 100 μc of leucine-4,5-H^3 per ml. After 30 and 60 minutes' incubation at 37°, the suspension was carefully layered over a dense solution of Ficoll in isotonic sucrose and centrifuged to yield a purified nuclear sediment and an interphase layer containing some whole cells.

Autoradiographs of the nuclei and of some intact cells from the same labeling experiment were prepared as described under Methods. The results are presented in Figs. 2 to 4, which show the grain distribution over thin sections (about 600 A thick) of the labeled cells and nuclei.

Fig. 2 is a representative view showing the distribution of radioactive protein in an intact cell. It is clear that, in the whole thymocyte, both nucleus and cytoplasm are very active in H^3-leucine uptake; on the average a total of 10.9 grains per cell was observed. The internal distribution of the grains is of some interest, because 7.1 grains appeared over the nuclei of these cells. Thus, much of the protein synthetic activity of the cell is localized in its nucleus (at least under these *in vitro* conditions). This finding is in agreement with early experiments on amino acid uptake by cells *in vivo*, in which the "residual" proteins of the chromosomes showed surprisingly high rates of synthesis and turnover (46).

Figs. 3 and 4 show the presence of radioactive protein in thin sections of free, isolated nuclei. Since amino acid incorporation occurred *in vitro* after isolation, this is unequivocal evidence for the synthesis of labeled proteins in the isolated nucleus. On the average, the free nuclei contained 3.7 grains per particle. When this is compared with the 7.1 grain average over nuclei in intact cells it is obvious that the isolated nucleus is not doing as well in protein synthesis as is a nucleus

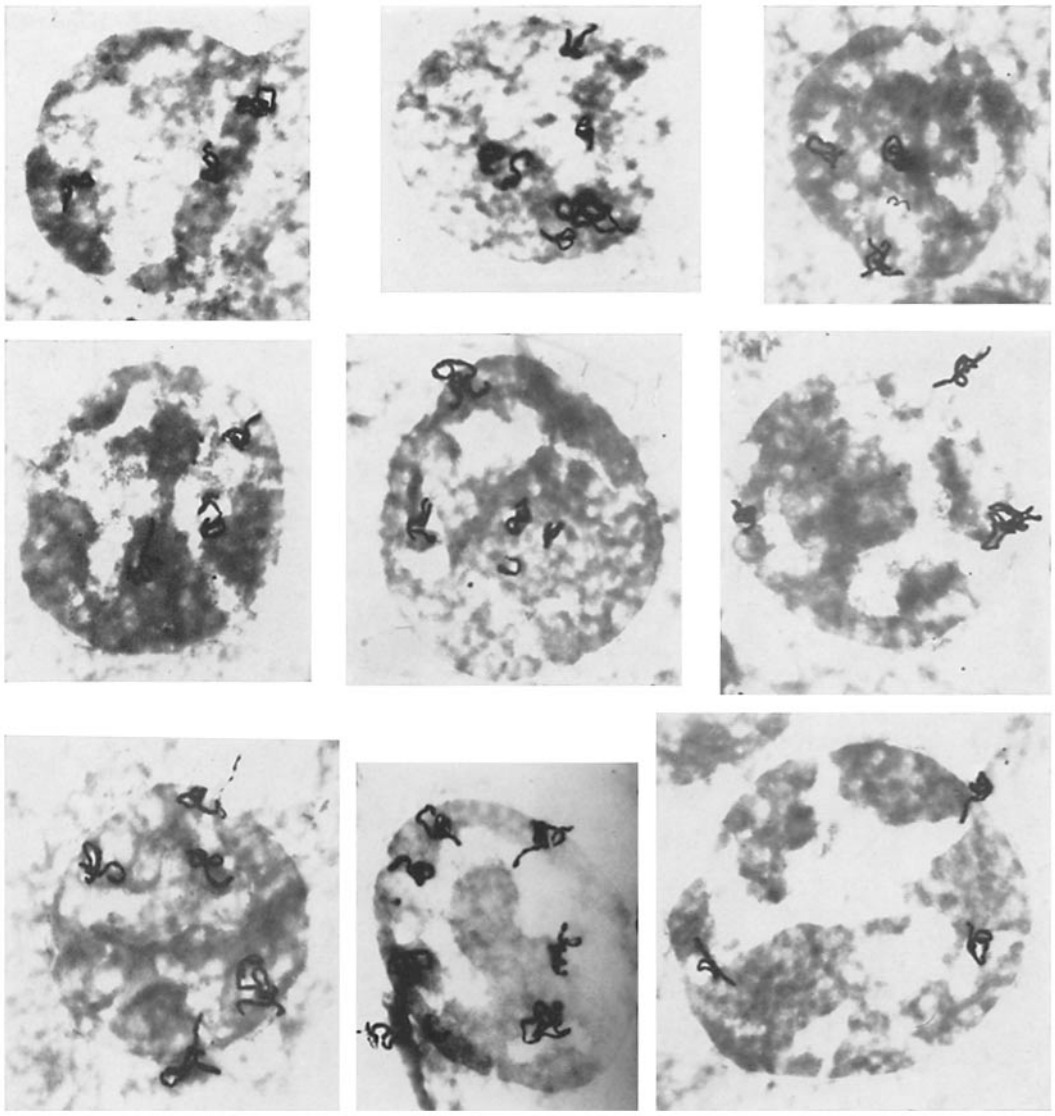

FIGURE 3 Electron microscope autoradiographs showing grain distributions over isolated cell nuclei following incubation *in vitro* with leucine-4,5-H^3 and subsequent purification in Ficoll. Note that most of the grains are centrally located over these thin sections (600 A) through the free nuclei. × 12,000.

in an unbroken cell. This is a direct verification of the biochemical evidence presented earlier that intact cell fractions are about 3 times more active in amino acid uptake than are isolated nuclei. Nevertheless, the retention of this much synthetic activity in an isolated subcellular particle is impressive.

(It is assumed in this discussion that the presence of H^3-leucine in the fixed and washed nuclei is an indication of protein synthesis. Chemical evidence that labeled amino acids become incorporated into peptide bonds was presented earlier (2); tryptic digestion releases at least 8 peptides containing radioactive leucine.)

It should be stressed that most of the grains over nuclei occur over the central area and not in a ring around the periphery, and it follows that amino acid incorporation by isolated nuclei represents *internal* protein synthesis and is not simply due to the activity of ribosomes on the

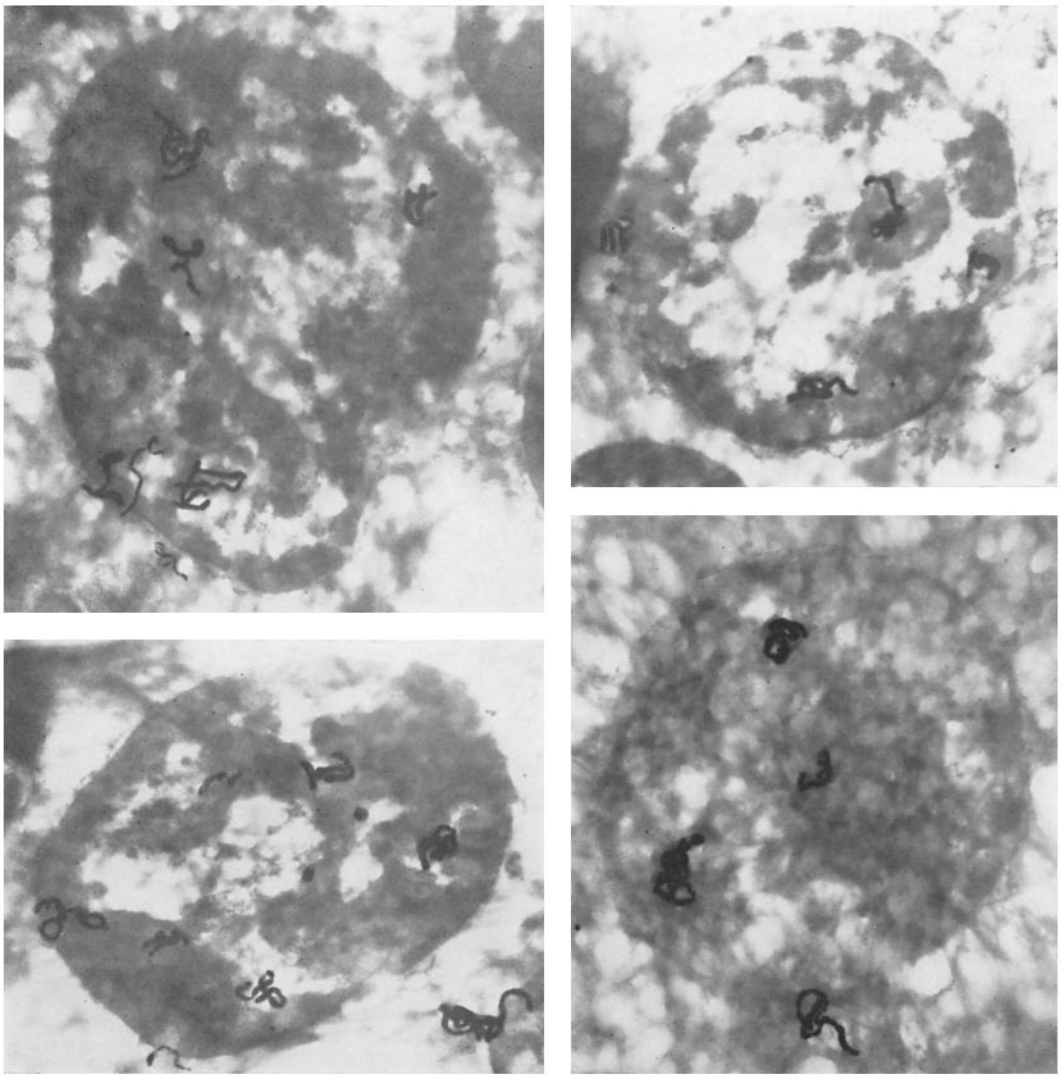

FIGURE 4 Electron microscope autoradiographs showing grain distributions over isolated cell nuclei purified in Ficoll after incubation with H^3-leucine. As in Fig. 3, a high proportion of the grains are localized over "condensed" areas of the chromatin. × 16,200.

outer or inner nuclear membranes. Of course, some grains can be seen in close proximity to the nuclear envelope, and they occasionally occur in cytoplasmic tabs adhering to some of the nuclei, but the presence of a tab is not a prerequisite for nuclear labeling. Many of the grains occur over "condensed" areas of the nuclear chromatin; this is of interest because this is not the case in RNA-labeling experiments. Autoradiography after incubations with H^3-uridine shows most of the radioactive RNA to be localized in the comparatively clear intranuclear spaces (61).

Few, if any, grains have been localized over nuclear invaginations following H^3-leucine uptake. Other autoradiographs using H^3-glycine as a protein precursor have yielded additional evidence for intranuclear protein synthesis, with grains centrally localized over thin sections of the isolated cell nuclei.

Thus, the results of high resolution autoradiog-

raphy using the electron microscope confirm in detail the work of Ficq and Errera (13) and our own autoradiographs of C^{14}-labeled thymus nuclei under the light microscope (2).

DISCUSSION

It was mentioned earlier that previous work on amino acid uptake by isolated thymus nuclei had made it exceedingly probable that free nuclei can synthesize protein autonomously, and that they utilize a biosynthetic pathway resembling that of the cytoplasm; *i.e.*, amino acid activation by ATP (38, 39), amino acid transfer to low-molecular-weight RNA's (38–39) and formation of peptide bonds on intranuclear ribosomal particles (31, 7).

Among the experimental facts supporting this conclusion, the following are especially significant:

(1) Amino acid–activating enzymes have been localized in thymus nuclei and in other nuclear preparations of high purity (38, 42). Since the nuclei were prepared from different tissues in non-aqueous media as well as in isotonic and hypertonic sucrose solutions (38), the intranuclear localization of the enzymes seems certain.

(2) Nuclei use endogenous ATP for amino acid activation (27). External ATP does not enter the nucleotide pool of the isolated nucleus, but is rapidly hydrolyzed instead (21).

(3) Leucyl-RNA complexes have been isolated from thymus nuclei and characterized chemically (43).

(4) Amino acyl–transfer RNA's have been shown to occur in nuclei (38) and to be synthesized there (44).

(5) Amino acid incorporation by thymus nuclear suspensions is *not* affected when ribonuclease is added to the medium (2), but cytoplasmic protein synthesis (*e.g.*, amino acid uptake by liver microsomes) is easily inhibited under these conditions (40, 41). The failure of ribonuclease to block nuclear protein synthesis is in line with the observation that ribonucleoprotein particles within the cell nucleus are not destroyed when RNase is added to the medium (31).

(6) Amino acid uptake into nuclear proteins requires a prior synthesis of RNA (2), and the latter process is DNA-dependent (9). As a result, amino acid uptake into nuclear proteins is markedly inhibited when deoxyribonuclease acts on the nuclei and, by removing the necessary DNA "primers," stops the synthesis of "messenger" RNAs (9). The removal of DNA also releases the histones which inhibit RNA, protein, and ATP synthesis (8), (but which do not leak out of the nuclei into the medium).

(7) Amino acid incorporation by nuclear suspensions proceeds best in the presence of sodium ions (2, 30), while cytoplasmic protein synthesis is strongly potassium-ion dependent (*e.g.*, reference 45).

(8) Low power autoradiography of nuclear suspensions after the uptake of C^{14}-labeled amino acids showed that the isotope was localized in (or around) the isolated nuclei (13, 2). However, it might be objected that because of the difficulty in distinguishing nuclei from small thymocytes (17), or because of the relatively long range of β particles from C^{14} and the low resolution of the light microscope, these autoradiographs do not prove the *intranuclear* localization of the isotope. The high resolution autoradiographs just presented, using H^3-leucine (with a short range β) and the electron microscope, should dispel these doubts about the validity of the earlier C^{14}-autoradiographs, and make it clear that thymus nuclei do, in fact, synthesize protein autonomously.

Amino acid uptake is not limited to thymus nuclei. It has been observed in nuclei from other cell types and from other species, *e.g.*, mouse AKR lymphoma (1), rat liver (54), a rat hepatoma (55), pea seedlings (56), and insect salivary glands (57). Low magnification autoradiographs showing the nuclear localization of the isotope have been published (55, 57, 60, 62) and they lend further support to the general conclusion that the presence of protein synthetic machinery within cell nuclei is a necessary and widespread phenomenon.

The authors wish to extend their thanks to Dr. Dan H. Moore for his generous permission to use the RCA EMU-3F electron microscope and darkroom facilities. We are also indebted to Mrs. Sarah C. Reynolds and to Mr. Barry L. Horowitz for their skillful and conscientious technical assistance.

This research was supported in part by a grant (GM-04919) from the United States Public Health Service.

Received for publication, July 23, 1963.

BIBLIOGRAPHY

1. ALLFREY, V. G., *Proc. Nat. Acad. Sc.*, 1954, **40,** 881.
2. ALLFREY, V. G., MIRSKY, A. E., and OSAWA, S., *J. Gen. Physiol.*, 1957, **40,** 451.
3. ALLFREY, V. G., and MIRSKY, A. E., *Proc. Nat. Acad. Sc.*, 1957, **43,** 821.
4. SIBATANI, A., DEKLOET, S. R., ALLFREY, V. G., and MIRSKY, A. E., *Proc. Nat. Acad. Sc.*, **48,** 471.
5. BREITMAN, T., and WEBSTER, G. C., *Nature*, 1959, **184,** 637.
6. WANG, T.-Y., *Biochim. et Biophysica Acta*, 1961, **49,** 108.
7. WANG, T.-Y., *Biochim. et Biophysica Acta*, 1961, **49,** 239.
8. ALLFREY, V. G., LITTAU, V. C., and MIRSKY, A. E., *Proc. Nat. Acad. Sc.*, 1963, **49,** 414.
9. ALLFREY, V. G., and MIRSKY, A. E., *Proc. Nat. Acad. Sc.*, 1962, **48,** 1590.
10. STERN, H., and MIRSKY, A. E., *J. Gen. Physiol.*, 1953, **37,** 177.
11. ALLFREY, V. G., and MIRSKY, A. E., *Science*, 1955, **121,** 879.
12. HOPKINS, J. W., Reactions Governing Amino Acid Incorporation into Proteins of the Cell Nucleus, Ph.D. Dissertation, The Rockefeller Institute, New York, 1960.
13. FICQ, A., and ERRERA, M., *Exp. Cell Research*, 1958, **14,** 182.
14. ALLFREY, V. G., and MIRSKY, A. E., Biochemical properties of the isolated nucleus, *in* Subcellular Particles (T. Hayashi, Editor), New York, The Ronald Press, 1959, 186.
15. FEULGEN, R., and ROSSENBECK, H., *Z. physiol. Chem.*, 1924, **135,** 203.
16. LITTAU, V. C., unpublished observations, 1962.
17. KODAMA, R. M., and TEDESCHI, H., *J. Cell Biol.*, 1963, **18,** 541.
18. SCHNEIDER, R. M., and PETERMANN, M. L., *Cancer Research*, 1950, **10,** 751.
19. ROOF, B. S., and AUB, J. C., *Cancer Research*, 1960, **20,** 1426.
20. FISHER, H. W., and HARRIS, H., *Proc. Roy. Soc. London, Series B*, 1962, **156,** 521.
21. MCEWEN, B. S., ALLFREY, V. G., and MIRSKY, A. E., *J. Biol. Chem.*, 1963, **238,** 758.
22. MCEWEN, B. S., ALLFREY, V. G., and MIRSKY, A. E., *J. Biol. Chem.*, 1963, **238,** 2571.
23. MCEWEN, B. S., ALLFREY, V. G., and MIRSKY, A. E., *J. Biol. Chem.*, 1963, **238,** 2579.
24. CHAUVEAU, J., MOULÉ, Y., and ROUILLER, C., *Exp. Cell Research*, 1956, **11,** 317.
25. HOLTER, H., and MØLLER, K. M., *Exp. Cell Research*, 1958, **15,** 631.
26. BIRNSTIEL, M. L., and HYDE, B. B., *J. Cell Biol.*, 1963, **18,** 41.
27. OSAWA, S., ALLFREY, V. G., and MIRSKY, A. E., *J. Gen. Physiol.*, 1957, **40,** 491.
28. ANDERSON, N. G., and BURGER, C. L., *Science*, 1962, **136,** 646.
29. FISCHER, W. D., and CLINE, G. B., *Biochim. et Biophysica Acta*, 1963, **68,** 640.
30. ALLFREY, V. G., MEUDT, R., HOPKINS, J. W., and MIRSKY, A. E., *Proc. Nat. Acad. Sc.*, 1961, **47,** 907.
31. FRENSTER, J. H., ALLFREY, V. G., and MIRSKY, A. E., *Proc. Nat. Acad. Sc.*, 1960, **46,** 432.
32. SEKIGUCHI, M., and SIBATANI, A., *Biochim. et Biophysica Acta*, 1958, **28,** 455.
33. JOHNS, E. W., and BUTLER, J. A. V., *Biochem. J.*, 1962, **82,** 15.
34. JOHNS, E. W., PHILLIPS, D. M. P., SIMSON, P., and BUTLER, J. A. V., *Biochem. J.*, 1960, **77,** 631.
35. PHILLIPS, D. M. P., and JOHNS, E. W., *Biochem. J.*, 1959, **72,** 538.
36. SCHWEITZER, G. K., and STEIN, B. R., *Nucleonics*, 1950, **7,** 65.
37. CARO, L. G., and VAN TUBERGEN, R. P., *J. Cell Biol.*, 1962, **15,** 173.
38. HOPKINS, J. W., *Proc. Nat. Acad. Sc.*, 1959, **45,** 1461.
39. ALLFREY, V. G., HOPKINS, J. W., FRENSTER, J. H., and MIRSKY, A. E., *Ann. New York Acad. Sc.*, 1960, **88,** 722.
40. ALLFREY, V. G., DALY, M. M., and MIRSKY, A. E., *J. Gen. Physiol.*, 1953, **37,** 157.
41. KELLER, E. B., and ZAMECNIK, P. C., *J. Biol. Chem.*, 1956, **221,** 45.
42. GVOZDEV, V. A., *Biokhimiya*, 1960, **25,** 920.
43. HOPKINS, J. W., ALLFREY, V. G., and MIRSKY, A. E., *Biochim. et Biophysica Acta*, 1961, **47,** 194.
44. CHIPCHASE, M. I. H., and BIRNSTIEL, M. L., *Proc. Nat. Acad. Sc.*, 1963, **49,** 692.
45. SACHS, H., *J. Biol. Chem.*, 1958, **233,** 643.
46. ALLFREY, V. G., DALY, M. M., and MIRSKY, A. E., *J. Gen. Physiol.*, 1955, **38,** 415.
47. BLOCH, D. P., and GODMAN, G. C., *J. Biophysic. and Biochem. Cytol.*, 1955, **1,** 17.
48. BUSCH, H., DAVIS, J. R., and ANDERSON, D. C., *Cancer Research*, 1958, **18,** 916.
49. HOLBROOK, D. J., IRVIN, J. L., IRVIN, E. M., and ROTHERHAM, J., *Cancer Research*, 1960, **20,** 1329.
50. ROTHERHAM, J., IRVIN, J. L., IRVIN, E. M., and HOLBROOK, D. J., *Proc. Soc. Exp. Biol. and Med.*, 1957, **96,** 21.
51. YARMOLINSKY, M. D., and DE LA HABA, G., *Proc. Nat. Acad. Sc.*, 1959, **45,** 1721.

52. ALLEN, D. W., and ZAMECNIK, P. C., *Biochim. et Biophysica Acta*, 1962, **55**, 865.
53. STARK, G. R., and SMYTH, D. G., *J. Biol. Chem.*, 1963, **238**, 214.
54. ZALTA, J. P., ROZENCWAJG, R., CARASSO, N., and FAVARD, P., *Compt. rend. Acad. sc.*, 1962, **255**, 412.
55. VORBRODT, A., *Bull. Acad. Pol. Sc.*, 1960, **8**, 89.
56. BIRNSTIEL, M. L., CHIPCHASE, M. I. H., and HAYES, R. J., *Biochim. et Biophysica Acta*, 1962, **55**, 728.
57. SIRLIN, J. L., and SCHOR, N. A., *Exp. Cell Research*, 1962, **27**, 165.
58. MIRSKY, A. E., and POLLISTER, A. W., *J. Gen. Physiol.*, 1946, **30**, 117.
59. HUBERT, M. T., FAVARD, P., CARASSO, N., ROZENCWAJG, R., and ZALTA, P., *J. Micr.*, 1962, **1**, 435.
60. LOGAN, R., and DAVIDSON, J. N., *Biochim. et Biophysica Acta*, 1957, **24**, 196.
61. LITTAU, V. C., FRENSTER, J. H., ALLFREY, V. G., and MIRSKY, A. E., *Proc. Nat. Acad. Sc.*, in press.
62. LOGAN, R., FICQ, A., and ERRERA, M., *Biochim. et Biophysica Acta*, 1959, **31**, 402.