INCORPORATION OF TRITIATED CYTIDINE INTO RIBONUCLEIC ACID BY ISOLATED PEA NUCLEI

JOON H. RHO, Ph.D., and MARGARET I. CHIPCHASE

From the Division of Biology, California Institute of Technology, Pasadena

ABSTRACT

A new method for the isolation of the nuclei from plant tissue has been devised. This method consists in passing the plant tissue through a set of spring-loaded, counter-rotating rollers and collecting the liberated nuclei in sucrose solution containing calcium ions. A semi-automatic machine which couples the rollers with a special tissue chopping device has been constructed. It has been shown that nuclei obtained in this way actively incorporate cytidine-H^3 into RNA. This incorporation is increased in the presence of nucleoside triphosphates and an energy-regenerating system.

INTRODUCTION

With the recognition of the part played by the nucleus in the hereditary process it becomes exceedingly important to study the isolated nucleus as a means of distinguishing between nuclear and cytoplasmic processes. Several different methods of isolating intact nuclei have been reported and these various procedures were reviewed by several workers (1–5). The high speed Waring blendor was commonly used in early work on isolation of cell nuclei. However, due to the severe damage to cell constituents and the accompanying low yield of nuclei, many workers have tried to replace it with homogenizers in which optimal gaps, shearing forces, tissue concentration, and time of homogenization have been varied in efforts to get maximal cell breakage with minimal nuclear damage (6–12).

In the present work, we report a new method for isolation of intact nuclei. This method produces a relatively pure nuclear fraction in high yield. That the nuclei so obtained are physiologically active is demonstrated by their ability to incorporate tritiated cytidine and uridine into nuclear RNA.

MATERIALS AND METHODS

Plant Tissues

Pea seedlings, *Pisum sativum*, were grown in vermiculite in the dark for 4 days at 25°C. Apical sections 1.0 to 2.0 cm in length were harvested, thoroughly washed with distilled water, and treated with 0.5 per cent chlorox for 3 minutes. Tissues were then rinsed with cold (4°C) distilled water and stored in the cold for approximately 30 minutes. Immediately before grinding, the tissues were rinsed with a 0.003 M tris[1]-0.003 M $CaCl_2$ solution.

Extraction of Nuclei

The nuclei were released from the cell by means of the specially designed apparatus[2] illustrated in

[1]*Abbreviations:* tris, tris (hydroxymethyl) amino methane; RNA, ribonucleic acid; DNA, deoxyribonucleic acid; ATP, GTP, UTP, 5′-triphosphates of adenosine, guanosine, uridine; CPM, counts per minute.

[2] The authors are indebted to Mr. Frank Ostrander, instrument maker, Division of Biology, California Institute of Technology, for the development and construction of this semi-automatic machine, and to Dr. Clifford Sato, for his original suggestion of the squeezing method.

Figs. 1 through 3. The machine consists of two main parts, the tissue-squeezing rollers and the tissue-chopping device. The frame and rollers are constructed of aluminum. The tissue is brought to the rollers on an 8½-inch band of 100 per cent nylon marquisette mesh running along the tissue delivery panel (a 9 × 35 inch aluminum plate) at the rate of 3 inches per minute. The nylon band is initially rolled onto the first spool which has a capacity of about 200 yards. The nylon band, carrying the tissue, then passes under the stainless steel chopping blade which chops the tissue into sections of about 1 mm length. The blade is driven by a pair of heart-shaped cams located under the delivery panel with a lift of 1 inch so that the cutting period for the blade is actually a small fraction of that for one complete revolution. They are driven by a 1/10 horse power Bodine AC-DC motor, and a variable speed, ranging from 50 to 250 strokes per minute, is obtainable with the aid of a variac. It should be pointed out that the plant tissues on the nylon cloth are not severed completely, but that the level of the blade is adjusted to leave a bottom layer of uncut tissue.

The finely chopped tissue is covered by a second layer of nylon mesh fed from a second spool and the mesh-encased tissue passes between two squeezing rollers. The squeezing rollers are cylinders 8⅞ inches long and of 1⅞ inches diameter. They are of aluminum and may be clearly seen in Fig. 2. The upper roller is spring-loaded whereas the lower roller is fixed. During normal operation they are adjusted to maintain 0.011-inch clearance. These rollers are driven independently by a 1/25 horsepower AC motor through a series of reducing gears, and one complete revolution occurs every 2 minutes. The chains used are ¼-inch pitch single rollerless chains. A triangular stainless steel scraper is constructed under the two rollers so as to funnel the nuclei-rich exudate into a beaker. The nylon mesh, encasing the plant debris, is then wound onto a pickup spool equipped with a slip clutch attached to the shaft so that the pulling force is automatically adjusted to the speed of the squeezing rollers.

As the tissues are squeezed between the rollers, homogenization medium is added simultaneously from a burette. The flow rate is adjusted to give a final concentration in the homogenate of 0.25 M sucrose-0.003 M $CaCl_2$-0.006 M tris, pH 7.2. This

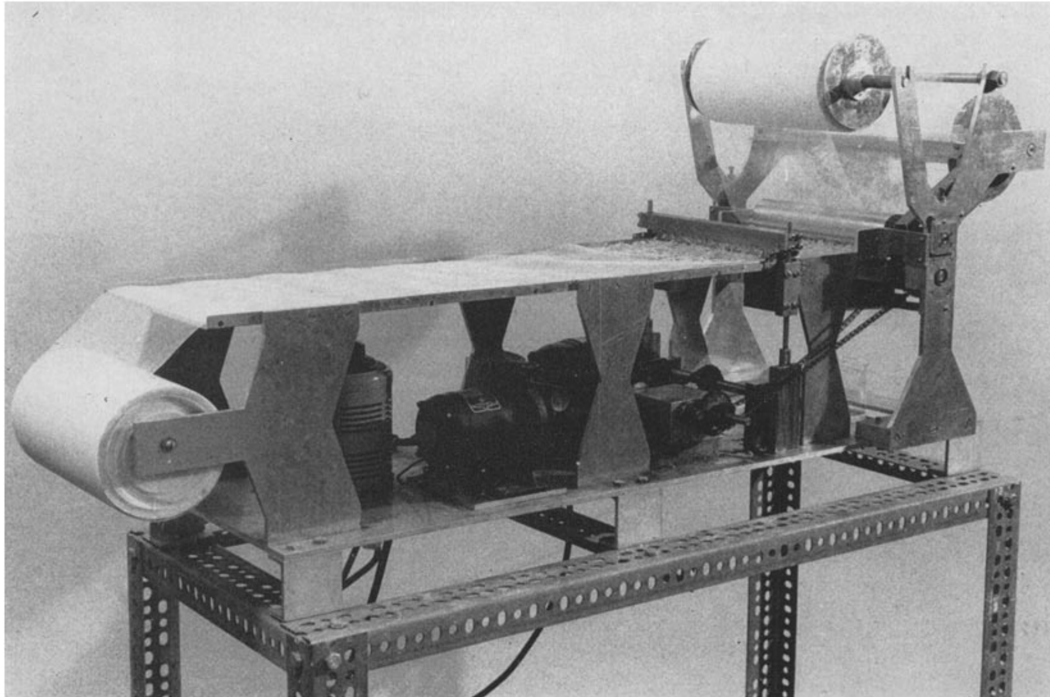

FIGURE 1

An over-all view of the "Enuclear Reactor" or "Pea-Popper." This figure illustrates the general construction of rollers and the three spools.

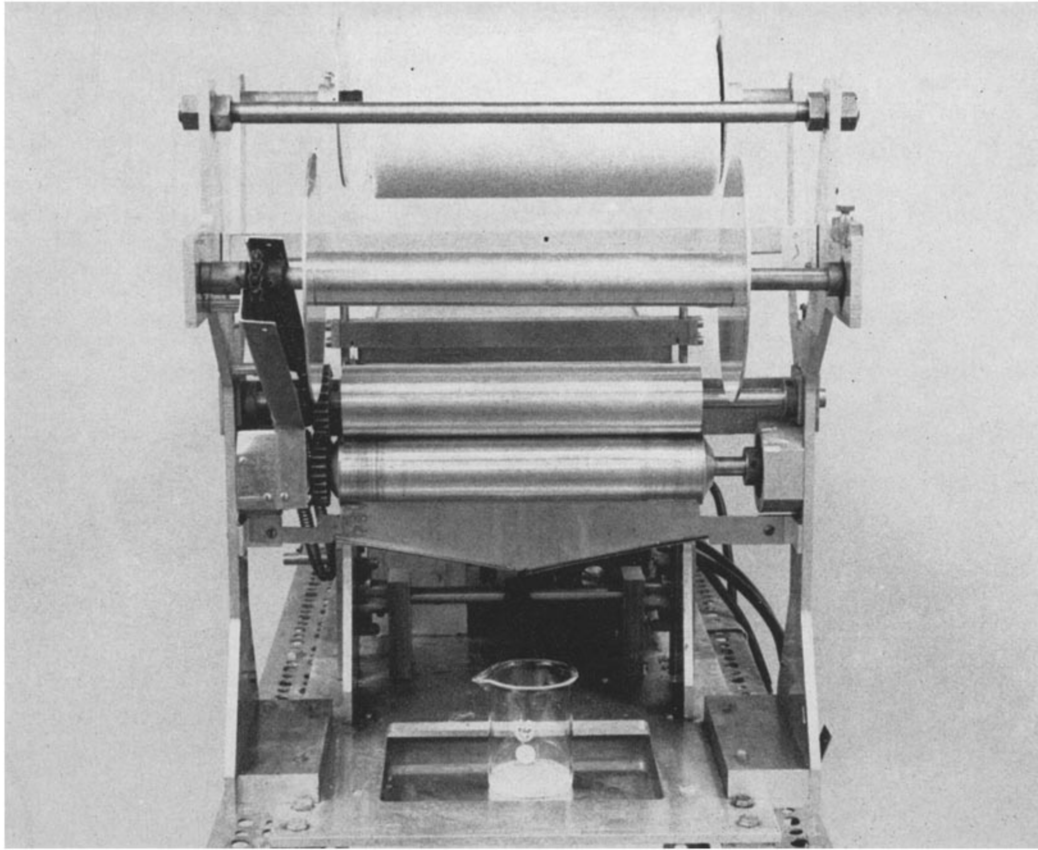

FIGURE 2

A side view of the machine. Under the two spools the two tissue-squeezing rollers are shown. The stainless steel scraper, constructed in a triangular fashion under the two rollers, funnels juice squeezed out of the pea tissues into a beaker.

medium buffers the liberated cell contents and also washes the extract off the squeezing rollers. The extract then runs into a beaker, chilled in ice, containing additional sucrose to give a final concentration of 0.75 M.

Isolation of Nuclei

The liberated nuclei are freed from larger contaminants by filtration through a double layer of "Miracloth" and collected by subsequent centrifugation at 350 *g* for 10 minutes in an International swinging-bucket-type centrifuge. All of the operations are carried out at 4°C. One kilogram of tissue can be processed in about 20 minutes.

After centrifugation, a pellet of starch granules can be observed at the bottom of the tube covered by a layer of nuclear material. The supernatant is removed by pipetting and discarded. The nuclear layer is then removed with a hypodermic syringe. The crude nuclear suspension may be further purified by relayering on 1.2 M sucrose solution and spinning at 450 *g* for 10 minutes. The purified nuclei constitute the pellet.

Incubation of Nuclei

Cytidine-H^3, used as a precursor for radioactive RNA, was obtained from New England Nuclear Corporation, Boston; its specific activity was 4,900 mc per mM. Isolated nuclei were incubated at 37°C in medium containing such cytidine-H^3. Controls were held at 0°C for equal times. Each sample for incubation contained about 3 to 4 mg of nuclear protein. Uptake of radioactive precursors was stopped by the addition of 100-fold excess unlabeled cytidine followed by an equal volume of 6 per cent $HClO_4$ solution.

Determination of RNA, DNA, and Protein

The nuclei were precipitated in 3 per cent $HClO_4$ for 2 hours at 0°C and washed according to the procedure of Ogur and Rosen (13). The pellet was washed 3 times in 3 per cent $HClO_4$ containing 100 × excess unlabeled cytidine, once with 70 per cent ethanol-0.1 per cent $HClO_4$, once with 70 per cent ethanol, twice with ethanol-ether (3:1) and, finally, once with 2 per cent $HClO_4$ (all at 2–4°C). The RNA in the washed precipitate was then hydrolyzed in 1 N $HClO_4$ for 60 hours at 0°C. The amount of RNA in the perchloric acid hydrolysate was estimated from the UV absorption at 260 mμ as determined by a Cary Recording Spectrophotometer. The residue was treated twice with 0.5 N $HClO_4$ to extract DNA, each time for 20 minutes and at 70°C. The DNA content of the combined extracts was estimated from optical density at 268 mμ. The residual protein was determined by the Biuret method according to the procedure of Gornall *et al.* (14).

Determination of Radioactivity

The radioactivity of tritium-labeled RNA or nucleotides was measured with a Packard liquid scintillation spectrometer. For this purpose, a 0.1 ml aliquot was mixed with 10 ml of the following mixture (15): 700 ml of toluene, 300 ml of absolute ethanol, 4 gm of 2,5-diphenyloxazole, and 100 mg of 1,4-bis-2-(5-phenyloxazolyl)-benzene.

Paper Electrophoresis

In some experiments the samples which had been incubated previously in a medium containing cytidine-H^3 were subjected to paper electrophoresis to determine the distribution pattern of radioactivity in the different nucleotides. The 3 per cent $HClO_4$-treated and 70 per cent ethanol-washed samples were hydrolyzed in 0.3 N KOH for 18 hours at 37°C (Davidson and Smellie) (16). After precipitation of potassium at pH 1 by perchloric acid the supernatant was adjusted to pH 3.5. Following the procedure described by Wallace and Ts'o (17), approximately 0.5 mg of RNA nucleotides contained in 0.5 ml was applied as a band 8 cm from one end of a strip of water-washed Whatman 3 mm paper (56 × 7.6 cm). The nucleotides were separated in 0.04 M citrate buffer (pH 3.5), by the application of a potential

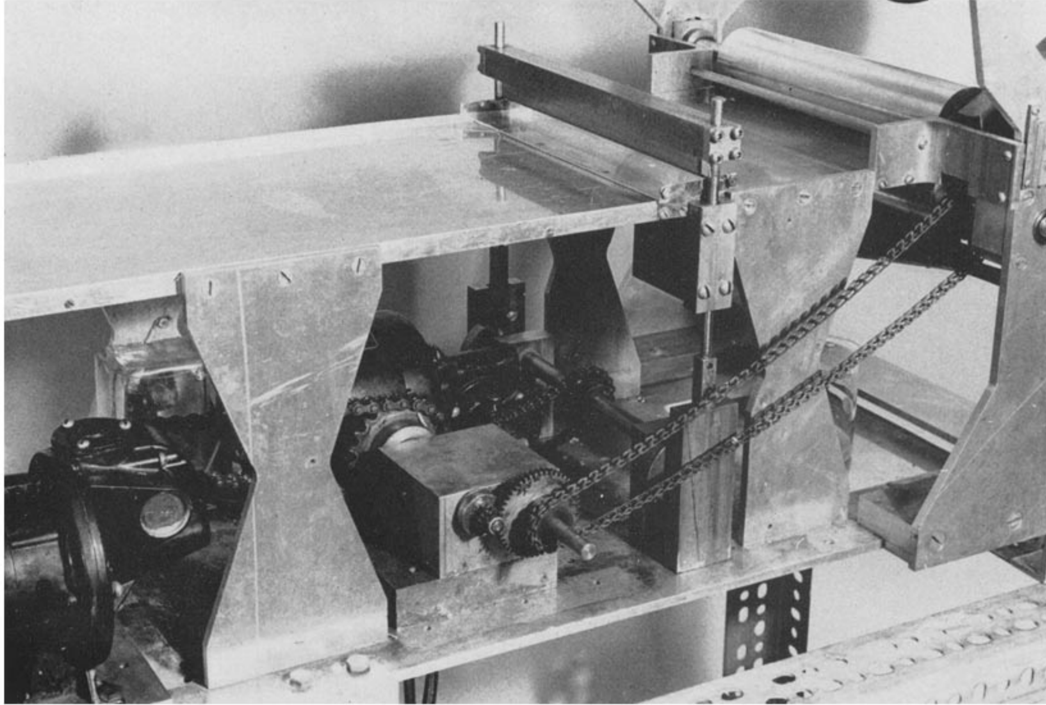

FIGURE 3

A close-up view of the chopping device. The blade is driven by a pair of heart-shaped cams which are located under the delivery panel as shown.

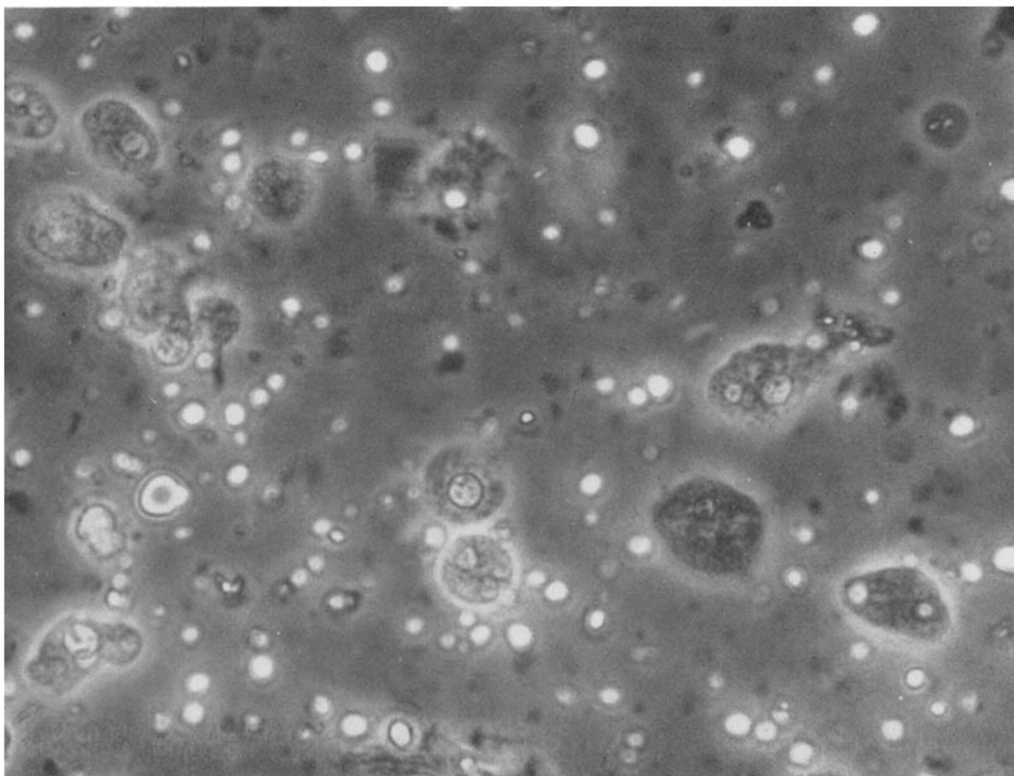

FIGURE 4

The non-purified nuclear preparation photographed by phase contrast micrography. Intact nuclei are shown with highly light-reflecting nucleoli in them. The most strongly light-reflecting particles are iodine-positive starch grains. The other dark particles are mostly acetocarmine-positive chromatine fragments. × 1,500.

Photo courtesy of Dr. Max Birnstiel.

gradient of 29 volts per cm. A blank strip was run simultaneously as a control. Nucleotide regions and the corresponding blank regions were cut from the strips, eluted with water, and made up to a known volume. The optical densities of these solutions were measured over the region 240 to 280 mμ and corrected for the paper blank. Radioactivity of aliquots of these eluates was then determined.

RESULTS

Isolation Media

From microscopic examination of the nuclei it was found that in sucrose concentrations below 0.25 M the nuclear membrane becomes distended, forming a "balloon-like structure" on the nuclear surface. At pH's below 6.0, the nuclei become distorted with eventual disruption, and above pH 8.0 they clump together in non-dispersible masses. The nuclei require the presence of divalent cations to maintain normal structure. In the absence of calcium or magnesium ions, they appear distorted and clumped. When 0.003 M $MgCl_2$ is added to the buffered sucrose medium there is still considerable clumping but some nuclei appear normal. However, in the presence of $CaCl_2$ nearly all the nuclei are found to be preserved; the optimal concentration of $CaCl_2$ in the tris-buffered sucrose medium is 0.003–0.004 M. The most suitable medium for isolation of the nuclei contains a final concentration of 0.25 M sucrose and 0.003 M $CaCl_2$ and is buffered with 0.006 M tris at pH 7.2 (18). Nuclei prepared in this medium appear normal for as long as several hours after preparation.

Purity and Yield of Nuclei

The purity of the nuclear preparations was examined under the light microscope by staining with iodine or acetocarmine. The major contaminant of the initial nuclear pellet was found to be starch, although some acetocarmine-negative and iodine-negative particles are present (Figs. 4 and 5). Nuclei purified by relayering over 1.2 M sucrose solution followed by centrifuging at 450 *g* for 10 minutes were freed of the majority of these contaminants, as is shown in Fig. 6. For routine activity determination the non-purified preparations were generally used. All nuclear preparations were completely free of intact cells.

To determine the yield of nuclei and liberation of DNA into non-nuclear fractions of the homogenate, the supernatant of the prepared nuclear fraction was fractionated further into subcellular components according to the procedures described by Ts'o and Sato (19). The supernatant was diluted to 0.2 M with respect to sucrose and centrifuged for 15 minutes at 5,000 *g* to obtain a second nuclear fraction. From the supernatant of this spin the mitochondrial fraction (40,000 *g* for 12 minutes) and ribosomal fraction (110,000 *g* for 90 minutes) were subsequently obtained. Total DNA of the tissue was determined from an equivalent amount of tissue ground in a mortar at 2–4°C with 0.4 M sucrose solution (0.5 ml per gram of tissue). The homogenate was filtered by pressing through cheese-cloth and the filtrate used for the determination of total DNA.

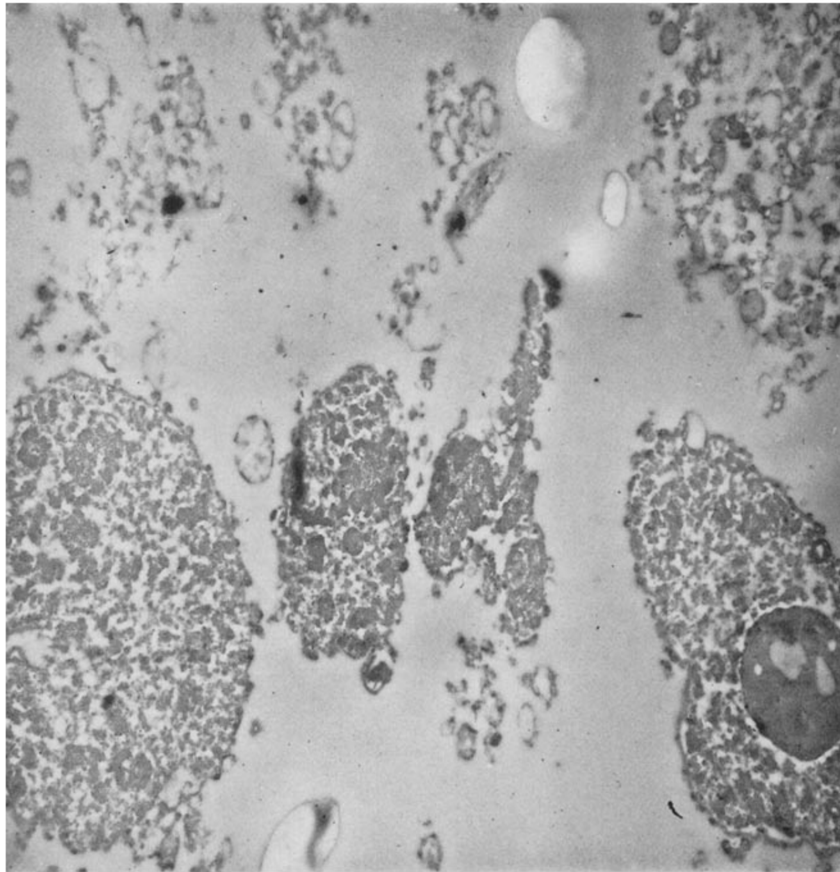

FIGURE 5

The electron micrograph of non-purified nuclear fraction. Grinding medium 0.3 M sucrose buffered with 0.01 M tris at pH 7. Fixed in veronal-buffered OsO_4 and embedded in methacrylate. × 6,500. Photo courtesy of Dr. Beal Hyde.

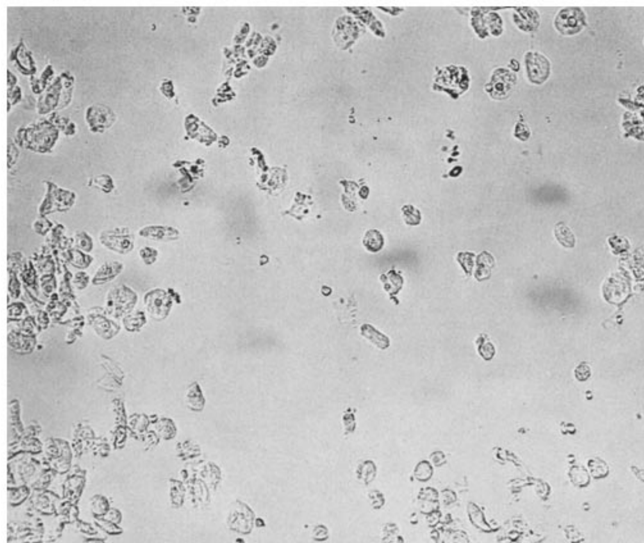

FIGURE 6

Isolated nuclei purified by relayering over 1.2 M sucrose solution and centrifuging at 450 *g* for 10 minutes. Purified nuclei were stained with iodine to detect dark-staining starch contaminants. × 250

The yield of nuclei obtained by the new method varied among different preparations possibly as a result of slight variations in the age and physiological conditions of the tissue. However, the variation did not exceed 5 to 10 per cent in terms of total tissue DNA recovery. In a typical experiment the yield of DNA in the first nuclear fraction was about 6 per cent of total tissue DNA, 2.5 per cent in the second nuclear fraction, 0.9 per cent in the mitochondrial fraction, and 1.9 per cent in the ribosomal particles. About 5 per cent of total tissue DNA was recovered in the supernatant fraction.

Incorporation of Cytidine-H³ into Nuclear RNA

When isolated pea nuclei were incubated with tritiated cytidine under appropriate conditions, a significant amount of incorporation of label into the acid-insoluble residue was observed. Maximal incorporation of cytidine-H^3, comparing media of differing pH, took place between pH 7.0 and 7.8 with an optimum near pH 7.0 (Fig. 7). The presence of calcium ions resulted in an increase in cytidine incorporation at low concentration (0.002 M) but calcium became inhibitory at higher concentrations (Fig. 8). These results are in agreement with the microscopic observation of optimal conditions for maintaining nuclear integrity. Low concentrations of magnesium and manganese exerted no effect on incorporation, whereas high concentrations were again inhibitory.

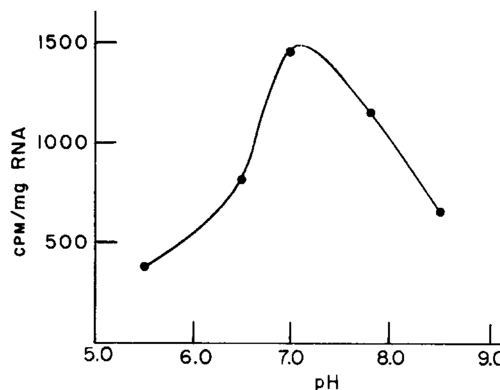

FIGURE 7

Effect of varying pH on cytidine-H^3 incorporation into isolated pea nuclei. The rate of cytidine-H^3 incorporation into nuclear RNA is plotted against the pH of the incubation medium. The incubation medium contained 0.2 ml of nuclear suspension and 0.8 ml of the following mixture: 0.2 M sucrose, 0.025 M tris buffer, 0.002 M $CaCl_2$, 1 μmole of ATP, 25 μc cytidine-H^3, and 1 μmole of uridine, adenosine, and guanosine. The pH was adjusted with KOH and HCl and checked throughout the entire incubation period. The nuclei were incubated at 30°C for 60 minutes.

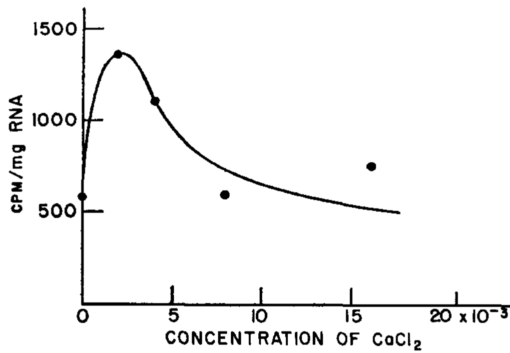

FIGURE 8

The effect of the $CaCl_2$ concentration of the incubation medium on the rate of cytidine-H^3 incorporation into the isolated pea nuclei. The rate of cytidine-H^3 incorporation in the various $CaCl_2$ concentration of the medium is plotted against the concentration of $CaCl_2$ in a 60-minute incubation period at 30°C. The incubation medium contained the following: 0.25 M sucrose, 0.025 M tris buffer (pH 7.6), 1 μmole ATP, 0.001 M $MgCl_2$, 0.0003 M $MnSO_4$, 25 μc cytidine-H^3, and 1 μmole of each of uridine, adenosine, and guanosine. Total volume, 1 ml.

The incorporation of cytidine-H^3 into nuclei was greatly enhanced in the presence of the remaining three nucleosides. The corresponding nucleoside triphosphates were two to three times more active than the corresponding nucleosides (Fig. 9). A further enhancement of incorporation was effected by the addition of phosphocreatine and phosphocreatine kinase (prepared from rabbit muscle according to the procedures described by Kuby *et al.*, 20) as an energy-regenerating system.

At 37°C incorporation proceeded rapidly for about 20 minutes and thereafter showed a gradual decrease in rate; in the presence of the energy-regenerating system and nucleoside triphosphates about 10 μμm cytidine-H^3 per mg of RNA were incorporated within 20 minutes. Under these optimal conditions, nuclei could also incorporate an equivalent amount of uridine-H^3. The addition of a complete amino acid mixture did not increase the incorporation of cytidine to any detectable extent.

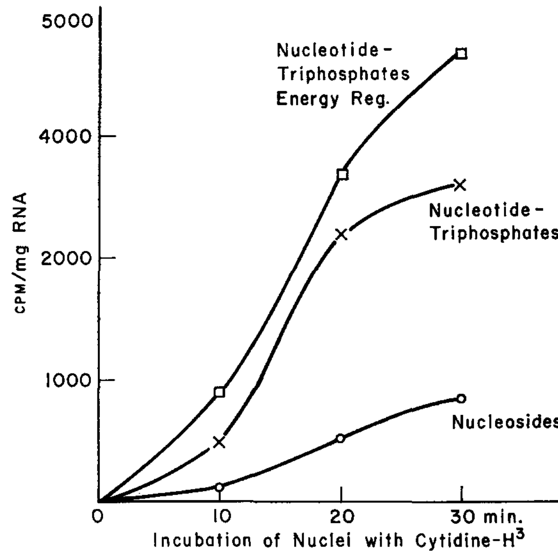

FIGURE 9

Comparative effects of the addition of various RNA precursors and energy-regenerating system on the rate of cytidine-H^3 incorporation into isolated nuclei. The specific activities of the nuclear RNA at different incubation times at 37°C are plotted against the time intervals. The basic incubation medium contained: 0.25 M sucrose, 0.025 M tris buffer (pH 7.7), 0.001 M $CaCl_2$, 0.0001 M $MgSO_4$, and 50 μc cytidine-H^3. In the experiment testing the effect of nucleosides, 0.0001 M of adenosine, uridine, and guanosine were added; as nucleoside triphosphates, 0.0001 M ATP, UTP, and GTP were used; and to study the effects of the addition of energy regenerating system, 0.005 M phosphocreatine and 100 γ per ml of phosphocreatine kinase were included. Total volume: 2 ml.

Identification of the Radioactive Product as RNA

The labeled product, isolated from the reaction mixture, is acid-insoluble (0.5 N TCA or 3 per cent $HClO_4$) and is extracted by treatment with 1 N $HClO_4$ at 0°C for 36 hours. Treatment with 0.3 N KOH at 37°C for 18 hours, conditions known to hydrolyze RNA, results in the formation of acid-soluble material. After incorporation of cytidine-H^3, the products formed by alkaline hydrolysis were separated by paper electrophoresis and were identified as the monophosphates of cytidine, uridine, adenosine, and guanosine. Cytidine monophosphate was the most heavily labeled, uridine monophosphate was slightly labeled, and adenosine and guanosine monophosphate contained only negligible amounts of the original radioactivity.

DISCUSSION

The isolation of nuclei from plant tissue is more difficult than the corresponding isolation from animal tissue since the forces necessary to break the plant cell walls by conventional methods are so great that there is inevitable destruction of many of the liberated nuclei. Furthermore, plant cells are much more resistant to disruption so that a large number of unbroken cells may be present as a contaminant in the resulting nuclear preparation. The tissue-disrupting machine avoids the above hazards to a considerable extent and a pure preparation of isolated nuclei results with a minimum of difficulty. The chopping device provides a large cut surface so that large quantities of nuclei may be gently squeezed from the cells without the damaging action of any severe shearing force. The rollers of the machine may be adjusted to provide optimal release of the nuclei without grinding during liberation. At the same time, the connection between individual cells remains intact, and the residual tissues and cell wall fragments are retained between the layers of nylon cloth. As a result, few whole cells are present in the nuclei-rich extract collected from the tissue and may be removed by subsequent filtration through Miracloth.

Since the nuclei are collected by very low speed centrifugation, the majority of the lighter contaminants such as mitochondria and ribosomes are removed, and, as can be seen from Fig. 4, a second centrifugation in sucrose solution yields nuclei of very high purity.

If the percentage recovery of total DNA is used as a measure of nuclear recovery, the yield is still low as compared to that reported by some other workers. Ts'o and Sato (19) report recovery of 93 per cent of the total DNA in the nuclear fraction of their tissue extract, whereas our two nuclear fractions represent only about 50 per cent of the total DNA of the homogenate. Stern and Mirsky (21) obtained 18 per cent of total tissue DNA in their purified nuclei from wheat germ compared with our 5 to 10 per cent. However, with the present method, a relatively pure, intact nuclear fraction may be obtained without further purification steps so that the nuclei may be incubated immediately after isolation and while they are still most active. It is our opinion that the purity and rapidity of isolation more than compensates for the somewhat lower yield.

A necessary precaution in the preparation of nuclei is to avoid the loss of any nuclear constituents which may be extracted from the nuclei during isolation. A comparison of the properties of aqueous and non-aqueous preparations of nuclei shows that there may be considerable loss of soluble enzymes and intermediates from nuclei prepared in aqueous media (4). While some materials of low molecular weight are lost during preparation of nuclei in sucrose, loss of either protein or nucleic acids is inconsequential since these nuclei show the ability to synthesize RNA as well as protein (22).

It has been pointed out that nuclear damage (probably resulting from the action of nucleolytic enzymes) may occur in the crude homogenate prior to isolation of the nuclei. Anderson (23) has reported that liver nuclei rapidly lose their contents if incubated at 37°C while still in the homogenate. Roodyn (24) has also observed that delay in fractionation can sometimes result in a marked redistribution of enzyme activity between nuclei and supernatant. Philpot and Stanier (10) have made similar observations and have found such damage to occur much more rapidly in nuclear fractions heavily contaminated with cell particles than in purified nuclear fractions. The reduced mechanical damage of cellular constituents offered by the present method coupled with immediate fractionation will undoubtedly minimize such degradative enzymic processes. The ability of such nuclei to form structural gels in alkali or high concentration of salt might be

considered as an indication that little autolytic degradation of nuclear structure has occurred (4).

When isolated pea nuclei are incubated in a tris-buffered sucrose medium containing tritiated cytidine, there is a rapid and considerable incorporation of isotope into nuclear RNA. When the cytidine-labeled, acid-insoluble product is subjected to alkaline hydrolysis followed by electrophoresis, the label is found to be present in cytidine monophosphate and to a lesser extent in uridine monophosphate. These results are highly indicative of incorporation into part of a polyribonucleotide chain. Thus, the radioactive product upon incubation was tentatively identified as RNA. Further evidence that this is a genuine incorporation and not mere adsorption of label is the increased rate of labeling brought about by the addition of the other three nucleosides or nucleotides. This was shown as an energy-requiring process since the addition of an energy-regenerating system increased the rate of labeling. Uridine was incorporated in a manner comparable to that of cytidine.

That RNA synthesis is not obligatorily coupled with protein synthesis may be derived from the fact that RNA synthesis is not enhanced by the presence of amino-acid mixture which is required in protein synthesis.

Report of work supported in part by Grant No. RG-5143, National Institutes of Health, United States Public Health Service, and Grant No. A-3102, United States Public Health Service. Dr. Rho is supported by the Visiting Scientists Program, National Academy of Sciences.

We wish to thank Professor James Bonner and Dr. Max Birnstiel for their valuable advice and interest in this work, and Mr. Robert Hayes and Mr. Frank Ostrander for their technical assistance.

Received for publication, February 1, 1962.

REFERENCES

1. Dounce, A. L., The Nucleic Acids, (E. Chargaff and J. N. Davidson, editors), 1955, **2,** 93.
2. Allfrey, V. G., The Cell (J. Brachet and A. E. Mirsky, editors), 1959, **1,** 193.
3. Brachet, J., Biochemical Cytology, New York, Academic Press, 1957, 26.
4. Siebert, G., and Smellie, R. M. S., *Internat. Rev. Cytol.*, 1957, **6,** 383.
5. Roodyn, D. B., *Internat. Rev. Cytol.*, 1959, **8,** 279.
6. Corper, H. J., and Cohn, M. L., *J. Lab. Clin. Med.*, 1936, **21,** 428.
7. Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, 1936, **114,** 495.
8. Wilbur, K. M., and Skeen, M. V., *Science*, 1950, **111,** 304.
9. Harris, E. S., and Mehl, J. W., *Science*, 1954, **120,** 663.
10. Philpot, J. St.L., and Stanier, J. E., *Biochem. J.*, 1956, **63,** 214.
11. Chayen, J., and Benfield, A. H., *Exp. Cell Research*, 1960, **20,** 172.
12. Emanuel, C. F., and Chaikoff, I. L., *Biochim. et Biophysica Acta*, 1957, **24,** 254
13. Ogur, M., and Rosen, G., *Arch. Biochem. and Biophysics*, 1950, **25,** 262.
14. Gornall, A. G., Bardwill, C. J., and David, M. M., *J. Biol. Chem.*, 1949, **177,** 751.
15. Rabson, R., and Novelli, G. D., *Proc. Nat. Acad. Sc.*, 1960, **46,** 484.
16. Davidson, J. N., and Smellie, R. M. S., *Biochem. J.*, 1952, **52,** 594.
17. Wallace, J. M., and Ts'o, P. O. P., *Biochem. and Biophys. Res. Comm.*, 1961, **4,** 125.
18. Allfrey, V. G., and Mirsky, A. E., *Proc. Nat. Acad. Sc.*, 1957, **43,** 589.
19. Ts'o, P. O. P., and Sato, C. S., *Exp. Cell Research*, 1959, **17,** 227.
20. Kuby, S. A., Noda, L., and Lardy, H. A., *J. Biol. Chem.*, 1954, **209,** 191.
21. Stern, H., and Mirsky, A. E., *J. Gen. Physiol.*, 1953, **36,** 181.
22. Rho, J., Birnstiel, M., Chipchase, M., and Bonner, J., *Fed. Proc.*, 1961, **20,** 147.
23. Anderson, N. G., *Exp. Cell Research*, 1953, **5,** 361.
24. Roodyn, D. B., *Biochem. J.*, 1956, **64,** 368.