# THE ISOLATION OF CELL NUCLEI IN NON-AQUEOUS MEDIA*

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The present paper describes in detail procedures for the preparation of cell nuclei from a diversity of animal tissues. These procedures are modifications of methods introduced by Behrens (1, 2) and applied, with some changes, by Dounce (3) to the study of intracellular enzyme and amino acid distribution in rat liver.

Since the Behrens procedure does not involve "homogenization" in aqueous media it is the only method available at present which permits the isolation of nuclei without loss of water-soluble nuclear components and without adsorptive contamination by serum and cytoplasmic proteins.

In this laboratory nuclei have been prepared from the erythrocytes of the chicken and goose, from the liver of the calf, horse, and rat, from calf and fowl kidney, from calf, beef, and horse pancreas, and from calf thymus, intestinal mucosa, and heart.

The results of a biochemical study of these nuclei are reported separately (4).

*Outline and Consideration of the Method.*—The steps in the isolation procedure are as follows: (1) the tissue is frozen, lyophilized, and minced, (2) the dry powder so obtained is suspended in petroleum ether and ground in a ball mill to fragment the cells and liberate the nuclei, (3) the nuclei are then separated from both lighter and heavier components of the ground tissue suspension by alternate sedimentations and flotations in cyclohexane–carbon tetrachloride mixtures of varying specific gravity.

In almost all cases, the nuclei have a characteristic and relatively high density which permits their separation from the blocks of free cytoplasm and the partly ground cells which form a large part of the ground tissue suspension. For if the suspension is adjusted to a specific gravity slightly lower than that of the nuclei, centrifugation will sediment the nuclei and float the lighter (cytoplasmic) components. And in organic media of higher specific gravities, the nuclei can be floated away from heavier cellular debris.

* Many of the results presented in this and the succeeding report (4) were given at the Jubilee Meeting of the Genetics Society of America in Columbus, Ohio, on September 13, 1950, and summarized in the volume Genetics in the 20th Century, (L. C. Dunn, editor), New York, Macmillan Co., 1951.

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In practice, the number of such successive sedimentations and flotations required to purify the nuclei depends mainly upon the difference in density between nucleus and cytoplasm and, secondly, upon the nuclear fraction of the ground cell suspension.

Both the density and the nuclear fraction vary from tissue to tissue. Calf thymus or pancreas nuclei, for example, are easier to prepare in the pure state than the nuclei of calf intestinal mucosa or liver; and calf liver, in turn, is a better source of nuclei than rat liver. Indeed, satisfactory preparations of rat liver nuclei could not be obtained in this laboratory unless the animals were first fasted. A similar observation was reported by Behrens in the isolation of liver nuclei of the guinea pig (1, 5). In this instance the depletion of liver glycogen during the inanition period led to a drop in cytoplasmic density which permitted the separation of the nuclei. In several tissues, however, (fowl reticulocytes, chicken, rat and fetal calf liver, turtle erythrocytes, and calf bone marrow) nuclear and cytoplasmic densities coincide, and satisfactory nuclear preparations could not be obtained in this laboratory.

The isolation procedures described in this report apply to tissue suspensions in which the densities of the components permit the separation of nuclei showing little or no cytoplasmic contamination.

In some instances in which the nuclear density coincides or nearly coincides with that of a cytoplasmic component, the latter may be removed by techniques which exploit differences in size. Small particles, for example, sediment more slowly than larger particles of the same specific gravity. A nuclear fraction contaminated by small granules can, therefore, be further purified by centrifuging under conditions in which the nuclei sediment but smaller particles remain in suspension. On the other hand, when the cytoplasmic contaminant is relatively large, it can be removed by filtration through fine wire gauze. This device is used in many of the preparations about to be described and is particularly effective in the removal of contaminating fiber from heart muscle nuclei.

In many tissues the isolation is complicated by the fact that the nuclei form only a small fraction of the ground cell suspension. Under these conditions, a straightforward separation of nucleus and cytoplasm cannot be achieved by sedimentation at specific gravities immediately below some fixed value characteristic of the nucleus. Instead, the nuclei are trapped and carried along by the other components of the suspension, For example, if a ground erythrocyte suspension is adjusted to a specific gravity of 1.32 and centrifuged, all cellular components are brought to the surface. Yet an appreciable fraction of chicken erythrocyte nuclei, once isolated, will sediment at specific gravities above 1.34. Obviously, the extent to which nuclei are trapped and carried along by other components of the ground cell suspension is a function of concentration. The entrapment can be minimized in two ways; (1) by working with very dilute

suspensions, or (2) by a stepwise removal of the cytoplasm, beginning with the lighter, fatty fractions and progressing to specific gravities slightly below those of the nuclei.

In practice, the second of these alternatives is the more useful, since it permits one to follow the purification, taking note of the nuclear losses, and, at the same time, it allows an estimate of nuclear density, needed for further steps in the isolation. The details of this stepwise removal of cytoplasm, the adjustment of specific gravities, and the staining procedures used to follow the purification are described in the section on fractionation procedure.

Although the nuclear specific gravity is fairly constant and reproducible in different preparations from the same tissue, it is not a single, sharply defined value. Instead it covers a range of about 0.3 to 0.5 unit. (Calf thymus nuclei, for example, have specific gravities between 1.37 and 1.41.) It follows, therefore, that in the lower part of the range, light nuclei will sediment together with heavier nuclei bearing cytoplasmic tabs. In practice, this mixture is discarded. Similarly, nuclei at the upper end of the specific gravity range are often discarded with heavy fiber and dense cytoplasmic debris. Thus, depending on the tissue, an element of selection is necessarily introduced which leads to nuclear preparations in which all nuclear densities are not represented. There does not appear to be a corresponding fractionation according to shape or size; nor do the heavier components of a nuclear preparation differ appreciably from the lighter in enzyme content.

In the original Behrens technique, nuclei are separated in benzene–carbon tetrachloride (or ether-chloroform) mixtures. Enzyme studies in this laboratory indicated that benzene inactivates some enzymes (*e.g.* adenosine deaminase) to a far greater extent than does cyclohexane. An attempt was also made to find a heavy solvent more suitable than carbon tetrachloride. However, the number of commercially available solvents of comparable density is limited. Substitution of the heavy solvent, dibromcyclohexane, for carbon tetrachloride led to complete inactivation of several enzymes considered. Therefore, for all isolations (with the exception of horse liver nuclei, where benzene was used) the organic solvents chosen were cyclohexane (sp.gr. 0.779) and carbon tetrachloride (sp.gr. 1.595).

The possibility remains, however, that the applicability of the method to enzyme studies of isolated nuclei can be broadened by the substitution of other organic solvents, or silicones, for the cyclohexane-$CCl_4$ mixtures used. The preparations obtained by the present procedure are comparable to the acetone-extracted powders commonly used in the investigation of many enzyme systems. The broad literature on acetone powders can be used as a guide in the selection of enzyme systems which are likely to resist inactivation during the Behrens procedure.

## EXPERIMENTAL

*Preparation of Tissues.*—

*Freezing.*—Most preparations deal with discrete organs; *e.g.*, calf or beef liver, thymus, pancreas, kidney, and heart. In these instances, the fresh tissue is cut into sections about 5 cm. in width, and frozen, (*a*) by storage at −26°C. for 18 to 24 hours, or (*b*) by submersion in liquid air for 10 to 20 minutes.

The latter procedure was introduced to check the possibility of protein migration from cytoplasm to nucleus during the comparatively slow freezing at −26°. If such migration occurs one would expect, (1) a widespread occurrence of soluble cytoplasmic proteins in the isolated nuclei, and (2) a difference in enzyme distribution between nucleus and cytoplasm for the same tissue frozen in different ways and then fractionated. Neither expectation is fulfilled. Many soluble proteins, like the myoglobin of beef heart tissue and the arginase of chicken kidney, do not occur in the nuclei prepared from these tissues; and no difference is observed in the distribution of arginase and uricase between nucleus and cytoplasm of calf liver frozen by the different procedures.

*Drying.*—Once frozen, the tissue sections are sliced into chips about 5 mm. in thickness and the chips are transferred immediately to 1 liter, wide mouthed lyophilizing flasks immersed in a dry ice–acetone mixture. (The slicing may be conveniently performed manually with a fixed blade, adjustable platform slicer of a type in common use as a kitchen utensil.)[1]

Six to seven hundred gm. of frozen tissue can be handled safely in two such drying flasks provided the water removal capacity of the lyophilizing unit employed exceeds 500 ml., or if the trapped moisture in smaller systems is removed frequently. The flasks are then immediately connected to the lyophilizing unit and maintained under vacuum for 60 hours. To avoid thawing, the drying is not accelerated by heat. On the contrary, there is a great deal of condensation and freezing of atmospheric moisture on the cold glass surfaces, and the flasks remain jacketed in ice throughout most of the drying period. The frozen dry tissue is shredded in a Waring mixer and the resulting dry powder stored at −16° until needed.

The preparation of rat or fowl liver and kidney is simplified by their small size. The organs are frozen at −26°, fragmented by a blow with a hammer, and lyophilized directly. After shredding in a Waring mixer, the tissue is stored at −16°C.

The heparinized blood of the goose or chicken is centrifuged in the cold as soon as possible after collection. The cells are washed three times with about four times their volume of cold 0.9 per cent NaCl solution and transferred to lyophilizing flasks. No hemolysis is observed during this procedure. The cell suspension

[1] The EVR-SHARP slicer, available at R. H. Macy Co., New York.

is rapidly frozen by immersion in a dry ice–acetone mixture and the flasks are then connected to the lyophilizing unit. If 150 to 250 ml. of the cell suspension is frozen as a film on the walls of a 1 liter flask, drying is complete within 24 to 30 hours. The dried cell mass is powdered in a mortar and kept at −16°C.

Intestinal mucosa may be frozen at −26° and sliced in the usual way, or it may be poured into lyophilizing flasks and frozen by immersion in $CO_2$-acetone. The frozen tissue is lyophilized for 48 to 60 hours, powdered in the Waring blendor, and stored at −16°.

The grinding and all steps in the fractionation procedure are carried out at 2–4°C.

*Grinding.*—A 100 gm. portion of the dried tissue powder is transferred to a porcelain milling jar[2] containing 450 ml. of petroleum ether (b.p. 60°) and 1400 gm. of irregular grinding stones (2 to 3 cm. in diameter). The jar cover is locked into position and the jar is mounted on its side on motor-driven rollers and rotated at about 110 R.P.M. for 44 to 48 hours.

The purpose of grinding is to convert a tissue suspension which, at the outset, is completely cellular to a mixture containing a high proportion of free nuclei. Its success depends on the relatively high resistance of the nuclei to the milling conditions. But because the nucleus is not entirely refractory to grinding, the process must be stopped before any extensive nuclear breakdown occurs. In practice, this end-point corresponds to a ground cell suspension in which only a fraction of the nuclei are completely devoid of cytoplasm. The rest of the suspension comprises blocks of free cytoplasm, intact and partly ground cells, nuclei with cytoplasmic tabs, pigment granules, and fiber. If the grinding is not carried to excess, the isolation of free nuclei from this mixture is not complicated by the presence of ground nuclear debris.

The fractionation procedures described in this report are based on the suspensions obtained under the above milling conditions. In grinding smaller amounts of tissue powder, *e.g.* 50 gm., the volume of petroleum ether, quantity of grinding stones, and size of the milling vessel may be reduced proportionately.

The possibility that cytoplasmic proteins are ground into the nucleus during the milling procedure was tested and found untenable. The experiments are described in detail in the section on criteria of nuclear purity.

*Filtration.*—To remove coarse fiber the ground suspension is passed either through a wire screen (35 mesh, 0.5 mm. apertures), or through a single layer of bandage cloth (Johnson and Johnson type I). Grinding stones and filter pads are washed twice with about 100 ml. of petroleum ether and the washings and filtrate are combined.

[2] Porcelain Mill Jar, Type A, 15 cm. diameter, Paul O. Abbé, Inc., Little Falls, New Jersey.

*Fractionation Procedure.—*

*Control Tissue Preparation.*—The main purpose of the fractionation procedure is to supply isolated nuclei in pure condition and, if possible, in high yield. Since, in enzyme studies, the nucleus is generally considered in relation to the cell in which it occurs, it is also desirable to have a tissue fraction which approximates the cell in its chemical and enzymic constitution. The steps in preparing such a fraction from the ground cell suspension are as follows:—

1. An aliquot of the filtered suspension (approximately $\frac{1}{10}$ of the total) is transferred to a glass tube of 100 ml. capacity and centrifuged at 900 *G* (2000 R.P.M.) for 20 minutes.
2. The supernatant fluid is discarded and the sediment is resuspended (with the aid of a high speed stirrer) in about 80 ml. of a petroleum ether–cyclohexane—carbon tetrachloride mixture (2 parts by volume of petroleum ether to 1 part of cyclohexane and 1 of $CCl_4$, sp. gr. 0.95 at 4°C.).
3. This suspension is centrifuged at 900 *G* for 20 minutes and the supernate is discarded. In most tissues this supernate has a high fat content and is opalescent.
4. The sediment is again suspended in the ternary solvent mixture and centrifuged as before. The opalescent supernate is again discarded.
5. The sediment is extracted once or twice more with the solvent mixture, as described, and the supernates, which are finally clear, are discarded.
6. The residue is then taken up in 80 ml. of the 2:1:1 solvent mixture and stored at 4°C. until the nuclear isolation is also accomplished.
7. At that time, the suspension is centrifuged, the supernate is discarded, and the sediment is dried *in vacuo* at room temperature.

In this way a preparation is obtained which is free of fat and coarse fiber, and which has been exposed to the same solvents for the same time as the isolated nuclei. In the study of enzyme localization in the nucleus, this material serves both as a measure of the enzyme content of the cell and as a control experiment for losses in activity due to lyophilization, grinding, and solvent extraction.

The "cell" preparation has, however, several limitations: (1) the connnective tissue is not entirely removed by filtration through wire screen to remove coarse fiber, (2) extraction with petroleum ether–cyclohexane–carbon tetrachloride mixtures does remove lipids which are intracellular in origin, and the possibility exists that proteins with lipid prosthetic groups are also extracted under these conditions, and (3) since the cells are no longer intact, enzyme systems which depend upon the proper orientation of their components may be damaged or destroyed, and the destruction may be selective.

To determine whether this treatment results in appreciable losses of free amino acids or small peptides both the "control tissue" preparation and minced fresh tissue were extracted with 50 per cent ethanol and the extracts were ana-

lyzed for their amino nitrogen content by the colorimetric ninhydrin reaction. The same result was obtained in both cases.

*Isolation of Nuclei.*—

The remaining $\frac{9}{10}$ of the filtered, ground tissue suspension is next treated to isolate the nuclei. The details of this stepwise removal of cytoplasmic components are described below for a diversity of tissues. These procedures may require slight modification in individual cases depending on natural variations in nuclear and cytoplasmic densities. The general reproducibility of the methods, however, is high, and minor changes in the recommended specific gravity adjustments will solve most isolation problems.

*Staining Procedures.*—A careful microscopic examination of the different fractions is essential in following the isolation of the nuclei. In all cases a drop of the tissue or nuclear suspension is spread on a microscope slide and dried at room temperature. To facilitate microscopic examination and accentuate the contrast between nucleus and cytoplasm, several staining solutions are used: (1) aqueous crystal violet, 1 mg./ml., (2) standard hematoxylin-alcoholic eosin, and (3) the acetoorcein-fast green mixture of Kurnick and Ris (6). A magnification of 440 is satisfactory for all stages in the isolation, and the purity of the final nuclear preparation is determined at a magnification of 900 to 1400.

The purification of the nuclei is best followed routinely by crystal violet staining. A drop of the dye solution (1 mg./ml.) is spread over the dried sample on the slide, a cover slip is applied, and the stained preparation is examined and appraised for its content of free nuclei. Under these conditions the nuclei stain an intense blue relative to light blue cytoplasm and colorless fiber; the difference in intensity of staining together with the characteristic nuclear morphology makes identification easy. As a rule there is no difficulty in distinguishing between free nuclei and nuclei with attached cytoplasmic tabs.

When examining the final nuclear preparation for cytoplasmic contamination either hematoxylin-eosin, or acetoorcein-fast green mixtures may be applied. The former is a particularly effective stain for avian erythrocyte nuclei, but is not as satisfactory for other tissues, such as thymus, in which nuclear-cytoplasmic contrast is not accentuated. (The absence of contrast may be related to treatment with organic solvents such as petroleum ether and carbon tetrachloride.) Erythrocyte preparations are stained with Harris' hematoxylin, followed by 0.5 per cent alcoholic eosin, in the usual way, and mounted in balsam. Under these conditions nuclei stain a deep purple; the cytoplasm is bright red.

The acetoorcein-fast green mixture stains chromatin red to violet; the cytoplasm is light green, and protein within the nucleus is also green. The green of the nucleus, however, is blended with the contrasting stain for chromatin;

cytoplasmic green is light and more uniform. Together with the characteristic nuclear morphology this stain makes it relatively simple to distinguish between a free nucleus and a nucleus with an attached cytoplasmic tab. Acetoorcein-fast green staining is effective for all tissues examined, in contrast to hematoxylin-eosin, and is rapidly applied. A drop of the dye mixture is spread over the dried nuclei, a cover slip is applied, and after 2 to 3 minutes, staining is complete.

*Chicken Erythrocyte Nuclei.*—An aliquot of the ground suspension is first removed to prepare the "tissue control" described earlier. The remainder of the suspension is transferred to 100 ml. glass centrifuge tubes and centrifuged at 900 *G* for 20 minutes. The supernate is discarded. The steps in the isolation of the nuclei from the sediment are as follows (all steps are performed at 2 to 4°C.):—

1. The sediment is resuspended with the aid of a high speed stirrer in about 700 ml. of a 1:1.5 cyclohexane–carbon tetrachloride mixture. A portion of the suspension is transferred to a glass cylinder (or centrifuge tube) containing a small hydrometer covering the range 1.200 to 1.425 in 0.005 unit graduations. The intercept of meniscus and scale (viewed with the eye at meniscus level) is the specific gravity of the suspension, and can be determined within 0.002 unit. The desired specific gravity for the first step in the isolation is 1.290, and the suspension can be adjusted to this density by the addition of small amounts of cyclohexane, if the specific gravity is originally too high, or of carbon tetrachloride, if the original density is low. Once adjusted, the suspension is transferred to 100 ml. glass centrifuge tubes and centrifuged at 2000 *G* (3000 R.P.M.) for 40 minutes.

The purpose of this first step is to float much of the free cytoplasm and sediment the other components of the suspension. Therefore, the supernatant fluid following the centrifugation should be opaque and not clear. If it is clear, the selected specific gravity, 1.290, is too low for floating the cytoplasm, and the total suspension must be remixed, readjusted to sp. gr. 1.293 to 1.295 by the addition of small amounts of carbon tetrachloride, and recentrifuged. On the other hand, if the supernate following centrifugation is not only opaque, but surmounted by a heavy crust, then the original setting, 1.290, is too high and should be lowered to 1.287 by the addition to the remixed suspension of small amounts of cyclohexane. This is done to minimize the loss of nuclei by entrapment when too much of the suspended material is carried to the surface.

If the supernate is satisfactory, *i.e.* opaque with a slight crust, it is decanted, stirred, transferred to another set of centrifuge tubes, and again centrifuged at 2000 *G* for 40 minutes. The supernate is now discarded.

2. All the sediments are then resuspended with rapid stirring in about 700 ml. of 1:1.5 cyclohexane–$CCl_4$ mixture. The suspension is brought to sp. gr. 1.300 by the addition of small amounts of cyclohexane or $CCl_4$ as required, and centrifuged at 2000 *G* for 60 minutes. The supernate is opaque and has a crust which generally adheres to the walls of the centrifuge tubes. The crust can be cut free with a small spatula without disturbing the sediment. The supernate and crust are decanted, remixed, transferred to another set of tubes, and centrifuged as before. The resulting supernate is discarded.

This step removes most of the remaining free cytoplasm and a portion of the intact or slightly ground cells.

3. All the sediments at sp. gr. 1.300 are resuspended in about 700 ml. of 1:1.5 cyclohexane-$CCl_4$ mixture; the suspension is brought to sp. gr. 1.310 and centrifuged at 2000 *G* for 60 minutes. The supernate, which contains many unground or partly ground cells, is discarded.

4. The sediments at sp. gr. 1.310 are again taken up in 700 ml. of 1:1.5 cyclohexane-$CCl_4$, the suspension is brought to sp. gr. 1.320, and centrifuged at 2000 *G* for 60 minutes. The supernate, which now contains partly ground cells and nuclei with large cytoplasmic tabs, is discarded.

5. The sediment at sp. gr. 1.320 is then resuspended in 500 ml. of 1:2 cyclohexane-$CCl_4$, brought to sp. gr. 1.390, and centrifuged at 2000 *G* for 60 minutes. The supernate, which contains the nuclei, is surmounted by a heavy crust which may adhere to the walls of the centrifuge tubes. The crust is detached from the walls with a small spatula, without disturbing the sediment, and the supernate and crust are decanted. Now the sediment, which contains lymphocyte nuclei and heavy mineral debris resulting from the grinding, is discarded.

6. Cyclohexane is then added to the supernate to lower the specific gravity to 1.327. The suspension is mixed by rapid stirring and centrifuged at 2000 *G* for 80 minutes. The supernate, which contains nuclei with large cytoplasmic tabs, is discarded. The sediment is again resuspended in about 400 ml. of 1:1.5 cyclohexane-$CCl_4$, brought to sp. gr. 1.327, and centrifuged for 80 minutes. The supernate is discarded.

7. The sediment at sp. gr. 1.327 is taken up in about 400 ml. of cyclohexane-$CCl_4$, brought to sp. gr. 1.333, and centrifuged at 2000 *G* for 80 minutes. The supernate, which contains nuclei with cytoplasmic tabs and some free nuclei, is discarded. The sedimented nuclear fraction is resuspended at sp. gr. 1.333 and recentrifuged, again discarding the supernate.

8. The nuclei are next suspended in about 300 ml. of 1:1.5 cyclohexane-$CCl_4$, adjusted to sp. gr. 1.338, and centrifuged at 2000 *G* for 80 minutes. The supernate, which contains both light, free nuclei and heavier nuclei with cytoplasmic tabs, is decanted. The sedimented nuclei are washed twice more at sp. gr. 1.338, following the above procedure, and discarding the supernates.

9. After the three washings at sp. gr. 1.338, the nuclei are almost free of cytoplasmic contamination. To remove the last traces of heavy debris, they are suspended in 200 ml. of 1:2 cyclohexane-$CCl_4$, brought to sp. gr. 1.390, and centrifuged at 2000 *G* for an hour. The supernate is carefully decanted, and the sediment is discarded.

10. The supernate is adjusted to sp. gr. 1.338 with cyclohexane and centrifuged for 80 minutes. The supernate, which contains a few tabbed nuclei, is discarded. The sediment, now entirely nuclear, is collected in about 100 ml. of petroleum ether and centrifuged at 900 *G* for 20 minutes.

The nuclei are then dried *in vacuo* at room temperature and weighed. (The "control tissue" preparation, which has been suspended in 2:1:1 petroleum ether–cyclohexane–$CCl_4$ throughout the nuclear isolation, is also centrifuged and dried *in vacuo* at this time.) The average yield of four preparations made in

this laboratory is 2.8 gm. of nuclei per 100 gm. of lyophilized cells. Careful microscopic examination of these preparations after staining and counterstaining showed negligible cytoplasmic contamination.

The appearance of the tissue suspension at different stages of the isolation procedure is shown in Figs. 1 to 5. The preparations are stained with hematoxylin-alcoholic eosin, under which conditions the nuclei appear purple and the cytoplasm red. Beginning with intact cells (Fig. 1), grinding in petroleum ether yields the mixed suspension shown in (Fig. 2), in which the red color of the cytoplasm predominates and most of the nuclei are bound to cell fragments. After treatment at specific gravities 1.290, 1.300, and 1.310 the nuclei form a larger part of the suspension and most of the free cytoplasm has been removed. Later in the procedure, after washing at sp.gr. 1.327, the suspension consists mostly of nuclei and tabbed nuclei (Fig. 3). Sedimentation at sp.gr. 1.338 leaves the nuclei and a few tabbed nuclei (Fig. 4). Repeated washings at sp.gr. 1.338 remove the last of the tabbed nuclei and give the final nuclear preparation (Fig. 5) which, morphologically, shows negligible contamination. A careful search of the slide may show a few cytoplasmic tabs amid hundreds of nuclei, but in a good preparation such tabs are extremely difficult to find.

*Goose Erythrocyte Nuclei.*—The isolation of goose erythrocyte nuclei from the ground cell suspension can be accomplished by following, without change, the procedure recommended for the preparation of the chicken erythrocyte nuclei. The nuclear preparations compare favorably in purity and in yield with those obtained from the red cells of the chicken.

*Calf Pancreas Nuclei.*—After removing an aliquot of the ground suspension for the "control tissue" preparation, the remainder is centrifuged at 900 *G* for 20 minutes and the supernate is discarded. The steps in the isolation of the nuclei from the sediment are as follows:—

1. The sediment is resuspended with rapid stirring in about 800 ml. of a 1:1 cyclohexane-$CCl_4$ mixture, centrifuged at 900 *G* for 20 minutes, and the supernate is discarded. The residues are washed twice more in the 1:1 solvent mixture as described, and both supernates are discarded.

2. The sediment is now taken up in about 700 ml of 1:1.5 cyclohexane-$CCl_4$; the suspension is adjusted to sp. gr. 1.335 and centrifuged at 2000 *G* for 60 minutes. The supernate, which contains both incompletely ground cells and free cytoplasm, is discarded.

3. The residue is suspended in 700 ml. of the 1:1.5 solvent mixture, the specific gravity is adjusted to 1.345, and the mixture is then passed through a wire screen (60 mesh, 250 micra apertures) to remove relatively coarse fiber. The filtrate is centrifuged at 2000 *G* for 60 minutes and the supernate is discarded.

4. The sediment at sp. gr. 1.345 is taken up in about 500 ml of 1:2 cyclohexane-$CCl_4$; the suspension is brought to sp. gr. 1.386 and centrifuged at 2000 *G* for an

hour. The supernate contains the nuclei and is carefully decanted. The sediment contains a mass of heavy fiber and some dense nuclei and is discarded.

5. Cyclohexane is added to the supernate with rapid stirring to bring the specific gravity to 1.355 and the suspension is centrifuged at 2000 *G* for 80 minutes. The supernate, which contains many nuclei with cytoplasmic tabs, fiber, and some free nuclei, is discarded. The residue is washed once more at sp. gr. 1.355 and the supernate is again discarded.

6. The sediment is taken up in about 400 ml. of 1:1.5 cyclohexane-$CCl_4$, brought to sp. gr. 1.360, and centrifuged at 2000 *G* for 80 minutes. The supernate contains some relatively light, free nuclei and heavier nuclei with cytoplasmic tabs. It is discarded.

7. The sediment at 1.360 is then suspended in about 200 ml. of 1:2 solvent mixture, the suspension is adjusted to sp. gr. 1.386 and filtered through wire screen (140 mesh, 105 micra apertures) to remove fiber. The filtrate is centrifuged at 2000 *G* for an hour. The supernate, which contains the nuclei, is carefully decanted, remixed, and recentrifuged. The sediments are discarded.

8. A little cyclohexane is added to the supernate to bring the specific gravity to 1.363 and the suspension is centrifuged at 2000 *G* for 2 hours. The supernate contains some tabbed nuclei and a little fiber. It is discarded. The sedimented nuclei are again washed at sp. gr. 1.363, in about 200 ml. of solvent mixture, and the supernate is discarded.

9. The sedimented nuclei are finally taken up in about 100 ml. of petroleum ether, filtered twice through fine wire screen (325 mesh, 44 micra apertures), and centrifuged at 900 *G* for 20 minutes. The nuclei are dried *in vacuo* at room temperature and weighed. The "control tissue" preparation is collected and dried at the same time.

About 2 gm. of nuclei are obtained from 100 gm. of lyophilized tissue. Microscopic examination shows very slight cytoplasmic contamination; *i.e.*, very few cytoplasmic tabs and rare traces of fiber.

*Fetal Calf Pancreas Nuclei.*—The above procedure, without modification, can be used for the preparation of nuclei from the ground suspension of fetal calf pancreas tissue. These nuclei show somewhat more contamination than those prepared from the tissue of the older animal.

*Horse Pancreas Nuclei.*—The same procedure gives good results in the isolation of nuclei from a ground suspension of horse pancreas tissue. These nuclei, stained with acetoorcein-fast green and magnified 1400 times, are shown in Fig. 6.

*Calf Thymus Nuclei.*—After removing an aliquot of the ground suspension for the "control tissue" preparation, the remainder is treated to isolate the nuclei.

1. The suspension is centrifuged at 900 *G* for 20 minutes and the supernate is discarded. The sediment is resuspended in 800 ml. of 1:1 cyclohexane-$CCl_4$ mixture and centrifuged at 900 *G* for 20 minutes. The supernate is discarded. The residue is

washed twice more with the 1:1 solvent mixture as described, discarding the supernates.

2. The sediment is next suspended in about 700 ml. of 1:1.5 cyclohexane-$CCl_4$; the specific gravity is adjusted to 1.312, and the suspension is centrifuged at 2000 *G* for 20 minutes. The supernate contains most of the free cytoplasm and some slightly ground cells. It is discarded. The residue is again taken up in 700 ml. of cyclohexane-$CCl_4$ at sp. gr. 1.312 and centrifuged as described. The supernate is again discarded.

3. The sediment is resuspended in about 700 ml. of 1:1.5 cyclohexane-$CCl_4$; the specific gravity is adjusted to 1.345, and the suspension is centrifuged at 2000 *G* for 60 minutes. The supernate contains many partly ground cells and nuclei with large cytoplasmic tabs. It is discarded.

4. The sediment at sp. gr. 1.345 is taken up in about 700 ml. of cyclohexane-$CCl_4$, brought to sp. gr. 1.358, and centrifuged at 2000 *G* for 60 minutes. The supernate is discarded.

5. The sediment at sp. gr. 1.358 is next suspended in about 700 ml. of 1:2 cyclohexane-$CCl_4$; the suspension is adjusted to sp. gr. 1.410 and centrifuged at 2000 *G* for 60 minutes. The supernate, which contains the nuclei, is carefully decanted. The sediment is discarded.

6. Cyclohexane is added to the supernate to bring the specific gravity to 1.368 and the suspension is centrifuged at 2000 *G* for 80 minutes. The supernate, which contains tabbed nuclei and fiber, is discarded.

7. The sediment at 1.368 is suspended in about 500 ml. of 1:2 cyclohexane-$CCl_4$, brought to sp. gr. 1.410, and centrifuged at 2000 *G* for an hour. The supernate is carefully decanted and the sediment is discarded.

8. A little cyclohexane is added to the supernate to lower the specific gravity to 1.373 and the suspension is centrifuged at 2000 *G* for 80 minutes. The supernate is discarded. The sediment is washed twice more at sp. gr. 1.373, using about 400 ml. of solvent mixture for each washing and discarding the supernates.

9. The sediment at sp. gr. 1.373 is taken up in about 400 ml. of 1:2 cyclohexane-$CCl_4$, adjusted to sp. gr. 1.378, and filtered through wire screen (140 mesh). The filtrate is then centrifuged at 2000 *G* for 80 minutes. The supernate contains many tabbed nuclei and is discarded. The sediment is washed thrice more at sp. gr. 1.378 using about 400 ml. of solvent for each washing. The supernates are discarded.

10. The sedimented nuclei are finally taken up in about 200 ml. of petroleum ether, filtered twice through wire screen (325 mesh), and centrifuged at 900 *G* for 20 minutes. They are dried *in vacuo* at room temperature and weighed. The "control tissue" preparation is collected and weighed at the same time.

About 23 to 25 gm. of nuclei are obtained from 100 gm. of lyophilized tissue. This yield is about 50 per cent higher than that reported by Behrens for calf thymus nuclei (1). These nuclei show negligible cytoplasmic contamination. A preparation, stained with acetoorcein-fast green and magnified 1400 times, is shown in Fig. 7. Immunological tests for serum protein contamination in thymus nuclei are described in the section on criteria of nuclear purity.

*Calf Heart Nuclei.*—After removing an aliquot for the "control tissue" preparation, the suspension is centrifuged at 900 *G* for 20 minutes.

1. The sediment is suspended in 800 ml. of 1:1 cyclohexane-$CCl_4$ mixture and centrifuged at 900 *G* for 20 minutes. The supernate is discarded. The sediment is washed twice more with the 1:1 solvent mixture as described, discarding the supernates.
2. The sediment is then taken up in about 700 ml. of 1:1.5 cyclohexane-$CCl_4$, brought to sp. gr. 1.325, and centrifuged at 2000 *G* for 60 minutes. The supernate is decanted, mixed, and recentrifuged. The supernate is now discarded.
3. The combined sediments are suspended in 700 ml. of cyclohexane-$CCl_4$ at sp. gr. 1.335 and centrifuged for an hour. The supernate is discarded.
4. The sediment at 1.335 is taken up in about 500 ml. of 1:1.5 cyclohexane-$CCl_4$, brought to sp. gr. 1.345, and centrifuged for 80 minutes. The supernate is discarded. By this time most of the free muscle fiber and many nuclei with large cytoplasmic tabs have been removed.
5. The sediment at 1.345 is then suspended in about 400 ml. of cyclohexane-$CCl_4$ at sp. gr. 1.355 and centrifuged at 2000 *G* for 80 minutes. The supernate is again discarded.
6. The sediment is taken up in about 400 ml. of cyclohexane-$CCl_4$, brought to sp. gr. 1.363, and filtered through 140 mesh wire screen to remove relatively coarse fiber. The filtrate is centrifuged for 2 hours, discarding the supernate. The sediment is washed twice more at sp. gr. 1.363, using 200 to 300 ml. of solvent for each washing and discarding the supernates.
7. The sediment at sp. gr. 1.363 is then suspended in 200 ml. of 1:2 cyclohexane-$CCl_4$, brought to sp. gr. 1.400, and filtered through 200 mesh wire screen. The filtrate is centrifuged at 2000 *G* for an hour and the supernate is carefully decanted. The sediment is discarded.
8. Cyclohexane is added to bring the specific gravity of the supernate to 1.360 to 1.363 and the suspension is centrifuged for 80 minutes. The supernate is discarded. The residue is washed twice more at sp. gr. 1.360 to 1.363, using about 100 ml. of solvent for each washing and discarding the supernates.
9. The nuclei are finally suspended in about 100 ml. of petroleum ether, filtered four or five times through 325 mesh wire screen, and centrifuged at 900 *G* for 20 minutes. They are dried *in vacuo* at room temperature and weighed.

About 1.5 gm. of nuclei are obtained from 100 gm. of lyophilized tissue. This is five times the yield reported by Behrens for calf heart nuclei (1). The isolated nuclei show very little cytoplasmic contamination. A preparation, stained with acetoorcein-fast green and magnified 1400 times, is shown in Fig. 8.

*Beef Heart Nuclei.*—The above procedure may be applied without modification to the preparation of nuclei from ground beef heart tissue. These nuclei, however, show more fibrous contamination than do nuclei prepared from calf heart.

*Calf Kidney Nuclei.*—After removing an aliquot of the suspension for the "control tissue" preparation, the remainder is centrifuged at 900 *G* for 20 minutes.

1. The sediment is resuspended in 800 ml. of 1:1 cyclohexane-$CCl_4$ mixture and centrifuged at 900 *G* for 20 minutes. The supernate is discarded. The sediment is washed twice more with the 1:1 solvent mixture and both supernates are discarded.
2. The sediment is then taken up in 700 ml. of 1:1.5 cyclohexane-$CCl_4$, brought to sp. gr. 1.325, and centrifuged at 2000 *G* for an hour. The supernate is discarded.
3. The sediment at 1.325 is resuspended in about 700 ml. of solvent at sp. gr. 1.335 and centrifuged for an hour, again discarding the supernate.
4. The sediment at 1.335 is taken up in about 500 ml. of 1:1.5 cyclohexane-$CCl_4$, brought to sp. gr. 1.350, and centrifuged at 2000 *G* for 80 minutes. The supernate is discarded.
5. The sediment is suspended in 500 ml. of solvent mixture at sp. gr. 1.365 and centrifuged as before. The supernate contains tabbed nuclei and fiber and is discarded.
6. The sediment at 1.365 is now taken up in about 400 ml. of 1:2 cyclohexane-$CCl_4$, brought to sp. gr. 1.410, and centrifuged at 2000 *G* for 60 minutes. The supernate, which contains the nuclei, is carefully decanted. The residues are discarded.
7. Cyclohexane is added to the supernate to bring the specific gravity to 1.370 and the suspension is centrifuged at 2000 *G* for 2 hours. The supernate is discarded, and the residue is again washed at sp. gr. 1.370, using about 300 ml. of solvent and discarding the supernate.
8. The sediment at 1.370 is suspended in 200 ml. of 1:2 cyclohexane-$CCl_4$, brought to sp. gr. 1.410, and centrifuged for an hour. The nuclei are carefully decanted, and the residue is discarded.
9. The nuclei are then brought to sp. gr. 1.373 and centrifuged at 2000 *G* for 80 minutes. The sediment is washed two or three times more at sp. gr. 1.373, using 200 ml. of solvent for each washing and discarding the supernates.
10. The nuclei are finally suspended in about 100 ml. of petroleum ether, filtered through 140, 200, and 325 mesh wire screens, and centrifuged at 900 *G* for 20 minutes. They are dried *in vacuo* at room temperature and weighed.

About 1.5 gm. of nuclei are obtained from 100 gm. of lyophilized tissue. The nuclei show very little cytoplasmic contamination, although there may be traces of fiber. A preparation stained with acetoorcein-fast green and magnified 1400 times is shown in Fig. 9. For contrast, the original ground tissue suspension stained in the same way, is shown in Fig. 10.

*Calf Kidney Cortex Nuclei.*—The preparation of nuclei from the kidney cortex suspension is very similar to that described above. The first four steps in the isolation are identical and the remainder of the isolation can be briefly summarized as follows:—

5. The sediment at sp. gr. 1.350 is washed twice at sp. gr. 1.360, using about 500 ml. of solvent mixture for each washing and discarding the supernates.

6. The sediment at 1.360 is suspended at sp. gr. 1.365 and centrifuged at 2000 *G* for 80 minutes.

7. The sediment at 1.365 is taken up in 400 ml. of 1:2 cyclohexane-$CCl_4$, brought to sp. gr. 1.405, and centrifuged for an hour. The supernate, which contains the nuclei, is decanted; the residue is discarded.

8. Cyclohexane is added to the supernate to bring the specific gravity to 1.363 and the suspension is centrifuged for 80 minutes. The sediment is washed twice more at sp. gr. 1.363, using about 300 ml. of solvent for each washing and discarding the supernates.

9. The sedimented nuclei are then floated in 200 ml. of cyclohexane-$CCl_4$ at sp. gr. 1.400 and filtered through 140 and 200 mesh wire screens.

10. The filtrate is brought to sp. gr. 1.363 and centrifuged at 2000 *G* for 80 minutes, discarding the supernate.

11. The nuclei are finally taken up in 100 ml. of petroleum ether, filtered twice through 325 mesh wire screen, collected by centrifugation, and dried *in vacuo* at room temperature. About 1.5 gm. of very clean nuclei are obtained from 100 gm. of lyophilized tissue.

*Chicken Kidney Nuclei.*—The ground tissue suspension is centrifuged at 900 *G* for 20 minutes and the supernate is discarded.

1. The sediment is washed three times with 800 ml. portions of 1:1 cyclohexane-$CCl_4$, in the usual way and the supernates are discarded.

2. The sediment is then suspended in 700 ml. of the 1:1.5 solvent mixture, brought to sp. gr. 1.320, and centrifuged at 2000 *G* for 60 minutes, discarding the supernate.

3. The sediment at 1.320 is taken up in about 600 ml. of cyclohexane-$CCl_4$ at sp. gr. 1.330 and centrifuged as before. The supernate is again discarded.

4. The residue is resuspended in 600 ml. of 1:1.5 cyclohexane-$CCl_4$, brought to sp. gr. 1.340, and centrifuged for an hour, discarding the supernate.

5. The sediment at 1.340 is next suspended at sp. gr. 1.353 and centrifuged for 80 minutes.

6. This sediment is taken up in about 400 ml. of cyclohexane-$CCl_4$ at sp. gr. 1.358 and centrifuged for 80 minutes.

7. The sedimented nuclei are then floated at sp. gr. 1.410 using about 200 ml. of solvent mixture, and filtered through 140 and 200 mesh wire screens.

8. The filtrate is brought to sp. gr. 1.358 and centrifuged for 80 minutes, discarding the supernate.

9. The nuclei are finally suspended in 100 ml. of petroleum ether, filtered twice through 325 mesh wire screen, collected by centrifugation, and dried *in vacuo*.

About 2 gm. of very clean nuclei are obtained from 100 gm. of lyophilized tissue. The absence of arginase activity in such nuclei is further evidence for the absence of cytoplasmic contamination, as described in the section on criteria of nuclear purity.

*Calf Liver Nuclei.*—The preparation of nuclei from calf liver tissue frozen in

liquid air and ground in the usual way is as follows: After removing an aliquot of the ground suspension for the "control tissue" preparation, the remainder is centrifuged at 900 *G* for 20 minutes.

1. The sediment is washed three times with 800 ml. portions of 1:1 cyclohexane-$CCl_4$ mixture, discarding the washings.
2. The residue is then suspended in 700 ml. of 1:1.5 cyclohexane-$CCl_4$, brought to sp. gr. 1.320, and centrifuged at 2000 *G* for an hour.
3. The sediment at 1.320 is next suspended at sp. gr. 1.335 and centrifuged as before, discarding the supernate.
4. The residue is then taken up in 600 ml. of 1:1.5 cyclohexane-$CCl_4$, brought to sp. gr. 1.345, and centrifuged for an hour.
5. The sediment at 1.345 is suspended in about 600 ml. of solvent mixture at sp. gr. 1.360 and centrifuged at 2000 *G* for 80 minutes. The supernate contains tabbed nuclei and fiber and is discarded. The residue is again washed at sp. gr. 1.360, as described, discarding the supernate.
6. The nuclei are then floated at sp. gr. 1.400 using about 400 ml. of cyclohexane-$CCl_4$, and filtered through 60 and 140 mesh wire screens.
7. The filtrate is brought to sp. gr. 1.365 and centrifuged at 2000 *G* for 2 hours. The sediment is washed twice more at sp. gr. 1.365, using about 300 ml. of solvent for each washing and discarding the supernates.
8. The nuclei are again floated at sp. gr. 1.400 using about 200 ml. of cyclohexane-$CCl_4$ mixture and filtered through 140 and 200 mesh wire screens.
9. The filtrate is brought to sp. gr. 1.365 and centrifuged for 80 minutes, discarding the supernate.
10. The nuclei are finally suspended in petroleum ether, filtered four or five times through 325 mesh wire screen, collected by centrifugation, and dried *in vacuo* at room temperature.

About 1.5 gm. of nuclei are obtained from 100 gm. of lyophilized tissue. Microscopic examination and enzyme studies show very little cytoplasmic contamination.

*Horse Liver Nuclei.*—Nuclear preparations from ground suspensions of normal and fasted horse liver were made using benzene–carbon tetrachloride mixtures. Since the nuclear density falls in a prolonged fast, the isolation procedures are not identical. The nuclei of normal tissue are prepared as follows: After removing an aliquot of the suspension for the "control tissue" preparation (which, in this case, is treated with 2:1:1 petroleum ether–benzene–carbon tetrachloride), the remainder is centrifuged at 900 *G* for 20 minutes.

1. The sediment is washed three times with 800 ml. portions of 1:1 benzene-$CCl_4$ in the usual way and the washings are discarded.
2. The residue is taken up in 700 ml. of 1:1.5 benzene-$CCl_4$, brought to sp. gr. 1.325, and centrifuged at 2000 *G* for 60 minutes. The supernate is decanted, mixed by rapid stirring, and centrifuged as before. The supernate is then discarded.

3. The combined sediments are then suspended at sp. gr. 1.335 and centrifuged for an hour. Again, the supernate is decanted, mixed, and recentrifuged. The final supernate is discarded.

4. The combined sediments at sp. gr. 1.335 are taken up in 600 ml. of 1:1.5 benzene-$CCl_4$, brought to sp. gr. 1.345, and centrifuged at 2000 *G* for 2 hours. The supernate contains tabbed nuclei and fiber and is discarded. The sediment is washed twice more at sp. gr. 1.343 to 1.348, using about 400 ml. of solvent and discarding the supernates.

5. The nuclei are then floated at sp. gr. 1.370, using about 300 ml. of benzene-$CCl_4$ mixture, and filtered through 60 and 140 mesh screens.

6. The filtrate is brought to sp. gr. 1.345 and centrifuged for 2 hours, discarding the supernate.

7. The sedimented nuclei are again floated at sp. gr. 1.370, using 200 ml. of solvent, and filtered through 140 and 200 mesh screens.

8. The filtrate is brought to sp. gr. 1.343 and centrifuged for 80 minutes. The sedimented nuclei are washed twice more in the specific gravity range 1.343 to 1.348 using about 200 ml. of solvent mixture and discarding the supernates.

9. The nuclei are finally suspended in 100 ml. of petroleum ether, filtered four or five times through 325 mesh screen, collected by centrifugation, and dried *in vacuo*.

About 1.2 gm. of very clean nuclei are obtained from 100 gm. of lyophilized tissue. A preparation of horse liver nuclei, stained with acetoorcein-fast green and magnified 1400 times, is shown in Fig. 11.

*Liver Nuclei from a Fasted Horse.*—The relatively low density of the nuclei following a prolonged fast requires modification of all but the first step in the above procedure. The remainder of the isolation can be briefly summarized as follows:—

2. The residue after washing with the 1:1 solvent mixture is suspended in 700 ml. of 1:1.5 benzene-$CCl_4$, brought to sp. gr. 1.315, and centrifuged at 2000 *G* for 60 minutes. The supernate is discarded.

3. The sediment is then suspended at sp. gr. 1.330 and centrifuged for an hour. The supernate is decanted, mixed, and recentrifuged. The final supernate is discarded.

4. The combined sediments at sp. gr. 1.330 are then taken up in about 400 ml. of 1:2 benzene-$CCl_4$, brought to sp. gr. 1.370, and centrifuged for an hour. The supernate is carefully decanted and filtered through 60 mesh wire screen.

5. The filtrate is brought to sp. gr. 1.335, centrifuged for 2 hours, and the supernate is discarded.

6. The nuclei are again floated at sp. gr. 1.370, using 200 to 300 ml. of solvent, and filtered through 140 and 200 mesh wire screens.

7. The filtrate is brought to sp. gr. 1.335 and centrifuged for 2 hours. The sediment is washed twice more in the specific gravity range 1.333 to 1.338, using about 200 ml. of solvent for each washing and discarding the supernates.

8. The nuclei are finally taken up in 100 ml. of petroleum ether, filtered twice through 325 mesh screen, collected by centrifugation, and dried *in vacuo* at room temperature.

About 1.2 gm. of very clean nuclei are obtained from 100 gm. of lyophilized tissue. Enzyme studies indicating the absence of cytoplasmic contamination are described in the section on criteria of nuclear purity.

*Rat Liver.*—A modified Behrens procedure for the isolation of nuclei from rat liver tissue is described by Dounce (3). In this laboratory satisfactory preparations of nuclei from normal rat liver could not be obtained, although fairly clean nuclei can be prepared if the animals are first fasted. The isolation procedure for fasted rat liver is essentially the same as that described above for fasted horse liver.

*Calf Intestinal Mucosa Nuclei.*—The following isolation procedure applies to both calf and fetal calf intestinal mucosa. After removing an aliquot of the ground suspension for the "control tissue" preparation, the remainder is centrifuged at 900 *G* for 20 minutes, discarding the supernate.

1. The sediment is washed three times with 800 ml. portions of 1:1 cyclohexane-$CCl_4$ in the usual way, discarding the washings.
2. The residue is suspended in 700 ml. of 1:1.5 cyclohexane-$CCl_4$, brought to sp. gr. 1.330, and centrifuged at 2000 *G* for an hour. The supernate is decanted, mixed, and recentrifuged. The final supernate is discarded.
3. The combined sediments are next suspended at sp. gr. 1.350 and centrifuged for an hour, discarding the supernate.
4. The sediment at 1.350 is taken up in 500 ml. of 1:2 cyclohexane-$CCl_4$, brought to sp. gr. 1.400, and centrifuged for an hour. The supernate, which contains the nuclei, is carefully decanted. The residue contains heavy "matrix" debris and is discarded.
5. Cyclohexane is added to bring the supernate to sp. gr. 1.360 and the suspension is centrifuged for 80 minutes. The supernate contains tabbed nuclei and fiber and is discarded.
6. The sediment at 1.360 is suspended in 400 ml. of solvent mixture at 1.370 and centrifuged for 2 hours. The supernate is discarded and the sediment is washed once more at sp. gr. 1.370 as described.
7. The nuclei are then floated at sp. gr. 1.400, using about 300 ml. of solvent, and filtered through 140 and 200 mesh wire screens.
8. The filtrate is brought to sp. gr. 1.375 and centrifuged for 2 hours. The sediment is washed twice more in the specific gravity range 1.373 to 1.378, using about 200 ml. of solvent for each washing and discarding the supernates.
9. The sediment is taken up in about 200 ml. of 1:2 cyclohexane-$CCl_4$, brought to sp. gr. 1.395, and centrifuged for an hour. The supernate is decanted, filtered through 325 mesh screen, and adjusted to sp. gr. 1.373.
10. The filtrate is centrifuged for 2 hours and the final supernate is discarded.
11. The sedimented nuclei are finally taken up in 100 ml. of petroleum ether, filtered twice through 325 mesh screen, collected by centrifugation, and dried *in vacuo* at room temperature.

About 3 gm. of nuclei are obtained from 100 gm. of lyophilized tissue. Careful microscopic examination after staining and counterstaining shows some contamination, mainly in the form of a colorless "matrix" which binds the nuclei in small clumps. Enzyme studies which indicate that the contamination is of the order of a few per cent are described below.

*Criteria of Nuclear Purity.—*

*Morphology.*—The first criterion of purity of a nuclear preparation is the appearance of the nuclei under the microscope. With the three staining reactions previously described and using high magnifications, the detection of cytoplasmic and other contaminants is simple and direct.

Where it exists, contamination generally consists of (1) small tabs attached to relatively large nuclei, (2) fiber, probably originating in connective tissue, blood vessel walls, etc., and (3) a relatively dense "matrix" material which binds nuclei together in small clumps and thus avoids the specific gravity separation. The latter impurity is sometimes the result of nuclear breakdown caused by overgrinding of the tissue suspension. In other cases however, *e.g.* intestinal mucosa, the "matrix" contamination appears to be cytoplasmic and cannot be removed by specific gravity separation alone.

Enzyme studies of intestinal mucosa nuclei indicate that the contaminant is present in only a few per cent; yet it is detected with ease under the microscope. Similar results with other nuclear preparations establish the high sensitivity of the staining reactions as a measure of nuclear purity. This sensitivity is not surprising, however, if one considers that hundreds of nuclei are sometimes examined in a single field and contrasting stains for cytoplasm emphasize the impurities.

Although careful examination of the nuclei throughout the isolation and at the end of the preparation is essential in forming an estimate of nuclear purity, it does not allow a quantitative measure of cytoplasmic contamination. The methods described below provide such quantitative data.

*Immunological Tests for Purity.*—A second criterion of nuclear purity is the absence of contaminating serum proteins. To test for such contaminants, antisera were prepared (in the rabbit) which would precipitate bovine serum albumin and bovine serum globulin (Armour's fraction II). Saline extracts of calf thymus nuclei, and of the "control tissue" preparation were then tested at different dilutions for their precipitin titers against these antisera. A comparison of the precipitin titers showed that the isolated nuclei contained less than 10 per cent of the serum protein concentration in the "tissue control." However, in calf thymus, nuclei comprise about 60 per cent of the tissue. It follows, therefore, that the serum protein content of the isolated nuclei is less than 5 per cent of the concentration in the non-nuclear portion of the tissue.

The same test was performed "in reverse" by preparing an antiserum (in the rabbit) to calf thymus nuclear extracts. This antinuclear serum was then tested for its precipitin titer against bovine serum albumin and bovine serum globulin. No precipitate was obtained against serum albumin and only exceedingly faint reactions were obtained with serum globulin.

*Enzymological Tests for Purity.*—The absence of serum proteins is one index of nuclear purity. An even more satisfactory criterion, however, is the absence of characteristic cytoplasmic proteins in the isolated nuclei. There are many cases in which a protein which is present in high concentration in the cytoplasm does not occur in detectable amounts in the nucleus. Beef heart nuclei, for example, do not contain myoglobin, since their total iron content (0.0098 per cent) is completely accounted for as non-heme iron. Yet myoglobin is one of the characteristic soluble proteins of beef heart tissue. Its absence from the nucleus means that a general migration of cytoplasmic components into the nucleus during the freezing of the tissue does not occur.

The absence of myoglobin in beef heart nuclei is to be contrasted with the occurrence of hemoglobin in fairly high concentration in the nuclei isolated from avian erythrocytes (4). To test the possibility that this nuclear hemoglobin represented cytoplasmic contamination which was ground into the nuclei (since it is not evident under the microscope, Fig. 5), calf thymus nuclei were prepared from a mixed suspension of thymus tissue and avian erythrocyte debris. The suspension was ground and fractionated in the usual way for the isolation of calf thymus nuclei. The isolated nuclei were white, in contrast to the dark brown coloration of erythrocyte nuclei, and their normal iron content (0.0066 per cent) indicated that no contamination by extraneous hemoglobin had occurred.

Further evidence for the purity of nuclear preparations is often provided by enzyme studies which compare the activity (in units per milligram) of the isolated nuclei to that of the "control tissue" preparation. The comparison is particularly striking in the following instances, in which the enzymes concerned occur in high concentration in the tissue.

*1. Catalase.*—There is no catalase activity detectable in nuclei isolated from calf kidney or chicken kidney.

*2. Arginase.*—The arginase activity of isolated chicken kidney nuclei is only 6.5 per cent of the activity of the tissue control.

*3. Uricase.*—There is no uricase activity detectable in nuclear preparations from calf liver and kidney. Horse liver nuclei (normal) show about 5 per cent of the uricase activity of the tissue control, and fasted liver nuclei show only 2 per cent of the total activity.

*4. Amylase.*—The amylase activity of beef pancreas nuclei is 7 per cent of that

measured in the tissue control. Horse pancreas nuclei have only 2.5 per cent of the total tissue activity.

*5. Lipase.*—Nuclei prepared from beef pancreas have 8 per cent of the lipase activity of the tissue control. Horse pancreas nuclei have only 1.4 per cent of the total tissue activity. These figures are in sharp contrast to the 50 per cent reported by Dounce (13) for beef pancreas nuclei prepared in citric acid. The latter figure is probably the result of selective nuclear adsorption of the enzyme from the aqueous tissue homogenate. This adsorption of cytoplasmic enzymes by the nucleus is one of the major disadvantages of aqueous isolation procedures.

*6. Alkaline Phosphatase.*—The alkaline phosphatase activity of calf thymus nuclei is only 1 per cent of the activity of the tissue control. Nuclear preparations from calf intestinal epithelium, which staining reactions show to contain some cytoplasmic contamination, have only 4 per cent of the total tissue activity.

An additional indication of nuclear purity is furnished by the comparison of nuclei prepared from normal and fasted horse liver tissue. The comparison shows that the enzyme content of the nucleus may vary, but the variation may occur in precisely the opposite direction to the changes observed in the tissue (4). For example, the esterase activity of the fasted tissue is 60 per cent higher than the normal; yet nuclei prepared from fasted liver show a fourfold diminution in their esterase activity relative to that of the tissue. Similarly, the arginase activity of the tissue is nearly doubled after prolonged fast. The relative arginase activity of the "fasted" nuclei, however, is only one-third of the normal value.

Thus, a great deal of the data of enzyme studies indicates that the nuclei are free of serious cytoplasmic contamination. Furthermore, since the enzymes considered are in some cases soluble, *e.g.* amylase and catalase, and in other instances insoluble, *e.g.* uricase, alkaline phosphatase, their absence in the nucleus supports the general conclusion that enzymes are not transported from cytoplasm to nucleus during the isolation. It follows that the Behrens procedure is an effective and reliable method for the study of intracellular enzyme distribution.

It is conceivable that the absence of certain enzyme *activities* in isolated nuclei may represent a complete selective destruction or selective inhibition of these enzymes when they occur in the nucleus; such inactivation or inhibition need not occur in the "control tissue" preparation. This possibility, however remote, cannot be disproved by measurements of relative enzyme activities. Nevertheless, the supposition of selective nuclear inactivation could not explain the absence of myoglobin in heart muscle nuclei, since this observation is based on an absolute measurement; *i.e.*, direct iron analysis.

*DNA Analyses of Nuclei and Tissue Preparations.—*

The desoxyribonucleic acid–phosphorus contents (DNA-P) of nuclei and "control tissue" preparations from diverse sources are listed in Table I. The DNA-P was determined colorimetrically by the diphenylamine reaction of Dische (7) applied to the hot 10 per cent perchloric acid extract of the nuclei, or tissue, after preliminary washings with 3:1 alcohol-ether and cold 2 per cent $HClO_4$.

Since the DNA is localized in the nucleus, the ratio of the DNA-P content of the tissue to that of the isolated nuclei gives the nuclear fraction of the

TABLE I

| Source of nucleus | (1) DNA phosphorus of nucleus | (2) DNA phosphorus of tissue | (3) Nuclear fraction of tissue: (2)/(1) |
|---|---|---|---|
| | *per cent* | *per cent* | *per cent* |
| Beef heart | 1.65 | 0.08 | 5 |
| Calf heart | 1.90 | 0.113 | 6 |
| Calf liver | 1.50 | 0.193 | 13 |
| Calf kidney | 1.70 | 0.344 | 20 |
| Calf kidney cortex | 1.93 | 0.330 | 17 |
| Calf thymus | 2.58 | 1.57 | 61 |
| Calf bone marrow | 2.34 | 0.301 | 13 |
| Beef pancreas | 1.71 | 0.163 | 9 |
| Equine pancreas | 2.20 | 0.328 | 15 |
| Equine liver, normal | 1.20 | 0.105 | 9 |
| Equine liver, fasted | 1.78 | 0.146 | 8 |
| Chicken kidney | 1.45 | 0.224 | 15 |
| Chicken erythrocytes | 2.55 | 0.345 | 14 |
| Goose erythrocytes | 2.34 | | |

tissue. These nuclear fractions are presented in column 3 of Table I. The treatment of the tissue preparation is such that the nuclear fraction is "corrected" for depot fat and coarse fiber. However, it is subject to error caused by removal of lipids from the cell. A striking variation in nuclear fraction is seen in different tissues, ranging from 5 per cent, in heart muscle, to 60 per cent in calf thymus.

*DNA and Protein Contents of Nuclei.—*

The DNA-phosphorus contents of the isolated nuclei are converted to percentage desoxyribonucleic acid and listed in Table II. As an approximation the rest of the nucleus can be considered to be made up of protein and ribonucleic acid. The latter can be determined colorimetrically by the orcinol reaction. The non-nucleic acid or protein portion of the nucleus is then obtained by

difference. The protein contents of the isolated nuclei, determined in this way, are listed in column 3 of Table II.

The ratio of protein to DNA, which is a measure of the protein content per nucleus, is given in column 4 of the table. This ratio varies from 2.8, in calf thymus, to 7.3 in normal horse liver. In all cases, it indicates a nuclear protein content which far exceeds the amount of protein measured in nuclei prepared by the citric acid procedures of Stoneburg (8) or Dounce (9).

Additional evidence for the relatively high protein content of nuclei isolated in non-aqueous media is obtained when these nuclei are extracted with dilute

TABLE II

*DNA and Protein Contents of Nuclei*

| Source of nucleus | DNA content | Protein content | Ratio protein/DNA | After extraction with citric acid | | |
|---|---|---|---|---|---|---|
| | | | | DNA content | Protein content | Ratio protein/DNA |
| | *per cent* | *per cent* | | *per cent* | *per cent* | |
| Calf thymus | 26 | 74 | 2.85 | 30 | 74 | 2.33 |
| Guinea pig thymus* | | | 8.4* | | | |
| Beef heart | 16.5 | 83.5 | 5.1 | | | |
| Calf heart | 19 | 80 | 4.2 | | | |
| Calf kidney | 17 | 79 | 4.6 | 25 | 73 | 2.9 |
| Calf liver | 15 | 85 | 5.7 | 22 | 74 | 3.35 |
| Calf bone marrow | 23.5 | 76.5 | 3.3 | | | |
| Beef pancreas | 17 | 81.5 | 4.8 | | | |
| Horse pancreas | 22 | 76 | 3.45 | | | |
| Horse liver | 12 | 88 | 7.3 | 22.5 | 74 | 3.3 |
| Horse liver, fasted | 18 | 82 | 4.55 | 27 | 72 | 2.7 |
| Mouse liver* | | | 16.9* | | | |
| Chicken erythrocytes | 25.5 | 74.5 | 2.9 | | | |

* Experiments of Pollister and Leuchtenberger.

citric acid and then analyzed for their nucleic acid and protein composition. The desoxyribonucleic acid content of liver, kidney, and thymus nuclei after citric acid extraction is given in column 5 of Table II. The corresponding protein contents are given in column 6, and the protein/DNA ratios are listed in column 7.

The DNA and protein contents of the extracted nuclei (with the exception of thymus nuclei) are the same as those found in corresponding nuclei prepared directly by the citric acid procedure. The data clearly indicate that dilute citric acid may remove from 18 to 55 per cent of the protein of the nucleus, depending upon its source.

Table II includes two entries for the protein/DNA ratios of guinea pig thy-

mus and mouse liver nuclei determined by microspectrophotometric analyses of single nuclei in tissue sections (10). These ratios are approximately three times higher than comparable ratios determined on isolated Behrens' nuclei. The probable reason for the discrepancy is the fact that the results of microspectrophotometric analysis commonly express DNA and protein concentrations in "arbitrary units." Since there is no evidence that these arbitrary units are identical for protein and DNA, the quotient of such units need not correspond to results obtained by quantitative chemical procedures.[3]

## DISCUSSION

One of the main reasons for isolating cell nuclei is to determine which of the diverse enzyme systems that characterize a cell occur in its nucleus. Before such detailed studies of isolated nuclei are begun, the minimum requirement is that the nuclear composition is not appreciably altered by the isolation procedure. This minimum requirement is not met in nuclei prepared in aqueous media. Such nuclei are subject to two major sources of error. First, proteins and other water-soluble nuclear components may diffuse out of the nucleus during the isolation and be discarded with the cytoplasm. The data presented in Table II indicate that as much as 55 per cent of the protein in the nucleus can be lost if the nuclei are prepared in dilute citric acid. The same criticism applies to nuclei prepared in sucrose solutions. For example, a careful preparation of sucrose nuclei from rat liver tissue in this laboratory (following the method of Wilbur and Anderson (11)) gave a product which contained no nucleoside phosphorylase. This is an enzyme we now know to be concentrated in the nucleus (4). Furthermore, "nuclear fractions" obtained in sucrose solutions do not meet the standards of purity of either citric acid or Behrens' nuclei.

The second criticism which can be made of aqueous isolation procedures is that soluble components of the cytoplasm and serum proteins may diffuse into the nucleus to be precipitated there as nucleic acid complexes. Recent reviews on intracellular enzyme distribution (12, 13) emphasize the high concentration of nucleotide phosphatases, *e.g.* ATPase, in nuclei isolated in aqueous media. Enzyme studies of nuclei prepared from many tissues by the procedures described in this report lead to the opposite conclusion (4). It should be emphasized that nuclei so isolated have been shown by many tests to be free of diffusion artifacts (as described in the section on criteria of nuclear purity).

The general conclusion which can be drawn from the data presented in this report is that nuclei isolated in non-aqueous media are better suited for the study of intracellular enzyme distribution than are nuclei prepared by "homogenization" techniques in citric acid or sucrose solutions. The number of enzymes that can be studied in this way is limited by the inactivation of some

[3] See addendum on page 554.

systems during the isolation of the nuclei. On the other hand, the wide experience with acetone-extracted tissue powders can be used as a guide in the selection of enzyme systems which are likely to resist inactivation during the isolation. Obviously, nuclei prepared in organic media cannot be used for studies of intracellular lipid distribution.

Earlier in this report it was mentioned that satisfactory preparations of normal rat liver nuclei could not be obtained in this laboratory. The "nuclear fractions" which were prepared showed appreciable contamination which could not be removed by specific gravity separation. The ribonucleic acid content of these "nuclear fractions" which is a fair measure of their cytoplasmic contamination, was, nevertheless, only a third of that reported by Dounce *et al.* in their isolated rat liver nuclei. No nuclear preparation obtained in this laboratory has an RNA content at all comparable to the 7.6 per cent reported by Dounce. Both calf and horse liver nuclei, for example, have only 1.5 per cent RNA, and fasted rat liver nuclei have only 1.2 per cent RNA. In general the RNA content of clean liver nuclei is only about a tenth of their DNA content, which ranges from 12 to 15 per cent depending upon the source. In our experience, the isolation procedures described by Dounce *et al.* for rat liver would lead to nuclear fractions showing gross cytoplasmic contamination. It should be emphasized, however, that the liver nucleus is only 8 to 13 per cent of the cell, and even a procedure which removes 90 per cent of the cytoplasm gives a nuclear preparation still showing about 50 per cent contamination.

## SUMMARY

1. A modified Behrens procedure is described for the isolation of nuclei from avian erythrocytes and from the liver, kidney, thymus, pancreas, heart, and intestinal mucosa of the calf or horse.

2. The purity of these nuclei has been established by staining reactions, enzyme studies, and immunological tests for serum proteins.

3. Evidence is presented to show that a transport of cytoplasmic proteins into the nucleus does not occur during the isolation.

4. Nuclei prepared in non-aqueous media contain considerably more protein and a very different enzyme composition from that observed in nuclei prepared by "homogenization" techniques in dilute citric acid.

5. The suitability of nuclei prepared in organic media for the study of intracellular enzyme distribution is discussed.

The authors are greatly indebted to Dr. Alexander L. Dounce, who generously provided us with a detailed description of the preparation of rat liver nuclei, and Walker carcinoma nuclei, by a modified Behrens procedure. This information was furnished to us before its publication (3) and was of great assistance in the formulation of the isolation procedures described in this report.

*Addendum.*—An alternate procedure to the analysis of isolated nuclei is the microspectrophotometry of single nuclei in tissue sections and in recent years there has come into being a large literature devoted to this subject. In its essentials the method uses a microscope, photoamplifier tube, and galvanometer to measure the light transmission of the nucleus after selective color reactions for nucleic acids and proteins. In the microscopic colorimetry of desoxyribonucleic acid (DNA) by Feulgen staining it has been shown that the extinctions measured have a relative, though not an absolute quantitative significance (Ris, H., and Mirsky, A. E., *J. Gen. Physiol.*, 1949, **33,** 125). This demonstration was possible only because the amounts of DNA in the nuclei tested microscopically were known from direct chemical analysis of the same nuclear species isolated (in bulk) in citric acid. Whether this proportionality between extinctions and DNA concentrations holds for all the nuclei so far analyzed microspectrophotometrically remains to be demonstrated.

It is the purpose of this addendum to point out that the minimum requirement of analytical chemistry, namely, that the method used be capable of analyzing a known sample, is not satisfied by *in situ* color reactions for nuclear protein. For example, Millon staining of thymus nuclei before and after extraction with $Hg^{++}$ in $H_2SO_4$ (to remove histone) indicates that histone comprises only 10 per cent of the total nuclear protein (10). Yet a direct chemical analysis of thymus nuclei, isolated by the procedures described in this report, shows that histone released in this way comprises 26 per cent of the nuclear mass and 35 per cent of the nuclear protein. The inescapable conclusion is that the microscopic Millon colorimetry of nuclear protein is not universally quantitative in either absolute or relative terms. Although the possibility exists that the method has relative quantitative significance in certain cases, it is the responsibility of those who use the method to demonstrate its validity in such cases.

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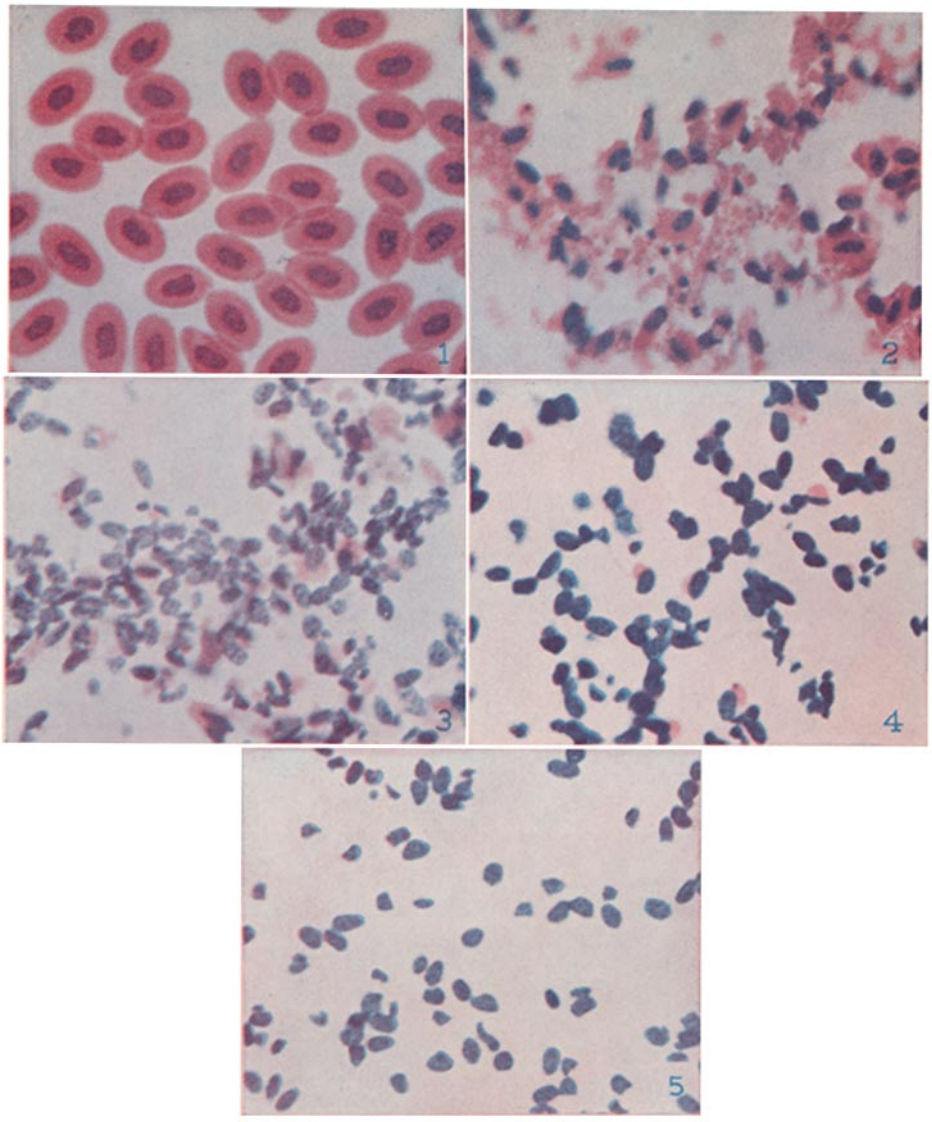
FIGURES




Stages in the isolation of chicken erythrocyte nuclei.

All samples stained with hematoxylin-alcoholic eosin. Magnification: × 1400.

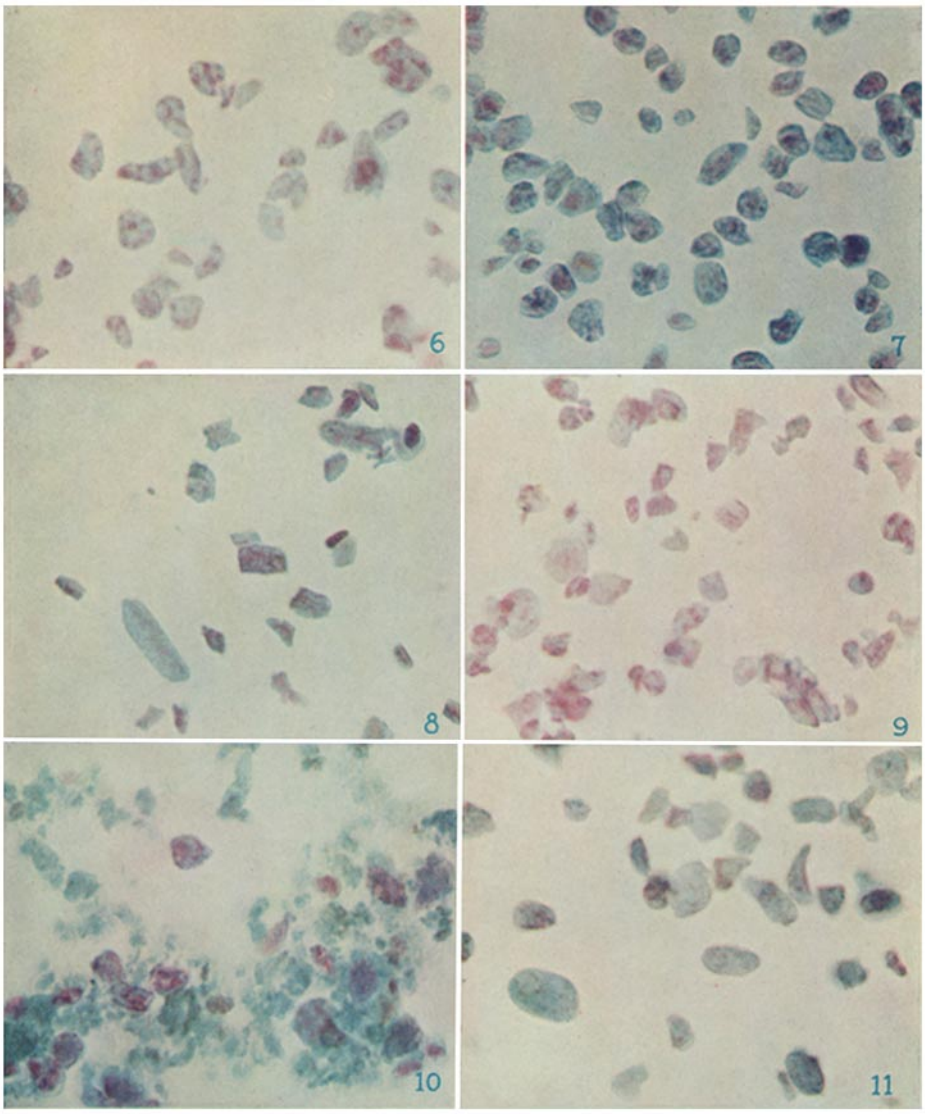
FIG. 1. Intact erythrocytes.

FIG. 2. Ground cell suspension before specific gravity fractionation.

FIG. 3. Residue after sedimentations in cyclohexane-$CCl_4$ mixtures at specific gravities 1.290, 1.310, and 1.327 to remove free cytoplasm and unground cells.

FIG. 4. Nuclear fraction after a single washing at specific gravity 1.338. Some tabbed nuclei still remain.

FIG. 5. Final nuclear preparation.




Isolated nuclear preparations.

All preparations stained with the acetoorcein-fast green mixture of Kurnick and Ris (6). Magnification: × 1400.

FIG. 6. Horse pancreas nuclei.

FIG. 7. Calf thymus nuclei.

FIG. 8. Calf heart nuclei.

FIG. 9. Calf kidney nuclei.

FIG. 10. Original ground tissue suspension from which nuclei (Fig. 9) were prepared.

FIG. 11. Horse liver nuclei.