A COMPARISON OF NUCLEAR DRY WEIGHTS DETERMINED BY CHEMICAL AND BY INTERFEROMETRIC METHODS*

By ARTHUR J. HALE, Ph.D., and ERNEST R. M. KAY,‡,§ Ph.D.

(From the Institute of Physiology and the Department of Biochemistry, University of Glasgow, Scotland)

Plates 22 and 23

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INTRODUCTION

Nuclei isolated from their cytoplasm have frequently been used in the examination of cell structure and function. The isolation techniques generally used have been reviewed by Schneider and Hogeboom (1) and more recently by Dounce (2). Most investigators have used nuclei isolated in aqueous media whereas a few have used those isolated in non-aqueous solvents. There is some discrepancy between the values obtained for certain constituents of the same tissue prepared by these two methods and it has recently been shown (3) that nuclei isolated in non-aqueous media contain more protein and ribonucleic acid (RNA) than do those isolated in aqueous media.

It is fairly easy to determine the mean dry weight of large numbers of nuclei isolated in aqueous media but difficult to determine it in those isolated in non-aqueous media. Thus any comparison of weight differences between nuclei prepared by these two methods is not an easy task. Interferometric (interference) microscopy provides a method of estimating the dry weight of small objects (4). The method is simple to use and gives accurate and easily reproducible results. With its aid we can weigh individual nuclei in small numbers.

Applying this newer microscopic method and modifications of the conventional isolation techniques we have examined nuclei isolated by the citric acid method, by the sucrose–calcium chloride method, and by the non-aqueous method in order to determine:—

(*a*) The mean nuclear dry weight (mass).

(*b*) The variation about the mean.

(*c*) The variation of dry weight with nuclear type.

(*d*) The variation of dry weight and nucleic acid content of the nuclei between different preparations.

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‡ Research Fellow of the National Cancer Institute of Canada.

§ Present address: Acadia University, Wolfville, Nova Scotia, Canada.

(*e*) The effect of aqueous extraction on non-aqueous preparations.

(*f*) The variation in dry weight with alterations in the morphology of the same type of nucleus.

(*g*) The effect of cytoplasmic contamination on the estimation of the dry weight of isolated nuclei.

Methods

1. Isolation of Nuclei.—Calf's thymus was used so that sufficient material would be available for the large number of manipulations carried out. It was obtained as soon as possible after the death of the animal and was frozen and kept at approximately −10°C. until required.

(*a*) Nuclei were prepared from a portion by the citric acid method described by Mirsky and Pollister (5) and by Smellie, Humphrey, Kay, and Davidson (6). These nuclei were examined and analysed immediately after isolation. A portion of the citric acid nuclei (CN) was frozen-dried (FDCN) and stored in a desiccator and used as required.

(*b*) Another sample of the tissue was carried through the non-aqueous method of preparation described by Kay, Smellie, Humphrey, and Davidson (3). These nuclei (NAN) can be stored dry and used as required.

(*c*) A further portion of the tissue was used for the preparation of nuclei according to the sucrose–calcium chloride method of Schneider and Peterman (7). These nuclei (SN) were examined and analysed immediately after preparation.

2. Chemical (Bulk) Methods of Nucleic Acid and Dry Weight Estimation.—(*a*) Aliquots of each preparation were carried through the following method for the determination of deoxyribonucleic acid phosphorus (DNA-P) and ribonucleic acid phosphorus (RNA-P) according to the procedure of Smellie *et al.* (6). A portion of the citric nuclei was treated with one-half volume of 30 per cent trichloracetic acid (TCA) and the precipitate washed twice more with 10 per cent TCA. A sample of NAN was weighed and extracted three times with 10 per cent TCA. In both cases the extracted residues were treated with successive portions of acetone, ethanol, ethanol-chloroform (3:1), ethanol-ether (3:1) and ether. The residues were dried and incubated overnight (18 hours) with 0.5 N KOH to hydrolyse RNA to the nucleotides. The alkaline digests were neutralised by the addition of 12 N perchloric acid. The resulting precipitate of DNA and protein was centrifuged down and washed twice with 0.7 N perchloric acid. The precipitate was dissolved in 0.1 N NaOH and the DNA-P content determined. The P in the washings containing the RNA nucleotides was determined by the Allen (8) method. Thus the percentage composition of a given weight of sample in terms of RNA-P and DNA-P was obtained.

(*b*) The dry weights of the CN and SN were estimated by counting the nuclei, in aliquots, using a haemocytometer, weighing the dry residues of similar aliquots, and thereby calculating the dry weights per nucleus. It was not possible to do this with the NAN as they would not disperse evenly in the suspension medium and thus could not be accurately counted. Their mean dry weight was obtained by the following method. The DNA-P content of a known weight of NAN was measured as described. Since the DNA-P per nucleus is the same regardless of the method of preparation (3) the number of nuclei in the known weight of sample can be determined by dividing the total DNA-P of the sample by the DNA-P per nucleus obtained from the CN preparation. Thus the mean dry weight of the NAN can be obtained by dividing the dry weight of the sample by the calculated number of nuclei present.

3. Interferometric Methods.—The method reported here was adopted after many observations had been made on nuclei isolated from several tissues and animals by a variety of visual methods of measuring optical retardation by means of interference microscopes.

According to Davies, Wilkins, Chayen, and LaCour (4) the dry weight of an object can be calculated from the formula

$$m = \frac{\phi A}{\chi} \tag{1}$$

where ϕ is the optical retardation (proportional to the phase change) in wave lengths (λ) produced by the object in the interference system, A is the projected area of the object in cm.^{2}, and χ is 100 α in which α is the specific refractive increment of the material in the object. In general χ can be considered to be 0.18 for most biological materials. Possible variations of χ in the material examined here will be considered later. When particulate objects are examined in a medium which has a refractive index (n) higher than that of water and which penetrates the interstices of the object then a correction factor must be applied to the formula given above. Thus

$$m = \frac{\phi A}{\chi} + (n_m - n_w)\frac{A}{\chi} t(1 - f) \tag{2}$$

in which t is the thickness of the object in cm., n_m, and n_w are the refractive indices of the medium and of water respectively, and f is the fraction of the object unoccupied by the particles in the object. The expression $t(1 - f)$ may be called the effective or optical thickness factor. This effective thickness may be calculated if the refractive index (n_p) of the object is known and if its optical retardation in any medium is known, since

$$\phi = (n_p - n_m)t(1 - f) \tag{3}$$

It has been found that the refractive index of fixed sperm heads is 1.54 (4) and that that of fixed thyroid colloid is also 1.54 (9, 10) and it has been stated (11) that this figure is applicable to most fixed microscopic specimens. Assuming that the refractive index of some of these nuclei is 1.54, since the optical retardation is measured in each one then the effective thickness can be calculated for each. As the correction factor is small and n_p may not be exactly 1.54 it is not worth while calculating the correction for each nucleus. We calculated several values for $t(1 - f)$ and found that it varied slightly around 2 μ. Thus a general correction for an effective thickness of 2 μ was applied to all nuclei examined in a medium with a refractive index different from that of water. If the effective thickness of the specimens is comparable then, merely by using formula 1 a comparison of the relative weights of two specimens examined in a medium of high refractive index can be made without calculating the thickness. In this case a "reduced" weight is obtained which is not influenced by errors arising in measuring the thickness.

The nuclei were examined in a Cooke-Dyson interference microscope using an objective of numerical aperature 1.3 and a × 10 ocular. Illumination, on the Kohler principle, was by means of a mercury vapour lamp from which the green band of 5461 A could be isolated by means of filters. The order of retardation of all the specimens was determined by examining them in "white light" with fringes in the field (Fig. 1). They were then examined and photographed on 35 mm. Kodak micro-file in green light. Each nucleus was placed in the fringe system in such a position that its retardation could be accurately measured by the fringe displacement method (4). In this method the photographic negative is scanned in a direct recording microdensitometer[1] (12), and the optical retardation calculated from the degree of displacement produced in the fringe system by the object. As the optical retardation varies across the surface of most of these nuclei attempts were made to get a true record

[1] Manufactured by Joyce, Loebel & Co., Newcastle-upon-Tyne, England.

of the average for each nucleus by taking three traces across it at different positions. The areas were measured by planimetry of the nuclear outlines. These were drawn at a fixed magnification on a photographic enlarger. The method is time-consuming but has an advantage over that of Caspersson, Carlson, and Svensson (13) in that a permanent remeasurable record is available which can be correlated with the area and the morphological features of the object at any time.

Nuclei were examined, in whatever medium was being used, on ordinary glass slides covered with conventional glass coverslips. Each specimen was ringed with cement to prevent evaporation of the medium during examination. Some degree of compression of the specimen can be obtained by allowing the mounting medium to evaporate for some time before ringing the preparation.

Fresh CN were examined in 0.01 M citric acid ($n = 1.333$). FDCN were examined in 0.01 M citric acid and in nonane ($n = 1.406$). NAN were examined in water, in 0.01 M citric acid and in nonane. SN were examined in water ($n = 1.333$).

Approximately 30 nuclei were examined in each sample.

TABLE I

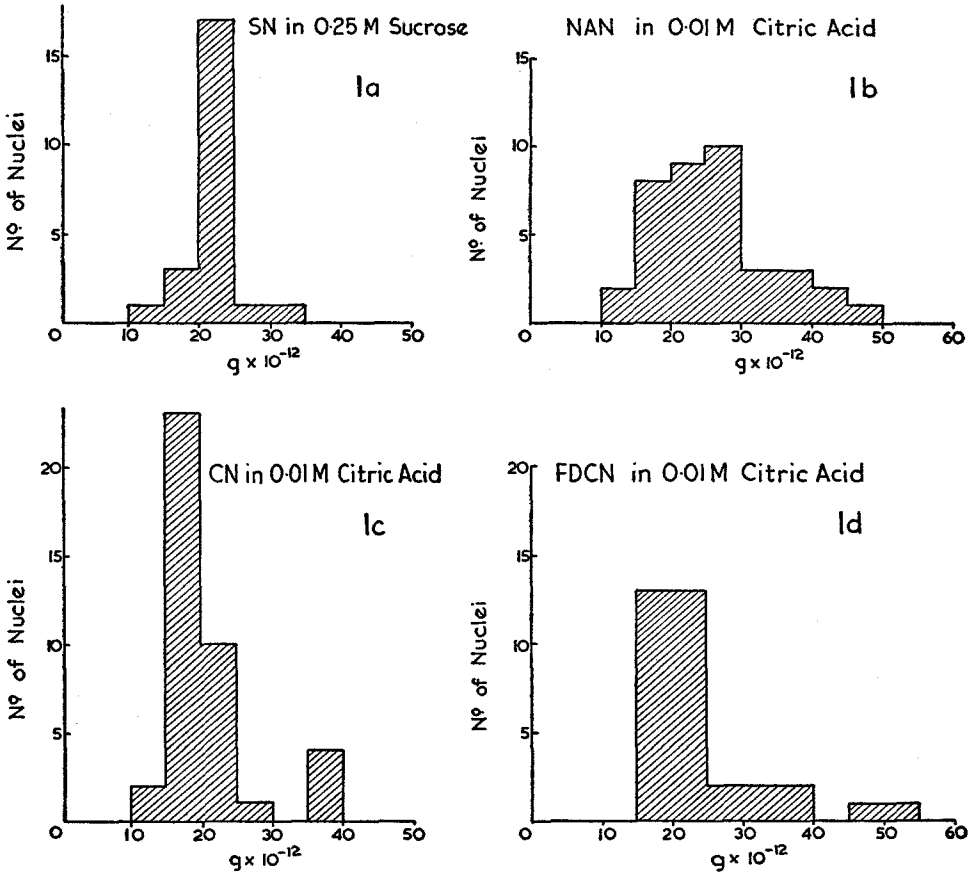
Mean Values in Picograms ($g \times 10^{-12}$) of the Dry Weight of Different Nuclear Preparations

(a) Type of nucleus	(b) Chemical value	(c) Interference value	(d) Mounting medium	(e) No. of nuclei	(f) Standard error
CN	19.1	19.8	0.01 M citric acid	39	0.900
FDCN		20.0	Nonane	35	0.914
FDCN		23.6	0.01 M citric acid	34	1.457
SN	23.0	21.1	0.25 M sucrose–$CaCl_2$	27	0.703
NAN	33.0	35.0	Nonane	30	2.562
NAN		25.5	0.01 M citric acid	38	1.361
NAN		17.7	Washed and mounted in 0.01 M citric acid	34	1.201
NAN		15.4	Washed and mounted in water	18	0.812

RESULTS

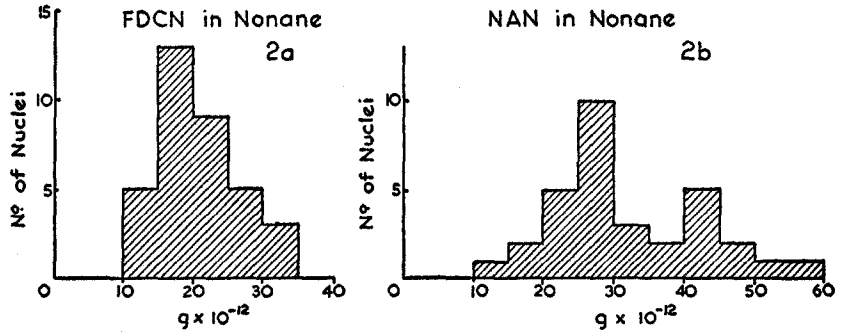
Table I gives the mean mass values for all the preparations examined. The values obtained by the bulk methods are given in column (*b*). The values obtained by interferometry are in column (*c*), the medium in which they were obtained in (*d*), and the number of nuclei examined by the latter method in column (*e*). The standard error of the mean is given in column (*f*). Text-figs. 1 to 4 show the results in the form of histograms. The mass values of the nuclei vary from 11 to 57 picograms ($g \times 10^{-12}$) depending upon the method of preparation. Text-fig. 1 *c* shows the weight distribution in fresh CN of the appearance shown in Fig. 2. The values vary from 13 to 26 picograms with a few higher values around 35 to 40 picograms. These higher values are given by larger nuclei of the type shown in Fig. 2. Text-fig. 1 *d* shows the distribution in CN which have been frozen-dried by a method which although efficient in removing the water from the specimen causes considerable morphological distortion of it. There is a similar pattern of distribution to that shown in Text-fig.

1 *c* despite the fact that the nuclei have a different appearance (Fig. 3). Text-fig. 1 *a* shows the distribution in SN examined in water. These nuclei are very large and have a low optical retardation. They have obviously become swollen by imbibition of water. If examined for some time they will be seen to swell and

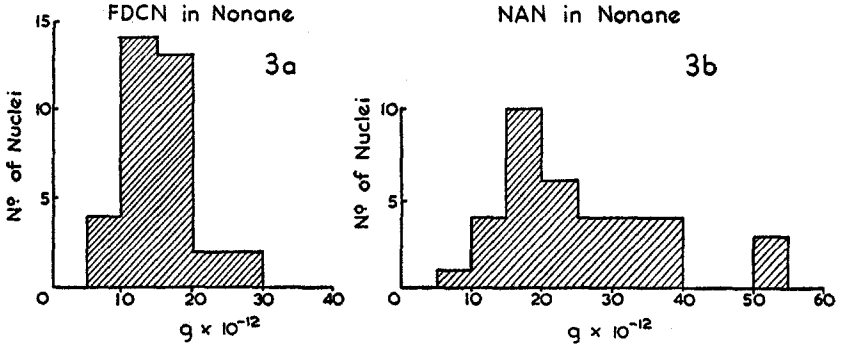


TEXT-FIG. 1. The distributions of dry weight values for sucrose–calcium chloride nuclei (SN), non-aqueous nuclei (NAN), citric acid nuclei (CN), and frozen-dried citric acid nuclei (FDCN) in the various media in which they were examined are shown in 1 *a*, 1 *b*, 1 *c* and 1 *d* respectively.

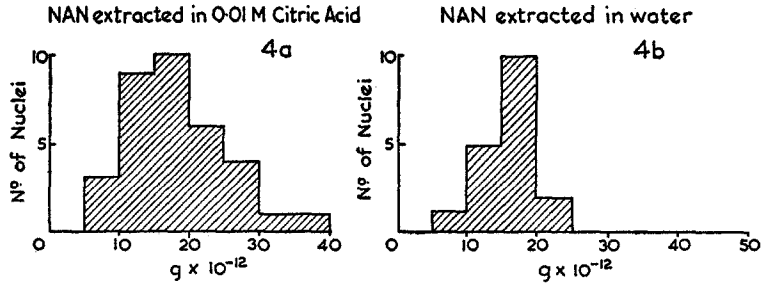
eventually rupture losing their contents into the medium. There can be considerable cytoplasmic contamination in this type of preparation as will be seen from Fig. 4. This contamination will prevent any accurate bulk estimation of values. If a cytoplasm-free preparation is chosen for microscopic examination, which is easily done, then again we get the same pattern of distribution of mass values despite the variation in morphology.



TEXT-FIG. 2. The distributions of dry weight values for frozen-dried citric acid nuclei (FDCN) and non-aqueous nuclei (NAN) examined in nonane are shown in 2 *a* and 2 *b*, respectively. The values given here are absolute, a correction having been applied for the thickness of the nuclei when the values were calculated.



TEXT-FIG. 3. The distributions of dry weight values for frozen-dried citric acid nuclei (FDCN) and non-aqueous nuclei (NAN) examined in nonane are shown in 3 *a* and 3 *b* respectively. The "reduced weight" values are given here; no correction has been made for the thickness of the nuclei when the values were calculated.



TEXT-FIG. 4. The distribution of dry weight values for non-aqueous nuclei (NAN) examined in 0.01 M citric acid (4 *a*) and water (4 *b*) after the sample had been extracted in these respective media.

Text-fig. 1 *b* shows the values for NAN which vary from 11 to 47 picograms with a distribution which is quite different from that of the CN samples. These nuclei are morphologically similar to those in the FDCN preparation. A possible explanation of this distribution might be that, when these NAN were mounted in an aqueous medium, then their water-soluble contents were leached out. Thus we obtained a variation between those which lost much and those which lost very little material in this way. To determine whether this was the case samples of the NAN and FDCN were reexamined in the non-aqueous medium nonane. Since this medium has a refractive index higher than that of water the calculated values are shown as absolute weights (Text-fig. 2) and "reduced" weights (Text-fig. 3) as explained above. When NAN are examined in nonane they have the appearance shown in Fig. 1. They look more like amorphous debris than nuclei. When examined in water they do however

TABLE II

Percentage Dry Weight Composition of Calf's Thymus Nuclear Preparations

	Percentage dry weight	
	NAN	CN
DNA-P	1.68	2.90
RNA-P	0.35	0.30
DNA	18.7	32.3
RNA	3.9	3.3
Protein	77.4	64.5
RNA-P/DNA-P	0.2	0.09
Protein/DNA	4.1	2.0

assume a nuclear appearance and since no cytoplasmic contamination can be detected it appears that all of the "debris" represents nuclear material. It is possible that any cytoplasmic contamination which may have been present could have gone into solution immediately after mounting the preparation. In our experience this type of solvent action, when occurring under a microscope coverslip, is a slow one and is easily detectable in an interference system. If small fragments of albumen are mounted in water and examined they will be seen to be surrounded by an area which is slightly darker or lighter than the remainder of the field. This area is due to the solution of albumen, of a refractive index higher than water, which surrounds the dissolving fragments. This process does not occur with NAN. These nuclei merely imbibe water until they burst.

NAN were examined after one sample had been shaken in a test tube with water and another with 0.01 M citric acid, to remove any water-soluble or acid-soluble material which might be in them. The distribution of the weight values

obtained is, in the citric acid–extracted sample, similar to that in other NAN preparations but the mean value is very low (Text-fig. 4). The distribution in the water-extracted sample is similar to that in CN preparations and the mean value of it is low. These citric acid–extracted nuclei are morphologically similar to FDCN and the water-extracted nuclei are similar to those isolated in sucrose.

Table II shows the content of DNA-P and RNA-P for the CN and NAN preparations as percentages of the dry weight. The content of DNA-P is appreciably higher in the CN and the ratio of RNA-P to DNA-P is consequently lower. The approximate values for DNA and RNA in this table were calculated from the above values and those given in Table I, assuming that the P content of RNA and DNA is 9 per cent.

DISCUSSION

It has previously been shown (3) that a considerable amount of protein and RNA can be lost from nuclei during their isolation in aqueous media. In the present investigation we have found that calf thymus CN contain about 45 per cent less material than do NAN on the basis of absolute dry weight differences. This corresponds with the values obtained for the amount of material extractable with aqueous solvents from NAN, as reported by Dounce, Tishkoff, Barnett, and Freer (14), Allfrey, Stern, Mirsky, and Saetren (15), and Kirkham and Thomas (16).

There is a close correlation between the interferometric mean dry weight values for fresh CN examined in citric acid, the FDCN examined in nonane, and the bulk mean dry weight value for CN. All these mean values lie between 19 and 20 picograms. Similarly, there is a close correlation between the mean interferometric value for NAN examined in nonane (35 picograms) and the mean bulk value (33 picograms) of the same preparation. There is also a reasonable correlation between the mean values obtained for SN by both methods: 23 picograms by the bulk method and 21.1 picograms by the interferometric method. The higher values in the bulk method may be due to the slight cytoplasmic contamination which was present in the preparation.

It has been shown (3) that part of the material lost in dilute citric acid is RNA since the percentage composition of RNA in CN does not rise appreciably when their absolute weight drops. Dounce (2) states that calf's thymus NAN contain about 74 per cent protein. Thus the lower weight values in the aqueous preparations are presumably due to loss of protein and RNA during isolation.

The percentage composition of DNA-P and RNA-P of the nuclei reported here and the ratio of RNA-P to DNA-P, correspond closely to those reported by Kay *et al.* (3) for the nuclei of rabbit's thymus.

Mellors, Stoholski, and Beyer (17) have reported the ratio of protein to DNA as being 4:1 in mouse chromosomes. In the present investigation this ratio

holds for those nuclei isolated in non-aqueous media (NAN) but is reduced to 2:1 for those nuclei isolated in aqueous media (CN) (Table II). These ratios are based on mean values of protein and DNA for a nuclear population and the individual values may vary considerably about that mean as we see from the present investigation.

The mean values for dry weight are significantly different between NAN in nonane and FDCN in nonane ($p < 0.002$). There is no difference between FDCN in nonane and CN in citric acid. The scatter of values in the NAN is significantly different from the scatter in the aqueous preparations. A comparison of the possible significance of the other results can be made from columns (*c*) and (*f*) of Table I. Despite the fact that we are comparing the result obtained by a method involving tens of nuclei with one obtained from a method involving millions the results show a very close correlation. A more rapid method of measurement in the microscope system would provide more values which would be useful in investigating the scatter obtained in the NAN to see if it would fall into a normal distribution.

Preliminary observations of appendix and intestinal mucosa show that in the latter tissue there is a very great variation of nuclear dry weight which is probably related to the presence of epithelial, fibroblast, lymphocyte, and other nuclei.

The nuclear dry weight values in the samples examined show a rather low and narrow distribution in the aqueous (CN, FDCN, and SN) preparations. The broader distribution to higher values in the NAN preparations corresponds to that reported by Hale (18) in living chick heart fibroblasts. The NAN values reported here range from 10 to 47 picograms and the chick heart fibroblast values varied from 10 to 40 picograms. Walker and Yates (19) and Davies (20), using microspectrophotometric techniques, have shown that chick heart fibroblasts contain around 5 to 15 picograms of DNA and that there is an increase from the lower to the higher value during interphase. Richards (21) has shown that there is a similar increase in dry weight during interphase. It may be that the distribution of dry weight values reported here, for fixed thymus NAN in interphase, corresponds to the pattern already described for living tissue culture nuclei in interphase. The lower and narrower distribution in the aqueous preparations would then represent the DNA fraction together with the smaller amount of protein which is found in the aqueous nuclei.

The occasional scattered higher values correspond to two types of nuclei. The larger of these is about 7μ in diameter and is finely granular or reticulated when seen in a citric acid preparation (Fig. 2). It is probably the nucleus of a reticular cell. Apart from these very large nuclei there is very little variation from the normal distribution of weight in the citric acid preparations despite the considerable variation in the appearances of the nuclei, *e.g.* in Fig. 2 the very small dense nuclei contain the same amount of material as the slightly

larger but partly reticulated ones. It is not possible to correlate variations in weight with any morphological characteristics in the SN, FDCN, or NAN preparations. Thus we are left in the position of being able to identify weight differences with cell type only in a preparation which is unsatisfactory for accurate quantitative measurements.

The second type of nucleus that gives high dry weight values is found in the non-aqueous preparation. It is small, irregular, and highly refractile and its apparent high dry weight value is discussed below.

Sources of Error in Measuring Mass Values

1. *Chemical Methods.*—When using CN which can easily be dispersed in citric acid and counted in a haemocytometer the main source of error is that inherent in any haemocytometric method. It is not possible to use this method with NAN as these nuclei will not disperse easily and thus cannot be counted accurately. Consequently the more indirect method described has to be used. This assumes that the DNA-P content per nucleus of CN and NAN is the same.

2. *Interferometric Methods.*—As has been pointed out (4) objects which are markedly ahomogeneous or highly refractile are not suitable for microscopic interferometry. Fresh CN are ahomogeneous but not highly refractile. Using the trace displacement method it is possible to get easily reproducible results. If a method of visual estimation of optical retardation is used, and several have been tried "*in extenso*," then reproducible values cannot be obtained. The sucrose-calcium nuclei are neither ahomogeneous nor highly refractile and are excellent objects for the method, but they are unstable when examined in water and are frequently contaminated by much cytoplasmic debris making the chemical methods unreliable. They could be examined in other media of a more desirable osmotic pressure but then a small correction would have to be made for the higher refractive index of that medium. The NAN are homogeneous but highly refractile. A few of them, which have already been mentioned, are so small and dense that the optical retardation at their centres is too great to permit measurement by the trace displacement method. Some of these can be flattened by compression under the coverslip and they can then be measured. With others this cannot be done.

A series of measurements were made on a few nuclei which showed that, with increasing flattening, the decrease in optical retardation of the nucleus was compensated by an increase in area which kept the measured dry weight constant.

Any variation in the value of χ between preparations might introduce a source of error. In the sucrose and fresh CN a value of 0.18 for χ can be safely assumed as these nuclei have their protein contents in a reasonably hydrous state. In the FDCN and NAN examined in citric acid the nuclei appear to have

imbibed a certain amount of water and again 0.18 appears to be a reasonable value to assume. In the FDCN and NAN examined in nonane these nuclei are assumed to be anhydrous; thus the value of χ may be appreciably lower. Davies *et al.* (4) state that it may vary between 0.17 and 0.15 for dry protein. Since any fall in χ will increase the calculated dry weight of an object then the values given for FDCN and NAN in nonane may be too low. Both sets of values will presumably be influenced to the same degree however and their relative relationship will not alter. The close correlation between the results obtained from these two preparations using the chemical and interferometric methods suggests that the value of 0.18 assumed for χ is not far wrong.

SUMMARY

1. The nuclei of cells from the thymus of the calf were isolated by three different techniques; the citric acid, the sucrose–calcium chloride, and the non-aqueous.

2. The mean dry weights of the nuclei were determined by chemical methods and by microscopic interferometry. There was a close correlation between the results from the interferometric and chemical methods.

3. The range of values about that mean was determined in each sample: the nuclei isolated in aqueous media contained approximately 45 per cent less material than those isolated in non-aqueous media.

4. The variations in dry weight with varying nuclear type are discussed.

5. The possible relationship between DNA content and dry weight is discussed.

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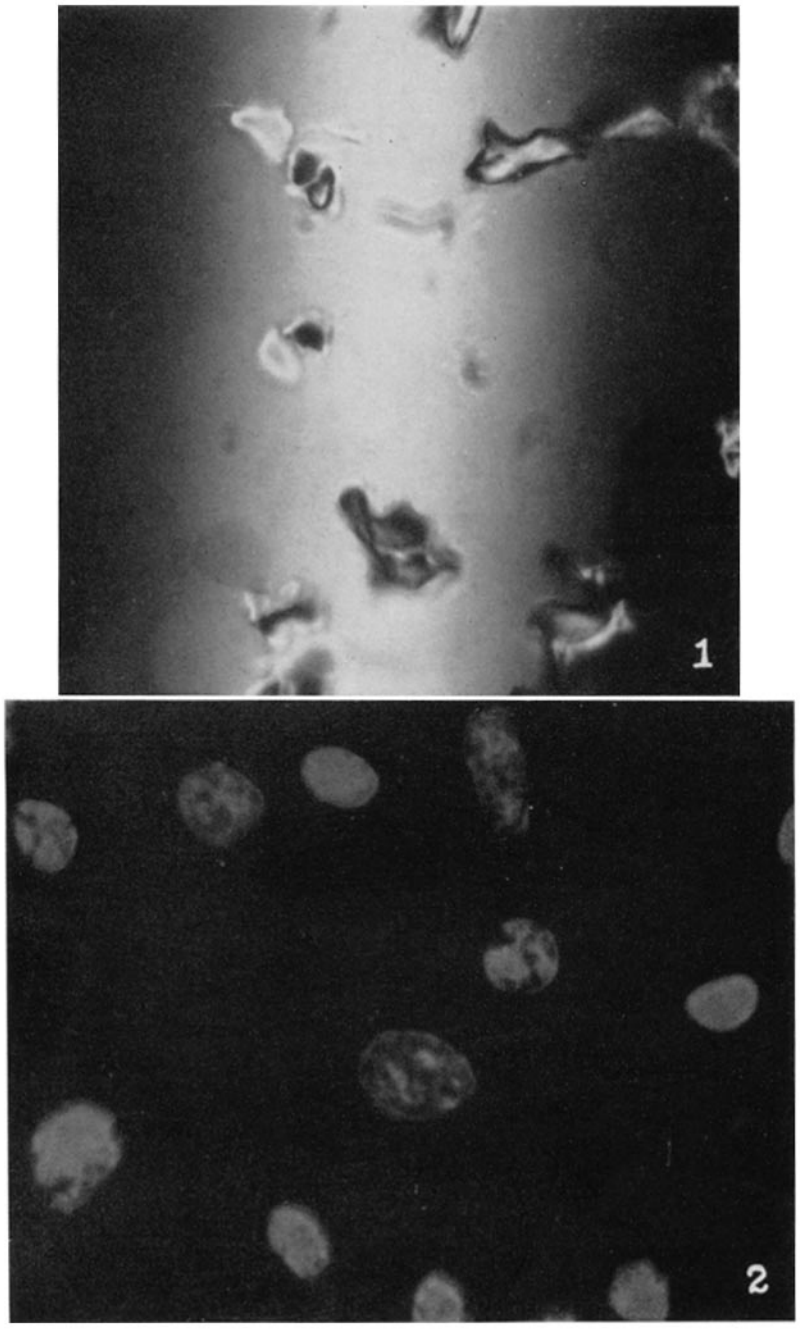
EXPLANATION OF PLATES

PLATE 22

FIG. 1. Non-aqueous nuclei (NAN) mounted in nonane and photographed with interference fringes in the field. × 950.

FIG. 2. Fresh citric acid nuclei (CN) mounted in 0.01 M citric acid and photographed at infinite fringe separation. × 2000.

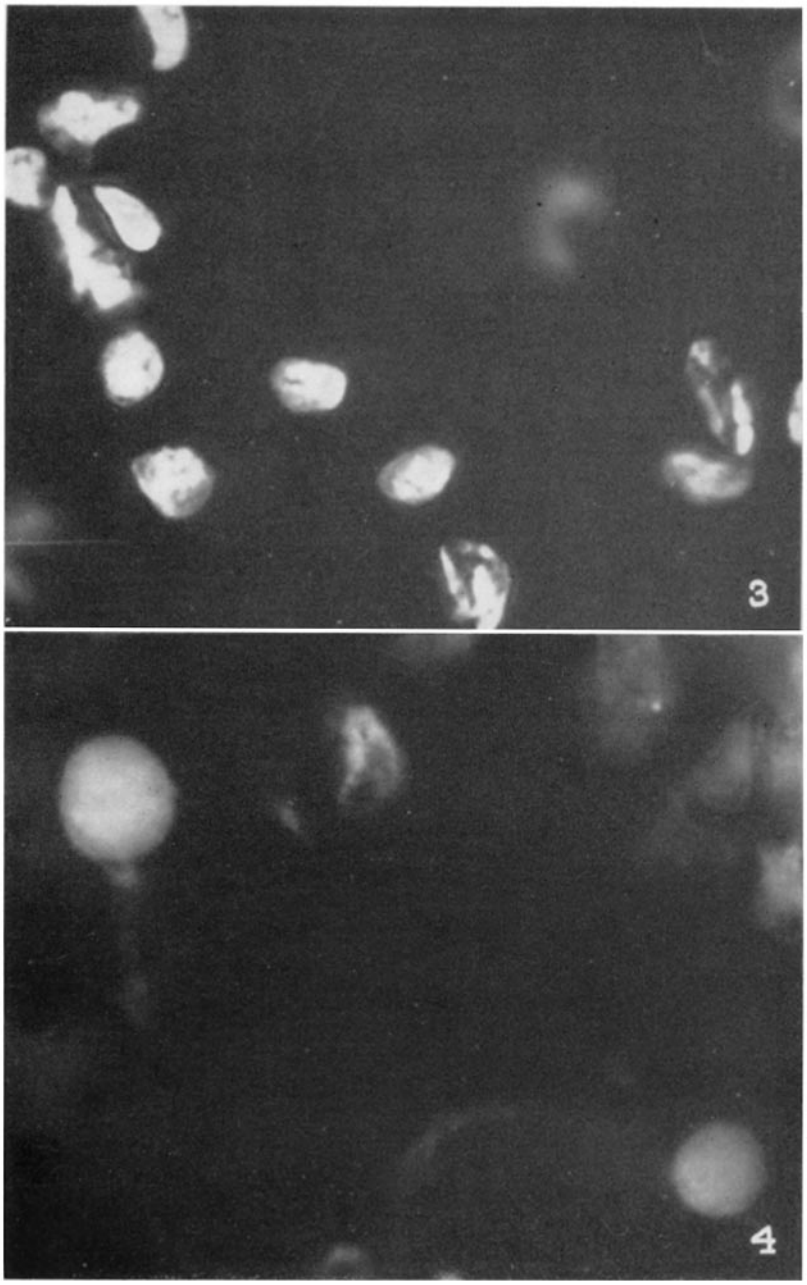


(Hale and Kay: Comparison of nuclear dry weights)

PLATE 23

FIG. 3. Frozen-dried citric acid nuclei (FDCN) mounted in 0.01 M citric acid and photographed at infinite fringe separation. × 2000.

FIG. 4. Sucrose nuclei (SN) mounted in 0.25 M sucrose and photographed at infinite fringe separation. × 2000.



(Hale and Kay: Comparison of nuclear dry weights)