MICROCHEMICAL DEOXYRIBONUCLEIC ACID DETERMINATION IN INDIVIDUAL CELLS

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ABSTRACT

A method for the quantitative determination of DNA in the 50 to 500 $\mu\mu$g. range is presented. Cells or cell nuclei are isolated individually from fixed tissue by means of micromanipulation. The tissue units in question are extracted in an oil chamber with deoxyribonuclease solution. The extracts are evaporated to dryness and redissolved to lens-shaped drops, the DNA contents of which are determined by a photographic-photometric procedure in ultraviolet light. Determinations on calf thymocytes and rat spermatids show a relatively good agreement with biochemical data. The present method tends, however, to give somewhat higher values than those reported earlier. The coefficient of variation for analytical values from test material is about ± 10 per cent. The method has been applied to cells from the axolotl, adults as well as tadpoles. Germ cells (spermatids and spermatocytes) do not show any evidence of a biological variation in DNA content. Cells from proliferating tissues give an increased spread of the DNA values. It could be shown, for epithelial cells, that there are at least two factors determining the DNA content of these cells. One is the fact that the cells are investigated at different phases of the mitotic cycle; the other is the fact that the DNA synthesis cycle occupies different ranges for different cells.

Cytochemical methods for DNA determination are based on measurements of a natural or induced light absorption in tissue sections. In spite of the success of some of these methods it is desirable in certain instances to use procedures in which the optical and physicochemical conditions are more favourable and thus decrease the methodological errors, which in the standard procedures often lead to uncertainties about the biological significance of recorded variations.

Pigon and Edström (16) extracted DNA from single protozoa and performed the determinations in the extracts, thus avoiding the major difficulties of *in situ* methods. This procedure has now been further developed to permit quantitative determinations in isolated single urodele cells or small groups of mammalian cells.

MATERIALS AND METHODS

Adults and tadpoles of the axolotl *Sideron mexicanicum*, calf thymus, and rat testes were used for these investigations. The tissues are fixed in Carnoy's solution for 90 minutes and embedded in paraffin after passing through ethanol and benzene. 25 to 40 μ sections are cut and mounted on 12 × 32 × 0.17 mm. coverglasses. For microdissection the preparations are deparaffinized with chloroform and hydrated with a 0.01 N acetic acid solution after passing through diethylether and 70 per cent ethanol, and placed, with the sections underneath, over a groove in a thick slide. The space below the coverglass is filled with liquid paraffin and the whole arrangement constitutes an oil chamber according to de Fonbrune (4). The micromanipulator of de Fonbrune equipped with two needles is used to separate and transport cells or cell nuclei. The manipulator is also used for

directing micropipettes during the later stages of the procedure.

Before the determinations of DNA, RNA is extracted with a ribonuclease solution in a concentration of 0.4 mg. crystalline pancreatic ribonuclease (Worthington Corporation, Freehold, New Jersey) per ml. 0.2 N ammonium bicarbonate solution adjusted to pH 7.6 with acetic acid (6). A prolonged incubation (overnight) was used for epithelial cells, which were found to be particularly resistant towards the action of ribonuclease.

For extracting DNA a solution of the following composition is used: 0.4 mg. of Worthington's crystalline pancreatic deoxyribonuclease per ml. 0.02 M phosphate buffer of pH 7.0, containing 0.003 M $MgCl_2$, 0.005 M hydroxylamin, and 0.1 per cent gelatin. Hydroxylamin is used to suppress any possible contaminating proteolytic activity (7), and the purpose of including gelatin is to prevent surface denaturation of the enzyme at water-oil interphases in the oil chamber. Only freshly dissolved enzyme is used. The buffer solution is kept in the refrigerator.

As a rule the cells or cell nuclei are extracted four times with the enzyme solution, using volumes (0.05 to 0.1 mμl.) of an order about ten times larger than that of the tissue units in question. All enzyme volumes are placed as individual drops in the chamber before use. The extracts are evaporated to dryness on a quartz glass, 24 × 30 × 0.5 mm., in an oil-free part of the chamber. Extracts originating from the same tissue unit are placed close together. After an extract has been delivered, the pipette is used for taking a new volume of enzyme solution to the same unit. In this way the pipette is washed after every extraction except the last one, and the washings are included with the new extracts. The present work has been carried out at room temperature (21–23°C.), although it was found later that a higher temperature (37°C), which accelerates the digestion, could be used with advantage. The whole deoxyribonuclease extraction procedure lasts for 4 to 6 hours.

The completeness of the extractions was ascertained in a number of cases by making further extractions with new enzyme solution and inspecting the extracts in the ultraviolet microscope. To investigate whether the enzyme is able to remove *all* DNA present, tests were carried out with 1 N perchloric acid for 15 to 17 hours at 21–23°C. Controls on untreated sections showed that this treatment removed all stainable DNA. From cells which had undergone the enzymatic extraction procedure, perchloric acid extracted ultraviolet-absorbing material corresponding to about 10 per cent of that present in the DNA extracts, when calculated on the basis of the ultraviolet absorption at 257 mμ. 10 per cent represents a maximal figure for undissolved nucleic acid, since dissolved protein degradation products also contribute to this figure. Methylene blue stainings (17) were also performed on some material, showing that all stainable DNA was removed.

TABLE I

Recovery of Nucleotides in Extracts from Fixed and Sectioned Calf Thymus

	mg.
Non-lipid dry weight	10.040
Nucleotides, calculated as adenylic acid (AMP) in ethanol-HAc extract	0.050
RNA removed by ribonuclease	0.245
DNA removed by the standard extraction procedure	1.420
DNA removed by further digestion with deoxyribonuclease	0.098
Remaining nucleotides extracted by 1 N perchloric acid	0.050

The extraction procedure was also checked by a macrochemical investigation. Pieces of calf thymus were fixed and embedded according to the routine procedure. They were sectioned at 15 μ and the sections were put in a centrifuge tube. Here they could be subjected to various extractions and the extracts could be collected after the sections had been spun down. The procedure used for the microextractions was followed as closely as possible. The sections were first deparaffinized with chloroform-ether, and subsequently dried and weighed. They were then extracted with 70 per cent ethanol and 0.01 N acetic acid. Some ultraviolet-absorbing material was obtained in the combined ethanol–acetic acid extracts. It was found to be dialyzable through a cellophane membrane, and it is known to contain a high proportion of adenine (5). Consequently, this absorption is probably chiefly due to free nucleotides. RNA and DNA were then extracted enzymatically with the solutions used for the microextractions for the corresponding periods at 22°C. The completeness of the removal of DNA was checked by subjecting the sections to renewed treatment with the enzyme solution at 37°C. for 2 hours. Finally the sections were extracted with 1 N perchloric acid for 19 hours at 22°C. Complete spectra were taken of all extracts in the Zeiss spectrophotometer. The results are shown in Table I. As can be seen from the table, the DNA extraction is complete to at least 90 per cent, which is in agreement with the microchemical tests. The DNA removed according to the standard procedure was also measured with the diphenylamine reaction (Dische), giving 1.350 mg., which may be compared with the amount calculated from the ultraviolet absorption, 1.420 mg.

The importance of extracting RNA before DNA

TABLE II

The Effect of Varying the Order of the Digestions with Ribonuclease and Deoxyribonuclease on the Amounts of Ultraviolet-Absorbing Material Recovered in the Different Fractions Extracted from Nuclei of Adult Axolotl Tissue

Values in $\mu\mu$g. per nucleus. Each figure represents the mean of seven determinations.

	Spermatocytes		Spermatids	
	RNA fraction	DNA fraction	RNA fraction	DNA fraction
Ribonuclease before deoxyribonuclease	50	181	15	50
Reversed order	19	225	6	53

is illustrated by an experiment shown in Table II, in which the material was divided into two groups, in one of which ribonuclease was applied before deoxyribonuclease, while the order was reversed in the other. It was found that, in the latter case, more absorbing substance is recovered in the DNA fraction than when the usual order is followed and RNA is extracted before DNA, which indicates that RNA is partly extracted by the deoxyribonuclease solution, if not removed earlier. This is probably not due to any contamination of deoxyribonuclease with ribonuclease, but is more likely an expression of the well known fact that RNA in Carnoy-fixed tissue is partly soluble in electrolyte solutions around neutrality (20) and consequently also in the buffer solution used for deoxyribonuclease. As RNA is removed quantitatively from Carnoy-fixed tissue with a buffered ribonuclease solution (5, 8, 19), contamination of the DNA fraction with RNA need not be considered if the right order is followed.

A series of experiments was carried out in order to investigate whether there is any loss of DNA to the RNA fraction. Groups of calf thymocytes were extracted with the ribonuclease solution applied for 1 hour as well as for 18 hours. Nucleic acid in amounts of 0.2 to 0.4 $\mu\mu$g. per thymocyte was extracted. Since thymocytes were found to contain about 8 $\mu\mu$g. of DNA and since, furthermore, the extracted amounts correspond to the quantities of RNA known to be present in calf thymocytes (18, 24), it is obvious that only insignificant amounts of DNA can be present in the RNA fraction, even when ribonuclease is applied for such a long time as 18 hours.

The dried DNA extracts are dissolved under liquid paraffin in small volumes of a liquid consisting of 6 parts of glycerol and 1 part water (*v/v*). Glycerol is used to bring the refractive index of the volumes close to that of the liquid paraffin covering them. The glycerol-water solution and the liquid paraffin have identical optical densities at 257 mμ (O.D. = 0.8 at 1 cm. light path). The extracts become lens-shaped and circular provided the quartz glass is kept clean and completely water-free. This is accomplished by keeping the cleaned glasses in a dry organic liquid like chloroform or decane for at least 2 days before use. Furthermore, the evaporations of the extracts should be carried out at a relatively low humidity (below 50 per cent). A series of control experiments showed that the specific optical density constant of DNA, depolymerized through deoxyribonuclease, was negligibly affected by glycerol in concentrations up to and above the present range (Table III). Blanks prepared by evaporating and dissolving enzyme solution in volumes of the size routinely used showed no measurable optical density at 257 mμ, and such are not needed.

The lens-shaped dissolved extracts are photographed in ultraviolet light at 257 mμ in the Köhler ultraviolet microscope together with a reference system (Fig. 1) and the plates are investigated by photometry in the same way as for quantitative RNA determinations (6). The small difference in refractive index between the extracts and the liquid paraffin does not give rise to any disturbance provided the extracts are well focussed.

The specific optical density constant used was derived from the values for deoxyribonuclease-digested calf thymus DNA, given by Laland *et al.* (9, 10). The optical density of 1 $\mu\mu$g./μ^2 at 257 mμ of that preparation is 2.57. An almost identical value (2.58) at pH 7.0 was obtained for a commercial sample of salmon sperm DNA (California Corporation for Biochemical Research, Los Angeles, CfP, N 13.57 per cent, P 8.3 per cent, Cl 0.1 per cent, arginine

TABLE III

The Optical Density of Deoxyribonuclease-Digested DNA in Solutions of Varying Glycerol Content

DNA Solution	Water	Glycerol	Optical density at 257 mμ and 1 mm. cuvette depth		
			Expt. I*	Expt. II*	Expt. III‡
ml.	*ml.*				
2	23	—	0.224	0.231	0.277
2	6	to 25 ml.	0.231	0.232	0.279
2	2	to 25 ml.	0.238	0.232	0.278
2	—	to 25 ml.	0.230	0.226	0.271

* DNA solution containing 1.25 mg. DNA and 0.2 mg. deoxyribonuclease per ml. buffered solution used in the standard procedure.

‡ As in expts. I and II but with 1.50 mg. DNA per ml.

0.08 per cent). The ϵP values (1) of the two preparations at 257 mμ after digestion with enzyme are 10.130 and 9640 respectively. Although it is desirable to give nucleic acid content in terms of phosphorus owing to the varying water content of nucleic acid reference preparations, we have chosen to give it in weight of the nucleic acid, since this will facilitate comparisons with other cytochemical work. Using our value for specific optical density constant (2.57) and knowing the correct ϵP value, one can easily recalculate any values given here to DNA phosphorus if desirable.

The nucleic acids are not the sole contributors to the absorption at 257 mμ in a tissue extract. Non-specific absorption constitutes an important part of the total absorption at 257 mμ in some standard procedures for nucleic acid determination, in which the nucleic acids are extracted with acids or alkali (12, 3, 23). Fortunately the situation is considerably more favourable in microextractions with nucleases. This is because the enzymes are of a high specificity and the contribution of non-specific dissolution to the ultraviolet absorption is unimportant, as has been shown earlier for RNA (5, 8) and demonstrated also for DNA in the macrochemical control experiment described earlier. The ultraviolet absorption curve for DNA enzymatically extracted from calf thymus is shown in Fig. 2 together with a curve recorded from the pure sample of digested commercial DNA (California Corporation) used in the determination presented above. The original curves have been recalculated to the same maximum values to make comparisons easier. As can be seen from Fig. 2, the two curves are practically identical. The fact that the tissue is particularly DNA-rich contributes of course also to the spectral purity of the extracted DNA. Also in the microextractions it is usually possible to use units with a high relative content of DNA, *e.g.*, by removing cytoplasm from nuclei before the extractions. Another factor of importance for the low extent of unspecific dissolution is to be found in the preparative technique, including fixation, embedding, and deparaffinization, which has a strong precipitating and denaturing effect on tissue proteins, resulting in their low solubility in the aqueous solutions used.

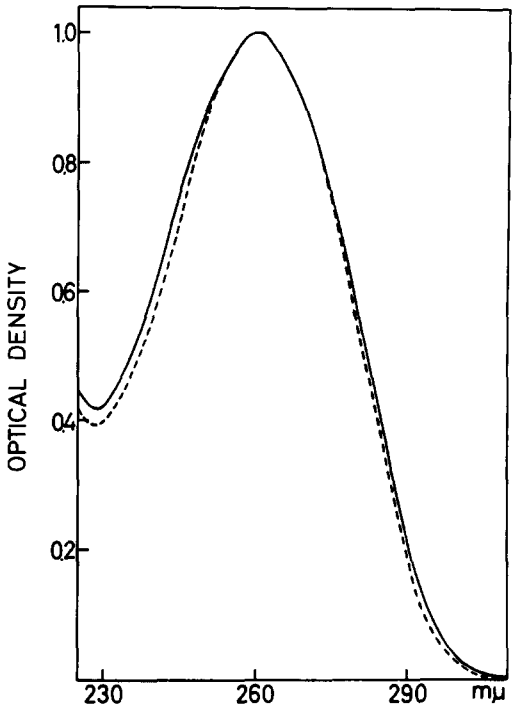

FIGURE 2

Ultraviolet absorption curves of deoxyribonuclease-digested pure DNA (solid line) and the material extracted with pancreatic deoxyribonuclease from fixed and sectioned calf thymus (dashed line). The original curves have been recalculated to give an optical density of 1.0 at the absorption maxima.

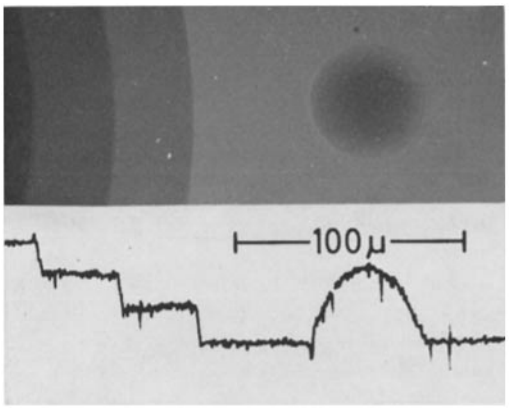

FIGURE 1

A DNA extract from a collection of 15 thymocytes containing 117 μμg. of DNA, photographed at 257 mμ together with a reference system (rotating sector), giving differences in optical density units of 0.1505 between successive steps. The corresponding photometer curve is shown below.

For the identification of certain types of cell nuclei, *e.g.* during mitosis, it is advantageous to use material which is stained during the dissections. The hydrated sections are stained with 0.01 per cent thionine blue in 0.01 N acetic acid for some minutes. Mitotic stages can then be clearly identified in sections of axolotl tadpole tail fins. The stain is removed from the isolated cells after they have dried, with 70 per cent ethanol, for 1 hour. As the optical density of thionine at 257 mμ is one-fourth of that in visible light, absence of visible colour indicates that

TABLE IV

Amounts of DNA in Calf Thymocytes and Rat Spermatids and Spermatocytes

Material	No. of cells per analysis	Mean value ± S.E.M	Coefficient of variation	Mean value per cells	No. of analyses
		μμg.	%	μμg.	
Calf thymocytes	15	121 ± 2.2	± 7	8.1	15
	10	77 ± 2.1	±10	7.7	13
	5	39 ± 1.4	±10	7.8	7
Rat spermatids	20	78 ± 3.3	±10	3.9	6
Rat spermatocytes	5	76		15.2	2

TABLE V

Amounts of DNA in Cells and Cell Nuclei from the Axolotl

Material	Mean value ± S.E.M.	Coefficient of variation	No. of analyses
	μμg.	%	
Spermatids, individual nuclei	48 ± 1.1	± 10	19
Spermatids, in groups of 10 nuclei	483 ± 8.2	± 6	12
Primary spermatocytes, individual nuclei	180 ± 3.2	± 6	10
Liver cell nuclei, tadpole	107 ± 2.3	± 12	32
Erythrocytes from the tadpole liver	112 ± 4.2*	± 16	18
Epithelial cells from the tadpole tail fin:			
Interkinesis, all layers	109 ± 5.3	± 32	43
Interkinesis, basal layers	101 ± 12.8	± 44	12
Interkinesis, superficial layers	106 ± 7.1	± 25	14
Telophase	64 ± 2.7	± 32	56
Early interkinesis	62 ± 1.8	± 25	72

* Two values, 195 and 200 μμg., rejected in the statistical treatment (see text).

there is no contribution from the stain to the absorption in ultraviolet light. In the present work the epithelium was treated in this way. In control experiments it was found that this treatment did not interfere with the digestions.

RESULTS

Calf thymocytes and rat spermatids and primary spermatocytes were used to test the method. Collections of cells were used, in the case of thymocytes small uniform cells, which constitute the majority of the cell population of the gland. The larger type was avoided, since as indicated by the results of Swift (21) large nuclei may be an indication of increased amounts of DNA (polyploidy). Table IV shows the results. The mean value for thymocytes, 7.7 to 8.1 μμg., though somewhat high, agrees reasonably well with values reported earlier: 6.4 to 6.8 μμg. by Vendrely and Vendrely (24), 7.15 μμg. by Mirsky and Ris (13), and 7.5 μμg. by Davidson and McIndoe (2). For rat nuclei values around 7 μμg. have been reported for different kinds of tissues (liver is an exception owing to polyploidy) by Thomson *et al.* (22), corresponding to a haploid value of 3.5 μμg., which is in relatively good agreement with the present results. From Table IV it can furthermore be seen that the coefficient of variation lies around ±10 per cent in the 40 to 80 μμg. range and decreases somewhat with larger DNA amounts.

The axolotl cells were found to be rich in DNA (Table V), in agreement with what is known in this respect about urodeles in general (14). Determinations on spermatids showed the haploid value to be 48 μμg. DNA. Primary spermatocytes contained DNA in amounts slightly less than four times this value. The coefficient of variation in the analyses of gonadal cells is of the same order as the errors of the method and there is consequently no evidence for a contribution by biological variation to this figure.

Cells and cell nuclei from proliferating tadpole

tissues, on the other hand, show evidence of a biological variation. The tissue with the highest mitotic rate, the epithelium, gives DNA values per cell with a coefficient of variation that lies much above the one caused by the methodological errors, and this effect is found also, though less pronounced, for proliferating erythrocytes (taken in the liver) and liver cell nuclei. This variation could be due to the fact that the analyses comprise cells in the course of synthesizing DNA.

In order to obtain a rough measure of the dispersion of values in the tadpole material the calculations have been carried out under the assumption that the populations show a normal distribution. As such a distribution does not obtain, the statistical data have only a restricted value, chiefly of interest for comparisons within the material. Two extreme erythrocyte values have been rejected from the statistical treatments. All data are graphically represented in Fig. 3.

According to current views the cells divide when they have attained a DNA value corresponding to four times that of the sperm, and consequently the values recorded for a dividing tissue should be distributed between the 2 C and 4 C values, *i.e.*, in the range from 95 to 200 $\mu\mu$g. (1 C being the DNA value of the sperm). As is evident from the frequency histogram in Fig. 3, this is not the case for the epithelial cells. The occurrence of values below the 2 C level, 96 $\mu\mu$g., can be explained in either of two ways. One possibility is that the cells lose DNA in the course of their differentiation and transport towards the surface as found by Pelc (15) in the esophagus epithelium of mice. In our material, however, we found no difference between basal and superficial layers of the skin (Table V). Another explanation for the findings might be that the cells divide before they have attained the 4 C amount. As interkinetic values below the 1 C level are rare, it follows that mitosis in such a case is taking place somewhere in the range between 2 C and 4 C. The validity of this assumption was investigated by analyzing cells in telophase and early interkinesis. Their DNA values were found to be distributed in the broad range between the 1 C and 2 C levels (Fig. 3 and Table V). The variation of the values (coefficient of variation ± 28 per cent in telophase) is too large to be caused by the errors of the method only, indicating that the epithelial cells start cell division at different levels in the 2 C to 4 C range. A strong support for this view could be obtained by investigating the degree of correlation between the amounts of DNA and its significance in sister cells at telophase. If the spread of values in telophase is due to the fact that the epithelium starts mitosis at different DNA levels, one would expect the DNA amount in a cell to be correlated with that in its sister cell. A prerequisite for this is of

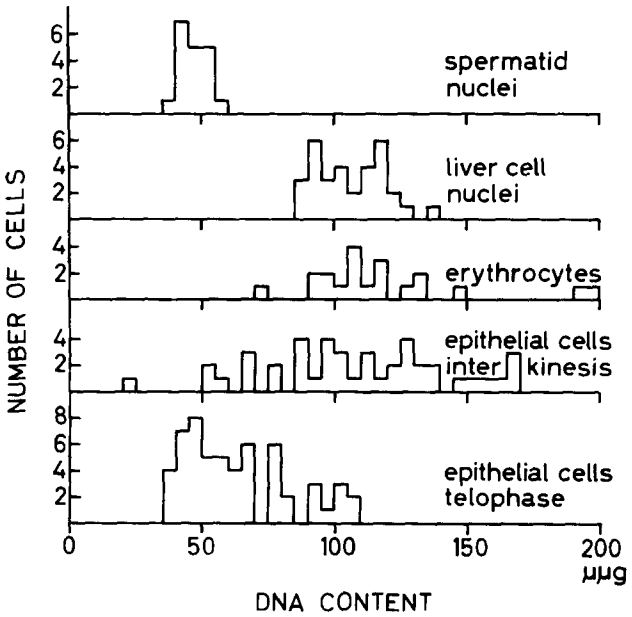

FIGURE 3

DNA amounts in various tissues from the axolotl. The spermatids are taken from adults; other cells from tadpoles.

course that no DNA synthesis take place in telophase, in which case similar sister cell values might be the result of identical times available for DNA synthesis. If this were the case, however, early interkinetic cells would show increased DNA amounts, which we did not find in this material. A correlation analysis of sister cells in telophase showed that they were correlated (28 pairs, correlation coefficient 0.84, $P < 0.001$), which in turn indicates that the variation between members of different pairs cannot be caused only by errors in the method. Consequently, in the rapidly dividing epithelium, the cells divide at levels between 2 C and 4 C, varying for different cells. Of the three dividing tissues investigated, only epithelium showed evidence of such a behaviour, whereas the values for erythrocytes and liver nuclei are compatible with the traditional view that division occurs at the 4 C level.

DISCUSSION

The results of the present work do not show an absolute agreement with values obtained through biochemical analyses, and our values lie somewhat in the upper range. As is evident from the varying macrochemical results obtained on similar material in different laboratories, however, it is possible that some of these results need revision. If the values of the present method should continue to show this trend in the future, this could be checked by a correction factor. It is more important that the random errors are small (for a cytochemical method) and that their size can be accurately assessed.

As compared with the microextraction technique for RNA determinations (6) the present method shows a lower accuracy. This is probably due to the fact that deoxyribonuclease works less efficiently than ribonuclease. This necessitates the use of more extraction fluid, resulting in larger and less dense ultraviolet-absorbing spots.

The sensitivity of the method makes it suitable for the analysis of single urodele cells but does not allow single-cell analyses of most species. It would be desirable to increase the accuracy as well as the sensitivity. The latter point presents no fundamental difficulty. With direct photometry in ultraviolet light instead of the photographic-photometric procedure it would also be possible to improve the accuracy somewhat.

Analyses on single spermatids and spermatocytes have given no indication of the existence of any larger biological variation in DNA amounts. Using microphotometric methods in ultraviolet light, Sandritter *et al.* (18) have reported a considerable variation for spermatids from other species. Further studies will have to decide whether the difference in results is due to species differences.

In the tissues showing proliferative activity, on the other hand, the coefficient of variation rises well above the one caused by methodological errors, in agreement with the results of other workers. This spread is particularly evident in the epithelium, where mitoses are most frequent, and is due to the fact that part of the population is synthesizing DNA in preparation for the next mitosis (21, 25). Our results indicating that the epithelium starts cell division at varying DNA levels between the 2 C and 4 C amounts are surprising, but it is possible that the results of earlier investigations are an expression of the same conditions. Thus, Leuchtenberger (11) shows a distribution curve of interkinetic values for normal human skin which encompasses a broad range on both sides of the 2 C point. In the epithelium studied by us there seem to be at least two causes of variation in DNA amounts. One is that the cells are investigated at different phases of the mitotic cycle; the other is that the DNA synthesis cycle occupies different ranges for different cells. The variations shown by liver cell nuclei and erythrocytes could, however, be explained by the occurrence of the first of these two factors only, indicating that the results cannot be generalized.

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