FEULGEN REACTION AND QUANTITATIVE CYTOCHEMISTRY OF DESOXYPENTOSE NUCLEIC ACID

I. ESTIMATION OF THE LOSS OF TISSUE DNA CAUSED BY FIXATION AND ACID HYDROLYSIS

by

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INTRODUCTION

An extensive application of the Feulgen reaction is found in recent literature on the determination of desoxypentose nucleic acid (DNA) content of cell nuclei by means of microspectrophotometry[1-9]. During the last decade many papers challenging the specificity of this reaction for DNA have been published[10-14], but they have always been criticized by other groups of investigators[15-23]; at present its reliability for localizing DNA within the cell is accepted almost universally among cytochemists when it is conducted under proper conditions and with appropriate controls.

As to the quantitative phase of this staining procedure, however, some authors are rather sceptical[20,24]. Since WIDSTRÖM[25] and CASPERSSON[26], the intensity of the reaction *in vitro* is known to be influenced considerably by many factors involved in the reaction procedure and now different authors are in agreement in pointing out the poor reproducibility of this reaction *in vitro* as well as *in situ*[1,8,27]. This was overcome by many of the investigators working in this field by taking values obtained under comparable conditions as indicating the relative quantity of potentially reactive substances[1,8,25-28]. Indeed, DI STEFANO[29] and RIS AND MIRSKY[28] have claimed that the Feulgen reaction can actually serve as a measure of the relative DNA content of cell nuclei, on the basis of their microspectrophotometric analyses of the reaction *in situ*. Such data have apparently been taken by most of the workers (*e.g.*, LEUCHTENBERGER[5], or PASTEELS AND LISON[8]) engaged in microspectrophotometric measurements of the Feulgen-stained materials as convincing evidence for the validity of the techniques which they have employed. However, it has recently been pointed out that all the results of microspectrophotometric measurements thus far reported are open to question, since an important source of serious error, the SCHWARZSCHILD-VILLIGER effect, involved in the course of photometric procedures, has invariably been overlooked[30-32].

Therefore, with respect to the quantitative nature of the Feulgen reaction *in situ*, it would seem unjustified to rely solely upon such information which could be obtained only *a posteriori* using microspectrophotometry.

Furthermore, there are contentions[20,24] about the significant loss of DNA from

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tissues which occurs during the course of hydrolysis involved in the procedure of the histochemical Feulgen reaction. In addition, some cytologists may wonder that formalin fixation used extensively in the quantitative Feulgen work might cause some loss of DNA.

The relative-quantitative nature of the Feulgen reaction may further be questioned from another corner. According to WIDSTRÖM[25] and CASPERSSON[26] the intensity of the Feulgen reaction *in vitro* is affected by proteins present in the reaction mixture; a remarkable effect of histone has been reported by SIBATANI[22].

Under these circumstances it would be obvious that detailed studies are required to explore such problems in order to know to what extent the Feulgen reaction could be used as a quantitative test for the DNA present in cell nuclei. The present paper opens a series of investigations directed to attack these problems, beginning with a gross chemical estimation of the possible loss of DNA from tissues during the course of fixation and mild acid hydrolysis involved in the Feulgen reaction *in situ*.

MATERIALS AND METHODS

Tissue homogenates. Several rat tissues characterized by various levels of DNA content as well as of the PNA/DNA ratio were used. Thus, livers, spleens, thymuses, and testes of different animals were homogenized with ice-cold physiological saline by an all-glass homogenizer to make the final tissue concentration 15 to 30%.

Fixation. 20 and 50% formalin and alcohol-formalin (9:1) were used as the fixative: 50% formalin has been frequently used in quantitative Feulgen studies since SWIFT[1]; alcohol-formalin fixation was adopted by LISON AND PASTEELS[8,9] for the same purpose; 20% formalin was included in order to examine whether the dissolution of tissue DNA into hydrolyzing medium is influenced by the concentration of formaldehyde employed in the fixation procedure. This type of test was conducted since the intensity of Feulgen reaction of thymus nuclei fixed with 20% formalin has been proved microspectrophotometrically to be markedly lower than that with 50% formalin[33]. In formalin fixation every 1 ml portion of the tissue homogenates was pipetted into a centrifuge tube along with 1 ml commercial formalin (*ca.* 40% formaldehyde), directly, or after diluting 2.5 times with distilled water, to make final concentration of formalin 50 or 20%, respectively. With alcohol-formalin (9:1) 10 ml of the fixative was added to 1 ml of the homogenate. After fixing for 3 hours with 50% formalin and for 24 hours with 20% formalin or alcohol-formalin, the mixture was centrifuged and the precipitate washed with 10 ml portions of distilled water until the supernate of the washing became Schiff-negative (8 times). The final precipitate was analyzed directly, or further subjected to hydrolysis. In the latter case, it was then washed with alcohol and ether, and dried at 70°.

Hydrolysis. Fixed and non-fixed materials were hydrolyzed with *N* HCl at 60° for 13 and 15 minutes, since microspectrophotometric measurements on rat tissues revealed that maximal intensity of the Feulgen colour is attained at such lengths of hydrolysis time[30,33]. With non-fixed materials, 1 ml of the homogenate was pipetted into a centrifuge tube along with 1 ml 2 *N* HCl. With fixed materials the dried samples as obtained above were suspended in 2 ml *N* HCl. The centrifuge tubes were then heated in a 60° water bath for the periods indicated. The temperature of the liquid contained in the tubes was raised above 50° within one minute and approached 60° within the next minute. The tubes were then removed from the bath, cooled immediately with ice-water and 8 ml portions of distilled water were added. They were then centrifuged, and the residues washed once with water. Control tubes without hydrolysis were treated similarly except for heating; instead, they remained the while in ice-water.

Analytical procedure. Fresh samples and fixation or hydrolysis residues were analyzed for total nucleic acid or DNA phosphorus by the method of SCHNEIDER[34]. Determination of total P was made according to LE PAGE[35], that of DNA P through diphenylamine reaction[36]. For this test a sample of DNA (Na salt) extracted from beef spleen according to PETERMANN AND LAMB[37] (P: 9.1%; N:P = 1.6) served as the standard. Its colour yield with the cysteine reaction[36,38] per μg P was the same as that of a DNA sample extracted from herring spermatozoa (received through the courtesy of Dr I. WATANABE, Institute for Science and Technology, University of Tokyo). As the latter may be considered free from PNA, the standard sample of DNA used is probably free from contamination with PNA. In preliminary experiments, recovery of DNA from hot trichloracetic acid extracts of tissues fixed in 50% formalin was tested with the diphenylamine and the cysteine reactions by adding known quantities of DNA to the extracts. It was thus found that the cysteine reaction, but not the diphenylamine reaction, is affected significantly by the extracts of the formalin-fixed tissues. The validity of applying the diphenylamine reaction to estimate the DNA content of formalin-fixed

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tissues thus seems to be warranted. Another trouble arises now with hydrolyzed tissues, because the colour yield of the diphenylamine reaction is progressively diminished by the hydrolysis with *N* HCl at 60° [39]. With extinctions for DNA P of the hydrolyzed tissues, corrections were made by dividing them with 0.970 and 0.965 for 13 and 15 minutes' hydrolyses, respectively. These factors were obtained during hydrolysis experiments with purified DNA[39]. The quantity of DNA P liberated from the tissue residue by the hydrolysis was obtained from DNA P contents of the non-hydrolyzed (control) and hydrolyzed tubes by difference.

Feulgen reaction. A procedure established by SIBATANI[39] for the test *in vitro* was employed: each 1 ml aliquot of the hydrolysis supernates of formalin-fixed tissue materials was neutralized with 1 ml *N* NaOH and then added with 6.5 ml 0.1 *M* glycine buffer of pH 2.28, 1 ml 15% sodium metabisulfite, and 0.5 ml Schiff reagent. Colours developed were measured with Beckman DU spectrophotometer after standing for 4 hours at room temperature. Control tubes with fixed tissues were warmed at 60° for 13 minutes with 2 ml distilled water and the supernates treated as above, but NaOH was replaced with distilled water. No colour was developed, assuring the absence of formaldehyde contamination. That the residue in these control tubes retained DNA quantitatively during the incubation was proved by Schneider analysis.

RESULTS

Results obtained are summarized in Tables I–III. From Tables I and II it can be seen that total nucleic acid and DNA, and hence PNA also, of different rat tissues are quantitatively retained throughout the course of formalin or alcohol-formalin fixation prolonged up to 24 hours. However, hydrolysis of fixed as well as non-fixed tissue materials with *N* HCl at 60° for 13 minutes decidedly give rise to dissolution of a part of DNA contained therein (Table III). Although the fixatives employed are effective in decreasing the magnitude of DNA loss from tissues, fractions of DNA lost from the fixed tissue residues are in most cases by no means negligible. They amount in average to some 8% of total DNA originally present in fresh or fixed tissues, but also figures remote from the mean value are obtained; fluctuation of the values is especially prominent with spleen. It is unlikely that there is any significant difference in the degree of dissolution of DNA among three fixatives employed or among different tissues tested. The rate of DNA solubilization seems further to be independent of the "concentration" of tissue DNA in the hydrolyzing suspension at least within the range of our experimentation. Usually longer hydrolysis (15 minutes) caused more loss than a shorter one (13 minutes). It should be noted, however, that the results of parallel determinations were not always consistent. For example, parallel experiments with liver and spleen were repeated twice; although in either case the samples of both tissues were fixed and hydrolyzed simultaneously and under exactly comparable conditions, values for the loss of DNA (13 minutes' hydrolysis), obtained consecutively in these two experiments, were nearly the same with liver, whereas the corresponding values for spleen differed markedly, not only from those for liver, but also from each other (Table III, column 5, lines 4, 6, 8, and 9). Another example emerges from comparing values of hydrolysis for 13 and 15 minutes. Here, the increments of the loss of DNA due to the prolonged hydrolysis are by no means consistent. It was impossible to answer the question how far these inconsistencies were contributed by unevenness of the fixing reaction, by lack of complete dispersion during the hydrolysis of the fixed homogenate, or by occasional errors in the determination.

Here, it may well be presumed that the extent of the hydrolysis with tissue sections adhered to a coverslip[40] and incubated in 60° *N* HCl for 13 minutes is somewhat greater than that with our tissue homogenates for 13 minutes, but not for 15 minutes, because in our experiments the temperature of the hydrolyzing mixture was approximately 60° within the first 2 minutes of heating.

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TABLE I

TOTAL NUCLEIC ACID CONTENTS OF SEVERAL RAT TISSUES AS DETERMINED BEFORE AND AFTER FIXING WITH 50% FORMALIN FOR 3 HOURS. EACH DETERMINATION WAS MADE WITH DUPLICATE TUBES

	mg total nucleic acid P in 100 gram fresh tissue		
	Liver	*Testes*	*Thymus*
Without fixation	124	65	221
With fixation	124	69	221

TABLE II

DNA CONTENTS OF SEVERAL RAT TISSUES AS DETERMINED BEFORE AND AFTER FIXING WITH VARIOUS FIXATIVES

Fixation	*mg DNA P in 100 gram fresh tissue*			
	Liver	*Spleen*	*Spleen*	*Thymus*
None	24.2	76.5	109	222
50% formalin (3 h)	—	76.5	—	—
20% formalin (24 h)	24.2	—	109	—
Alcohol-formalin (9:1) (24 h)	23.9	—	109	222

TABLE III

FRACTIONS OF DNA LOST FROM NON-FIXED AND FIXED RAT TISSUE HOMOGENATES IN THE COURSE OF FEULGEN HYDROLYSIS IN *N* HCl AT 60° FOR 13 AND 15 MINUTES. FIGURES FOR 15 MINUTES ARE GIVEN IN PARENTHESES

Fixation	*Tissue*	*DNA P content of hydrolyzing suspension* $\mu g/ml$	*DNA P solubilized by hydrolysis*		*Feulgen reaction of hydrolysis supernate*
			$\mu g/ml$	*Per cent*	E_{548}
None	liver	36.1	9.7	26.9	
None	spleen	109	19.5	17.9	
None	thymus	167	38	22.8	
50% formalin	liver	38.1	4.2	11.0	
			(4.2)	(11.0)	
50% formalin	liver	35.7	3.8	10.6	
			(5.5)	(15.3)	
50% formalin	spleen	76.5	16.5	21.6	
50% formalin	spleen	94.5	1.5	1.6	
			(5.5)	(5.8)	
50% formalin	thymus	176	18	10.2	0.192
			(21)	(11.9)	(0.286)
50% formalin	thymus	160	10	6.3	0.120
			(8)	(5.0)	(0.157)
20% formalin	liver	36.1	2.4	6.6	
20% formalin	spleen	109	11	10.1	
20% formalin	thymus	167	21	12.6	
Alcohol-formalin (9:1)	liver	36.1	2.4	6.6	
Alcohol-formalin (9:1)	spleen	109	3	2.8	
Alcohol-formalin (9:1)	thymus	167	12.5	7.5	

Feulgen reactions of the hydrolysis supernates are markedly increased by the prolongation of the hydrolysis, but this may, at least in part, be due to the colour augmentation caused by the extended hydrolysis of DNA. But the outcomes from the Feulgen reaction should not be referred with too much weight, because proteins transferred into the supernates may have exerted appreciable interference. At any rate, it must be expected from these data that the Feulgen hydrolysis conducted for the peroid of as long as 13 minutes causes some loss of DNA from fixed tissues, which may not proceed with exactly comparable rates in individual cases even when treated quite similarly. The proportion of DNA retained within the hydrolyzed preparation may, therefore, fluctuate to a certain extent from case to case (or from nucleus to nucleus in one and the same section), but usually the range of such fluctuation may not be very wide, so that in the *relative* microspectrophotometric measurement of Feulgen-stained DNA the error caused by the hydrolysis loss of DNA would not exceed 10%. In certain instances, however, it might nevertheless amount, at least, to as large as 20%, as can be anticipated from our figures for spleens fixed with 50% formalin.

DISCUSSION

Although the mechanism of the DNA dissolution caused by the acid hydrolysis is not understood in full detail[20], it is almost certain that the extent of the dissolution is partially conditioned by the behaviour of the protein bound to DNA during the course of the hydrolysis[24]. Indeed, desoxypentose nucleohistone is precipitated by 50% formalin, but the sodium salt of highly "associated" DNA entirely fails to do so; it has further been observed in a preliminary experiment that by the Feulgen hydrolysis DNA is much more readily liberated from non-fixed nucleohistone than from a formalin-fixed sample. It seems that in this case the formalin fixation renders histone less soluble in acid media, so that DNA resists dissolution more strongly.

For tissue DNA the situation is much the same. Under identical conditions of hydrolysis non-fixed tissues lose more DNA than those fixed with formalin or alcohol-formalin. ROBERTS AND ANDERSON[41] have noted that non-fixed nuclei of liver cells give stronger Feulgen reaction than fixed nuclei, but our experiments with thymus homogenate gave an opposite result. The STEDMANS[24] showed that about 27% of nucleic acid P was detached from Carnoy-fixed beef liver nuclei by the Feulgen hydrolysis for 10 minutes. Although some part of this figure is undoubtedly contributed by nuclear pentose nucleic acid (PNA) which is more readily detached from the cell by acid hydrolysis[20, 42–44], this value is rather comparable to our figures obtained with non-fixed tissues. It is known, however, that nuclear histone retains its solubility in dilute mineral acid after being fixed with Carnoy but not with formalin and other fixatives[24, 29]. Now it should be noted, as shown above, that even fixation with the latter class of fixatives can not effect a complete inhibition of the DNA dissolution from tissues during the course of the Feulgen hydrolysis which should give maximal colour intensity of the reaction *in situ*. Thus, our conclusion is essentially the same as STEDMANS'[24] or ELY AND ROSS'[20] as to whether quantitative recovery of cellular DNA is possible with the histochemical Feulgen reaction.

This opinion seems to be inconsistent with the view of LESSLER[23], who failed to detect any significant loss of DNA contained in gelatin blocks during the Feulgen hydrolysis for up to 20 minutes. However, the thickness of the gelatin blocks used was at least more than 1.63 mm. Under such

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conditions the diffusing out of DNA is undoubtedly minimized, so that the LESSLER's experiment may not be taken to be comparable to the case of tissue sections.

Meanwhile, THOMAS[45] has shown by paper chromatography of DNA hydrolysates that by a hydrolysis in *N* HCl at 60° for 16 minutes almost all the purines of DNA are liberated, while solubilization of thymic acid is still negligible. However, in THOMAS's experiment the concentration of DNA in the initial hydrolyzing mixture was relatively high (may be higher than 0.5% if calculated from figures indicated in his diagram). According to our experience attainment of the complete dissolution of DNA realized by the Feulgen hydrolysis is considerably retarded by the increase of DNA concentration, because DNA of higher concentration is precipitated by acid, forming heavy coagulation which resists the hydrolysis more strongly. Indeed, 0.02%, but not 0.5%, DNA is completely brought into dissolution by a Feulgen hydrolysis for 25 minutes. It is thus likely that under the conditions employed by Thomas, rate of the solubilization of hydrolyzed DNA is more reduced than in the case of hydrolyzing microscopic preparations. It appears, however, rather curious that, in a similar experiment of ERRERA *et al.*[46] using 0.2% DNA, the overall situation is exactly comparable to the results of THOMAS, whereas a decidedly higher rate of DNA dissolution was proved in our experiment with non-fixed thymus, which was hydrolyzed with a "concentration" of DNA only slightly lower than that employed by ERRERA *et al.* (1.7 mg/ml; see Table III).

Now we come to the data of microspectrophotometric measurements presented by DI STEFANO[29] and RIS AND MIRSKY[28], which tend to suggest that no appreciable amount of thymic acid polynucleotide is liberated from nuclei during hydrolysis until attainment of maximal intensity of the Feulgen colour, or even for longer hydrolysis. DI STEFANO's argument is based upon the methyl green staining of nuclei the intensity of which showed no significant difference between non-hydrolyzed nuclei and those hydrolyzed for 12 minutes. However, there is a number of uncertainties about his data, namely, the intensity of methyl green staining may be affected by the impurity of methyl green used (DI STEFANO is stated to have omitted the necessary purification of methyl green[47]), by proteins co-existing with DNA, or by "disaggregation" of DNA caused by the hydrolysis[5,46-54]. Consequently, we can not have any confidence in his conclusion drawn from the data obtained without any consideration of such factors. It may be that the apparent agreement of the two extinction values in DI STEFANO's experiment is fortuitous. It should further be considered that if the SCHWARZSCHILD-VILLIGER (S–V) effect is not eliminated, what is most likely in his photometric procedure, transmittance obtained with nuclei characterized by higher absorption would occasionally tend to be leveled off on a certain value (about 15% and frequently higher)[31,32]; in fact, in the diagram given by DI STEFANO[29] the extinctions for methyl green of the nuclei in question (*ca.* 0.42; transmittance about 38%) are by far the highest among extinction values included.

A similar situation may be found in the data of RIS AND MIRSKY[28]. First it must be pointed out that in the Feulgen reaction *in vitro* with purified DNA the length of time required for the Feulgen hydrolysis to give maximal colour intensity is some 15 minutes or even longer[20,23,39], whereas in RIS AND MIRSKY's data the intensity of the Feulgen stain ceases to increase significantly after being hydrolyzed for longer than 8 minutes with calf nuclei isolated with citric acid and fixed with Carnoy, remaining practically constant up to 15 minutes. This suggests either that the loss of thymic acid which occurred to an appreciable extent during the course of the prolonged hydrolysis may have exactly been compensated for by the concomitant increase in the Feulgen-reactivity of the DNA remaining within the nuclei, or that by virtue of the S–V effect the extinction values could not exceed a certain limiting value. The first alternative seems, however, less likely, not only because of the very low probability (a fortuitous coincidence of 6 values), but also because in another experiment of the same authors with a formalin-fixed rat liver section, the intensity of the Feulgen stain remained

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constant from 13 to 25 minutes, while in our microspectrophotometric experiments (free from S–V effect) with quite similar material hydrolyses continued beyond 15 minutes resulted a significant diminution in the intensity of the Feulgen stain[33].

On the other hand, if we calculate from the figures given by RIS AND MIRSKY[28] the actual mean extinction of the tetraploid nuclei of rat liver, assuming the average diameter of these nuclei 6 μ on the basis of our own measurement with materials of the same type, we obtain a value of 0.42, which is quite approximated to the highest values found in the DI STEFANO's data[29] (see above). The calculation is $E = \frac{12}{3.14 \times 3^2} = 0.42$, where 12 is $E \times$ area given by RIS AND MIRSKY. In contrast to this, extinction values of similar materials obtained by ourselves with an apparatus, with which the elimination of S–V effect is warranted[40,55], are usually around 1.0[33]. As for the figures of RIS AND MIRSKY[28] on *calf* liver nuclei, we obtain 4 μ for the diameter of the nucleus if we assume that also in this case the mean actual extinction was 0.42. The diameter so computed is somewhat smaller than that of the average diploid nucleus of *rat* liver encountered in the *formalin-fixed* section. However, RIS AND MIRSKY have used in this case *citric acid-isolated* nuclei, and such material would certainly tend to shrink to some extent by losing an appreciable fraction of the proteins[56]. Thus, it seems to us by no means unlikely that the apparent discrepancy between the conclusions of RIS AND MIRSKY and ours is largely due to the failure in their measuring the transmittance of the Feulgen-stained nuclei correctly with the equipment they used[57]. If so, another question arises with respect to how RIS AND MIRSKY[28] could reach an impressive parallelism of the two series of independent measurements of the relative and absolute amount of DNA contained in a single nucleus, by microspectrophotometry of the Feulgen stain on the one hand and by gross chemical analysis on the other hand, on somatic nuclei of different animals. Also the extinction value of *ca.* 0.42 as estimated above is somewhat too low to be expected reasonably for the highest limiting value of the extinction to be obtained with microspectrophotometry accompanied by the S–V effect.

An attempt should now be made to search for procedures which may cause a less considerable loss of DNA. This may be done along three lines: by shortening the period of hydrolysis; by selecting milder conditions for hydrolysis; and by introducing some reagent which may minimize the solubilization of DNA from fixed materials.

As to the period of hydrolysis, BRACHET[15], POUYET[42], and THOMAS[45] have noted that Feulgen hydrolysis for 5–10 minutes conducted with non-fixed or fixed tissues failed to liberate more than negligible amount of desoxypentose- or thymine-containing material into the hydrolysate. However, at such lengths of hydrolysis time the Feulgen reaction of DNA *in vitro* as well as *in situ* is on the way to reach its attainable maximum[20,30,33,39], so that the magnitude of the error may be increased prominently. In fact, in our microspectrophotometric measurements of the nuclear Feulgen stain the variation coefficient or relative error was minimal at the hydrolysis for 13 minutes where the colour intensity attained its maximum value, and it increased rather regularly at both sides of this hydrolysis period[33].

According to OVEREND[27], treating DNA in a weakly acidic medium (pH 2.0) at 100° for 2.75 minutes and precipitating the partially hydrolyzed DNA with addition of alcohol give a material completely non-soluble and strongly Feulgen-positive. We have worked under similar conditions (pH 2.28 at 100°) and found that maximal colour intensity of the Feulgen reaction *in vitro* is attained at a hydrolysis for 2.5 minutes[39]. Moreover, we have obtained evidence with rat liver and thymus homogenates that the hydrolysis under the same conditions caused no appreciable loss of DNA from formalin-fixed tissues. The Feulgen colour developed with such materials is, however, whether it is developed *in vitro* or *in situ*, remarkably less intense than that produced by the usual method of hydrolysis[33,39]; and especially in the case of the reaction *in situ*, it is too weak to allow precise measurements of the nuclear diameter. More disappointing is the procedure of obtaining apurinic acid as reported recently by CHARGAFF *et al.*[58]

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(hydrolysis with dilute HCl of pH 1.6 at 37° for 26 h), because sections failed to colour to any recognizable degree after prolonged warm incubation.

It might seem promising to make an attempt to inhibit the DNA dissolution by treating tissues with lanthanum salt[59]. But it was observed by cytochemical techniques[44] that the removal of PNA from fixed cells through the Feulgen hydrolysis[20,42,43] can be reduced neither by the addition of lanthanum acetate to formalin used for the fixation[59,60], nor by that to *N* HCl used for the hydrolysis of formalin-fixed sections*, so that the effectiveness of using this agent to minimize the DNA dissolution appears rather questionable.

Thus we have at present no satisfactory means to prevent the DNA loss caused by the Feulgen hydrolysis as the prerequisite for the microspectrophotometric determination of DNA. The implication of this argument is, however, not so serious as it appears first. Although the extent of DNA loss may never be the same for all the nuclei in one and the same section, the variation caused by this factor would fall well within the range of the error involved in the microspectrophotometric measurement with nuclei. But when we will compare the results of the measurements on separate sections, the situation becomes somewhat more serious. Even if we may be contented with measuring relative values only, the estimated values of the total nuclear DNA content must be expected to fluctuate, within a certain limit, from section to section prepared separately with materials of the same type, or even with one and the same material. The error due to the non-equal loss of DNA in different preparations would usually be not so large, but occasionally it might attain to at least 20%. Furthermore it is by no means unlikely that the fluctuation in the extent of DNA loss to be observed among sections of one and the same tissue fixed separately is not less than that to occur among sections originating from separate tissues. Therefore, any photometric measurements of Feulgen-stained cells should preferably be repeated several times with separate materials before attempting to interprete them, in order to eliminate the possibility that differences among values obtained with various objects are simply due to an unequal hydrolysis loss of DNA from nuclei, which may well occur by chance for every type of materials.

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SUMMARY

Nucleic acids are retained quantitatively in rat tissues fixed with formalin or alcohol-formalin. Appreciable fractions of desoxypentose nucleic acid (DNA) are liberated from non-fixed as well as fixed tissues during the course of Feulgen hydrolysis for 13 minutes. The loss of DNA is decreased decidedly by the preliminary fixation, 8% on average, showing an irregular fluctuation which seems to be independent of the fixatives or tissues employed.

The results of different investigators are discussed, and the validity of the Feulgen reaction as a quantitative-cytochemical test of DNA is examined under the light of the conclusion reached finally.

* This is rather natural because PNA is degraded largely to mononucleotides by the Feulgen hydrolysis for 10 minutes (J. DIRKX, *Biochim. Biophys. Acta*, 8 (1952) 194).

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RÉSUMÉ

Au cours de la fixation par formol ou par alcool-formol, les tissus du Rat retiennent les acides nucléiques quantitativement. Des quantités appreciables de l'acide désoxypentosenucléique (ADN) sont libérées, par l'hydrolyse de Feulgen pendant 13 minutes, dans les tissus frais ainsi que dans les tissus fixés. Au cours de l'hydrolyse suivante la fixation, il y a perte d'ADN, d'environ 8% en moyenne. Il y a une fluctuation irrégulière, qui semble indépendente des fixateurs ou des tissues employés.

Les résultats des autres auteurs, ainsi que la validité de la réaction de Feulgen comme un technique cytochimique pour la détermination quantitative de l'ADN ont été discutés.

ZUSAMMENFASSUNG

Im Laufe der Fixierung mit Formol oder Alkohol-Formol bleiben die Nucleinsäuren in den Geweben der Ratten quantitativ erhalten. Merkliche Mengen der Desoxypentosenucleinsäure (DNS) werden jedoch durch die Feulgen-Hydrolyse während 13 Minuten aus frischen sowie fixierten Geweben abgenommen. Die vorläufige Fixierung wirkt eine ausschlaggebende Verminderung der Auflösungsrate von DNS. Ihr Verlust reicht aber dann noch zu etwa 8% im Durchschnitt, weist ferner eine regellose Schwankung auf, welche von der Art der gebrauchten Gewebe oder Fixierungsflüssigkeiten unabhängig zu sein scheint.

Es werden die Resultate verschiedener Verfasser diskutiert, und die Anwendbarkeit der Feulgen-Reaktion als eine quantitativ-cytochemische Bestimmungsmethode der DNS wird im Licht der erzielten Schlussfolgerung erörtert.

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