FEULGEN REACTION AND QUANTITATIVE CYTOCHEMISTRY OF DESOXYPENTOSE NUCLEIC ACID

V. CHEMICAL DETERMINATION OF COLOUR INTENSITY OF THE REACTION *IN SITU**

by

ATUHIRO SIBATANI**

Microbial Diseases Research Institute, University of Osaka, Osaka (Japan)

In a previous report[1], it was demonstrated, by means of microspectrophotometry (MSP), that the change in protein content of cell nuclei does not affect the intensity of the Feulgen reaction *in situ*. Since, however, this line of attack is an indirect one, it appeared desirable to duplicate the experiment with direct chemical determination of relative concentration to desoxypentose nucleic acid (DNA) of the Feulgen stain developed in cell nuclei.

Much difficulty has been encountered in experiments of this type. The methods available for the chemical determination of two nucleic acids in fresh animal tissues are completely unusable in the case of Feulgen-stained nuclei; for, apart from the difficulty due to the presence of fuchsin, Feulgen hydrolysis renders the DNA, partly at least, acid-soluble[2], and the treatment with Schiff reagent of acid-hydrolyzed DNA makes the desoxypentose moiety non-reactive to Dische diphenylamine test and presumably to other reactions characteristic of desoxysugars, owing to the reaction of its aldehyde with SO_2, a component of the Schiff reagent. Thus, neither SCHMIDT-THANNHAUSER[3], nor SCHNEIDER method[4] can be employed for the estimation of DNA in the present case. Another difficulty emerges now from the situation that the Feulgen pigment possesses light-absorbing properties which differ from those of the native fuchsin dye and is further liable to partial decomposition on exposing to such drastic treatment as may be required to release DNA from the protein residue.

Under these circumstances, attempts have been made to extract Feulgen-stained tissues or nuclei with hot perchloric acid (PCA), and to measure the absorption due to bases of hydrolyzed DNA and fuchsin in the extract at respective absorption maxima, taking appropriate precaution to errors caused by pentose nucleic acid (PNA) remaining in cells after the hydrolysis and by variation in degree of the decomposition of the Feulgen stain.

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** Present address: Departments of Cytochemistry and Histology, Yamaguti Medical School, Nakaube, Ube, Yamaguti-Ken (Japan).

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MATERIAL AND METHODS

Materials and procedures employed are essentially the same as in previous communications[1,2,5,6]. Nuclei were isolated according to MIRSKY AND POLLISTER[7]. Histone-poor nuclei were prepared by extracting isolated beef liver nuclei with 0.2 *N* HCl. Fixation with 20 or 50% formalin and colour development of tissue homogenates and isolated nuclei were carried out in centrifuge tube. For complete removal of formaldehyde, fixed materials were treated overnight with 1.5% sodium metabisulfite and then washed with water. Unstained controls were hydrolysed, but not allowed to contact with Schiff reagent or SO_2. Whole tissue homogenates were defatted with hot alcohol-ether (3:1), prior to Feulgen hydrolysis, in order to remove plasmal. Aqueous solution of pure Feulgen pigment, free of the excess Schiff reagent, was prepared by extracting Feulgen-stained and routinely washed histone-poor nuclei of beef liver overnight with distilled water in the cold.

RESULTS

In Feulgen-stained nuclei, fuchsin dye is linked to DNA through desoxypentose aldehyde group unmasked by the release of purines[8]. If these nuclei are subjected to partial hydrolysis with hot PCA as in the procedure of OGUR AND ROSEN[9] or of SCHNEIDER[10] for nucleic acid determination, fuchsin dye as well as DNA is to be dissolved, the linkage between fuchsin and DNA either being broken or remaining intact*. To study the extent of such a decomposition of the Feulgen stain, optical properties of the hot PCA extracts were compared with those of intact Feulgen pigment and of basic fuchsin. PCA extracts were neutralized with NaOH and all samples to be compared were adjusted on the same level of $NaClO_4$ concentration. These pigments are purple to pink at pH 2.28 (maximum: 550–560 mμ) and yellow in strong H_2SO_4 (maximum: 430–440 mμ)**. Results of a typical experiment are listed in Table I.

It is seen from this table that the absorption peak of the Feulgen pigment is shifted slightly towards longer wavelengths than that of basic fuchsin either at pH 2.28 or in sulphuric acid. There is further a great difference between the two pigments in relative intensity of the colours exhibited at pH 2.28 and in sulphuric acid, as evidenced by the ratio E_{560}/E_{430}. Judging from this ratio, and also from the colour taken in dilute acid (blue with Feulgen pigment and colourless with fuchsin), heating with PCA causes a marked breakdown of the Feulgen pigment to regenerate the native fuchsin colour. In another experiment it was demonstrated that with 0.75 *N* PCA, heating at 100° for 15 minutes does not bring about such an extensive decomposition as can be attained with 1.5 *N* PCA. But even under the latter conditions, the decomposition of the Feulgen pigment may not be complete, because faint blue coloration appears in weak acid.

Hot PCA extracts (1.5 *N*, 90°, 15 min) were prepared from formalin-fixed rat liver nuclei hydrolysed but unstained (control) and from those stained with mixture F[1], and mixed with equal volume of 50% H_2SO_4. Absorption maximum of the extract from unstained nuclei, mainly due to the pyrimidine bases of DNA, was located at the wavelengths of 268–270 mμ (Fig. 1, A). This agrees quite well with that of apurinic acid reported by TAMM *et al.*[11], although the latter was measured with neutral solution. The extract from stained nuclei also showed a peak at the same wavelength, but it had another one at 430–440 mμ, owing to its yellow colour in sulphuric acid (Fig. 1, B). Likewise, the basic fuchsin, in the same solvent, exhibited a peak at 430–440 mμ, but

* Attempts to detach the fuchsin moiety from the Feulgen-stained nuclei without affecting DNA were unsuccessful.

** It was proved that the colour of fuchsin dye in both neutral and acidic reactions is not affected by a preliminary heating in 1.5 *N* PCA at 90° for 15 minutes.

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it showed an additional peak at 260 mμ, its extinction value at 268 mμ being about 40% of that at 432 mμ (Fig. 1, C). Extinction value at 432 mμ of the extract from unstained nuclei was quite low, amounting to only 1–2% of that at 268 mμ. So, if Feulgen pigment were degraded completely to basic fuchsin by heating with PCA, and if PNA were quantitatively removed from the cell by the Feulgen hydrolysis[12], we could calculate the relative amount of fuchsin to DNA in Feulgen-stained nuclei, using extinction values at 268 and 432 mμ of the curves A and C of Fig. 1. Such an attempt was made in a previous paper[13], but the validity of this method might be questioned, since complete decomposition of the Feulgen stain developed *in situ* can not be attained even by prolonged heating of the nuclei with 1.5 *N* PCA.

TABLE I

OPTICAL DETERMINATION OF FEULGEN PIGMENT, BASIC FUCHSIN, AND HOT PERCHLORIC ACID EXTRACTS OF FEULGEN-STAINED BEEF LIVER NUCLEI*

Material	E_{550}/E_{580} *in pH 2.28 buffer*	E_{430}/E_{440} *in strong* H_2SO_4	$\frac{E_{580}\ (buffer)}{E_{430}\ (H_2SO_4)}$
Feulgen pigment	0.92	0.91	2.32
Basic fuchsin	1.09	1.03	0.77
0.75 *N* PCA extract (100°, 1 min) of Feulgen-stained nuclei	0.96	0.96	1.48
1.5 *N* PCA extract (100°, 11 min) of Feulgen-stained nuclei	1.04	0.97	0.90

* All samples contain PCA in the same concentration. 1 ml of a neutralized solution of respective pigments was added with 3 ml of either 0.1 *M* glycine buffer of pH 2.28 or 50% H_2SO_4 (1 vol. of conc. H_2SO_4 plus 1 vol. of H_2O).

Under these circumstances, the ratio $R = E_{268}/E_{432}$ of the PCA extract of Feulgen-stained nuclei mixed with strong H_2SO_4 can be regarded to be inversely proportional to the reaction intensity in the first approximation: let f_{432} and f_{268} be the extinctions, at 432 and 268 mμ respectively, due to the fuchsin moiety in a sample of the Feulgen pigment extract, and d_{268} the extinction at 268 mμ contributed by the DNA moiety in the same sample. If we neglect the very slight absorption of the DNA moiety at 432 mμ, we get

$$R = \frac{E_{268}}{E_{432}} = \frac{d_{268} + f_{268}}{f_{432}}.$$

Here, f_{268}/f_{432} could be assumed to be constant in a given medium, and d_{268}/f_{432} may be used as the measure of relative concentration of DNA to fuchsin. And, from curves B and C of Fig. 1, it may be

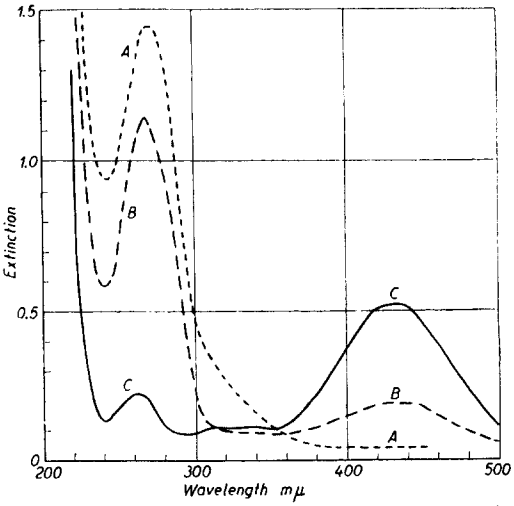

Fig. 1. Absorption spectra of basic fuchsin and of hot PCA extracts of hydrolysed-unstained and Feulgen-stained nuclei of rat liver, all being in strong sulphuric acid. A. Hydrolysed but unstained nuclei: 2 ml 1.5 *N* PCA extract (90°, 15 min) of nuclei (density arbitrary) + 2 ml 50% H_2SO_4. B. Feulgen-stained nuclei: treated as A. C. Fuchsin: 2 ml 0.0025% basic fuchsin in 1.5 *N* PCA + 2 ml 50% H_2SO_4.

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postulated that f_{268}/f_{432} is much smaller than d_{268}/f_{432}. So we can compare R's of different samples to get the idea on the variation in the intensity of Feulgen reaction *in situ*.

Accordingly, the procedure for determining the relative concentration of DNA to fuchsin in the Feulgen-stained material was established as follows: aqueous suspension in appropriate density of Feulgen-stained material (2 ml) is mixed with equal volume of 3 N PCA and heated at 90° for 15 minutes*. The centrifuged residue is washed once with 1.5 N PCA (2 ml). Combined supernatants are mixed with one or two volumes of 50% H_2SO_4**. Extinctions are measured at 432 and 268 mμ against a blank prepared by mixing 1.5 N PCA and 50% H_2SO_4 (1:1 or 1:2 in volume).

Several measurements were made with thymuses and livers of young rats, using whole homogenates and isolated nuclei. Results are listed in Table II. In general, duplicate determinations conducted with different concentrations of the extracted stain gave excellent agreement of R values. With the whole homogenates, the liver gave higher R values than the thymus. This, however, could well be expected if a part of the PNA remained in the stained tissue residue[14]. Attempts were made to test this possibility with extracts of control nuclei. But it was found that orcinol and diphenylamine tests could not be applied to our material, owing to the formalin fixation and the heating with stronger PCA***. So the sugar reactions were abandoned and a test of more indirect nature was introduced. In this, the principle utilized in a preceding paper[1] was applied again; if the change in R values between liver and thymus were due to the "protein effect", staining mixtures BM and B[1] might give opposite results in respect of which one of the two tissues used gives higher R values. Actually however, both mixtures gave quite similar results, so that it appeared more likely that the noted difference was caused by the contamination of PNA.

TABLE II

RELATIVE INTENSITY OF FEULGEN REACTIONS OF LIVERS AND THYMUSES OF RATS AS ESTIMATED BY OPTICAL DETERMINATION OF HOT PERCHLORIC ACID EXTRACTS OF THE STAINED MATERIAL IN STRONG SULPHURIC ACID

Material	*Staining solution*	*Volume ratio of extract to 50% H_2SO_4*	$R = E_{268}/E_{432}$		
			Liver	*Thymus*	*Liver/Thymus*
Whole tissue	BM	1	8.18	6.30	1.30
Whole tissue	BM	0.5	4.58	3.07	1.49
Whole tissue	BM	0.5	4.87	3.48	1.40
	B	0.5	4.72	3.36	1.40
Isolated nuclei*	F	1	8.08	7.77	1.04

* Ratio of E_{268} of 1.5 N PCA extract of unstained controls (total of 5 ml) mixed with equal volume of 50% H_2SO_4 to mg total N of the defatted residue (protein) was 2.92 for liver and 4.65 for thymus.

* The washed residue is coloured in light grey, and on neutralizing the medium the colour turns into faint pink. But if observed microscopically nuclei look colourless, so that the dye must have been released nearly quantitatively by this procedure.

** Increase in volume of H_2SO_4 added results in enhancing extinction at 432 mμ relative to that at 268 mμ.

*** One should have conducted such control tests with PCA of lower concentration to get the ratio of ultraviolet absorption to diphenylamine reaction.

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An experiment with isolated nuclei was carried out to exclude the disturbing effect of PNA[15]. Agreement of R values between the two tissues was rather satisfactory, while the nucleic acid/protein ratio of the nuclei was about 1.6 times higher with thymus than with liver, as estimated from the ratio of E_{268} of the PCA extract to protein N in control nuclei. These results, then, strongly suggest that the difference in relative concentration of protein to DNA in cell nuclei does not necessarily result in the change in the colour yield of Feulgen reaction *in situ*.

Experiments with histone-poor nuclei might be of interest in this respect. But the removal of histone causes a marked release of DNA from the nuclei during the whole reaction procedure (see above and ref.[5]). This may tend to obscure the eventual change in the colour yield of the *in situ* reaction due to the presence in nuclei of "free" DNA. So we could not expect much information from experiments of this type.

DISCUSSION

When one attempts to utilize a given histochemical reaction specific to a certain substance, as a tool of quantitative cytochemistry in conjunction with MSP, one must ascertain that this reaction proceeds at least relative-quantitatively not alone in test-tube with pure sample of the substance in question but also in fixed cells in which other co-existing substances, mainly proteins, might interfere with the reaction. Such an interference, due to cellular proteins, of the reaction between nucleic acids and basic dyes has been demonstrated repeatedly to occur with DNA as well as PNA[16-20]. It is rather questionable that the effect of cellular substance on any kind of histochemical reactions can be evaluated correctly with model experiments in test-tube. This is not only because the cellular system is far from simple and hence difficult to reproduce experimentally, but also because the effect observed with the reaction developed in solution does not necessarily hold with the precipitated system such as fixed cells[1,21].

Under these circumstances, it is most desirable to ascertain the (relative) quantitative reactivity of cellular substance with direct chemical determinations on the fixed cells subjected to the histochemical reaction. This is, however, by no means an easy task. As outlined in this paper, there are a number of difficulties to be encountered in such an attempt: cellular material may be altered by fixation and subsequent treatment involved in the reaction procedure, so that it may no longer behave in biochemical analyses like the same substance originating in fresh biological material; coloured compound must first be released from the stained cells without affecting, or together with, reactants in cells, but the procedure required to this must or may tend to destroy the reaction product; further the introduction of reagents into the reaction system *in situ* might cause a severe interference to subsequent determination which is difficult to overcome. So, it is quite probable that the estimation of the colour yield can not be conducted even in a relative measure, not to speak of an absolutely quantitative one.

Chemical determination of the colour yield of the histochemical Feulgen reaction, as submitted in this communication, has encountered all such difficulties. We have failed to establish a method which permits to estimate, in quite an unequivocal way, the colour yield of the Feulgen reaction *in situ*. But as far as the method proposed here could show, there was no indication that the Feulgen reaction of the citric acid-isolated cell nuclei was affected significantly by the change in their relative abundance of protein to DNA. It is, of course, not pertinent to generalize the conclusion drawn from the

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present investigation. But it should be admitted that the present experimentation did contribute to reinforcing the evidence obtained from MSP[1] in respect of the evaluation of the "protein effect" on the Feulgen reaction *in situ*.

SUMMARY

A method of chemically estimating the intensity of the histochemical Feulgen reaction was proposed. There was no difference in the relative quantity of fuchsin and desoxypentose nucleic acid (DNA) in the pigment developed *in situ* between liver and thymus nuclei of the rat, which differ from each other in the protein-DNA ratio.

RÉSUMÉ

On a proposé une méthode pour évaluer chimiquement l'intensité de la réaction histochimique de Feulgen. La teneur relative en fuchsine et en acide désoxypentosenucléique (ADN) du pigment développé *in situ* est la même pour les noyaux du foie et ceux du thymus de Rat, qui diffèrent les uns aux autres dans le rapport de la protéine à l'ADN.

ZUSAMMENFASSUNG

Eine Methode zur Schätzung der Intensität der histochemischen Feulgen-Reaktion wurde ausgearbeitet. Die relative Menge von Fuchsin und Desoxypentosenucleinsäure (DNS) im *in situ* entwickelten Farbstoff verändert sich zwischen Rattenleber- und -thymuskernen nicht, die sich im Verhältnis von Protein zu DNS voneinander unterscheiden.

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