ACTA HISTOCHEM. CYTOCHEM. Vol. 8, No. 4, 1975

FEULGEN NUCLEAL REACTION III. A CONTRIBUTION TO THE STUDY OF THE ADSORPTION ISOTHERM AND STOICHIOMETRY OF FEULGEN'S NUCLEAL REACTION *IN SITU*

TOSHISUKE HIRAOKA

Department of Biology, School of Medicine, Shiga University of Medical Science, Moriyama City, Shiga 524

Received for publication October 29, 1975

In the hope of gaining some insight into the staining mechanism of the Feulgen nucleal reaction *in situ*, the staining reaction of three kinds of hydrolyzed nucleoprotein-containing films with Schiff's reagent was studied from both the physicochemical and the chemical points of view. The establishment of an approximate equilibrium state in staining was assumed, and the mode of the adsorption isotherm in this state was examined. The Feulgen nucleal reaction in the heterogeneous phase conforms to Langmuir's adsorption isotherm and thus exhibits a saturation value in staining. Based on this fact, the stoichiometry of the reaction was determined. The saturation value of the stoichiometry was proved to be four.

In spite of the common use of the Feulgen nucleal reaction *in situ* for the detection and the quantification of deoxyribonucleic acid (DNA) in the cyto- and histochemical fields (18), only little has been known of the physicochemical and chmical characteristics of this reaction (5,9,11,15). As the nucleal reaction *in situ* proceeds in the heterogeneous phase, the staining reaction of three kinds of hydrolyzed deoxyribonucleoprotein (DNP)-albumin films with Schiff's reagent was studied as a cytochemical model for the *in situ* reaction. In such a case, the quantitative aspects are properly accessible by studying the reaction in an equilibrium state. With this in mind, the establishment of an approximate equilibrium in staining was assumed, and the mode of the adsorption isotherm and the stoichiometry were determined. The knowledge of these quantitative aspects, physicochemical and chemical, must contribute to the better understanding of the mechanism of the Feulgen nucleal reaction *in situ*.

MATERIALS AND METHODS

Preparation of raw materials: Crude DNP was prepared from the testes of *Scomberomorus niphonius* (*Teleostei, Pisces*) after the method of Pollister and Mirsky (16), deoxyribonucleohistone (NH) from calf thymus after the method of Kay, Simmons and Dounce (12), and isolated nuclei from calf thymus after the sucrose

Dedicated to Professor Hideo Takamatsu in memory of his retirement.

method of Allfrey *et al.* (1). Egg albumin was obtained commercially from E. Merck, Germany.

Preparation of substrate films for staining: Three kinds of substrate films were used for staining: *Scomberomorus* DNP-albumin films, calf thymus NH-albumin films, and albumin films incorporated with isolated calf thymus nuclei. The former two kinds of films were prepared as described in the previous paper (10). The latter was prepared as follows: Ten *mg* of dried nuclei were suspended in ten *ml* volume of 50% ethanol. A half *ml* aliquot of the evenly dispersed suspension was mixed with an equal volume of a 1 *mg/ml* albumin solution in a centrifuge tube, acidified with trichloracetic acid and kept cool for an hour; thus albumin was precipitated together with the isolated nuclei added. The resulting precipitate was spun down, washed twice with cold water, twice with ethanol, and then dried overnight at 30°C.

Hydrolysis: The substrate film was hydrolyzed with one *ml* of 5.0 N HCl at 20°C for exactly 70 minutes (4,7). This procedure gave an optimal hydrolysis of the film to produce the maximum staining intensity on subsequent treatment with Schiff's reagent. The hydrolyzed film was washed twice with cold water, freed of excess washing fluid by the use of absorbent paper, and was ready for staining.

Staining Procedures: Several kinds of Schiff's reagent differing in fuchsin concentrations were prepared. They varied in fuchsin concentrations from 50 *μg/ml* to 10.0 *mg/ml*, while all contained 0.1 N HCl and 20.0 *mg/ml* $NaHSO_3$ in final concentration. Here, the term "fuchsin concentration" of the reagent is used as defined in the previous paper (11).

The hydrolyzed film was treated with one *ml* each of the reagents in a tightly stoppered tube in the dark at a constant temperature. The staining was carried out with agitations at regular intervals, to insure that the film was stained evenly.

For the purpose of determining the equilibrium position of the staining, one *ml* of the Schiff reagent of 10.0 *mg/ml* fuchsin concentration was used as the reagent. The staining was carried out for a time period ranging from 15 minutes to 5 hours at 20° and 40°C. For the purpose of studying the adsorption isotherm and stoichiometry of the staining, the hydrolyzed film was stained with one *ml* each of the Schiff reagents of different fuchsin concentrations until an approximate staining equilibrium was reached, namely, for two and a half hours at 15°C, for two hours at 20°C or for one hour at 40°C. The removal of excess reagnet from the stained film was carried out as described previously (10).

Chemical analyses of stained film: In the cases of studying the equilibrium and adsorption isotherm of the staining, the stained film was analyzed for the amount of combined fuchsin after the fuchsin extraction method developed earlier by the present author (10). In the cases of studying the stoichiometry, the stained film was analyzed concurrently for both combined fuchsin and phosphorus. A minute amount of fuchsin due to non-specific staining and the quantity of phosphorus originating from the albumin were corrected to obtain the fuchsin amount due to the nucleal reaction and the apurinic acid phosphorus amounts (10). The amount of phosphorus was analyzed according to the method of Barton (2), after incineration of the film (13).

RESULTS

I. Establishment of approximate staining equilibrium

In order to gain some insight into the quantitative aspects of a staining reaction in the heterogeneous phase, an analysis in the equilibrium state is necessary. With this in mind, our preliminary approach was directed toward finding the equilibrium state, if any, in the staining reaction under investigation.

The stained film was analyzed for the amount of combined fuchsin, and this amount was expressed in terms of a percentage exhaustion of the external reagent used, namely, the percentgae ratio of the fuchsin combined with the film at a given time period to the initial amount of fuchsin in the reagent. In Fig. 1, the percentage exhaustion for the staining reaction of hydrolyzed *Scomberomorus* DNP-albumin films was plotted against the reaction time. The staining proceeded hyperbolically or exponentially (19,5), reached a plateau for various durations, and again diminished gradually. As explained later, this plateau was assumed as "an approximate equilibrium" in the present paper.

An approximate equilibrium was reached after two hours of staining at 20°C and after one hour of staining at 40°C, the maximum percentage exhaustion attained being 0.12% at 20°C and 0.11% at 40°C. A similar time course of staining was also obtained with hyrdolyzed calf thymus NH-albumin films and with hydrolyzed calf thymus nuclei-albumin films. The maximum percentage exhaustion reached was 0.31% at 20°C for the former film and 0.33% at 20°C for the latter one.

II. Conformity to Langmuir's adsorption isotherm of Feulgen's nucleal reaction in heterogeneous phase

In order to gain some insight into the staining mechanism of hydrolyzed substrate film with Schiff's reagent, the information on the distribution of fuchsin between the film and the external reagent in the state of equilibrium is of fundamental significance. Such knowledge of fuchsin distribution can be adequately expressed in the form of the adsorption isotherm. Here, the term "adsorption"

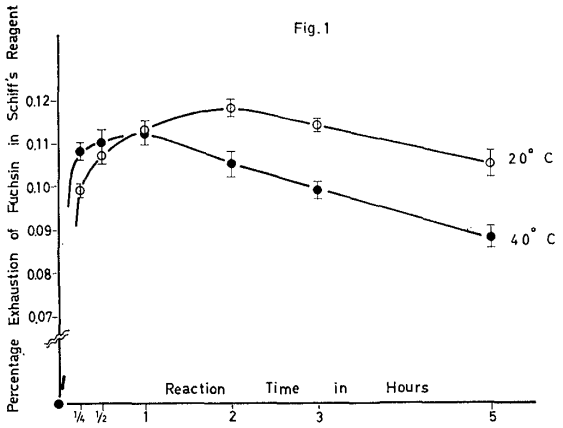

FIG. 1. Diagram showing the approximate equilibrium in the staining reaction of hydrolyzed *Scomberomorus* DNP-albumin films with Schiff's reagent at 20°C (hollow circles) and at 40°C (solid circles). Each circle represents the mean value of 5 determinations, and two short bars lying on the upper and lower sides of the circle, the standard deviation.

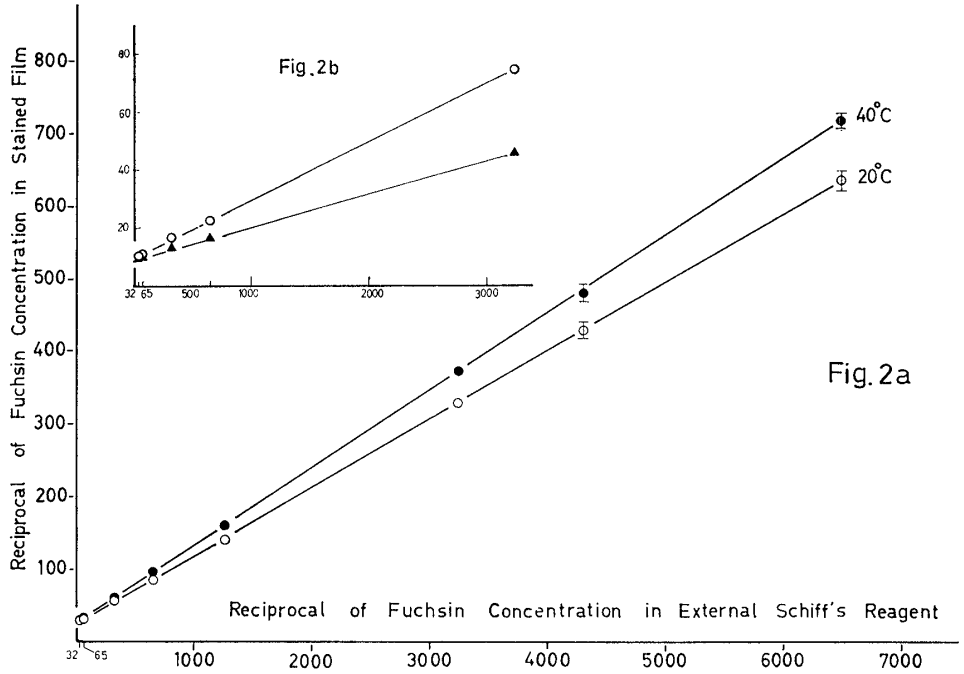

FIG. 2. Diagrams showing the conformity to Langmuir's adsorption isotherm of Feulgen's nucleal reaction in the heterogeneous phase. Fig. 2a: Hydrolyzed *Scomberomorus* DNP-albumin films at 20°C (hollow circles) and at 40°C (solid circles). Fig. 2b: Hydrolyzed calf thymus NH-albumin films at 20°C (hollow circles) and hydrolyzed calf thymus nuclei-albumin films at 20°C (solid triangles). Each circle or triangle represents the mean value of 5 determinations, and the standard deviation was within 5%.

is used according to the definition of Vickerstaff (19), and means the take-up of a dyestuff by the solid phase from the external liquid one.

The hydrolyzed substrate film was stained with the Schiff reagent of different fuchsin concentrations until an approximate equilibrium was established, and was analyzed for the amount of combined fuchsin. From the amount found, we calculated the fuchsin concentration in the stained film in equilibrium with the external reagent used. This was expressed in terms of *m moles* per *g* film, while the fuchsin concentration of the external reagent was expressed in terms of *m moles* per *ml*, taking the molecular weight of para-fuchsin to be 323.8 (3). Plotting the reciprocal of the fuchsin concentration in the stained film against the reciprocal of the fuchsin concentration in the external reagent used, a straight line relationship was obtained (Figs. 2a and 2b). This straight line relationship implies that the Feulgen nucleal reaction in the heterogeneous phase conforms to Langmuir's adsorption isotherm. When these straight lines in Figs. 2a and 2b are extrapolated to the axis of ordinates, we obtain the limiting values of fuchsin concentration in the stained films, which are in equilibrium with the Schiff reagent of infinite fuchsin concentration. These values may be called the "saturation values" of staining. The saturation value was 25 for *Scomberomorus* DNP-albumin films, 10 for thymus NH-albumin films and 9.6 for thymus nuclei-albumin films. All these situations are briefly represented by the following formula: $\frac{1}{(F)_n} = \frac{1}{C(F)_s(F)_r} + \frac{1}{(F)_s}$; where $(F)_n$ denotes the concentration of fuchsin bound with the

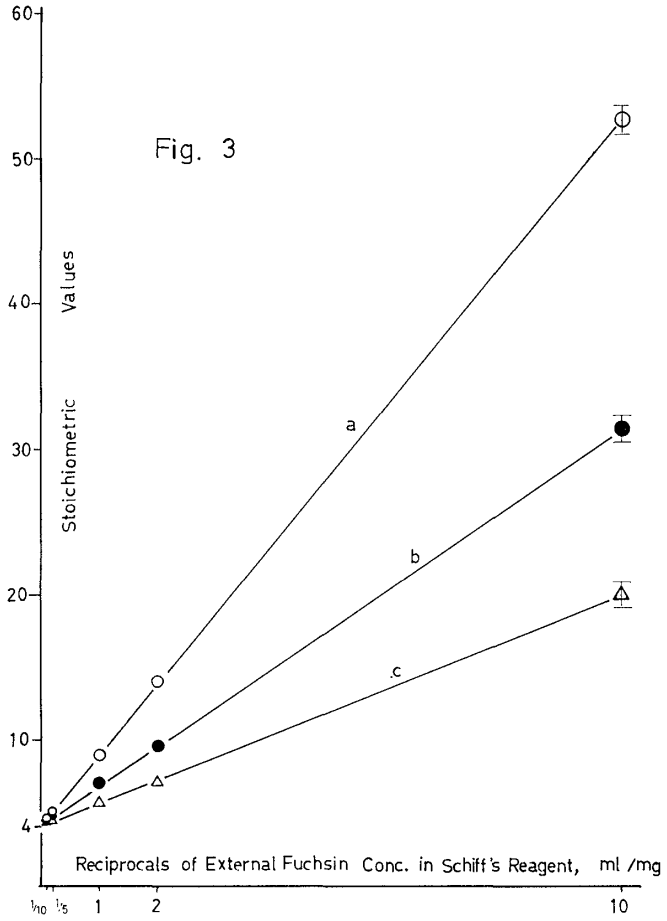

FIG. 3. Diagram showing the stoichiometry of Feulgen's nucleal reaction in the heterogeneous phase at 15°C. Line *a*: Hydrolyzed *Scomberomorus* DNP-albumin films. Line *b*: Hydrolyzed calf thymus NH-albumin films. Line *c*: Hydrolyzed calf thymus nuclei-albumin films. Note that Lines *a*, *b* and *c* meet together at a definite point of 4 on the ordinate axis. Each circle or triangle represents the mean value of 5 determinations, and the standard deviation was within 5%.

hydrolyzed substrate film, in equilibrium with the external Schiff reagent of fuchsin concentration $(F)_r$; $(F)_s$ the saturation value of $(F)_n$; and C a constant (19). To show an instance, in Fig. 2a the mean values of C were found to be 268.5 with a mean error of 1.7 at 20°C, and 228.8 with a mean error of 4.2 at 40°C. A similar conformity to Langmuir's adsorption isotherm has been reported to occur in the dyeing of wool, silk, casein fibre and nylon with some acidic dyes (8,17).

In Fig. 2a, the straight line for the reaction at 40°C showed a greater angle of inclination than that for the reaction at 20°C, the former having an angle of inclination of 33.7 degrees, while the latter had that of 31.0 degrees. Therefore, the distribution of fuchsin in an approximate equilibrium between the hydrolyzed substrate film and the external reagent is more favourable for the latter as the temperature rises. This fact means that this staining reaction is exothermic, or in other words, that the heat of staining in this reaction is negative. We can calculate the heat of staining according to the following formula: $\Delta H^\circ = -\frac{RTT'}{(T'-T)} ln \frac{(F)_{T'}}{(F)_T}$;

where ΔH° is the standard heat of staining; T and T′ are two absolute temperatures concerned; R is the universal gas constant, that is, 1.987 *cal/mole·degree*; and $(F)_T$ and $(F)_{T'}$, are the fuchsin concentrations of the external reagent, which give the same fuchsin concentration of the stained film in an equilibrium state at T and T′ respectively (19). The mean value of the heat of staining obtained was −1159 *cal/mole*, with a mean error of −5 *cal/mole*.

III. Stoichiometry of Feulgen's nucleal reaction in heterogeneous phase

Since the Feulgen nucleal reaction in the heterogeneous phase conforms to Langmuir's adsorption isotherm, the reaction shows a definite value of saturation in staining. This fact permits us to determine the stoichiometry of the reaction.

The stained substrate film, which was in approximate equilibrium with the Schiff reagent of different fuchsin concentrations at 15°C, was analyzed for the amounts both of the combined fuchsin and apurinic acid phosphorus. From these amounts the stoichiometric value, or number of apurinic acid phosphorus atoms per fuchsin molecule, in the stained film was calculated. Plotting the stoichiometric value against the reciprocal of the fuchsin concentration of the external Schiff reagent, a straight line relationship was obtained for the staining reaction of all three kinds of hydrolyzed substrate film with Schiff's reagent. Fig. 3 shows the mean values of five determinations, obtained with hydrolyzed *Scomberomorus* DNP-albumin films (Line *a*), with hydrolyzed calf thymus NH-albumin films (Line *b*) and with hydrolyzed calf thymus nuclei-albumin films (Line *c*). Extrapolating these three lines to the axis of ordinates, they meet at a definite point of four on the axis. This value is understood to be the saturation value of stoichiometry, which will be reached when the hydrolyzed substrate film is in equilibrium with the Schiff reagent of infinite fuchsin concentration.

CONCLUSION

The staining reaction studied in the present investigation proceeds in the heterogeneous phase, that is, the dye molecules of the reagent are taken up by the solid phase of the substrate film from the external liquid one. In this respect, it may be regarded as a model system for the cyto- and histochemical staining reactions *in situ*. As this model system includes many chemical constituents other than hydrolyzed DNP and fuchsin derivatives, some kind of simplification is necessary for analytical treatment. For this reason, only the fuchsin moiety was used as a marker, and its behavior was followed in this investigation. This simplification proved to be effective enough to make a rough sketch of the reaction mechanism concerned.

In our preliminary approach, the time course of staining in the heterogeneous phase was examined to determine the staining equilibrium. An equilibrium state of a staining reaction, in the exact sense, is a state wherein the ratios of both the staining and the destaining processes are equal; hence a steady state or plateau of staining is attained (19). Unfortunately, an equilibrium state in the exact sense was not found in the reaction studied. Instead, a plateau for various durations and an ensuing diminution of staining were encountered. The establishment of the plateau may be taken to mean that the staining reaction is close to its true

equilibrium, though the ensuing diminution implies that some deterioration of the stained substrate concurs with the staining process (Autohydrolyse, 14). Based on this assumption, this plateau was taken as "an approximate equilibrium" in the present paper.

The physicochemical and chemical evidence obtained in the present study seems significant in permitting some insight into the mechanism of the Feulgen nucleal reaction *in situ*. This evidence suggests that the reaction proceeds at least in two steps. The nucleal reaction in the heterogeneous phase conforms to Langmuir's adsorption isotherm. This fact means that, in the first step, the molecules of Schiff's reagent are adsorbed on the hydrolyzed substrate film selectively only at specific reactive sites. The overall energy of adsorption was found to be -1159 *cal/mole*. As to the chemical nature of these reactive sites, it seems highly probable that they are the aldehydic reactive groups, which are engendered by hydrolysis, and no others (6,15). In the second step, the adsorbed Schiff reagent molecules undergo some chemical reaction involving covalent formation with the aldehydic reactive groups (*cf.* 5,6,9,11). The stoichiometric evidence obtained in this and the previous study contributes to a better understanding of this chemical reaction. Hitherto, various values of stoichiometry have been reported for the Feulgen nucleal reaction in the heterogeneous phase (*cf.* 9). These inconsistent values may be brought about by variations in staining conditions, but the idea of saturation value presented here eliminates such complexities and leads to a clear-cut understanding of the stoichiometry. In the present study, the saturation value of stoichiometry for the Feulgen nucleal reaction in the heterogeneous phase was found to be four. The typical colored product of the nucleal reaction *in vitro* (fraction *i*) has been examined stoichiometrically. The saturation value of six was obtained for the non-dialyzed sample, that of eight for the dialyzed one, and the theoretical saturation value of four by extrapolation (11). Since the greatest common divisor of all these saturation values, practical and theoretical, is two, it seems highly probable that each adsorbed Schiff's reagent molecule reacts with the reactive aldehydic groups of apurinic acid or hydrolyzed DNA in the form of di-substitution. Further consideration of how these saturation values in stoichiometry are brought about in the Feulgen nucleal reaction *in situ* and *in vitro* will be given elsewhere.

As mentioned above, the Feulgen nucleal reaction *in situ* exhibits a definite saturation value in staining. This fact permits us to explore a new microspectrophotometric method of determining DNA amounts in terms of a fundamental unit of mass.

REFERENCES

1. Allfrey, A. G., Mirsky, A. E. and Osawa, S.: Protein synthesis in isolated nuclei. *J. Gen. Physiol.* 40; 451, 1957.
2. Barton, C. J.: Photometric analysis of phosphate rock. *Anal. Chem.* 20; 1068, 1948.
3. Conn. H. J.: Biological stains. 6th edition, Williams & Wilkins Co., Baltimore & Maryland, 1960.
4. Deitch, A. D., Dieter, W. and Richart, R. M.: Conditions influencing the intensity of the Feulgen reaction. *J. Histochem. Cytochem.* 16; 371, 1968.

5. Duijndam, W. A. L., Hermans, J. and van Duijn, P.: Application of the method of kinetic analysis of staining and destaining processes to the complex formed between hydrolyzed deoxyribonucleoprotein and Schiff's reagent in model films. *J. Histochem. Cytochem.* 21; 729, 1973.
6. Feulgen, R. und Rossenbeck, H.: Mikroskopisch-chemischer Nachweis einer Nukleinsäure von Typus der Thymonukleinsäure und die darauf beruhende elektive Färbung von Zellkernen in mikroskopischen Präparaten. *Hoppe. Seylers Z. physiol. Chemie* 135; 203, 1924.
7. Fox, D. P.: Some observations of the cold hydrolysis technique for staining plant tissues by the Feulgen reaction. *J. Histochem. Cytochem.* 17; 266, 1969.
8. Giles, C. H., Mac Ewan, T. H., Nakhwa, S. N. and Smith, D.: Studies in adsorption. Part XI. A system of classification of solution adsorption isotherms, and its use in diagnosis of adsorption mechanisms and in measurement of specific surface areas of soilds. *J. Chem. Soc. London* 3973, 1960.
9. Hardonk, M. J. and van Duijn, P.: A quantitative study of the Feulgen reaction with the aid of histochemical model system. *J. Histochem. Cytochem.* 12; 752, 1964.
10. Hiraoka, T.: Feulgen nucleal reaction I. Quantitative extraction of fuchsin from Feulgen-stained nucleoprotein. *J. biophys. biochem. Cytol.* 3; 525, 1957.
11. Hiraoka, T.: Feulgen nucleal reaction II. Approaches to the understanding of mechanism of *in vitro* reaction. *Histochemie* 35; 283, 1973.
12. Kay, E. R. M., Simmons, N. S. and Dounce, A. L.: An improved preparation of sodium desoxyribonucleate. *J. Am. Chem. Soc.* 74; 1724, 1952.
13. King, E. J.: The colorimetric determination of phosphorus. *Biochem. J.* 26; 292, 1932.
14. Milovidov, P. F.: Physik und Chemie des Zellkernes. Erster Teil. Protopl. Monogr. 20 Bd., Gebrüder Borntraeger, Berlin, 1949.
15. Overend, W. G. and Stacey, M.: Mechanism of the Feulgen nucleal reaction. *Nature* 163; 538, 1949.
16. Pollister, A. W. and Mirsky, A. E.: The nucleoprotamine of trout sperm. *J. Gen. Physiol.* 30; 101, 1946.
17. Skinner, B. G. and Vickerstaff, T.: The absorption of acid dyes by wool, silk, casein fibre and nylon. *J. Soc. Dyers Col.* 61; 193, 1945.
18. Vendrely, R. and Vendrely, C.: The results of cytophotometry in the study of desoxyribonucleic acid content of the nucleus. *Int. Rev. Cytol.* 5; 171, 1956.
19. Vickerstaff, T.: The physical chemistry of dyeing. 2nd edition, Oliver & Boyd, London, 1954.