A CYTOCHEMICAL STUDY OF THE L.E. BODIES OF SYSTEMIC LUPUS ERYTHEMATOSUS

I. Nucleic Acids*, ‡

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Plates 60 and 61

(Received for publication, April 24, 1957)

Since its first identification by Hargraves *et al.* (1) the L.E. cell phenomenon has been repeatedly described as an alteration pathognomonic of systemic lupus erythematosus. The basic identity of the L.E. body of the blood with the "hematoxylin bodies" in the tissues can now hardly be doubted. At the time of their first recognition by Gross (2) and Ginzler and Fox (3), the hematoxylin-stained bodies were thought to originate from cell nuclei, a judgement which has since been confirmed (4); for the L.E. bodies, either of blood or in the tissues, are the products of a peculiar alteration of the constituents of cell nuclei. Recent studies by Klemperer (5) and Gueft and Laufer (6) have indicated that the L.E. body and its tissue counterpart, the hematoxylin body, are of more than diagnostic significance, and that indeed they are an important link in the chain of pathogenetic events in systemic lupus erythematosus.

The discovery of a factor in the serum of patients with systemic lupus erythematosus capable of provoking this specific change in leukocytes (1, 7–10) and the histochemical characterization of hematoxylin bodies (4–6) and L.E. bodies (11) augured a better insight into the pathogenesis of this malady. The hematoxylin bodies were reported by Klemperer and co-workers (4, 12) to contain partially depolymerized deoxyribonucleic acid (DNA), and this was subsequently also reported of the L. E. cell (11). It was therefore hypothesized that a disturbance of nucleic acid metabolism affecting the nuclei of cells of "mesenchymal" derivation occurs in systemic lupus erythematosus, and is characteristic of the disease (4–6, 12–14). Since really significant elevation of circulating deoxyribonuclear depolymerase (DNase) could not be found in patients suffering from systemic lupus erythematosus, and because the L.E. factor of the serum could not be correlated with DNase activity, (5, 13, 15, 16) other explanations for the supposed degradation of DNA of affected cells were investigated.

* Aided by grants from the Jane Coffin Childs Fund for Medical Research, the Damon Runyon Fund for Cancer Research, and an institutional grant of the American Cancer Society, Inc., to Columbia University.

‡ Presented in part to a meeting of the American Association of Pathologists and Bacteriologists, Cincinnati, April 26, 1956.

The studies of Kurnick *et al.* (13, 14) led them to conclude that depolymerization of DNA in the L.E. cell was due to release of an intracellular DNase from an intracellular inhibitor of DNase (17) by the L.E. factor of the blood. There is no evidence that this inhibitor is inactivated by the L.E. factor itself (16), and it has therefore been further postulated that the L.E. globulin facilitates the ingress of serum proteases into the cell, which in turn effect destruction of the inhibitor of intracellular DNase (18, 19).

Many of our conceptions of the pathogenesis of the tissue changes occurring in systemic lupus erythematosus have thus been based upon histochemical analysis of the L.E. and hematoxylin bodies. In the work of Klemperer *et al.* (4) the state of DNA in the hematoxylin bodies was inferred from photometric measurement of relative Feulgen and methyl green staining. Interpretation of methyl green staining has since been clarified by demonstrating that methyl green binding by DNA is influenced by factors other than the polymerization of DNA.

The cytochemical investigations presently reported were undertaken with the object of reassessing and extending our information concerning the composition of the L.E. bodies.

Materials and Methods

L.E. bodies and cells were compared cytochemically with blood lymphocytes in concentrated preparations made in three different ways. The "ring" method of Snapper and Nathan (20) yielded an abundant source chiefly of phagocytosed bodies in single leukocytes or in rosettes (Fig. 1). A technique devised by Davis and Eisenstein (21), in which dried leukocytes, concentrated in buffy coat serve as substrate cells for the action of the L.E. factor of the plasma, was employed for obtaining free L.E. bodies in great profusion (Fig. 2). Preparations were incubated at 37°C. for 5, 10, 15, 20, and 30 minute intervals. In some instances, fresh viable leukocytes were added with the L.E. plasma to give typical phagocytosed (ingested) L.E. cells and rosettes in large numbers (Fig. 2). The method of Lee (22) in which *atabrine* (quinacrine, an acridine derivative) renders concentrated suspensions of leukocytes susceptible to the action of the L.E. factor of the plasma, was used to provide large numbers of free L.E. bodies derived from normal buffy coat or from lymphocytes of lymphocytic leukemia (Fig. 3), as well as conventional L.E. cells. In all cases, lymphocytes from the same or similar blood as that used for substrate cells served as control material.

Sera were obtained from three patients with characteristic clinical features of systemic lupus erythematosus. *Patient A. S.* (Presbyterian Hospital No. 176042) a 25 year old white female, developed a febrile illness at the age of 18, with a butterfly rash and arthralgias, for which she was hospitalized and treated with steroids and ACTH. Her serum gave positive L.E. preparations at this time. During the following 7 years she suffered several remissions and exacerbations. Positive L.E. preparations were obtained on several occasions. During an exacerbation of her illness in September, 1956, most of the blood used in these studies was drawn. She died of intercurrent infection in March, 1957, after a course of 7 years. No autopsy was obtained. *Patient V. J.* (Presbyterian Hospital No. 271568) is a 29 year old colored female who became sick at the age of 27, with fever and arthralgia. Splenomegaly and proteinuria were noted. Her serum gave positive L.E. preparations at the time of her first illness and has since repeatedly been found to be active. She had an exacerbation with polyserous effusions 4 months after her first illness and is in remission at the time of writing. Steroid therapy is being administered. *Patient M. F.* (Mount Sinai Hospital) is a 24 year old white female who devel-

oped fever, arthralgias, a typical butterfly rash and evidences of nephropathy at the age of 19. Her serum gave positive L.E. preparations at this time, and has continuously been found to be strongly active since, yielding L.E. cells in titres as high as 1:64 on some occasions. She has suffered numerous recurrences of her illness with fever, arthralgias, and pleurisy the principal features. Steroid therapy has been constantly given.

Preparations generously contributed by Drs. I. Snapper and D. Nathan were made with serum derived from well authenticated cases of systemic lupus erythematosus (See reference 20). Substrate cells and control sera were obtained from several sources. Normal plasma and buffy coat cells were prepared from the blood of patients with unrelated illnesses or from healthy volunteers. Preparations of lymphocytes were made from the blood of several patients with chronic lymphatic leukemia having leukocyte counts in excess of 100,000 per cu. mm.

The smears were fixed in methanol, and stained with Wright's stain. Suitable bodies or cells were carefully mapped to permit relocation of the same objects in subsequent manipulations.

Methyl green staining was carried out as prescribed by Pollister and Leuchtenberger (23) in the phenol-glycerin solution of methyl green (National Aniline, C.I. 685) purified by chloroform extraction. The pH of this solution is 4.2. Preparations were stained for 45 minutes at 56°C., rinsed quickly in ice-cold distilled water, blotted, and differentiated in tertiary butyl alcohol for 18 to 20 hours. In some experiments, this material was destained after measurement of methyl green binding, and submitted to the Feulgen reaction. Where the conventional Feulgen reaction was employed, a hydrolysis time of 8 minutes was allowed. In one group of experiments, the Feulgen reaction was done as modified by Bloch and Godman (24), in which trichloracetic acid is substituted in equinormal concentration for HCl in the hydrolysis bath, the Schiff's reagent and the bleaches. The hydrolysis time in this case was 14 minutes.

To evaluate the binding of a cationic and an anionic dye by lupus bodies, methanol-fixed preparations were stained in methylene blue and orange G in final concentration of 5×10^{-4} M in phosphate or acetate buffers of ionic strength 0.01, in a graded series of pHs from 2.9 to 7.3. (See references 25 and 26)

Acetylation of protein amino groups was performed by exposure to pure acetic anhydride at room temperature for 2 hours. Specific removal of ribonucleic acid was effected by incubation in 0.05 per cent crystalline protease-free ribonuclease (Worthington) in doubly distilled water, (pH 6.5) at 37°C. for 1 hour. Deoxyribonuclease (Worthington) 0.01 per cent made up in a buffered gelatin solution at pH 7.5 was used to remove DNA. Both nucleic acids were extracted from some specimens by exposure to 5 per cent trichloracetic acid at 90°C. for 15 minutes. The effect of immersion in 0.01 N HCl at room temperature for 24 hours on methyl green uptake was studied in some preparations.

Quantitative cytophotometric determinations (see references 27–29) of the apparent relative concentrations and/or amounts of DNA as revealed by methyl green or the Feulgen color were made using a microspectrophotometric apparatus incorporating some of Moses' (27) and Pollister's (30) modifications of the basic design of Pollister and Moses (31). The methyl green color was measured at a wave length of 633 mμ and the Feulgen at 568 mμ isolated by a Bausch and Lomb monochromator from a ribbon filament Tungsten source. The L.E. bodies and lymphocyte nuclei were mounted in fluid of closely matched refractive index and were treated as regular discoid objects, a constant plug approximately 3.5 μ in diameter being circumscribed for photometric measurement. Due care was observed to keep the field of illumination small, to reduce light scatter. Relative amounts were calculated by multiplying the optical density (extinction) of the plug by the product of the nuclear diameters. Measured values are reported in arbitrary units of apparent amounts of dye per body or nucleus. It is important to note that arbitrary units of one set of determinations are not necessarily com-

parable with those obtained in another set of determinations in which somewhat different conditions may have prevailed, although ratios can always be compared from experiment to experiment in this report. Arbitrary units are of course, comparable within any experiment.

RESULTS AND COMMENTS

DNA is one of the principal constitutive elements of cell nucleus and one of its most stable components. The mature leukocyte nuclei, from which L.E. bodies are derived, are regarded as cells with the typical diploid charge of DNA (32) which in man amounts to about 7.3 pcg. per cell (33). Cytochemically, DNA is demonstrable by the natural absorption of its bases in the ultraviolet, by the affinity of its available phosphoryl groups for cationic dyes, and by the Feulgen reaction given by its deoxypentose.

The Affinity of Methyl Green for DNA.—

The basic dye methyl green (septamethylpararosaniline), under certain conditions, can combine selectively with DNA (23, 34–37). Experiments with various nucleic acid preparations *in vitro* led to the conclusion that methyl green combines specifically with polymerized DNA (34), and in definite stoichiometric proportion with the DNA orthophosphate groups, 1 mole of dye being bound per 10 atoms of P (35) presumably in the formation of a dye-nucleate salt. Depolymerization was found to depress methyl green uptake *in vitro* and degraded DNA was not stained (34, 38). Methyl green staining of fixed nuclei which was found to be highly reproducible (23) was therefore proposed and employed for quantitative microphotometric determinations of DNA in histological preparations (23, 38–42). Pollister and Leuchtenberger (23), observing that treatment with hot water depressed methyl green binding in sections without loss of nucleic acid or of protein, invoked Kurnick's experiments (34) and concluded that methyl green stainability could be used to detect changes in state or molecular configuration of DNA, "in the nature of depolymerization," in suitably prepared histological material. This explanation was applied to the observed depression of nuclear methyl green stainability following irradiation (41–43, but see 44), and the lowered methyl green binding by the hematoxylin bodies and L.E. cells was the basis of the interpretation that depolymerization of DNA occurs in systemic lupus erythematosus (4–6, 11).

The selectivity and stability of the DNA methyl green combination have been supposed to depend upon a specific spatial relation of the anionic groups in the DNA bihelix to the dibasic dye molecule, any disturbance of which, such as would occur in depolymerization would be reflected in a reduction of methyl green binding (23, 34, 35, 38).

Not only their propinquity, but more importantly, the availability of the phosphate groups for chemical union governs the combination of methyl green and other basic dyes with DNA. Kurnick and Mirsky (35) and Kurnick (39) recognized that added lanthanum ion or histone competes with methyl green by preempting the phosphoryl groups of DNA; indeed the competitive inhibition of other cations to the formation of dye nucleate complexes is a generally recognized phenomenon (25, 28, 45, 46). In a study of the conditions affecting methyl green stainability of nucleic acids in models and in tissues, Alfert (47) stressed the importance of protein interference;

i.e., blocking of stainable groups of both nucleic acids by protein. Such masking of stainable groups has indeed been recognized as a general occurrence in tissues (48–50). Methyl green is much more sensitive to protein interference than other basic dyes (46, 47). It is thus apparent that depression of staining of DNA in nuclei with methyl green, even when carried out with the precautions of Kurnick (39, 51) cannot by itself afford unequivocal evidence of depolymerization of DNA. The positively charged ϵ-amino and guanidyl groups of proteins which compete with the basic dye for available phosphoryl groups of nucleic acid, can be destroyed or blocked by acetylation, reaction with nitrous acid, or chloramine-t (47, 52–54).

TABLE I

Mean Amounts of Methyl Green and Feulgen Dye Bound in L.E. Cells

Successive measurements of the same lymphocytes and L.E. cells after the treatments indicated. The L.E. cells, mostly phagocytosed, were obtained from "ring" L.E. preparations (20) employing normal donor leukocytes as substrate. The TCA-Feulgen method of Bloch and Godman (24) was used; 15 L.E. cell inclusions were measured in this case.

	Lymphocytes	L.E. cells
(No. measured)	(30)	(33)
Methyl green	10.7 ± 0.3	3.3 ± 0.3
Methyl green after acetylation	11.6 ± 0.4	7.0 ± 0.8
TCA-Feulgen	11.0 ± 0.4	6.5 ± 0.8
$\frac{\text{Post-acetylated methyl green}}{\text{Methyl green}}$	1.08	2.15
$\frac{\text{Feulgen}}{\text{Methyl green}}$	1.03	2.00
$\frac{\text{Feulgen}}{\text{Post-acetylated methyl green}}$	0.95	0.93

Methyl Green Binding of L.E. Bodies.—

To assess the influence of acetylation on methyl green binding by DNA in L.E. bodies, the amount of dye capable of being bound to nucleic acid in the same bodies was measured microphotometrically before and after acetylation of protein basic groups. Table I summarizes the results obtained on preparations made using the "ring" method of Snapper and Nathan (20) in which the inclusions of classical L.E. cells and free bodies in early stages of phagocytosis were measured. Buffy coat controls providing lymphocytes were incubated in normal serum for the same time period. In these lymphocytes, following acetylation, there is approximately a 10 per cent rise in methyl green binding capacity. In lupus bodies, however, blocking of protein basic groups in this way brings about a greater than twofold (more than 100 per cent) average increase in methyl green binding capacity of their DNA. It would thus appear that somewhat over half of the stainable nucleic acid phosphate in lupus bodies

is bound or masked by protein, while less than one-tenth of the nucleic acid phosphate is preempted in this way in the lymphocyte. Almost identical results were obtained in series of measurements using free L.E. bodies derived from substrate lymphocytes from lymphatic leukemia in atabrine (22) preparations (Table II) and in other preparations made by the "ring" method, the average postacetylated methyl green:methyl green ration in these determinations being 1.9. In "ring" preparations both free bodies and phagocytosed bodies gave comparable methyl green and postacetylated methyl green values. The effect of acetylation on methyl green binding by L.E. bodies is illustrated in Figs. 4 to 8.

TABLE II

Mean Amounts of Methyl Green and Feulgen Dye Bound in L.E. Bodies Derived from Lymphocytes

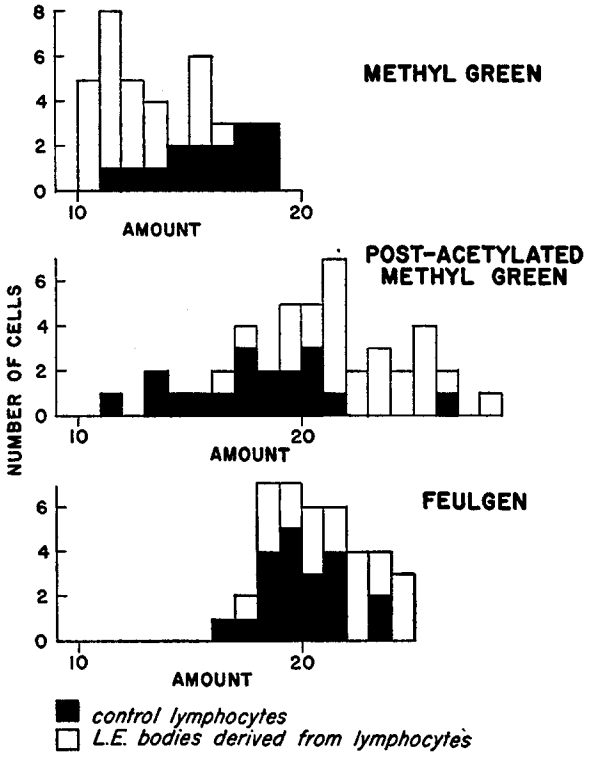
Measurements of free (non-phagocytosed) L.E. bodies in atabrine L.E. preparations (22) employing lymphocytes from a patient with chronic lymphatic leukemia as substrate.

	Lymphocytes	L.E. Bodies
(No. Measured)	(20)	(20)
Methyl green	16.7 ± 0.5	11.9 ± 0.3
Methyl green after acetylation	17.8 ± 0.8	23.1 ± 0.6
Feulgen	19.9 ± 0.4	21.3 ± 0.5
$\frac{\text{Post-acetylated methyl green}}{\text{Methyl green}}$	1.06	1.94
$\frac{\text{Feulgen}}{\text{Methyl green}}$	1.19	1.79
$\frac{\text{Feulgen}}{\text{Post-acetylated methyl green}}$	1.12	0.92

Methyl Green–Feulgen.—

The Feulgen reaction for DNA, which depends upon revelation of aldehyde groups on the deoxypentose sugar exposed by acid hydrolysis, is relatively insensitive to those changes of state or configuration of the DNA molecule, or its relation to protein, that affect basic dye binding. Feulgen measurements of DNA therefore provide a useful baseline or standard of reference against which other staining properties can be compared. In the measurements reported by Klemperer *et al.* (4) and Lee *et al.* (11) the methyl green: Feulgen ratios of hematoxylin bodies and L.E. cell inclusions were found to be greatly decreased as compared with nuclei, owing to the observed depression of methyl green staining. The resultant abnormally low methyl green: Feulgen (high Feulgen: methyl green) ratios of the hematoxylin bodies which were interpreted at that time as indicative of depolymerization of DNA have been shown in the foregoing experiment to be due, in some part, to protein interference.

It is pertinent to inquire whether there is any residual decrease in methyl green binding in L.E. bodies which cannot be accounted for by the competitive effect of protein. If there were, it might suggest some change in the DNA itself. To assess this, the methyl green binding of L.E. bodies after acetylation of protein basic groups was compared with the amount of DNA revealed by the



TEXT-FIG. 1. Histogram illustrating (*a*) the diminished capacity of L.E. bodies to bind methyl green, as compared with lymphocytes, (*b*) the increase of methyl green binding capacity of L.E. bodies by acetylation to values approximating (and somewhat exceeding) those of lymphocytes submitted to acetylation and (*c*) the similar amounts of Feulgen—revealed DNA in both lymphocytes and lupus bodies.

Feulgen technique in the same bodies. The results of such measurements, using L.E. bodies prepared by the "ring" and the atabrine methods, are given in Tables I and II, and Text-fig. 1. These data show that after acetylation the relative amounts of methyl green and Feulgen revealed DNA are similar, and that the Feulgen:post-acetylated methyl green ratios of lupus bodies and lymphocytes alike tend to approach 1.0 and in any case become similar (0.95 and 0.93 in Table I; 1.12 and 0.92 in Table II). Entirely analogous ratios were

obtained using preparations of free L.E. bodies made by the Davis and Eisenstein (21) method. It is therefore concluded that there is no significant decline of methyl green staining of DNA in L.E. bodies which cannot be accounted for by protein interference, and that the DNA is not *detectably* "depolymerized" or altered in state in systemic lupus.

Comparison of the amounts of Feulgen-revealed DNA in lymphocytes and the phagocytosed lupus bodies respectively employed in the experiment tabulated in Table I would seem to point to some loss of DNA from the latter, from average amounts of 11.0 units in the lymphocyte to 6.52 units in the L.E. cell inclusion. However, neither the actual magnitude of this change, nor its significance can be assessed from the data of Table I. Although each L.E. body measured was geometrically regular and of approximately similar size, it cannot be known whether a given body is derived from a whole nucleus,

TABLE III

Mean Amounts of DNA Revealed by the Feulgen Reaction in L.E. Bodies

Measurements of free (non-phagocytosed) L.E. bodies prepared from dried normal leukocytes according to the method of Davis *et al.* (21).

	Lymphocytes	L.E. Bodies	
(No. measured)	(20)	(20)	(20)
Incubation time	—	5 min.	30 min.
Feulgen	14.6 ± 0.3	14.7 ± 0.3	13.4 ± 0.3

from fusion of some of the lobes, or from a single lobe of a polymorphonuclear leukocyte. Therefore, while concentration measurements and the Feulgen–methyl green ratio and their comparisons are wholly meaningful, comparison of total amounts of DNA in whole nuclei and these particular L.E. bodies is questionable. Valid determination of total amounts of DNA in L.E. bodies as compared with whole nuclei can only be made on free uningested L.E. bodies whose origin from whole nuclei can be assumed. Such measurements are shown in Tables II and III from which it can be ascertained that there is no loss in the total amount of DNA in the formation of L.E. bodies from whole leukocyte nuclei. After relatively long (30 minute) incubation of the substrate leukocytes with the L.E. factor (Table III) there appears to be a small decrease in the average amount of DNA in the free bodies, but additional data are required to determine whether this represents a significant trend. After bodies have been phagocytosed for long periods and presumably submitted to the prolonged action of intracytoplasmic proteases and nucleases of the polymorphonuclear leukocyte (14, 17), there is a visible decline in affinity for all stains in them, but free uningested bodies show no such changes in a comparable time span.

Effects of Ribonuclease Digestion and Acid Extraction.—

Methyl green when employed as prescribed, with certain precautions, (23, 39, 46, 47, 51) may be selective for DNA in tissues, but it is not always dependably specific. Under certain conditions it is apparently capable of staining RNA (47). To examine the possible influence of non-specific staining of nucleic acids, methyl green binding before and after ribonuclease digestion, and after acetylation, was measured microphotometrically, using ring prepa-

TABLE IV

Mean Amounts of Methyl Green and Feulgen Dye Bound by L.E. Bodies after Various Pretreatments

Successive measurements of methyl green binding of the same lymphocytes and L.E. bodies after the treatments indicated. Free L.E. bodies prepared from dried normal leukocytes according to the method of Davis *et al.* (21) were measured. Different preparations of the same kind were used for the Feulgen measurements.

	Stain	Lymphocytes	L.E. Bodies
(No. measured)		(20)	(19)
No pretreatment	Methyl green	15.5 ± 0.7	10.2 ± 1.1
Ribonuclease	Methyl green	12.5 ± 0.5	7.7 ± 0.9
RNase followed by acetylation	Methyl green	14.4 ± 0.4	12.7 ± 1.3
Ribonuclease	Feulgen	19.2 ± 0.6	20.2 ± 0.4
$\frac{\text{Post-RNase methyl green}}{\text{Methyl green}}$		0.81	0.76
$\frac{\text{Post-acetylated methyl green}}{\text{Post-RNase methyl green}}$		1.15	1.65
$\frac{\text{Feulgen}}{\text{Post-acetylated methyl green}}$		1.33	1.59

rations of Snapper and Nathan (20) and freshly phagocytosed bodies made by the method of Davis *et al.* (21). The results of the latter appear in Table IV. There is a decline in methyl green staining of L.E. bodies in excess of 25 per cent following ribonuclease digestion. Acetylation increases the methyl green uptake of the remaining ribonuclease-resistant nucleic acid by about 65 per cent, and the resultant post-RNase postacetylated methyl green:post RNase methyl green is 1.65. In "ring" preparations it is 1.88. The magnitude of this rise, while less than that of the material charted in Tables I and II, is nevertheless of comparable dimension. Owing to loss of methyl green stainable material resulting from the action of ribonuclease, the Feulgen:postacetylated methyl green ratios are considerably higher than those obtained from measurement of material not so treated. Thus, ribonuclease effects a lowering of methyl green uptake, and the residual stainable nucleic acid, *i.e.* DNA, is masked in

part by protein. However, ribonuclease treatment has no effect on Feulgen staining (Table IV). Extraction of histone was proposed by Kurnick (39, 51) as prerequisite to quantitation of the methyl green stain, on the assumption that this basic protein was the sole or principal competitive substance interfering with methyl green binding in nuclei. To this end methanol-fixed smears of lymphocytes and free L.E. bodies were exposed to dilute (0.01 M) HCl after their methyl green uptake had been measured, and were then restained and remeasured (Table V). After acid extraction lymphocyte nuclei displayed a small (about 5 per cent) increase in amount of methyl green bound; L.E.

TABLE V

The Effect of Extraction with Dilute Acid on the Concentrations of Methyl Green Bound by L.E. Bodies

Successive measurements of methyl green binding by lymphocytes and free (non-phagocytosed) L.E. bodies prepared from dried normal leukocytes according to the method of Davis *et al.* (21) before and after extraction.

	Lymphocytes	L.E. bodies
(No. measured)	(20)	(20)
No pretreatment	0.32 ± 0.02	0.23 ± 0.02
0.01 N HCl for 20 hrs	0.33 ± 0.01	0.23 ± 0.02
$\frac{\text{Methyl green after acid}}{\text{Methyl green}}$	1.06	0.99

bodies showed none. Masking of stainable groups in DNA by histone would thus hardly seem to be an important factor governing methyl green binding in this material. Extraction of all nucleic acids completely extinguished methyl green staining, even after extracted preparations were deaminated by acetylation, indicating that even acidified proteins accept no methyl green in this material.

Controlled Dye Binding.—

Determination of the pH at which the basic thiazine dye methylene blue and the acid azo dye orange G are no longer capable of being bound to any structure, and the apparent extent of their uptake at various hydrogen ion concentrations sometimes affords useful qualitative information concerning the nature of the substances present, or their associations (25, 26, 45, 55).

The extinction point was taken to be that at which coloration could not be detected with the aid of a complementary filter. Methylene blue staining of leukocyte nuclei was extinguished at pH of about 4.0; that of most free L.E. bodies was no longer apparent at pH 5.0. Orange G staining of unextracted nuclei could hardly be detected at pH of 2.0; coloration of some L.E.

bodies was noted at 2.9, above which no orange G staining could be detected. The L.E. bodies, both free and uningested, were distinctly metachromatic (reddish violet) after methylene blue staining down to pH 5.0; nuclei were orthochromatic (blue). The metachromasy was not abolished by pretreatment with ribonuclease, or by extraction for 3 hours in a mixture of equal parts of methanol and chloroform. Removal of all nucleic acids by hot trichloracetic acid abolished metachromasia, and raised the methylene blue extinction point to 6.8. Treatment with deoxyribonuclease abolished all basophilia up to pH 6.5.

The higher methylene blue extinction point exhibited by L.E. bodies as compared with nuclei probably reflects the competitive influence of cationic groups of the protein in the former, which is evidently greater than in leukocyte nuclei. The greater number or activity of protein basic groups in L.E. bodies can also be inferred from the methyl green binding experiments. Basic dye staining below pH 5.0 is attributable to those phosphate groups of nucleic acid that are accessible; staining at higher pH, as that seen after extraction of nucleic acids, is due to protein acidic groups, presumably carboxyl radicals. The staining of L.E. bodies by orange G at higher pH than that of nuclei is further evidence of the greater numbers of available protein basic groups in the L.E. bodies.

Metachromasia in the L.E. bodies is probably not due to acidic lipide, nor is it readily explainable by the presence of acid mucopolysaccharide. The data do not permit a definite conclusion as to whether their nucleic acid is responsible for the observed metachromasy.

DISCUSSION

Apart from considerations of the amounts of material that can be made available for analysis, cytochemistry, because it permits selection and treatment of individual cells and units, affords a practically feasible approach to an examination of the composition of L. E. and hematoxylin bodies. Although quantitative determination in cytophotometry is generally limited by the number of usable color-developing reactions that have been worked out, methods for the colorimetric demonstration of nucleic acids have been given most careful study (27, 46, 51, 55–58 *inter alia*). The L.E. body itself affords a most favorable object for microspectrophotometric measurement: it can usually be obtained in regular geometrical form (circular discs) in smears; it is almost perfectly homogeneous, thus eliminating distributional error arising from dispersal of chromophore packets (27–29), its optical densities with the color tests employed fall within those limits (about 0.2 to 1.2) prescribed for accurate measurement and for avoidance of errors due to stray light (27, 29, 58).

The factors concerned in basic dye binding, in particular the binding of methyl green to nucleic acid are more complex than were envisaged when the dye was first proposed (23, 39) for quantitative cytophotometric use. The in-

fluences of fixation (46, 51, 56) degree of polymerization (23, 34) or altered steric configuration (34, 38–42), electrostatic forces dependent upon molecular size (59), pH (45, 51), ion competition (45, 46), and associated protein (46–50) on the uptake of dye by nucleic acid have become increasingly well known. Methyl green can be used as a selective stain for DNA only when all these factors governing the dye equilibrium are controlled or accounted for. Chief among these, other conditions being constant, is the relationship of nucleic acid to protein. To conclude, however, that for quantitative use there are too many variables to give a reliable photometric estimate of the amount of DNA (46, 56) is in effect to discard much useful information. On the contrary, our knowledge of the principal influences that condition quantitative methyl green binding, and our ability to alter them experimentally can be exploited microspectrophotometrically to yield data on the relationship of DNA with protein and the nature of the latter, and on the changes in the nucleoprotein complex in the cell cycle (50, 60). The presently reported experiments show that following the conversion of nuclei which takes place in lupus, methyl green uptake by DNA is markedly impaired owing to augmented protein interference. The restoration of methyl green stainability and of the nuclear methyl green: Feulgen ratio which is observed when protein competition is counteracted, indicates that there has been no cytochemically demonstrable change in the molecular configuration of DNA or its degree of polymerization. About half of the methyl green-stainable groups of the DNA present in classical L.E. bodies are covered or masked by protein groups which are either not present or are in different relation to DNA in the nuclei from which the L.E. bodies originate. That this protein is not histone is apparent from the extraction experiments. Indeed, although it might be expected that these basic proteins would be chiefly responsible for competition with methyl green in nuclei (35, 51), and it has been stated that ". . . proteins, other than the basic histones and protamines . . . are not bound by salt linkages with the phosphoric acid groups (*i.e.* they are bound at other sites) and therefore do not compete with methyl green" (51) this has not been found to be the case experimentally. In interphase nuclei in methanol- or formalin-fixed material histone appears to be less effective in depressing methyl green binding than protein of the higher type, such as chromosomal residual protein (50). Basic staining of nucleic acid in tissue exposed to fibrinogen and other proteins (46, 61 *inter alia*) is reduced or abolished. When used under controlled conditions it is likely that variations in methyl green binding mainly involve changes in amount or kind of adjacent proteins or in their relationship to DNA. There are reasons for doubting that the methyl green:Feulgen ratio can be used to reveal major changes in polymerization of DNA in fixed tissues (46, 47) although possibly some changes in steric configuration might be indicated if reduced methyl green affinity could not otherwise be accounted for.

The possibility that the decline of methyl green staining in the lupus bodies that results from incubation with ribonuclease might not be attributable solely to loss of stainable nuclear RNA was considered. Although the amount of RNA in cell nuclei is not known with certainty (62) the RNA of human leukocyte nuclei probably does not exceed 5 per cent of the nuclear nucleic acid if they are analogous to similar cells of other species (63). There is nearly a 25 per cent drop in methyl green staining of ribonuclease-treated nuclei. In order to ascertain whether ribonuclease treatment permitted a persistent intracellular deoxyribonuclease to act, a phenomenon like that encountered by several authors (64–66) in fixed tissues, microphotometric measurements of enzyme–treated and control Feulgen-stained preparations were made. Since these showed that the amounts of DNA were unaffected by ribonuclease treatment, it may be that depression of methyl green stainability was due to loss of stainable RNA, and possibly to the influence of basic protein groups formerly combined with RNA. However, were nuclei and bodies to adsorb enzyme protein during incubation in ribonuclease solution, their binding of methyl green could be depressed in the manner observed here.

Metachromasia with thiazine dyes occurs in the presence of chromotropes of relatively high molecular weight having an acidic function. The metachromasy exhibited by the L.E. bodies stained with thiazine dyes was unexpected since the normal nuclei were not metachromatic. It might be analogous to the metachromasy observed by Carnes *et al.* (67) in nuclei of fresh cells suspended in dilute toluidine blue, or to that seen in stained sections of frozen dehydrated material.

The specific nuclear change that is brought about in susceptible cells by the factor of lupus erythematosus is quite unique, and cytochemically does not resemble other types of regressive change. In pyknosis, for example, there is condensation of chromatin with increased methyl green basophilia of DNA (68, 69) consequent on loss of non-histone protein (40, 68), while karyolysis involves concomitant loss of the entire nucleoprotein complex. With respect to DNA the formation of the L.E. body entails neither loss nor detectible alteration in its state or degree of polymerization. This may not preclude the occurrence of some steric rearrangements in the DNA molecule, but there are presently no cytochemical means of discovering these. Rather, with the disintegration of structural chromatin in the lupus transformation, a large proportion of the DNA-binding sites become preempted through augmented combination or association with protein in manner and amount quite different from that of the original nuclei. It is evident that there is no necessary reason for supposing, as has been done in the past (4–6, 11–14, 18, 19) that the destructive nuclear process in lupus acts primarily or especially on the DNA. Rather, the data compel attention to the alterations in the protein moiety of the altered nucleoprotein and their relation to the nucleic acid, and not

to changes in the nucleic acid itself. With engulfment of the L.E. body by phagocytes, its deposition in the tissues, or with time, secondary lytic changes, which may have nothing to do with the pathogenesis of the lupus transformation of nuclei itself, ultimately take place. Deoxyribonucleodepolymerase might then participate in this degradation process. A distinction has therefore been made in this study between free L.E. bodies and bodies which had been incorporated into viable phagocytes for periods sufficiently long to allow their digestion.

SUMMARY

The composition, with respect to nucleic acids, of the L.E. bodies resulting from the action of the plasma of patients with systemic lupus erythematosus on substrate leukocyte nuclei in different kinds of preparations was compared microspectrophotometrically with that of control lymphocyte nuclei. Binding of the basic dye methyl green to DNA was uniformly found to be depressed in L.E. bodies as compared with control nuclei. Since Feulgen-revealed DNA, which served as a standard of reference, was relatively unchanged in amount, the Feulgen:methyl green ratios of L.E. bodies were higher than those of lymphocyte nuclei. Acetylation, which covers basic groups of proteins, was found to increase methyl green uptake by DNA of L.E. bodies to values approximating those of control nuclei, with consequent revision, after acetylation of the Feulgen:methyl green ratios of L.E. bodies to values similar to those of lymphocyte nuclei. Ribonuclease was found to reduce methyl green staining; extraction with dilute acid had little effect. These data have been interpreted to indicate (*a*) the presence in L.E. bodies of DNA-associated proteins whose basic groups compete with the cationic dye for binding sites of DNA and so inhibit methyl green staining, and (*b*) the DNA itself is not detectibly altered in state or degree of polymerization. Photometric comparison of the mean Feulgen-stainable DNA content per L.E. body with that of control nuclei showed that DNA is not lost in the L.E. transformation of nuclei.

The authors are grateful to Professor I. Snapper and Dr. D. Nathan of the Beth El Hospital, Brooklyn, for generously providing L.E. preparations made with the "ring method" originated by them, to Dr. S. Lee of the Mount Sinai Hospital, New York for making available instructions and preparations of the "atabrine" method, and to Drs. B. Davis and W. Mautner for details of the dried substrate method. The authors are greatly indebted to Dr. Paul Klemperer for his valuable advice and unfailing encouragement.

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EXPLANATION OF PLATES

PLATE 60

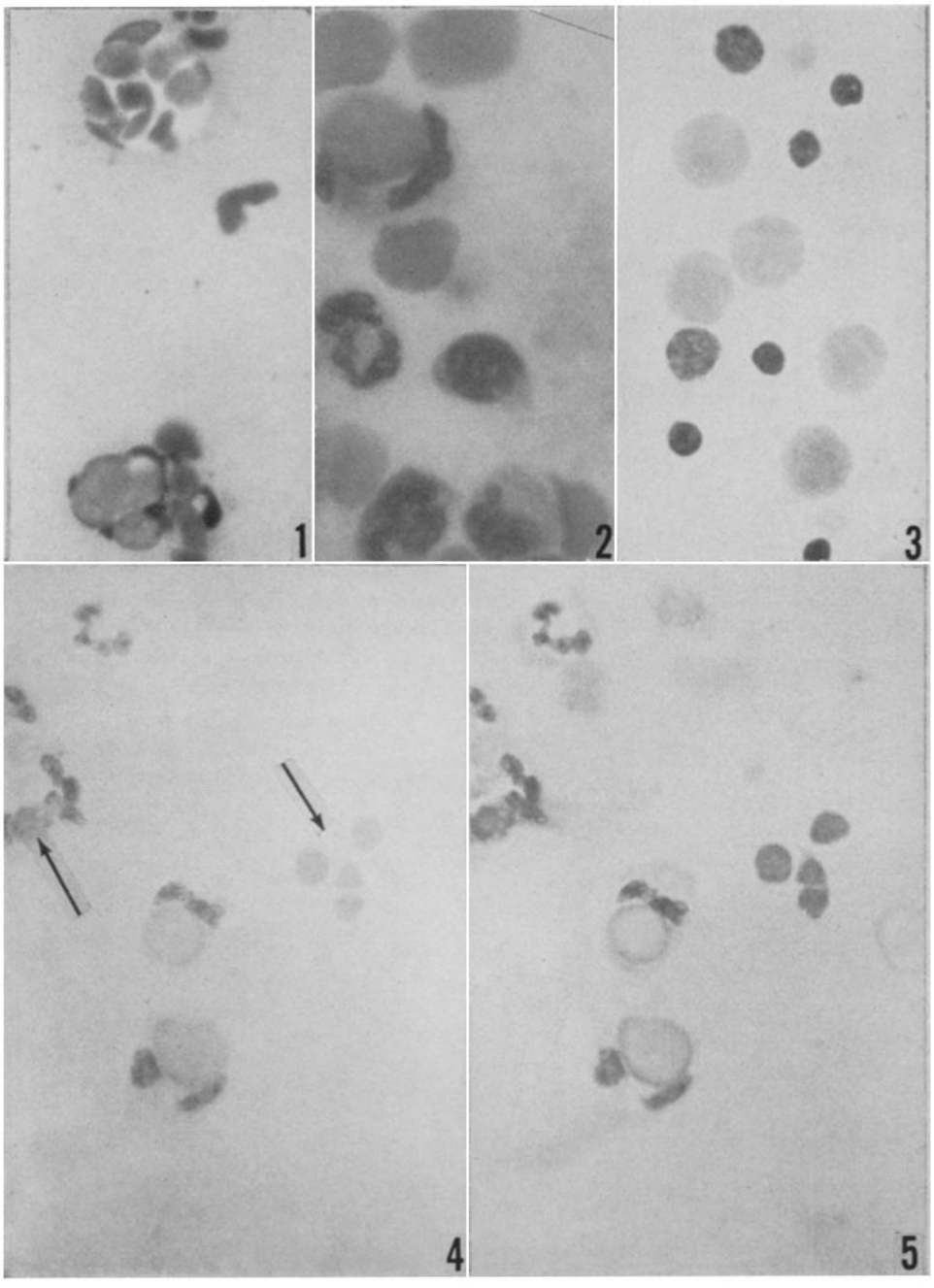
FIG. 1. Rosettes and free (non-phagocytosed) L.E. bodies prepared according to the method of Snapper and Nathan (20). The geometrically regular bodies were chosen for measurement of amounts. Wright's stain. × 900.

FIG. 2. L.E. cells, free L.E. bodies, and polymorphonuclear leukocytes in a preparation made from dried substrate leukocytes according to Davis *et al.* (21). Wright's stain. × 1300.

FIG. 3. L.E. bodies and lymphocytes in a preparation made from the lymphocytes of a patient with chronic lymphatic leukemia, using the atabrine method of Lee (22).

FIG. 4. L.E. cells and free L.E. bodies (arrows) in a dried substrate preparation (21) stained with methyl green. Methyl green, red filter. × 1300.

FIG. 5. Same field as that shown in Fig. 4 after acetylation and restaining with methyl green. There is intensification of staining in the free L.E. bodies and inclusions. Methyl green, red filter. × 1300.



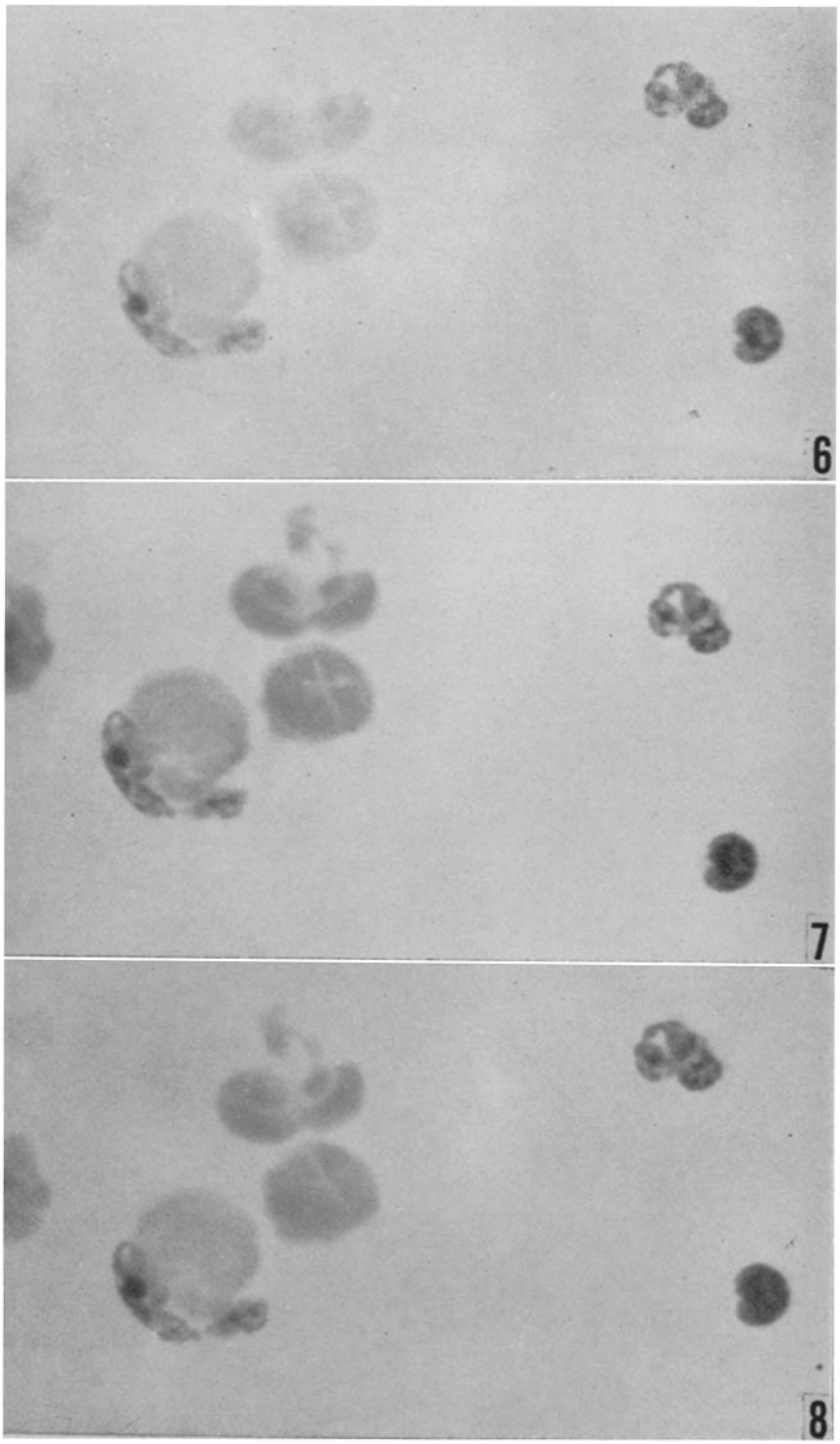
(Godman and Deitch: Systemic lupus erythematosus. I)

PLATE 61

FIG. 6. L.E. cell, free L.E. bodies and leukocytes in a dried substrate preparation (21) stained with methyl green. Red filter. × 1300.

FIG. 7. Same field as that shown in Fig. 6 after acetylation and restaining with methyl green. The enhancement of staining, especially in the L.E. bodies is evident Red filter. × 1300.

FIG. 8. Same field stained by the Feulgen reaction. Green filter. × 1300.



(Godman and Deitch: Systemic lupus erythematosus. I)