THE RELATIONSHIP OF THE "*SPHERE CHROMATOPHILE*" TO THE FATE OF DISPLACED HISTONES FOLLOWING HISTONE TRANSITION IN RAT SPERMIOGENESIS

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ABSTRACT

Cytochemical, radioautographic, and microspectrophotometric studies bearing on the relationship of histone transition to the origin and development of the protein and RNA components of the "*sphère chromatophile*" in the developing spermatogenic cells of the albino rat are presented. These studies show that the *sphère chromatophile* has many features in common with somatic nuclei: it contains histonelike basic proteins rich in lysine, with lesser amounts of arginine. No evidence is found for the presence of a protamine in this granule. The *sphère chromatophile* is rich in RNA, but contains no DNA. The failure of a positive reaction with basic protein stains, unless the RNA is first removed, indicates either a chemical bonding or a very close association between the RNA and basic protein. The basic protein and RNA components of the *sphère chromatophile* appear to have different origins in the cell. A sequence of stages in the development of the lysine-rich basic protein component of this structure commences with the appearance of tiny grains in those spermatid nuclei which are beginning to replace lysine-rich histones with arginine-rich histones. Subsequently, similar-staining cytoplasmic grains appear, which coalesce to form the *sphère chromatophile* in the cytoplasm. Labeling studies show that the basic protein component is synthesized at about the time of the last premeiotic DNA (and histone) synthesis. The results of the microspectrophotometric measurements support the idea that the basic protein lost from the spermatid nucleus is the source of the basic protein in the *sphère chromatophile*.

INTRODUCTION

Friedrich Miescher (1871, 1897) and Albrecht Kossel (1884) reported that protamines could be recovered from mature salmon sperm, but that histones are present in the immature testis. Alfert (1956), using his alkaline fast green staining technique (Alfert and Geschwind, 1953), conducted the first cytochemical studies on the *individual* testicular cells and found that the replacement of somatic histone by arginine-rich protamine occurs in the elongated spermatid. Such a histone transition has since been the subject of considerable study (Alfert, 1957, 1958; Bloch and coworkers, 1960, 1962 to 64; Das et al., 1964 *a,b*; Monesi, 1964 *a*, and others). It is not surprising that emphasis has been on the nature of the newly synthesized histones, since these remain with the sperm nucleus at least up to the time of fertilization. The fate of the displaced histones has received no attention whatever. Some evidence has now accumulated

(Vaughn, 1965 *a,b*) which indicates that these may aggregate in the cytoplasm during rat spermiogenesis, where they persist for many days in the form of a discrete nucleoprotein body, called the "*sphère chromatophile*": a component of the residual body formed by the maturing sperm.

Near the end of stage VIII (see Fig. 1 for designation of stages), the mature spermatozoa are released into the lumen of the seminiferous tubule; the bulk of the cytoplasmic elements are sloughed off (Brown 1885; Regaud 1901 *a,b*), resulting in residual cytoplasmic bodies (see structure *RB* in Fig. 1), which are often phagocytosed by Sertoli cells (Regaud, 1901 *a,b*; Kingsley-Smith and Lacy, 1959; Lacy, 1960). Regaud showed that each residual body contains a large safranophilic mass, the *sphère chromatophile*. This structure contains RNA (Daoust and Clermont, 1955; Kingsley-Smith and Lacy, 1959); protein (Brown, 1885; Sud, 1961); and glycogen (Firlit and Davis, 1965). The granule arises, at least in part, from a fusion of the numerous RNA-containing spermatid granules (Regaud, 1901 *a*; Daoust and Clermont, 1955; Sud, 1961), first reported by von Ebner (1888), and subsequently referred to as the von Ebner granules. Sud (1961) reported that the *sphère chromatophile* does not stain with the alkaline fast green method for basic proteins, but that it does stain with acid dyes and with the Sakaguchi reagent for arginine. Surprisingly, he concluded from these observations that this structure contains no histones, but rather protamines.

Because of the relatively small amount of von Ebner and *sphère chromatophile* granular material present in the testis, the isolation, extraction, and chemical characterization of these bodies would be quite difficult. The purpose of the experiments presented here is to describe certain cytochemical, radioautographic, and microspectrophotometric data which bear on the problem of the fate of the displaced somatic histones following histone transition.

MATERIALS AND METHODS

PREPARATION OF MATERIAL: Male albino rats (*Rattus norvegicus*, var. *albinus*) were killed by a sharp blow to the head. The testes were cut into small pieces and fixed either in Carnoy's fixative (3:1 ethanol–acetic acid) for 3 hr, or in 4% formaldehyde at pH 7.0 for 12 to 18 hr. Formaldehyde-fixed material was rinsed overnight in running tap water, dehydrated in a graded ethanol series, cleared in benzene, and em-

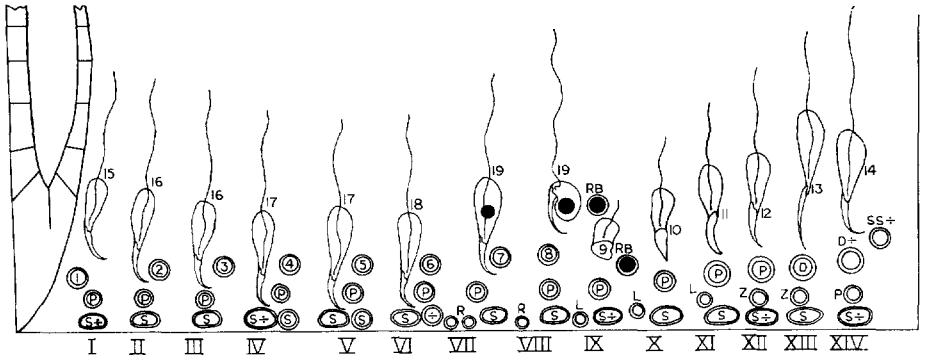


FIGURE 1 Schematic longitudinal section through one cycle of a rat seminiferous tubule. Roman numerals denote stages of the cycle of the seminiferous epithelium; Arabic numerals, stages of spermiogenesis. The "stages" are those described by Leblond and Clermont (1952 *a, b*). Note that spermatids at a given stage of their development are always associated with spermatogonia and spermatocytes at characteristic stages of their own development.

S and *S*÷, spermatogonia and dividing spermatogonia
R, resting primary spermatocytes
L, Z, P, leptotene, zygotene, and pachytene primary spermatocytes, respectively
D, diplotene primary spermatocytes
D÷, diakinesis and first meiotic division in primary spermatocytes
SS÷, secondary spermatocytes and second meiotic division
1–19, spermatids in spermiogenesis
RB, residual bodies; the dark structure within each one is the *sphère chromatophile*

For simplicity, Sertoli cells are not shown.

bedded in paraffin. Carnoy-fixed material was rinsed for 1 day in several changes of 95% ethanol, passed through absolute ethanol, benzene, and embedded in paraffin. Sections 2 to 3 μ in thickness (radioautography) or 5 μ (cytochemistry and microspectrophotometry) were mounted on albuminized slides.

STAINING PROCEDURES: Unless otherwise stated, both formaldehyde- and Carnoy-fixed materials were employed. Nucleic acid staining reactions used were the Feulgen method for DNA (Feulgen and Rossenbeck, 1924; as modified by Bloch and Godman, 1955), and azure B, which was used only on Carnoy-fixed material (Flax and Himes, 1952). Protein stains employed were fast green at pH 2.0 (Swift, 1959), and naphthol yellow S (Deitch, 1955) for proteins in general; fast green at pH 8.1 (Alfert and Geschwind, 1953), and bromphenol blue at pH 2.3 (Bloch and Hew, 1960) for basic proteins; the Sakaguchi reaction for protein-bound arginine (Sakaguchi, 1925; as modified by McLeish et al., 1957, and Deitch, 1961), and the dinitrofluorobenzene reaction for protein-bound lysine (Sanger, 1945; Danielli, 1950, 1953; as modified by Bloch et al., 1964). Polysaccharides were stained by the method of Hotchkiss (1948). Sections to be stained by the Feulgen-alkaline fast green method were treated as follows.

1. Bring slides to water.
2. Hydrolyze (12 min if Carnoy-fixed; 25 min if formaldehyde-fixed) in 1 N trichloroacetic acid at 60°C.
3. Stain in trichloroacetic acid–Schiff's reagent for 45 min at room temperature.
4. Three 5 min rinses in sulfite bleach (prepared with trichloroacetic acid).
5. Three 10 min rinses in 70% ethanol. Then rinse in water.
6. Stain for 30 min in pH 8.1 fast green, 0.1% aqueous solution.
7. Differentiate for 5 min in two rinses of absolute methanol.
8. Clear in xylene and mount in Permount, (Fisher Scientific Company, Pittsburgh, Pennsylvania).

Results: DNA stains deep magenta; stable non-DNA-associated histonelike proteins stain green.

BLOCKING OF LYSINE: Deamination was carried out according to Van Slyke (1911), with minor modifications. Alternatively, material was acetylated according to the method of Monné and Slautterback (1951), with slight modifications.

EXTRACTION OF RNA: RNA was removed by treatment of the sections for 5 hr with 10% perchloric acid at 20°C (Woods, 1959), or by treatment with 0.02% ribonuclease (Worthington Corporation, Freehold, New Jersey) at pH 6.5, 37°C for 2 hr (Swift, 1959).

RADIOAUTOGRAPHY: Tritiated thymidine (Schwarz Bioresearch, Mount Vernon, N. Y. 6.6 c/mmole, 1 mc/ml; Schwarz, 3.0 c/mmole, 1 mc/ml; Schwarz, 1.9 c/mmole, 1 mc/ml) was used as a precursor of DNA; tritiated lysine (Nichem. Inc., Bethesda, Md. 0.71 c/mmole, 5 mc/ml) was used as a precursor of protein; tritiated cytidine (Schwarz, 1.0 c/mmole, 1 mc/ml) as a precursor of RNA (with suitable control experiments with ribonuclease). The appropriate isotope was diluted with 0.9% NaCl, and injected into the posterior peritoneal cavity, in a single dose. The animals were then maintained in the laboratory for periods ranging from 30 min to 864 hr (36 days). The radioactive tissue sections were stained by the periodic acid–Schiff method of Hotchkiss (1948), occasionally preceded by 1 N HCl hydrolysis as for the Feulgen method, covered with Kodak AR 10 stripping film at room temperature (about 24°C), dried for several hours in a current of clean air, and stored in sealed black plastic boxes containing desiccant (Drierite), in the refrigerator. The slides were developed in Kodak D-19 developer for 6 min, cleared in Kodak fixer for 15 min, and rinsed for 1 hr in several changes of distilled water, all at 18°C (at this point occasional slides were stained through the emulsion with pH 4.1 toluidine blue, according to Prescott and Bender (1962)), and then either air-dried, or dehydrated through graded alcohols, cleared in xylene, and mounted in Shillaber's refractive index oils or Permount.

MICROSPECTROPHOTOMETRY: Microspectrophotometric readings were carried out on a Canalco Ultramicrospectrophotometer, using a Zeiss microscope. The plug method of Swift (1950) was utilized in the determination of the ratios of lysine:DNA and lysine:RNA. To minimize the possible sources of error, once an optical plug had been established through a cellular structure and the light advanced to a spot on the phototube, the readings at both wavelengths were made without moving this optical plug or the spot employed on the phototube. Background was then recorded for each wavelength (again, without changing the location of the spot on the phototube from its previous position). The two wavelength measurements were made by using the standard methods of Patau (1952) and Ornstein (1952).

As Swift (1950) has shown, in the classical case of a homogeneous, spherical-shaped nucleus with an optical plug passing through its center, the amount of DNA present is given by:

$$\mathrm{DNA}_{\mathrm{nucleus}} = \tfrac{2}{3} KE\pi r^2 = \tfrac{2}{3} KEA$$

where K = extinction coefficient, E = the extinction, A = area of a section passing through center of sphere, r = radius of the sphere.

In developing spermatid nuclei, however, one encounters a number of irregular shapes, and the classical formula does not apply. In the dinitrofluorobenzene-Feulgen analyses of developing spermatid nuclei, the following relationship was employed:

$$\text{Amount lysine in nucleus } N = \frac{K_{\text{lysine}}}{K_{\text{DNA}}} \times \frac{E_{\text{lysine}N}}{E_{\text{DNA}N}} \times \text{Amount DNA in nucleus } N.$$

The amount of DNA in the rat nucleus has been measured biochemically by Cunningham et al. (1951) in diploid thymus and spleen nuclei, and found to be 6.1×10^{-12} g. Therefore, a haploid spermatid nucleus would contain 3.05×10^{-12} g of DNA, or 1.02×10^{-14} moles of DNA nucleotide. Bloch et al. (1964) have worked out the extinction coefficients for dinitrofluorobenzene (DNFB) at 400 mμ and Feulgen at 570 mμ (although the latter is approximate; for a rigorous treatment, it should be independently established for each staining run):

$$K_{\text{DNFB}} \text{ at } 400 \text{ m}\mu = 1.84 \times 10^{-7} \text{ moles}/EA,$$

$$K_{\text{Feulgen}} \text{ at } 570 \text{ m}\mu = 2.3 \times 10^{-7} \text{ moles}/EA.$$

(In these cases, A is in cm^2).

With these values, the amount of lysine in the various nuclei can be calculated.

STAGING OF CELLS: In order to facilitate identification of the various types of spermatogenic cells encountered in the rat testis, we employed the method developed by Leblond and Clermont (1952 *a*, *b*) for the staging of the spermatogenic cycle into some 14 different stages. Three adjacent sections were mounted on three different slides, and the center section stained by the periodic acid–Schiff (PAS) method. This center section was then observed under a dissecting microscope at a magnification of 15, and the position of the tubules was recorded by photographing the section. The slide was then examined under oil immersion, and each tubule was staged according to Leblond and Clermont. The two adjacent sections were stained as desired and the stages of the cells in question on these slides determined by comparing the sections to the PAS-staged photograph.

OBSERVATIONS

Cytochemistry

In cytochemical studies on formaldehyde- and Carnoy-fixed rat testes sections, not only nuclei but also numerous large granules near the lumen of the stage VIII seminiferous tubules stain intensely with the alkaline fast green (Fig. 2) and bromphenol blue method (Fig. 22). After the literature was consulted (Regaud, 1901 *a*; Kingsley-Smith and Lacy, 1959), each of these granules could be readily identified as a *sphère chromatophile* within a residual body of spermatid cytoplasm. Positive results with pH 2.0 fast green and with naphthol yellow S indicate the presence of protein. When deamination or acetylation precedes staining with alkaline fast green or bromphenol blue, these stains no longer bind to this granule (Figs. 3 *a*,*b*). In order to obtain information on the specific amino acids responsible for these results, sections were subjected to the 1-fluoro-2,4-dinitrobenzene method for protein-bound lysine, and also to the Sakaguchi reagent for protein-bound arginine. Both of these staining procedures result in a positive reaction in the *sphère chromatophile*. This granule stains with azure B; however, this staining is prevented by pretreatment with 10% perchloric acid or with ribonuclease. Application of the Feulgen method for DNA gives a negative result. These granules fail to stain with alkaline fast green or bromphenol blue unless the section is first treated with 5% trichloroacetic acid, as in the alkaline fast green method,[1] or with ribonuclease. Apparently the azure B stainable material (RNA) and the stainable basic groups of the protein are very closely associated. The results of these and other cytochemical studies are summarized in Table I.

Development of the "Sphère Chromatophile"

In studying the development of the *sphère chromatophile* serial sections were prepared, one section stained with periodic acid–Schiff reagent (for staging of the cells) and the two adjacent sections stained either with azure B or with the Feulgen-alkaline fast green technique. Homogeneously distributed cytoplasmic RNA (in the form of very finely dispersed grains as revealed by azure B staining) fills the cytoplasm in the elongating spermatids. As spermatid maturation pro-

[1] It is interesting that if sections are hydrolyzed for 15 min at 90°C in 5% trichloroacetic acid (as in the alkaline fast green method), fast green stainability of the *sphère chromatophile* is limited to the large bodies of stage VIII. That this limitation is due to loss of stainable material is shown by the fact that in many sperm at stages *earlier* than stage VIII (see Figs. 6 and 8) cytoplasmic granules stain green by the Feulgen-alkaline fast green method (which employs only short 60°C hydrolysis in 1 N trichloroacetic acid) and by the picric acid–bromphenol blue method for basic proteins.

TABLE I

Cytochemistry of Mature Stage VIII Sphère Chromatophile

Staining procedure	Reactive material	Results
Naphthol yellow S or pH 2.0 fast green	Proteins in general	S
Alkaline fast green (acid hydrolysis)	Basic proteins	S
Picric acid–bromphenol blue	Basic proteins	S
Alkaline fast green or bromphenol blue (after ribonuclease treatment)	Non-DNA-associated basic proteins	S
Alkaline fast green or bromphenol blue (after Feulgen staining)	Non-DNA-associated basic proteins	S
Alkaline fast green or bromphenol blue (after deoxyribonuclease treatment)	DNA-associated basic proteins	NS
Alkaline fast green or bromphenol blue (without prior acid hydrolysis and also without ribonuclease or deoxyribonuclease)	Non-nucleic acid-associated basic proteins	NS
Alkaline fast green or bromphenol blue (following deamination or acetylation)	Basic proteins, rich in arginine	NS
1-Fluoro-2,4-dinitrobenzene	Protein-bound lysine	S
Sakaguchi reaction	Protein-bound arginine	S
Azure B	Nucleic acids	S
Azure B (following ribonuclease)	DNA	NS
Azure B (following deoxyribonuclease)	RNA	S
Feulgen Reaction	DNA	NS

In all the above procedures, both Carnoy and formaldehyde fixations were employed, except for the azure B sections, where only Carnoy fixation was used. An S result denotes binding of the dye; NS denotes no detectable binding.

ceeds to stages 17 to 18 of spermiogenesis, fewer finely dispersed cytoplasmic RNA grains and more of a larger variety of RNA granules (von Ebner's granules) appear in the cytoplasm. Still later, by early stage 19, most of the cytoplasmic RNA is localized in the *sphère chromatophile*, although the cytoplasm also contains von Ebner granules. The *sphère chromatophile* moves toward the basement membrane during stages IX to X and soon loses its azure B stainability (Figs. 4 to 14).

The results of the Feulgen-alkaline fast green study show the first stainable cytoplasmic structures evident only at about early stage 19 (Fig. 6); prior to this stage, no alkaline fast green stainable granules are present in the elongate spermatid cytoplasm. The von Ebner granules, for example, do not stain, confirming Sud's earlier report (1961). From early stage 19 onward, the development of the alkaline fast green stainable *sphère chromatophile* is identical with that of the azure B stainable component, except that the von Ebner granules do not stain with alkaline fast green (Figs. 4 to 14). Note especially that cytoplasmic binding of alkaline fast green commences at a much later time in the developing spermatid than does azure B stainability of the multiple von Ebner granules.

Adjacent sections were also used to facilitate identification of stages in the case of picric acid–bromphenol blue staining. The nuclei of elongating stage 13 spermatids contain numerous tiny intensely staining grains; the cytoplasm does not stain. This is the stage during which histone transition begins (see later observations and also Discussion). Stage 14 spermatid nuclei are homogeneously stained, and no nuclear grains are to be seen. However, the cytoplasm shows a few intensely staining grains, usually quite near the nucleus. During stages 15 to early 16, a large number of such grains is scattered throughout the cytoplasm of the elongated spermatids. By stage 17, the bulk of these grains is present in the posterior spermatid cytoplasm; few grains appear elsewhere in the cytoplasm. In this region, during stages late 18 to early 19, the *sphère chromatophile* first appears. Many tiny grains surround this larger granule. By stage VIII, only the larger granule is seen. These results are shown in Figs. 15 to 24. When alkaline fast green or picric acid–

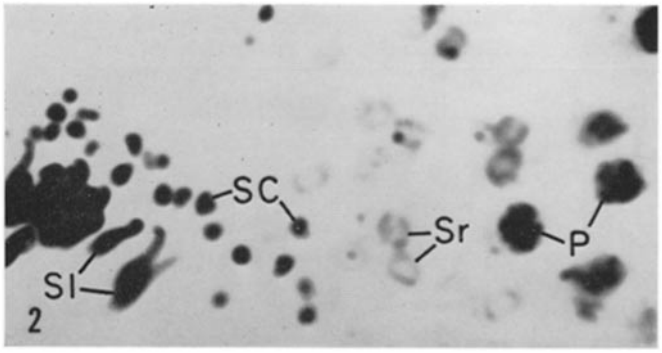
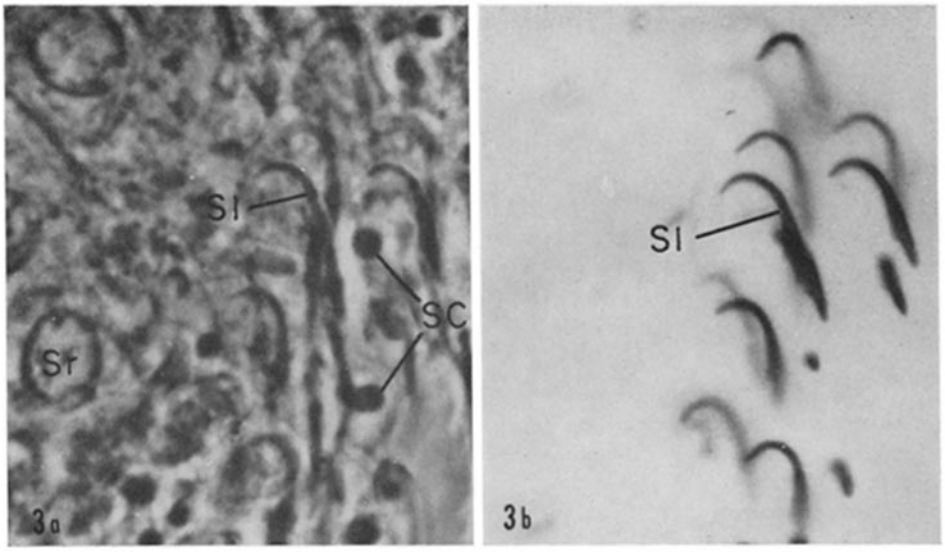


FIGURE 2 Bright-field photomicrograph, stage VIII, showing a small area of a sectioned rat seminiferous tubule. All nuclear types: pachytene primary spermatocytes (*P*), round spermatids (*Sr*), and elongate spermatids (*Sl*), as well as the *sphère chromatophile* bodies (*SC*), bind the dye. Alkaline fast green technique; formalin fixation. × 1000.



FIGURES 3 *a* and 3 *b* Phase-contrast and bright-field photomicrographs, stage late VII, showing a small area of a sectioned rat seminiferous tubule. Only the elongate spermatid nuclei (*Sl*) are stained. Round spermatids (*Sr*) and *sphère chromatophile* bodies (*SC*) do not stain. Alkaline fast green after deamination technique; Carnoy fixation. × 3000.

bromphenol blue staining is preceded by deamination or acetylation, elongate spermatid nuclei prior to stage 13 do not stain. Stage 13 nuclei stain very faintly. More stain is retained in stage 14 nuclei, and later nuclei remain deeply stained; no cytoplasmic granules stain.

An experiment was designed to put these observations on a semiquantitative basis. A spectral absorption curve, determined with the microspectrophotometer, showed an absorption maximum of 620 mμ for alkaline fast green–stained nuclei. The positions of stained nuclei somewhat separated from nearby nuclei were noted at each stage between 12 and 19. The amount of fast green in these nuclei was then determined, using the two wavelength method. A rectangular field of light was directed to the phototube. The data are given in Table II. The reason for the apparent decrease in the amount of dye bound after stage 16 is not known. However, it may be due to the obvious metachromasy of the stain in this material, which may, in turn, be due to physical changes within the spermatid nuclei themselves. It is interesting that these data also show that synthesis of the arginine-rich histones (or their stable complexing to DNA) begins at stage 13 and ends at about stage 16, a period of about 66 hr (estimated from Clermont et al., 1959). Ap-

parently, histone transition is a rather drawn-out process here. Monesi (1964 *a*) has found, in the mouse, that incorporation of arginine-H^3 into spermatid arginine-rich histones also occurs over several stages of the cycle.

Autoradiography

In the thymidine-H^3 experiments, prepared in order to time spermatogenesis, animals were killed at ½, 2, 20, 76, 140, 402, and 864 hr after injection. The doses of radioactive tracer administered were 1 μc/g of rat in all but the last two, where the dosage was 2 μc/g. The two shortest times show that the last premeiotic DNA synthesis, as revealed in the radioautographs, (and presumably histone synthesis as well, see below) occurs in the "resting" primary spermatocytes at stages VIII through early IX. Two hours after injection of the isotope, 96% of all stage VIII resting primary spermatocyte nuclei is labeled. After 864 hr, this thymidine label is found in the greatly elongated nuclei of early stage VIII spermatids (which have each formed a large cytoplasmic *sphère chromatophile*). The results of these timing experiments are in good agreement with those of Clermont et al. (1959), who used thymidine-H^3 to elucidate the duration of each of the 14 stages of the cycle.

In the lysine-H^3 experiments, injected animals were killed after similar periods. The doses administered were 2 μc/g of rat in all but the last experiment, where the dosage was 8 μc/g. In none of these experiments, except the last, are silver grains observed above the *sphère chromatophile*. In the 402 hr experiment, grains are commonly observed lying over the residual body around the edges of the *sphère chromatophile*, but not over the latter. Incorporation into surrounding nuclei and cytoplasm of many cell types shows that lysine is available for incorporation into protein. In the 2 hr lysine experiment, the resting primary spermatocyte nuclei at stages VIII and early IX are heavily labeled; supposedly, some of this labeling is due to incorporation into newly synthesized histones, in conjunction with the DNA being synthesized at this time. After 864 hr, the radioautographs show heavy incorporation of lysine-H^3 over the *sphère chromatophile*, with scarcely any silver grains lying over the remainder of the residual body. These results are shown in Figs. 25 to 31.

In the cytidine-H^3 experiments, injected animals were killed at ½ hr after injection. The dose of tracer administered was 5 μc/g. The radioautographs show no incorporation over the *sphère chromatophile*, although many nuclei and also the cytoplasm of most cell types do incorporate the isotope. This lack of incorporation of an RNA precursor by the *sphère chromatophile* after ½ hr is consistent with results reported by Monesi (1964 *b*), who found that the mouse residual body becomes labeled with RNA precursors only after several days.

TABLE II

Comparison of the Relative Amounts of Nuclear Arginine-Rich Histone in Some of the Spermiogenic Stages

Stage of spermiogenesis	12	13	14	late 15	early 16	16	late 16	early 19	late 19
Stage of the cycle	XII	XIII	XIV	late I	early II	II	early III	early VII	early VIII
Number of nuclei measured	—	—	2	4	3	3	4	4	5
Relative amounts of fast green	None	Trace	27.1	78.8	164.6	101.1	124.3	108.7	76.1
			13.8	32.7	198.2	115.6	128.6	122.2	88.9
				46.3	142.4	130.4	84.6	94.6	84.7
				89.5			105.3	70.8	88.9
									100.9

Microspectrophotometry, Using the Plug Method

A method of measuring the ratio of basic protein to RNA was developed, based on the double-staining technique of Bloch and coworkers. A section of Carnoy-fixed testis was stained by the azure B method for RNA. An adjacent section

was stained by the dinitrofluorobenzene (DNFB) method for protein-bound lysine. These slides were then compared so that the same tubule region would be used in both. Large, well stained, homogeneous-appearing *sphère chromatophile* bodies were selected for determination of spectral absorption curves. The position of the body used for obtaining the DNFB curve was recorded. This DNFB-stained slide was then double-stained with the azure B method for RNA (Flax and Himes, 1952). The same *sphère chromatophile* used in determining the DNFB curve was relocated, and the DNFB–azure B spectral absorption curve was determined. These absorption curves are shown in Fig. 32.

One of two adjacent 3 μ sections of Carnoy-fixed

Figs. 4 through 14 show the seminiferous epithelium of the rat at different stages of the cycle, and are presented in pairs, stained with either the azure B or the Feulgen-alkaline fast green technique, for purposes of comparison. Carnoy fixation; 5 μ sections.

R, resting primary spermatocyte
P, pachytene primary spermatocyte
Sr, early spermatid with spherical nucleus
Sl, later spermatid with elongate nucleus
B, basement membrane of seminiferous tubule
SN, Sertoli cell nucleus
SC, *sphère chromatophile*
V, von Ebner granules

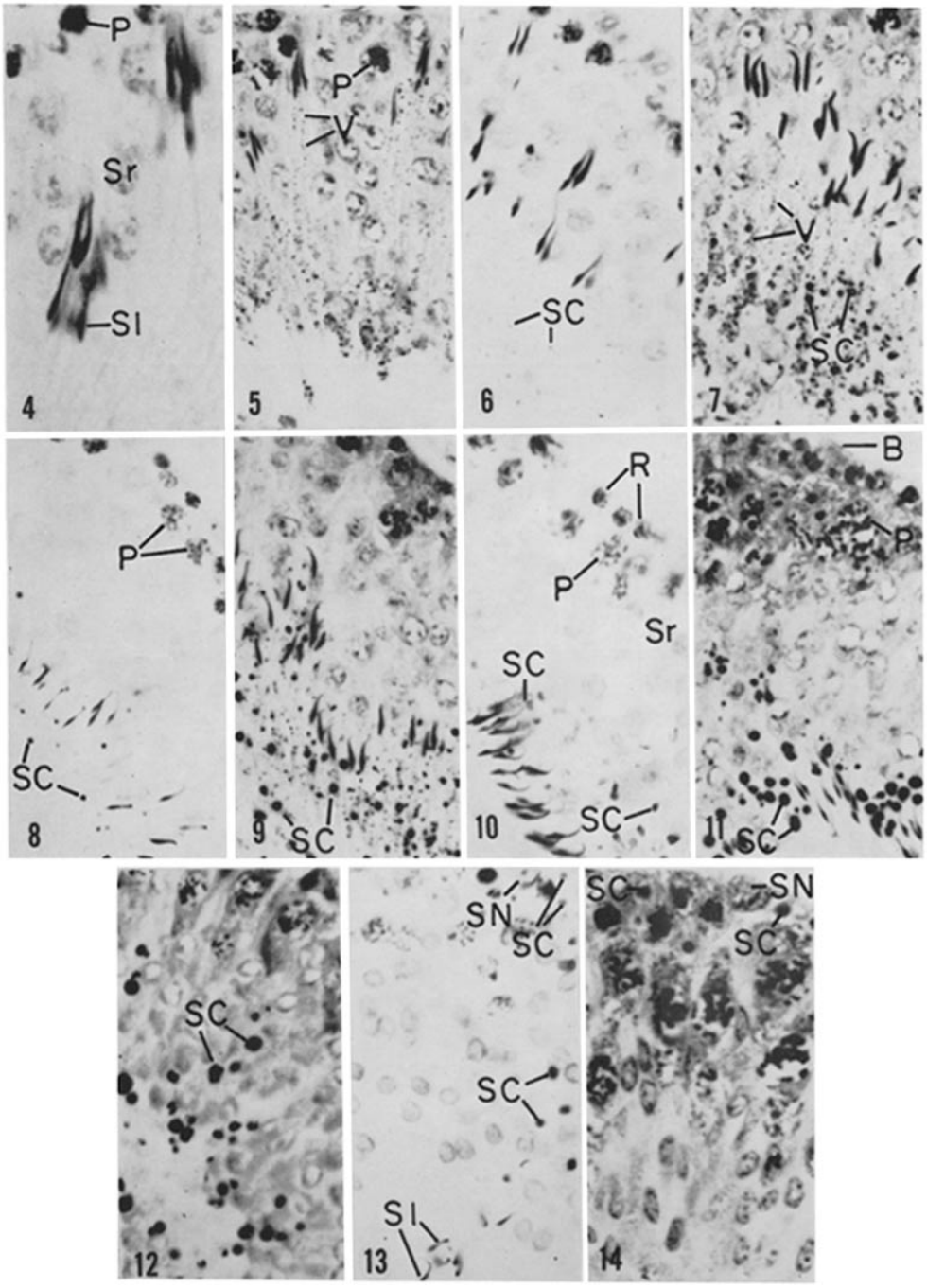
FIGURES 4 and 5 Bright-field photomicrographs, stage VI, showing part of two sectioned tubules. The section of Fig. 4 is stained with the Feulgen-alkaline fast green technique; that of Fig. 5, with azure B. All nuclear types, i.e. primary spermatocytes, round spermatids, and elongate spermatids, shown in Fig. 4 are stained magenta for DNA. No cytoplasmic structures bind fast green; the image of the cytoplasm in Fig. 4 is due to refractility. Von Ebner spermatid cytoplasmic granules are heavily stained in Fig. 5, as are all nuclear types. Fig. 4, × 1000; Fig. 5, × 800.

FIGURES 6 and 7 Bright-field photomicrographs, stage early VII, showing part of two sectioned tubules. The section of Fig. 6 is stained with the Feulgen-alkaline fast green technique; that of Fig. 7, with azure B. In addition to the magenta-stained nuclear types in Fig. 6, the *sphère chromatophile* is the only cytoplasmic structure which stains green for basic protein. In Fig. 7, not only the *sphère chromatophile*, but also the von Ebner granules, stain. The von Ebner granules are not stained in Fig. 6. Fig. 6, × 600; Fig. 7, × 800.

FIGURES 8 and 9 Bright-field photomicrographs, stage VII, showing part of two sectioned seminiferous tubules. The section of Fig. 8 is stained with the Feulgen-alkaline fast green technique; that of Fig. 9, with azure B. The *sphère chromatophile*, larger now, is stained in both sections, and is nearer the elongate spermatid nucleus than at the earlier stages. Fig. 8, × 600; Fig. 9, × 800.

FIGURES 10 and 11 Bright-field photomicrographs, stage VIII, showing part of two sectioned tubules. The section of Fig. 10 is stained with the Feulgen-alkaline fast green technique; that of Fig. 11, with azure B. The *sphère chromatophile*, stained in both sections, continues to move toward the basement membrane. The elongate spermatids will soon be released from the seminiferous epithelium. Fig. 10, × 600; Fig. 11, × 800.

FIGURES 12, 13, and 14 Bright-field photomicrographs showing part of three sectioned seminiferous tubules. Figs. 12 and 13, stained with the azure B and with the Feulgen-alkaline fast green techniques, respectively, show stage IX; Fig. 14, stained with the azure B technique, shows stage X. The elongate spermatids have been released from the seminiferous epithelium, although a few are still seen in the lumen of the tubule shown in Fig. 13. The residual bodies, each containing a *sphère chromatophile*, continue to move toward the basement membrane, and some are seen lying very near Sertoli cell nuclei in Figs. 13 and 14. By stage X, the sphere binds less azure B and less fast green, and is presumably losing its RNA and basic protein; the early spermatid nuclei have begun to elongate. Fig. 12, × 800; Fig. 13, × 600; Fig. 14, × 800.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testis was then stained by the periodic acid–Schiff method for staging of the tubules. Comparison of the adjacent section, double-stained by the DNFB-azure B method, facilitated identification of the stages of the cycle present in the various tubules. This double-stained section was mounted in Shillaber's oil, of refractive index 1.592, and cytoplasmic *sphère chromatophile* and von Ebner bodies of all measurable sizes between stages early VII to IX were read on the microspectrophotometer. In these readings, the plug method was employed exclusively. Each body was read at 400 mμ (for determination of amount of bound DNFB) and at 550 mμ (for determination of amount of bound azure B). The diameter of each body and the stage of the cycle were also recorded. This process was repeated for 87 different granules of various sizes. The extinctions at 400 mμ and at 550 mμ, and finally the ratio E_{400}/E_{550}, were calculated for each reading. The data are given in Tables III and IV. These results are plotted as ratio of extinctions against the size of the granule (Fig. 33). The extinctions of the DNFB and of the azure B are separately plotted against the size of the granule (Fig. 34). In these curves, the line is in each case drawn by the method of least squares to obtain the regression.

An experiment was also designed (Bloch and

Figs. 15 through 24, showing the appearance of a small part of the seminiferous epithelium of the rat at successive stages of the cycle, have been stained with the picric acid–bromphenol blue technique, for the localization of basic proteins. Carnoy fixation; 5 μ sections.

FIGURE 15 Bright-field photomicrographs, stage XIII. Elongate spermatid nuclei (*Sl*) contain many intensely stained granules. The spermatid cytoplasm does not bind bromphenol blue. A primary spermatocyte nucleus is also shown. × 950.

FIGURE 16 Bright-field photomicrograph, stage XIV. Elongate spermatid nuclei now stain homogeneously. However, spermatid cytoplasm now contains a few granules (*G*) which bind bromphenol blue. Part of a primary spermatocyte nucleus appears at the top of the micrograph. × 1800.

FIGURES 17 and 18 Phase-contrast and bright-field photomicrographs, stage I, of the same area of one sectioned tubule. The elongate spermatid cytoplasm is now filled with tiny stainable granules (*G*). The light dots in the phase-contrast photomicrograph are due to granules which are out of focus. × 1800.

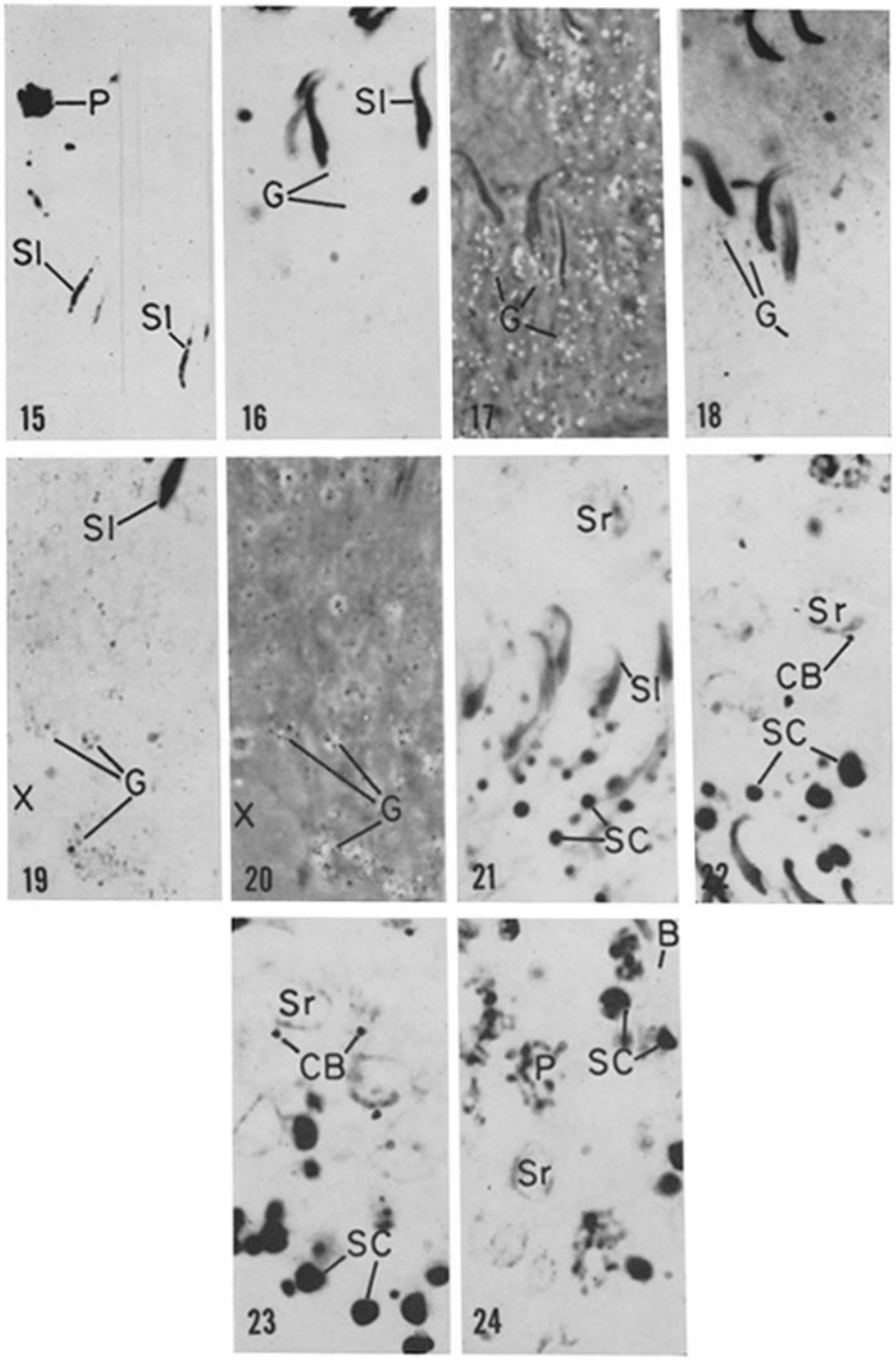
FIGURES 19 and 20 Bright-field and phase-contrast photomicrographs, about stage V, of the same area of one sectioned tubule. Although a few stained granules are scattered indiscriminately throughout the elongate spermatid cytoplasm, most of these granules (*G*) are localized in a tight cluster in the part of the cytoplasm adjacent to the lumen (*X*) of the tubule. × 1800.

FIGURE 21 Bright-field photomicrograph, stage VII. The *sphère chromatophile* (*SC*), which binds the bromphenol blue, is now well formed. Several are seen in this section. A round spermatid nucleus (*Sr*) appears at the top of the photomicrograph. × 1800.

FIGURE 22 Bright-field photomicrograph, stage VIII. The *sphère chromatophile*, now nearly completely formed, has moved considerably closer to the basement membrane, and lies near the anterior part of the elongate spermatid nuclei. × 1800.

FIGURE 23 Bright-field photomicrograph, stage IX. The elongate spermatids have been released from the seminiferous epithelium; the *sphère chromatophile* bodies (*SC*) remain, and continue to move toward the basement membrane of the tubule. Also shown in this photomicrograph are four chromatoid bodies (*CB*), lying in the round spermatid cytoplasm very near to the nucleus. The role of these structures is not known. × 1800.

FIGURE 24 Bright-field photomicrograph, stage early X. The basement membrane (*B*) can be seen at the top right corner of the micrograph. The *sphère chromatophile* bodies have moved very close to the basement membrane now. Several primary spermatocyte nuclei are shown. × 1800.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Brack, 1964) for determination of the amount of protein-bound lysine in the developing spermatid nuclei. Spectral absorption curves were first determined for Feulgen-stained nuclei and for DNFB-stained nuclei. The DNFB-stained slide was then stained by the Feulgen method for DNA, using trichloroacetic acid hydrolysis, and the nucleus used in the determination of the DNFB spectral absorption curve was relocated. The spectral absorption curve for this DNFB-Feulgen double-stained nucleus was determined, using the plug method as before. These absorption curves are shown in Fig. 35.

Periodic acid–Schiff staged nuclei, double-stained with the DNFB-Feulgen technique, were then measured on the microspectrophotometer, using the plug method, to obtain their extinctions at 400 mμ and at 560 mμ, and the ratio E_{400}/E_{560} was calculated for each measured nucleus. About 20 nuclei were so measured at each stage from stage 7 spermatids to stage 17 spermatids. Employing the method of calculation for determination of the number of moles of nuclear protein lysine (see Materials and Methods):

$$\text{Moles of nuclear lysine} = \frac{K_{\text{lysine}}}{K_{\text{DNA}}} \times \frac{E_{\text{lysine}}}{E_{\text{DNA}}} \times \text{moles nuclear DNA},$$

the results entered in Table V and plotted in Fig. 36 were determined.

DISCUSSION

The cytochemistry of the *sphère chromatophile* is at first glance remarkably similar to that of somatic nuclei: both stain with alkaline fast green or bromphenol blue for histonelike basic proteins; this reaction is abolished after deamination or acetylation. In the case of nuclei, such stainability so long as it is restricted to the chromatin, is generally attributed to histone proteins (Alfert and Geschwind, 1953; Bloch and Hew, 1960; Das et al. 1964 *b*). In addition, the similarity between the chemical nature of nuclei and that of *sphère chromatophile* bodies is carried further by similar reactions of both to the Sakaguchi and 1-fluoro-2,4-dinitrobenzene tests.

A failure of nuclei to stain with alkaline fast green after deamination or acetylation is generally attributed to the blocking of the epsilon amino groups of lysine in the basic protein, but not the guanidine group of arginine (Olcott and Fraenkel-Conrat, 1947; Klotz, 1950). Ability to stain is due to the presence of a sufficiently high ratio of arginine to lysine. This being the case, replacement of histones rich in lysine by arginine-rich histones in the elongated spermatid nuclei must

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Figs. 25 through 31 show the lysine-H^3 labeling pattern of the rat seminiferous epithelium after various periods of exposure to isotope.

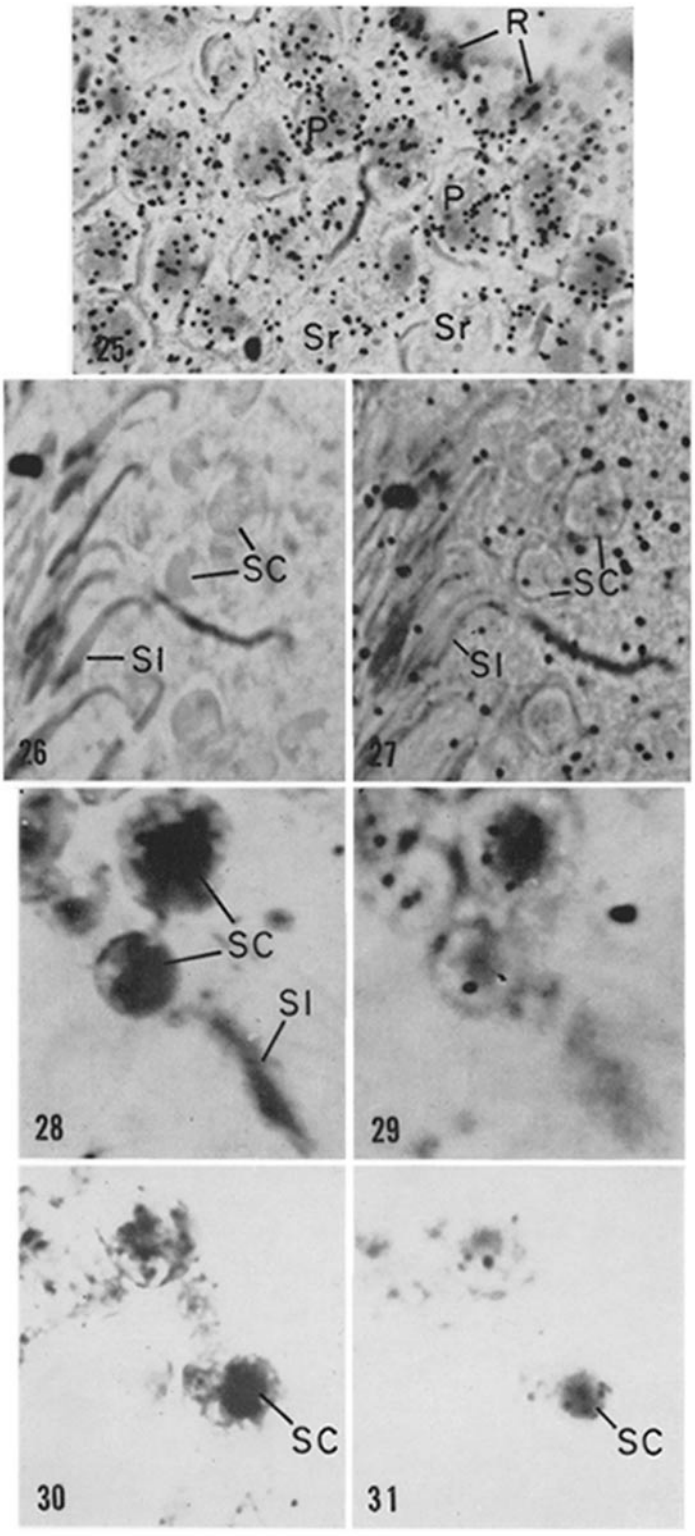
R, "resting" primary spermatocyte
P, pachytene primary spermatocyte
Sr, early spermatid with spherical nucleus
Sl, later spermatid with elongate nucleus
SC, *sphère chromatophile*

FIGURE 25 2 hr lysine. Phase-contrast photomicrograph, stage VIII. Focus is on silver grains lying over pachytene primary spermatocytes and resting primary spermatocyte nuclei. Only an occasional grain appears over a round spermatid nucleus. Feulgen stained; formaldehyde fixation.

FIGURES 26 and 27 2 hr lysine, stage VIII. Focus is on the tissue in Fig. 26; on silver grains in Fig. 27. Silver grains are not present over the *sphère chromatophile* or the elongate spermatid nuclei. Feulgen–toluidine blue technique; Carnoy fixation.

FIGURES 28 and 29 402 hr lysine, stages VIII to IX. Focus is on the tissue in Fig. 28; on silver grains in Fig. 29. Silver grains are present over the residual body around the periphery of, but not directly over, the *sphère chromatophile*. The elongate spermatid nucleus is not labeled. Feulgen–toluidine blue technique; formaldehyde fixation.

FIGURES 30 and 31 864 hr lysine, stage VIII. Focus is on the tissue in Fig. 30; on silver grains in Fig. 31. Silver grains are present over the *sphère chromatophile*, but not over the remainder of the residual body. Toluidine blue technique; formaldehyde fixation.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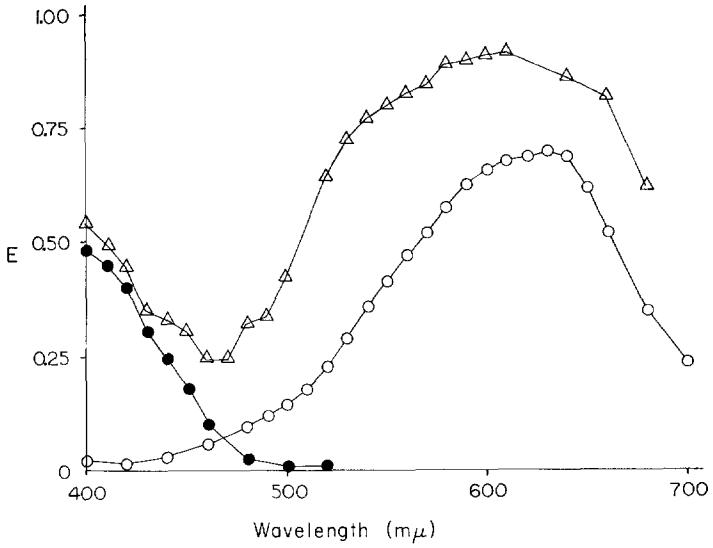

FIGURE 32 Absorption spectra of *sphère chromatophile* stained with the dinitrofluorobenzene (DNFB) and the azure B techniques. A single body was used for determining all three curves. —●— DNFB;—○— azure B; —△— DNFB–azure B.

TABLE III

DNFB-Azure B Double-Stain Data on the Sphère Chromatophile

Stage of cycle	early VII	VII	VIII	IX
Granule diameter range	4.0–6.0	5.0–10.5	7.0–12.0	10.0–12.0
Number of granules measured	12	28	40	7
Mean ratio $\frac{E_{400}}{E_{550}}$*	0.59 ± 0.06	0.71 ± 0.13	0.82 ± 0.13	0.85 ± 0.13

* Standard deviations are given.

begin at stage 13, in the same nuclei in which the lysine-rich basic protein grains are first seen. Since neither somatic nuclei nor the *sphère chromatophile* stain with alkaline fast green or bromphenol blue after deamination or acetylation, it is concluded that the stainability of the *sphère chromatophile* with these dyes is due to the presence of basic proteins rich in lysine, relative to arginine. Because these bodies stain with alkaline fast green after trichloroacetic acid hydrolysis, they presumably do not contain protamines, for these should be leached from the bodies during such hydrolysis (Alfert and Geschwind, 1953; Alfert, 1956). The fact that the *sphère chromatophile* stains with azure B, but not if staining is preceded by treatment of the tissue with ribonuclease or 10% perchloric acid, confirms the presence of RNA. The sphere does not contain DNA, since it is not stainable either with the Feulgen reaction for DNA, or with azure B after treatment with ribonuclease or 10% perchloric acid.

The observations that the *sphère chromatophile* bodies fail to stain with alkaline fast green or with bromphenol blue unless they have been previously treated with acid, or with ribonuclease, should not be construed to mean that the stainable basic groups are bound to the RNA by salt-type bonds, as is the case in the nuclear bonding of DNA to most[2] histones. This is admittedly a possibility, and Kaufmann et al. (1951) have suggested this as an explanation for similar phenomena. Swift (1953) has proposed an alternative explana-

[2] Nuclear histones have recently been shown to be also bound to RNA, by nonionic bonds (Huang and Bonner 1965).

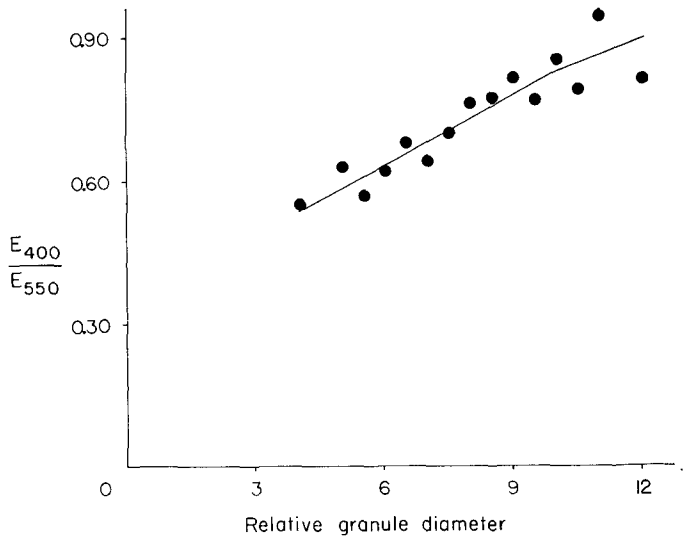

FIGURE 33 The ratios of the extinctions at 400 mμ and 550 mμ, which express relative ratios of protein lysine to RNA, are plotted against the relative diameter of a series of *sphère chromatophile* bodies.

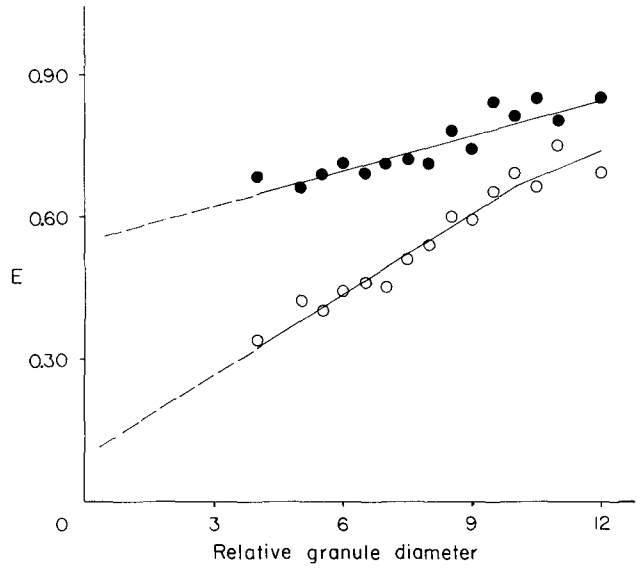

FIGURE 34 The extinctions at 400 mμ, which express relative amounts of protein lysine, and the extinctions at 550 mμ, which express relative amounts of RNA, are each separately plotted against the relative diameter of a series of *sphère chromatophile* bodies. —○— E_{400}(DNFB); —●— E_{550} (azure B).

tion as to why stainability of the histonelike basic proteins requires removal of nucleic acid: it may be due to the repulsion of the anionic fast green molecules by the negatively charged phosphate groups of the RNA, thus preventing the dye molecules from coming into contact with the basic groups of the basic protein. The nature of the association between the RNA and the basic proteins of the sphere cannot be decided from the present data.

The development of the *sphère chromatophile*, as revealed by staining studies, can readily be appreciated by studying the micrographs of azure B and Feulgen-alkaline fast green stained testes sections (Figs. 4 to 14), and the bromphenol blue sequence (Figs. 15 to 24). The azure B–stainable component (RNA) is thought to arise primarily in the cytoplasm of the developing spermatid by coalescence of the finely granular cytoplasmic grains, as described by Regaud (1901 *a*), Daoust and Clermont (1955), and Sud (1961). These grains become larger by stages late 17 to 18, and are then called von Ebner's granules. As the von Ebner granules coalesce with each other, they

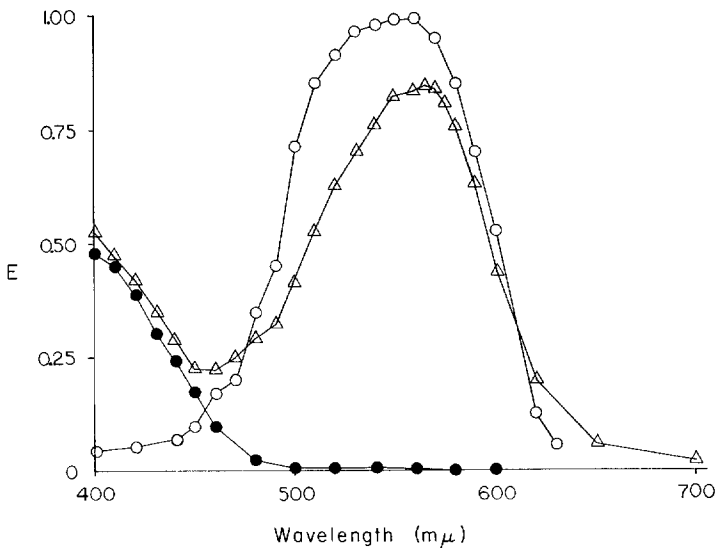

FIGURE 35 Absorption spectra of spermatid nuclei stained with the dinitrofluorobenzene (DNFB) and the Feulgen techniques. A single nucleus was used for determining all three curves. —●— DNFB; —○— Feulgen; —△— DNFB-Feulgen.

TABLE IV

DNFB-Azure B Double-Stain Data on the Sphère Chromatophile, Organized According to Granule Diameter

Relative granule diameter	Number of granules measured	Mean ratio $\frac{E_{400}}{E_{650}}$ *
4.0	3	0.52 ± 0.02
5.0	6	0.63 ± 0.03
5.5	7	0.57 ± 0.07
6.0	3	0.62 ± 0.10
6.5	4	0.68 ± 0.08
7.0	5	0.64 ± 0.06
7.5	5	0.70 ± 0.08
8.0	9	0.76 ± 0.11
8.5	3	0.77 ± 0.05
9.0	10	0.81 ± 0.12
9.5	5	0.77 ± 0.01
10.0	10	0.85 ± 0.11
10.5	3	0.79 ± 0.26
11.0	7	0.94 ± 0.16
12.0	7	0.81 ± 0.08

* Standard deviations are given.

eventually form one component of the *sphère chromatophile* in the cytoplasm of the stage early 19 elongate spermatid. At this time, von Ebner granules continue to coalesce with the *sphère chromatophile*, increasing its size. By late stage 19, the RNA component of this structure is complete, and the residual body separates from the remainder of the spermatid. The residual body then passes toward the basement membrane, and soon thereafter its RNA and basic protein components are lost. These later stages in the life of the *sphère chromatophile* were not, however, investigated in this present work. These observations on the development of the RNA component of this granule are in complete accord with those of Regaud (1901 *a*), Daoust and Clermont (1955), and Sud (1961).

The results of the alkaline fast green studies alone might suggest that the basic protein component of the *sphère chromatophile* arises rather abruptly, at about early stage 19. After this, the growth and fate of this structure (as revealed by Feulgen-alkaline fast green staining studies) is nearly identical with that of the RNA component described above.

However, the results of the picric acid–bromphenol blue study (Figs. 15 to 24) shed more light on the origin of the basic protein component of the sphere. These studies suggest that this component is originally nuclear. It is thought to be significant that the localizations of bromphenol blue and of azure B stainable cytoplasmic granules are different during the maturation of the spermatid. These observations, and those ob-

TABLE V

DNFB-Feulgen Double-Stain Data on Spermatid Nuclei

Stage of cycle (measured stage of spermiogenesis in parentheses)	Mean ratio* $\frac{E_{400}}{E_{560}}$	Moles lysine‡ ($\times 10^{14}$)	Moles lysine‡ / Mole DNA (nucleotide)
VII (7)	2.51 ± 0.19 (22)	2.05 (±0.19)	2.00 ± 0.19
IX (9)	2.66 ± 0.42 (18)	2.17 (±0.42)	2.13 ± 0.42
X (10)	2.24 ± 0.29 (23)	1.83 (±0.29)	1.80 ± 0.29
early XI (11)	1.97 ± 0.26 (26)	1.61 (±0.26)	1.58 ± 0.26
XII (12)	1.27 ± 0.16 (27)	1.04 (±0.16)	1.02 ± 0.16
XII–XIII (12–13)	0.74 ± 0.15 (25)	0.60 (±0.15)	0.59 ± 0.15
late XIV (14)	0.66 ± 0.14 (20)	0.54 (±0.14)	0.53 ± 0.14
I (15)	0.88 ± 0.13 (22)	0.72 (±0.13)	0.71 ± 0.13
II (16)	0.99 ± 0.25 (16)	0.81 (±0.25)	0.79 ± 0.25
III (16)	0.90 ± 0.16 (22)	0.73 (±0.16)	0.72 ± 0.16
V (17)	0.90 ± 0.17 (20)	0.73 (±0.17)	0.72 ± 0.17

* Standard deviations are given; number of nuclei measured is in parentheses.

‡ Calculated from formulae given in Materials and Methods; standard deviations are shown.

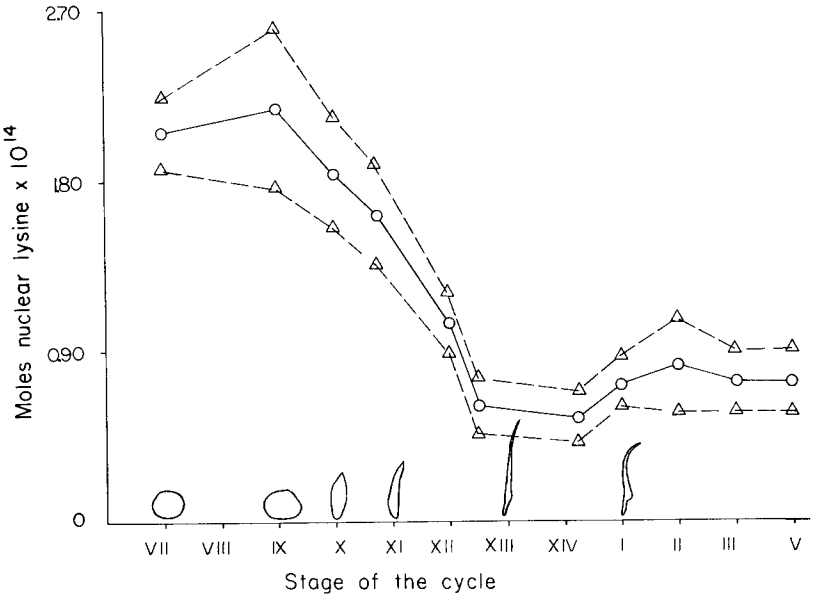

FIGURE 36 The number of moles of spermatid nuclear protein lysine during spermiogenesis are plotted against the stage of the cycle. The curve is calculated from data obtained on DNFB-Feulgen double-stained spermatids. ----- Limits of standard deviation.

tained by microspectrophotometry, indicate that the RNA and basic protein components of the *sphère chromatophile* may have different origins in the cell.

The data gathered in the present paper, relating to histone transition and the development of the *sphère chromatophile*, are partially summarized in Fig. 37.

The fact that the smaller (von Ebner's) azure B–positive granules at stages late 17 to 18 do not stain with alkaline fast green or bromphenol blue for basic proteins, whereas the larger granules do (stage early 19 onward), suggests three hypotheses to account for the origin of the basic protein component of the *sphère chromatophile*: (*a*) the alkaline fast green stainable basic proteins

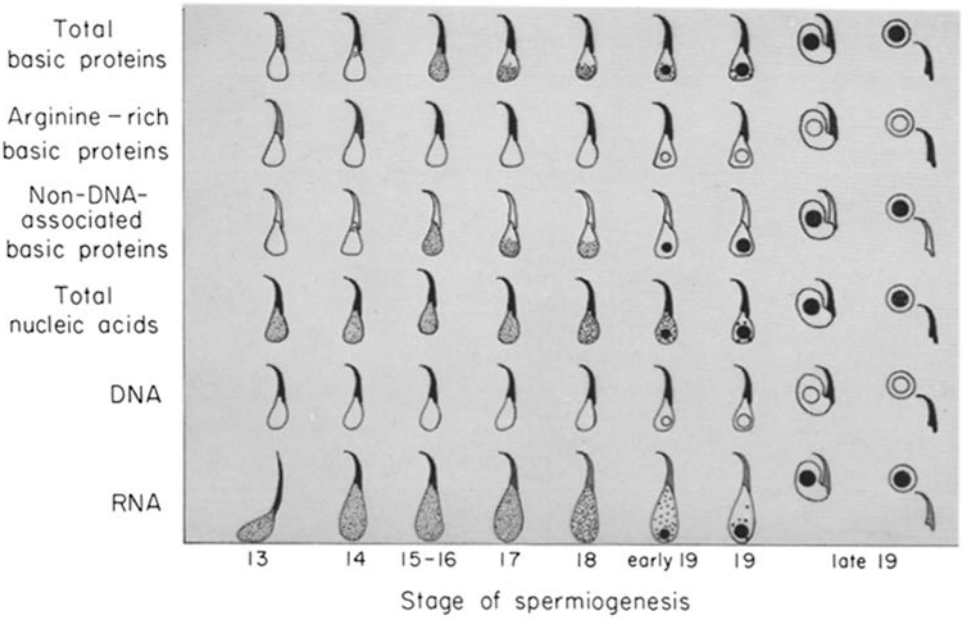

FIGURE 37 Summary of cytochemical observations on the origin and development of the RNA and basic protein components of the *sphère chromatophile* in the rat spermatid cytoplasm. Histone transition begins at stage 13.

are synthesized *in situ* rather late in the RNA-containing sphere (at around stage early VII); or (*b*) these proteins are transferred to the sphere from some other site of synthesis, cytoplasmic or nuclear; or (*c*) these proteins, while in the smaller von Ebner granules (prior to stage VII), are not stable to the fixation and ensuing treatments, and are washed from the cells, leaving the RNA granules behind. Then, by stage early VII, as the granule becomes larger, the basic proteins are rendered stable. However, since the picric acid–bromphenol blue method is known to stabilize even very labile protamines (Bloch and Hew, 1960), the third hypothesis is considered doubtful. The first hypothesis is also unlikely, since Monesi (1964 *b*) reports that mouse residual bodies incorporate labeled precursors into RNA only after many days.

That this granule shows no incorporation of lysine-H^3 until 864 hr indicates that the *sphère chromatophile* does not synthesize its own lysine-rich basic proteins *in situ*. Incorporation into surrounding cytoplasm and nuclei (Fig. 25) shows that lysine is available for incorporation into protein.

The generally accepted view (Regaud, 1901; Daoust and Clermont, 1955; Sud, 1961) that the RNA component of the residual body, i.e. the *sphère chromatophile*, arises by coalescence of the tiny cytoplasmic ribonucleoprotein granules (presumably ribosomes), and the fact that ribosomes have repeatedly been shown to contain basic proteins (Ts'o et al., 1958; Crampton and Petermann, 1959; Setterfield et al., 1960; Butler et al., 1960; Spahr, 1962; Waller, 1964), and the observation that ribosomal aggregates in some spore-forming fungae stain with bromphenol blue and with alkaline fast green (Vaughn, unpublished)[3] make the postulation of a cytoplasmic origin for the basic protein component of the *sphère chromatophile* the most tempting possibility of the three hypotheses. Since the experiments using lysine-H^3 radioautography do not decide the case in an unambiguous manner, a new approach was sought using microspectrophotometry: one would predict that the ratio of basic protein to RNA would remain about the same during increase in size of the von Ebner and *sphère chromatophile*

[3] Whitfield and Youdale (1965) have recently reported cytoplasmic alkaline fast green staining in mouse fibroblasts, which they attribute to ribosomal aggregates.

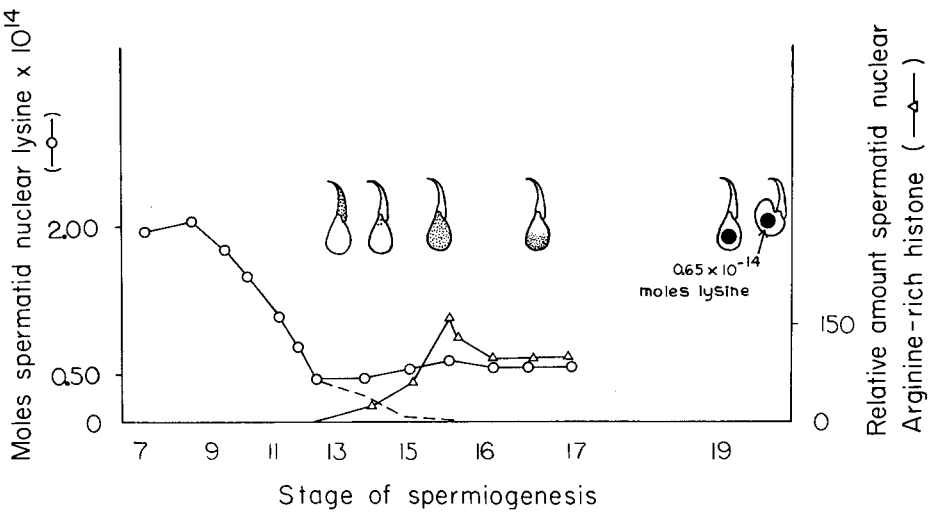

FIGURE 38 Summary of events leading to development of *sphère chromatophile* basic protein component. The broken line curve is based on assumption of a 1:1 replacement of somatic histone by arginine-rich histone, and on Table V.

bodies if these arise by coalescence of ribosomes. If, however, the basic protein component of the developing *sphère chromatophile* has a nonribosomal source, then one would predict that the ratio of basic protein to RNA in the developing granule might not remain constant, but that it might *increase* with increasing granule size. (The assumption is made that, with increase in granule size, the relative distribution of RNA and protein lysine does not change within the granule.)

The double-staining technique, which allows for the determination of the ratio of protein lysine to RNA, was designed to decide between these two predictions. The results on *sphère chromatophile* bodies double-stained with the DNFB–azure B technique (Fig. 33) show that the ratio E_{400}/E_{550} *increases* with the stage of the cycle and with increasing granule diameter. The separate plots of E_{400} and E_{550} against granule diameter (Fig. 34) show that this increase is due largely to accumulation of protein lysine. When the curve for DNFB extinction is extrapolated (Fig. 34), it runs almost to the origin: the smallest granule (unmeasurable on the microspectrophotometer) would then contain little, if any, protein lysine. Extrapolation of the azure B curve shows that even the smallest granule (again unmeasurable) would have a relatively high RNA content. Since the largest granules contain both lysine and RNA, and since the smallest granules contain largely RNA, and accepting the view that cytoplasmic ribonucleoprotein (RNP) granules coalesce to form the *sphère chromatophile*, we must conclude that the RNA and lysine in this structure are coming from different sources. It thus appears that as the granule increases in size, by accretion of material, protein is added faster than RNA. These observations are compatible with the cytochemical observations. Since the basic proteins apparently do not come from the cytoplasmic RNP granules, they must arise from some other source.

Support for the hypothesis of a nuclear origin of the *sphère chromatophile* basic proteins is seen in the finding of silver grains over this structure in the 864 hr lysine experiment. The observation that the resting primary spermatocyte, 864 hr earlier, does incorporate lysine and thymidine (incorporation of the former presumably due in part to histone synthesis), is compatible with the interpretation that the *sphère chromatophile* basic proteins have their origin in the nucleus of the resting primary spermatocyte.

If the basic proteins of the *sphère chromatophile* have their origin in the nucleus, loss of spermatid nuclear protein lysine would be expected prior to the formation of the sphere. Fast green staining after deamination (Table II) does indicate a decrease in the lysine to arginine ratio during stages 13 to 16. Studies on DNFB-Feulgen—stained material (Table V and Fig. 36) suggests

that loss of protein lysine occurs during two phases. The first, occurring during stages 9 to 12, is characterized by an absolute loss of 70% of the nuclear protein lysine. The lost material is probably not histone, since little if any change in alkaline fast green staining occurs during this period. Loss of histone lysine during a second phase is somewhat problematical: it is inferred from the observation of no net change in the protein lysine of the nucleus during a period in which arginine-rich histone, which also contains appreciable lysine, accumulates. It is thought that this gain is compensated by a loss of protein lysine in the small granules eliminated during this latter period. These relationships are summarized in Fig. 38.

There is indirect evidence to support the idea that much of the protein of the *sphère chromatophile* may be derived from histone eliminated from the nucleus after stage 13. These nuclei have not yet begun to accumulate arginine-rich histone. At this stage the nuclei contain 0.60×10^{-14} moles of protein lysine and, like the *sphère chromatophile*, are fast green positive. If the histones contained therein are completely replaced, and represent the material found later in the *sphère chromatophile*, a comparable amount of lysine would be expected in the latter body. If we assume that stage 13 spermatid DNA is at this time still completely covered with histone, and that the nuclear lysine present at this stage is largely in histone, then 0.60×10^{-14} moles is, by inference, the quantity of lysine in the histone of the early spermatid, prior to histone transition. A calculation of the number of moles of protein lysine in a mature stage VIII *sphère chromatophile* was made on the basis of the data in Table IV, using the formula:

$$\text{Moles } \textit{sphère chromatophile} \text{ lysine} = \tfrac{2}{3}\ KEA$$

(The area of a section through the center of a large stage VIII sphere is 0.6×10^{-7} cm^2). Taking the highest $E_{400\ m\mu}$ value encountered at stage VIII, (which is 0.887), the maximum number of moles of lysine is calculated to be 0.65×10^{-14}, which is nearly identical with the value derived above for the early spermatid nucleus, affording further circumstantial evidence for a nuclear origin of the basic proteins in the *sphère chromatophile*.[4]

If the basic proteins of the *sphère chromatophile* do indeed come from the nucleus, this study does not provide any conclusive evidence as to whether or not they are histones, i.e., that they are ever associated with DNA. Nuclear ribosomes (Wang, 1962) and nucleoli (Horn and Ward, 1957; Bloch and Hew, 1960; Birnstiel and Hyde, 1963) have been shown to contain basic proteins. Setterfield et al. (1960) and Vaughn (unpublished data) have shown that ribosomal basic proteins, when present in sufficient quantity, can give a positive alkaline fast green reaction.

Isolation and characterization of the basic proteins of the *sphère chromatophile*, and comparison of these with the histones extracted from germ cell nuclei, may help to settle the question which still exists. With regard to the latter, Lindsay (1966) has recently demonstrated that the electrophoretic mobilities of chicken liver ribosomal and nuclear basic proteins are nearly identical.

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[4] An argument along slightly different lines arrives at the same conclusion. It is known that there are 1.02×10^{-14} moles of DNA nucleotide in the haploid rat sperm nucleus. The ratio of phosphate groups of DNA to the combined basic groups of histone is close to 1.0 (Davison and Butler, 1956; Vendrely et al., 1960). Biochemical determinations often reveal the molar ratio of lysine to arginine in mammalian somatic histone to be about 1.6-1.8 (Davison, 1954; Crampton et al., 1955; Davison and Butler, 1956). Therefore, if the early stage 13 spermatid DNA is completely covered with histone, there must be about 0.62 to 0.68×10^{-14} moles of lysine in this histone. This value is again very close to that derived above for the moles of lysine in the *sphère chromatophile*.

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