AN ANALYSIS OF THE INACTIVATION OF THE FROG SPERM NUCLEUS BY TOLUIDINE BLUE*

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Many years ago Hertwig (1) and Dalcq (2) showed that trypaflavine has the property of inactivating the frog sperm nucleus without affecting the extranuclear parts of the cell. Following an appropriate treatment with the dye, the sperm cells still retain their capacity to activate eggs, but contribute no chromosomes to the resulting embryos, which then develop as haploids with the maternal chromosomes only. These early investigations were concerned mainly with the use of the dye as a tool in studying the role of the nucleus in embryonic development. The nature of the reaction between the dye and the sperm nucleus was not itself analyzed at that time and, so far as we know, has not since received any serious attention. Yet, as we shall try to show later on, this reaction presents an unusual opportunity for a quantitative study of chromosome chemistry in relation to chromosome function.

Since the time of Hertwig's and Dalcq's investigations considerable chemical work has been done on the reactions between basic dyes and nucleic acids. Therefore, in the present study it was desirable at the outset to test several dyes in order to see whether any of them offer advantages over trypaflavine as specific inactivators of the sperm nucleus.

The dyes tested were toluidine blue, methylene blue, thionine, acriflavine (trypaflavine), pyronin, methyl green, and crystal violet. All except the triphenylmethanes (methyl green and crystal violet) were found to inactivate the sperm nucleus without damaging appreciably the extranuclear parts. The thiazin dyes were particularly effective. For example, an appropriate treatment with toluidine blue inactivated the sperm nucleus completely, leaving the sperm otherwise unaffected, so that eggs inseminated with the treated sperm developed uniformly as typical gynogenetic haploids. Other dyes mentioned above as being effective could produce the same result, but none offered advantages over toluidine blue and it was therefore selected for the experiments reported here.

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Given such a specific effect as that obtained with toluidine blue, the way appeared open to begin a quantitative study of the reaction between the dye and the sperm nucleus. The particular advantages of the dye-sperm system for this type of study are that both dye concentration and sperm number can be easily measured, and, most important, the effect of a given amount of dye on the function of the sperm nucleus can be determined by observing the development of normal eggs inseminated with the treated sperm. Various types of biochemical information concerning chromosome function could be obtained with this material. For example, a measurement of the number of dye molecules which must be bound by the sperm nucleus in order *just* to inactivate it should provide us with an estimate of the number of combining groups essential for the functioning of the chromosomes in development. Our efforts along this line so far have been confined largely to the development of a procedure for estimating the uptake of dye by sperm cells. This involved preliminary experiments on the influence of pH on the reaction between dye and sperm, on the reversibility of this reaction, and on the localization of the dye within the sperm cell. With this information at hand a simple procedure for measuring dye uptake was worked out, and an estimate was obtained of the number of dye molecules required for complete inactivation of the sperm nucleus in development.

Materials and Methods

Experiments on the effects of dyes on the sperm nucleus were done in a constant temperature room (18°) with sperm and eggs of *Rana pipiens*, according to the following general procedure: A sperm suspension was first made by macerating two testes in 3 to 5 ml. of dilute Ringer's solution (8.7 per cent of full strength = 0.01 total molarity). After 15 to 25 minutes the discrete pieces of testis tissue were removed, the suspension was centrifuged in an international clinical centrifuge for 10 minutes at full speed. The supernatant fluid was discarded and the sperm cells were resuspended in a measured volume of 0.01 M Ringer's solution—usually 2 to 5 ml. The few remaining discrete masses in the suspension were removed with a fine pipette, and a sperm count was then made in a standard blood-counting chamber.

In some experiments the dye, dissolved in 0.01 M Ringer's solution, was mixed directly with this stock suspension (in pyrex tubes) to give a sperm count of 10,000 to 15,000 cells per c. mm. Samples of the dye-sperm mixture were then diluted 1 to 10 after appropriate intervals before being used for inseminating eggs. In other experiments the stock sperm suspension was first diluted with 0.01 M Ringer's solution, and then mixed with dye solution to give a final count of 1000 to 1500 per c. mm. This suspension was used without further dilution for insemination. Periods of exposure of sperm to dye, counted as the time between mixing sperm and dye and the time of insemination, ranged from 5 minutes to 60 minutes or more. At each interval eggs were stripped from a pituitary-stimulated female into a 1 ml. sample of the sperm suspension. 15 minutes after insemination the eggs were washed free of sperm suspension. 1 to 3 hours later the egg mass was cut up into groups of 4 to

6 eggs and distributed to finger bowls containing spring water—roughly 50 eggs per bowl. From observations made on the development of these eggs it was possible to determine to what extent the activities of nuclear and extranuclear parts of the sperm were impaired by a given dye treatment. The criteria used for this purpose are described in the first section of the experimental part of the paper.

Special methods used in measuring dye uptake by sperm cells and in determining the effect of pH on the reaction between dye and sperm are given in the appropriate sections of the experimental part of the paper.

The dye used in all the experiments reported here was Toluidine blue O, purified, zinc-free, C.I. No. 925, lot No. 12441 (National Aniline). The dye content was stated to be 90 per cent, in correspondence received from the manufacturer. An independent determination of dye content, made by comparing the extinction coefficient of the commercial dye with that of the perchlorate of the dye, also gave a value of 90 per cent. Stock aqueous solutions (10^{-3} M) kept in pyrex volumetric flasks and periodically diluted to 10^{-5} M with 0.01 N HCl and measured in the Beckman spectrophotometer, showed no change in optical density at the absorption maximum (630 mμ) during storage for 1 to 8 weeks. Stock solutions were therefore sometimes used for 2 to 3 weeks and were just as effective as were freshly made up solutions. Dilutions of the stock solution were always made just before the diluted solution was mixed with the sperm suspension.

EXPERIMENTAL

The nature of the effects of toluidine blue on sperm cells can best be presented by describing a typical experiment. In this experiment a dilute sperm suspension was mixed with dye and buffer at 0 time to give a final dye concentration of 2×10^{-7} M; sperm count, 1560/c.mm.; and pH 8.1. At times ranging from 7 minutes to 60 minutes after mixing, sperm samples were removed and used for inseminating eggs. The development of these eggs is summarized in Table I and Figs. 1 and 2.

The large majority of eggs cleaved normally, showing that the sperm tail, acrosome, and middle piece (*i.e.* those parts concerned with activation and the formation of normal cleavage centers) were not significantly affected by dye exposures of 7 to 40 minutes, and only slightly affected by a 60 minute exposure. By contrast, the effects on the sperm nucleus were pronounced. The result of a 7 minute dye treatment of the sperm was that over 90 per cent of the inseminated eggs developed a variety of severe abnormalities during gastrulation and neurulation (see Figs. 1 and 2 B). In these embryos ectoderm cell size was variable and generally intermediate between the sizes of corresponding cells as seen on diploid and haploid controls. This suggests that a short exposure to the dye produces partial inactivation of the sperm chromosome complex, and an irregular distribution of the remaining chromosomes during cleavage—a condition known to result in abnormal and arrested development (3).

With longer exposures of sperm to dye there was a sharp drop in the number of abnormal embryos and a concomitant sharp rise in the number of eggs developing as typical haploids (Fig. 1). The latter could be recognized easily by their characteristic form (Fig. 2 C), and the uniformly small size of their ectoderm cells—criteria previously shown to be reliable indications of haploidy (4, 5). Furthermore, chromosome counts made on smears of 10 haploid em-

TABLE I

Development of Eggs Inseminated with Toluidine Blue–Treated Sperm

(1)	(2)	(3)	(4)	(5)	(6)	(7) Abnormal embryos		(8) Typical haploids		
Exposure of Sperm to dye	Total No. of eggs	Uncleaved	Abnormal cleavage	Normal cleavage	Normal stage 18 (diploids)	No.	Per cent of normally cleaved eggs	No.	Per cent of normally cleaved eggs	Per cent of total eggs
min.										
0 (control)	92	1	1	90	88	2	2	0	0	0
7	118	1	2	115	8	105	91	2	2	2
15	125	0	1	124	0	28	23	96	77	77
25	90	2	1	87	0	7	8	80	92	89
40	125	2	4	119	0	4	3	115	97	92
60	87	2	10	75	0	3	4	72	96	83

In this experiment the dye concentration was 2×10^{-7} M; sperm number was 1560 per c. mm.; and pH was buffered at 8.1 with 0.01 M tris(hydroxymethyl)-aminomethane.

Following insemination with dye-treated sperm, the eggs were first observed within 24 hours and classified as to type of cleavage (columns 3, 4, 5). 3 days later, when the controls had developed to late tail bud stage (stage 18 of Shumway's (25) series), the embryos were classified as to type of development (columns 6, 7, and 8). At this stage the haploids are already distinctly different in appearance from diploids and from abnormal embryos. Furthermore, differences in ectodermal cell size are most easily seen at this time (26). At later stages of development the differences in form become accentuated (see Fig. 2), but the ectodermal cells are not so clearly outlined and their sizes are therefore more difficult to estimate in living embryos.

bryos from the present experiment gave the established haploid value of 13 for perfectly clear figures, and 12 to 14 for equivocal figures. A final test of the inactivation of the sperm nucleus was done using *Rana catesbeiana* sperm. It is well established that the cross, *catesbeiana* ♂ × *pipiens* ♀, is uniformly lethal. The hybrids are arrested at the beginning of gastrulation and die 2 to 3 days later without developing further (6–8). When *catesbeiana* sperm cells were treated with toluidine blue (one experiment) this lethal effect was abolished, and 90 per cent of the normally cleaved eggs developed as typical haploids—a result similar to that obtained when the *catesbeiana* sperm nucleus is inactivated with large doses of x-rays (6, 8).

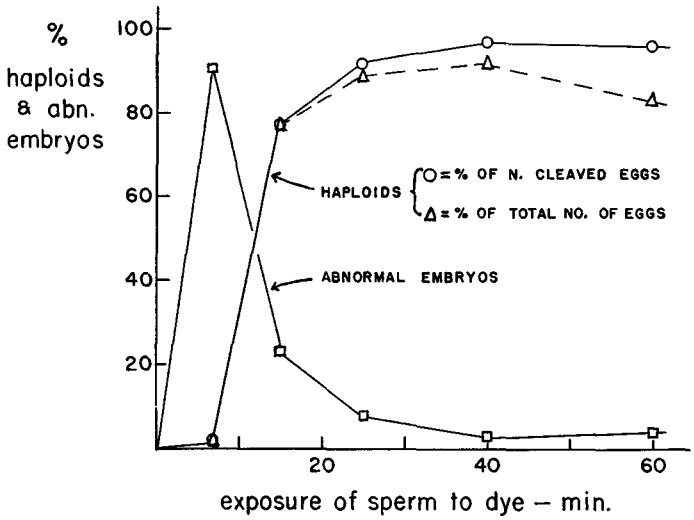

FIG. 1. Development of eggs inseminated with sperm treated for different lengths of time with toluidine blue. The proportion of eggs developing as typical haploids represents the proportion of sperm cells in which the dye has brought about a complete inactivation of the nucleus. The proportion of eggs developing abnormally represents the proportion of sperm cells with partially inactivated nuclei. Data from Table I.

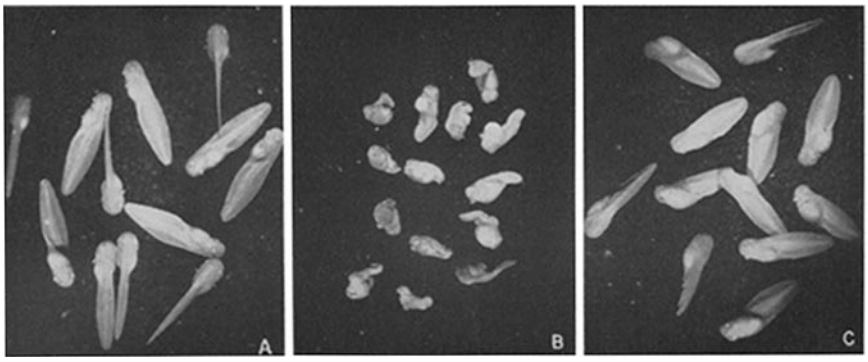

FIG. 2. Appearance of embryos derived from eggs inseminated with toluidine blue–treated sperm. All embryos are 9 days old. A, Sperm untreated; embryos are normal diploids. B, Sperm treated for 10 minutes with 5×10^{-6} M dye in 10 per cent Ringer's solution; embryos are very abnormal and unviable. C, Sperm treated for 60 minutes with 5×10^{-6} M dye. Embryos are typical haploids.

The evidence given above demonstrates that toluidine blue, applied to sperm cells under properly controlled conditions, inactivates specifically the sperm

nucleus without appreciably affecting the functions of the extranuclear parts of the cell. The cytological mechanism of this inactivation has not yet been studied in any detail, but we may suppose it to be similar to that described for trypaflavinized sperm by Dalcq (2) and for x-irradiated sperm by Briggs, Green, and King (8). Dalcq showed that the trypaflavine-treated sperm enters the egg, but that the sperm nucleus then remains condensed and fails to fuse with the egg nucleus. During cleavage the sperm chromatin is more difficult to follow, but it appears to be irregularly distributed and eventually lost to the cytoplasm. Our own studies show that x-rayed sperm chromatin behaves in much the same way. It is found in only a small number of cells, from which it appears to be eliminated during blastula stages, leaving the embryo to develop as a typical gynogenetic haploid. Both the x-rayed and dye-treated chromatin may preserve to some extent its capacity to increase in amount during cleavage, particularly when the egg nucleus is removed and cleavage occurs with the treated sperm chromatin only (8). However, this chromatin has no effect on cleavage rate, plays no role in differentiation, and therefore may be regarded as inactivated so far as effects on development are concerned.[1] Some of the properties of the reaction between dye and sperm, leading to this inactivation of the sperm nucleus, are shown by the following experiments.

Effect of pH on the Inactivation of the Sperm Nucleus by Toluidine Blue

An effect of pH on the reaction between dye and sperm nucleus was noted by Hertwig (1) who showed that trypaflavine was much more effective as a nuclear inactivator at a pH of about 8.2 than at pH 5.5. In order to define more exactly the relationship between pH and inactivation rate, we have measured the effectiveness of toluidine blue as an inactivator of the sperm nucleus over the whole of the pH range which can be tolerated by the sperm. Before these experiments could be done it was first necessary to test a series of buffers and to select only those buffers and pH values which leave the sperm normally viable; *i.e.*, capable of fertilizing eggs and giving rise to normal diploid embryos. The buffers selected were (1) acetate at pH 4.6, 5.1, 6.0; (2)

[1] Basic dyes are known to produce both chromosome breaks (23) and mutations (24). It seems possible, therefore, that multiple breaks and translocations may be involved in the inactivation of the frog sperm nucleus by toluidine blue. It is also possible that the spinning out of the sperm chromosomes—a process normally occurring following entrance of the sperm nucleus into the egg—may be inhibited by some mechanism which is independent of chromosome breaks in the usual sense. Evidence on these points might be obtained from cytological studies of eggs containing partially inactivated sperm nuclei.

phosphate at pH 6.1, 7.1, 7.6, 7.9; (3) tris(hydroxymethyl)-aminomethane[2] (Gomori (9)) at pH 8.0, 8.4, 8.8; and (4) borate at pH 8.5, 9.0, 9.6, 10.1. For each buffer series an experiment was done in which sperm cells, suspended in 0.01 M Ringer's solution, were mixed with buffer to give a final buffer concentration ranging, in different experiments, from 0.004 M to 0.01 M. The pH measurements were made with the glass electrode either directly on the sperm-buffer mixture or on control buffer-Ringer mixtures, depending on the volume of sperm suspension available. After exposures to buffer for times ranging from 10 to 60 or 90 minutes, sperm samples were removed and tested on eggs in the usual way.

The results of these experiments showed that at pH values ranging from 6.0 to 9.6 none of the buffers affected the sperm during the test period of 60 to 90 minutes. At pH 5.1 (acetate buffer) the sperm cells remained normally viable for 25 to 30 minutes but thereafter their capacity to fertilize eggs dropped sharply. However, the eggs that were fertilized developed as normal diploids. At pH 4.6 the fertilizing power of the sperm was lost completely within 10 to 20 minutes. At the upper end of the range (borate buffer) the results showed that the sperm cells were unaffected by 20 to 25 minutes' exposure to pH 10.1, while an exposure of 60 minutes reduced fertilizing power to roughly half its control value. As in the case of the pH 5.1 acetate buffer, those eggs that were fertilized following the longer exposures of spermatozoa to the high pH developed as normal diploids.

These experiments establish a pH range of 5.1 to 10.1 within which the sperm cells remain normally viable for 25 minutes or more—a sufficiently long period for carrying out the studies on the toluidine blue inactivation of the sperm nucleus as a function of pH. The results of these studies are summarized in Table II. In each experiment sperm cells were exposed to dye at 2 or 3 different pH values and for intervals ranging from 7 minutes to 60 minutes. At the end of each interval a sample was removed from each sperm suspension, and 70 to 140 eggs were stripped into it. The percentage of these eggs developing as typical haploids represents the percentage of sperm cells with nuclei completely inactivated by the dye. These percentages, plotted against time of exposure of sperm to dye, give for each experiment a set of curves like those shown in Fig. 3. A rough estimate of the relative effectiveness of the dye at the different pH values can be obtained by comparing the percentage of haploids developing from eggs inseminated with sperm suspensions treated for a given length of time with dye. For example, in Experiment 2 (Fig. 3) this comparison is made for eggs inseminated with the 10 minute sperm samples,

[2] I am indebted to Dr. Morris Spirtes of this Institute for a purified sample of this chemical, prepared by recrystallizing the commercial product twice from hot ethanol after passing the ethanol solution through activated charcoal.

and indicates that at this time 3 per cent of the sperm nuclei are completely inactivated at pH 6.1; 39 per cent at pH 7.1; and 91 per cent at pH 7.9. These are the values listed in line 2 of Table II. All other experiments summarized in this table were done in the same way. Analysis of these results provides the following information. First, the concentration of dye necessary for pro-

TABLE II

*Effect of pH on the Inactivation of the Sperm Nucleus by Toluidine Blue**

Exp. No.	Dye concentration	Time of exposure of sperm to dye	Buffer	pH	Haploids
	M	*min.*			*per cent*
1	5×10^{-6}	24	Acetate	5.1	13
			Diluted Ringer's	6.9	70
2	5×10^{-6}	10	Phosphate	6.1	3
				7.1	39
				7.9	91
3	1×10^{-6}	10.5	"	7.5	5
				7.9	88
4	1×10^{-7}	30	"	7.4	0
				7.8	11
				8.1	41
5	2×10^{-7}	15	"	8.1	13
				8.3	35
6	2×10^{-7}	7.5	Tris(hydroxy-methyl)-aminomethane	8.0	1
				8.4	56
				8.8	90
7	5×10^{-8}	7	Borate	8.9	0
				9.5	19
				10.0	40

* The sperm number in the dye-sperm mixtures was 15,000 and 10,000 per c. mm. in experiments 1 and 2 respectively. In all other experiments it was 1200 to 1600 per c. mm.

ducing significant degrees of inactivation within, say, 7 to 30 minutes, was found to decrease strikingly as pH was raised. Thus, for the lower part of the pH range (*ca.* 5 to 7) the effective dye concentration was about 5×10^{-6} M, in the intermediate range (*ca.* 7 to 8.5) it was 1×10^{-6} to 1×10^{-7} M, and in the upper region (8.5 to 10.0) it was about 5×10^{-8} M. Secondly, within each of these pH ranges the rate of inactivation produced by a given dye concentration was found always to increase as pH was raised. The general

conclusion, then, is that the rate of inactivation of sperm nuclei by toluidine blue is very sensitive to pH changes, increasing steadily as pH is raised from 5.1 to 10.0, with no sign of an optimum within this range.

The most probable interpretation of these results is that the increase in inactivation rate with pH is due, not so much to an effect on the reaction between dye and sperm nucleoprotein as such, as to a change in the rate of penetration of the dye into the sperm head. It has been known for a long time that the penetration of basic dyes into cells is proportional to the concentration of dye existing as free (uncharged) base (10, 11). At low pH values

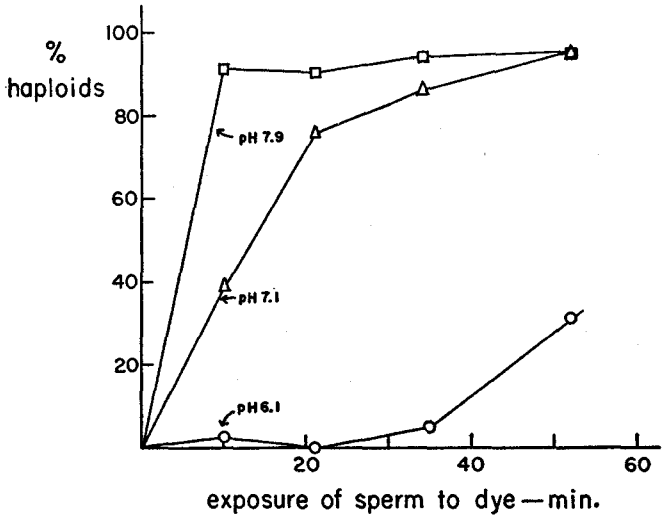

FIG. 3. Graph illustrating for one experiment (Experiment 2, Table II) the effect of pH changes on the rate of inactivation of sperm nuclei by toluidine blue. For further description see text.

where the dye is largely in the cationic form, there is little penetration unless the cell is injured, whereas in alkaline solution the dye is largely converted into free base and passes readily into cells, even though the interior of the cell may remain at a lower pH than that of the medium (11). This situation may very well hold for the dye-sperm system. The interior of the sperm head, consisting principally of nucleoprotein (12), should be well buffered and should change relatively little in pH as the pH of the weakly buffered medium is altered. As the pH of the medium is raised, more and more of the dye is converted into free base and can enter the sperm head. Once it is within the sperm head at a lower pH, the dye presumably is changed back into its cationic form. In this form it is assumed to displace protein from combination with nucleic acid and to be bound mainly to the phosphoric anions of the nucleic

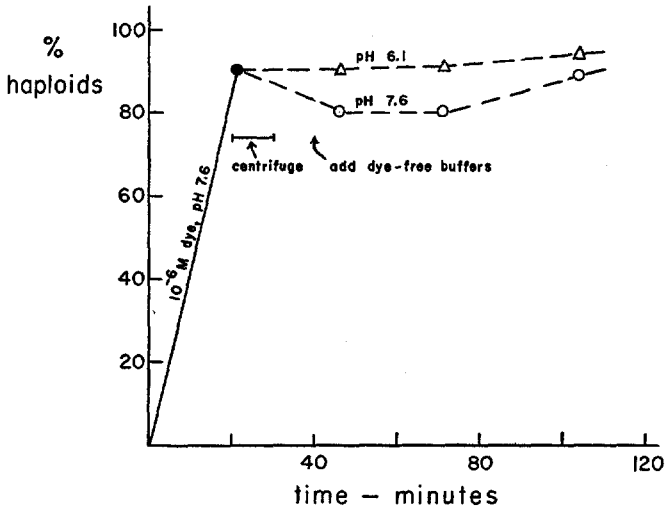

FIG. 4. Test of reversibility of toluidine blue inactivation of sperm nuclei. Sperm cells were exposed 20 minutes to 1×10^{-6} M dye at pH 7.6; then were centrifuged, resuspended in buffer at pH 7.6 and pH 6.1, and tested on eggs at the intervals shown on the graph. Each point represents 83 to 144 test eggs. Total number of eggs used in the experiment = 877.

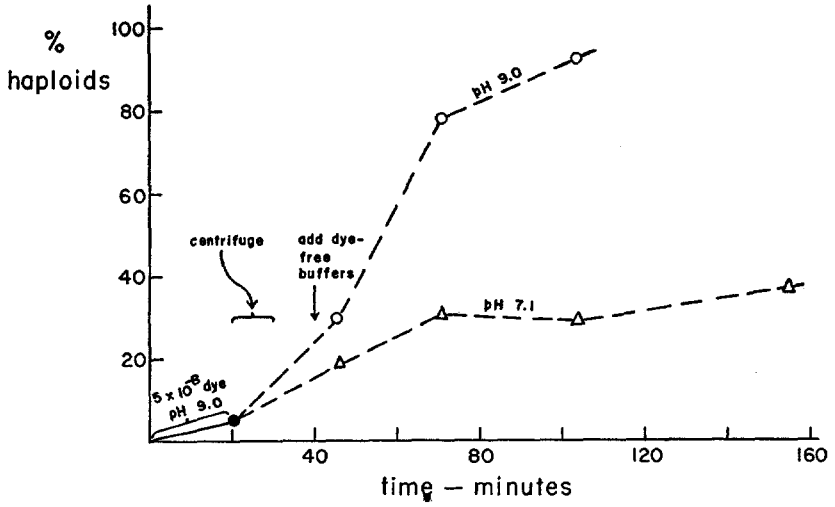

FIG. 5. Test of reversibility of toluidine blue inactivation of sperm nuclei. Sperm cells exposed 20 minutes to 5×10^{-8} M dye at pH 9.0, centrifuged, resuspended in buffer at pH 9.0 and pH 7.1, and tested on eggs in the usual way. Each point on the graph represents 85 to 150 test eggs. Total number of eggs = 930.

acid (see Discussion). The latter type of reaction may also be affected by such pH changes as do occur within the sperm head. For example, a shift to higher pH values would increase the net negative charge on the nucleoprotein and would result in an increase in dye-binding rate provided that an adequate amount of dye remained in the cationic state.

Experiments on Reversibility of the Inactivation of Sperm Nuclei by Toluidine Blue

In these experiments the spermatozoa were first exposed to toluidine blue at a given pH until the nuclei of a known proportion of the cells were inactivated. The cells were then washed once by centrifugation and were resuspended in dilute Ringer's solution buffered at (*a*) the original pH, and (*b*) at a pH 1.5 to 1.9 units lower than the original. This washing procedure eliminated almost all of the dye in the medium—the maximal amount that could remain in the tubes being about 1/100 the minimal effective concentration at the original pH and 1/1000 the minimal effective concentration at the lowered pH. Following removal of the dye from the medium, the resuspended sperm cells were tested on eggs in the usual way at regular intervals in order to determine whether there was any reversal of the nuclear inactivation.

The results of these experiments are summarized in Figs. 4 and 5. In the first experiment (Fig. 4) the sperm nuclei were completely inactivated in 90 per cent or more of the sperm cells. Following removal of the dye and resuspension of the cells at the same pH (7.6), there was a slight reduction in the proportion of cells with completely inactivated nuclei. However, this decrease was followed by an increase to the original level, and cannot be regarded as evidence for reversal of inactivation. When the sperm cells were washed and resuspended at a lower pH (6.1), there was also no indication of reversal of inactivation.

In the second experiment the exposure of sperm cells to dye was limited so that it produced complete inactivation of the nuclei in only 5 per cent of the cells. The cells were then washed, resuspended at the initial pH (9.0) and at a lower pH (7.1), and were subsequently tested on eggs in the usual way. The results (Fig. 5) show clearly that there is no reversal of inactivation. On the contrary, the evidence indicates that inactivation continues even after removal of dye from the medium, the process occurring more rapidly at pH 9 than at pH 7. This would suggest that molecular alterations resulting in inactivation continue to occur for some time after a given amount of dye is taken up by the nucleus.

Microscopic Observations on Toluidine Blue–Treated Sperm

The specificity of the effect of toluidine blue on the sperm nucleus suggests that the nucleus binds the dye preferentially. The following microscopic

observations were made in order to see whether detectable amounts of dye are, in fact, bound by the living sperm cell under the conditions of these experiments, and to determine how it is distributed within the cell.

Three sets of observations were made. They gave the same result and will be described together. The sperm suspensions were treated for 20 to 40 minutes with 1×10^{-5} M or 5×10^{-5} M dye at a pH of about 6.9, and were then centrifuged and resuspended in 10 per cent Ringer's solution. Samples were then observed under a water immersion objective at a total magnification of about 1000 diameters. When first observed the cells were still motile, although less so than were the untreated controls. A rather faint, but none the less definite, bluish color was seen in the nuclei of all spermatozoa. This color appeared to be uniformly distributed throughout the nucleus. (In one experiment we saw in some sperm heads a few clear, unstained spherical bodies or vesicles, but otherwise the dye appeared uniformly distributed in these as well as in other sperm nuclei.) The extranuclear parts of the sperm were completely lacking in blue color, so far as could be seen. However, small amounts of dye might not be visible in structures as delicate as these. Careful observations on control suspensions failed to reveal any blue color in either nuclear or extranuclear parts of the sperm.

It should be noted that in these experiments the sperm cells were exposed to the dye for considerably more than the minimal time required for inactivation of the nucleus, and undoubtedly took up much more than the minimal effective amount of dye—an amount which is probably not detectable under the microscope (see following section). The main value of the observations described here is to show, in a qualitative way, that the dye is taken up by the nucleus, in which it appears to be uniformly distributed, and is not bound by extranuclear structures in microscopically observable amounts.

Measurement of Dye Uptake by Sperm Cells

The next step in the investigation was to measure the amount of dye taken up per sperm cell—the main objective of this being to determine (*a*) the minimal number of dye molecules which must be bound in order just to inactivate the sperm chromosome complex, and (*b*) the maximal amount of dye that can be bound before extranuclear parts of sperm are affected. In principle these measurements of dye uptake are simple. A dye solution is mixed with a sperm suspension and after an appropriate interval the sperm cells are centrifuged out, and the amount of dye they have taken up is determined by measuring the diminution in the dye content of the supernatant spectrophotometrically. The sperm cells are resuspended in dilute Ringer's solution and tested on eggs in the usual way in order to determine the frequency of nuclear inactivation produced by the amount of dye taken up.

The actual steps in the measurement can be illustrated by describing a typical experiment. In this experiment (No. 2) 6 *Rana pipiens* testes were cut up in 3.5 ml. of 10 per cent Ringer's solution. After about 20 to 30 minutes the pieces of testis tissue were removed and the sperm suspension was twice centrifuged and resuspended in fresh 10 per cent Ringer's solution in order to eliminate turbidity in the supernatant. Following the second washing, 0.1 ml. of the suspension was removed for counting, and 1.2 ml. was pipetted into a 3 ml. pyrex centrifuge tube to be mixed later with dye. The remainder of the suspension was centrifuged once again in order to provide (1) a control supernatant strictly comparable with the fluid phase of the above sperm suspension, and (2) control sperm, which were tested on eggs to make sure that the routine of handling produced no damage in itself.

The control supernatant (1.2 ml.) and the test sperm suspension (1.2 ml.) were each mixed rapidly with 0.4 ml. of 2×10^{-5} M toluidine blue. An equivalent proportion of 10 per cent Ringer's solution was added to another sample of the control supernatant to provide a blank for the spectrophotometric measurements. 10 minutes later the three tubes were centrifuged (10 minutes). The sperm supernatant and the fluid from the other tubes were each pipetted into 1/10 volume of 0.1 N HCl. Acidification was essential because at neutral pH the dye is rapidly adsorbed on the walls of the Lowry microcells used for the spectrophotometric measurements.[3] These measurements were done in the Beckman spectrophotometer at 630 mμ, the absorption maximum for toluidine blue. In the experiment being described the supernatant of the dye-sperm mixture (cell 1) gave a density reading (D) of 0.061 compared with a D of 0.116 for the mixture of dye with control supernatant (cell 2)—both taken against the control supernatant blank (cell 3). When read against a water blank the values were 0.081 for cell 1, 0.136 for cell 2, and 0.020 for cell 3. Following correction of these values for the volume of acid added, the density difference between cells 1 and 2 was 0.061. From this value the dye bound by the sperm could be determined on the basis of a previously established plot of dye concentration against D. Using acidified solutions this plot is linear for dye concentrations below 1×10^{-5} molar, and is the same whether the dye is in water or in a control supernatant. (Supernatants with $D > 0.020$ do, however, affect the slope of the curve and for this reason should not be used.) In the experiment being described the reduction in dye concentration brought about by the sperm was 1.85×10^{-6} molar.

[3] The possibility of significant errors arising through adsorption of dye on glassware had to be considered throughout this investigation. For example, in the Lowry microcells, which have a large surface/volume ratio, dye concentration can be reduced by adsorption to about 70 per cent of its initial value in about 30 minutes. Adsorption can be prevented by (*a*) acidifying the dye solution; or (*b*) by pretreating glassware with dye solution and washing out excess (unadsorbed) dye with water; or (*c*) by coating glassware with dri-film 9987 (General Electric Co.). In these experiments adsorption onto the microcells was prevented by acidifying the dye solution, as mentioned in the text. Pipettes used for measuring dye solutions were usually coated with dri-film. Other glassware was pretreated with an appropriate dye solution when this was advisable, as in the case of flasks containing very dilute solutions of dye.

The sperm number was 17,500 per c. mm. From these values it can be calculated that each sperm cell took up 1.1×10^{-16} mole of dye. The biological tests showed that these sperm cells retained the capacity to fertilize eggs, which then developed as typical haploids, indicating that a complete inactivation of the sperm nuclei had been produced by the dye taken up.

The main source of error in the procedure outlined above is probably in the determination of sperm number. Counts made on duplicate samples differed from each other by ±0 to ±4.7 per cent (average = ±3.4 per cent). In addition, the suspensions contained some immature sperm cells. These represented a minority (*ca.* 10 to 30 per cent) of the total cell number and appeared microscopically to take up dye more slowly than did the sperm nuclei. They were not considered in the calculations of dye uptake which may, therefore, indicate somewhat higher values than the true ones.

TABLE III

Measurements of Toluidine Blue Uptake by Sperm Cells

Experiment No.	Sperm	pH	Dye uptake per sperm $\times 10^{-16}$ M	Development				
				Total eggs	Normal cleavage		Typical haploids	
					No.	Per cent	No.	Per cent of normally cleaved eggs
1	*Pipiens*	*ca.* 7	0.5	191	153	80	140	91
2	"	" "	1.1	162	134	83	124	93
3	"	" "	1.5	116	110	95	94	85
4	*Catesbeiana*	" "	2.0	332	95	29	80	84
5	"	" "	2.5	94	75	80	27	36*
6	"	" "	3.6	108	10	9	10	100
7	*Pipiens*	" "	5.9	No tests. Sperm relatively immobile				
8	"	9.4	5.4	274	0	0	0	0

* The low percentage of typical haploids recorded in this experiment does not mean that the sperm nuclei were not completely or nearly completely inactivated by the dye. The majority of the abnormal embryos (not listed as haploids) consisted of small, haploid size, cells, and were probably haploids. A significant proportion (22 per cent) of the diploid controls (*pipiens* ♀ × *pipiens* ♂) also developed abnormally in this experiment.

The results of all measurements of dye uptake are summarized in Table III, which includes also the analysis of the development of eggs inseminated with the dye-treated sperm. These results show that 0.5 to 1.5×10^{-16} mole of dye per sperm produces complete or nearly complete inactivation of the nuclei without impairing appreciably the capacity of the sperm to activate eggs (Experiments 1, 2, and 3). When larger amounts of dye are taken up the fertilizing power of the sperm decreases, indicating that in addition to inac-

tivating the nucleus the dye is now beginning to damage the extranuclear structures as well.

These measurements indicate that the maximal amount of dye that can be bound without producing deleterious effects on extranuclear structures is about 1.5×10^{-16} M per sperm. On the basis of our microscopic observations it may be concluded that most, if not all, of this is bound in the nucleus. The measurements do not define the minimal amount of dye which must be taken

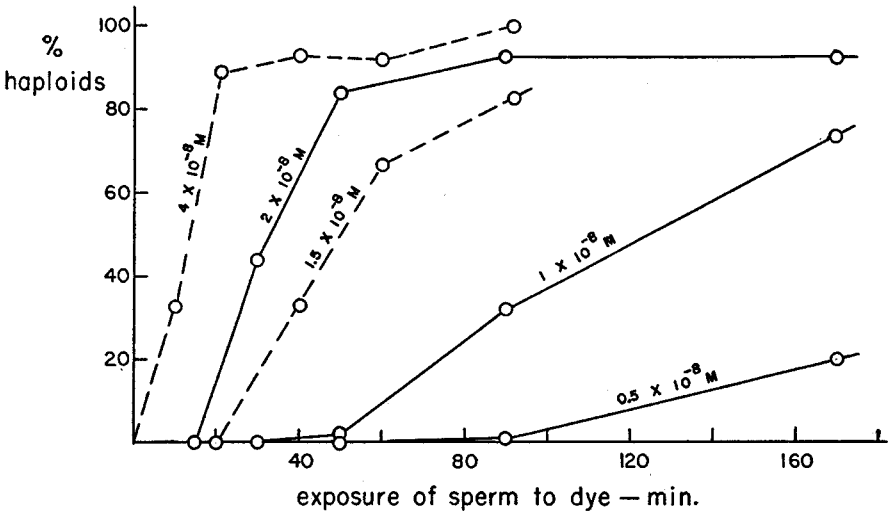

FIG. 6. Determination of minimal concentration of toluidine blue effective for inactivation of sperm nuclei. This graph summarizes the results of two experiments, based on a total of 2848 test eggs. In Experiment 1 (broken lines) the sperm cells (1460 per c. mm.) were exposed at pH 9.4 to 4.0, 1.5, 0.5, and 0.1×10^{-8} M toluidine blue. The two lowest concentrations failed to inactivate the sperm nuclei within 92 minutes and are not included in the graph. In Experiment 2 (solid lines) the sperm cells (1500 per c. mm.) were exposed at pH 9.4 to 2.0, 1.0, and 0.5×10^{-8} M dye. The proportion of sperm cells with completely inactivated nuclei is indicated by the number of haploids developing from the test eggs.

up in order just to inactivate the nucleus. The smallest measured amount of dye taken up (0.5×10^{-16} M per sperm) still produced complete inactivation of the nuclei in practically all sperm cells. By refining the technique and by using quite thick sperm suspensions it would be possible to measure considerably smaller degrees of dye binding. However, for our present purpose of defining roughly the minimal amount required for nuclear inactivation, it seemed advisable to use an easier indirect method. This method simply involves exposing a series of sperm samples to increasing amounts of dye, ranging from ineffective to fully effective amounts. The minimal effective amount

can be located in such a series, and from it one can calculate the minimal effective dye uptake per sperm cell, assuming that all the available dye is taken up. This assumption appears to be valid for the following reasons: In the first place, it has already been shown that the biological effects of dye on sperm nuclei are irreversible. This is compatible with irreversible binding of the dye but does not, of course, prove it to be the case. Secondly, we have done one experiment showing that sperm cells can, in fact, remove all observable amounts of dye from solution. This is Experiment 8 (Table III), in which a sperm suspension was mixed with toluidine blue to give a dye concentration of 4.9×10^{-6} M and a sperm number of 8850 per c. mm. at pH 9.4. After 10 minutes' exposure of sperm to dye, plus a 10 minute centrifuging period, the supernatant fluid appeared colorless to the eye, and in the Beckman spectrophotometer it gave no specific absorption at 630 mμ. In other words, the sperm had reduced the dye concentration of the surrounding medium from 5×10^{-6} M to less than 0.2×10^{-6} M—the minimal concentration spectrophotometrically detectable under the conditions of the experiment.

Two experiments were done to determine the minimal effective amounts of toluidine blue. In both of these the dye sperm mixtures were buffered at pH 9.4—a pH which is sufficiently high so that the rate of reaction between dye and sperm is very rapid, and which still allows the sperm to survive for about 3 hours. Other details concerning the experiments are given in the legend to Fig. 6, in which the results are summarized graphically. From these results it appears that, in mixtures containing 1500 sperm cells per c. mm., the minimal dye concentration which will inactivate the majority of the sperm nuclei is 1.0×10^{-8} molar. If all the available dye is bound by the sperm—as seems probable on the basis of considerations given above—then the dye bound per sperm cell is 6.7×10^{-18} mole. This is roughly 1/7 of the smallest uptake measured spectrophotometrically, and 1/22 of the largest uptake that could be tolerated by the sperm without damage to its extranuclear parts (see Table III). Actually, the minimal effective uptake of dye per nucleus may be somewhat smaller than the amount indicated by these experiments, for what we are measuring here is the minimal amount required for inactivating the majority of the sperm nuclei. Some of these will have taken up just the minimal effective amount of dye, and some will have taken up more than this amount. However, the actual minimal effective uptake cannot be much less than 6.7×10^{-18} mole, for when the amount of dye available per sperm is reduced to half this value (see Fig. 6) only 20 per cent of the sperm nuclei are inactivated.

DISCUSSION

In the preceding section of this paper we have shown that the minimal amount of toluidine blue required in order just to inactivate the sperm nucleus is about 6.7×10^{-18} mole, or 4×10^{6} molecules. This indicates that there are

roughly 4 million binding sites in the nucleus which, when blocked by dye molecules, somehow prevent the sperm chromosomes from participating in the development of the egg.

The first question to be considered now has to do with the nature of these sites. We know that the dye can be seen within the affected nucleus, and that it cannot be detected in the extranuclear structures which, as we have already indicated, remain relatively unimpaired functionally. Thus the proteins and other substances of the extranuclear sperm parts are not combining with appreciable amounts of dye. The same may be assumed for the proteins of the nucleus, which are quite basic and are not likely to combine with a basic dye (13). This indicates that the dye is bound mainly to nucleic acid.

The binding of basic dyes by nucleic acids or nucleoproteins has been extensively studied and is generally thought to occur through interaction between the positively charged dye cations and the negatively charged phosphoric acid groups of the nucleic acid (14–17). In the case of the nucleoproteins this reaction results in the displacement of protein from its combination with nucleic acid (13). Presumably the dye cations compete successfully with the cationic groups of the protein for binding at the phosphoric acid sites of the nucleic acid.

Adopting this view of the reaction we may assume that in the nucleus of the living sperm cell the dye also is bound by the phosphoric anions of nucleic acid, displacing cationic protein groups, and resulting somehow in chromosome inactivation. It is of interest now to estimate what proportion of the total number of phosphoryl groups must be bound in order just to inactivate the nucleus. The total number of groups in the DNA of a frog sperm nucleus can be calculated from the DNA determinations of Mirsky and Ris (18). For the frog, these determinations are reported for the red blood cell (15.0×10^{-9} mg. per nucleus), and for the hepatic cell (15.7×10^{-9} mg. per nucleus). Since the haploid sperm contains about one-half as much DNA as do diploid somatic cells we may assume that the DNA content of the sperm nucleus is about 7.7×10^{-9} mg. Taking the theoretical P content of DNA as 9.9 per cent, the P content of the DNA of a single sperm nucleus will be 7.6×10^{-10} mg., or 2.5×10^{-14} mole. As we have already mentioned, the minimal amount of dye which must be bound in order to inactivate the nucleus is about 6.7×10^{-18} mole. From these values it is calculated that the binding of only 1 out of every 3700 phosphoric acid groups will bring about the inactivation of the sperm chromosomes.

This degree of dye binding is very small indeed compared with that reported for the *in vitro* binding of basic dyes by nucleic acid solutions. In the latter case the equilibrium dye–P ratio may be 1:1 for such dyes as crystal violet (14) or toluidine blue (19); or 1:13 in the case of the binding of methyl green by polymerized DNA (20). Thus, in the living frog sperm only a very small

proportion of the potentially available phosphoryl groups needs to be bound in order to inactivate the nucleus.

Whether there is anything special about the character or distribution of these groups is, of course, a speculative matter. It may be that they correspond to secondary phosphoric acid groups, which are very small in number compared with the primary groups, have a dissociation constant of 6.0 to 6.5 (21), and are presumably located only at the ends of polynucleotide chains. These groups appear to bind dye more strongly than do the primary groups (16, 17), and thus may be the sites which are preferentially bound in frog sperm.

The distribution of the dye-binding sites within the nucleus is certainly widespread, judging from the uniformly stained appearance of the nuclei of dye-treated sperm. If we assume that the distribution is random on the molecular level we can calculate the size of the hypothetical DNA unit which is "inactivated" by interaction with a single dye molecule. This unit will contain about 3700 P atoms. With 1 P atom per nucleotide, the average molecular weight of which may be taken as 330, it is calculated that the DNA unit involved has a molecular weight of 1,200,000 (or some multiple of this if more than 1 P atom is bound per DNA unit). This value is about the same as some of the higher values reported for the molecular weight of DNA (22).

Whether there is actually a DNA unit of this size in the nucleus of the living frog sperm is, of course, quite conjectural. The distribution of the dye binding sites may not be random, as it was assumed to be for the purposes of the calculation, and much work remains to be done on the chemistry of the reaction and on the nature of the effects on chromosomes before dependable information of this kind can be obtained. However, it is clear from these studies that only a small number of dye molecules are involved in the inactivation of the sperm nucleus, and that regardless of the exact nature and distribution of the dye binding sites, the size of the functional units which are directly or indirectly affected must be large. Further characterization of these units may be expected from studies on the dye sperm system, or on some other system which, like it, allows for quantitative estimates of chemical reactions with chromosomal constituents, and at the same time provides a test for the effects of these reactions on the activity of the chromosomes.

SUMMARY

Toluidine blue, applied to frog sperm under appropriate conditions, inactivates specifically the sperm nucleus, leaving the extranuclear parts of the cell undamaged. Thus, the dye-treated spermatozoa stimulate eggs to cleave normally, but contribute no chromosomes to the resulting embryos, which develop as typical gynogenetic haploids.

The concentration of dye required to produce this inactivation varies with pH. Measurements made over the entire pH range which can be tolerated by

sperm cells showed that in the lower part of the range (5 to 7) the effective dye concentration was about 5×10^{-6} M; in the intermediate range (7 to 8.5) it was 1×10^{-6} to 1×10^{-7} M; and for the higher pH values (8.5 to 10.0) it was about 5×10^{-8} M. Using sperm suspensions containing 1500 cells per c. mm. these concentrations of dye produced specific inactivation of the sperm nuclei within 7 to 60 minutes at 18°C.

Tests of the reversibility of the inactivation were made by transferring the sperm from the dye to a dilute Ringer's solution after a known degree of inactivation had been produced. Following removal of the dye the sperm cells were tested on eggs over a period of 2 hours. During this time there was no indication of a reversal of the inactivation.

Microscopic observations of sperm treated with 10^{-5} M or 5×10^{-5} M dye show that the dye is taken up by the sperm nucleus, which is faintly but definitely stained. The dye appears to be uniformly distributed in the nucleus, while extranuclear structures remain unstained.

Measurements of the amount of dye bound per sperm nucleus indicate that the minimal quantity required for complete inactivation is about 6.7×10^{-18} mole, while the maximal amount which can be bound without injury to extranuclear structures is about 1.5×10^{-16} mole. The value obtained for the minimal requirement (6.7×10^{-18} mole $= 4 \times 10^{6}$ molecules) suggests that there are roughly 4 million binding sites in the nucleus which, when blocked by dye molecules, somehow prevent the sperm chromosomes from participating in the development of the egg.

Note Added to Proof.—Since this paper was submitted for publication K. Drebinger (*Arch. Entwcklngsmechn. Organ.*, 1951, **145,** 174) has reported that basic dyes which damage the sperm nucleus in the presence of diffuse daylight, fail to do so in the dark. We have confirmed this result, finding in one experiment that when sperm cells are exposed to 10^{-6} M toluidine blue (pH 7.6) in the dark there is no inactivation of the sperm nuclei. In another experiment we exposed sperm cells to 10^{-6} M toluidine blue for 25 minutes and then washed them free of dye by two centrifugations, all operations being done in the dark or in red light. At this point the nuclei were still unaffected by the dye. The sperm cells were then exposed to the type of diffuse light that was used in all our experiments; *i.e.*, a mixture of northern daylight and fluorescent ceiling light. Within 11 minutes the majority (70 per cent) of the sperm nuclei were completely inactivated. Thus it appears that the dye enters the sperm head in fully effective amounts in the dark, but that no reaction occurs between the dye and the contents of the sperm nucleus until the sperm cells are exposed to light.

REFERENCES

1. Hertwig, G., *Anat. Anz.*, 1924, suppl., 223.
2. Dalcq, A., *Arch. Biol.*, 1931, **41,** 143.
3. Fankhauser, G., *J. Exp. Zool.*, 1934, **68,** 1.
4. Porter, K. R., *Biol. Bull.*, 1939, **77,** 233.

5. Briggs, R., *Growth*, 1946, **10,** 45.
6. Rugh, R., *Proc. Am. Phil. Soc.*, 1940, **83,** 607.
7. Moore, J. A., *J. Exp. Zool.*, 1941, **86,** 405.
8. Briggs, R., Green, E. U., and King, T. J., *J. Exp. Zool.*, 1951, **116,** 455.
9. Gomori, G., *Proc. Soc. Exp. Biol. and Med.*, 1946, **62,** 33.
10. Harvey, E. N., *J. Exp. Zool.*, 1911, **10,** 507.
11. Irwin, M., *J. Gen. Physiol.*, 1927, **10,** 927.
12. Pollister, A. W., and Mirsky, A. E., *J. Gen. Physiol.*, 1946, **30,** 101.
13. Mirsky, A. E., and Ris, H., *J. Gen. Physiol.*, 1951, **34,** 475.
14. Feulgen, R., *Z. physiol. Chem.*, 1913, **84,** 309.
15. Michaelis, L., *Cold Spring Harbor Symp. Quant. Biol.*, 1947, **12,** 131.
16. Cavalieri, L. F., and Angelos, A., *J. Am. Chem. Soc.*, 1950, **72,** 4686.
17. Cavalieri, L. F., Angelos, A., and Balis, M. E., *J. Am. Chem. Soc.*, 1951, **73,** 4902.
18. Mirsky, A. E., and Ris, H., *Nature*, 1949, **163,** 666.
19. Hermann, H., Nicholas, J. S., and Boricious, J. K., *J. Biol. Chem.*, 1950, **184,** 321.
20. Kurnick, N. B., and Foster, M., *J. Gen. Physiol.*, 1950, **34,** 147.
21. Gulland, J. M., Jordan, D. O., and Taylor, H. F. W., *J. Chem. Soc.*, 1947, 1131.
22. Davidson, J. N., The Biochemistry of the Nucleic Acids, John Wiley and Sons, Inc., New York.
23. Battaglia, E., *Pubb. stazione zool. Napoli*, 1950, **22** suppl., 125.
24. Witkin, E., *Cold Spring Harbor Symp. Quant. Biol.*, 1947, **12,** 256.
25. Shumway, W., *Anat. Rec.*, 1940, **78,** 139.
26. Briggs, R., *J. Exp. Zool.*, 1947, **106,** 237.