PRELIMINARY REPORT ON THE EFFECT OF PHOSPHORIZED OLIVE OIL ON THE SPERMATOGENESIS OF ABRAXAS GROSSULARIATA

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ONE TEXT FIGURE AND FOUR PLATES (TWENTY FIGURES)

CONTENTS

INTRODUCTION

The normal spermatogenesis of animals is a subject now largely worked out. In recent years there has been a call for experimental work on the cytoplasmic inclusions, and the writer and his associates have attempted by x-radiation to bring about experimentally changes in the cell inclusions during spermatogenesis. The most interesting work along these lines came from a series of experiments on the x-radiation of

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the larvae of Abraxas grossulariata(7), and in the present paper are given the results of experiments of presumed phosphorus poisoning on the same type of material.

As will be seen, the present experimental work has produced interesting results, and the method should prove of value for further work on the effect of poisons and various narcotic substances on normal cell growth.

The writer cordially thanks Dr. Emil Werner, of the Chemistry School, Trinity College, Dublin, for much valuable information, and Dr. J. W. Bigger, of the Bacteriology School, for making a supply of diphtheria toxin. In this work the writer was assisted by Miss H. Douglas, who made some of the smears and injected some of the larvae.

PREVIOUS WORK

E. V. Cowdry(3) has collected the various references in the literature to the pathology of the Golgi apparatus, to which the reader may refer. Various substances, such as pilocarpine, lead, strychnine, and phosphorus, have been tried on mammalian somatic cells. Such of these researches as refer especially to the present investigation will be discussed in a later communication, but, so far as is known, there is no previous work along the present lines on invertebrate germ cells. Cowdry, in his article on the Golgi apparatus(3), gives a number of figures of the effects of phosphorus on acinar cells of the guinea-pig's pancreas. This author describes the progressive disintegration and final disappearance of the Golgi apparatus in animals exposed to phosphorus poisoning, and his pupil, W. J. M. Scott(8), working on the mouse, comes to the conclusion that the mitochondria also show pathological changes under the same circumstances. Scott states that the mitochondria form agglutinated masses, which fuse to form droplets possessing the characteristics of lipoid. Scott does not say whether he used oil-alone controls, which one would think necessary.

TECHNIQUE AND MATERIAL

The material consisted of larvae of Abraxas grossulariata at their final instar.

Excess phosphorus was heated in olive oil, with stirring, and after cooling, the top part was decanted and used for injections. In a number of experiments this phosphorized oil was diluted by one-half and one-third with untreated oil, or used full strength. The injection was carried out by means of glass pipettes with rubber bulb, the point of the pipette being in the form of a fine needle. The glass needles were prepared by pulling out tubing in a gas flame, producing a point fine enough to pass through the body wall of the larva, but without bending perceptibly. If the needle was too fine, the oil would not flow quickly enough, and also the tip tended to bend.

The larvae were injected toward the front end of the body and, so far as possible, the needle was passed only through the body wall. It was impossible to say, however, whether the gut was uninjured in every case, owing to the twisting movements of the larvae as they were held by fine forceps. Various specimens of these pipettes produced different-sized drops when the bulb was squeezed for a short time. It was therefore never possible to be certain that each batch of larvae got the same dose.

In figure A is a drawing of a larva, with the tip of a pipette, and a drop of average size. It will be noted that the dose of phosphorus the animals received must have been quite small.

In this type of work three sets of controls were necessary. First, larvae merely pricked with a glass needle; secondly, larvae injected with oil alone, and then normal uninjected larvae. So far as the amount of material allowed, such control experiments were carried out several times. The oil-alone controls were pretty extensive, as also were the normal uninjected specimens. Only a few larvae simply pricked and not injected were used, as the supply of material began to run short. While oil-alone controls were indispensable in

this type of work, one would not imagine that such would be necessary except as a formality of experimentation, as it would not be expected that a benign fatty substance like olive oil in such minute quantities would be toxic to the larvae. However, some of these controls appeared to show perhaps a little more degeneration of spermatic cells than is usual, and as with degenerating germ cells, certain minor 'lag' phenomena(5, 7) could be discovered. More of this will be mentioned below.

These larvae are remarkably hardy. Out of the many hundreds injected, only three or four died. If injected when hungry, the larvae begin feeding quite soon after being placed

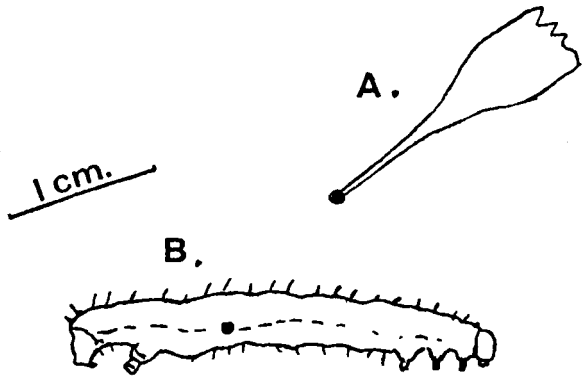

Fig. A A. Point of pipette, showing average drop. B. Caterpillar of Abraxas, with averaged-sized drop near center of body.

on food plant. But in those larvae incubated at 32°C. there was a fairly high mortality after some hours.

Practically all the first experiments were done at room temperature in warm weather, the thermometer registering from 19° to 22°C. Afterward, a temperature of 32°C. was tried. This was too high. It is indicated that a temperature of 22°C. is about right. In both the 18°C.-to-22°C. material and the 32°C. experiments changes began about eleven and one-half to nineteen hours. A sufficiency of experiments was not carried out under strictly controlled temperature. The difficulty here was that it did not seem worth while trying

to do this, unless the caterpillars were carefully picked for size and weight and some method of injecting exactly the same amount of oil into each worked out. Some such technique is very desirable, but under the conditions in which Miss Douglas and the writer were working and with the time at their disposal, a more elaborate technique could not have been used.

Nothing whatever is known as to the fate of the drops of olive oil after injection, so that no information can be given as to how the phosphorus passes through the thick testicular capsule and so to the spermatic cells. It is hoped at some future date to investigate this problem by means of colored oil.

NOMENCLATURE(6)

The germ cells of Lepidoptera contain two separate categories of cytoplasmic inclusions, *Golgi bodies* and *mitochondria,* which show clearly in stained sections and usually in stained smears and which may be seen intravitam.

The Golgi bodies form the acrosome or head tip of the spermatozoon. Consequently, they are often called *acroblasts* (H. D. King). Another term proposed some years ago for the Golgi bodies is *dictyosomes* (Perroncito). The newer nomenclature of Parat is not used, but a separate *vacuolar system* of Parat is present, but does not show in the smears which form the basis of the present description.

THE SMEAR AND THE SECTION

Two types of permanent preparation were used throughout this work—smears and sections of testes. All the figures in the plates relate to smears, and some words of explanation are necessary. Smears can be, but rarely are, as perfect cytologically as sections of testes carefully fixed, upgraded, and embedded. The reasons for the imperfections of smears are that the smearing process can introduce distortion of the inclusions and that the delicate cells are directly exposed to the fixing fluid, which, if not of the proper tonicity, will produce artefacts. In other words, little reliance can be placed

on the correctness of the shapes and sizes of the various cell inclusions in smears. As in sections of badly fixed testes, but much more so in smears, the mitochondria in one and the same smear may be vesicular, hollow tubes, solid filaments, or a matted mass. Only examination of fresh material and comparison with really good stained sections enable one to avoid making mistakes. In some of the figures on plates 2 to 4 imperfectly fixed mitochondria are drawn as seen.

Smears are used in this section of the work, because one is sure that the whole cell is under examination, and the phenomena herein described are so marked as to show in smears. In smears, however, the Golgi vesicle often shrinks and then appears as a stained dot, the delicate threads of the Nebenkern shows either as a solid or a 'bubbly' mass. But apart from the fact that in the smear the whole cell is under examination, there is another important advantage—the smear takes less time to prepare. There is no embedding—the average smear as made by the writer and Miss Douglas taking four days; viz.: Champy, two days; wash under tap, two hours; 70 per cent alcohol,[2] one night; iron alum, one day; haematoxylin, one night. The testes for sectioning had to be upgraded carefully, followed by sectioning and drying out of the slides, which altogether took a week, even if there was no delay in each process.

The author considers that these smears could have been improved, possibly so as to be generally as reliable as sections, by experimenting with the strength of the Champy and by prior osmic-vapor fixation. There is still the objection that during the smearing process, and the consequent flattening of most of the cells, distortion usually takes place. In very few of the Champy smears did the centrioles and flagella stain. These appear clearly in smears fixed in Bouin's fluid.

MAJOR AND MINOR ABNORMALITIES IN GERM CELLS OF LEPIDOPTERA (5, 7)

It is well known that in normal testes certain minor abnormalities may occur. These are practically always due to

[2] If this part is left out, patchy smears result.

'lag effects' traceable to incipient degeneration, and do not occur in young testes. When a large number of spermatozoa have been formed, those cells still in process of metamorphosis tend to grow a little abnormally. Consequently, what the writer calls *minor abnormalities* appear; the nucleus may lag behind the cytoplasmic elements in development, or may even fail to keep its proper position in the cell, ultimately producing the so-called oligopyrene, or apyrene spermatozoa. The commonest abnormality is found in spermatids, where lag effect in the nucleus and normal growth in the acrosome produce a disharmony in the various processes bringing about spermatozoon formation. In old testes, when the caterpillars are pupating or very near this period, lag effects in spermatocyte divisions, producing several nuclei, instead of one, from the telophase chromosomes, are common. With the exception of the latter phenomenon, these minor changes are often difficult for any but the expert to recognize. In figure 4, for instance, is an oil-alone control showing an advanced acrosome at *A*. This cell is slightly abnormal. In the following pages, what the writer would call *major changes,* not yet found in any type of control, are described.

THE PHOSPHORUS-AND-OIL-INJECTED MATERIAL

a. Earliest changes in Golgi bodies of spermatocytes

These changes appear in smears at about sixteen hours after injection, but can be found in sections as early as eleven and one-half hours. In cells in which the mitochondria lie to one side, leaving a clear space, as occurs quite often, some of the dictyosomes or Golgi bodies may be seen to be in process of running together, to form usually one aggregation of granules, as depicted in figures 6 and 7. At first the apparently crescentic nature of each participant is still preserved, but as the aggregations become more easily found, that is, stain more easily, the body shown in figure 8 becomes formed. In other words, the osmiophile parts of the Golgi bodies unite and form a reticulum. Inside this (the chromophobe part) a dark bead soon appears (fig. 9). This latter

process, so far as is known, takes about three hours, but the time can only be judged roughly. But in the nineteen-hour material some of these bodies are relatively enormous, as shown in figure 13. They are usually about the size shown in figure 12, but vary considerably. In any case these bodies must have grown in size, and have increased in staining ability. Nothing like them has been described before by any author who has studied the normal spermatogenesis of Lepidoptera. In a good many cases the cell produces two Golgi-body aggregates, as shown in figures 6 and 13. In a given nest of spermatocytes almost all the cells contain one of these bodies, which may be called abortive acroblasts, but almost always one can find a few cells in which none is to be discovered.

Now the growth of the cell and the formation of the chromosomes of the maturation division go on otherwise as usual, and the primary spermatocyte *enters division quite normally* as shown in figure 14, the large abortive acroblast being carried over to one or the other daughter cell, and never dividing. In figure 15 is an otherwise normal second spermatocyte telophase showing the enormous acroblast in one cell. Finally, about one out of every four spermatids contains an overgrown acroblast, the other cells usually being apparently quite normal. In figures 17 to 20 is such a group. Now, in spite of this acroblast formation, not all spermatocyte Golgi bodies are used up, and there is usually enough of the normal acroblasts left to form the normal Golgi vesicles at this period in the spermatid (*GB* in fig. 18).

b. The spermatid

In figures 1 and 2 are normally forming and formed spermatids. The Golgi elements appear to be rings or vesicles surrounding the nucleus, and at telophase all the elements lie near the latter, so that the newly formed spermatid has the appearance shown in figure 2.

In figures 16 to 20 are five spermatids, of which two (figs. 17 and 18) look fairly normal. In the others abortive acro-

blasts (*AA*) can be seen. These range from fairly small specimens, as seen in figure 20, to very large ones, as in figure 17, and may range from banana-shaped, as in figure 20 (and fig. 15), to subspherical, as in figure 19. In a large group of spermatids, as has been mentioned, only about one in four contains abortive acroblasts of such a large size.

Turning now to figures 10 and 11, we see two more interesting types. In figure 10 there are four abnormal acroblasts, *X*, *Y*, and *Z*, and some normal ones at *GB*. The abnormal one at *Z* has become fixed to the nucleus and has been accepted, but those at *X* and *Y* are too far away. The two examples at *X* are in process of being absorbed and stain much less intensely than the others.

In figure 11 the abortive acroblast has become attached to the spermatid nucleus, but a fairly normal acroblast is present at *GB*.

In subsequent stages the abortive acroblasts fail to keep their position at the head of the cell, and drift into the tails of the lengthening spermatozoa.

c. Changes in the mitochondria

In some of the sectioned testes not described in this paper, the mitochondria of many spermatocytes, even in well-fixed and stained material, existed as peculiar tubular forms. Closer attention was drawn to these by the discovery of unusual bodies of the same appearance in spermatids in smears as depicted in figure 11 at *MX*. Each of these elongated structures appeared hollow and contained in a widened area near the middle a granule (*G*). The nature of these was not at first understood, but the writer now regards them as formed from mitochondria which are precociously secreting the tail sheath substance or 'central substance' of Bowen (1). In figure 14 similar tubular forms are seen, in a smear preparation, mixed up with normal granular mitochondria.

DISCUSSION

a. Critique of method

Now the results described in this paper might be due to the following causes: *a*) Trauma of the body wall by pricking. *b*) Effect of olive oil. *c*) Effect of phosphorus. *d*) Accidental pricking of the gut, and escape of digestive juices into haemocoel. It may seem that an elimination of a number of these possibilities would be easy. While this is in general quite true, it is not easy completely to eliminate all such factors and prove that the phosphorus alone causes the changes which this paper describes. It was found that the changes in the germ cells were at their best at about sixteen hours after operation. Control batches were killed before, at, and after this period, and examined. No control testis ever showed the remarkable swelling of the spermatocyte Golgi bodies, and we seem justified in believing that the major changes described in this paper are due to phosphorus.

This may seem quite sound, but the difficulty is that in such controls one can never be quite sure that the abortive acroblasts have not suddenly appeared and been absorbed.

In addition, however, we tried olive oil in which sulphur had been melted and stirred and also injections of diphtheria toxin. No major changes were noted after injection of such substances.

Not all the testes dissected from phosphorus-treated caterpillars showed changes. Nor did all the spermatocyte nests show equal changes. The reason why certain caterpillars in a positive batch were negative for the changes in the germ cells was assumed to be that the injected drop of oil ran out with the slight effusion of haemocoel fluid as the glass needle withdrew. This could be seen to occur in a number of cases.

Regarding the last-mentioned possibility, namely, that injury to the gut released fluid which itself caused the changes, it is again difficult absolutely to eliminate such a possibility. Specimens in which the gut had been deliberately run through showed no changes at eighteen hours.

Finally, many of the Abraxas testes harbored bacteria. These were found alike in controls and experimentals, and naturally could not be eliminated. In all the normal controls, prepared for this and the previous work on Abraxas(7), and in a large collection of material prepared during some years past, the author has never found definite changes caused by such bacteria. Cells simply crammed with bacteria undergo mitosis quite normally and in step with non-infected sister cells neighboring. This is probably by virtue of the spindle bridges between the sister cells of a group.

b. Comparison between the effects of phosphorus and x-radiation

The fact that abortive acrosome formation takes place both after x-radiation and phosphorus poisoning suggests strongly that this effect is due to incipient cell necrosis in both cases. But there are a number of differences. In the x-radiated material the abortive acroblast formation rarely takes place away from the nuclear membrane, the dictyosomes at first adhering to the latter and only later dropping off(7). In the phosphorus material, however, the abortive acroblasts very rarely adhere to the nucleus, the process taking place in the cytoplasm often far removed from the nucleus.

There is another difference, which seems important. The abortive acroblasts in the phosphorus material *grow quite often to a very large size.* That is, the size of the abortive acroblasts is not determined by the number of dictyosomes which contribute, but secondarily by actual growth. This was never found in x-rayed material.

An explanation of these facts, which seems likely, is that x-rays age the whole cell, and the spermatocyte nucleus after such treatment is more 'differentiated' than is the nucleus of phosphorus-treated material, where nuclear changes happen, if at all, much later, and then only after intense disturbance in the cytoplasm.

c. The effects of phosphorus on the mitochondria and the Golgi bodies

Two questions will at once arise in the mind of the reader of this account: What happens in ordinary cell necrosis, and could the remarkable changes in both mitochondria and Golgi bodies be caused by other toxic substances?

In the first place, as mentioned on page 265, ordinary necrosis is common in insect testes, when pupation or emergence is at hand. But in no case has the writer ever found such large abortive acroblasts. As to the effects of other toxic materials like arsenic, alcohol, chloroform, strychnine, etc., no experiments have at present been carried out, except in the case of diphtheria toxin and sulphur, which gave no result by the method used.

Until the effects of arsenic, antimony, lead, etc., have been tried, it would be unprofitable to attempt to discuss the pharmacological and cytological issues of this type of investigation. It is quite safe to write at present that in this material the Golgi bodies were more sensitive to phosphorus than were the mitochondria, and that abnormalities in the amphiastral figure and chromosomes were practically non-existent in phosphorus-injected material at the same stage when, in x-radiated testes, the well-known lagging effects in the amphiastral figure and chromosomes were common.

SUMMARY

In this paper it has been shown that phosphorized olive oil has a special effect on the cytoplasmic bodies of the male germ cells of Abraxas grossulariata. It is possible to bring about those processes which should normally take place in the spermatid, so that they precociously occur in the spermatocyte.

This 'stimulation' brings about the running together of a number of Golgi bodies in one part of the cell, so that relatively enormous acroblasts are produced. These acroblasts may grow considerably and then pass over whole to one of the daughter cells at mitosis, so that spermatids are formed

containing very large whole acroblasts, which are non-functional, and which finally drift down the forming tails of what may be otherwise normal spermatozoa.

It has been possible to show that the acrosome of the Abraxas spermatozoon is purely the product of the Golgi bodies, and is not due in any way directly to activity of the nucleus, as has been insisted upon by Vejdovsky(9).

In a smaller number of cases the mitochondria became affected at the same period (eleven to sixteen hours) and produced tubes with a contained granule, supposed to be homologous with Bowen's 'central substance,' or the sheath substance of the sperm tail, again a case a precocious secretion as with the Golgi elements.

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DESCRIPTION OF PLATES

ABBREVIATIONS

A, acrosome
AA, abortive acroblast
CHR, chromosomes
G, secreted granule of mitochondria
GB, Golgi body
GBR, Golgi-body remnant
M, mitochondria
MX, modified mitochondria
N, nucleus
NBK, Nebenkern
X, *Y*, *Z*, abortive acroblasts in various stages

All drawings from smears made as stated on page 264.

PLATE 1

EXPLANATION OF FIGURES

1 to 3 Spermatid stages from oil-alone controls.
4 Spermatid showing acrosome more advanced than rest of the cell.
5 Phosphorus-treated, incubated at 32°C. Abnormal.

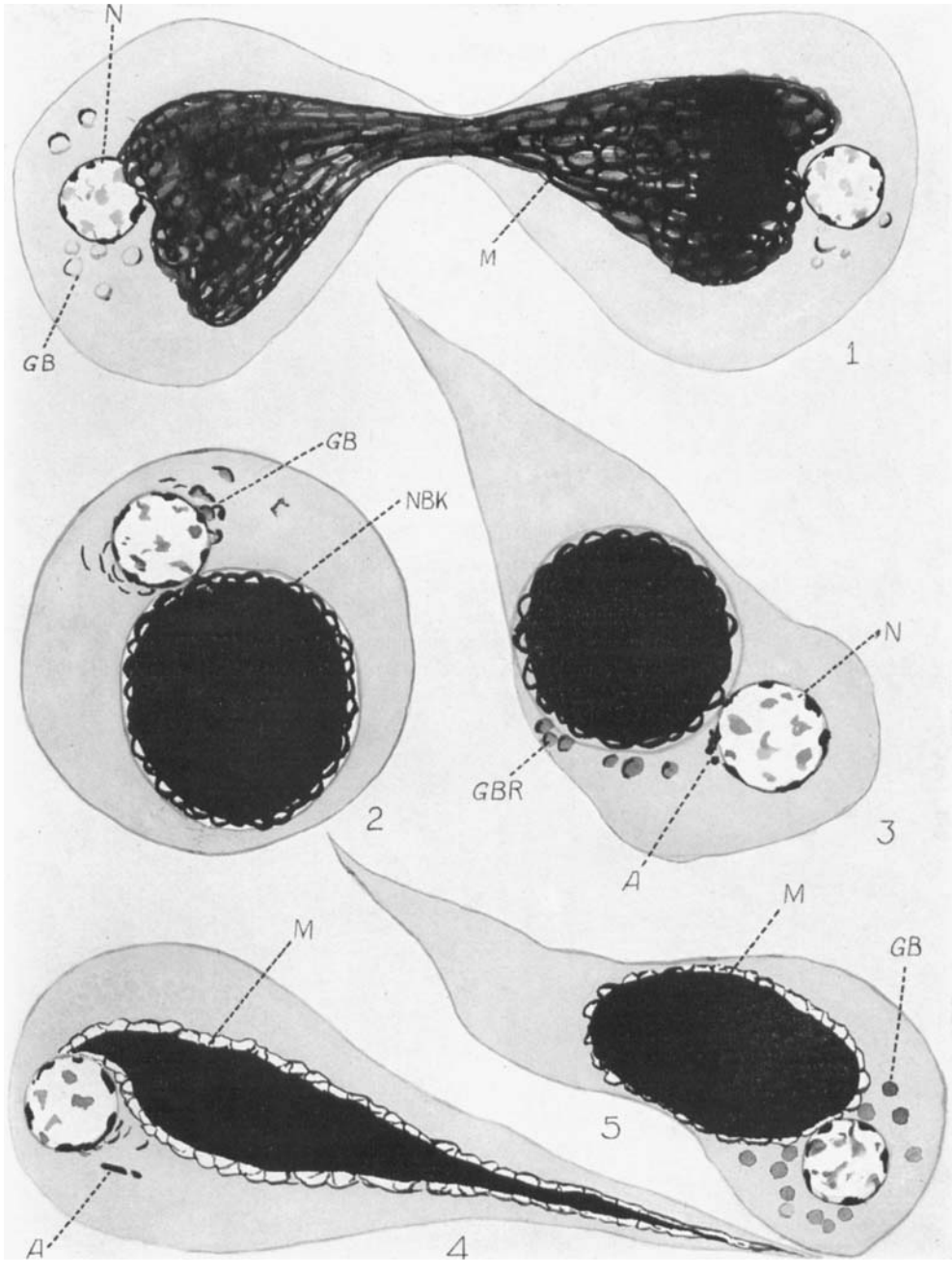

N
M
GB
1
GB
NBK
2
N
GBR
3
A
M
GB
5
M
A
4

PLATE 2

EXPLANATION OF FIGURES

Half-strength phosphorus-oil treated.

6 and 7 Stages in running together of Golgi bodies of spermatocyte to form together abortive acroblasts (AA^1, AA^2, AA^3).

8 and 9 Abortive acroblasts at later stage.

10 and 11 Abnormal spermatids showing acroblasts, abortive (X, Y) and successful (Z), and normal Golgi bodies, and large abortive acroblast adhering to nucleus in figure 11. Supposed abnormal mitochondria at MX.

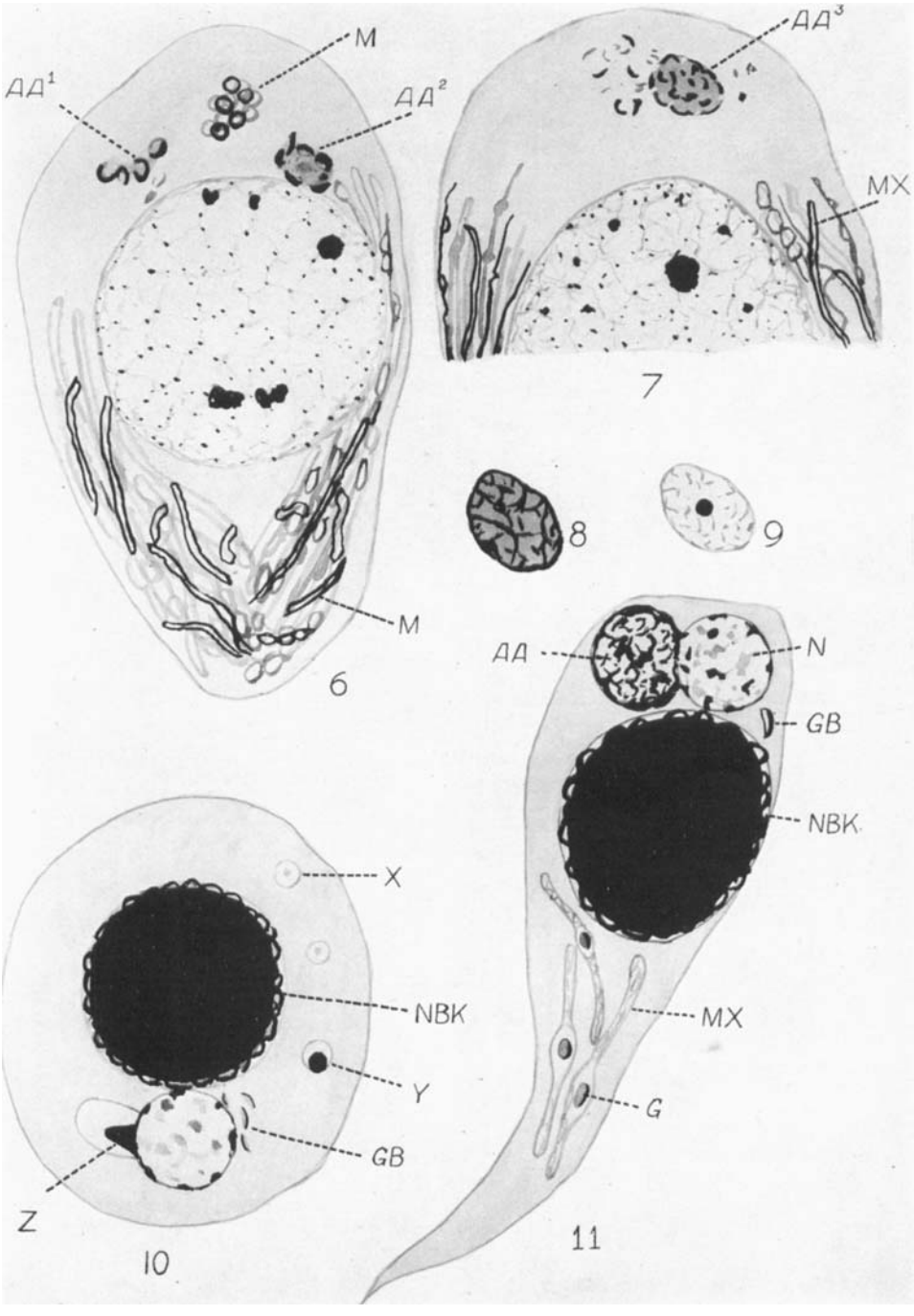

M
AA1
AA2
M
6
AA3
MX
7
8
9
AA
N
GB
NBK
MX
G
11
X
NBK
Y
GB
Z
10

PLATE 3

EXPLANATION OF FIGURES

All phosphorus-treated, undiluted oil.

12 to 14 Spermatocytes in different stages, with abortive acroblasts.

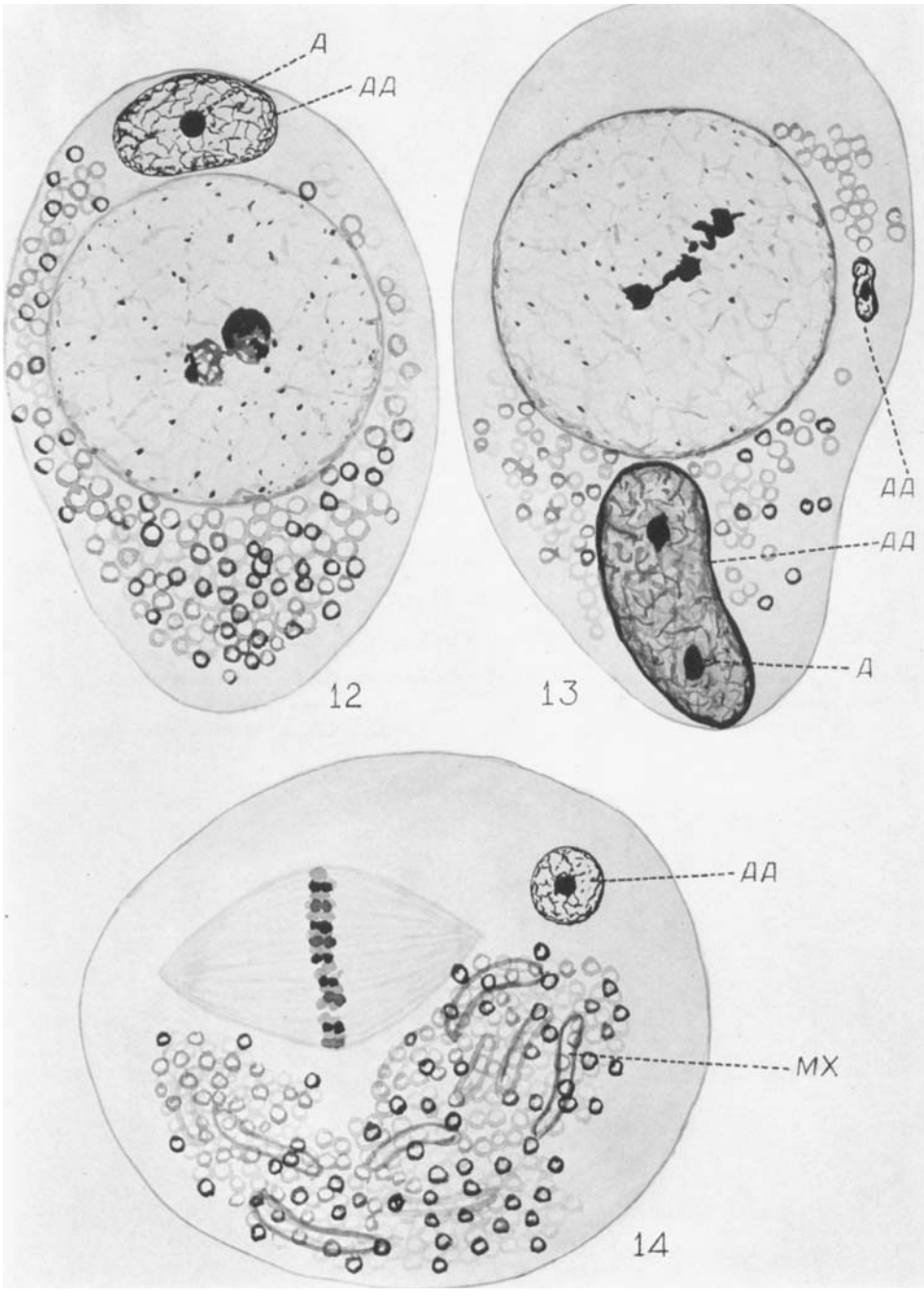

A
AA
12
AA
AA
A
13
AA
MX
14

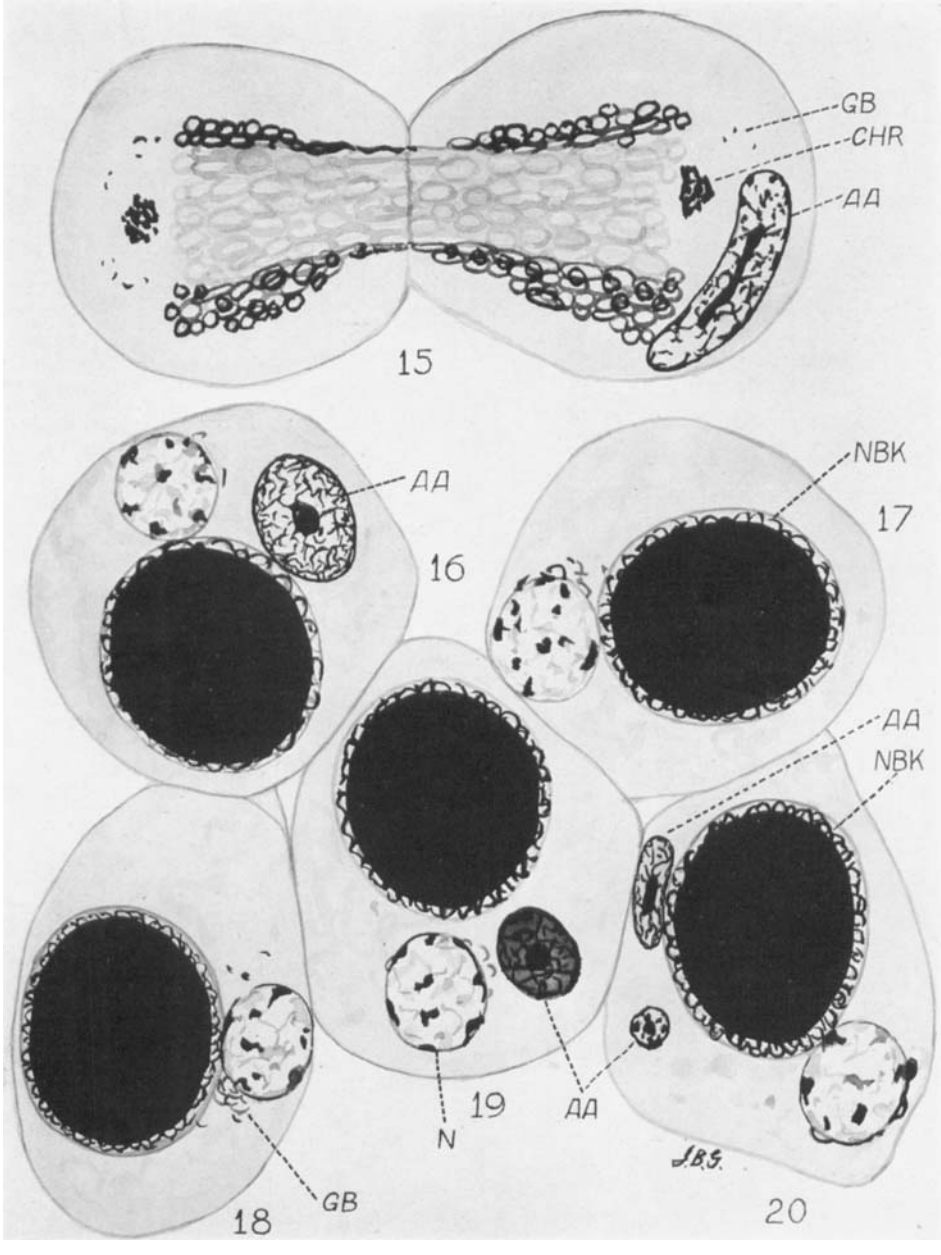
PLATE 4

EXPLANATION OF FIGURES

All phosphorus-treated, undiluted.

15 Telophase of second maturation division, showing abortive acroblast in one daughter cell.

16 to 20 Group of spermatids, some with, some without abortive acroblasts.


GB
CHR
AA
15
AA
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NBK
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AA
NBK
N
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AA
GB
18
20