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METABOLIC DNA IN TIPULA OLERACEA*

By

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With 18 Figures in the Text

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Introduction

Seven main sources of evidence support the assumption that DNA is the essential carrier of genetic information. (1) Its location in chromosomes. (2) The relation between DNA content and chromosome number. (3) The relative metabolic stability of DNA. (4) The complementary structure of the molecule. (5) The effects of mutagenic agents on DNA. (6) Transformation in bacteria. (7) The role of DNA in virus reproduction.

Of this evidence, metabolic stability, is perhaps the least well established.

DNA synthesis is known to occur at a definite period of the cell life cycle. The amount of DNA doubles during preparation for mitosis, and gets reduced to half in direct relation to the anaphase distribution of chromosomes. DNA is only synthesized in chromosomes which prepare for mitosis or undergo replication leading to polyteny or endopolyploidy. These results have lead to the assumption that the amount of DNA remains constant throughout division and that DNA is metabolically stable. They do not exclude, however, a certain amount of metabolism in DNA. If such a metabolism occurred in small amounts at specific chromosome segments it could not easily be detected by the methods which were used to establish the behavior of DNA in whole nuclei.

In the oocytes of several *Tipula* species *(Diptera)* besides the chromosomes and the nucleolus there is a Feulgen positive body. This body is formed in the divisions preceding meiosis in contact with the sex chromosomes, enlarges before pachytene, but suddenly disintegrates at diplotene. The body is not present in the nucleus by metaphase I (BAUER 1932, 1952, BAYREUTHER 1952, 1956). The Feulgen positive body appears suddenly in the secondary oogonia in the resting nucleus of the two daughter cells that result from a mitotic division. In the next

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division no new body is formed but the one present is included in one of the daughter cells. Two more divisions follow and sixteen cells are formed. Of these sixteen cells only two possess the two original bodies formed. Of these two cells one becomes an oocyte the other a nurse cell. The 14 cells without body also become nurse cells. The final result is that only one of fifteen nurse cells possesses the body but that all oocytes contain it. This sequence of events was described by BAUER (1952).

The studies of BAUER and BAYREUTHER on this particular nuclear body did not involve any other chemical test than the Feulgen reaction. The tritium technique is particularly suitable to check whether DNA synthesis actually occurs in the body and to study its metabolic behavior. This investigation describes the results of the incorporation of tritiated thymidine into the nuclei of larvae of *Tipula oleracea*.

Material and methods

Autoradiography. — *Tipula oleracea* is a fly with $2n = 6 + XX$ in the female and XY in the male. The larvae go through 4 instars before becoming pupae. Larvae at the fourth instar were injected with tritiated thymidine. This is the period when the last divisions preceding meiosis take place and when the initial stages of meiosis are found. Tritiated thymidine was obtained from Schwarz BioResearch, Inc., Mount Vernon, N.Y., with a specific activity of 1.88 curie/mM. During injection the larvae were kept in position by covering part of the body with rubber strings fixed with pins to solid paraffin. The injection was given with a thin glass needle connected with a tuberculin syringe graduated in 0.01 ml. The operation was performed under a large field binocular microscope. Each larvae received 0.02 ml. injected directly into the body cavity at the third posterior segment. No anesthesia was used. The hole made by the glass needle was very small and there was no or very little loss of fluid. Of 132 animals injected only six died.

Squashes. — The larvae were taken from the culture and cut with a pair of scissors close to the anus. The ovaries were removed under a large field binocular microscope and fixed in acetic-alcohol 1:3 for 15 to 30 minutes. After passage through water, they were hydrolyzed in N HCl at 60° for 10 minutes. Passed through water (1 minute) and stained in fuchsin-sulphurous acid for two hours. The material was squashed in 45% acetic acid, the cover slips were removed by the dry ice technique and the slides were passed through the alcohol series: 100%, 70%, 35% to water. AR-10 stripping film from Kodak Ltd., England, was applied and the preparations were exposed for 24 hours in boxes kept at 0—4° C. Kodak D-19 and F-6 were used for development and fixation, respectively. After that the slides were washed, dried and studied by placing immersion oil directly over the film.

Larvae. — Two series of injections were performed. In both cases the animals used were at the first, second, third and fourth days of the fourth larval instar. This is the period when the Feulgen positive body is formed in the ovaries. The animals were fixed 2 and 4 days after injection, the period of time which was considered suitable to get the body labelled. Besides this material used for squashes, the ovaries of 20 larvae were sectioned following the procedure described below for the pupae ovaries.

Pupae. — Over 50 larvae were allowed to develop into pupae with a view to study the disintegration of the Feulgen positive body that occurs at this period of life. Fixations were made of animals between 1 and 7 days after they initiated the pupal stage. After 7 days of pupal life the animals normally become adults. The ovaries are at this period much larger and the eggs get hard. The eggs were fixed in acetic-alcohol 1:4. Subsequently they were dehydrated in 100% alcohol, transferred by steps to mixtures of ethanol-butyl alcohol, and embedded in paraffin. The sections were cut with a Reichert rotary microtome at 2 microns. After sectioning, the slides were passed through xylol and the alcohol series to water. Hydrolysis and staining followed in the same way as for the squashes. After fuchsin-sulphurous acid the slides were rinsed in SO_2 water (3 changes) passed through distilled water and AR-10 applied.

Spectrophotometric measurements. — A highly sensitive recording microspectrophotometer designed by CHANCE, PERRY, ÅKERMAN and THORELL (1959) was used in this investigation. In this apparatus the light received from the microscope is then reflected with a 45° mirror into a system of vibrating mirrors which move in a vertical direction and flash the light from two positions of the image intermittently upon an adjustable aperture. A photo tube and an amplifying circuit transmit the signal to a recorder which plots the difference between "reference" and the "measure" singals. This reference is almost equal to the optical density difference. Other details of the apparatus and of the technique used in chromosome measurements are found in LIMA-DE-FARIA (1961). The photo cell aperture corresponds to an area at the plane of the object equal to 0.7×0.7 microns. This allows us to study the DNA content of chromosome segments which are only 0.7—1.0 micron long. The photometric measurements were made in *Tipula* nuclei from Feulgen squash preparations made after the technique described above.

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Results

In the anaphases that precede the onset of meiosis, *i.e.*, that take place in the oogonia, the body is relatively small and displays various shapes. It is often spherical, or egg shaped but it may also have the form of a cube or a parallel piped (Figs. 1—4). At this stage its diameter is circa 1.5 microns. At the onset of meiosis, the body becomes much larger now having a diameter of 6 microns. Its shape is now that of a large sphere (Figs. 10—12).

During the oogonial divisions the body is best seen during anaphase. At prophase and metaphase the body is not always distinguishable from the equally Feulgen-stained chromosomes due to its small size. At anaphase it is usually in contact with the chromosomes of one group. The autoradiographic preparations show the body and the chromosomes heavily labelled (Figs. 3 and 4). This means that in this case the body is synthesizing DNA at the same time as the chromosomes. The body is large enough to be easily resolved by the tritium beta particles.

Larvae which had initiated the fourth instar 1 day before injection and were fixed 48 hours after contact with the tritiated thymidine show the body labelled both in oogonial anaphases and in oocytes (Table 1).

This evidence and the fact that the body more than triplicates its diameter during oocyte formation is a good indication that it goes on synthesizing DNA during the four interphases that occur between its formation and leptotene (Fig. 1). Moreover, older larvae, at the fourth

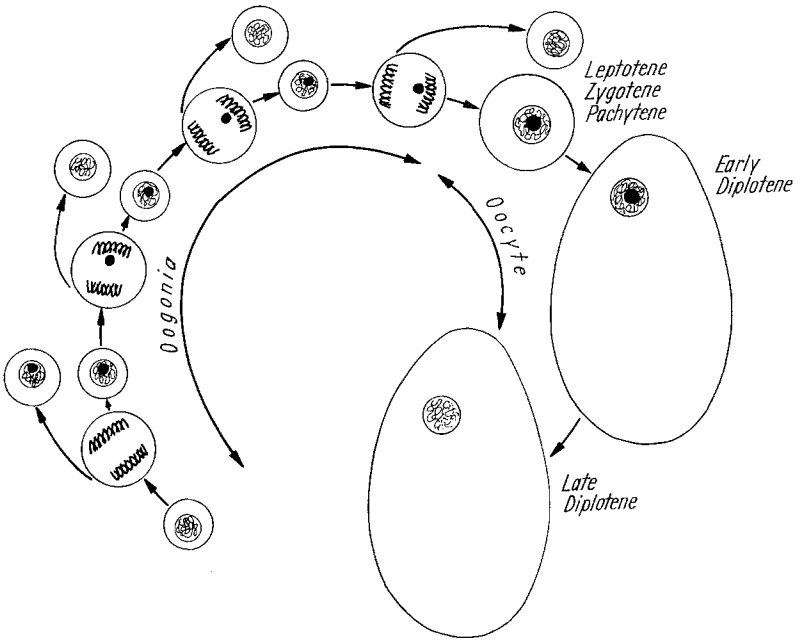


Fig. 1. The life cycle of the Feulgen positive body from formation in the oogonia to disintegration at late diplotene in the oocyte. The body is represented as a black circle. The events during the oogonial divisions are based on the work of BAUER (1952). The body and the other cellular components are not drawn to scale

Table 1. *Occurrence of labelling in the Feulgen positive body after injection of H^3-thymidine into larvae of Tipula oleracea*

Age of larvae since they entered IV instar	Number of preparations studied	Period between H^3-injection and fixation	Body during mitotic stages of the oogonia	Body in oocytes
1 day old	3	2 days	not identifiable	labelled
	9	4 days	labelled	labelled
	5	2 days	labelled	labelled
2 days old	3	2 days	labelled	labelled
	5	2 days	not identifiable	labelled
3 days old	20	2 days	not identifiable	unlabelled
	5	2 days	not identifiable	unlabelled
	5	2 days	labelled	labelled
4 days old	18	2 days	not identifiable	labelled
	5	2 days	labelled	labelled

day, have still many oogonial anaphases with the body labelled (Table 1). The animals are not fully synchronized in their development but it is most improbable that this is just the first oogonial division following the formation of the body.

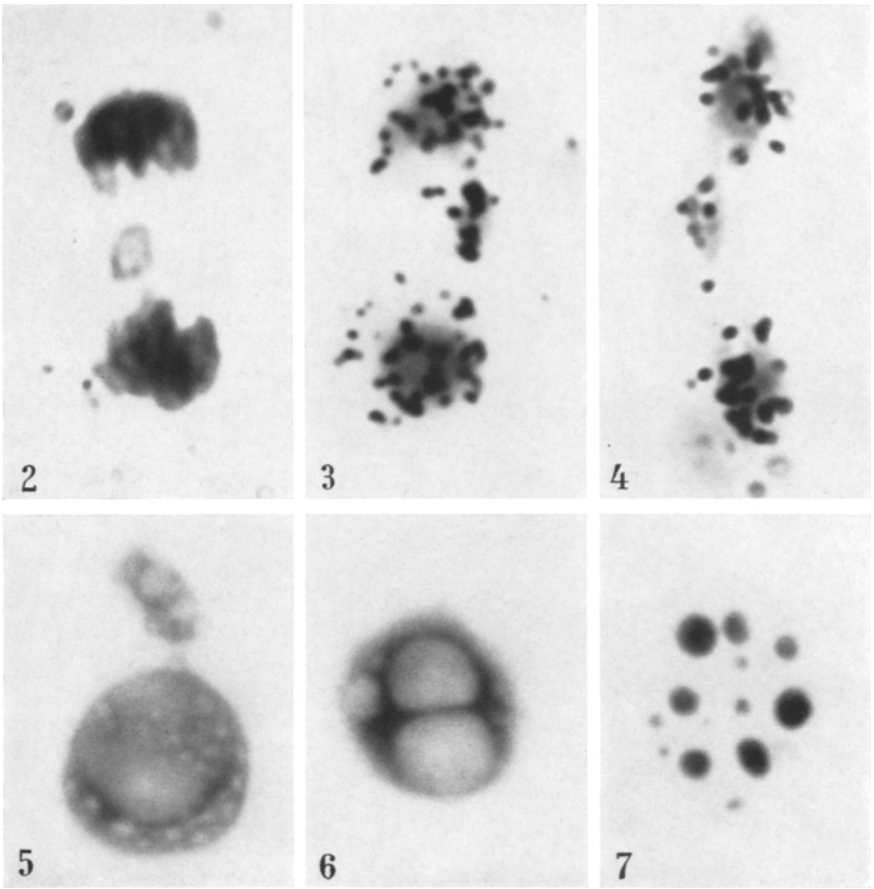
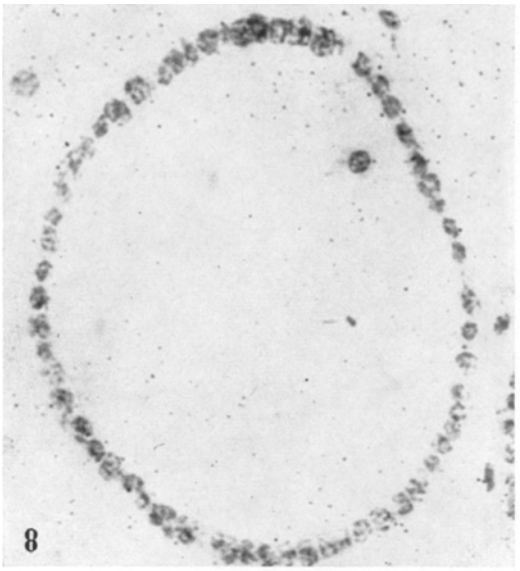
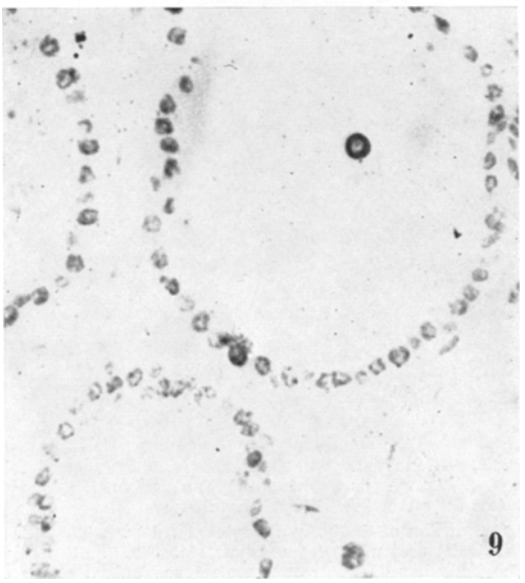


Fig. 2—4. Oogonial anaphases. Fig. 2, Anaphase showing the Feulgen positive body halfway between the poles. Figs. 3 and 4. Anaphases showing the Feulgen positive body and the chromosomes fully labelled with H^3-thymidine

Figs. 5—7. Three stages in the disintegration of the body at diplotene. Fig. 5. The body shows a mass of Feulgen positive material protruding from its surface. Fig. 6. The body exhibits large vacuoles. Fig. 7. The body has disintegrated into minor Feulgen positive spherules

The asynchrony of DNA synthesis of the Feulgen positive body in relation to the chromosomes is particularly distinct at prophase of meiosis. In leptotene-pachytene cells four categories of nuclei are found in the preparations: (1) Nuclei not labelled (Figs. 10—12), (2) nuclei





Figs. 8 and 9. The body in the oocyte at diplotene

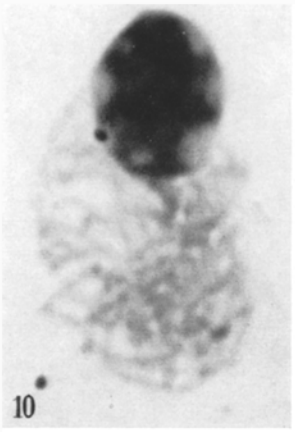
labelled in the body and not in the chromosomes (Figs. 13—15), (3) nuclei labelled in both body and chromosomes, and (4) nuclei labelled only in the chromosomes (Figs. 16—18). This means that there is a period during which the chromosomes synthesize DNA alone, a period where body and chromosomes synthesize simultaneously and a third period where the body synthesizes DNA when the chromosomes are not active.

As the prophase of meiosis proceeds the body starts to get very vacuolized and during diplotene it suddenly disintegrates (Figs. 1, 5—7). To follow the disintegration process, sections were made of ovaries of pupae at various periods of development (Table 2). The eggs at this stage are relatively large and the body is seen strongly stained in the nucleus where the chromosomes are very weakly stained (Figs. 8—9).

Figs. 10—12. Three nuclei at pachytene showing the large Feulgen positive body. Note its size in relation to the chromosomes

Figs. 13—15. Nuclei at pachytene showing incorporation of tritiated thymidine into the body but not into the chromosomes (at another focus than the silver grains)

Figs. 16—18. Nuclei at the same stage as Figs. 13—15 showing incorporation of H^3-thymidine into the chromosomes but not into the Feulgen positive body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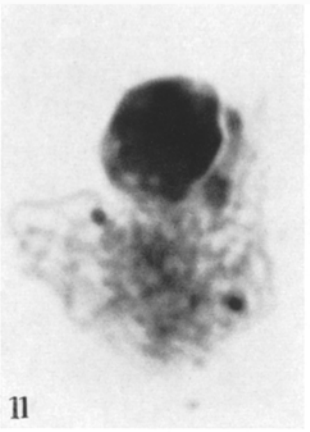
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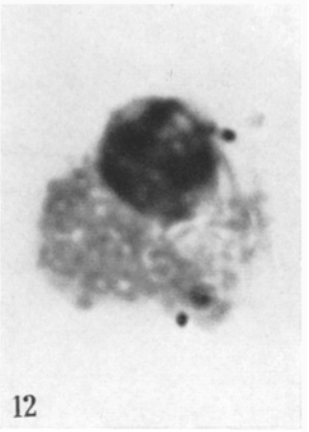
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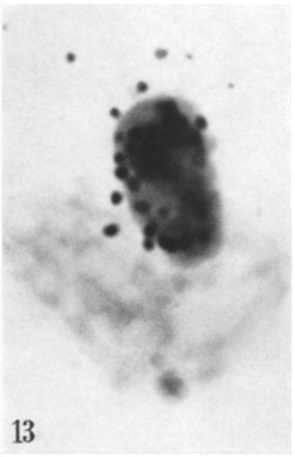
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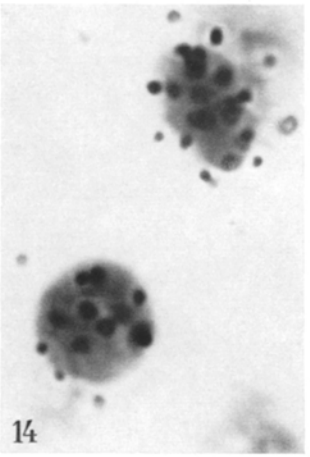
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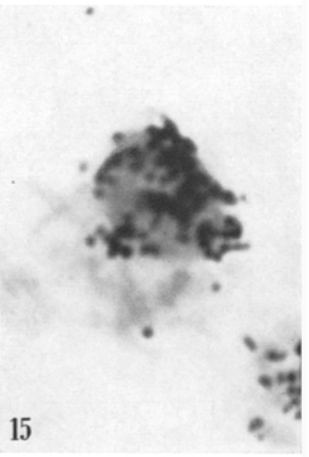
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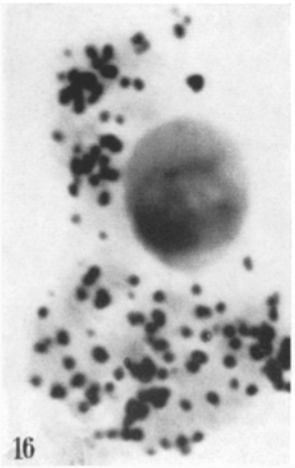
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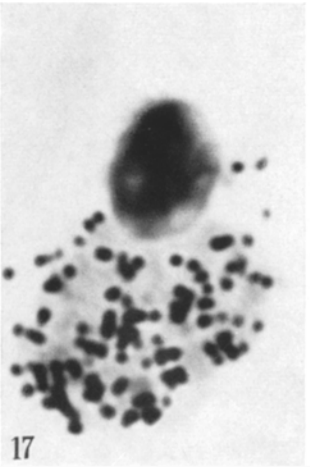
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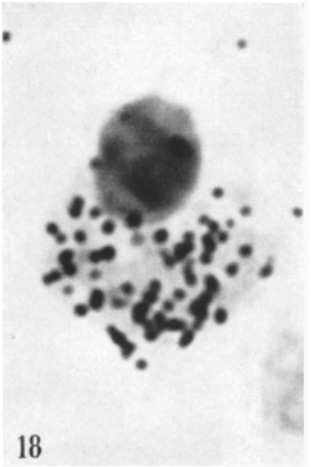
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Table 2. *The disintegration period of the Feulgen positive body in pupa oocytes at the diplotene stage*

Pupa no.	Age in days	Feulgen positive body	Preparation number																Total	Yolkgranules in cytoplasma
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16		
1	1	unlabelled	1	4	10	18	14	20	26	23	12	5	5						138	not conspicuous
		labelled	0	0	0	0	2	0	0	0	0	0	0						2	
2	2	unlabelled	11	10	30	14	3												68	not conspicuous
		labelled	0	1	1	0	0												2	
3	2	unlabelled	6	20	19	20	4												69	not conspicuous
		labelled	3	6	3	2	2												16	
4	2	unlabelled	11	17	26	17	4	2											77	not conspicuous
		labelled	0	0	0	0	0	0											0	
5	2—3	unlabelled	7	5	6	7	8	16	10	8	12	11	13	8	8	7	10	8	144	not conspicuous
		labelled	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	2	
6	2—3	unlabelled	0	6	6	4	8	7	0	0									31	not conspicuous
		labelled	0	0	0	0	0	0	0	0									0	
7	3—4	unlabelled	0	10	10	8	18	18	13	0									77	not conspicuous
		labelled	0	0	0	0	0	0	0	0									0	
8	3—4	unlabelled	1	2	6	14	11	8	2	1									45	not conspicuous
		labelled	0	0	0	0	0	0	0	0									0	
9	3—4	unlabelled	2	0	0	2	1	1	0	0	1	1	2	1	0	1	0		12	conspicuous
		labelled	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		0	
10	3—4	unlabelled	0	0	0	1	1	2	6	1									11	conspicuous
		labelled	0	0	0	0	0	0	0	0									0	
11	4	unlabelled	0	0	0	5	0	1	2	0	4								12	conspicuous
		labelled	0	0	0	0	0	0	0	0	0								0	

12	4	unlabelled	0	0	6	1	1	6	7	2									23	not conspicuous
		labelled	0	0	0	0	0	0	0	0									0	
13	5	unlabelled	2	2	0	5	1	0	1	1									12	very conspicuous
		labelled	0	0	0	0	0	0	0	0									0	
14	5	unlabelled	1	1	1	0	1	0	0	0									4	very conspicuous
		labelled	0	0	0	0	0	0	0	0									0	
15	5	unlabelled	0	0	2	1	0	0	1										4	very conspicuous
		labelled	0	0	0	0	0	0	0										0	
16	6	unlabelled	2	2	0	2	1	0											7	very conspicuous
		labelled	0	0	0	0	0	0											0	
17	6	unlabelled	0	1	0	0	1	0											2	very conspicuous
		labelled	0	0	0	0	0	0											0	
18	7	unlabelled	0	0	0	0	0	0	0	0	0	0							0	very conspicuous
		labelled	0	0	0	0	0	0	0	0	0	0							0	

As seen from Table 2 the body disintegrates between the third and fourth days of pupal life. By the end of the fourth day the body has disappeared from most oocytes, it is only still present in small oocytes which are retarded in their development. By the fifth and sixth day the body is nearly absent and in seven-day-old pupae, just the day before the animals become adults, the body finally vanishes. At the same time that the body disintegrates in the nucleus a large mass of yolk granules appears in the cytoplasm. These granules, which are mainly constituted by protein, become conspicuous between the third and fourth day and are very distinct after that period (Table 2).

When the body disintegrates at diplotene the tritiated thymidine is released into the nucleus. The migration of the tritiated thymidine can hardly be checked due to the following reasons. (1) The number of bodies labelled in the pupa ovaries is low (Table 2). Only in pupa no. 3 did it amount to 16 (Table 2). Like this the chance to see a labelled body in the process of

disintegration is small. The disintegration process also seems to be fairly rapid. (2) When the body disintegrates the labelled material becomes easily diluted. At this stage the body is a sphere 6 microns in diameter but the nucleus is as large as 50 microns in diameter and the cytoplasm approximately 120 microns in diameter. This means that once the labelling is released either in the nucleus or the cytoplasm it gets so diluted that it becomes indistinguishable from background radiation.

The amount of DNA released is not small compared to the nuclear DNA. This was studied by making spectrophotometric measurements

Table 3. *Extinction values of 0.7 × 0.7 micron regions of Feulgen stained chromosomes and of Feulgen positive body at pachytene*

Nucleus	Chromosomes left side			Feulgen positive body					Chromosomes right side			Ratio body-chromosome
1	0.082	0.140	0.110	0.256	0.328	0.274	0.284	0.240	0.087	0.078	0.082	2.9
2	0.122	0.110	0.116	0.495	0.409	0.468	0.387	0.443	0.087	0.082	0.116	4.2
3	0.198	0.177	0.146	0.468	0.432	0.409	0.347	0.347	0.156	0.171	0.128	2.5
4	0.060	0.099	0.082	0.256	0.248	0.240	0.233	0.198	0.060	0.068	0.092	3.1
5	0.128	0.105	0.110	0.347	0.366	0.357	0.302	0.456	0.092	0.140	0.092	3.3
6	0.051	0.071	0.078	0.586	0.568	0.586	0.538	0.602	0.087	0.064	0.116	7.4
7	0.087	0.110	0.146	0.538	0.538	0.586	0.508	0.508	0.105	0.051	0.087	5.5
8	0.134	0.078	0.099	0.538	0.586	0.443	0.456	0.468	0.099	0.156	0.140	4.2
9	0.122	0.105	0.071	0.409	0.456	0.538	0.495	0.482	0.128	0.122	0.122	4.2
10	0.212	0.156	0.110	0.508	0.508	0.538	0.522	0.468	0.219	0.146	0.071	3.3
11	0.082	0.044	0.092	0.482	0.319	0.495	0.443	0.456	0.128	0.110	0.051	2.9
Total	1.278	1.195	1.160	4.883	4.758	4.934	4.515	4.668	1.248	1.188	1.097	—
Mean	0.116	0.109	0.105	0.444	0.432	0.448	0.410	0.424	0.113	0.108	0.100	4.0

of the body and of the adjacent chromosomes within the same nucleus. As Table 3 shows the Feulgen positive body contains on the average 4 times more DNA per unit area than the chromosomes. For each nucleus five measurements were made within the body and three on either side at randomly chosen sites in the chromosomes. The measurements were made at pachytene. At this stage the area of the body is 28.3 square microns and that occupied by the chromosomes is on the average 78.5 square microns. Minor deviations due to the geometry of the body and of the chromosomes have been taken into consideration, but did not affect the calculations. Since the body contains four times more DNA per unit area than the chromosomes its total amount of DNA is of a higher order of magnitude than that present in all the chromosomes. In other words the body contains approximately 59% of the DNA of the whole nucleus, and this DNA is suddenly released and becomes available either to the chromosomes or other cellular components.

Discussion

Recently reports have been published suggesting that tritiated thymidine may be incorporated into nuclei not preparing for division or which were not turning polyploid (PELC 1959, PELC and GAHAN 1959, LACOUR and PELC 1958, PELC and LACOUR 1959). These results were received with a certain scepticism because the evidence presented was based on a correlation difficult to establish with certainty. GALL and JOHNSON (1960) repeated some of their experiments, with a view to checking this interpretation and could not find support for an incorporation in absence of subsequent nuclear division. They draw the conclusion that since the uptake of H^3-thymidine accompanies DNA synthesis and precedes mitosis, there is no reason to postulate the occurrence of metabolic DNA in the mouse vesicle, as claimed by PELC and coworkers.

Another source of evidence furnishes, however, good evidence of the occurrence of metabolic DNA. In two species of *Diptera*, certain chromosome bands show a disproportionate local increase of DNA content related to puff formation. The large mass of DNA accumulated at the puff disappears subsequently. The loss is accompanied by a swelling and disintegration of the DNA globule. Spectrophotometric measurements, enzyme digestion and autoradiography used on the same chromosomes confirm this metabolic behavior of DNA at specific loci (BREUER and PAVAN 1955, RUDKIN and CORLETTE 1957, FICQ and PAVAN 1957). In *Rhynchosciara* the 2-fold increase in DNA content measured with the help of the U.V. in puff II would add less than 1 per cent to the total nuclear DNA (RUDKIN and CORLETTE 1957). In this case the amount of metabolic DNA is only a minimal fraction of the total nuclear DNA. In *Glyptotendipes* the variation in DNA content found in puff region I amounts only to 10 per cent of the DNA of the entire chromosome where the puff occurs (STICH and NAYLOR 1958). This is also a small fraction of the total DNA of the nucleus. In *Tipula oleracea* the situation is much more extreme, since the body contains approximately 50 per cent of the total nuclear DNA.

The disintegration of the body takes place at a stage where the cytoplasm of the oocyte is becoming packed with protein granules. At the same time the chromosomes are going to condense in preparation for metaphase I and the spindle is going to be formed. This is a period where the egg is growing rapidly and where a strong physiological activity is taking place.

What is, however, of significance is that this large amount of DNA is released, and it becomes available to the chromosomes or to cytoplasmic components such as protein. It can in this way carry its own genetic message to other cell structures.

Summary

In *Tipula oleracea* females 2n = 6 + XX *(Diptera)* a Feulgen positive body is present in the oogonia and oocyte nuclei. This body appears in the nucleus at the oogonial divisions that precede meiosis. It gets larger by leptotene, attaining a diameter of 6 microns, and at diplotene suddenly disintegrates. By metaphase I the body is not seen.

Injection of tritiated thymidine into the larvae leads to a heavy labelling of the Feulgen positive body. The body is found to synthesize DNA at a different period of time from the chromosomes, and there is an intermediate period when the synthesis of the two nuclear structures overlaps.

The tritium labelled thymidine is released from the body between the third and fourth day of pupal life. At this time the yolk granules in the cytoplasm become particularly conspicuous.

When the body disintegrates the labelled material becomes easily diluted. The volume of the nucleus and of the cytoplasm are sufficiently large to dilute this material in such a way that it easily becomes indistinguishable from background radiation.

Spectrophotometric measurements of the body reveal that it contains four times more DNA per unit area than the chromosomes. The area of the body is 28.3 square microns and that of the chromosomes 78.5 square microns. This means that the amount of DNA in the body is of a higher order of magnitude than that found in all the chromosomes.

This large amount of DNA becomes suddenly available either to the chromosomes or other cellular components. DNA can carry its own genetic information to other cellular components.

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