Acta Botanica Neerlandica **12** (1963) 302–314

THE INFLUENCE OF GLUTATHIONE AND GLUTATHIONE ANTAGONISTS ON MEIOSIS IN EXCISED ANTHERS OF LILIUM HENRYI[1])

A. S. RODRIGUES PEREIRA AND H. F. LINSKENS

(*Botanical Institute of the University of Nijmegen, Nijmegen*)

(*received May 9th, 1963*)

ABSTRACT

Young anthers, in early prophase and in the premeiotic mitotic stage, were excised and cultivated under aseptic conditions on media containing glutathione and/or several sulfhydryl antagonists.

1. Meiotic induction in excised anthers, not yet in prophase, is possible and is not dependent on addition of glutathione.
2. The formation of the wall structures of young microspores is dependent on the availability of sulfhydryl groups. Formation is inhibited reversibly by p-chloromercuribenzoic acid and irreversibly by cystine and iodoacetic acid.
3. It is suggested that inhibition of cross wall formation and separation of microspores in cultured anthers may be due to a lack of energy or substrate reserves.

INTRODUCTION

Since the initial intriguing speculations of RAPKINE (1931) on the rôle of glutathione in the mitotic cycle of urchin eggs, the conviction has been held that thiol groups play an important and perhaps even a dominant part in cell division. Experiments by MAZIA (1960), SAKAI and DAN (1958) and KAWAMURA and DAN (1958) have indicated that the mitotic apparatus in its original labile status predominantly contains $-SH$ bonds, which, with advancing mitosis, are converted to $S = S$ bonds. However, the way in which these bonds work is still not clear.

STERN (1959) and ZIMMERMAN (1960) observed that the thiol groups, besides having a structural function in the mitotic apparatus, may also play an important part in the metabolism of the dividing cell. STERN (1958) showed that soluble thiols accumulated in early phases of meiosis with a peak in zygotene. There was a second maximum at microspore mitosis and a minimum during a prolonged period between the two maxima, i.e. during the period of cell wall formation. LINSKENS and SCHRAUWEN (1963) confirmed the time of the first peak; they also found a second maximum, but at a much earlier stage than STERN, i.e. immediately after telophase II, at the beginning of cell wall formation, with a sharp minimum at anaphase II.

[1]) IVth contribution in the series "Physiologische Untersuchungen zur Reifeteilung".

In these present studies we have investigated the significance of sulfhydryl compounds for the induction and progress of meiosis.

The technique of sterile culture of anthers, developed, among others, by TAYLOR (1950), SPARROW *et al.* (1955) and in particular by VASIL (1957, 1959, 1960) has been used. In the nutrient media used by these previous workers sulfhydryl compounds were absent. Development of anthers was only observed if at the moment of excision they were in a stage not exceeding leptotene or zygotene. In general, cultures were discontinued at the tetrad stage; division of one-celled microspores was not reported. In all these previous experiments the androecium was dissected from the flower bud and the anthers severed from one another. In a recent publication HOTTA and STERN (1963) describe a modified procedure. From the young flower bud they eliminated all leaves surrounding the anthers and cut the axis of the flower a few millimeters below the receptacle. They then placed the intact group of anthers in a small tube containing 0.1 ml of nutrient solution. These cultures lasted for more than 40 days, and microspore mitosis was easily obtained. Attempts to culture the microspore mother cells after having them squeezed out, so that the effect of tapetum and wall layers might be eliminated, have also not been reported.

In view of the above facts our object was to study, with the help of tissue culture methods, the following: (a) the progress of meiosis and, possibly, the formation of microspores in the isolated androecium; (b) the progress of meiosis in excised anthers as compared to that in isolated androecia; (c) the influence of glutathione on the course of meiosis, in particular with a view to investigating the possibility to induce meiosis in anthers that are still in the premeiotic stage; (d) the influence of sulfhydryl antagonists on meiosis.

MATERIALS AND METHODS

Plants of *Lilium henryi*, clone 1 (LINSKENS, 1956) were cultured in an unheated warehouse and under normal field conditions in the Nijmegen university botanical garden. There is no strict correlation between bud length and stage of development of the anther (ERICKSON, 1948; LINSKENS, 1958; LINSKENS and SCHRAUWEN, 1963), which remains unchanged during the whole flowering season. Therefore every morning some buds were picked and, by means of a rapid iron-acetocarmine squash, the stage of development of the anthers was determined. Afterwards, buds of the required length and stage were collected. Handling and sterilizing of anthers followed the procedure of VASIL (1959). Out of every bud one anther was taken in order to determine the stage of development at the beginning of the experiment. Excised anthers were cultivated in petri dishes of 5 cm diameter; entire androecia were cultivated in pyrex tubes (10 × 1 cm), each of them contained 5 ml of the nutrient medium.

The meiotic stages are indicated as stage A through R, according to LINSKENS (1956). Cultures were started several stages before and

after onset of prophase: sporogenous tissue (stage A), resting nucleus (B), early prophase (C), pachytene (D), diplotene (E). Buds in stages A and B were about 11 mm long, those in the latter stages were about 15 mm. In some cases we started from older buds, about 22 mm, that were in stages varying from metaphase I (G) to that of young tetrads (K).

VASIL's modified medium was used, the stock solutions having the following composition:

(I) *Major element solution* (in g/l): magnesium sulphate, 7 H_2O, 3.6; calcium nitrate, 4 H_2O, 2.6; potassium nitrate, 0.8; potassium chloride, 0.65; sodium dihydrogen phosphate, H_2O, 1.65;

(II) *Trace element solution* (in mg/l): manganese sulphate, 4 H_2O, 3000; zinc sulphate, 7 H_2O, 300; boric acid, 1500; copper sulphate, 5 H_2O, 25; ammonium molybdate, 2 H_2O, 25; cobalt chloride, 25; 0.5 ml concentrated sulphuric acid (sp. gr. 1.84).

(III) *Ferric citrate solution*: 2.5 g/l.

(IV) *Vitamin and amino-acid solution* (in mg/100 ml): niacin, 25; thiamin hydrochloride, 20; pyridoxine hydrochloride, 15; calcium pantothenate, 5; glycine, 150.

All solutions were prepared with quartz double-distilled water. Solutions (I)–(III) were sterilized by autoclaving at 120° C for 20 minutes; solution (IV) was sterilized by filtration through a 0.45 micron millipore filter (Millipore Filter Corporation, Bedford, Mass., U.S.A.), with the help of an apparatus as designed by NITSCH and NITSCH (1957).

The final medium consisted of:

solution I–100 ml, solution II–1 ml, solution III–1 ml, solution IV–5 ml, gibberellic acid, 20 mg, indoleacetic acid, 1 mg, ribonucleic acid, 50 mg, agar, 9.5 g, sucrose, 10 g; all made up to 1 litre. With the exception of solution (IV) all components were autoclaved together; pH was adjusted to 5.7 with the help of 0.01 N NaOH, before autoclavation. Solution (IV) was added after the autoclaved medium had cooled to approximately 40° C. This medium will be called the basic medium (BM).

The glutathione solution was prepared fresh every two days; the stock solution, containing 12.5 mg/ml, was sterilized by filtration and stored at −10° C. Of this solution 1 ml was added to 50 ml of basic medium.

The cultures were stored at 15° C under diffused laboratory light. Each experimental series consisted of 20 petri dishes or tubes with 5 anthers in each, and was repeated at least twice. Observations were made after a fortnight, at which moment the meiotic stage of every anther was determined with the help of an iron-acetocarmine squash.

The following compounds were used as *sulfhydryl inhibitors* (BENDALL 1962):

the mercaptide forming agent p-chloromercuribenzoic acid (PCMB), applied in final concentrations of 10^{-3}, 10^{-4} and 10^{-5} M;

the oxidizing agent cystine, applied in a final concentration of 10^{-3} M;

the alkylizing agent iodoacetic acid in final concentrations of 10^{-3}, 10^{-4} and 10^{-5} M. These compounds were added to the medium before autoclaving.

In tables it is shown which part of the original number of 20 buds or 100 anthers respectively reached the highest meiotic stage observed. The two ways of notation confirm each other, although it is evident that the latter is more reliable. In histograms the percentages were plotted for all stages observed. In general, the earliest stage found was that in which tetrads parted into one-celled microspores; the latest one was that in which the microspores fully presented the typical wall structure (stages L and R respectively).

Experimental results

1. *Influence of kind of explant — (Meiosis in excised anthers compared to development in excised androecia).*

It is conceivable that meiosis in isolated anthers may stop earlier than if the receptacle with anthers and pistil is implanted into the medium. Experiments summarized in Table 1 show that, with one

Table 1

Meiosis in single anthers of L. HENRYI *as compared to that in androecia.*

Medium according to VASIL (1959), modified (BM) and the same with glutathione 250 mg/l (GSH). Explants were made at early prophase (C). Figures are percentages of the number of buds found to be at the stages indicated. D-pachytene; K-tetrads; M- 1-celled microspores, membrane not visible; P-microspore wall fully structured, pigment yellow; Q-pigment orange.

Exp. No.	Medium	Stage of excision	Duration of experiments	Anthers			Androecia		
				No. of buds	Most advanced stage	%	No. of buds	Most advanced stage	%
1	BM	C	10 days	11	K	20	10	K	50
2	BM	C	14	7	M	10	7	K	100
3	BM + GSH	C	8	19	D	40	10	K	30
4	BM + GSH	C	14	20	P	80	20	Q	60
5	BM	C	14	19	P	40	20	P	60

exception, development was more advanced in the androecia, i.e. there were more anthers that reached the highest stage observed. In no case was a higher stage than Q attained, i.e. fairly complete microspore with fully developed wall structure. Division of the microspore nucleus was not induced in the fourteen days the experiments lasted.

Further details of experiments 4 and 5 are presented in Figs. 1a and b. Thus, it appears that if a certain treatment promotes meiosis, the whole histogram is shifted in the direction of the higher stages.

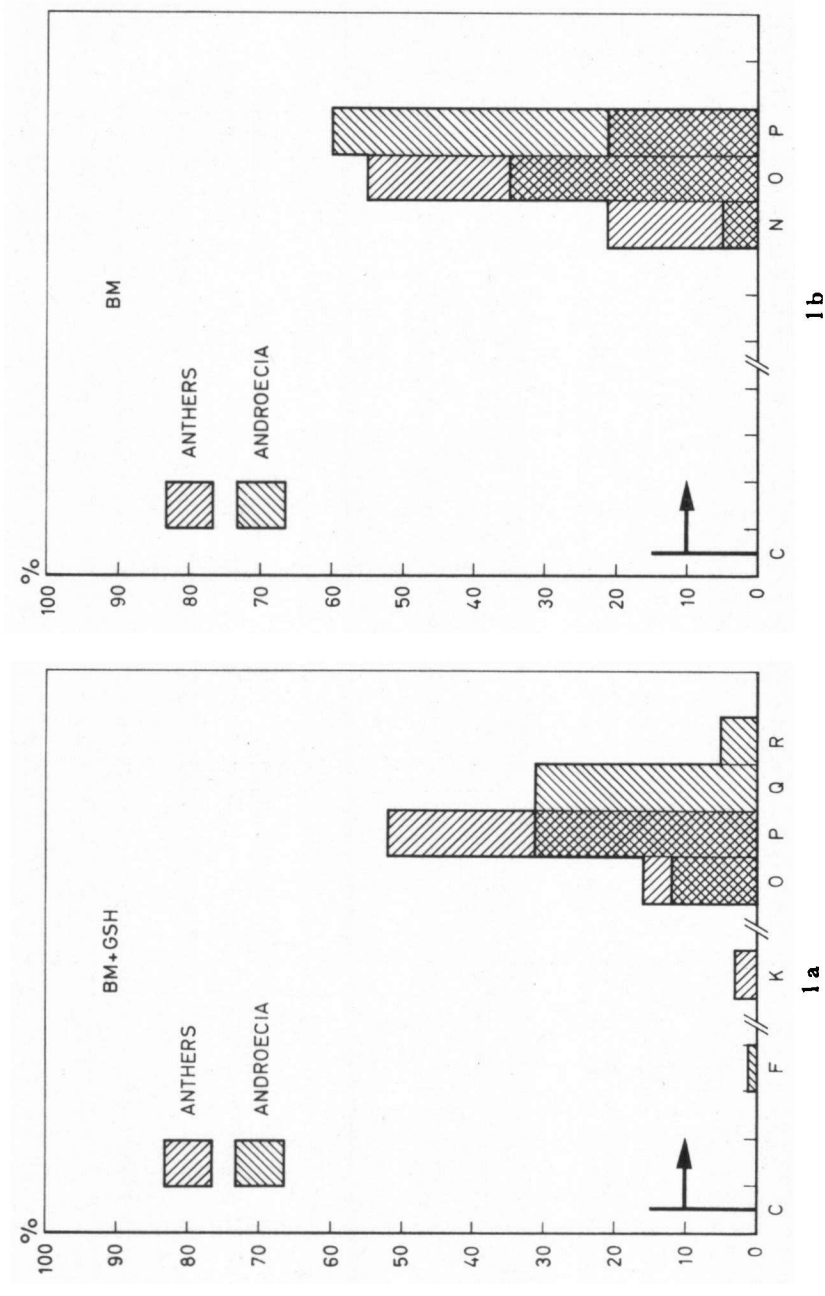

Fig. 1. The rate of meiosis in single anthers and androecia of *L. henryi*, excised at early prophase. a. Exp. 4; b. Exp. 5. Conventions as in Table 1.

The promotive influence is thus exerted in every stage between K and Q.

2. *Influence of glutathione*

Experiments presented in Table 1 also indicated the promotive effect of glutathione. The influence of GSH in the medium was studied in greater detail; anthers that had been excised in early prophase at bud length 15 to 19 mm and anthers that were still in the preceding stage of mitosis, stage A with bud length approximately 11 mm, were treated. The results are presented in Tables 2 and 3.

TABLE 2

Influence of glutathione on progress of meiosis in anthers of L. HENRYI *excised in early prophase (C).*

Glutathione concentration 250 mg/l. Duration of experiments 14 days. Figures are percentages of buds or anthers resp. found at the stages indicated. O-microspore wall clearly structured. Other conventions as in Table 1.

Exp. No.	Explant	Stage of excision	Basic medium			Basic medium with glutathione		
			No. of buds	Most advanced stage	%	No. of buds	Most advanced stage	%
6	androecium	C	20	P	60 (buds)	20	Q	55 (buds)
7	androecium	C	30	P	4.7 (anth.)	30	P	10 (anth.)
8	anthers	C	18	O	79 (buds)	18	P	80 (buds)

TABLE 3

Influence of glutathione on induction of meiosis in anthers of L. HENRYI, *excised in the premeiotic stage (A).*

G- metaphase I; H- interphase. Other conventions as in Table 2.

Exp. No.	Explant	Stage of excision	Basic medium			Basic medium with glutathione		
			No. of buds	Most advanced stage	%	No. of buds	Most advanced stage	%
9	androecium	A	20	K	3 (anth.)	20	H	1 (anth.)
10	androecium	A	20	H	10 (buds)	20	H	5 (buds)
11	anthers	A	7	O	57 (buds)	14	O	7 (buds)
12	anthers	A	10	G	2 (anth.)	13	O	5 (anth.)

From Table 3 it appears that to a limited extent it is possible to induce meiosis in isolated anthers that are in the premeiotic stage.

In 50 to 75 % of the young anthers excised in stage A there was no sporogenous tissue left after 14 days, the contents having entirely degenerated. Another 15 % of the cells were still recognizable, but they were autolyzed and no longer stainable. There is no clear-cut promotive influence of glutathione neither on the induction of meiosis, nor on its progress. In anthers isolated in leptotene (stage C), however,

this influence is manifest, as appears from Table 2. In experiments 6 to 8 microspores were further developed on the medium containing glutathione. But it is important to note, that even on this medium microspores did not reach cell division.

3. *Cultivation of microspore mother cells*

Following a suggestion by VASIL (1959) we have squeezed out young microspore mother cells and cultured them in Carrel's flasks on liquid basic medium with glutathione. The flasks were about 4 cm in diameter and contained approximately 3 ml of the nutrient solution. At the beginning of the experiments the cells were in early prophase, in late prophase, or in metaphase or beyond. They retained their healthy appearance and their stainability throughout the 14 day period of the experiments, but showed no sign of development. In some instances they even appeared to have retrograded to an earlier stage. Cells, cultivated in a medium containing 0.5 % instead of 1 % sucrose degenerated; addition of a pistil extract in citrate buffer of pH 5.8 or of DNA in a concentration of 12.5 mg/l had no effect.

4. *Influence of sulfhydryl antagonists*

a) *Experiments with p-chloromercuribenzoic acid.* As shown in Table 4, PCMB in low concentrations slightly inhibits the progress of meiosis in both excised anthers (Exp. 14) and androecia (Exp. 13 and 15),

TABLE 4

Influence of p-chloromercuribenzoic acid (PCMB) and glutathione (GSH) on microspore formation in isolated anthers of L. HENRYI *excised in early prophase (C).*

Concentration of glutathione 250 mg/l. Concentration of PCMB in Exp. 13 and 14, 10^{-5} M; in Exp. 15, 10^{-3} M. Duration of experiments 14 days. The number of microspores found at the stages indicated is given as percentage of the total number of buds or anthers. Other conventions as in Table 1.

Exp. No.	Explant	Stage of excision	Basic medium			Basic medium with PCMB		
			No. of buds	Most advanced stage	%	No. of buds	Most advanced stage	%
13	androecium	C	20	P	60 (buds)	20	P	5 (buds)
14	anthers	C	19	O	79 (buds)	18	O	10 (buds)
15	androecium	C	30	P	4.7 (anth.)	19	O	5 (anth.)

Exp. No.	Explant	Stage of excision	Basic medium with GSH			Basic medium with PCMB and GSH		
			No. of buds	Most advanced stage	%	No. of buds	Most advanced stage	%
13	androecium	C	20	Q	55 (buds)	20	P	60 (buds)
14	anthers	C	20	P	80 (buds)	20	P	30 (buds)
15	androecium	C	30	P	10 (anth.)	19	O	15 (anth.)

isolated in early prophase. The inhibition only concerns the formation of cell wall structures of young microspores and is reversed by glutathione. In every experimental series there were pollen mother cells that were to varying degrees autolyzed. Their contents often were resorbed to such an extent that it was no longer possible to obtain cellular, or even stainable, material from the anthers. On basic medium, with or without glutathione, about one third of all anthers appeared to be in this condition. Addition of PCMB to the medium caused a sharp increase that was not reversed by simultaneous administration of glutathione.

Experiments were also carried out with anthers at a more advanced stage of meiosis at bud length 22–24 mm (Table 5). The ultimate

TABLE 5

Influence of p-chloromercuribenzoic acid (PCMB) and glutathione (GSH) on microspore formation in isolated anthers of L. HENRYI *excised at advanced stages of meiosis.*

Concentration of PCMB 10^{-5} M; B- diplotene; F- diakinesis; L- tetrads breaking up; R- fully developed microspores. Other data as in Table 1. Number of buds 20.

Exp. No.	Explant	Stage of excision	Basic medium + PCMB		Basic medium + PCMB + GSH	
			Most advanced stage	%	Most advanced stage	%
16	androecium	E/K	Q	60 (buds)	R	20 (buds)
17	anthers	F/K	Q	10 (anth.)	Q	24 (anth.)
18	androecium	L	Q	35 (anth.)	Q	40 (anth.)

stage attained on a medium with PCMB was more advanced than when starting from early prophase. Application of glutathione increased the rate of development, but microspores did not proceed to mitosis.

The results of experiment 13 are more fully shown in Fig. 2. It appears that cell wall inhibition and promotion are not restricted to any definite stage, but can occur during any period of cell wall formation; thus PCMB shifts the histogram maxima to lower stages and glutathione moves them back to higher ones.

b) *Experiments with cystine.* Cystine at 10^{-3} M also inhibited meiosis. This effect was not reversed by glutathione. A typical experiment is presented in Fig. 3. The inhibition manifests itself by decreasing the rate of development of the young microspores; but, the number of anthers whose contents were resorbed or autolyzed, was not increased.

c) *Experiments with iodoacetic acid.* This antagonist was already slightly toxic at the lowest concentration used (10^{-5} M) and still more at 10^{-4} and 10^{-3} M. Very few anthers showed any recognizable meiotic stage. Besides, in anthers that retained their stainability meiosis was retarded. The inhibition by iodoacetic acid was not reversed by simultaneous application of glutathione.

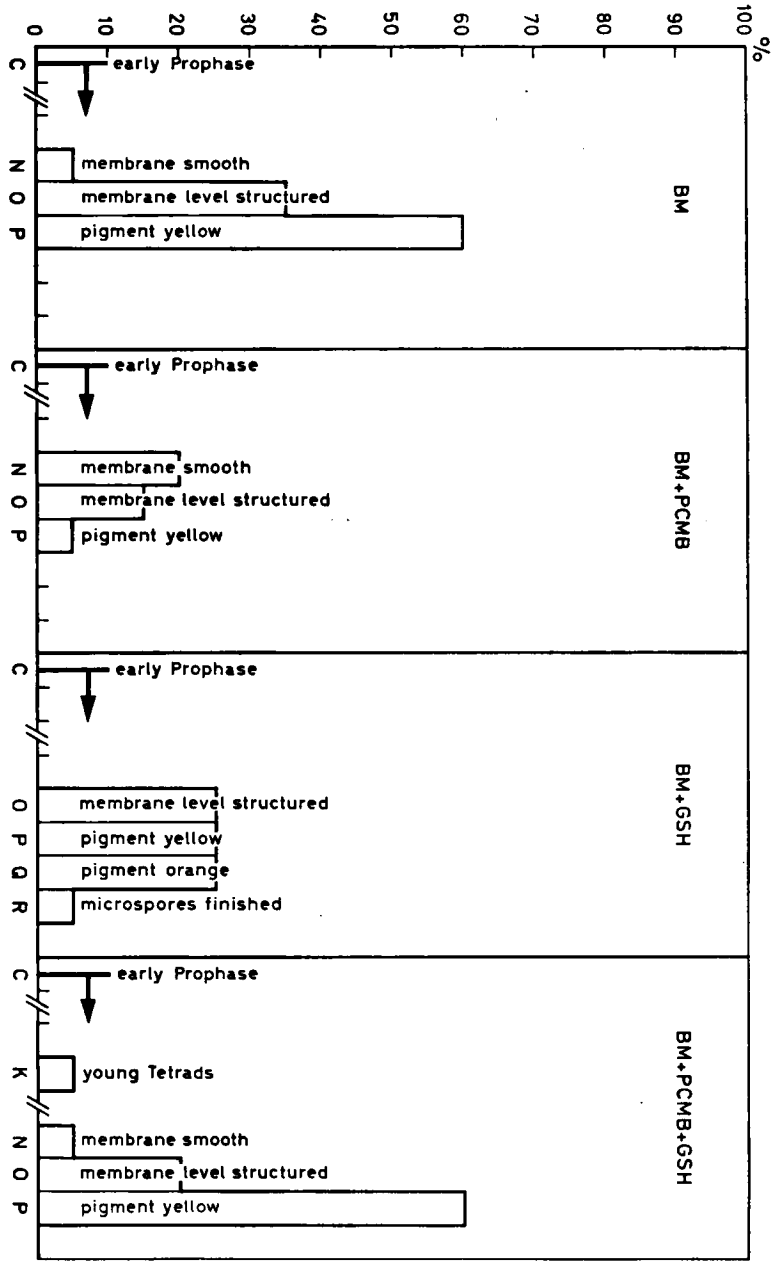

Fig. 2. Influence of p-chloromercuribenzoic acid and glutathione on meiosis in excised androecia of *L. henryi*. Concentrations of PCMB and GSH 10^{-5} M and 250 mg/l resp. Other conventions as in Table 4.

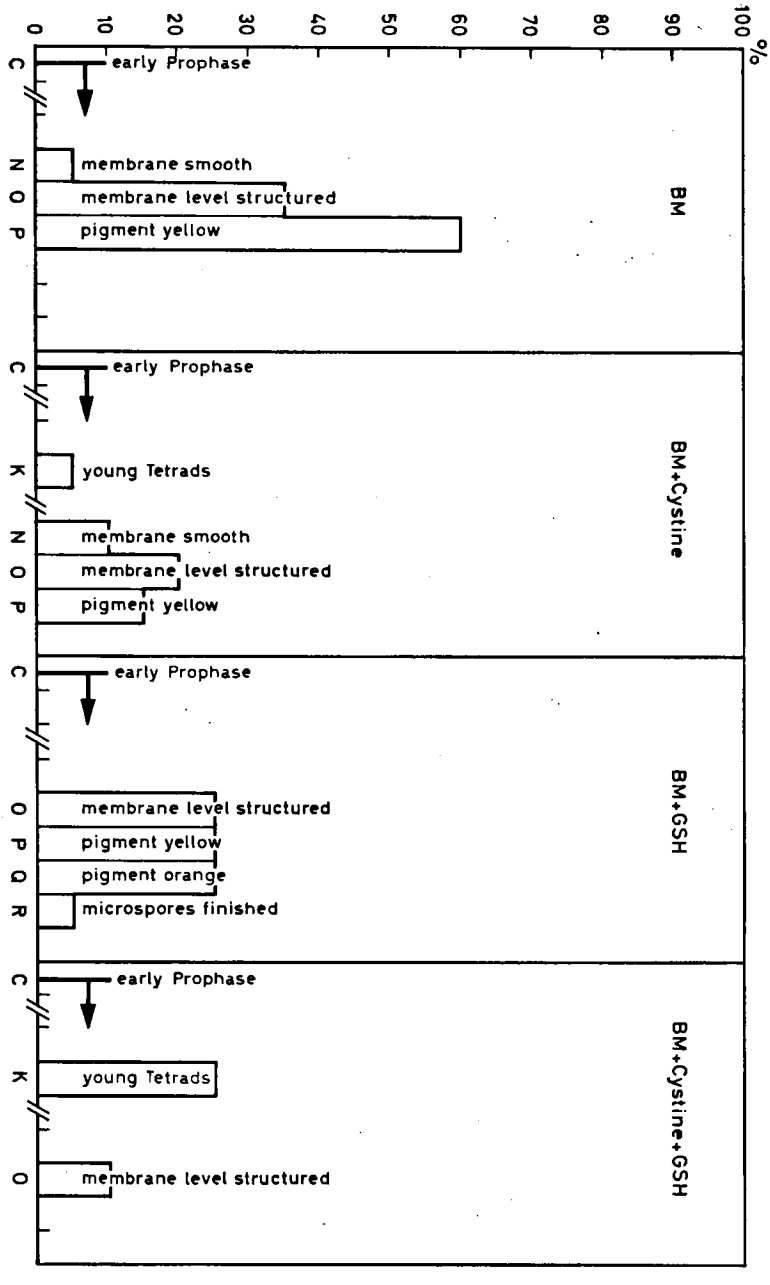

Fig. 3. Influence of cystine and glutathione on progress of meiosis in isolated anthers of *L. henryi*, excised at early prophase (C). Duration of experiment 14 days.

Discussion

The fact that in lily anthers a strong synchronization takes place of meiotic processes and microspore development suggests a chemical induction of these important developmental processes. Nucleic acids are suggested as of primary importance in this induction. Changes in DNA and RNA level in anthers and the appearance of nucleotides at the time of depression at the onset of prophase and the beginning of the crumbling of the tapetum cells have been observed (Linskens, 1958; Stern, 1960). We have also confirmed results of Vasil (1959, 1960), that RNA—as a part of the medium—is unable to induce meiotic processes in excised anthers; however, we did observe a promotive effect of this substance on the progress of meiosis. Neither Vasil, nor any other author, added glutathione to the medium when culturing excised anthers. The present study continued the observation that a cyclic variation takes place in soluble sulfhydryls during mitosis and meiosis of sporogenous tissue (Stern, 1958; Linskens and Schrauwen, 1963); we found that anthers cultivated on a basic medium with GSH were in a more advanced stage of meiosis than anthers on the control medium.

Both on the basic medium and on the medium with glutathione a certain percentage of the cells remaining at early stages autolyzed or the anthers were otherwise degenerated. The more advanced the meiotic stage at excision the more healthy the appearance of the cells after a fourteen days period of cultivation. In anthers excised in metaphase or beyond hardly any cell appeared to be autolyzed. Since the percentage of degenerated cells was not decreased by addition of glutathione nor increased by application of PCMB and iodoacetic acid, this phenomenon of anther autolysis is apparently due to a lack of some other substance or radical than thiols or sulfhydryl groups.

The promotive effect of glutathione on meiosis was particularly clear in anthers that were excised in early prophase; it was expressed in formation of the cell wall structure (stages N through R). It was only during this period of development that thiol groups seemed to act as a limiting factor. This is exactly the period in which, according to Stern (1958) soluble sulfhydryl concentration in lily anthers is at its minimum.

The inability of glutathione to lessen the percentage of cells degenerating at the tetrad stage is shown in experiments with very young anthers (Table 3). Development stopped either at interphase/telophase or at the much later stage in which the membrane already had its definite structure; this was observed on both the basic medium with and without glutathione. Generally in these very small anthers not even the primary cell wall was formed. According to Hotta and Stern (1963) wall formation is especially affected by the antagonist ethionine. With ethionine there seemed to be a certain correlation between the inhibition of chromosome reversion to the interphase tage—which means that the chromosomes remained in the aggregated

state—and the fact that the cells failed to form walls. WALKER and DIETRICH (1961) found an inhibition of cross wall formation upon sugar starvation or application of kinetin; they attributed this to depletion of energy reserves. In our experiments the percentage of cells degenerating at the tetrad stage was not affected by PCMB, but strongly increased both on the medium containing cystine and on that containing iodoacetic acid—much as was the case with cells remaining in early prophase that were discussed above. Therefore, a lack of energy reserves, increased by the effect of oxidizing or alkylizing agents can probably be a causative agent at both stages.

The fact that glutathione reverses PCMB inhibition suggests that the effect of the former substance is really due to its —SH group and not to any other part of the constituating aminoacids. Glutathione does not exert any promotive effect on meiosis in anthers isolated in the premeiotic stage. However, this does not necessarily mean that meiotic induction is not brought about by GSH; it is in fact more probable that at this stage the substance is not yet limiting (cf. the results by STERN, 1958 and 1959 and LINSKENS and SCHRAUWEN, 1963).

Development of squeezed-out microsporocytes does not continue, not even of those at an advanced stage of meiosis. From this it seems that some non-sulfhydryl factor in the tapetum cells, or transferred from the vegetative tissues, was lacking. Extracts of ripe and unripe anthers had no effect on microsporocyte development.

The general conclusion we would like to draw from experiments reported here is: that for a coordinated development of the induction and a normal course of meiosis at different stages, different levels of chemical constituents are necessary. Until now we know that nucleic acids, resp. nucleotides as well as soluble thiols are important. Nevertheless it seems not to be reasonable to assume that the concentration of —SH groups is the inducing factor to effect reduction of the cellular components (STERN, 1959). Also a linkage between the rôle of the sulfur in cell division and the nucleic acid metabolism has to be discussed (HASE, MIHARA and TAMIYA, 1961). A complex cell-phenomenon such as meiotic division cannot be dependent on a single chemical substance. The influence from the vegetative organs of the plant proceeding to meiosis is not to be neglected. In the same way we may suppose that the normal functioning tapetum is not the limiting factor in the induction of meiosis but only one of the sources of molecular gradients in the complex leading to meiosis.

For future experiments it seems reasonable to look for a less complex organism, that can be cultured on a simple medium and from which in an easier manner synchronized material can be obtained.

ACKNOWLEDGEMENT

The authors gratefully acknowledge the technical assistance of Miss. C. Garsten.

REFERENCES

BENDALL, F. 1962. In: Modern methods in Plant Analysis VI, H. F. LINSKENS and M. V. TRACEY, Eds. (Berlin, Göttingen, Heidelberg): 424.
ERICKSON, R. O. 1948. Am. J. Bot. **35**: 729.
HASE, E., S. MIHARA and H. TAMIYA. 1961. Plant and Cell Physiol. **2**: 9.
HOTTA, Y. and H. STERN. 1963. J. Cell Biol. **16**: 259.
KAWAMURA, N. and K. DAN. 1958. J. biophys. and biochem. Cyt. **4**: 615.
LINSKENS, H. F. 1956. Ber. dtsch. bot. Ges. **69**: 353.
———— 1958. Acta Bot. Neerl. **7**: 61.
———— and J. A. M. SCHRAUWEN. 1963. Biologia Plantarum (Praha). In press.
MAZIA, D. 1959. In: The Cell. J. BRACHET and A. MIRSKY, Eds. (New York): 250.
NITSCH, J. P. and C. NITSCH. 1957. Am. J. Bot. **44**: 555.
RAPKINE, L. 1931. Ann. physiol. physiochim. biol. **7**: 382.
SAKAI, H. and K. DAN. 1958. Exptl. Cell Res. **16**: 24.
SPARROW, A. H., V. POND and S. KOJAN. 1955. Am. J. Bot. **42**: 384.
STERN, H. 1958. J. biophys. and biochem. Cytol. **4**: 157.
————. 1959. In: Sulfur in Proteins. R. BENESCH *et al.*, Eds. (New York): 391.
TAYLOR, J. H. 1950. Am. J. Bot. **37**: 137.
VASIL, I. K. 1951. Phytomorphology **7**: 138.
————. 1959. J. exp. Bot. **10**: 399.
————. 1960. Memoirs of the Indian bot. Soc. **3**: 94.
WALKER, G. W. R. and J. F. DIETRICH. 1961. Can. J. Genetics and Cytol. **3**: 170.
ZIMMERMAN, A. M. 1960. Exptl. Cell Res. **20**: 529.