INHIBITION OF PROTEIN SYNTHESES DURING MEIOSIS AND ITS BEARING ON INTRACELLULAR REGULATION

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ABSTRACT

Several parameters of meiosis have been studied in cultured anthers of *Trillium erectum*. The accessibility of labeled substrates to meiotic cells and the fate of these substrates in relation to meiotic stage have been determined. Evidence has been adduced for the synthesis of RNA and protein during the meiotic cycle well after chromosome duplication. The effect of interfering with systems directly or indirectly connected with protein formation has been studied by means of chloramphenicol, 8-azaguanine, 5-methyltryptophan, and ethionine. Administration of these reagents at different intervals in the cycle elicits correspondingly different responses thereby indicating a periodicity in the activities of different systems. The following processes have been shown to be affected in these experiments: chromosome segregation, chromosome morphology, cytokinesis, wall synthesis, and enzyme appearance. The possibility of experimentally altering the normal sequence of events has also been shown.

INTRODUCTION

In studies of meiosis, chromosome pairing and segregation are features of primary interest; DNA replication, on the other hand, is commonly regarded as a secondary feature. This difference in emphasis is due to the fact that the distinctiveness of meiosis lies in the mechanics of chromosome behavior and not in the biosynthetic pattern of DNA replication. Although the latter has been infrequently studied in meiotic systems (7), it is presumed that regulation of DNA synthesis is much the same in meiotic and mitotic systems. The divergence in behavior of meiotic and mitotic chromosomes appears to have its origin in mechanisms other than those underlying DNA replication. To be sure, beyond certain excellent studies of the spindle body (4), there is hardly enough known about mitotic segregation to serve as a basis for a comparative biochemical study of the two processes. Commonly, it is assumed that the biosynthetic requirements of chromosome segregation are fulfilled prior to the onset of prophase and that the unfolding of the division cycle is a natural consequence of the properties of the preformed aggregate. The assumption is open to question principally because the possibilities of experimentally identifying sequential biosynthetic processes during the brief interval of "active" mitosis are extremely limited.

The comparatively long interval of time covering the pairing and movements of meiotic chromosomes provides an obvious advantage for a study of this problem. The principal obstacle to such studies lies in the suitability of meiotic tissues for biochemical studies. The microsporocytes of many plants which have so long proved suitable for cytological study have proved to be distressingly unsuitable for physiological experiments. There have been many attempts to culture

anthers *in vitro* for this very purpose (12, 15, 17), but none of these attempts have had complete success. The most encouraging results were obtained by Sparrow using the plant *Trillium erectum*, and undoubtedly much of his success was due to the fact that meiosis occurs in an otherwise dormant organism and at a temperature close to 0°C (8). We have followed Sparrow's lead, and in so doing have been able to conduct the experiments here reported.

EXPERIMENTAL APPROACH

In connection with studies of *Trillium*, it must be emphasized that once experiments are designed they remain fixed for the season. Repeats or alterations of plans are possible only for the succeeding year. Since we had no prior knowledge about the parameters we intended to examine, it was impossible to anticipate relationships which became evident later and which now beg more precise inquiry. The gaps in this study will be obvious, but the results, even as they are, provide interesting pointers on the nature of the meiotic process.

The hypothesis adopted in this study was made on the basis of previous results indicating that DNA replication is associated with a sharp periodicity in the appearance of pertinent enzymes and substrates (11). The idea that this periodicity represented a sequence of sharply timed inductions of protein synthesis appeared to be most attractive especially because such inductions would implicate immediate gene participation in the unfolding of the mitotic cycle. Since the paucity of material available precluded any direct chemical demonstration of *de novo* synthesis in the case of each of the enzymes found in the sequence, it was decided to test the plausibility of the idea by use of reagents which affect either RNA composition, protein synthesis, or protein composition. The reagents chosen to fulfill these functions were, corespondingly, azaguanine, chloramphenicol, and the amino acid analogues, 5-methyltryptophan and ethionine. Convenience rather than theoretical considerations dictated the choice of the amino acid analogues. In studies of lily microspore mitosis, which will be reported later, we were able to demonstrate a fairly precise relationship between the time of administering these inhibitors and the failure of the enzymes to appear in the microspores. These studies followed the experiments with *Trillium*. When studies of *Trillium* meiosis were planned, the aim was similar,—to determine whether there existed a sequence of specific protein syntheses in association with the meiotic cycle. The experiments were begun after the meiotic cells had completed their DNA synthesis, but since no information was available on the kinds of proteins which were synthesized following chromosome replication, less specific effects were sought: (*a*) influence of the reagents on the rate of meiotic development as a guide to the requirement of meiosis for synthesis of protein; (*b*) relationship between time at which the reagent was administered and the specificity of effect to establish whether syntheses were sequential; and (*c*) dissociability of events as an indicator of regulatory mechanisms. Some general aspects of these studies were recently reported at a symposium (12).

METHODS

Trillium anthers were cultured by removing the leaves and floral parts surrounding the anthers, cutting the axis of the flower a few millimeters below the surface and placing the intact group of anthers in a small tube containing 0.1 ml of nutrient solution. Operations were performed under aseptic conditions, the outer whorl of leaves being first sterilized by immersion of the bud in a solution of 5 per cent Chlorox. The tubes were fitted with a cotton plug and covered with Parafilm to reduce moisture loss. Cultures thus prepared were maintained in a refrigerator at approximately 3–5°C. Individual experiments consisted of removing single anthers at different times and testing for parameters of interest. Since there are 6 anthers per bud, all closely synchronized in development, it is possible to make observations at 6 intervals.

Generally, the anthers were cultured in an inorganic medium, Hoagland's solution. This appeared to be adequate provided the anthers were not detached from the floral axis. The capacity of the microsporocytes to take up reagents from the medium was tested by means of isotopic compounds. At given intervals the microsporocytes were squeezed out of the anther, washed, and sedimented in sucrose solutions (2). Although appreciable amounts of reagent are taken up by the somatic tissues surrounding the microsporocytes, the metabolic course of the reagents in these tissues was not followed. The following concentrations of inhibitors were used: azaguanine, 0.1 mg/ml; chloramphenicol, 100 μg/ml; ethionine,

2 mg/ml; 5-methyltryptophan, 2 mg/ml. In examining for reversals or competitive effects equimolar concentrations of the normal component were used. Propionocarmine smears were used for cytological studies. The capacity of the cells to phosphorylate thymidine was determined by a procedure previously described (3).

the microspores reached the binucleate condition. No obvious cytological aberrations were noted. These control tests could not be run entirely in advance of others because of the seasonal cycle; hindsight alone justified the usefulness of the other experiments.

The outstanding effect of thus culturing anthers

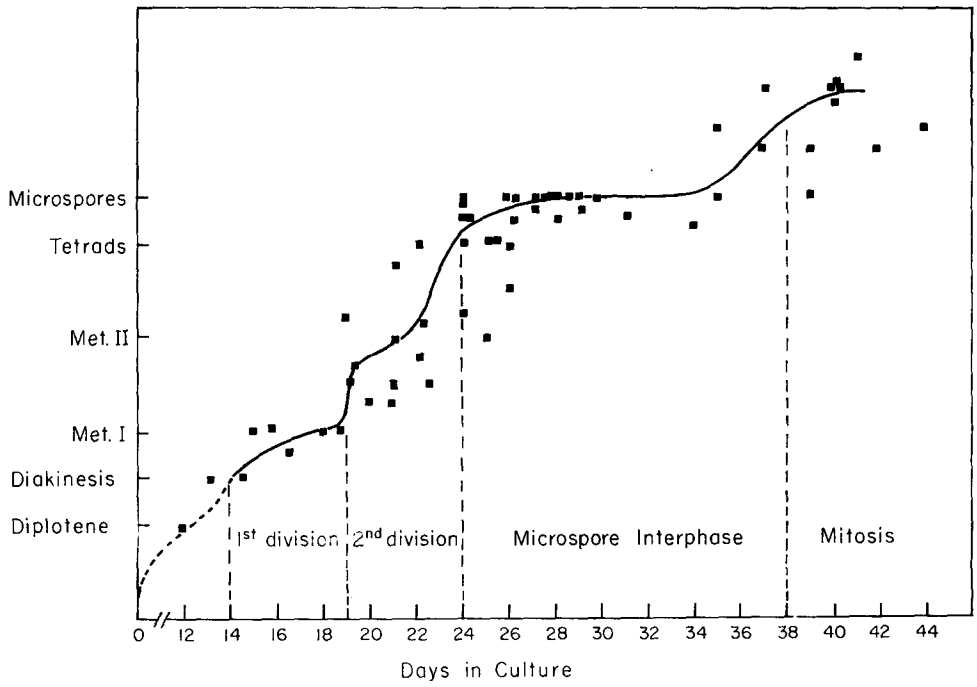


FIGURE 1

The rate of meiotic development in cultured anthers of *T. erectum*. Temperature was maintained at 3–5°C. Points represent stages of individual anthers at times after culture began. Overlapping points are not indicated. It is most probable that all buds were not at identical stages of development when culturing began. Synchrony among the 6 anthers of a single bud is excellent. A total of 6 observations could therefore be made for each series.

RESULTS

The suitability of the medium was tested with 20 different buds. In addition to cytological smears, the ability of the microsporocytes and microspores to phosphorylate thymidine was followed because of earlier demonstrations that under normal conditions the appearance of this enzyme is limited to 1 to 2 days and only at microspore interphase. It will be seen from Fig. 6 that a sharp periodicity persists in the cultured anthers. Although in some cases the supply of anthers was exhausted before microspore mitosis was complete, in the 9 buds which were followed for sufficient lengths of time, appears to be an acceleration of their rate of meiotic development. The total cycle is shortened to a period of approximately 40 days (Fig. 1) which is considerably shorter than that under normal conditions of storage (9). There are a number of possible explanations for this behavior but none of them would appear to have a significant bearing on the observations here made.

Uptake of Isotopically Labeled Compounds

Since one of the drawbacks to techniques of anther culture is the poor absorption of reagents through the anther filament, a series of experi-

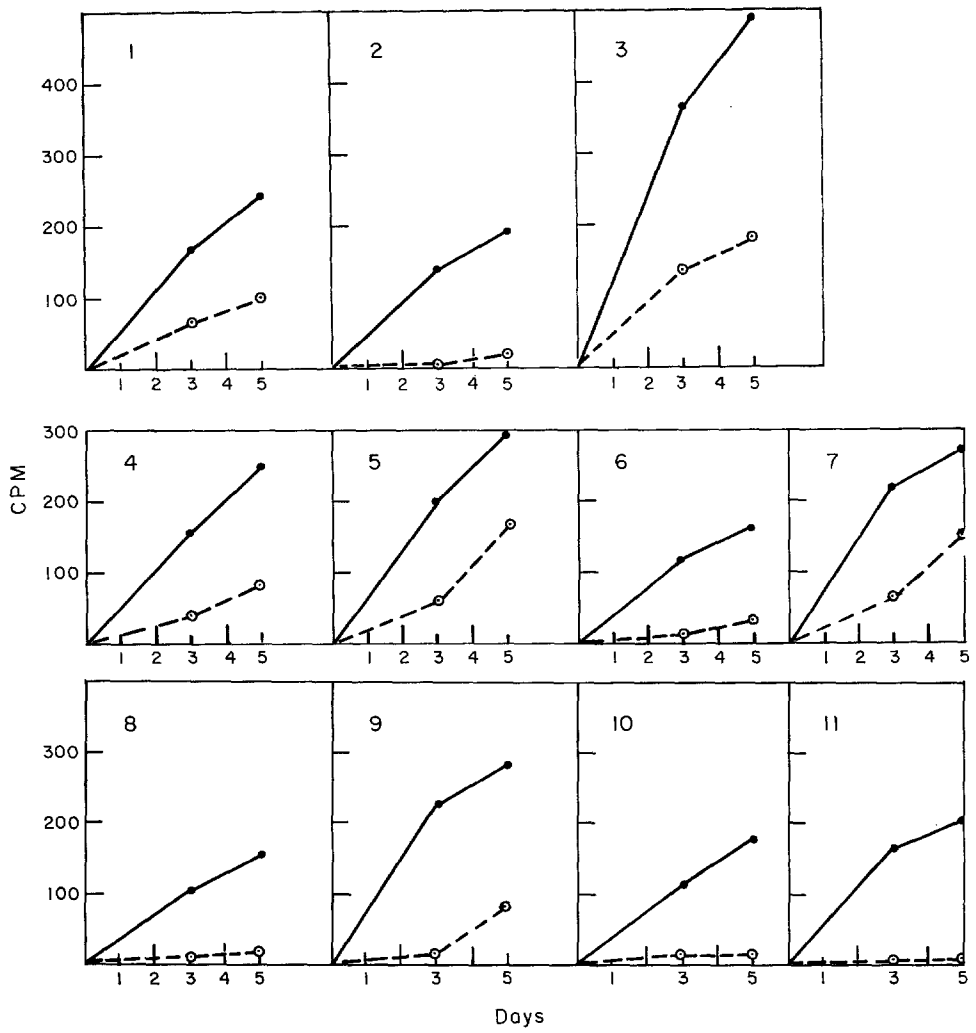


FIGURE 2

The rate of uptake of isotopically labeled compounds by microsporocytes of *T. erectum*. Solid lines represent the acid-soluble fraction; dotted lines, the acid-insoluble. Each point is the count in the microsporocytes of 5 anthers in 1 to 4 and of 10 anthers in the remainder. Total protein of such samples is of the order of 100 to 300 μg. The stage indicated is that found at the beginning of incubation.

1 to 4: C^{14}-thymine, 47,000 CPM added per bud. 5: Diplotene. 6: metaphase II-anaphase II. 7: anaphase II-tetrads.

8 to 11: C^{14}-leucine, 17,000 CPM added per bud. 8: Pachytene. 9: Diplotene. 10: metaphase II-anaphase II. 11: Tetrads.

ments was run with various isotopically labeled compounds in order to determine the efficiency of the present system with respect to reagent uptake. The results made evident several features of the process: (*a*) The total amount of isotope taken up by the cells is, within broad limits, independent of whether or not the compound absorbed is being simultaneously utilized in macromolecular syntheses. Examples of this are to be found in Fig. 2: the graphs have been selected in order to show that acid-soluble pools may be at much the same level in microsporocytes which are not synthesizing nucleic acid or protein as in those which are. (*b*) The kinetics of uptake are such that over a comparatively broad interval of development there is no marked increase in the

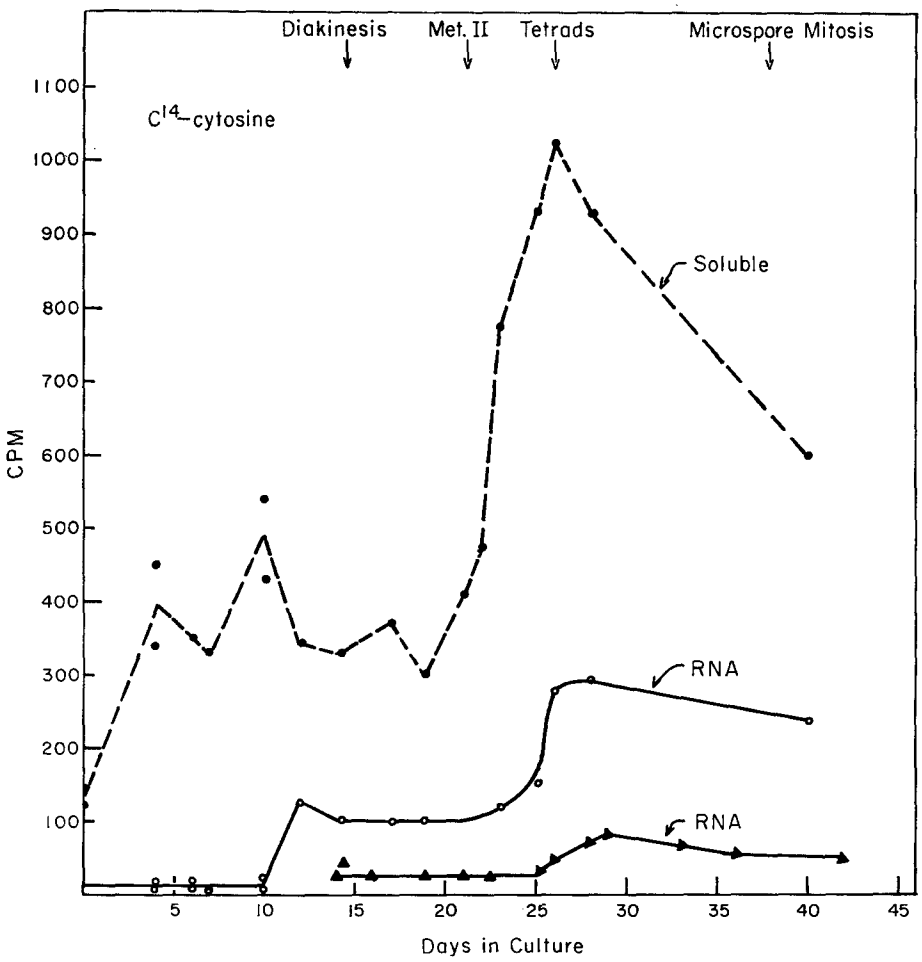


FIGURE 3

The accumulation and incorporation of C^{14}-cytosine by microsporocytes of cultured anthers under conditions of continuous exposure. 13,000 CPM were added to each bud and 10 anthers used for each determination. Dotted line represents acid-soluble fraction; solid line, RNA.

acid-soluble pool after 5 to 7 days of exposure. An example of this is provided in Fig. 3. A large number of anthers were exposed to 13,000 CPM of C^{14}-cytosine per bud in early leptotene. Groups of 12 anthers were removed at successive intervals and the distribution of label in the microsporocytes determined. After 20 days the total counts in the acid-soluble and RNA fractions are about the same as after 5 days; it is only at the time of tetrad formation that a new and comparatively vigorous increase in uptake occurs. (*c*) Synthesis of macromolecules is periodic. Microsporocytes which have been continuously exposed to label show only 2 intervals of RNA synthesis (around pachytene-diplotene and tetrad formation). The same pattern is evident in the series exposed from early leptotene and that exposed at a later stage (Fig. 3). As would be expected, only one interval of DNA synthesis is found and this occurs during microspore interphase (Fig. 4). Periodicity was also evident in cultures which had been exposed to label for a 5 day interval at different stages in the meiotic cycle (Fig. 5). On the whole this procedure is less satisfactory since the interval is long enough to cover the later meiotic stages (Fig. 1). It is nevertheless apparent, using C^{14}-leucine as a label, that the two periods of RNA synthesis are matched by two of protein synthesis (Fig. 5).

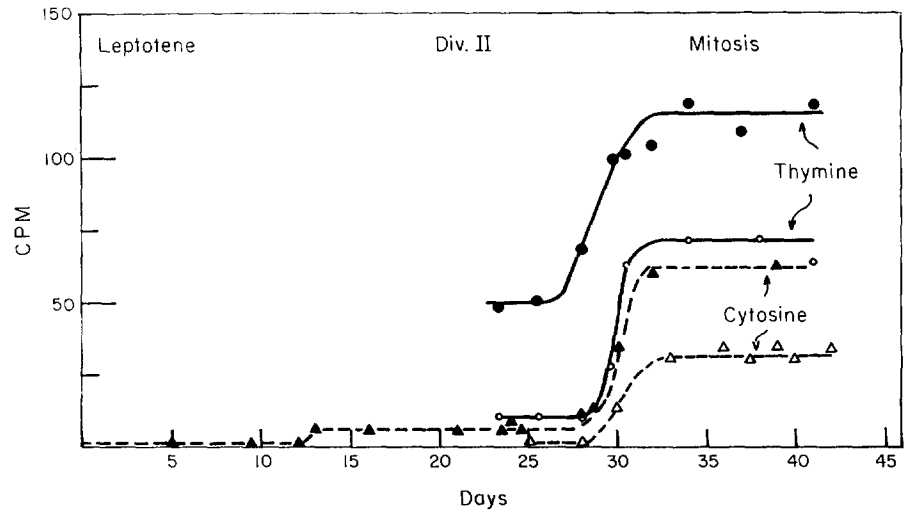


FIGURE 4

The incorporation of C^{14}-thymine and C^{14}-cytosine into DNA of developing microsporocytes of cultured anthers under conditions of continous exposure to label. Each point represents count in 10 anthers exposed to a total of 30,000 CPM. The beginning of the line indicates time at which culture of all the buds used in constructing the curve was begun.

After removing acid-soluble components from the microsporocytes the tissue was twice extracted with 0.2 N NaOH for 20 minutes at 70°C. DNA was precipitated by acidification, the residue twice washed with cold 5 per cent trichloracetic acid (TCA) and the DNA reextracted with 5 per cent TCA for 20 minutes at 100°C. This extraction was performed twice. TCA was removed from the extracts by use of ethyl ether. Counts were made in the usual way.

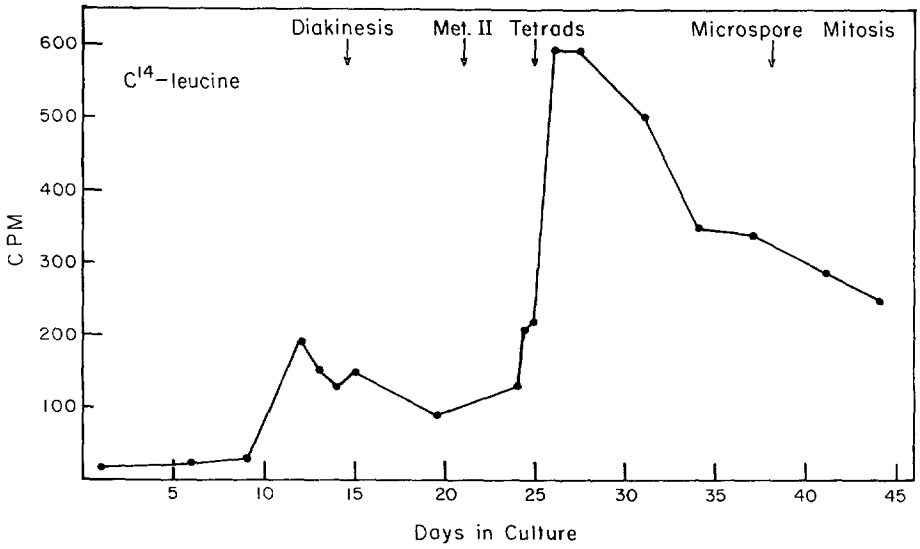


FIGURE 5

Rate of incorporation of C^{14}-leucine into proteins of microsporocytes at various stages of the meiotic cycle. Each point represents the total incorporation by the microsporocytes of 10 anthers exposed to 17,000 CPM of label for 5 days. The stage was determined at the onset of each exposure. "Protein" represents the alkaline-soluble material remaining after removal of RNA and DNA as described under Fig. 4.

To set the above results in perspective of the cultured system, some comparison between the somatic and germinal tissues is desirable. In terms of mass, the microsporocytes constitute 2 to 4 per cent of the explant. That the somatic tissues are essential to the normal performance of the microsporocytes is too evident to bear mention, although the fact that the small knob of tissue below the floral axis has proved effective in fulfilling the requirements for satisfactory culture serves to emphasize the point. The role of these tissues is beyond the scope of this study and, indeed, beyond our immediate interest. We have proceeded on the assumption that macromolecules are not transferred from somatic tissues to the microsporocytes, an assumption consistent with evidence thus far accumulated (the work of Takats, (13), in particular, bears admirably on this point). In these studies, therefore, the principal concern has been that the labeling presumed to occur in the acid-insoluble fraction of the microsporocytes arises from somatic contamination even though cytological observations show no evidence for it. A comparison of labeling in somatic and germinal tissues in early or preleptotene is given in Table I. It may be seen that, irrespective of the labeled precursor used or the extent to which it is incorporated into the somatic tissues, there is virtually no label to be found in the RNA and protein of the microsporocytes, and the same holds true for cultures which have been continuously exposed to label until some time in pachytene. A different type of test was made with C^{14}-arginine during leptotene-zygotene. Three groups of anthers were exposed for 5 days to either 3,000, 26,000, or 42,000 CPM, the specific activity being the same in all cases. At the end of this period the counts in the acid-soluble pool of the microsporocytes were 220, 220, and 240, respectively; the acid-insoluble counts were negligible. Thus, the fraction of amino acid taken up by the microsporocytes appears to depend upon the amount administered: the larger the amount, the lower the percentage. The result is consistent with the observations of Taylor (personal communication) that the specific activity and not the total amount of label is critical in radioautographic studies of these tissues.

TABLE I

Uptake and Distribution of Labeled Compounds in Somatic and Germinal Cells of Explants

Compound	Per cent label absorbed	Tissue	Acid-soluble (CPM)	Acid-insoluble (CPM)
C^{14}-leucine	75	Somatic	2940	6339
		Microsporocytes	205	20
C^{14}-arginine	99	Somatic	53,962	3195
		Microsporocytes	307	4
C^{14}-uracil	33	Somatic	54,069	839
		Microsporocytes	409	10

Counts are totals for two explants (12 anthers) which had been exposed to label for 5 days. "Per cent label absorbed" is calculated from the total counts present in the tissues plus counts remaining in mediums. All microsporocytes were in preleptotene or early leptotene through the experiment.

Taken together, the labeling experiments permit the following conclusions: (*a*) Reagents present in culture media do reach the microsporocytes and microspores at all stages of development. (*b*) Utilization of the accumulated reagents by the microsporocytes is a function of meiotic stage. (*c*) Protein and RNA synthesis occurs during the meiotic cycle well after DNA synthesis has been completed and at a time when chromosome condensation is already under way. This conclusion is supported by radioautographic studies of Taylor (14).

The Action of Inhibitors

If the evidence for RNA and protein synthesis during the meiotic cycle is correct, then reagents

interfering with these two processes should also interfere with the progress of the cycle. This is, in fact, the case, and the effects of the inhibitory reagents used may be classed under the following headings: (*a*) delay in meiotic development by all of the reagents (Fig. 6); (*b*) specific cytological upsets arising from the stage at which inhibitor administered; (*c*) specific cytological upsets arising from the nature of the inhibitor; (*d*) dissociation of the component events of meiosis. In thus listing the observed effects the possible influence of derangements in the metabolism of somatic tissues has been omitted. The point is a vexing one in any attempt at interpretation of the results and cannot be ignored. Without far more sophisticated techniques of study, direct proof that the effects of a particular inhibitor are due solely to its action within the microsporocyte is excluded. There are nevertheless some observations that provide circumstantial pointers. The first of these is that morphologically there is no apparent degeneration of somatic tissues even in those explants which had been maintained in culture along with inhibitor for 90 days. Of the 81 anthers remaining in as many culture tubes for final sampling, only 4 were degenerate. It so happened that the degenerate ones all belonged to series in which development to the microspore stage occurred, whereas those anthers in which microsporocytes were inhibited in early meiosis appeared healthy. The second observation is that occasionally microsporocytes within an anther fall into two extreme groups with respect to their response to a particular inhibitor; usually, when this occurs, all anthers on the bud behave similarly. Thus, in the case of ethionine, for example, one group proceeded to the microspore stage whereas the other remained for 90 days in early meiosis. Effects such as these are difficult to explain by a generalized indirect effect of somatic upsets. It is much simpler

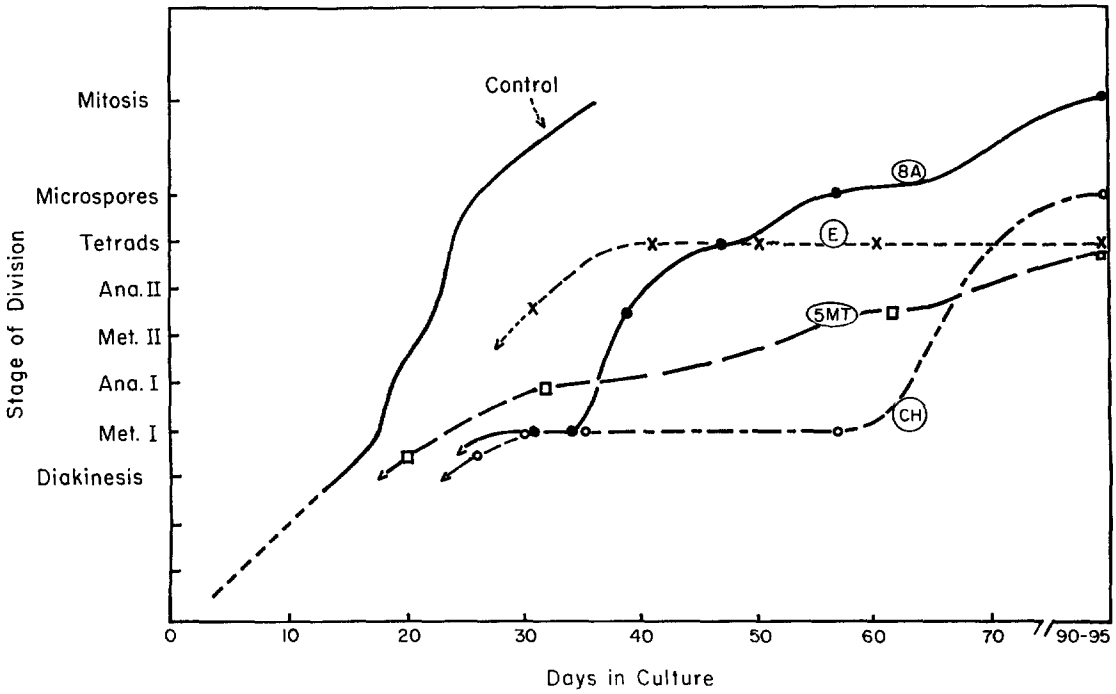


FIGURE 6

Effects of reagents on meiotic rate in cultured anthers. 8A = 8-azaguanine; E = ethionine; 5MT = 5-methyltryptophan; CH = chloramphenicol. Cultures were all begun during leptotene-zygotene stages and these are marked as zero time. First point in each curve marks first cytological observation. It may be noted that only 8-azaguanine–treated preparations entered microspore mitosis. These are sample curves each representing a single series of anthers.

to suppose a difference in the action of ethionine within the microsporocyte at a stage when the timing of action is critical.

1) Delay in Meiotic Development

Although all the inhibitors have the same general effect when administered at the leptotene-zygotene stage (Fig. 6), they also show certain differences with respect to the capacity of the microsporocytes to overcome the initial inhibitory action and the extent to which development proceeds thereafter. The cytological aberrations which ensue are excluded from these considerations. All cells tested overcame the initial effects of azaguanine, and, if cultured for 90 days, underwent microspore mitosis. Only 2 out of 24 chloramphenicol-treated groups reached microspore mitosis; the remainder were arrested at microspore interphase or much earlier in certain cases (to be discussed later). 5-methyltryptophan–treated cells behaved much the same as chloramphenicol-treated ones, but on the addition of tryptophan the microspores underwent mitosis. Ethionine-treated cells did not proceed beyond the tetrad stage unless methionine was added, and, even then, none of the microspores were observed to undergo mitosis.

Endogenous reversal of the effects of azaguanine is most readily explained by the acid-soluble pool of purine derivatives formed in the adjacent somatic tissues which would serve to dilute the analogue; chloramphenicol cannot be thus diluted. It would appear that neither 5-methyltryptophan nor ethionine are as readily diluted endogenously as is azaguanine. It appears likely that the irreversible effect of ethionine in blocking microspore mitosis is due to its incorporation into some persistent protein; 5-methyltryptophan, by contrast, is presumed to block protein synthesis but not to be incorporated.

2) Specific Cytological Effects in Relation to Time of Inhibitor Addition

A. ADDITION OF INHIBITORS DURING LEPTOTENE-ZYGOTENE STAGES: There is an interval during leptotene in which the presence of inhibitors (excepting azaguanine) virtually suppresses meiotic development over the 90 day period tested. Since cytological criteria are lacking to define this interval precisely, it is impossible to elaborate on this point. Of the 74 series tested, 9 such arrests were observed of which 7 were "partial," that is, the microsporocytes within an anther fell into the two extreme groups referred to earlier (Fig. 8, *B* and *D*).

Upset of divisions I and II: The most common disturbance arising from the presence of inhibitors during leptotene-zygotene is a failure of chromosome segregation at meiosis I. There is no indication as to whether this failure is due to the malfunction of the spindle apparatus or of the centromeres, but the result is uniform: the bivalents which normally come apart in anaphase movement dissociate into their component chromosomes and remain unoriented in the cell (Fig. 7). No single course is followed in response to the abnormal situation. What is clear—and probably significant—is that in no case have we been able to identify with certainty the formation of a single metaphase plate consisting of the 10 individual chromosomes. This is not because the mechanism governing the second meiotic segregation has also been disrupted. It is common to find equational divisions at anaphase II in cells which have had a 1st division failure (Fig. 7); unequal divisions in cells thus affected are the exception rather than the rule. Thus, although cells in which bivalents have dissociated without anaphase separation resemble superficially mitotic cells, there appears to be a regulatory mechanism which prevents such cells from following a normal mitotic cycle. Instead, chromosomes separate into two or more groups and then undergo a 2nd division. Occasionally two metaphase plates have been observed (Fig. 7) but not enough of them to permit any generalizations. The extent to which unequal divisions reduce the viability of the cells affected is not known, but, on the whole, it would appear that they reach the tetrad stage and, frequently, the microspore stage.

The microscopic appearance of tetrads and microspores is not a simple guide to the previous history of the cells. Other processes, which also affect their morphology, will be discussed shortly. Extreme differences are easy to diagnose. Thus, where 4 unequal nuclei are present in a tetrad, the cause is obvious, but this, for reasons given above, is not common. In some series the effect of a failure at first division followed by an equational second division is obvious in the numbers of differently sized pairs of nuclei at the tetrad stage (Fig. 7). Gross differences in microspore size are also reliable evidences of previous failures in meiotic divisions and these have been frequently

FIGURE 7

Effect of reagents presumed to interfere with normal protein formation on meiotic segregation.

In legends for Figs. 7 to 10; reagent used and approximate time of addition indicated in parentheses.

5MT, 5-methyltryptophan	8A, 8-azaguanine
T, tryptophan	G, guanine
E, ethionine	C, chloramphenicol
M, methionine	

A . (5MT + T; Leptotene-Zygotene). Upset of metaphase I after 28 days in culture. Groupings of chromosomes as well as random distributions may be seen. Series reached microspores about 60 days later but no mitoses found. × 400.

B . (8A; Leptotene-Zygotene). Bivalents have come apart and most have formed a single plate. Two plates have frequently been seen, but not one containing all 10 chromosomes. Fixed after 40 days in culture. Microspore mitosis occurred 50 days later. × 735.

C . (5MT; Leptotene-Zygotene). Groupings of dissociated bivalents. Reached tetrad stage. × 570.

D . (C; Zygotene-Diplotene). Cytokinesis after unequal segregation. See Fig. 7, *N* for microspore stage. × 670.

E . (C; Leptotene-Zygotene). Failure of metaphase I after 30 days in culture. In one cell, bivalents have not yet dissociated; in others they have begun to do so. Dissociation of bivalents is therefore not a precondition to metaphase I failure. See Fig. 8, *C* for persistence of effect. × 820.

F . (8A; Leptotene-Zygotene). Same slide as Fig. 7, *B*. Equal 2nd division following unequal 1st. This is the most common sequence. Note planes of division; normally these would parallel cell plate. × 450.

G . (8A; Leptotene-Zygotene). Same series as above but 10 days later. 2nd division lags. Note again equal 2nd following unequal 1st and thick wall formation. × 600.

H, I . (5MT; Leptotene-Zygotene). "Tetrad" formation after 60 days culture. Note consequence of equational division of 3 chromosome groupings. Most cells on slide had two groupings following metaphase I failure. Same group of treatments as Fig. 7, *C*. × 625.

J . (8A; Leptotene-Zygotene). Same preparation as Fig. 7, *B*. Note failure of synchrony in members of diad. The effect is seen occasionally. × 500.

K . (8A; Leptotene-Zygotene). Low power view of 1st division upset after 40 days culture. Only small pressure applied in fixing preparation. Note variations in distribution of chromosomes. × 280.

L . (5MT; Leptotene-Zygotene). Same preparation as Fig. 7, *C*. Tripolar meioses infrequently seen. × 450.

M . (8A; Leptotene-Zygotene). Same as Fig. 7, *B* but 10 days later. Unequal 2nd divisions have been infrequently seen. Note thick walls which have formed. This is also evident in other photographs and would appear to arise from a selective effect of inhibitor at time of administration. The opposite effect of 8A may be seen in Fig. 9. × 480.

N . (C; Zygotene-Pachytene). Variations in microspore size at mitotic metaphase due to difference in chromosome number arising from disturbances in meiosis. This is same series as Fig. 7, *D*, 60 days later. The normal haploid number is 5. × 500.

O . (5MT + T; Zygotene-Diplotene). Demonstration similar to Fig. 7, *N* except for difference in reagent. Without tryptophan mitosis does not occur. Note weakly staining areas on chromosomes and compare with Fig. 10 (*B*, *D*, and *H*). Early effects of this treatment shown in Fig. 7, *A*. × 700.

P . (8A + G; Leptotene-Zygotene). Variability in microspore size at prophase after 35 days culture. The effect is due to a fusion of cells. In presence of guanine, 8A is much less effective in retarding meiosis but a common result is failure of wall formation at tetrad stage and subsequent fusions of microspores. Same effect may be produced with 8A alone if administered in late 2nd division. (Fig. 10). × 230.

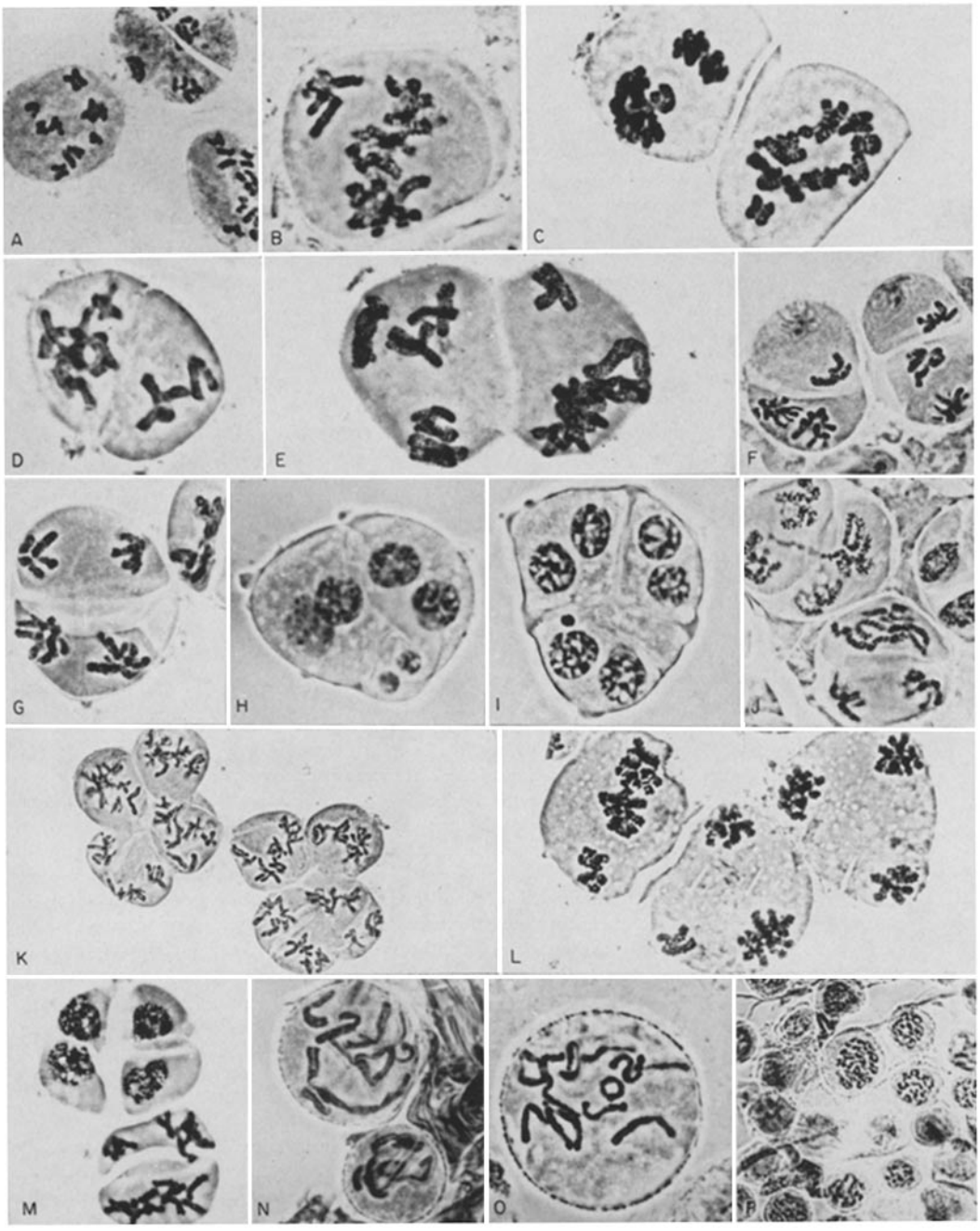


FIGURE 7

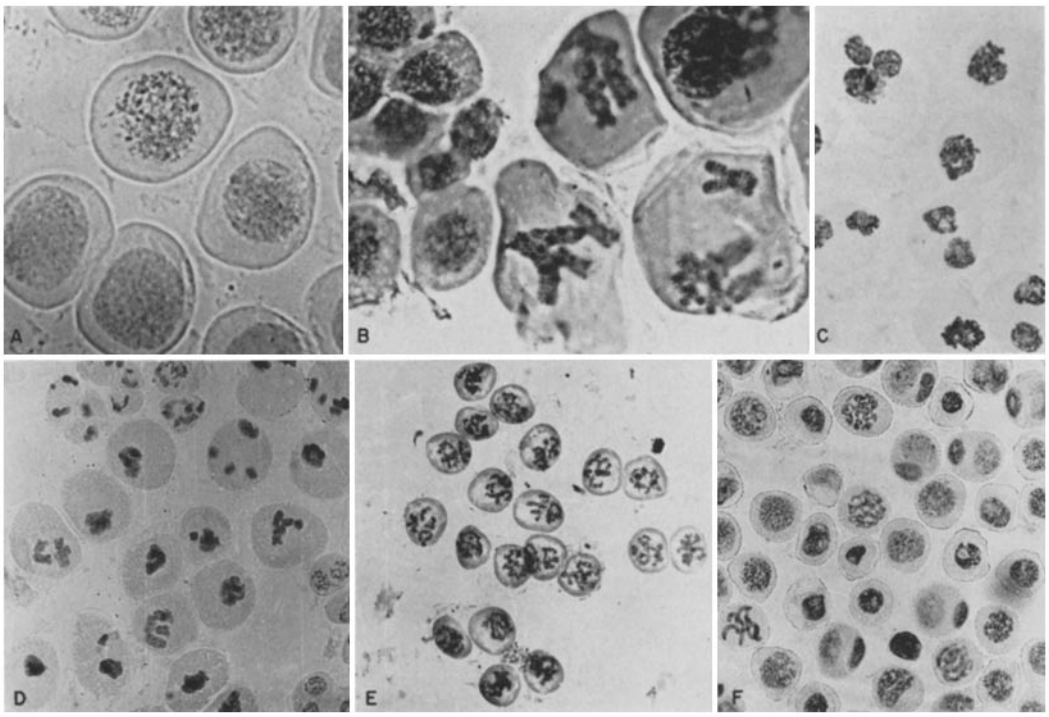


FIGURE 8

Some persistent effects of reagents on meiotic process. The persistence of material deficiencies in chromosomes due to 5MT has been shown in Fig. 10. No persistent effects have been noted with 8-azaguanine except where unequal segregations of the chromosomes occurred.

A . (E; Leptotene). Leptotene microsporocytes after 90 days of culture. A slight wall formation has occurred but otherwise the cells appear much the same as originally. The effect seems to be due to the presence of ethionine at a very early stage of meiosis but no exact data on this point are available. × 370.

B . (C; Zygotene-Pachytene). Metaphase I cells and microspores in same anther after 30 days of culture. The juxtaposition on a smear is rare. Extreme differences such as these were taken as evidence against any general effect of the reagents on the anther as a whole in producing division upsets. × 480.

C . (C; Leptotene-Zygotene). Meiotic cells arrested either at telophase I of meiosis (single nuclei) or telophase II. The diads have separated and no wall formed. Note the persistence of condensed chromosomes in telophase I. Photograph taken after 90 days of culture. × 400.

D . (E; Leptotene). Persistent metaphase I after 90 days of culture. Here the morphology of the chromosomes has been affected. As in the case of Fig. 8, *A*, it is presumed that ethionine reached the cells at very early leptotene. × 180.

E . (E; Diplotene-Diakinesis). Microspores in which chromosomes have failed to reaggregate after 90 days in culture. The series reached tetrad stage 50 days earlier. × 220.

F . (8A; Leptotene-Zygotene). Apparently normal microspores at various stages of mitosis after 90 days in culture. The series reach the microspore stage 50 days earlier. Apart from long delay, no other effects observed. × 220.

observed in azaguanine-treated preparations because they, unlike chloramphenicol-treated ones, develop into microspores. Chromosome counts at mitotic metaphase are not extremely helpful since the cells are poorly synchronized at that stage and relatively few metaphases are found in any one smear. It is of course easy to identify chromosome numbers which are much greater than haploid (Figs. 7 and 9), but in the relatively small microspores even the metaphase chromosomes are tightly packed and, although tempting, it is difficult to be convinced that less than the haploid number is present.

Comparatively few cells have been observed in which the twenty chromatids lie randomly dispersed. Unequal 2nd divisions following equational 1st divisions have also been seen infrequently. Examination at closer intervals of time would have to be made however before any significant conclusions could be drawn from these few observations.

Cytokinesis and planes of division: It is almost a rule that treatment of microsporocytes with azaguanine during leptotene-pachytene leads to upsets in cytokinesis. Such upsets are expressed in a number of ways and it is more meaningful to discuss them in the next section in connection with the dissociability of meiotic events. At present it is sufficient to note that this is a prominent consequence of treating the cells with inhibitors of protein synthesis. Associated with such upsets are departures from the normal planes of division. Frequently first anaphase groups are not widely separated and various planes of division result (Figs. 7 and 9).

Arrest of 2nd division: Normally, meiotic cells of *Trillium* show no stage which can be appropriately called telophase I. Chromosomes remain coiled after anaphase I and enter into division II without any detectable interphase. However, telophase figures are often seen in chloramphenicol-treated cells. The same holds true for cells treated with azaguanine at a later stage (Table II). Thus, the most common effect of these inhibitory substances on the second division is that of total arrest. Yet, although diads

TABLE II

Effect of Adding Azaguanine at Stages Beyond Zygotene

Stage at which inhibitor added	Segregation upset	Ultimate stage reached after days indicated	Microspore mitosis after days indicated
Pachytene	(Divisions I and II)	Tetrads (17)	−(57)
Diakinesis	(Telophase I)	Tetrads (29)	−(57)
Diakinesis–Metaphase I	(Telophase I)	Tetrads (29)	−(57)
Metaphase I	—	Microspores (29)	−(57)
Metaphase II–Telophase II	—	Microspores (29)	*
Telophase II–Tetrads	—	Microspores (10–18)	+(40–68)
Tetrads	—	Microspores (10–14)	+(40–68)

The days listed represent the interval of time between addition of inhibitor and property noted. The last column also indicates the time at which the last anther in the series was examined. Thus, it is conceivable that cells which had not reached the microspore stage or undergone mitosis might have done so if cells had been permitted to develop for longer periods of time. Although all preparations which reached mitosis are listed as microspores, see text for a discussion of aberrations. Explanation of * is also to be found in the text in connection with the pronounced sensitivity of cells at this stage to injurious effects. The extent of delay in reaching the different meiotic stages may be readily seen by comparing these data with the curves in Fig. 1. Telophase I signifies an arrest at this stage. Under normal conditions telophase I does not occur.

were common in some series during the middle intervals of observation, they were infrequent when later preparations were examined. Only in one chloramphenicol series did the diad condition persist after 90 days (Fig. 8) and it is doubtful, to judge from their appearance, that such cells were capable of further development. It seems reasonable to conclude from our observations that meiotic cells are so patterned as to resist reversion to a normal interphase state without prior separation of chromatids.

Chromosome structure: All the inhibitors used affected the structure of the chromosomes to a variable extent. This is particularly true of the amino acid analogues. Despite the extreme effects which were often evident with these reagents, there were relatively few instances of aberrations in chromosome segregation. The bearing of changes in chromosome morphology will therefore be more advantageously discussed in relation to the dissociability of meiotic events.

B. ADDITION OF INHIBITORS LATER THAN LEPTOTENE-ZYGOTENE: Observations in this connection have been made only with azaguanine. The results obtained, which are summarized in Table II, are all the more interesting in light of the fact that all cells treated with azaguanine during leptotene-zygotene underwent mitosis

Cells which were treated between pachytene and metaphase I either did not reach the microspore stage or, if they did, failed to undergo mitosis. Thus, irrespective of the degree to which the azagunaine persisted when added at the beginning of culture, there must be a later interval which responds differently to the effect of interference with RNA synthesis than the earlier one

FIGURE 9

Cytokinesis and wall formation in relation to chromosome behavior in presence of inhibitory reagents. The examples reproduced in this plate are intended to illustrate that the failure or occurrence of cytokinesis and wall formation are dissociable from other facets of chromosome behavior. Particularly noteworthy is the fact that wall synthesis may be induced without reversion of chromosomes to metaphase condition.

A . (8A; Leptotene-Zygotene). Wall formation in microsporocytes following failure of metaphase I. Same series as Fig. 7, *B* but 10 days later. Walls have not been stained and are seen as light areas. × 560.

B . (8A; Leptotene-Zygotene). Anaphase II in microsporocytes without intervening cytokinesis. Notice departure from normal with respect to division planes. See note in Fig. 7, *G*. This slide in series different from that of preceding one. × 750.

C, D . (E; Zygotene-Diakinesis). Aberrant planes of cytokinesis and extensive wall synthesis. Note lack of any correlation between position of nuclei and lines of wall formation. Ethionine markedly affects either wall formation or chromosome reaggregation depending probably on time of administration. No ethionine-treated cells entered microspore mitosis. × 870.

E . (E; Leptotene-Zygotene). Heavy wall formation in tetrads. Chromosomes did not uncoil following completion of meiosis. × 600.

F . (E; Leptotene-Zygotene). Same as above. × 1050.

G . (8A; Leptotene-Zygotene). Microspores without walls formed after 35 days of culture. Walls were evident 8 days later. × 750.

H . (8A; Anaphase II-Telophase II). "Tetrads" in mitotic prophase. Principal effect of azaguanine added at this stage is to inhibit wall formation without inhibiting mitosis. Frequently nuclei fuse, the cell plate disappearing. × 530.

I . (8A; Anaphase II-Telophase II). Lack of synchrony in nuclei of "tetrads." Note one prophase grouping but separate nuclei in other half of "tetrad." × 670.

J . (8A; Anaphase II-Telophase II). Microspore anaphase polyploid due to fusions. Same preparation as above. × 800.

K . (8A; Diakinesis-Metaphase I). Prophase in tetrads 17 days after azaguanine added. Wall formation and dissociation of cells into individual microspores inhibited. Fusion may occur as in Fig. 9 *I* and *J*. × 560.

L . (8A; Diakinesis-Metaphase I). Same preparations as Fig. 9 *K*. Note arrest of division II. Telophase I does not normally occur in *Trillium*. Also note prominent nucleoli, an unexplained feature of cells thus treated. × 380.

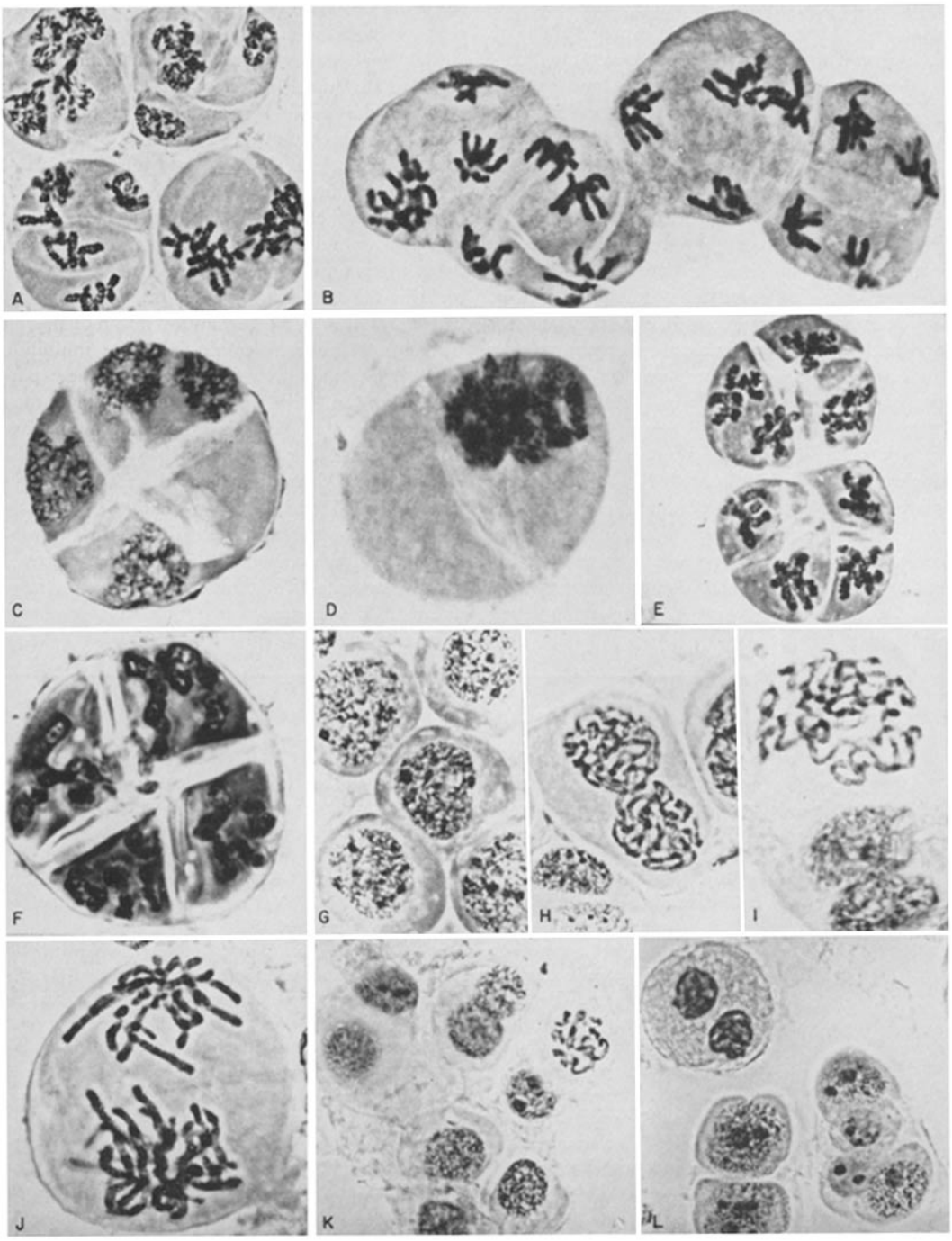


FIGURE 9

All the inhibitory effects could not have originated in the earlier stages of meiosis. The point at which the inhibitors acted in this series of experiments can only be approximated although it is certain that they could not have acted prior to the stage indicated. Cells which have reached pachytene are still susceptible to aberrations in the 1st and 2nd divisions. Those which have reached diakinesis can be arrested at telophase I. Beyond this stage a new set of effects appears. Three series of anthers treated at the time of 2nd division responded in a way which was not encountered in all the other treatments. In each of these there appeared a mixture of abnormal and normal microspores. The abnormal cells are best described as degenerate; the normal ones underwent mitosis. There was no consistent proportion of normal cells in each anther of a series. On the whole, this group behaved as though there were a narrow interval of time during which inhibition of RNA or protein synthesis had extreme effects on the cells. Apart from this unusual set of effects, the most common consequence of treating cells at the termination of 2nd division is an inhibition of wall formation and frequently of cytokinesis. Development may proceed in one of several ways (Fig. 9). Failure of wall formation in tetrads is often accompanied by a fusion of nuclei. Diads derived in this way are common and are obviously distinct from those which arise owing to an arrest at telophase I. Such diads undergo mitosis in much the same way as the haploid microspores. Tetrads may also fail to dissociate into microspores. Such failure is, however, clearly traceable to an inhibition of the mechanism associated with wall development. Unlike those cells in which the tetrads do not develop beyond that stage as a result of treatment with azaguanine at earlier stages, the individual cells in the tetrads may enter into mitosis. Synchrony of the four cells may or may not prevail (Fig. 9). It must be emphasized that inhibition of wall formation is not restricted to these particular groups. Cells treated at earlier stages may also show similar effects, but the effects are neither so widespread nor are they so persistent. At the end of the 90 day culture period, cells which had been exposed earlier to an inhibitor generally regain the capacity to form cell wall. In all probability, had the cells in the group been cultured for longer periods of time cell walls would have formed. One significant feature of these observa-

FIGURE 10 (*opposite*)

Effects of reagents on chromosome structure and behavior. The three principal effects observed are (*a*) uncoiling of chromosomes, (*b*) absence of stainable material in chromosomes, (*c*) failure of chromosomes to reaggregate. Effects (*a*) and (*c*) produced by azaguanine but are transient. 5MT and ethionine frequently produce persistent effects (Fig. 8).

A . (5MT + T; Zygotene). Microspore mitosis 90 days after treatment. Note thinness of chromosomes and pale areas. This is typical effect of 5MT. Tryptophan must be added for cells to enter microspore mitosis. × 750.

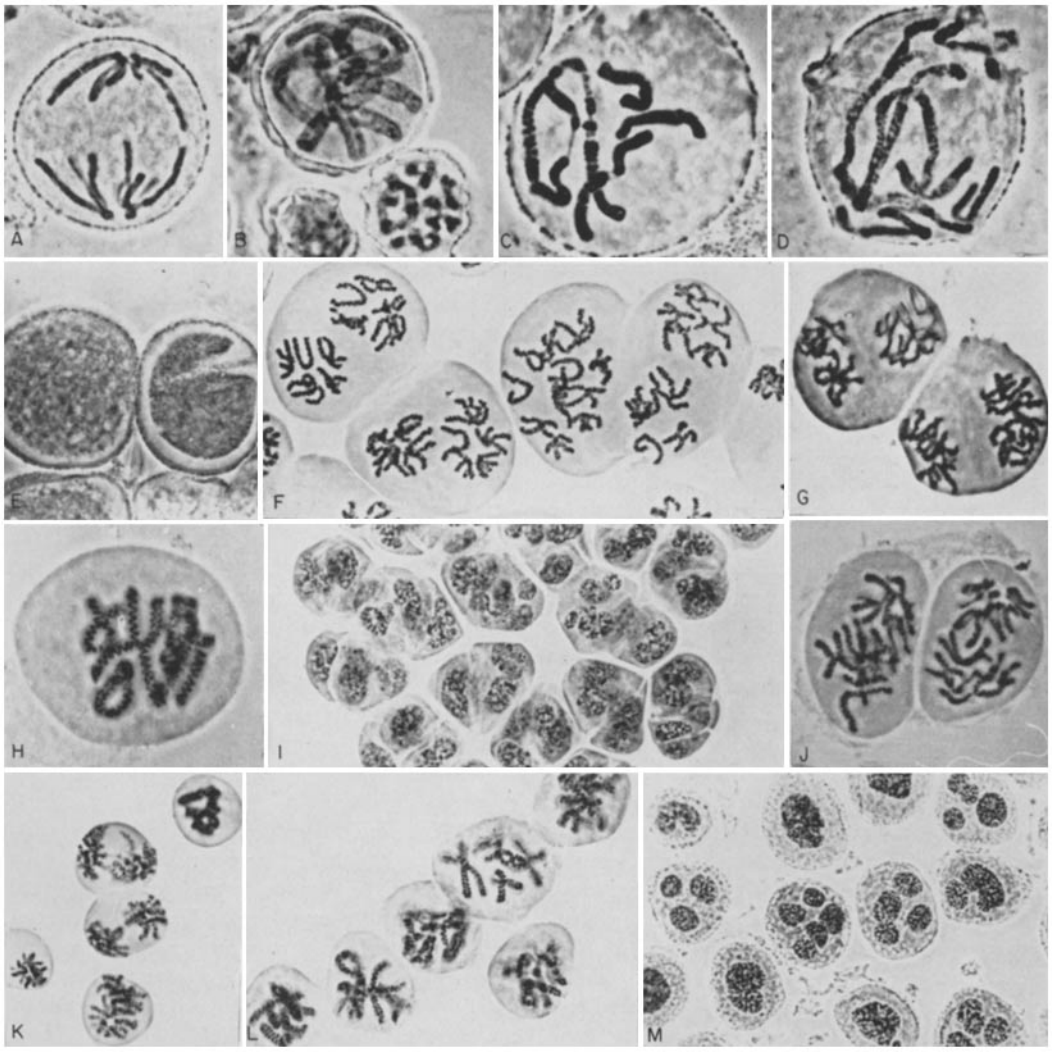
B . (8A + G; Leptotene-Zygotene). Contrast chromosome/cytoplasm volume ratio with A. Difference in microspore size due to fusions arising from failure of wall formation at tetrad stage (Fig. 7 *P*.). Note presence of wall indicating transient nature of wall inhibition. × 750.

C, D . (5MT + T; Zygotene-Diplotene). Absence of stainable material in metaphase chromosomes at microspore mitosis. Same preparation as Fig. 7, *O*. × 1250.

E . (8A; Tetrads). Swollen microspore nuclei 6 days after addition of azaguanine. The effect disappears. Mitosis observed 30 days later. × 630.

F, G . (C; Leptotene-Zygotene). One typical effect of chloramphenicol on chromosome appearance during metaphase I–anaphase I of meiosis. Contrast this with different series exposed to chloramphenicol (Fig. 7, *E*) where chromosomes have normal appearance but meiosis I upset. In general morphological abnormalities do not necessarily coincide with abnormalities in disjunction. Note that 5 chromosomes are present in each anaphase group. *F*, × 540; *G*, × 430.

H . (5MT + T; Leptotene-Zygotene). One of typical effects at metaphase I. Photographed after 60 days of culture. Unlike the transient effect of azaguanine (not



shown here) in which chromosomes extend on unspiralling, there appears to be little extension but rather an absence of matrical substance. Much later stage of this treatment shown in Fig. 8, *C* and *D*. × 600.

I . (8A; Leptotene-Zygotene). Multinucleate tetrads arising in part from unequal segregation and in part from failure of chromosomes to reaggregate. Compare with Fig. 10, *M*. × 340.

J . (5MT; Leptotene-Zygotene). "Uncoiled" chromosomes at anaphase I. Many of the cells in this series segregated abnormally (later stage in Fig. 7, *H*). × 430.

K . (5MT; Leptotene). "Uncoiled" chromosomes at anaphase I after 50 days of culture. Occasionally coils get trapped in segregation (upper cell). This is in series different from that of Fig. 10, *J*. The persistent effect of chloramphenicol may be seen in Fig. 8, C. × 260.

L . (5MT + T; Leptotene-Zygotene). Low-power field of cells in same preparation as Fig. 10, *H*. × 340.

M . (8A; Leptotene-Zygotene). Multinucleate microspores due to failure of chromosomes to reaggregate. Meiotic divisions were mainly normal. The individual nuclei ultimately fuse. Microspores undergo normal mitosis. Contrast with Fig. 8, *E*. × 540.

tions is again the evidence that wall formation and cytokinesis must be specified at an interval of time different from that governing the other facets of chromosome behavior.

Dissociability of Specific Events

1) CHROMOSOME MORPHOLOGY: One question which arises as result of our observations is the extent to which the normal segregation of chromosomes depends upon their morphological state. With all the inhibitors used there appeared in one group or another chromosomes which were either highly condensed or appreciably uncoiled (Figs. 7 and 10). The effects persisted least with azaguanine, somewhat longer with chloramphenicol, and in some respects were irreversible when induced with ethionine or 5-methyltryptophan. The different forms do not appear randomly in preparations, but, here too, we lack a sufficiently close control over time of treatment to assign the origins of the observed effects to any particular interval. It can only be said that condensed or partially unspiralled chromosomes have been consistently observed in anthers treated with one of the inhibitors at, or earlier than, pachytene. In some series of anthers one or another of the forms may be present in virtually all of the cells in 1st or 2nd division; in others there may be a mixture of the two; in still others, they are virtually absent. The appearance of these extremely different forms is not identical for all reagents. In chloramphenicol-treated preparations, for example, the chromosomes in anaphase I are frequently stringy (Fig. 10) whereas with azaguanine the coils are obvious. Metaphase I chromosomes exposed to 5-methyltryptophan frequently have a distinctive "uncoiled" appearance. In these it appears as though some of the chromosomal matrix is lacking (Fig. 10). But, these various points of difference and similarity aside, there is no striking correlation between aberrant segregation and the morphology of the chromosome. Highly uncoiled anaphase chromosomes may get entrapped with one another and in this way interfere with normal migration, but, short of such an effect, uncoiled chromosomes may segregate normally (Fig. 10). There is a far greater coincidence of failure at metaphase I and the presence of highly condensed chromosomes; in some preparations nearly all the cells with condensed chromosomes failed to segregate at 1st division. This may however be no more than a coincidence of two effects, as this relationship was not universal. Thus, although it is clear that the reagents used affect chromosome morphology we can at present infer little about the cause and only note that chromosome morphology at the microscopic level probably bears only a secondary relationship to segregation. This is strikingly illustrated with 5-methyltryptophan. Anthers cultured in the presence of this compound alone do not enter microspore mitosis. They do, however, if tryptophan is also added to the medium. The metaphase chromosomes of microspores previously treated in this way are generally thin, and under high magnification it can be seen that chromosome material is lacking (Fig. 10). In such cases it is clearly apparent that all of the material within the chromosome cannot be essential to mitotic development. It is of interest that we have observed no disruption of the linear integrity of the chromosomes despite the extremity of some of the effects, and to this extent our results are consistent with the view that linear integrity is a function of the DNA chain (16).

One result of general significance arises out of the behavior of microsporocytes treated with ethionine. This compound when present at certain intervals markedly inhibits the capacity of the chromosomes to revert to the interphase state. The inhibition does not always persist, but, where it does, it is apparent that the cells may continue to develop in an otherwise normal fashion. Tetrads may synthesize wall material even though all the chromatids have an anaphase II configuration (Fig. 9). In one series, virtually all the microspores after 90 day culture retained the individuality of the chromosomes even though the latter were much thinner than in meiosis (Fig. 8). Such behavior raises a fundamental point concerning the capacity of cells to perform syntheses when chromosomes are in an aggregated state (5).

2) CYTOKINESIS AND WALL FORMATION: In earlier descriptions of meiotic upsets the dissociability of cytokinesis from other meiotic events was already implied. We may summarize our observations by saying that there is no obligatory relationship between the times at which cytokinesis may occur after anaphase I and the developmental stage of the cell. Planes of cytokinesis may vary and they may surround several nuclei, one nucleus, or no nuclei at all (Fig. 9). They may be formed at interphase or during

division. There appears to be only one strict regulatory relationship: the prevention of cytokinesis prior to metaphase I. Much the same applies to wall formation which has already been discussed in relation to the effects of ethionine.

3) ENZYME ACTIVITY: Microspores of anthers in control cultures, like those of *in situ ones*, exhibit a single and brief period during which the enzyme, thymidine phosphorylase, is active (Fig. 11). There is no special significance attached to this particular enzyme except for its being a member of the DNA synthesizing system. Other members behave similarly (11) but this is a convenient one to measure. Although a large number of enzyme assays were made, the results are deficient in one respect. The peak of enzyme activity is relatively brief and, in order to locate it, measurements must be made at close intervals. With control cultures an anther could be removed and once its stage was determined an approximate schedule for 5 other assays could be set up. With treated cultures, it should be clear, from the long delays in meiotic development, that no possibility existed for any kind of short-time scheduling. The activities obtained were therefore no more than randomly timed measurements. If high peaks of activity existed, the probability of finding them was small, and this is clear even in the case of those anthers in which the microspores underwent mitosis (Fig. 11). On the other hand, the results made certain relationships apparent. With chloramphenicol, suppression of enzyme was absolute in 22 out of 24 series. This is consistent with the observation that only 2 out of the 24 reached microspore mitosis. With azaguanine, suppression varied, but the appearance of enzyme activity did not necessarily coincide with development of mitotic figures when the inhibitor was added at telophase II or later. This is understandable in light of our experiments with lily since one can inhibit mitosis at an interval later than that required to inhibit

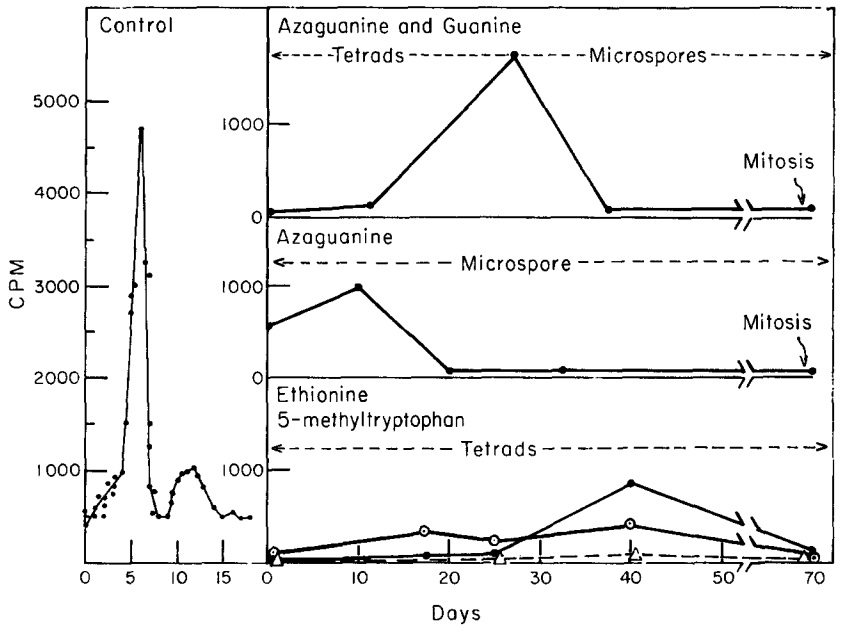


FIGURE 11

Periodicity of thymidine phosphorylase in microspores of cultured anthers. Conditions referred to under Methods. Ordinates represent CPM of thymidylic acid formed. The unforeseen limitations of obtaining adequate numbers of points for treated anthers are discussed in text. The dotted line representing 5-methyltryptophan in the lower right graph is typical for this reagent and chloramphenicol. With one or two exceptions, which may be related to the recovery evident by resumption of microspore mitosis, thymidine phosphorylase activity is below the levels of detectability in cells exposed to these two reagents.

the formation of the enzyme. What is quite apparent from ethionine-treated cells is that the enzyme may appear in early tetrads and that it may linger for extended periods of time. No ethionine-treated cells entered mitosis. Thus, at the molecular level, just as at the microscopic level, a process which has all the earmarks of induced synthesis may be severely dislocated in time without any obvious tie to other events within the cell.

DISCUSSION

Four features of the results here obtained lend themselves to a coherent explanation: the common effects of all reagents used; the specific differences between the effects of each of the reagents; the distinctiveness of effects as a consequence of the meiotic stage at which reagents are administered; and the dissociability of component events of meiosis.

1) COMMON EFFECTS: Leaving aside aspects of reversibility or frequency of occurrence, it is clear that the four reagents are all capable of inducing the following effects: delay of meiosis, upset of meiosis I without affecting meiosis II, and upset of meiosis II without previous upset of meiosis I, alterations in chromosome morphology (particularly uncoiling and condensation), inhibition of enzyme function. Thus, each of the effects observed is due either to an interference with different processes selectively acted on by correspondingly different reagents but all yielding the same net effect, or to an interference with the same process but *via* different mechanisms. There is no evidence upon which to base a choice between these alternatives. For the sake of simplicity, however, it would be preferable to suppose that all the reagents act on the same system. If so, then the obvious common denominator is synthesis of protein. The evidence that amino acids are incorporated into cells after initiation of meiosis is consistent with this conclusion.

2) DISTINCTIVE EFFECTS: If protein synthesis is the target of these reagents then one would expect certain differences between the actions of each of the reagents. In the case of amino acid analogues, the effectiveness of specific compounds should mirror to some extent the relative frequency with which the corresponding normal constituents are found in proteins. In so far as 5-methyltryptophan interferes more effectively with meiosis I than does ethionine, the expectation is fulfilled. Similarly, the marked effect of 5-methyltryptophan in reducing the amount of stainable material in the chromosomes and of ethionine in preventing the reconstitution of interphase nuclei points to selective effects of these reagents presumably on proteins. There is also an instructive contrast at the molecular level between ethionine and chloramphenicol. The latter virtually suppresses the appearance of thymidine phosphorylase, whereas ethionine-treated cells show presence of the enzyme at cytological stages which normally are not associated with appreciable enzyme activity.

3) TIME OF REAGENT ADDITION: Irrespective of the mode of action of the different reagents, it is clear that specificity of effect derives in part from the stage of the cycle at which the reagents are administered. It is also clear that the cytological upsets are caused prior to the time that they become manifest. Although it has been impossible in this first general study to pin-point the intervals responsible for specific effects, it is apparent that the presence of the different reagents at the time that observed meiotic failures occur is insufficient to cause such failures. Separation of bivalents and inhibition of anaphase I must be caused some time before diakinesis. Some group of processes essential to microspore mitosis can be inhibited in the latter part of meiosis II but not at a stage when tetrads are formed. Wall formation and dissociation of tetrads into microspores may be inhibited at a slightly later stage without inhibiting the mitotic process. Thus, it is clear on the one hand that the reagents themselves are not immediately involved in the disturbances provoked, and, on the other, that the processes essential to normal meiotic development are not all specified prior to the cytological changes which mark the termination of interphase. It is at best a tenuous proposition to link the action of these reagents with a particular mechanism; there are many ways in which the balance of meiotic events may be upset (18). Yet, despite this, it is preferable to provide a working and testable hypothesis than to take refuge in the excuse of complexity. The hypothesis, already implied in preceding paragraphs, is that the development of the meiotic cycle depends upon a sequence of protein syntheses which occur after the chromosomes have replicated and in part also after the chromosomes have undergone condensation.

4) DISSOCIATION: The dissociability of meiotic events is not only consistent with the above hypothesis but provides argument for it. Were all the effects randomly distributed among the cultured anthers, or were each of the effects due entirely to the nature of the reagent used, this might not be so. But, the fact is that a given series of anthers behaves in much the same way, and that the uniformity of response is partly due to the time at which the reagents reach the cells in question. The principal obstacle in extending this argument is that we do not yet have any reliable criterion by which to divide the developmental time axis into meaningful subunits. Gross divisions have been made but these do not cover the kinds of sequences we presume to operate in meiosis and which to some extent have been shown to exist in connection with microspore mitosis (11). The obstacle is not at all insuperable and will be overcome when the various effects noted are translated into molecular terms. When we know the times at which the formation of specific molecules may be inhibited and the times at which such molecules normally appear, a meaningful subdivision of the time axis will become apparent.

To tie the various speculations together, it is only necessary to resort to orthodoxy and to claim that if proteins are synthesized at different times then genes must also be active at different times. On this basis, internal regulation of the meiotic cycle must proceed by direct participation of gene action. To this extent and in this sense, the meiotic cycle is encoded in the gene string. The discovery by Rhoades and associate (1, 6) of single gene mutants which specifically and exclusively affect different facets of meiosis in corn is a major piece of evidence for this interpretation. The critical issue, which can only be resolved by experiment, is whether a meiotic sequence is possible if essential proteins are all simultaneously specified at an interval prior to the unfolding of the cycle. Biochemical studies of division have tended in favor of the latter view. There is no doubt that a process such as cytokinesis may occur at least in some marine eggs even in the absence of a nucleus (10) but it is patently impossible to test the proposition in this way for events involving the chromosomes. The point of view preferred here is that extrachromosomal events regulate the periodicity of gene action, and that the genes concerned are not members of a single operational unit, since component processes are dissociable. Concerning the mode of gene regulation it can only be said that the problem is as open with respect to cell division as it is with respect to differentiation.

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