PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Biochemistry of Meiosis [and Discussion]

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Biochemistry of meiosis

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The process of meiosis in *Lilium* falls into four physiological stages – prezygotene, zygotene, pachytene, and post-pachytene. Each of these stages has distinctive metabolic characteristics. Commitment to meiosis occurs during the prezygotene interval at about the time when S-phase replication is completed. The activities following commitment are essential to synapsis inasmuch as perturbations of cells during that interval have subsequent effects on synapsis and crossing over. Just before the initiation of synapsis, a distinctive lipoprotein complex appears in the nucleus. The complex most probably functions in the process of pairing. Zygotene is marked by the delayed replication of specific intercalary segments of chromosomal DNA (Z-DNA), the replication being a necessary condition for ongoing synapsis. The replication occurs in the lipoprotein complex in the presence of a reassociation protein (r-protein). Z-DNA segments would appear to have other meiotic functions inasmuch as the replicated segments remain unligated to the body of chromosomal DNA until the beginning of chromosome disjunction. The pachytene interval is marked by an activation of endonucleolytic activity. The enzyme produces single-stranded nicks in the DNA at specific loci. These loci consist of moderately repeated segments, about 100–200 base pairs long. Extracellular agents, such as radiation, cause random nicking regardless of the meiotic stage at which they are applied. Localized nicking and repair are thus unique features of meiosis. The temporal segregation of metabolic activities concerned with pairing and crossing over and their operation in special chromosome regions constitute the most prominent features of the biochemical events associated with meiosis.

This presentation has a narrow target; it is addressed only to those biochemical events in a meiocyte that are presumed to be directly related to chromosome pairing, crossing over, and perhaps marginally, to disjunction. The distinctive metabolism of a meiocyte is only partly due to the achievement of recombination; in higher organisms the products of meiosis must ultimately differentiate into functional gametes. Meiocyte metabolism encompasses processes which may be accessory or incidental to meiosis, but which are nevertheless essential to the longer term needs of gametogenesis. The formation of callose walls in the microsporocytes of flowering plants, and the amplification of ribosomal DNA during oogenesis are familiar examples (Stieglitz & Stern 1973; Gall 1969). In some cases, a distinction is easy to draw between the two categories of metabolic activity; in other cases, the boundary between the two is blurred, and an uncertain search for significance obscures rather than clarifies the nature of meiotic metabolism (Ghatnekar, Lima-de-Faria, Rubin & Menander 1974). Since very few organisms are amenable to biochemical analysis of meiosis, the danger of confusing accessory and essential functions is substantial. Our hope is that we have at least avoided major obfuscation.

Two general, and perhaps obvious, conclusions emerge from our studies of meiosis in *Lilium*. The first of these is that meiosis is mediated by a succession of metabolic phases, each of which

has its characteristic set of metabolic processes. Within an individual meiocyte, overlapping of these sets, if it occurs at all, must be minimal. The second conclusion is that chromosomal DNA contains specific regions with specialized meiotic functions in addition to those which house meiotic genes that are conventionally transcribed. Meiosis thus constitutes an interaction between a specially programmed set of metabolic activities and a specially developed set of chromosomal regions. Although analogies with recombination in microbial systems are inviting and instructive, it is virtually certain that only selected regions of a chromosome respond to the metabolic events in a meiocyte which involve synapsis and crossing over. Time and place are the theme of meiotic physiology; a specific time during meiosis for particular metabolic events to occur, and a specific place in the chromosome where a particular event is expressed.

Genome size is almost certainly a critical factor in determining the characteristics of meiotic organization within a particular group of organisms. If a basic or minimal plan of meiosis could be defined it would be seen to be progressively modified with increasing size of the genome. It is evident that the intensity of exchanges between homologues is more or less inversely related to genome size (Nei 1968). *Lilium*, for example, has 36 reciprocal exchanges per meiosis whereas yeast is estimated to have 75 (Hurst, Fogel & Mortimer 1972). Yet, the lily genome is 6000 times as large as the yeast genome. Although a rough proportionality exists between frequency of exchange and distance within a specified region of chromosome, such proportionality rarely applies to the entire chromosome. The absence of genetic map distance in heterochromatin is well known but, as emphasized strongly by Jones and co-workers, a non-random distribution of crossovers is the rule rather than the exception (Jones 1974; Perry & Jones 1974). Despite its regularity, the capacity to undergo crossing over is not shared equally by all segments of a chromosome. Indeed, some segments have no share. The exclusion of an appreciable fraction of the genome from crossing over most probably imposes special requirements on the structural organization of meiotic chromosomes and also on the organization of meiotic metabolism. These requirements must ultimately be reflected in a hierarachy of arrangements extending from the intense recombinational activities in bacteriophage reproduction to the very limited recombinational activity during microsporogenesis in *Lilium*. Certain basic molecular mechanisms probably traverse the hierarchy, but superimposed on these is a combination of structural and regulatory devices whose nature and size must be strongly influenced by the magnitude and particular organization of the genome which they are intended to serve.

Since our principal aim is to provide a biochemical account of chromosome pairing and crossing over, mechanisms that effectively limit recombination need to be given special attention. It is difficult for us to assess the extent to which this has been achieved. Because of its very large genome size, the biochemistry of meiosis in *Lilium* may turn out to be a caricature of the more common type of meiotic organization if, in fact, a 'common type' exists. Divergences in chromosome organization, such as are made evident by the contrast in proportion of unique sequences between mammals and many flowering plants may have been accompanied by corresponding divergences in achieving genetic recombination. Our conceptual approach to meiosis has been based on the *arbitrary* assumption that a 'common type' does exist, an assumption to be validated or discarded by future studies. At best, the *Lilium* caricature may point to some universal features of complex meioses by exaggeration. If so, the exaggeration will not be identified in this study. Our immediate experimental aim has been to achieve an *internally* consistent description of meiosis based on the different experimental approaches used. The

results of many of these approaches have been fully described elsewhere and their details will not be repeated here. Some of the experiments, however, are novel and these are recorded in adequate detail.

Results and discussion

From a physiological standpoint, meiosis may be divided into four successive stages. The boundaries between them are difficult to define precisely, because of the unavoidable overlapping that occurs in the analysis of individual stages. Nevertheless, each stage may be confidently assigned a distinctive set of biochemical characteristics. The first stage extends up to the initiation of homologue synapsis and will be referred to as the 'prezygotene interval'. Its beginning is deliberately left undefined, partly because claims about the extent of premeiotic influences on meiotic events remain controversial, but largely because there has been little biochemical characterization. The subsequent intervals of zygotene, pachytene, and post-pachytene are well defined cytologically and we consider them to house the functions of pairing, crossing over and disjunction.

Each of the four stages may be distinctively characterized by its protein formation (including enzyme activities), and susceptibility to disruptive effects of various physical or chemical agents. The evidence that these distinguishing properties are directly related to chromosome pairing and exchange is circumstantial but strong. We will present meiotic metabolism as a series of successive and separable processes that extend over a relatively long interval of time, the temporal pattern being essential to achieve crossing over and disjunction in organisms with large, complex genomes.

(*a*) *The prezygotene interval*

The first prominent biochemical characteristic of premeiosis is the extended duration of the S-phase which can be 100–200 times that of somatic cells. The discovery and analysis of the phenomenon is due primarily to Callan and co-workers (1973) who demonstrated that the lengthened S-phase is due to a much reduced frequency in the number of initiation points for DNA replication. This reduction may be correlated with the widespread evidence from a variety of sources for different degrees of premeiotic chromosome contraction, including extensive transient heterochromatinization during the S-phase (Stern, Westergaard & Wettstein 1975). Just how S-phase extension, in general, relates to the meiotic activities of chromosomes remains to be explored, but two particular characteristics of late premeiotic S-phase can be specifically related to meiosis. In *Lilium* microsporocytes, termination of the S-phase leaves about 0.3–0.4% of chromosomal DNA unreplicated; these unreplicated segments will be referred to as 'Z-DNA' because their replication occurs during zygotene in coordination with chromosome pairing. Termination of S-phase is also coincident with an irreversible commitment of cells to meiosis. Meiocytes may be induced to replicate their Z-DNA and enter a mitotic division up to the time that they terminate their S-phase, but not much later (Ninnemann & Epel 1973; Parchman & Roth 1971). Although physical and chemical perturbations of the meiocytes immediately following S-phase frequently affect chromosomal synapsis, the events initiating synapsis can no longer be reversed. A striking, but probably incidental, feature of the metabolic change is the loss of sensitivity to blue light following S-phase termination (Ninnemann & Epel 1973).

Among the biochemical factors underlying commitment to meiosis is a set of changes in nuclear lipoprotein. These changes begin during leptotene and are nearly completed when the

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

meiocytes reach zygotene. A prominent index of these changes is the behaviour of purified suspensions of nuclear membrane material prepared by the procedure of Kashnig & Kasper (1969). Material prepared from somatic cells segregates into two fractions when banded on a discontinuous sucrose gradient. When prepared from cells in meiotic prophase, the material segregates into three bands, the additional one having a relatively high buoyant density (Hotta & Stern 1971*a*). The lighter bands represent vesicles from the inner and outer nuclear membranes; the heavier band consists of lipoprotein but the structural source of this fraction is not yet known. Its general importance to meiosis is nevertheless emphasized by the fact that it is as abundant in mammalian spermatocytes as it is in lily microsporocytes. Evidence will be presented relating the heavy lipoprotein fraction to chromosome pairing. In anticipation of this discussion, it should be pointed out that long standing cytological evidence for unusual behaviour of the nuclear membrane at the leptotene–zygotene interval is abundant, particularly with respect to the phenomenon of cytomixis (Bopp-Hassenkamp 1959).

In *Lilium*, chromosomes proceed through leptotene to zygotene with segments still unreplicated. The modification of nuclear lipoproteins is accompanied by a modification of chromosomes due to the acquisition of axial or lateral elements (Moens 1968). Both modifications are unique to meiosis and all three features developed during this interval interact in association with the synapsis of homologues.

(*b*) *The zygotene interval*

Regardless of the number of prezygotene events contributing to the machinery of pairing, it is important to recognize that the actual pairing as defined by light and electron microscopy begins at zygotene and not earlier. Moreover, as best as we can judge from the experimental data, the process of juxtapositioning homologues is different from the process that follows immediately to effect a stabilization of chromosome pairs. The supposition that the structure maintaining chromosome pairs is also responsible for their alignment is an attractive simplification, but without experimental foundation. An alternative supposition, equally unsupported by experiment, is that the completed synaptonemal complex is a consequence rather than a cause of the juxtapositioning. Our evidence nevertheless indicates that matching and pair stabilization are separable events. A precise matching is not always essential to synaptonemal complex formation, as is now evident from a number of studies (Gillies, Rasmussen & Wettstein 1973).

At least three major factors in the metabolism of zygotene cells appear to be involved in chromosome pairing: the previously discussed lipoprotein complex, a DNA-binding protein capable of facilitating DNA renaturation, and replication of the still unreplicated stretches of Z-DNA (Hecht & Stern 1971; Hotta & Stern 1971*a*). Their presumed common function is supported by the fact that the three components are physically associated during all or part of zygotene, but not after the completion of pairing. Interference with the association, but not with the individual components, by administration of colchicine during leptotene–zygotene causes asynapsis; there is, however, no major effect on the rate of progression through meiotic prophase (Hotta & Shepard 1973). Inhibition of Z-DNA replication (Ito, Hotta & Stern 1967) or of lipid synthesis (unpublished) arrests synapsis and also the progression of cells through zygotene. None of the treatments affects regions of the chromosome that have already undergone synapsis. It appears as though the lipoprotein complex were a matrix for effecting chromosome matching but not the agent for maintaining matched pairs. Maintenance, as will be discussed later, apparently depends upon continued synthesis of protein.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

(i) *The lipoprotein complex*

The only distinguishing feature thus far established for the novel lipoprotein fraction is its exclusive presence in nuclei at meiotic prophase and its buoyant density which is higher than the inner and outer membranes of the nucleus. This feature is a common characteristic of at least male meiotic nuclei, a fact which adds considerable force to the belief that it has an important meiotic function. Its presence in meiocytes spans the critical zygotene and pachytene stages, but its function after zygotene, if any, is unclear. It may remain a passive component of the nucleus until its removal at diakinesis on dissolution of the nuclear membrane. Unlike the replicating Z-DNA which is transiently associated with the lipoprotein complex, no comparable association exists for DNA synthesized during pachytene (Hecht & Stern 1971). In our published studies of Z-DNA lipoprotein association, its relation to the heavy lipoprotein component was unknown (Hotta & Stern 1971*a*). It is now clear that Z-DNA appears in that fraction. The heavy lipoprotein fraction from rat spermatocyte nuclei is also associated with a small amount of DNA, but as yet, we have no way of ascertaining whether this DNA is comparable to the Z-DNA of *Lilium*.

Also present in the lipoprotein fraction is the DNA-binding protein whose properties are discussed below. This protein is associated with yet another protein that has the capacity to bind colchicine (Hotta & Shepard 1973), a capacity which is of particular interest in light of the effectiveness of colchicine in preventing synapsis without affecting the progress of other meiotic activities. A small but real time lag exists between synthesis of the binding protein and its presence in the lipoprotein complex. In the presence of colchicine much of the binding protein remains free (Hotta & Shepard 1973). Since that effect is the only identifiable biochemical consequence of colchicine administration, it has been supposed that it is the route by which colchicine interferes with chromosome synapsis. Interference with lipid synthesis by exposing leptotene cells to the drug 'cerulenin' inhibits Z-DNA replication and also meiotic progress. The action of the drug is severe inasmuch as it is almost impossible to reverse the inhibitory effect of the drug on progression through division, a characteristic not shared by mitotic cells. Cells that have entered meiosis appear to be unusually sensitive to inhibitors of lipid synthesis, such as cerulenin, and this sensitivity may be due to the meiotic presence of the heavy lipoprotein fraction.

(ii) *The* 'r-*protein*'

A principal feature of the DNA-binding protein associated with the heavy lipoprotein fraction is its ability to catalyse the reassociation of single-stranded DNA (Hotta & Stern 1971*a*). Since such catalysis is the equivalent of facilitating strand matching, a primary requirement for chromosome alignment, we are labelling the protein as a reassociation or 'r-protein'. We consider this function to be more relevant to the meiocyte than the unwinding function assigned to the gene 32-protein (Alberts 1970); the 'r-protein' is not prominent during premeiotic S-phase as would be expected if it were functioning in DNA replication. This being the case, a role of the r-protein in aligning homologous DNA stretches is the more attractive alternative. An intensive analysis has recently been made of the binding proteins present in rat spermatocyte nuclei (Mather & Hotta, in preparation). A major outcome of this analysis is to reinforce the interpretation proposed here. The protein is primarily meiotic despite the many other binding proteins that are common to somatic and meiotic cells. Moreover, in tracing the

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

behaviour of the r-protein, it has been found to contain phosphorylatable sites. In both *Lilium* and rat, phosphorylation of r-protein with protein kinase suppresses its capacity to bind DNA and to facilitate DNA–DNA reassociation; dephosphorylation with alkaline phosphatase restores the binding capacity, but with appreciable loss of specificity toward single-stranded DNA.

As yet, there is no direct way to test for the *in vivo* rôle of the r-protein. It is nevertheless reasonable to conclude tentatively that its role is concerned with synapsis of chromosomes. Its virtually exclusive presence in meiocytes during prophase, and its effectiveness in catalysing DNA reassociation are major factors in the conclusion. Moreover, not only is the protein absent, or nearly so, during S-phase, but there is no demonstrable association between DNA synthesis during pachytene and the lipoprotein complex; the membrane bound r-protein may therefore have no direct role in recombination. On the other hand, its close association with replicating Z-DNA which is essential to synapsis, and the asynaptic consequence of disturbing the r-protein–lipoprotein association argue for a direct role in the pairing of chromosomes. Such a rôle would cover the process of alignment but it would not cover the stabilization of that alignment.

(iii) '*Z-DNA*'

The centrepiece of zygotene metabolism in *Lilium* is the late replicating Z-DNA. Although no direct evidence has yet been adduced in support of a specific meiotic function, it is the one molecular component that links chromosome organization with distinctive aspects of zygotene metabolism. Since much has been written on the characteristics of Z-DNA replication, it will be sufficient to summarize them very briefly here. DNA synthesis during zygotene is well separated in time from the premeiotic S-phase and can, therefore, be monitored in the absence of S-phase activity. It is a delayed semi-conservative replication which is recognizable as a cluster of radioactive satellite bands in a CsCl gradient (Hotta & Stern 1971*b*). Replication occurs in transient association with the lipoprotein complex (Hecht & Stern 1971). This transient association is paralleled by a transient effectiveness of colchicine in suppressing chromosome synapsis (Shepard, Boothroyd & Stern 1974). Once formed, synaptonemal complexes are unaffected by either colchicine treatment or inhibition of DNA synthesis. The argument for Z-DNA being involved in homologue alignment but not in the maintenance of alignment is identical with the one already advanced for the r-protein.

The apparently distinctive relationships of Z-DNA to chromosome structure during meiotic prophase make it highly plausible to suppose that Z-DNA sequences have a specific rôle in meiosis. In the early studies of its behaviour it became apparent that its banding as a satellite on isopycnic centrifugation was inconsistent with its dispersed distribution within and among the chromosomes, assuming, of course, that satellites consist of highly repeated sequences, as is the case with centromeric heterochromatin. The actual distribution of Z-DNA has not yet been directly determined. Its dispersion among the chromosomes has been inferred from two lines of analysis. Partial inhibition of Z-DNA synthesis was found to lead to a fragmentation of all chromosomes, presumably due to nuclease activities at the regions of incomplete replication (Ito *et al.* 1967). The generalized effect was taken to indicate a generalized distribution. In later studies, radioautography of microsporocytes exposed to [^{3}H]thymidine label during zygotene showed a sparse but non-localized distribution of grains over diakinesis and metaphase chromosomes (Ito & Hotta 1973).

Major evidence for an unusual relationship between Z-DNA and chromosome organization

was obtained in consequence of analysing its reassociation kinetics (Hotta & Stern 1975). In common with other DNA satellites of nuclear origin, it was expected to behave as a highly repeated sequence but, instead, it behaved as a collection of unique sequences. To explain the satellite behaviour of Z-DNA in CsCl gradients would have required a release of clusters of unique sequences uniformly high in GC content by random shearing of the DNA during extraction. Such selective shearing seemed most improbable in view of the evidence for a dispersed distribution of the Z-DNA. A more probable explanation was the pre-existence of interruptions bracketing the Z-DNA stretches either because the Z-DNA was present as episomic material or because single-stranded interruptions were generated during its delayed replication and persisted during meiosis. Recent analyses demonstrate that under very gentle conditions of extraction, Z-DNA cosediments with bulk nuclear DNA but that in DNA extracted after termination of meiosis, it does not behave as a satellite regardless of conditions of extraction (Hotta & Stern 1976). Thus, were it not for its delayed replication and facile release by shearing, it could not have been recognized as any more exceptional than other regions of the genome having a relatively high GC content.

In a very recent study we have been able to show that the facile detachment is due to the presence of single-stranded interruptions in the newly replicated strands of DNA; physical continuity of the DNA is provided for by the intactness of the old strands. Two principal questions arise from this observation: Do the unreplicated Z-DNA regions exist as single strands in each of the chromatids following premeiotic S-phase and are there other kinds of distinguishing properties that mark the Z-DNA regions as unique elements of the chromosomes?

Z-DNA stretches, when detached from the bulk of nuclear DNA but otherwise unfragmented, are of the order of 10^4 base pairs in length (Hotta & Stern 1976). Since they are not clustered in the chromosomes but are probably interspersed to a considerable degree among the bulk of nuclear DNA, it is clear that their persistence as duplex structures during the interval between S-phase and zygotene compels a very close juxtapositioning of sister chromatids. It is, therefore, important to note that all attempts thus far made to demonstrate the presence of single-stranded regions during meiosis have been negative except, perhaps, for the pachytene interval. It has been possible to show that the position of Z-DNA extracted from prezygotene cells and centrifuged to equilibrium in a neutral CsCl gradient is identical with the position of Z-DNA after replication. Since the DNA is unlabelled if extracted before zygotene its position in the gradient had to be determined by hybridization with labelled Z-DNA sequences (Hotta & Stern 1976).

A more sensitive, though less specific, method to identify the presence of single-stranded DNA is to treat randomly labelled DNA with Hg^{2+} and to analyse its isopycnic profile in a $CsSO_4$ gradient (Oishi 1968). As shown in figure 1, no label was detected under the curve of a deliberately denatured preparation of DNA, nor was there any difference observed in the profiles of DNA prepared from somatic and meiotic cells. Sonication of the DNA (figure 1*b*) resulted in a slight shift of the profile toward the denatured position. The shift was small and could have been entirely or partially a product of fragmentation. However, even if the shift were to be accounted for entirely by the presence of single-stranded tails in otherwise duplex fragments, the displacement was such that no more than about 5% of the tailed duplexes could have been single-stranded nor could such duplexes represent more than 2–3% of the total DNA. Moreover, if DNA is prepared from cells at different meiotic stages, the highest proportion of duplexes with single-stranded tails occurs during pachytene and not during the

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

interval between the S-phase and zygotene (figure 2). The highest estimates of single-stranded regions are insufficient to account for the existence of Z-DNA as single-stranded stretches during the prezygotene interval unless the values obtained for somatic and postmeiotic DNA are entirely disregarded. We conclude that sister chromatids are joined at all the Z-DNA regions, each region containing a duplex strand that has yet to undergo replication. The delayed repli-

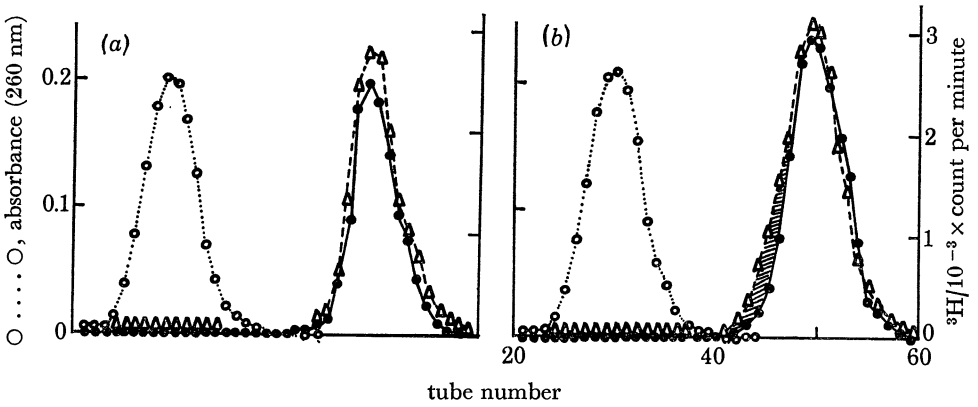

FIGURE 1. Single-stranded DNA in *Lilium* microsporocytes. DNA was prepared from microsporocytes at different meiotic stages by standard procedures from glycerin : sucrose nuclei (Hotta & Stern 1965). The average molecular mass of the preparations was 2×10^7. The DNA was dissolved in 2 × SSC and labelled with ^{3}H-dimethylsulphate for 48 h at room temperature according to the procedures of Smith, Armstrong & McCarthy (1967). The specific activity was 450–2600 min^{-1}/μg^{-1} DNA. The DNA was placed in a $HgCl_2$-Cs_2SO_4 gradient according to Oishi (1968). In so doing, the DNA was dialysed for 6 h or more against 0.02 M Na_2SO_4:0.01 M sodium borate buffer, pH 8.8. $HgCl_2$ was then added slowly to the dialysed preparation until a molar ratio of Hg/P = 0.25 was reached. Cs_2SO_4 was added to a refractive index of 1.3855 and the sample centrifuged to equilibrium at 35000 rev/min for 72 h at 25°C. O . . . . O, Alkali denatured *Lilium* DNA; ●——●, the position of DNA labelled *in vivo* and serves as a reference; △, microsporocyte DNA extracted at different stages of meiosis and labelled *in vitro*. The profiles shown in (*a*) were the same for all stages of meiosis. The profile (△) in (*b*) is for pachytene DNA following 1 min sonication. The shaded area indicates the maximum amount of SS-DNA that might be present.

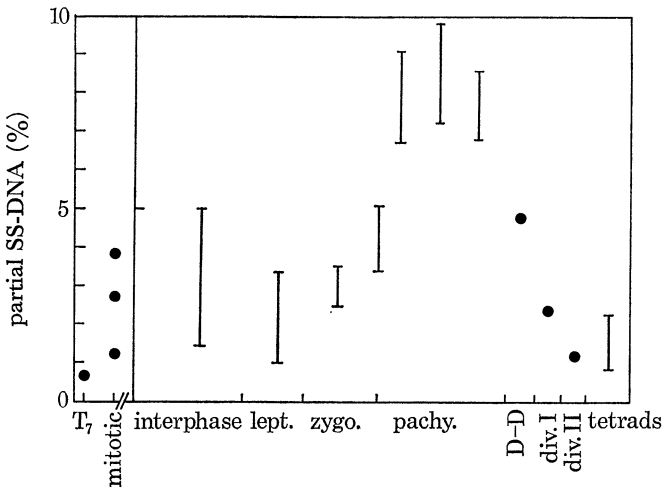

FIGURE 2. The proportion of partially single-stranded DNA at different stages of meiosis. The data were obtained as described in figure 1, except that the preparations were sonicated for 2 min to maximize the yield of apparent SS-DNA. Percentage values are derived from the ratio of shaded area/total area under curve. Each of the bars represents the range of values obtained with three or more separate measurements. Single experiments are indicated by dots. For comparison, sonicated DNA prepared from bacteriophage T7 and from somatic tissue are included. 'Interphase' includes premeiotic S-phase.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

cation of Z-DNA thus serves to hold sister chromatids in very close association between the completion of S-phase and the beginning of zygotene. Whether such association is a trivial by-product of the delayed replication or whether it is a precondition for initiation of synapsis is unknown.

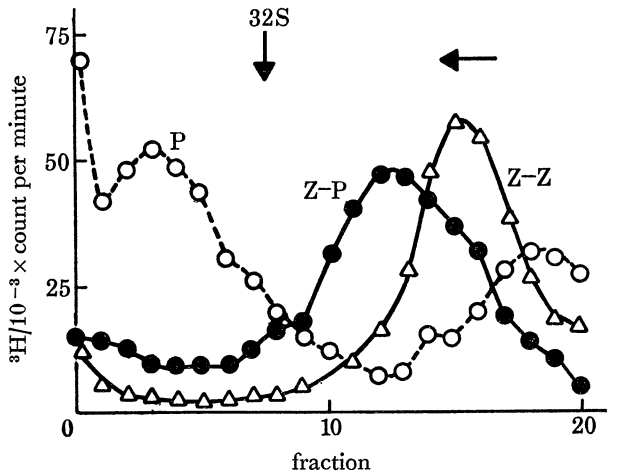

FIGURE 3. Sedimentation profiles of DNA prepared from isolated nuclei previously treated with S1 nuclease. Nuclei were isolated by the glycerin : sucrose procedure (Hotta & Stern 1965) and suspended in 0.1 M sodium acetate buffer (pH 5.0), 3 mM $MgCl_2$, 2 mM $ZnSO_4$, 0.15 M NaCl. 0.2 ml of the suspension containing 10–20 μg of DNA equivalents was treated with S 1 nuclease (enough to solubilize 0.3 μg T7 DNA in 10 min) at 45 °C for 10 min. The reaction was stopped by chilling and addition of EDTA to a concentration of 5 mM. The DNA prepared from the nuclei was layered over a neutral glycerol gradient (Hotta & Stern 1974) and centrifuged for 15 h at 24000 rev./min in an SW 27 rotor (Spinco). P, DNA prepared from nuclei that had been isolated from cells labelled during pachytene. A virtually identical profile was obtained using cells labelled at S-phase. Z–P, Cells labelled during zygotene but harvested at pachytene. Z–Z, Cells labelled during zygotene and harvested at the same stage.

There is now abundant evidence that the Z-DNA regions have distinctive responses to nuclease activities and must, therefore, be differently structured from the rest of the chromosome. During zygotene and pachytene these stretches are readily released from bulk DNA by briefly treating isolated nuclei with S1 nuclease. As shown in figure 3, the treatment releases Z-DNA almost quantitatively as a 15–20 S component. Under these conditions, bulk DNA is only slightly affected as is DNA which is undergoing repair replication during pachytene. The identity of the fragments released by the S1 nuclease is demonstrable in several ways. If tested on a CsCl gradient, the fragments band in the regular Z-DNA position. If assayed for C_0t behaviour, the fragments behave like unique sequences. It is perhaps of general interest to chromosome organization that the 10^4 base pairs stretches of unique sequences are either uninterrupted or infrequently interrupted by repeats. Whether they are flanked by repeated sequences is a question that is only now being answered by analysing post-meiotic DNA. It might be supposed that the preferential attack of S1 nuclease on Z-DNA regions reflects a general susceptibility of the regions to nucleases. This is not so. Micrococcal nuclease is indifferent to these regions and digests all fractions of chromatin to an equal degree. By contrast, Z-DNA regions are highly resistant to DNaseI activity if the enzyme is added to isolated nuclei. As shown in figure 4, no more than 50 % of Z-DNA gets digested whereas 80–90 % of bulk DNA or of pachytene-labelled DNA is hydrolysed to acid-soluble form.

It would be difficult to fit the different characteristics of Z-DNA regions into a simple structural scheme. On the other hand, it would be equally difficult to disregard these distinctive properties from the standpoint of meiotic function. Z-DNA represents about 0.3–0.4 % of the lily genome, consists almost entirely of unique sequences, and is distributed among all the chromosomes in 10^4 base pairs stretches. These stretches, which have been visualized by some as the axial elements of the chromosomes (Sorsa 1975), signify their distinctiveness in meiosis even during the prezygotene interval when their unreplicated but duplex condition hold sister chromatids in very close contact. The process of synapsis in the subsequent interval is also somehow tied to Z-DNA behaviour since its progress depends upon synchronous replication of Z-DNA strands. The paired condition of homologues probably also depends, either immediately or ultimately, on the organization of the chromosomes in the Z-DNA regions. At the nucleotide level, zygotene replication is itself incomplete, the newly formed Z-DNA strands not being ligated to their neighbouring DNA for 5–6 days, when disjunction occurs. To recapitulate, the structure of the chromosome or chromatin in the region of Z-DNA has features which make it distinguishable from other parts of the genome. The sensitivity of the gapped sites bracketing Z-DNA to S1 nuclease (figure 3), and the relative insensitivity of Z-DNA regions to DNaseI are shared neither by the regions undergoing repair replication nor by the DNA uniformly labelled during premeiotic S. To be sure, such differences disappear if nuclei are deproteinized.

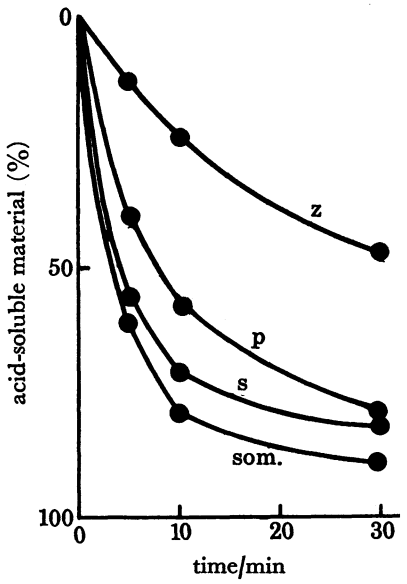

FIGURE 4. Differential sensitivity of isolated nuclei to DNaseI. Nuclei were isolated as in figure 3 from cells labelled during premeiosis (s), zygotene (z), pachytene (p), or from somatic tissue (som.). Suitable amounts of nuclei were suspended in 0.01 M tris buffer (pH 7.2), 3 mM $MgCl_2$ and treated with DNaseI (20 μg/ml) for the times indicated at 25 °C. Acid soluble material and residual DNA were determined at the ends of incubation periods.

It might be easier to relate the distinctive susceptibilities and other properties of the Z-DNA regions, if the extent to which they represent the unique sequences of the lily genome were known. A reasonable minimum value is about 3 % since it is unlikely that the total complement of unique sequences in *Lilium* is greater than 10 % (Hotta & Stern 1975). Such information is important because, as discussed earlier, the size of the lily genome is such that only a very small fraction of it is involved in crossing over. How selective that fraction is remains unknown, but

if it is assumed that crossing over is primarily addressed to regions containing unique sequences, an assumption for which there is no evidence, then the extent to which Z-DNA represents these sequences would be a critical factor in any speculation.

(*c*) *The pachytene interval*

Leaving aside the details of the transition process between zygotene and pachytene for which the experimental data lack adequate resolution, meiotic metabolism during pachytene contrasts sharply with that during zygotene. Although chromosomes remain paired through pachytene, it is doubtful that the meiocytes retain the ability to effect pairing at that stage. If arrested in protein synthesis during early zygotene, cells resume formation of homologous pairs after the source of inhibition is removed (Parchman & Stern 1969). By contrast, cells thus arrested at late zygotene resume progression through meiosis, becoming partially or entirely achiasmatic because of their inability to reconstitute the chromosome pairs that apparently had fallen apart in the absence of protein synthesis (Parchman & Stern 1969). Continued protein synthesis appears to be essential to the maintenance of pairs; pachytene cells seem capable of maintaining but not of forming chromosome pairs.

Unlike zygotene, pachytene DNA synthesis is entirely of the repair-replication variety. Various methods have been used to characterize pachytene DNA synthesis and all of them have yielded the same answer. BdUrd substitution, sensitivity to hydroxyurea, testing of old and new strands for the presence of label, determining the relative abundance of pachytene labelled sequences at different meiotic stages – all these approaches have consistently indicated that semiconservative replication is lacking. The evidence that meiocytes are programmed to perform repair replication at pachytene is unambiguous. Chandley and co-workers have found a similar situation to prevail in mammalian spermatocytes (Kofman-Alfaro & Chandley 1970). Such metabolism is clearly compatible with the view that crossing over occurs during this interval. It will be seen, however, that repair replication *per se* is an inadequate characterization of the distinctive features of pachytene metabolism.

The stimulus to repair replication is provided by the activation of an endonuclease at the termination of zygotene. Whether the enzyme is synthesized *de novo* has not been determined but its properties are different from the endonuclease found in somatic cells (Howell & Stern 1971). Evidence for the actuality of endonuclease activity *in vivo* is provided by two sets of data. *In vitro*, the enzyme is specific for double stranded DNA but makes only single-stranded nicks. Sedimentation profiles of DNA prepared from cells at different stages of meiosis indicate that DNA nicking occurs only at pachytene and that such nicking is entirely single-stranded in nature (Hotta & Stern 1974). A second source of evidence comes from the properties of the nick. Meiotic endonuclease has been shown to cleave internucleotide linkages so as to leave 3′-phosphoryl and 5′-hydroxyl groupings (Howell & Stern 1971). We have recently analysed the nature of free ends at different meiotic stages by the combined use of polynucleotide kinase and alkaline phosphatase. The results indicate that at all stages save pachytene the number of 5′-phosphoryl ends is slightly in excess of the number of 5′-hydroxyl ends. At pachytene, on the other hand, the number of 5′-hydroxyl ends is twice that of the 5′-phosphoryl ones (table 1). The two types of evidence, though not providing direct proof, strongly suggest that the endonuclease is the source of the DNA nicks which are formed at pachytene.

Endonuclease activation is probably regulated by the achievement of effective pairing during zygotene. This conclusion is prompted by the observation that in the achiasmatic lily hybrid,

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Black Beauty, very little DNA nicking and very little DNA repair-replication occurs. Since the amphidiploid undergoes normal chiasma formation, we assume that the diploid suffers from a regulatory and not a genetic upset. This is supported by the further observation that in other lily cultivars, cells rendered achiasmatic by treatment with colchicine, also suffer an appreciable reduction in the level of repair-replication. If this regulatory mechanism does operate, it must do so at the level of the bivalent. The achiasmatic hybrid, Black Beauty, has an average of one bivalent per cell. Since the level of DNA nicking and of repair-replication is no more than 10 % of that found in the parental species, it is unlikely that such activities occur in all the chromosomes. The levels observed are more compatible with the view that they reflect events confined to the occasional chiasmatic bivalent. Regulation at the bivalent level is compatible with cytogenetic data from various sources indicating compensatory regulation of chiasma frequencies between chromosome arms (Rhoades 1968). It is also compatible with the behaviour of a recessive mutant in *Hypochoeris* in which a particular pair of homologues is capable of synapsis but not of chiasma formation (Parker 1975).

TABLE 1. 5′-PHOSPHORYL AND 5′-HYDROXYL GROUPS IN DNA DURING MEIOSIS

	pmol/mg DNA						
end group	premeiotic	leptotene	pachytene	pachytene (irrad.)	pachytene (achiasmatic)	diakinesis	telophase
5′-phosphoryl	0.85	0.38	0.23	(0.72)	0.06	0.1	0.08
5′-hydroxyl	0.67	0.18	0.45	(0.64)	0.05	0.08	0.07
5′-p/5′-OH	1.3	2.1	0.5	(1.1)	1.2	1.3	1.1

Nuclei prepared by the glycerin : sucrose procedure (Hotta & Stern 1965) from cells at the stages indicated were reacted with polynucleotide kinase either directly or following treatment with alkaline phosphatase. If treated with alkaline phosphatase, 5–10 units of enzyme were added to 0.5–1.0 mg DNA equivalents of nuclei suspended in 1 ml of 0.5 M sucrose, 0.1 mM EDTA, and 0.01 M tris buffer, pH 7.5. After incubating for 15 min at 37 °C, the suspensions were diluted with 0.5 M sucrose, 3 mM $MgCl_2$ and 1 mM $CaCl_2$. They were centrifuged at 4000 rev./min for 5 min to sediment the nuclei and the operation repeated to wash them. They were then resuspended in 0.5 ml of 0.3 M sucrose, 0.01 M tris buffer (pH 7.5), and 3 mM $MgCl_2$ to which were added 2.5 nmol γ-labelled ATP (17 μCi) + 50 units of polynucleotide kinase. The suspension was incubated at 37 °C for 15 min. DNA was purified from the reaction mixtures and radioactivity determined. 5′-hydroxyl groups were calculated from the radioactivity of nuclei not exposed to alkaline phosphatase and 5′-phosphoryl groups were calculated from total phosphorylation after phosphatase treatment. The cultivar Bright Star was used in these experiments. Achiasmatic pachytene values were obtained from Black Beauty nuclei. The irradiated pachytene values were obtained from *L. speciosum* nuclei and the values normalized to Bright Star.

Although regulation of endonuclease activity in turn regulates the principal activity of pachytene cells, repair-replication, it is evident that repair-replication by itself is not a unique property of the pachytene cell. Indeed, repair-replication activity may be induced in meiocytes at all stages of meiosis except for the intervals of 1st and 2nd division, when the rate of replication is very low, probably because of chromosome compaction. The net level of DNA synthesis at any meiotic stage is, of course, dose dependent, but the lag between damage and onset of repair is not outstandingly different from stage to stage. There is, in contrast, a major difference in sites of repair between endogenously and exogenously stimulated cells. Radiation-induced repair is distributed more or less uniformly among all DNA fractions regardless of stage of treatment; pachytene repair is localized in special regions of the chromosomes. It has been shown in earlier studies that pachytene repair-replication occurs preferentially in regions of moderate repeats (Smyth & Stern 1973). The localization is due to the distribution of pre-existing lesions and not to a generalized resistance to nicking in other parts of the chromosomes.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Cells irradiated during pachytene no longer show a restricted distribution of repair sites; the net pattern is a combination of the two repair activities. This is illustrated in figure 5 by the C_0t curves for total *Lilium* DNA and for DNA labelled at pachytene under normal conditions or in response to radiation damage. In Black Beauty, which has negligible pachytene repair-replication, synthesis stimulated by irradiation tracks the absorbency curve. In chiasmatic types, the pattern of synthesis is intermediate between the absorbency curve which reflects DNA as a whole, and the curve for normal pachytene repair. As would be expected, with increase in radiation dose, the endogenous pachytene component decreases progressively.

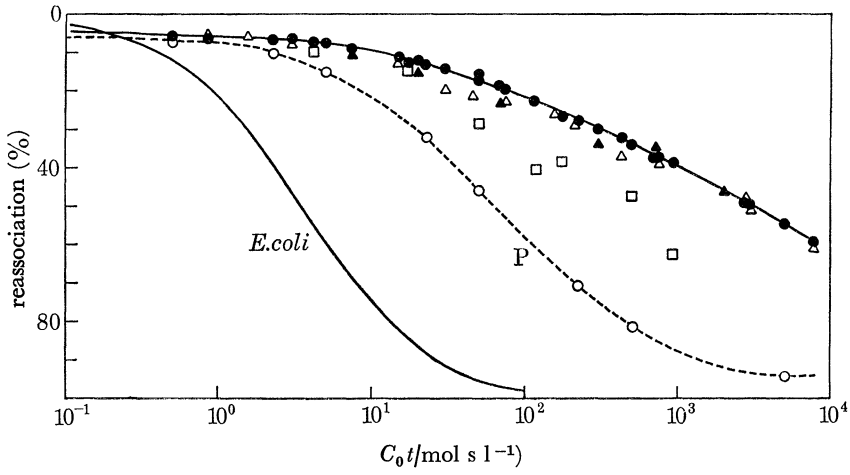

FIGURE 5. C_0t analysis of DNA prepared from cells labelled at pachytene without or following X- or u.v. irradiation. P, Control profile for pachytene labelled DNA in cultivar Bright Star. ——, Reassociation behaviour of total DNA; ●—●, radioactivity of cells X-irradiated during interphase with 250 r to 1 kr; △, X-irradiated pachytene cells of cultivar Black Beauty (250 r to 1 kr); ▲, u.v.-irradiated ($1–4 \times 10^{-2}$ J/cm^2) pachytene cells, Black Beauty; □, X-irradiated pachytene cells of Bright Star (250 r to 1 kr). All cells were incubated for 2 days in presence of [^{3}H]thymidine (20 μCi/ml). The purified DNA was sonicated for 10 min to 300–400 nucleotide fragments. Radiation-induced labelling at pachytene in Black Beauty tracks the profile of total DNA whereas in Bright Star, it falls between total and unirradiated pachytene. This is so because endogenous pachytene labelling is near-absent in achiasmatic Black Beauty.

Evidence suggestive of special chromatin organization in the regions of pachytene repair has been obtained in the course of analysing micrococcal nuclease digests of isolated nuclei. Except for pachytene, DNA labelled at the various meiotic stages is recovered primarily in the 200 base pair component, as is the total DNA. Endogenous pachytene label, but not exogenously induced label, is found in the minor and smaller 70–100 base pair fraction. A characteristic result is shown in figure 6. The atypical distribution is obtained even after periods of incubation lasting two days. It shifts to the normal distribution only after termination of pachytene replication. Such susceptibility to the action of micrococcal nuclease may be generally characteristic of regions undergoing replication but, if so, these regions are maintained in the susceptible state for relatively long intervals in the case of endogenous pachytene repair, but turn over rapidly in the case of other syntheses. As yet, we have no information on the constitution of the repair regions.

Significant to meiotic metabolism is the fact that the repair activity of pachytene does not include the zygotene stretches to any appreciable degree. The evidence is shown in figure 7. By making the strands synthesized during premeiotic S-phase heavy with BdUrd, the distribution of subsequent syntheses between the two strands of the duplexes may be traced at the

termination of meiosis. It may be seen that whereas pachytene label is present in both strands, zygotene label is attached to the heavy strands as would be expected for a delayed replication. Despite the persistence of gaps in the zygotene regions, such gaps are unavailable to the pachytene repair system. A mutual exclusion appears to exist between the stretches presumptively associated with pairing and those associated with crossing over. The mutual exclusion is further

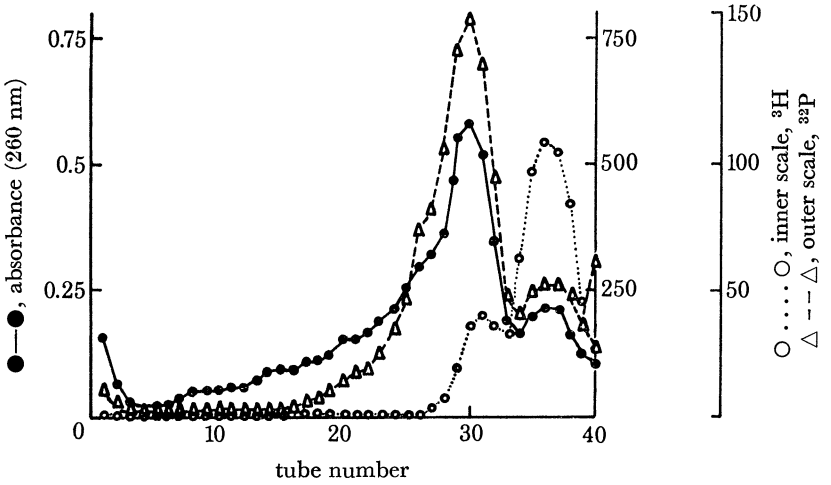

FIGURE 6. Distribution of endogenous and radiation-induced DNA label in pachytene cells following treatment of isolated nuclei with 300 units of micrococcal nuclease for 10 min at 37 °C. The DNA was purified and 0.2–0.3 mg DNA was placed on a 10–30 % glycerol gradient and centrifuged in an SW 41 (Spinco) rotor at 30000 rev./min for 20 h at 4 °C. Direction of sedimentation to the left. The endogenous pachytene label is ^{3}H; the radiation-induced label is ^{32}P.

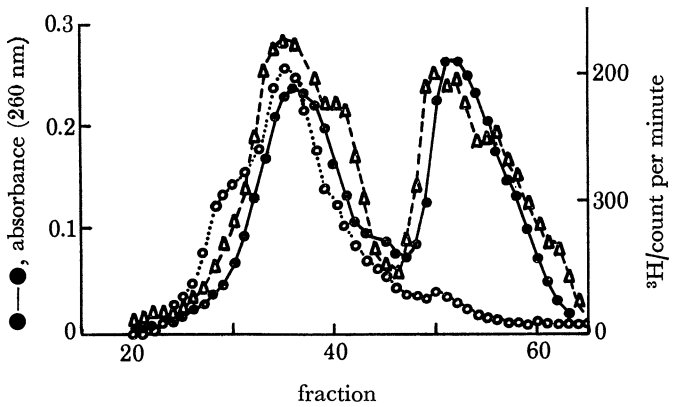

FIGURE 7. The partitioning of zygotene and pachytene label between new and old DNA strands. Buds at premeiotic S-phase were cultured *in vitro* (Shepard *et al.* 1974) in the presence of 5 mM BdUrd. Microsporocytes were explanted at leptotene and cultured in the presence of 2 mM BdUrd until early zygotene. They were then transferred to a medium containing 20 μCi/ml of [^{3}H]deoxyadenosine + 10 μg/ml of thymidine and cultured for 3 days to the termination of zygotene. Cold deoxyadenosine and thymidine were then added to a concentration of 10 μg/ml and the cells cultured for 10 more days to the termination of meiosis. Pachytene cells were labelled by the same procedure except that the deoxyadenosine was increased to 25 μCi/ml and 1 mM hydroxyurea was added to inhibit any semi-conservative labelling. Nuclei and DNA were isolated in the standard manner (Hotta & Stern 1965; Hotta & Stern 1971 *b*). The purified DNA was denatured in alkali and centrifuged in a CsCl solution containing 0.3 M NaOH in a no. 40 angle rotor (Spinco) for 72 h at 35000 rev./min. The profiles for zygotene (○) and pachytene (△) have been combined into one figure; ●—●, total Bu-substituted DNA.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

emphasized by the absence of any significant redistribution of zygotene label between old and new strands in the course of pachytene metabolism.

Additional considerations and conclusions

Termination of pachytene is almost coincident with the termination of meiotic metabolism as defined in the Introduction. In general, the salient biochemical characteristics of meiotic prophase disappear with the conclusion of pachytene. The r-protein is no longer detectable. DNA-associated enzymes – ligase, polynucleotide kinase, endonuclease – drop sharply. These changes are compatible with the termination of activities essential to crossing over. There is nevertheless one event that makes it necessary to qualify the nature of the termination. Gaps flanking Z-DNA stretches do not become sealed until some time after the pachytene interval, possibly during the first division (Hotta & Stern, 1976). It is unlikely that the delayed ligation is of a trivial nature, and we therefore speculate that it has a bearing on chiasma resolution and/or chromosome disjunction. If the speculation is correct then a very critical role must be envisaged for Z-DNA in the mechanics of chromosome behaviour in *Lilium* microsporocytes. It would be of interest to know – and we are indeed attempting to obtain the information – how Z-DNA is organizationally related to the moderately repeated sequences that become labelled during pachytene. The distribution of these two categories of DNA within the chromosome, both in relation to the genome as a whole and in relation to one another, would reveal much about the organization of chromosomes for meiosis.

Leaving aside the very difficult question of how to identify the different biochemical events of *Lilium* meiocytes in meiosis of other organisms, some significant conclusions may be drawn about the biochemistry of meiotic events in *Lilium*. The metabolism of a meiocyte is distinctly differentiated along the temporal axis of development. That differentiation reflects the orderly succession of metabolic intervals which are essential to the achievement, first of synapsis, and then of crossing over in the complex eukaryotic chromosomes. The processes of synapsis and crossing over are not intermingled, at least to the extent that each of these involves the synthesis of different DNA sequences. Synapsis is a separate event which apparently regulates the triggering of a distinctive set of processes that involve molecular pairing, nicking and recombination. If any significance is to be attached to the metabolic pattern of a cell, it must be concluded that the pronounced switches in metabolic pattern during meiosis reflect a need to modify chromosome behaviour in progressive and discrete steps so as to achieve the ultimate events of crossing over and disjunction.

A profound relationship undoubtedly exists between the temporal structuring of meiotic metabolism and the physical organization of the chromosome. The evidence for the existence of discrete regions in the chromosome having specific meiotic functions is unequivocal even if the details of the functions cannot be fully described. The existence of specific regions in a chromosome which, depending on their nature, include or exclude crossing over, is not at all at variance with the broad cytogenetic data on meiotic recombination. On the contrary, there is ample evidence for the exclusion of chromosomal regions such as heterochromatin from crossing over, and so too for a very high degree of chiasma localization in some chromosome arms (Jones 1974). There is also evidence at a different level of chromosome organization in *Neurospora* for regional controls of crossing-over frequency (Catcheside 1975). How much of our observations in *Lilium* can be applied to the interpretation of the various cytogenetic observations remains, of course, an open question. The autoradiographic evidence obtained by Riley &

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Bennett (1971) for DNA synthesis during the latter stages of wheat meiosis could be construed as a demonstration of the late ligation of zygotene stretches in *Lilium*. We would like to believe that a significant fraction of the observations can be fruitfully applied to other organisms.

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Discussion

G. Simchen (*Department of Genetics, The Hebrew University of Jerusalem, Israel*). Dr Stern has stated that commitment to meiosis in lily occurs at G_2, after the completion of the main round of premeiotic DNA synthesis. In these experiments meiosis was assessed cytologically, namely by observing chromosome pairing and disjunction/haploidization. In yeast, however, commitment to meiotic recombination occurs *during* the premeiotic S, probably at its very early stages. Cells that are plated on vegetative medium at this time form recombinant, though diploid, colonies. Thus commitment to meiotic haploidization occurs only later, after the premeiotic S, at a time comparable to commitment in lily. I suggest that if one looks at commitment to meiotic recombination in lily rather than commitment to meiotic chromosome behaviour, one would find the former to occur during the period of premeiotic DNA synthesis, like in yeast, but these recombinant cells will revert to mitosis. Meiotic recombination in diploid cells of lily could be assessed by changes in DNA, DNA enzymes or other proteins that normally occur in pachytene, or by recombination of cytological markers.

H. Stern. It is conceivable, and even probable, that commitment to crossing-over occurs during premeiotic S-phase in *Lilium*, as it does in yeast. The key question is whether that commitment is irreversible, as it appears to be in yeast. If so, microsporocytes reverting to mitosis would undergo crossing-over without benefit of microscopically visible chromosome pairing. This could be tested in individuals having at least one pair of heteromorphic chromosomes. Such irreversible commitment is nevertheless difficult to reconcile with the well-known studies of Henderson and of Peacock who showed that crossing-over could be eliminated by perturbing meiocytes during zygotene or early pachytene well after completion of S-phase.

Professor H. Rees (*University College of Wales, Aberystwyth*). Dr Stern has shown that the DNAs synthesized during zygotene and pachytene are of quite distinctive composition; unique sequence DNA on the one hand, repetitive on the other. Is there evidence to show whether the quality of the DNA synthesized at these stages is similar in different species?

H. Stern. As yet, we have no evidence that patterns of meiotic DNA synthesis resembling those in *Lilium* occurs in other genera besides *Trillium*.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

M. Hultén, (*Regional Cytogenetics Laboratory, East Birmingham Hospital*). Professor Lindsley has described some *Drosophila* prepachytene and pachytene meiotic mutants with a reduced crossover frequency and change in crossover distribution. This finding of a correspondence between reduced frequency and change in distribution pattern of crossing over events fits nicely with the observation in the human male where oligochiasmatic males seem to have an altered chiasma distribution within chromosome arms (Hultén & Lindsten 1973; Hultén, Solari & Skakkebaek 1974). An increased parental consanguinity indicates these men represent autosomal recessive meiotic mutants, some of which apparently of the prepachytene type since they have an abnormal synaptonemal complex (Hultén *et al.* 1974; Ferguson-Smith, personal communication). Professor Stern – I would like to ask you two questions. Firstly, have you had the opportunity to carry out any of the biochemical investigations you told us about on any prepachytene and pachytene meiotic mutants in lily? Secondly, did you observe an aberrant chiasma distribution in the Liliaceous plants in which you induced a reduced chiasma frequency by colchicine treatment? Thirdly, I think it would be very interesting to investigate some meiotic mutants in mammals, e.g. in man, e.g. to measure quantitatively the DNA binding reassociation protein in low chiasmata men. Would this be technically difficult?

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H. Stern. (1) To my knowledge, meiotic mutants have not yet been identified in *Lilium*. (2) We did not analyse chiasma distribution in colchicine-treated plants. (3) We hope to achieve some biochemical analyses of meiosis in the mouse and thus make possible a biochemical analysis of meiotic mutants in mammals. Measurement of r-protein in itself poses no technical difficulties, but collecting pachytene spermatocytes from low chiasmata men is a technical challenge that exceeds our reach.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES