MUTATIONAL MECHANISMS IN DROSOPHILA

by

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SUMMARY

Chemically induced rearrangements and 'point mutations' seem to require a primary effect on the DNA followed by secondary effects. In rearrangement formation, the primary effect consists of particular lesions that lead to chromosome breakage. This primary effect will be followed either by non-repair (cell death), repair (restitution) or mis-repair (rejoining of broken chromosome ends) to give a rearrangement of the chromosomal DNA. The production of 'point mutations' also consists of particular lesions followed by secondary effects, but little is known about their secondary effects in eukaryotes. If, as seems likely, repair enzymes play a role, it is expected that they will be different from those which allow reunion of broken chromosome ends.

The response of chemically-induced sex-linked recessive lethals and translocations to storage (when treated Drosophila melanogaster sperm is stored in inseminated females) differs. The frequency of sex-linked recessive lethals remains unaffected by storage while that of rearrangements increases strikingly. At neutral pH and 37°C, O^6 alkyl-guanine is released at a negligible rate in vivo (Margison and O'Connor, 1973). This lesion is considered to be the primary "effect" in the production of point mutations; this may be the reason why there is such a small or non-existent storage effect and point mutations, and also why this lesion is least likely to lead to chromosome breakage in a system (mature spermatozoa) where no enzymatic repair is occurring. The indications are that most chromosomal

aberrations are not due to O^6-alkylations (Newbold *et al.*, 1980) N7-alkyl guanine hydrolyses spontaneously at a much higher rate. At neutral pH and 37°C, N7-alkyl guanine has a half-life of 6 days (Margison and O'Connor, 1973). These lesions are therefore more likely to lead to chromosome breakage in a system where no enzymatic repair is occurring. Both of these lesions are released at a higher rate *in vivo* (7-alkyl guanine and O^6-alkyl guanine both having a half-life of 1 day) from rat liver (neutral pH, 37°C) indicating that they are removed enzymatically. A comparison between the rates of depurination of the different alkylated bases, and the rates of appearance of sex-linked recessive lethals and chromosome breakage events may shed some light on the mechanism by which these mutagenic events occur.

Experiments were carried out in an attempt to determine the nature of the potential breaks and the processes whereby they matured into actual breaks in *Drosophila melanogaster*. This was done by carrying out a detailed study of the kinetics of the storage effects and of the effects of different conditions, on the kinetics.

In order to carry out this investigation, a better definition of the conditions under which the storage effect could be observed was attempted. This involved looking at the effect of different concentrations of yeast, different concentrations of sugars and different sugar sources in the storage medium related to the survival of females and pattern of egg-laying with time. This arose from the problems encountered with DES.

Subsequently the effect of mutagen concentration and temperature on the rate at which the breaks open was observed. Breaks were measured by observing the frequency of translocations between chromosomes 2 and 3. An attempt was made to relate the frequency the translocations observed to the level of depurination of N7-ethyl guanine after different times of storage of EMS-treated mature spermatozoa of Drosophila melanogaster. An alternative method was to look at the frequency of dominant lethals, which gave a measure of the frequency of un-rejoined breaks.

An attempt was also made to establish the nature of sex-linked recessive lethals in genetic terms, since some mutagens produce a slight storage effect in this heterogeneous class of damage.

CONTENTS

ABBREVIATIONS

DES	Diethyl sulphate
Df	Deficiency (deletion)
DMS	Dimethyl sulphate
Dp	Duplication
El	Ethylene imine
FF	Formaldehyde food
HAM	Honey-agar medium
In	Inversion
kbp	kilobase pairs
M-5	Muller-5
MMC	Mitomycin-C
MMS	Methyl methane sulphonate
NMNG	N-methyl-N-nitro-N-nitrosoguanidine
NMNU	N-nitroso-N-methyl urethane
Rp	Repeat
SDM	Standard Drosophila medium
slrl	sex-linked recessive lethals
TEM	Triethylene melamine
T/Tr	translocations
*/M-5	multiply marked tester strain (see page 29 for details)

CHAPTER 1. INTRODUCTION

INTRODUCTION

1.1 COMPARISON BETWEEN THE GENETIC EFFECTS OF ALKYLATING AGENTS AND X-RAYS

In the early 1940's, Auerbach and Robson showed that a chemical agent could induce mutations in the germ line cells of Drosophila melanogaster (Auerbach and Robson, 1944 and 1947). In their experiments, nitrogen mustard, an alkylating agent, induced up to 24% sex-linked recessive lethals (slrl) compared to a spontaneous frequency of <1% (Auerbach, 1948). The investigation of the possible mutagenic effects of nitrogen mustard was prompted by the simple observation that exposure of humans to the chemical resulted in skin burns similar in character to those produced by X-rays, an established mutagen in Drosophila (Muller, 1927, 1928a and b) and in maize (Stadler, 1928). An analysis of the mutagenic effects of nitrogen mustard revealed that as well as having an inhibitory effect on mitotic activity, it was capable of producing the same array of mutagenic effects as X-irradiation, i.e. dominant lethal and visible mutations, recessive sex-linked and autosomal lethals, recessive visibles, large and small deletions and translocations (Auerbach and Robson, 1947; Nasrat et al., 1954-55). Using the frequency of induced slrl as a device to compare equivalent doses of the two mutagens, they demonstrated that X-rays and mustard gas differed in their mutagenic behaviour in two main respects:

TABLE 1.1. A variety of alkylating agents administered to different organisms have been shown to differ in their mutagenic behaviour as compared with X-rays. Some of these are listed below.

Alkylating Agents	Organism	Reference
El	Drosophila	Slizynska, 1973
EMS	Drosophila	Bishop and Lee, 1969
	"	Brink, 1970
	E. coli	Schwartz, 1963
	Maize	Amano and Smith, 1965
FF	Drosophila	Slizynska, 1957, 1963, 1973
TEM	Drosophila	Slizynska, 1973
Alkane sulphonic esters/ Alkyl methane sulphonates	Drosophila	Fahmy and Fahmy, 1957, 1961
	Barley	Ehrenberg et al., 1966
	"	Heslot et al., 1959.

A more comprehensive list may be found in Ross (1962) or in de Serres and Shelby (1981).

(i) a relative shortage of large chromosomal rearrangements (inversions, large deletions, translocations) and an excess of small rearrangements (small deficiencies and duplications) were found after mustard gas treatment compared with X-rays.

(ii) a high incidence of "delayed" mutation was noted after mustard gas treatment, i.e. the appearance of the mutation was separated from the time of treatment by one or more cell cycles (these were detected as mosaics). X-rays failed to produce delayed mutations.

These differences have also been found to hold true for a variety of alkylating agents administered to different organisms, as listed in Table 1.1. It was also noted that the relative shortage of mustard gas induced rearrangements was more pronounced in the case of translocations (interchromosomal rearrangements); this shortage decreased as the dose of mustard gas increased, more so for deletions than for translocations (Auerbach and Robson, 1947).

Three hypotheses were put forward to explain these observed effects:

(i) alkylating agents were weaker mutagens than X-rays, therefore they produced fewer chromosome breaks (Lamy, 1947-48).

(ii) alkylating agents were not weaker mutagens but the breaks they produced were less likely to rejoin to give rearrangements either because

(a) restitution was favoured, or

(b) they remained open much longer and thus were 'lost' as dominant lethals before rearrangements could be formed (Auerbach, 1948).

(iii) alkylating agent induced breaks and X-ray induced breaks, once formed, had the same rejoining ability. However, in contrast to X-ray lesions, the primary chemical lesions matured only slowly into open breaks. These 'potential breaks' could pass through mitosis, and open up at a later stage (Auerbach and Robson, 1947).

There were obvious difficulties in distinguishing, by experiment, the first hypothesis from part (a) of the second hypothesis, although mustard gas had been shown to be efficient at breaking the chromosomes of plants (Darlington and Koller, 1947).

Evidence of the ability of X-rays to break chromosomes came from plant work (Sax, 1938) - single, unrestituted breaks can be seen as such in irradiated cells, or as bridges in the next anaphase. Unrestituted breaks gave rise to dominant lethals, mainly through loss of chromosomes by bridge formation; but there are additional causes of dominant lethality and dose effect curves for dominant lethals include these as well. In Drosophila, however, the frequency of single chromosome breaks could not be measured directly. Muller (1940) found that at low and moderate doses of X-rays, the frequency of single chromosome breaks,

as measured by dominant lethals and, more accurately by viable chromosome losses, followed approximately linear kinetics. This was indirect confirmation of the single-hit interpretation of chromosome breakage by X-irradiation of Drosophila and plant chromosomes (Stadler, 1932).

For equivalent doses of X-rays and mustard gas (measured by slrl frequency) the frequencies of dominant lethals were determined (Nasrat et al., 1954-55). At low X-ray doses, when most dominant lethals were due to single breaks (Muller, 1940) the frequency of mustard gas induced dominant lethals equalled that of X-rays. At high X-ray doses, when an increasing proportion of dominant lethals was formed by viable two-break rearrangements (kinetics of dose-response curve for rearrangements was intermediate between linear and the square law - '3/2 power' law in Auerbach, 1976, p.94) the frequency of mustard gas induced dominant lethals exceeded that of X-rays. This result is not unexpected if, at high doses of mustard gas, potentially viable rearrangements are 'lost' as unrejoined breaks, thus boosting the frequency of dominant lethals. This work supported part (b) of the second hypothesis.

A special version of this hypothesis was put forward by Auerbach and Robson (1947)(see hypothesis (iii)). This third hypothesis - invoking the potential break - had already been debated by X-ray workers (Muller, 1932; Stadler, 1932). Muller (1940) considered that primary events (actual or potential) accumulated independently of length

of exposure, fractionation of exposure and dose of irradiation, and that their final number determined the number of structural changes produced. He also suggested that primary events combined, usually in twos, to produce rearrangements as secondary effects. The detection of an exchange between a treated paternal and an untreated maternal chromosome indicated that these 'secondary effects' took place but after fertilisation, at least in Drosophila (Sidky, 1939 in Muller, 1940; Schalet, 1956).

A rearrangement, such as a translocation, requires two independent breakage events. If the probability of a break occurring after a given dose of mutagen is p, then the probability of two breaks occurring is p^2. p is linearly related to dose; therefore the frequency of rearrangements requiring two independent breaks should increase with the square of the dose (Auerbach, 1976, p.126). If this holds true, and there is full interaction between all breaks, the following example should maintain:

If a dose of X-rays yields 4% translocations and a dose of alkylating agents yields 1% translocations, they must each have produced 20% ($0.2^2 = 0.04$) and 10% ($0.1^2 = 0.01$) breaks respectively. If these breaks do not interact, a combined dose of X-rays and alkylating agent should yield 4% + 1% = 5% translocations; however, if all breaks interact, the combined effect of the two treatments should yield $(0.3)^2 = 0.9$ or 9% translocations.

Oster (1958) exposed Drosophila males to X-rays and mustard gas in succession, and vice versa. The mustard gas treatment alone produced fewer translocations than the X-ray treatment alone. When the two treatments were applied in succession, the frequency of translocations was far greater than additive, i.e. if chemical breaks rejoined with chemical breaks, and X-ray breaks with X-ray breaks. The frequency was approximately that expected if any break had the potential to rejoin with any other break. Thus it was demonstrated that there was no intrinsic difference between an X-ray break and a mature chemical break.

1.2 STORAGE EFFECTS

A common conclusion emerging from two independent lines of research - that of the mutagenic effects of UV-irradiation in micro-organisms and the mutagenic effects of chemicals in Drosophila - was the existence of the premutational lesion preceding the production of a mutation. The concept of a premutational lesion which could lead to a break, was applied to the problem of the underlying cause of the shortage of large rearrangements compared to small rearrangements after chemical treatment, and to the delayed expression of chemical damage in the following manner.

Storage experiments with Drosophila proved the hypothesis that primary chemical lesions open slowly to form real breaks (e.g. Slizynska, 1969; Snyder, 1963; Watson, 1964). Female Drosophila have sperm storage organs (the two spermathecaeand the ventral receptacle) in which sperm is

TABLE 1.2 Types of structural changes and their frequencies per hundred spermatozoa (Slizynska, 1969).

Treatment	Sample	Type	Changes in all Spermatozoa n	C/M	Frequencies per 100 spermatozoa T	ln	T or ln	Rp	Df	Total	Rp and Df among all changes (%)
TEM	U	C	15	0.5	3.2	1.8	0.4	6.0	0.0	5.3	55.3
		M	32		1.8	0.4	0.0	6.0	3.2	11.4	
	L	C	21	2.6	5.2	3.6	2.1	0.0	0.0	10.8	24.1
		M	8		0.0	0.5	0.0	2.1	1.5	4.1	
	H	C	37	37.0	25.6	15.4	6.4	0.0	0.0	47.4	2.6
		M	1		0.0	0.0	0.0	0.0	1.3	1.3	
FF	U	C	5	0.1	1.0	1.0	0.5	0.0	0.0	2.4	76.1
		M	41		1.0	1.9	0.0	10.6	6.3	19.8	
	H	C	24	0.5	2.3	2.3	0.4	0.0	0.0	5.1	57.1
		M	46		0.8	0.4	0.0	4.9	3.6	9.7	
X-rays	U	C	53	7.6	13.5	9.5	3.5	0.0	0.0	26.5	10.0
		M	7		0.5	0.0	0.0	1.0	2.0	3.5	

C = complete changes; M = mosaic changes; U = unstored; L = stored at 12°C; H = stored at 25°C

deposited after insemination. Eggs are fertilised from this store of sperm just prior to their being laid. Thus sperm utilised at a time several days after insemination is considered to have been stored for the corresponding length of time. According to the hypothesis, stored sperm should contain a higher proportion of mature rather than potential breaks. Slizynska (1969) carried out a detailed cytological examination of female larvae whose fathers had been treated with TEM. She found that after storage for a period of 7-9 days, the spectrum of rearrangements resulting from TEM treatment became remarkably similar to that produced by X-rays. The results are shown in Table 1.2. The shortage of rearrangements before storage was interpreted as being due to only a limited number of breaks being open at one time, and to a tendency for breaks to open simultaneously in the same chromosome but at different times in different chromosomes, resulting in a high proportion of intra-chromosomal changes plus a low proportion of inter-chromosomal ones. Breaks which opened simultaneously after the first mitosis in the zygote and which formed a rearrangement would appear as mosaics and would not be detected in standard genetical tests, but would be detected in a cytological examination. The ability of chemicals to induce a high proportion of mosaics compared to X-rays was also attributed to a delay in the opening of breaks and gave an indication that chemical lesions can pass through replication of the DNA. No storage effect was found when X-irradiated males were used.

The detailed cytological analysis carried out by Slizynska (1957, 1963, 1969, 1973) greatly added to the evidence for a real difference between chemical and X-ray induced effects. In her analysis of salivary gland chromosomes of female larvae whose fathers had been fed as larvae on formaldehyde food (Slizynska, 1957) she found:

(i) a high proportion of mosaics

(ii) an excess of repeats and deficiencies over other structural changes

(iii) a shortage of translocations

(iv) fewer breaks in heterochromatic regions of the DNA than in the euchromatic regions.

All types of structural changes known to be produced by X-irradiation were also found among the formaldehyde food induced rearrangements. These differences were essentially the same as those seen for mustard gas compared with X-rays, that is:

'a larger proportion of intra-chromosomal rearrangements over inter-chromosomal ones and a delayed expression of damage'.

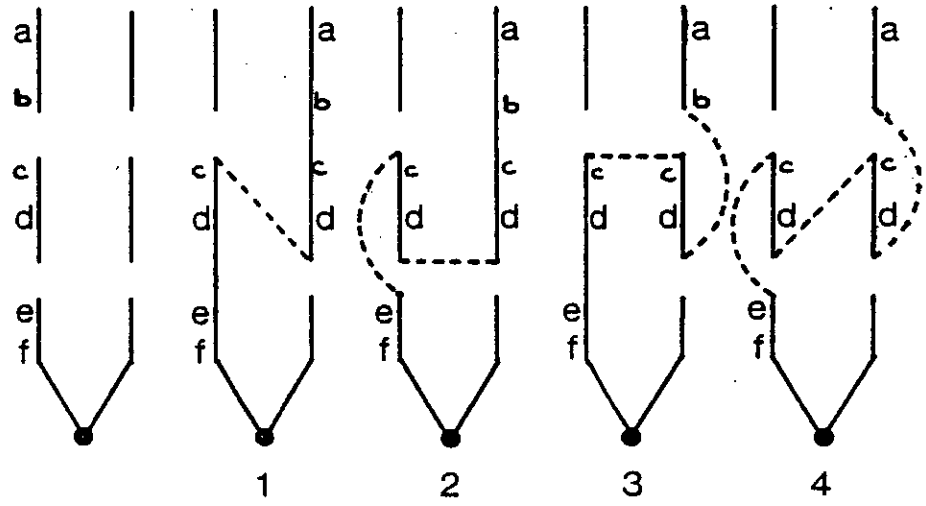
These results were explained in the following manner:

(i) X-ray induced breaks are fixed at the time of treatment; the fixation of chemically-induced breaks does not occur immediately. Most of the X-ray breaks (∿90%) restitute while the remainder form rearrangements. Only a small proportion of chemical breaks are

immediately available for rejoining (repair or mis-repair); breaks which open after the first cleavage division will, if mis-repaired, produce mosaic re-arrangements. This was shown by the lack of mosaic rearrangements induced by X-ray treatment, and the high proportion of mosaics after chemical treatment. Storage allows time for open breaks to accumulate (repair does not take place during storage - see page 24 for evidence). This was reflected by a decrease in mosaic and an increase in complete rearrangements detected after storage of chemically treated sperm (Slizynska, 1969 and 1973).

(ii) Chemically induced small deficiencies and duplications were detected in excess over large rearrangements (translocations, deletions). This was originally thought to indicate a different mechanism from the breakage-fusion events by which large rearrangements arise. Slizynska (1957, 1963, 1969, 1973) carried out an anlysis of the structural changes detected in the salivary glands of F1 larvae whose fathers had been chemically treated. Duplications were found in abundance: they are extremely rare after X-ray treatment. The frequency of the chemically-induced duplications decreased with storage. Most duplications are detected as mosaics together with the complementary deficiency. An analysis of the banding pattern of the duplications/deficiencies led Slizynska (1963) to

Fig.1.1. The mechanism underlying the formation of a repeat.



All types of repeat have the same complementary deficiency (ab−ef). Dotted lines mark the new rejoinings.

(Slizynska, 1963)

suggest a possible mechanism for their occurrence - theoretically, four different types of repeat are possible, each requiring two breaks at identical sites along the chromosome. All four types were detected. Figure 1.1 shows how the four types could arise from breakage and fusion between homologous chromatids. Slizynska realised that the special conditions required for the formation of a repeat were created if potential breaks opened during replication of the DNA. These conditions are a chromosome comprised of two chromatids with breaks at identical sites, as yet unrepaired. The high frequency of duplications after chemical treatment and its decrease with storage supported the idea that replication was the final stimulus for near mature breaks to open and restitute/rejoin in unstored sperm.

In genetic tests carried out by Nasrat et al. (1954-55), all of the deletions (intra-chromosomal rearrangements) but none of the translocations (inter-chromosomal rearrangements) induced by mustard gas treatment were shown to involve the X-chromosome. The X-chromosome is known to replicate asynchronously compared to the autosomes - it is generally considered late replicating (Galton and Holt, 1964) although less obviously so in Drosophila. Nasrat et al. (1954-55) also found that more translocations than large deletions were 'lost' after chemical treatment of adult Drosophila males. These results, shown in Table 1.3, supported the

idea that two potential breaks were more likely to open simultaneously and rejoin when they had occurred in the same chromosome than in different chromosomes. Slizynska (1973) saw this as indicating a connection between chromosome replication in the zygote and the way in which rearrangements are formed (see page 10, and Fig. 1.1) and a possible connection between replication and maturation of breaks in unstored sperm. Storage of sperm reduced the difference between the frequency of intra- and inter-chromosomal rearrangements. Any difference in joining ability between breaks in different chromosomes tended to decrease with storage suggesting that mature breaks were no longer susceptable to the stimulus of replication.

The shortage of heterochromatic (he-) breaks detected in the giant salivary gland chromosomes of Drosophila was attributable to the chromosomes themselves. Only euchromatic (eu-)- eu and eu-he rejoinings are scorable; he-he rejoinings cannot be detected. In plant cells, all such breaks can be detected: chemical treatment produced more he-breaks than did X-rays (Kihlman, 1966). If the same is true for Drosophila, the observed shortage of he-breaks in unstored sperm (Slizynska, 1973) must be due to a shortage of eu-he rejoinings and not a shortage of he-breaks. Storage increased the frequency of detectable he-breaks detected as eu-he rejoinings. Storage thus reduced any difference in rejoining ability between breaks in different chromatin. Asynchrony of replication between eu- and

TABLE 1.3. Comparison between the frequencies of large rearrangements in the progeny of males exposed to mustard gas or X-Rays

Dose (in % slrl)	Deletions			Ratio (approx.)	Translocations			Ratio (approx.)
	n	Expected (X-ray induced)	Observed (M-G induced)		n	Expected (X-ray induced)	Observed (M-G induced	
5[1]	8331	21	2	10:1	2655	54	5	11.1
7[1]	4523	20	4	5:1	3858	123	12	10.1
7[2]	5052	18	9	2:1				
9[2]	6635	41	16	3:1				
9[2]					816	56	7	8:1
10[1]	4926	31	14	2:1	3060	172	26	7:1
15[2]					981	98	21	5:1
Mean	29467	131	45	3:1	11370	503	71	7:1

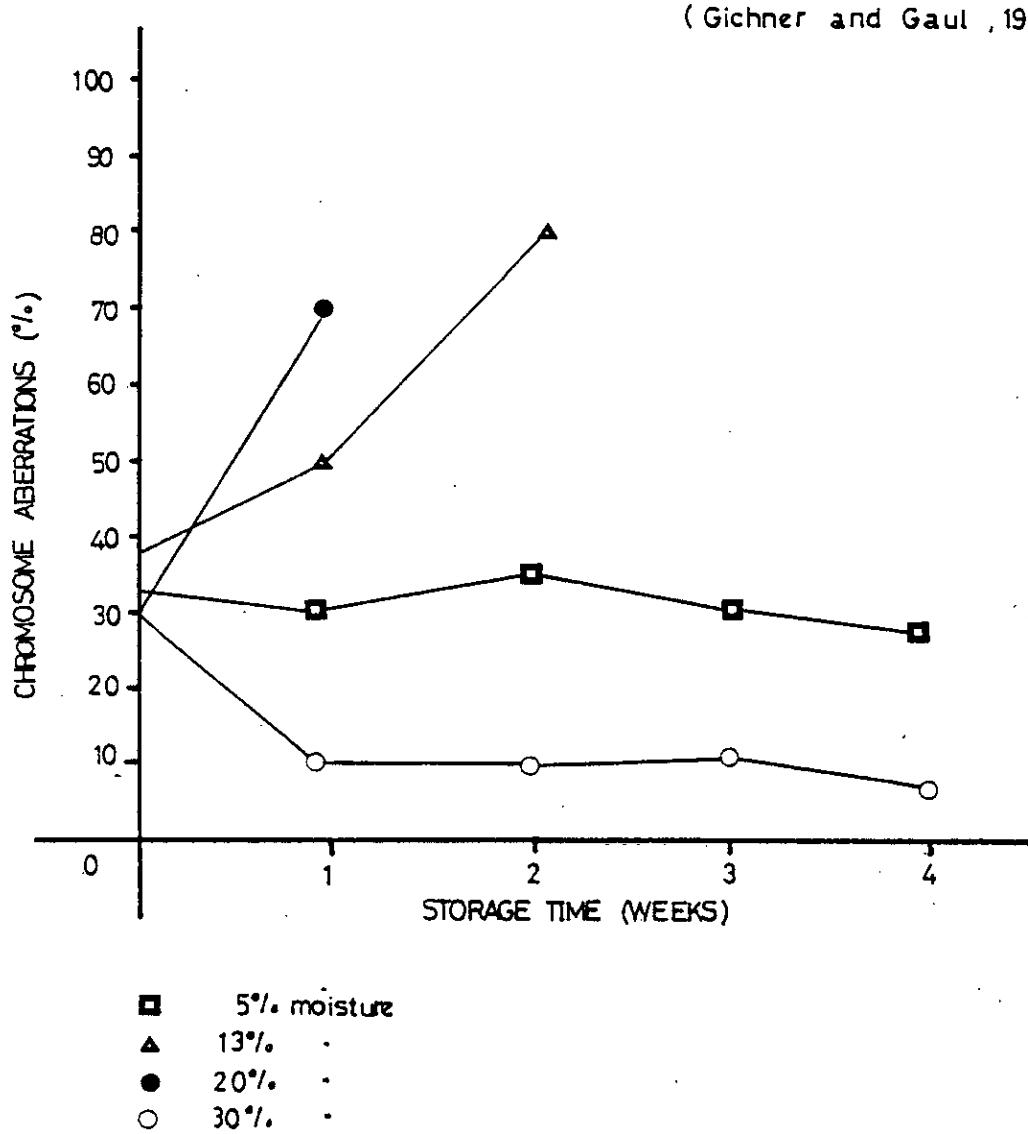
1 After Nasrat et al. (1954-55)
2 After Auerbach and Robson (1947)
slrl frequency determined in tests on aliquots of the treated males
n represents the number of X-chromosomes in the deletion test; the number of chromosome sets in the translocation test.

Expected values calculated from a dose of X-rays yielding the same frequency of sex-linked values.

Translocations involved chromosomes 1, 11, 111; in the last experiment chromosomes 11 and 111 only.

Fig 1.2 Frequency of aberrant anaphases (fragments and/or bridges) after storage at various moisture contents of EMS-treated barley seeds.

(Gichner and Gaul , 1971)



heterochromatin has also been reported (Back, 1976), with heterochromatin generally considered to be late-replicating.

These observations add weight to the hypothesis that chromosome replication stimulates the opening and rejoining ability of potential chromosome breaks.

While rearrangements were shown to increase with storage, intra-chromosomal changes scored as s1r1 did not, or no more than would be expected if, particularly at higher doses, a small proportion of lethals was connected with rearrangements which would be subject to a storage effect. The different response of s1r1 and chromosome breaks to storage after chemical treatment was the first clear indication of an essential difference between them.

A storage effect, comparable to that seen in Drosophila, has also been observed in plants (Gichner and Gaul, 1971). This has been shown to be dependent on moisture content during storage (see Fig. 1.2). When EMS-treated barley seeds were kept dormant for up to 4 weeks before allowing germination, the frequency of aberrant anaphases was found to increase with storage at 13% and 20% moisture content, to initially decrease at 30% moisture content then level off, and to remain fairly constant at 5% moisture content. In the latter case metabolic processes were more or less suspended, i.e. repair processes would be inhibited. The decrease at 30% could be attributed to repair processes when metabolic processes were not inhibited. However, inhibition of repair would not seem sufficient to account for continually

increasing amounts of genetical damage during storage of partially dehydrated seeds. As repair does not appear to take place before storage (treated but unstored seeds do not show different frequencies of aberrancies dependent on moisture content) one would not expect, even with total inhibition of repair, that the frequency of breaks would be higher in stored compared with unstored seeds (Gichner and Gaul, 1971). Slizynska (1973) suggested that there must be an additional source of new mutational changes that appear during storage of barley seeds. Alternatively, repair could be responsible for the decrease of damage in seeds if the rates of repair were higher than the rates with which new breaks were produced during storage. Thus repair processes can be taken into account only as one factor modifying the storage effect in seeds, but not as the cause of them.

In Drosophila, storage effects are present even though there are no detectable repair processes in spermatozoa (Muller and Settles, 1927; Proust, Sankaranarayanan and Sobels, 1972). Other factors, such as storage effects caused by residual mutagen can be discounted because:

(a) there is such a strong storage effect on rearrangements but not on slrl induced in Drosophila.

(b) the rates with which changes accumulate would appear to be too large to be specifically caused by traces of mutagen.

1.3 ALKYLATING AGENTS: MECHANISM OF REACTION

Current understanding of the mechanism of chromosome breakage and the nature of the potential break is limited even though changes occurring at the molecular level in DNA are capable of detailed description. Our knowledge of chromosome structure has increased with recent technological advances in EM and light microscopy but as yet has not shed any light on chromosome breakage. Like gene mutations, chromosome aberrations can be induced by a variety of agents. In particular, alkylating agents have been shown to break both the DNA (Lawley, 1966) and the chromosomes (Sax, 1938; Evans and Scott, 1969). The chemistry of their reactions with DNA has been well studied.

Alkylating agents have been defined as agents which add an alkyl group to macromolecules of biological importance. They can be represented by the general formula

$$R : Y$$

where $R = C_nH_{2n+1}$ (alkyl radical/carbonium ion)

Y = any other group, e.g. OH

The general equation for an alkylation reaction is

$$R : Y + X^- \longrightarrow R : X + Y^-$$

The extent of the displacement reaction will depend on the energy characteristics of the entities involved: Y becomes more or less detached together with the electrons which constituted the chemical bond, the positively charged R group then seeks electrons. Alkylating agents are thus electrophilic reagents which combine with electron rich or

nucleophilic centres.

The transfer of the R group can take place by two mechanisms known as sn_1 and sn_2.

(i) $\underline{sn_1 \text{ type reaction}}$.

$$R{:}Y \xrightarrow{(i)} \underset{(solvent)}{R^+} + \underset{(solvent)}{Y^-}$$

$$R^+ + X^- \xrightarrow{(ii)} R{:}X + solvent$$

(i) is a slow rate determining step which is reservible.

(ii) is a fast reaction, the R^+ rapidly reacting with any electron-rich centre in the system.

This is a unimolecular reaction because only one molecule, the alkylating agent R:Y, is undergoing covalency change in the rate determining step. If the concentration of Y^- is increased, the rate determining step will be reversed. sn_1 agents attack all nucleophiles and consequently yield a variety of products.

(ii) $\underline{sn_2 \text{ type reaction}}$

$$R{:}Y + X^- \longrightarrow \underset{\text{transition complex}}{[-X^{\delta-} \cdots R \cdots Y^{\delta-}]} \longrightarrow R{:}X + Y^-$$

Complete separation of R and Y does not occur, instead there exists a transition state with R loosely combined with both X and Y, the effective transfer of R^+ from Y to X occurring as bonds are simultaneously formed and broken. This is a bimolecular reaction because, in the rate determining formation of the transition complex, two molecules are undergoing covalency change. The rate of the reaction is dependent upon the concentration of the displacing group X^-

TABLE 1.4. Alkylating agents: relationship between s-value reaction type and functionality

CHEMICAL	s-VALUE	REACTION TYPE	FUNCTIONALITY
MMS	0.88	sn_2	monofunctional
DMS	0.86	sn_2	"
EMS	0.67	sn_1/sn_2	"
DES	0.65	sn_1/sn_2	"
MNU	0.42	sn_1	"
iPMS	0.28	sn_1	"
ENU	0.26	sn_1	"
mustard gas cation	0.95	sn_2 value but sn_1 kinetics	bifunctional

and upon the relative nucleophilic capacities of the groups X^- and Y^-. sn_2 agents show a strong preference for the most nucleophilic sites.
(A detailed review of the general chemistry of alkylating agents can be found in Ross, 1962).

Two attempts have been made to express the rates of these substitution reactions, e.g. the Swain-Scott constant (s) (Swain and Scott, 1953) and the more complex parameter equation developed by Edwards (1954) which gives a closer agreement with experimentally determined rate values. An agent with a high Swain-Scott s-factor reacts via an sn_2 mechanism while one with a low Swain-Scott s-factor reacts via an sn_1 mechanism. The s-value of an agent is sometimes quoted when comparisons are being made between a range of alkylating agents. Typical sn_1 agents are NMNU, NMNG, aromatic nitrogen mustards, iPMS (low s-value). Typical sn_2 agents are DMS, MMS, methyl bromide (high s-value). Some agents enter into both types of reaction, e.g. EMS, DES. A list of some alkylating agents and their s-values is given in Table 1.4.

If an alkylating agent is to be effective, it should be able to diffuse to and react with distant target sites. These requirements are met if the agent reacts either by (a) a moderately fast sn_1 mechanism, or (b) by an sn_2 mechanism sufficiently slow and with a low substrate constant (s) value so that the alkylating agent is not lost by irrelevant reactions <u>en route</u> to its target. Compounds

which react by an sn_1 mechanism cannot react at a rate faster than that of controlling ionisation stage; therefore, the rate cannot be increased by using a powerful nucleophile whereas that of sn_2 agents can be so increased.

1.4 ALKYLATING AGENTS: STERIC CONSIDERATIONS AND FUNCTIONALITY

There are also steric considerations to be taken into account, i.e. the site of the base in regard to the wide and narrow groves of the DNA helix. N-7 and 0^6 of Guanine, and the N-7 of Adenine are situated in the wide groove and as such are easily accessible to invading alkylating agents. N-3 of Adenine and Guanine are situated in the narrow groove of the helix which can limit their accessibility because large molecules may be physically unable to fit into the small groove. Protein components of eukaryote DNA, e.g. histones and protamines, may limit the mutagenic interaction or may themselves be sites of attack. There are also sites along the sugar-phosphate backbone of DNA which are susceptible to attack. No reaction is anticipated, nor has any been demonstrated, with the hydroxyl group in the sugar moities; however, a reaction with the phosphate groups is possible but appears to be non-mutagenic (Singer, 1975).

Alkylating agents can also be classified according to the type of alkyl group (e.g. a methyl or ethyl group) and the number of alkyl groups that a single molecule can donate. This is known as the functionality of an agent. The degree

of functionality cannot be inferred simply from the number of alkyl groups carried by a compound; it is determined by the number of alkyl groups available for alkylation, e.g. the alkane sulphonates:

EMS - one ethyl group)
) both are monofunctional agents
MMS - two ethyl groups)

There is no correlation between an agent's functionality and its reaction mechanism, e.g. EMS acts by an sn_1/sn_2 intermediate mechanism whereas MMS is an sn_2 agent (see Table 1.4).

Early experiments with closely related chemical compounds, one monofunctional and one polyfunctional, suggested that there was no apparent storage effect on translocations induced by monofunctional agents (Ratnayake, 1968; Watson, 1964 and 1966). They were interpreted in support of the idea that monofunctional agents were less effective than polyfunctional agents at breaking chromosomes. However, it was later demonstrated that the time needed for monofunctional agents to show any storage effect was a matter of weeks rather than days (Sram, 1970a,b). Although the storage effect takes longer to appear, storage eventually minimises any difference in breakage ability to the level at which it was observed pre-storage. This storage effect was seen for chromosomal aberrations but not for point mutations. Polyfunctional agents have an ability to cross-link between neighbouring guanines on the same or opposite DNA strands in prokaryotic and mammalian cells (Flamm et al., 1970) and in bacteriophage (Lawley et al., 1969) when 1 in 25 alkylations

TABLE 1.5 Sites in DNA open to attack by alkylating agents.

BASE	POSITION OF ATTACK	BASE	POSITION OF ATTACK
Guanine	N-1 N^2 3 → N-3 2 → N-7 C-8 1 → O^6	Adenine	3 → N-1 3 → N-3 3 → N-7 N^6 C-8
Cytosine	3 → N-1 ⋯→ N-3 N-7 ⋯→ N^4 N^6	Thymine	O^6 1 → O^4 3 → N-3

⋯→
⟶ Sites most likely to give rise to mutations if alkylated.

1-3 Indicates the importance of the site in terms of its likelihood to give rise to a mutation (1>2>3).

All of these sites are to some extent negatively charged.

results in cross-linking of opposite strands of DNA. The occurrence of double-strand breakage following depurination (see section 1.5(i)) has been shown to occur more rapidly following treatment with a bifunctional mustard gas than with a monofunctional derivative (Laurence, 1963). The storage effect on chromosome breakage also occurs more rapidly with polyfunctional agents than with monofunctional ones. These two observations have been explained by the cross-linking ability of polyfunctional agents.

Some monofunctional agents are excellent chromosome breakers (e.g. Smith and Lofty, 1955; Swanson and Merz, 1959). Experiments comparing the ratio of translocations to lethals induced by two closely related compounds showed that the monofunctional agents were at least as potent mutagens as their polyfunctional 'partner' (Nakao and Auerbach, 1961; Watson, 1964 and 1966). This agreed with cytological data (Slizynska, 1969 and 1973): non-mosaic rearrangements (i.e. those that are detectable in standard genetic tests) were four times as frequent after males had been treated with E1 (monofunctional) than after a mutagenically equivalent dose of TEM (polyfunctional). Thus the ability to form cross-links was shown not to be essential for an alkylating agent to induce chromosome breakage.

1.5 ALKYLATING AGENTS: REACTIONS WITH DNA

Alkylating agents are, then, essentially electrophilic agents with several possible sites of reaction along a DNA

TABLE 1.6. Reaction Products detected in alkylated DNA

	REACTION MECHANISM	
	sn_1	sn_2
Major Reaction Products	7 me-guanine (7 me-G) 3 me-A O^6 me-G phosphototriesters	7 me-guanine (7 me-G) 3 me-A
Minor Reaction Products	3 me-G 7 me-A	3 me-G 7 me-A trace quantities of other species

molecule. Those sites open to attack are listed in Table 1.5.

Virtually all potentially susceptible positions of the normal bases of DNA are alkylated by one or other alkylating agent under one or another set of reaction conditions (Miller and Miller, 1969; Singer, 1975). The major reaction products have been identified. These are shown in Table 1.6.

(i) 7-alkyl guanine

N-7 is strongly nucleophilic and therefore can increase its rate of reaction with sn_2 agents (more powerful electron donors) such as MMS, DMS and EMS. It is the most frequent point of attack and is effectively alkylated by both strong and weak mutagens. Early theoretical studies suggested that an ionised guanine should have a tendency to mispair with thymine instead of cytosine (Krieg, 1963b; Lawley and Brooks, 1961) yielding GC ——→ AT transitions. In practice this effect on base-pairing has not been detected: poly (U-me^7-G) fails to induce misincorporation as a template for either transcription (Ludlum, 1970) or translation (Wilhelm and Ludlum, 1966); 7 me-dGTP has been shown to substitute well for dGTP in the *in vitro* DNA polymerase reaction (Hendler *et al.*, 1970). Therefore 7-alkyl guanine is not considered mutagenic *per se*, even though it is a major reaction product. Alkylation at N-7 has been shown to lead to an increased rate of hydrolysis of the glycosidic bond at N-9 (Lawley, 1957), which can lead to loosening of

the bond between the base and the sugar-phosphate chain leading to a gradual leaching out of a free alkylated base leaving an apurinic gap. When alkylated bacteriophage DNA is mildly heated to specifically promote depurination, lethal but not mutagenic hits were introduced (Krieg, 1963a). Although it was considered as a possible mechanism (Bautz and Freese, 1960) depurination is now thought not to have any significance in the production of point mutations. Frame-shifts caused by this lesion are also a possible mechanism of mutagenesis, as is depurination leading to scission of the chain with the eventual possibility of double strand scission (Lawley and Brookes,1963). Depurination leading to strand breakage is a conceivable candidate for the potential lesions that, in Drosophila and plants, mature gradually into chromosome breaks.

(ii) 3 alkyl-adenine

sn_2 agents produce slightly higher amounts of 3-methyl adenine but are less mutagenic and carcinogenic than sn_1 agents. Therefore this lesion has been considered unimportant in alkylation mutagenesis (Lindahl, 1982) even though it is one of the major alkylation products. It may be that it is a potentially lethal or inactivating lesion.

(iii) O^6 alkyl-guanine and O^4 alkyl-thymine

O^6 is weakly nucleophilic and therefore more strongly attacked by sn_1 agents, e.g. NMNU, NMNG, EMS. O^6 lesions are implicated in mutagenesis which could explain why EMS is such a potent mutagen in terms of inducing intragenic

changes. When O^6-G is alkylated, it becomes a hydrogen donor instead of an acceptor such that G-T base pairing becomes a possibility (Gershman and Ludlum, 1973). Present experimental evidence reveals an excellent correlation between mutagenesis and alkylation of both O^6-G and O^4-T (reviews by Lawley, 1974; Singer, 1975). An *in vitro* system using RNA polymerase showed that UMP preferentially paired with O^6-me G as opposed to AMP or CMP. O^4-T, although infrequently alkylated, can also be shown to pair with G due to an extra bonding possibility (Drake and Baltz, 1976). Direct mispairing therefore seems to be one of the mechanisms by which alkylating agents induce mutagenesis. *In vivo*, alkylation directed mutagenesis has been shown to specifically generate AT ⟶ GC transitions in bacteriophage DNA (Krieg, 1963b). A mis-repair mechanism may be involved in repairing those alkylations not seen to give rise to a good mis-pairing scheme.

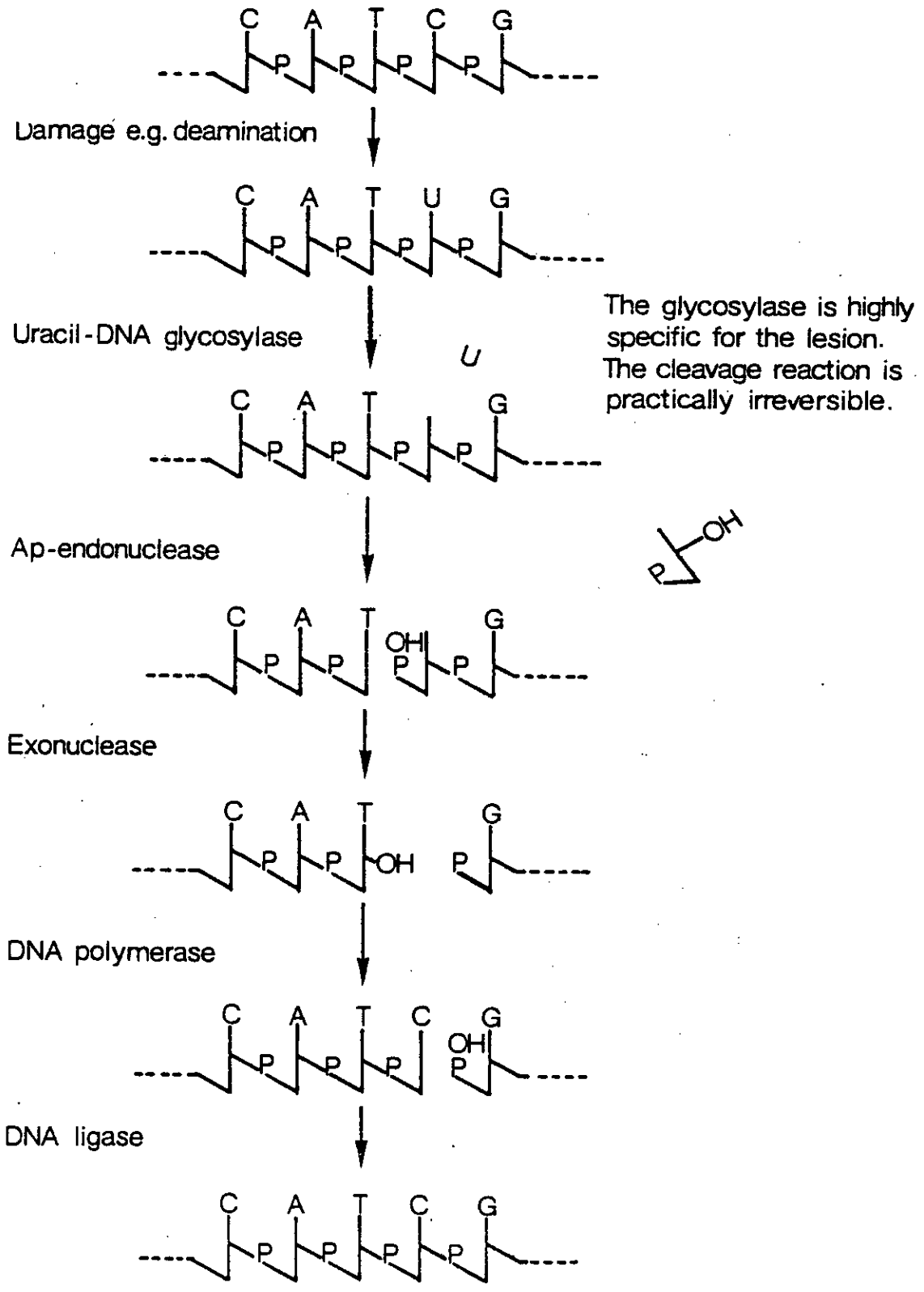
1.6 FATE OF ALKYLATED DNA

Alkylated DNA is known to suffer one of two fates:

(i) eventual hydrolysis of the damaged base leading to depurination or depyrimidation which is non-enzymatic (Lindahl and Nyberg, 1972; Lawley and Brookes,1963; Strauss and Hill, 1970).

(ii) enzymic removal of the damaged base by cleavage of the sugar-base bond (Lindahl, 1974).

Fig.1.3 Base excision repair of DNA



(Lindahl , 1979)

(i) Hydrolysis

DNA purine residues alkylated at N-3 or N-7 are positively charged and thus rapidly released by hydrolysis. The most common methylated purines can be ranked according to the rate at which they are released. In a descending order of rate of release, this ranking is: 7 me-A > 3 me-A > 7 me-G > 3 me-G >> 0^6 me-G (Lawley and Warren, 1976). This appears to hold true both *in vivo* and *in vitro*. Hydrolysis may be a possible explanation for the observed storage effects seen in *Drosophila*.

(ii) Enzymatic Removal

Removal of alkylated bases, as a step in an excision repair mechanism by specific DNA-glycosylases, is seen to further enhance depurination (see Fig. 1.3). Those glycosylases isolated to date are highly specific for one particular lesion, e.g. 3 methyl-adenine glycosylase (Lindahl, 1979; Laval, 1977; Brent, 1977). It seems likely that base excision repair is restricted to a small number of commonly occurring lesions with a separate DNA glycosylase for each lesion.

Three of the common derivatives of alkylation have similar half-lives both *in vivo* and *in vitro*, and therefore do not seem to be actively excised.

7-methyl guanine (Lawley and Warren, 1976)

7-methyl adenine (" " " ")

phosphotriesters (Shooter and Slade, 1977)

However, both 0^6-methyl guanine (Lawley and Orr, 1970) and

3-methyl guanine (Lawley and Warren, 1976) as well as 3-methyl adenine are liberated *in vivo*, the latter at a much higher rate than the first two.

There are indications that the same is true for ethylated derivatives (Lawley and Warren, 1975).

Enzymatic removal has been shown to occur at a rate 8-10 times faster than that of hydrolysis for 3-methyl adenine residues (Margison and O'Connor, 1973). Wolff (1960) held the view that repair or mis-repair of broken chromosomes required protein synthesis, and that spermatozoa lacked the relevant repair enzyme(s) which was (were) provided in the ovum or zygote. Several workers have provided evidence for this generally accepted idea that repair processes are lacking in mature sperm of *Drosophila*. Muller (1940) observed that neither fractionation of X-ray dose, nor storage of X-rayed spermatozoa had any effect on the frequency of translocations. Proust, Sankaranarayanan and Sobels (1972) used actinomycin-D to inhibit maternal repair processes acting at the stage of pronucleus formation. These processes are required for the repair (restitution) or mis-repair (leading to formation of rearrangements) of chromosome breaks which had been induced in X-rayed spermatozoa. This treatment was shown to decrease the frequency of translocation (mis-repaired breaks) while that of dominant lethals (unrepaired breaks) increased concomitantly. More recently, repair mutants have been isolated in *Drosophila* laboratories (Boyd and Setlow, 1976), these have

been used to verify the findings of Proust et al. (1972). Current opinion holds that, in Drosophila spermatogenesis, most, if not all, transcription ceases before meiosis and that the considerable post-meiotic translation is directed by pre-meiotically transcribed message (Brink, 1968). If repair enzymes, or their mRNA, are present in spermatozoa, it seems unlikely that they will be present in sufficient amounts to furnish repair of the extensive damage induced by a mutagen. It would therefore seem unlikely that the storage effect could be attributed to such an activity.

Chain breakage has been shown to occur preferentially at apurinic sites (Lindahl and Anderson, 1972). Basic proteins, such as histones and protamines, promote the rate of chain breakage at such sites (McDonald and Kaufman, 1954). This may be of relevance in explaining the storage effect.

1.7 THE PROJECT

7 methyl-guanine has been implicated in increased rates of depurination leading to chain breakage whereas 0^6-methyl guanine has been implicated in mispairing leading to base changes. These are the two major mutagenic lesions for alkylating agents operating via the sn_1 or the sn_2 mechanism. 7-methyl guanine spontaneously hydrolyses at much higher rates than 0^6-methyl guanine; therefore with storage, depurination resulting from this hydrolysis may enhance the probability of chromosome breakage while slightly diminishing

the mutagenic effects of O^6-methyl guanine in terms of base changes. If this holds true, one could expect that the greater the degree of alkylation of the DNA, the greater should be the response to storage, i.e. the greater the possibility of a strand-breakage event and also the greater the probability of a chain-breakage event due to alkylation occurring in close proximity on opposite strands, presuming that alkylation is a random process.

Although repair mechanisms do not operate in sperm, they will come into play when fertilisation takes place. These repair mechanisms may be affected by the degree of damage, e.g. at low doses there may be a threshold of alkylation below which translocations will not occur, at high doses repair mechanisms may be 'saturated' by extensive damage to the DNA. Depurination can be increased by raising the physiological temperature. This may or may not affect the frequency of observed rearrangements. Other, as yet unknown, factors must also be considered. Sex-linked recessive lethals are a heterogeneous class of mutants - an attempt to define this class more precisely and to observe the effects of storage on the different members of this class (e.g. base changes, small deletions) should prove useful.

The aim of this project was to attempt to determine the nature of the potential breaks and the processes whereby they mature into actual breaks. This was done by carrying out a detailed study of the kinetics of the storage effects and of the effects of different conditions on the kinetics.

In order to carry out this investigation, a better definition of the conditions under which the storage effect could be observed was attempted. This involved looking at the effect of different concentrations of yeast, different concentrations of sugars and different sugar sources in the storage medium related to the survival of females and pattern of egg-laying with time. This arose from the problems encountered with DES.

Subsequently the effect of mutagen concentration and temperature on the rate at which the breaks open was observed. Breaks were measured by observing the frequency of translocations between chromosomes 2 and 3. An alternative method was to look at the frequency of dominant lethals, which gives a measure of the frequency of un-rejoined breaks.

An attempt was also made to establish the nature of sex-linked recessive lethals in genetic terms, since some mutagens produce a slight storage effect in this heterogeneous class of damage.

CHAPTER 2. MATERIALS AND METHODS

MATERIALS AND METHODS

2.1 STOCKS

A. Stocks Used

1. Oregon-K Stock

A wild-type stock of Drosophila melanogaster, which has been maintained in this laboratory for several years. Its spontaneous mutation rate, measured by slrl frequency, is known to be approximately 0·2% (Kilbey et al., 1981).

2. 0-1; bw; st stock y sc^{s1} ln^{49} sc^{8}, bw; st

This stock can be used to test simultaneously for both sex-linked recessive lethals (slrl) and translocations involving the Y, II and III chromosomes. The genotype of the X-chromosome of this stock is y sc^{s1} ln^{49} sc^{8}. The long inversion sc^{8} contains within it the inversion ln^{49} and the entire X-chromosome is inverted with the recessive marker yellow (y) at its base. The two inversions suppress crossing-over along the whole length of the X-chromosome. In addition, chromosome II carries the recessive marker brown (bw) while chromosome III carries the recessive marker scarlet (st). The bw and st markers, when homozygous, interact to produce a phenotypically white-eyed fly.

3. bw; st stock

This stock was used solely for the detection of translocations. The autosomal recessive markers bw and st are carried on chromosomes II and III respectively. As with stock 2, this stock was phenotypically white-eyed.

4. Muller - 5 stock (M-5). sc^{s1}, B, ln-s, w^a, sc^8

This stock was used to determine the frequency of slrl in the majority of experiments. The X-chromosome carries the following markers: the dominant Bar (B) and the recessive white apricot (w^a), which effect the shape and colour of the eye; also two inversions sc^{s1} and inversion-s (ln-s), the latter contained the former. These inversions completely suppress crossing over along the whole length of the X-chromosome.

5. lnsc stock. sc^{s1}, ln49, sc^8

This is essentially a wild-type stock which carries an inversion on the X-chromosome to suppress crossing-over. The genotype of this stock is sc^{s1}, ln49, sc^8. As with the 0-1; bw; st stock, the long inversion sc^8 contains within it the inversion ln49, and the entire X-chromosome is inverted: the two inversions suppress crossing-over along the whole length of the X-chromosome. The stock was used in the specific locus test when it was essential to suppress crossing-over between the treated paternal X- and the untreated maternal X-chromosome present in heterozygous F_1 daughters. Preliminary tests by other workers in this laboratory have shown that spontaneous and EMS-induced mutability in these males is similar to that of Ork ♂♂ (Shukla and Auerbach, 1981).

6. Multiply-marked X stock

This stock supplied the tester ♀♀ for the specific locus test. The stock carries a multiply marked X-chromosome

TABLE 2.1. Inheritance of X- and Y-chromosomes when an XY♂ is crossed with an attached-X ($\hat{X}\hat{X}$Y)♀

♂ \ ♀	$\hat{X}\hat{X}$	Y
X	$\hat{X}\hat{X}$X *	XY ♂
Y	$\hat{X}\hat{X}$Y ♀	YY **

* inviable or highly abnormal and sterile.

** inviable.

and, as a balancer, a M-5 chromosome which may be used to estimate frequency of induced slrl as in the M-5 test. The M-5 chromosome suppresses crossing-over between the tester chromosome and itself. A stock homozygous for the multiply marked X shows greatly reduced fertility, hence the maintenance of a heterozygous stock. The markers on the X-chromosome are:

y = yellow body

w = white eyes

sn = singed bristles

m = miniature wings

g = garnet eyes

f = forked bristles

car = carnation eyes

The genotype of the flies used to maintain the stock was:

$$\frac{y\ w\ sn\ m\ g\ f\ car}{M\text{-}S}\ ♀♀ \qquad \frac{y\ w\ sn\ m\ g\ f\ car}{Y}\ ♂♂$$

7. Attached X-stock

The X-chromosome was multiply marked but these markers were of no significance in this work. The importance of the stock was that the mothers would donate a Y-chromosome to their sons, whereas normally they donate an X; the X being donated by the father, as shown in Table 2.1. This meant that treated paternal X chromosomes could be scored for recessive or dominant visible mutations in the hemizygous males of the F_1 generations.

TABLE 2.2. Chronology of D. melanogaster development at 25°C

By hour	By Days (Approx)	Stage	
0	0	Egg laid	Egg/larval period 5 days
0-22	0-1	Embryo	
22	1	Hatching from egg (1st instar)	
47	2	First moult (2nd instar)	
70	3	Second moult (3rd instar)	
118	5	Formation of puparium	Pupal period 4-2 days
122	5	"Prepupal" moult (4th instar)	
130	5½	Pupa: eversion of head, wings and legs	
167	7	Pigmentation of pupal eyes	
214	9	Adult emerges from puparium with creased and folded wings	
215	9	Wings unfold to adult size	

M.W. Strickberger (1962)

B. Maintenance of Stocks

Stocks 1, 2, 3, 4, 5 and 7 were maintained without selection. For stock 6, the appropriate males and females were selected at either every or every other generation. All stocks were kept in bottles of standard Drosophila Medium (SDM) at 25°C with continuous lighting. Under these conditions, the generation time was approximately 10 days. as shown by Table 2.2.

2.2 FOOD MEDIA

A. Standard Drosophila Medium (SDM)

The recipe for SDM is:

Maize meal	10%	e.g. 500g
Agar	1.3%	65g
Flaked yeast	1.5%	75g
Treacle	8.6%	430g
Proprionic acid	0.3%	15ml
Nipagin	0.06%	3g
Distilled water	100%	5,000ml

For the best results, the maize meal was soaked in a small volume of the distilled water for about 30 minutes. The remaining ingredients (except the proprionic acid and nipagin) were then mixed in, care being taken to slowly add the water to prevent lumps from forming. These were then brought to the boil and allowed to simmer for a few minutes, with continuous stirring. The mixture was allowed to slightly cool before the proprionic acid and nipagin was added. It was then dispensed into heat-sterilised glass milk bottles or glass vials; these were covered with a sheet of cotton

gauze and allowed to cool. Once the food had set, cotton wool stoppers were inserted and the bottles/vials allowed to cool further before either immediate use, or storage in a cold-room at 4°C. Before use, bottles were wiped out to reduce excess moisture, and a folded tissue was inserted into the food. If taken from the cold-room, bottles and vials were allowed to acclimatise at room temperature before use.

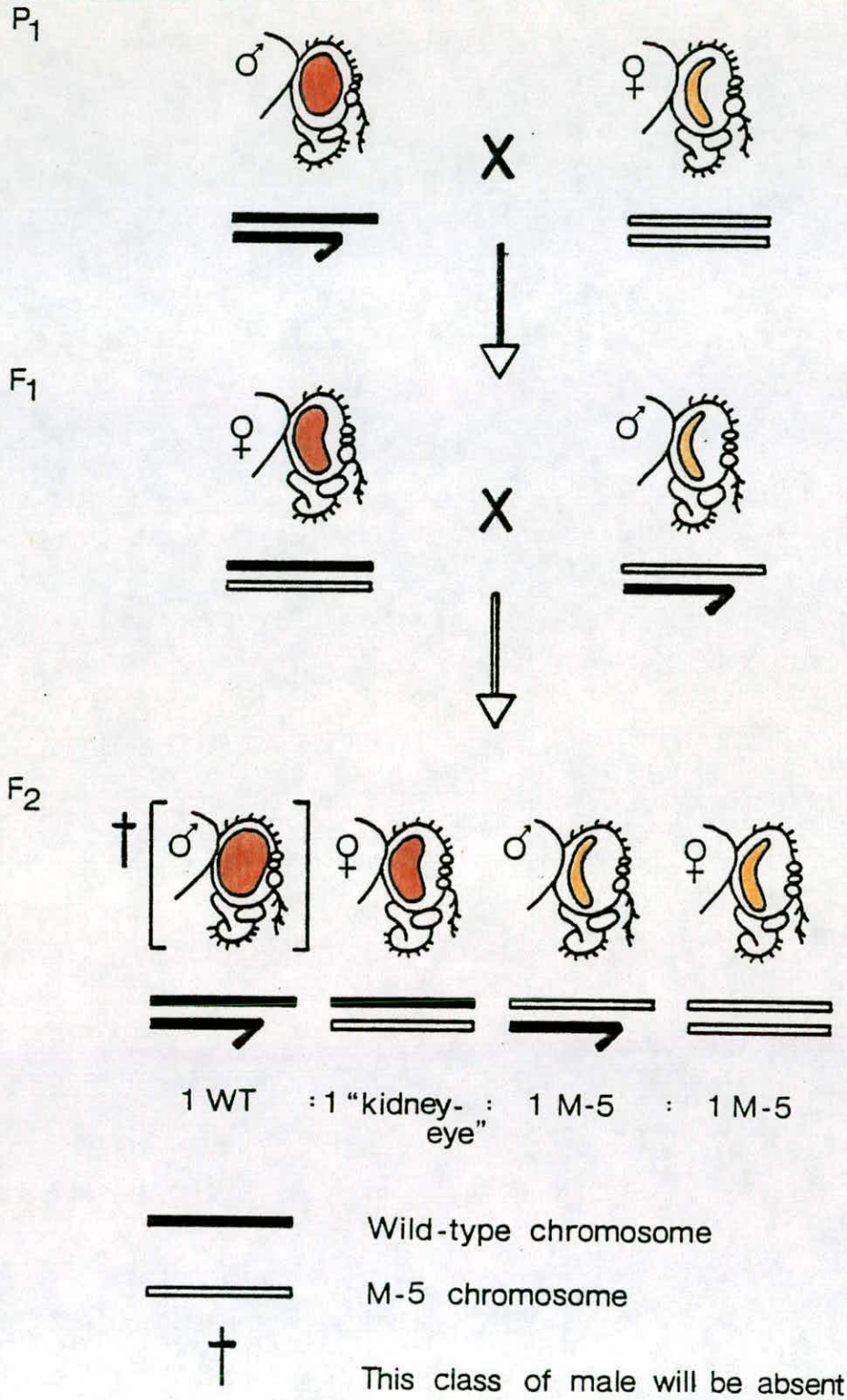
B. Honey-agar Medium (HAM)

The recipe for HAM is:

Agar	2%
Honey	5%
Nipagin	0.06%
Distilled water	100%

The dry ingredients were mixed together with the distilled water, care being taken to add the water slowly to prevent lumps from forming. The honey was then added. The mixture was gently heated, and stirred continuously until the honey had dissolved. It was then brought to the boil and allowed to simmer for 1 minute. It was then dispensed into heat-sterilised glass milk bottles, each bottle covered with a square of gauze secured with an elastic band, and allowed to cool. When it had set the food was placed at 4°C overnight. Before use, bottles were allowed to acclimatise at room temperature, they were wiped out to remove excess moisture and a 1cm wide filter paper ring inserted. The ring sat on the food and was a precautionary device

Fig.2.1. Test for sex-linked recessive lethals in Drosophila.



used to prevent flies from inserting themselves down cracks which appeared between the food and the sides of the bottle.

2.3 GENETICAL TESTS

A. Test for sex-linked recessive lethals (slrl) or Muller-5 (M-5) test

It is not possible to score all mutations in an experiment, therefore in order to measure mutagen success or failure, a representative class of mutations has been chosen which detects the more common types of gene mutations (i.e. recessive lethals). The tester chromosome used in the test for sex-linked recessive lethals (slrl) was the M-5 or Basc X-chromosome. It carried the following markers:

Marker	Phenotype	Chromosomal Location
Bar (B)	bar-eye in shape	1-57.0
white-apricot (w^a)	pinkish-white eye colour	1-1.5

plus a series of inversions to suppress crossing over of a lethal from the treated chromosome into its homologue when in the heterozygous state in the F_1. The genotype of the stock used was sc^{s1} B ln-S w^a sc^8 (see list of stocks for details). The marker genes allowed easy recognition of the different classes of flies in the F_2 cultures. The test for slrl can be represented as shown in Figure 2.1.

The test required two generations. The method of scoring (absence or presence of a whole class of flies from a culture) minimised any personal bias in the scorer.

Absence of the XY class of ♂♂, which carried the treated paternal X-chromosome, signified the induction of a lethal mutation. The XX class could be used to carry the lethal, either for retesting for maintenance of a lethal stock, or for further genetic analysis.

The detection of sex-linked recessive lethals in Drosophila

A culture was scored as "lethal" if the wild-type class of ♂♂ was absent and there were at least 7M-5 ♂♂ (8 including the F_1 father). Theoretically, the chance of misclassification was then reduced to <1%.

Probability of seven ♂♂ being M-5 = $1/2^7$

= 1/128.

Cultures not satisfying these criteria were re-tested. F_1 ♀♀ carrying a lethal often have reduced fertility and produce small F_2's - this was the reason, in the majority of cases, that a retest was necessary. Occasionally, the presence of a single WT ♂, or a disproportionate ratio between the two classes of ♂♂, also necessitated retests. If a lethal had been induced, the chromosome carrying the lethal would also be present in half of the F_2 ♀♀. These ♀♀ would have one M-5 X-chromosome and one WT X-chromosome: thus they would have kidney-shaped eyes and be easily identifiable. Any lethal could be kept for further analysis by mating these ♀♀ with M-5 ♂♂.

Treatment of ♂♂ was only a convenient way of treating their germ cells. Heterozygosity for a lethal may delay

larval development; it was therefore important to standardise the time after eclosion when the F_1 flies were collected. Unless stated otherwise, F_2 cultures were set up using flies that had hatched between days 1-4 after the F_1 flies started hatching. It was also important to score the F_2 cultures within 8 days after the emergence of the first progeny, to avoid any overlap of the F_3 generation.

A low and very constant mutation frequency of ∿ 0·2% slrl/X-chromosome/generation minimises any need to perform parallel control experiments (Kilbey et al., 1981).

In the initial experiments, both with DES and EMS, the P_1 matings were single-pair matings, i.e. 1♂ x 1 or 2♀♀. This was done to establish the frequency of slrl (and Tr) for the different doses of chemicals used and it ensured that any possible cluster effect (which would have enhanced the frequencies obtained) would be detected. Once the frequencies were established, and because P_1 single pair matings were very time consuming, the matings were carried out en masse, in bottles.

Although this test is widely used as a means of establishing the induction of mutations, it should be remembered that lethals are a heterogeneous class in the following respects:

(i) they involve an unknown number of genes - estimates of up to 1,000 loci have been quoted. The euchromatic X makes up ∿20% of the Drosophila genome. Approximately 1,000 bands are seen in the salivary gland giant X-

chromosome and from this it has been estimated that 600-900 loci can mutate to produce a recessive lethal (Vogel et al., 1981).

(ii) they comprise point mutations as well as small and large rearrangements, especially deficiencies, therefore the interpretation of mutation experiments may be difficult.

Two mutagenic treatments which produce similar lethal frequencies may differ considerably in their effects if the lethals in one case consist mainly of deficiencies but in the other of point mutations. Similarly, an increase in mutation frequency has a very different significance depending on whether all types of lethal have increased proportionally, or whether the whole increment is made up of one type only, be it point mutations or rearrangements.

B. Test for Translocations between autosomes II and III

Reciprocal translocations in Drosophila can be detected, in the F_2 generation after treatment, by a standard method using recessive autosomal markers. The two markers used in these experiments were:

Marker	Chromosomal Location	Phenotype
brown (bw)	2-104.5	light-brownish wine eye colour; red eye pigments are lacking
scarlet (st)	3-44.0	eyes bright vermillion, darkening with age; brown eye pigments are lacking

The double mutant $\frac{bw}{bw}$; $\frac{st}{st}$ is white-eyed.

The test can be represented as shown in Figure 2.2.

The rationale of the translocation test was as follows:

(i) previously unlinked markers appeared linked (pseudolinkage)

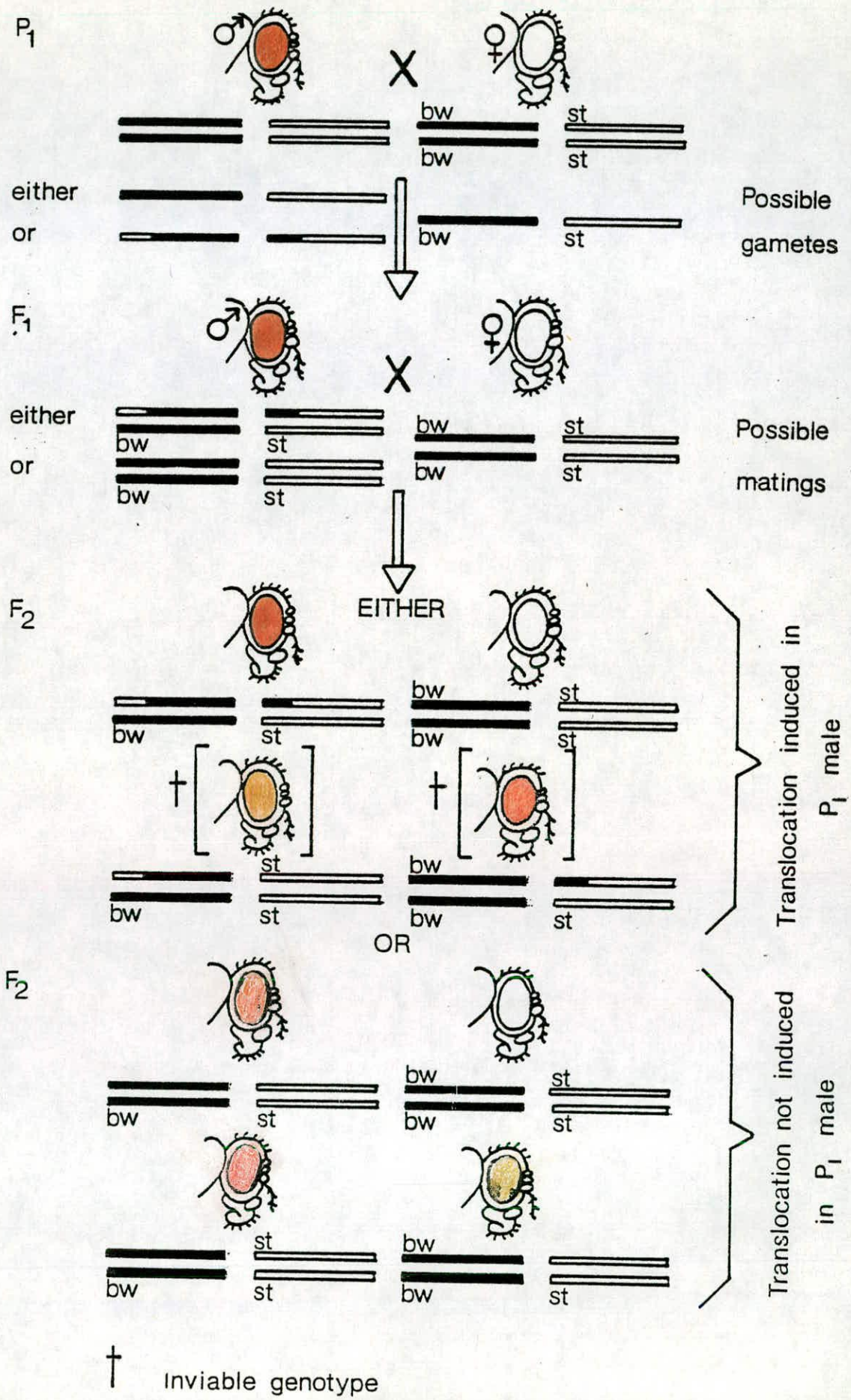
(ii) the absence of crossing-over in ♂♂ preserved the intact translocation in the ♂ carrier.

(iii) each F_1 ♂ = one treated chromosome set; therefore the scale of the experiment was determined by the number of F_1 ♂♂ used.

(iv) aneuploidy (dp/df) disturbs the genic balance which, in Drosophila, is usually lethal in the zygote.

F_2 cultures were scored, using CO_2 to anaesthetise the flies, within 4 days of the F_2 cultures beginning to hatch. As scarlet eyes darken with age, this made it easier to distinguish between scarlet and wild-type eye-colour. Arbitrarily, cultures with less than 20 flies in which all four eye colours were not seen were retested, as were all cultures giving a positive result. This minimised any possibility of misclassification. 40 II-III translocations recovered from daughters of irradiated ♂♂ were all fertile in heterozygous ♂♂: this showed that males carrying such translocations are fertile (in Lindsley and Tokuyasu, 1980). Only complete II-III translocations were scored. The majority of translocations between the X-chromosome and either of chromosomes II or III are thought to be made sterile if more than a very small portion of the X is translocated (in Lindsley and Tokuyasu, 1980): this class was

Fig. 2. 2. Test for reciprocal II-III translocations in Drosophila.



therefore not included in the test.

Spontaneous translocations occur with a frequency of about 0.04% which is why control experiments are usually unnecessary, and why this method is considered a reliable and sensitive tool for detecting breakage and rejoining events in treated chromosomes.

C. Tests for small deletions: Specific Locus Test

This test allowed the detection of recessive visible mutations in the F_1 generation following chemical treatment of the $P_1\sigma$. It is a useful test when characterising the spectrum of mutations induced by an already proven mutagen, as detected by the slrl test.

The tester ♀♀ for the specific locus test carried a multiply marked X-chromosome and, as a balancer, a M-5 chromosome. The markers on the X-chromosome were

y w sn m g f car (designated * in this thesis)

Marker	Phenotype and Chromosomal Location
y	yellow (1-0.0). body colour yellow; hairs and bristles with yellow tips.
w	white (1-1.5) eyes pure white. WT alleles not completely dominant to w.
sn	singed (1-21.0) bristles twisted and shortened, hairs wavy. sn/sn ♀♀ are sterile although it is possible to obtain fertile sn alleles.

Marker	Phenotype and Chromosomal Location
m	miniature (1-36.1) wing size reduced such that wings are only just slightly longer than the abdomen. In poor cultures (e.g. overcrowding) wings may become divergent and stringy.
g	garnet (1-44.4) eye colour deep purplish red but in young a more pinkish tone which darkens to brownish in old flies.
f	forked (1-56.7) bristles are shortened, gnarled and bent with ends split or sharply bent. Hairs are similarly affected but this can only be seen under high magnification.
car	carnation (1-62.5) eye colour dark ruby; body shape and proportions rounded.

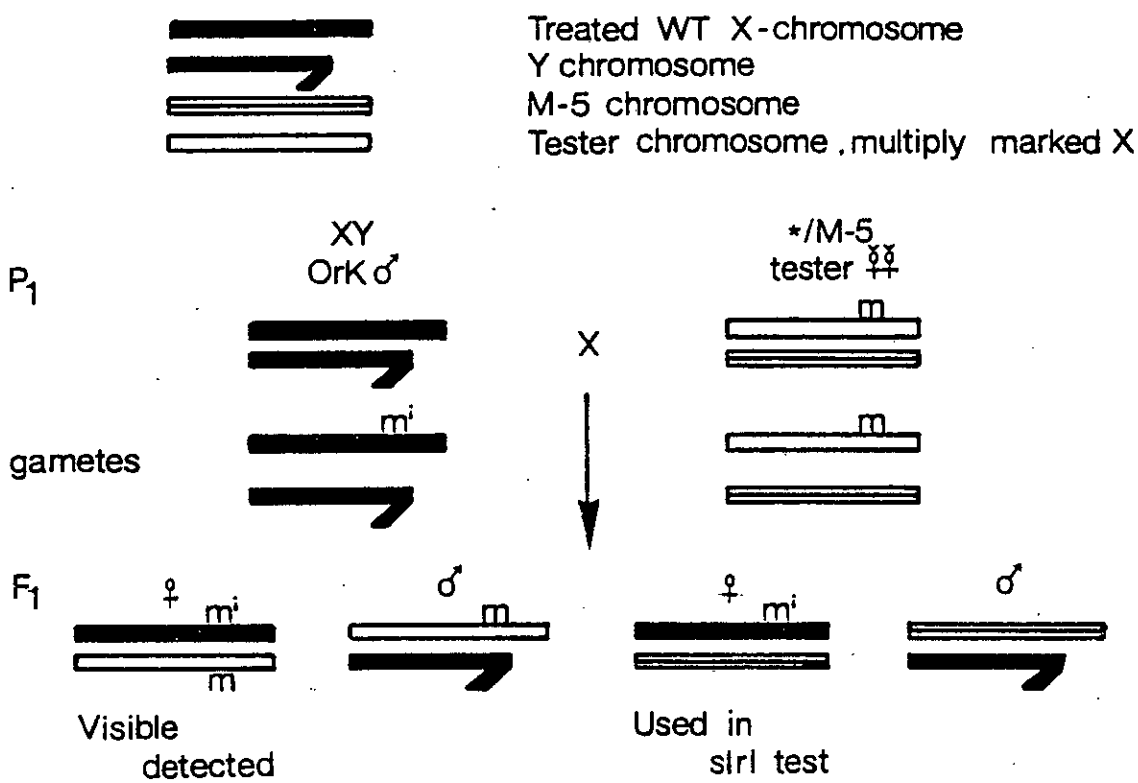
(a more detailed description of markers can be found in Lindsley and Grell, 1968).

The rationale of the test was as follows:

If a mutation was induced (either spontaneously or by chemical treatment) in the ♂ gamete, which manifested itself as a visible at any of the marked loci in the tester ♀, this mutation could be scored in the F_1 generation. Dominant mutations could also be detected, e.g. bar eyes (B). All visible mutants could be tested for transmission of the mutant (i.e. was it a gonadic mutant as well as a somatic mutant?) and for ♂ viability (did the induced mutation also cover a "vital" locus?).

(i) Detection of visible mutations

Expectation: if a visible mutation has been induced in a ♂ gamete, it can be detected in F_1 ♀♀, e.g. if m was induced in a treated ♂ (m^i):

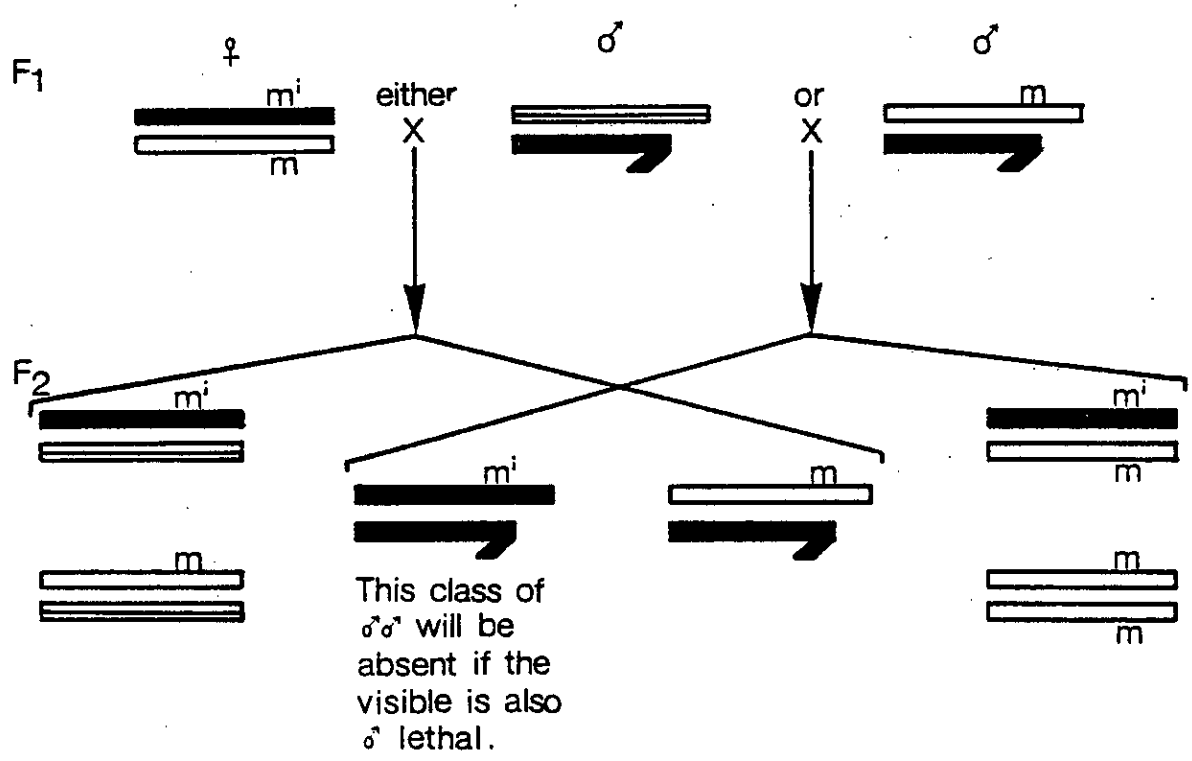


(ii) Test for viability of visible

The expectation of a test for viability of the visible and its transmission depend upon whether the mutation was a complete or a mosaic. There will be crossing-over in the F_1♀♀ but it was thought that this would not matter as we were

looking at single visible mutations. The presence or absence of, for example, miniature and either the ratio of miniature ♂♂ : ♀♀ or the ratio of miniature ♂♂:WT♂♂ in the F_2 determined whether it was also a gonadic mutant and whether it was a viable visible or a ♂ lethal. However, crossing-over did prove to be a problem if the induced mutation affected eye-colour because there was interaction between the eye-colour mutants. This made analysis of results difficult. To avoid this problem, Insc ♂♂ were used in place of Ork ♂♂.

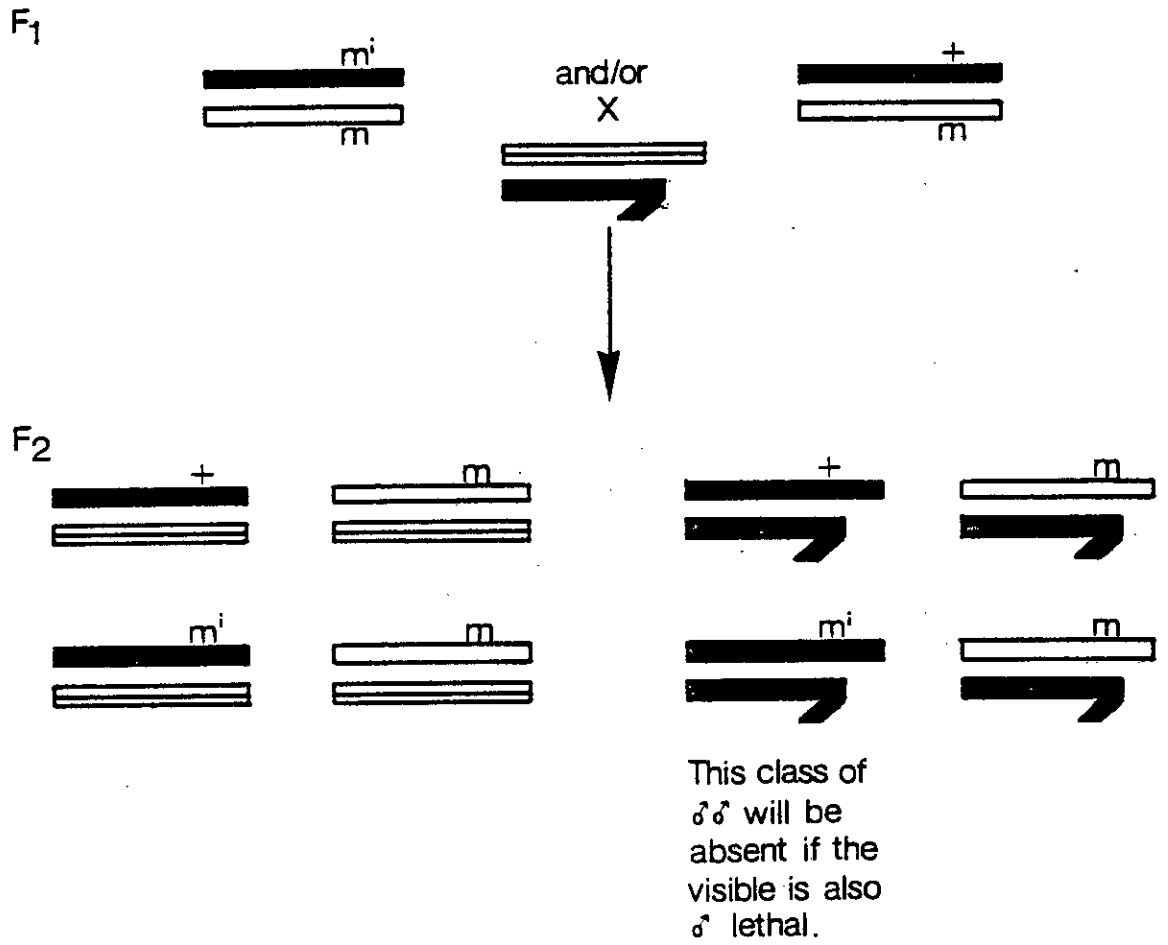
(a) Completes:



- If a ratio of 2♀♀:1♂ was seen then the visible was also a ♂ lethal.
- If a ratio of 1♀:1♂ was seen then the visible was ♂ viable, but all ♂♂ must be m.
- If ½♂♂ were WT for the marker and ½ were mutant, it was deduced that the F_1 ♀ was a somatic mutant but not a gonadic mutant.

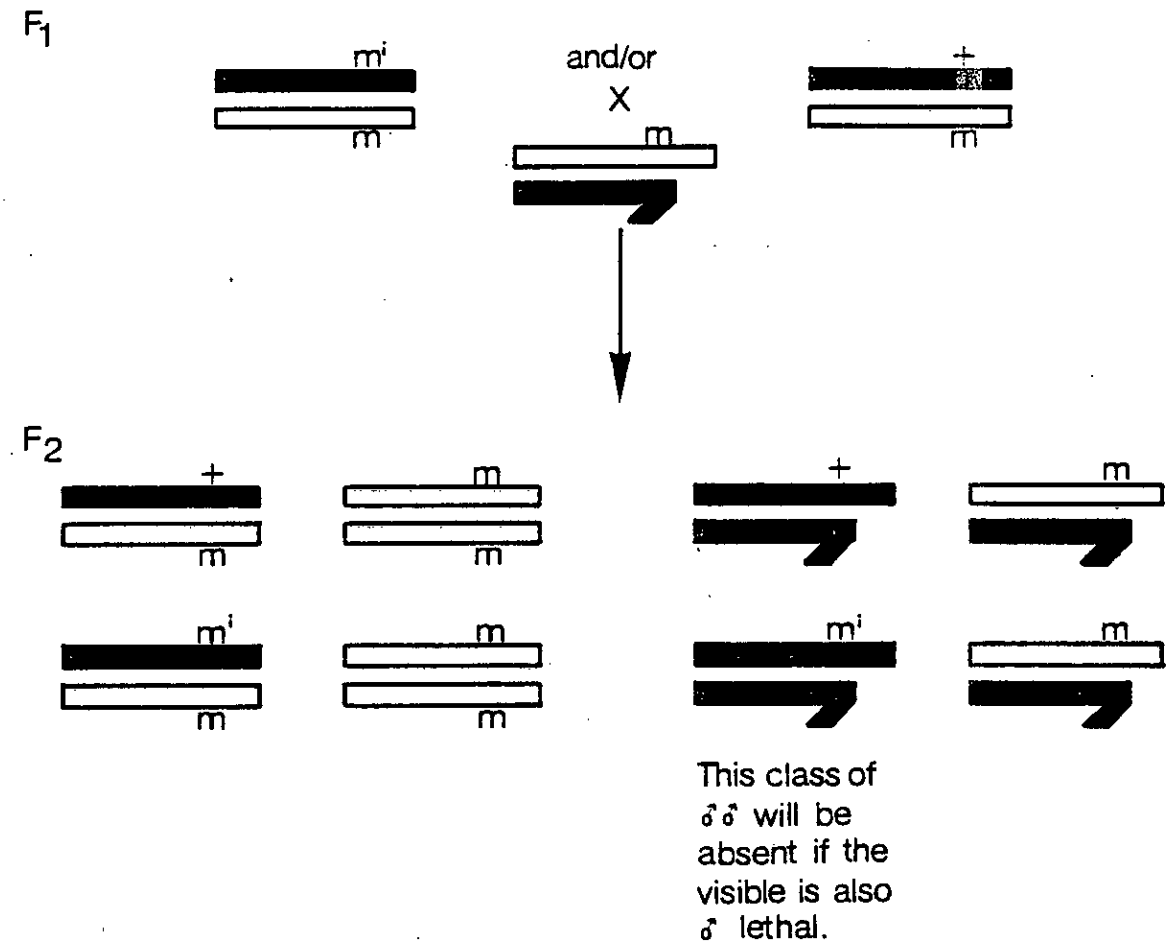
(b) Mosaics (assuming 50:50 mosaicism of the gonads)

(i)



- If a ratio of 3m♂♂:1WT♂ was seen then the visible was a gonadic mutant and ♂ viable.
- If a ratio of 2m♂♂:1WT♂ was seen then the visible was a gonadic mutant and ♂ lethal.
- If a ratio of 1m♂:1WT♂ was seen then the visible was a somatic mutant only.

(ii) Alternatively



- If a ratio of 3m♂♂:1WT♂) then the visible was gonadic
) and ♂ viable
  3m♀♀:1WT♀)

- If a ratio of 2m♂♂:1WT♂) then the visable was gonadic
) and ♂ lethal
  3m♀♀:1WT♀)

- If a ratio of 1m♂ :1WT♂) then the F_1♀ was deduced to be
) a somatic mutant but not a
  1m♀ :1WT♀) gonadic mutant

Both of these 'expectations' applied only to cases where the mosaic was a ½:½ mosaic. Anything less than this would not be readily detected.

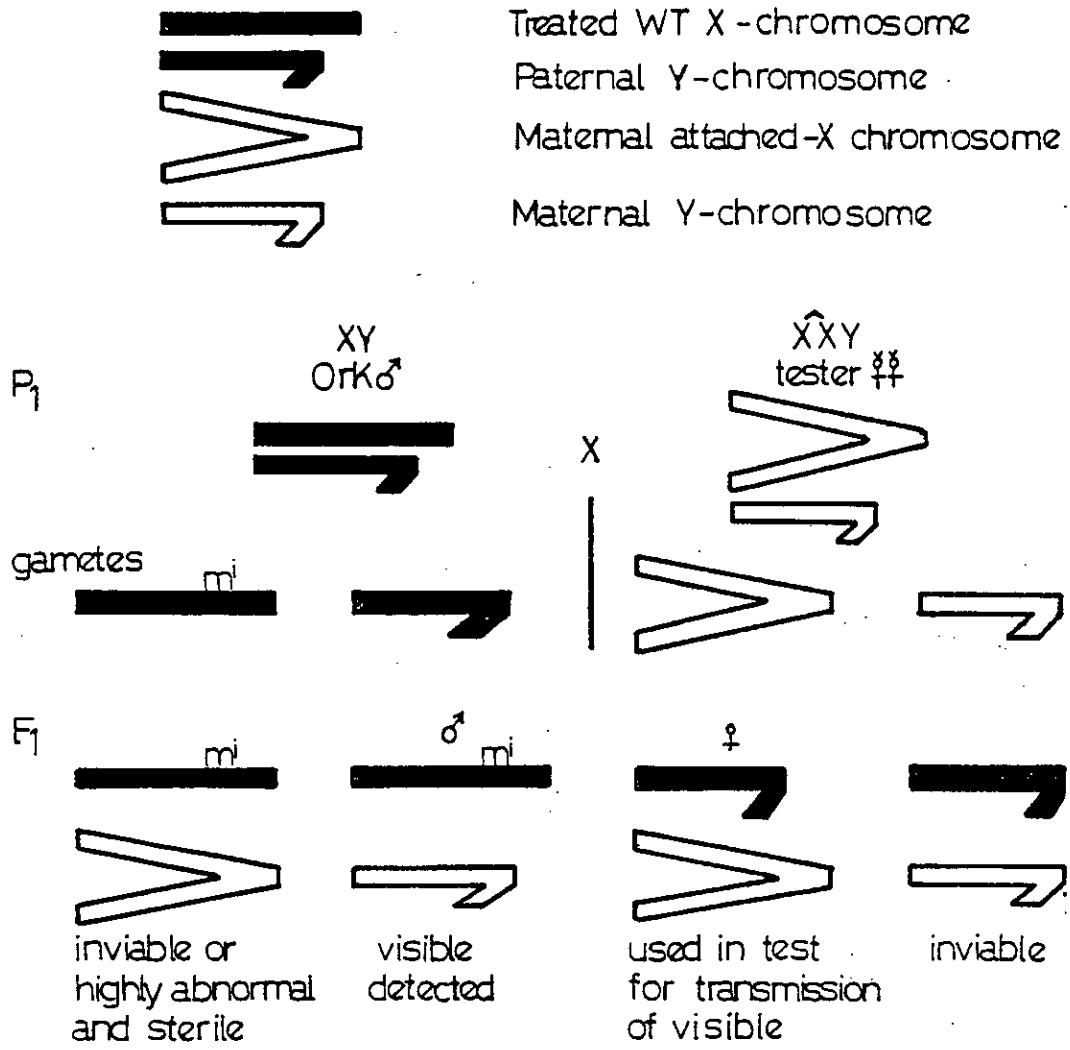
D. Test for small deletions: Attached-X test

Sex-linked visible mutations can be detected in the F_1 of treated ♂♂ that have been mated to ♀♀ with attached-X chromosomes. Such ♀♀ transmit their two X-chromosomes to their daughters and their Y-chromosome to their sons. Thus the sons inherit their X-chromosome from their father and any recessive visible induced in a spermatozoan X-chromosome will be detected.

Both the attached-X tests and the specific locus tests were open to more personal error than the recessive lethal test, or the translocation test. Each mutated chromosome was only represented by a single individual rather than a whole culture, therefore errors due to choice and selection may have played an important role. These disadvantages were countered by comparing frequencies of the same visible mutations detected either in heterozygous ♀♀ (as in the specific locus test) or in hemizygous sons of attached-X ♀♀ (as in the attached-X test).

(i) Detection of visible mutations

Expectation: if a visible mutation was induced in a ♂ gamete, it could be detected in F_1♂♂.



Any mutant phenotypes detected in the F_1♂♂ were further tested for their transmission to the next generation (i.e. were they gonadic as well as somatic?). This was easily done: brother-sister matings were set up, the mutant being crossed to attached-X ♀♀. If the mutation was gonadic,

the cross should yield all mutant sons (complete mutation) or some mutant sons and some WT sons (mosaic mutation).

Interpretation of the Specific Locus and Attached-X Tests

These genetic tests were used to estimate the proportion of small deletions among chemically induced point mutations in Drosophila. The criteria used allowed the detection of deletions that were large enough to include a viable visible mutation as well as a sex-linked lethal. The visible mutations scored needed, on practical grounds, to be specified in order to compare frequencies of visible mutations scored in the two tests and indeed this was a prerequisite for the specific locus test. The efficiency of this test could be increased by increasing the number of visible marker genes. Although time consuming and tedious to perform, because of the rarity of visible mutations, the detection of visibles using the seven listed markers was not difficult, and it was far simpler to perform this type of genetic test than to carry out cytological analysis of salivary gland chromosomes. However, personal bias was inevitable in this type of scoring, the results being dependent on the judgement of the individual as to the type or degree of abnormality which constituted a mutant.

Mutations scored in heterozygous females, as in the specific locus test, may have been due to intragenic changes or to small deletions; if the latter included at least one vital gene then they would be lethal in males.

Mutations scored in hemizygous males, as in the

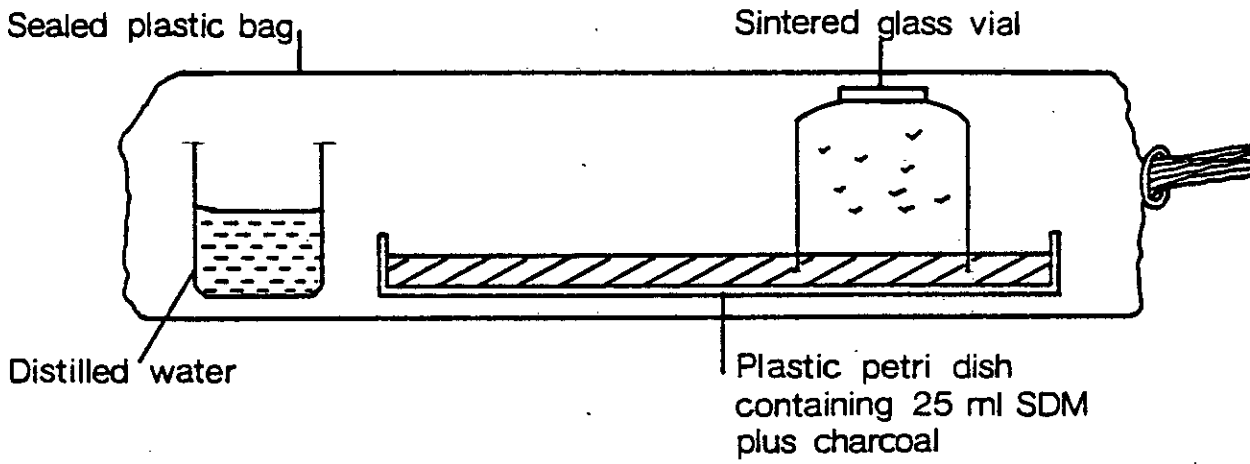
attached-X test, were considered mainly due to intragenic changes. If they were due to small deletions then the deletions must have been too small to cover a vital gene in proximity to the visible one scored. In Drosophila even very small deficiencies are normally lethal in hemizygous or homozygous conditions. Estimates that deletions of 76 bands or more are lethal have been given (Slizynska, 1957).

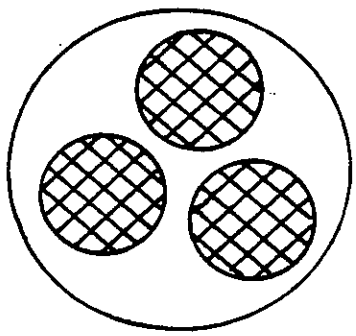
The higher the proportion of deletions among visible mutations, the greater will be the excess of visibles detected in ♀♀ over these detected in ♂♂. A corresponding proportion of visibles detected in ♀♀ should be lethal in ♂♂; this could be established by progeny tests on the mutant ♀♀. The ratio of slrl to ♂-viable visibles was expected to increase with the proportion of deletions among visible mutations.

Account has to be taken of mosaicism when interpreting the results.

It should be noted that most mutant flies are more or less inferior to normal ones in developmental speed, competitive ability during larval life, and general vigour. The frequency with which they appear is considerably influenced by variations in culture conditions and population density, and these variations can never be fully controlled. Thus cultures were scored for visibles from the first day of hatching until the culture had been exhausted of F_1 progeny.

Fig.2.3. Hatchability test – A test for dominant lethality





(Escaped) Drosophila's eye view of petri dish.

Egg-laying is encouraged and counting of eggs made easier if the surface of the agar is first scored, to give a grid, as above.

E. Hatchability test - A test for dominant lethals

The tests for dominant lethality were carried out in the following way: the egg-laying "chambers" consisted of a sintered glass vial and a plastic petri dish containing 25ml SDM plus charcoal as shown in Figure 2.3.

The test was set up by placing a specified number of ♀♀, either with or without ♂♂ depending on whether the test was conducted pre- or post-storage. The ♀♀ were allowed to lay eggs for 12-18 hours at 25°C, the time being dependent on their fecundity. After this time the vial was moved on to the next egg-laying area (3 per plate) without anaesthetising the flies. Eggs hatch ∿22 hours after they have been laid therefore to obtain an accurate"pre-hatch" egg count it was decided that counting should take place no later than 18 hours after the test had been initiated.

After the first egg-count, the plates were returned to the 25°C incubator and a second egg-count, to determine the number of unhatched eggs, was carried out 24 hours later and checked again after a further 24 hour interval. This permitted calculation of the frequency of egg-hatching. Comparing a "treated-chromosome" batch with a control batch gave a measure of relative dominant lethality (RDL).

$$RDL = 100 - \left(\frac{h^t}{h^c} \times 100\right)$$

where h^c = % hatchability for the control group

h^t = % hatchability for the treated group

The significance of any difference between any two

"treatments" could then be calculated using a modified χ^2 test. The tests for dominant lethals were quick and easy to perform but their meaning may be ambiguous. A dominant lethal has been defined as having an ability to cause the death of an organism when it is present in a single dose. Most dominant lethals are thought to be due to chromosome breakage events of various types, i.e. inviable rearrangements or unrestituted chromosome breaks (see Chapter 1, page 3). The majority of Drosophila workers have used hatching from the egg as opposed to eclosion from the pupa as the criteria for deciding whether or not a dominant lethal was present. However, eggs may not hatch for many reasons:

(i) non-genetic damage - the eggs may not have been fertilised. One of the most common reasons for non-hatchability after irradiation of immature germ cells is non-fertilization (possibly due to blocking of spermatogenesis). Chemicals are more likely than irradiation to have direct effects on the gametes or to interfere with mating performance of the flies or malfunction of sperm. These problems could be partly overcome by analysing unhatched eggs cytologically.

(ii) There may also be failure of syngamy or damage to the centromere which the sperm introduces into the zygote. This could mean that development would never get under way. None of this is dominant lethality, as it has been defined. Again cytological examination would be the only way to establish whether development had

proceeded at least to the first cleavage division, but it is extremely laborious. It cannot establish the genetical nature of the dominant lethal - this can only be done indirectly.

The usefulness of such a test has been questioned because:-

(i) there is often considerable variation between control experiments - the rates of unhatched eggs obtained from unhatched flies usually varying between 3% and 10%.

(ii) the test is not considered to be sensitive - this is its major drawback and makes it of only limited practical value in the detection of genetic damage caused by chemicals in Drosophila.

Hence caution must be exercised when interpreting the results from such a test. Any evidence for significantly increased egg hatchability in a treated vs control series should ideally be backed up with other tests such as those detecting either visible or lethal mutations, chromosomal rearrangements or chromosomal loss.

2.4 USE OF BROOD ANALYSIS AND STORAGE EXPERIMENTS

A. Brood Analysis

The "brooding" technique consists of translating the spatial pattern of spermatogenesis into a temporal pattern of successive broods. The following table shows which sperm stages can be expected to be present in the ejaculate of males from 1-10 days after treatment:

TABLE 2.3. Summary of Results: TIMING OF SPERMATOGENESIS IN DROSOPHILA MELANOGASTER USING TRITIATED THYMIDINE TO LABEL THE GERM CELLS (Chandley and Bateman, 1962).

Day after injection*		1	2	3	4	5	6	7	8	9	10	11
Stage	spermatogonia mitosis	Spermatocytes (mature)				meiosis	Spermatids		SPERM			
in		TESTIS							Seminal Vesicle		EJACULATE	
Days after irradiation**	10	9	8	7	6	5	4	3	2	1	0	

* Day after injection of (^{3}H) on which the stage indicated is heavily labelled.

** Above results interpreted as the number of days after irradiation or other treatment of any stage to the appearance of sperm, matured from that stage, in the ejaculate.

On each successive day following injection, Chandley and Bateman dissected testes of sample males. Serial sections were autoradiographed in order to determine which germ cells were labelled, and their position within the testis was established. From the ninth day onwards, smears were made of sperm from the ventral tube of inseminated ♀♀. These were examined for the first appearance of labelled sperm in the ejaculate. Most ♂♂ were presented with two fresh ♀♀ every day. In ♂♂ kept unmated until the day of sampling, the rate of maturation of spermatocytes and spermatids was the same as in continuously mated ♂♂, but labelled sperm was not detected in the ejaculate until Day 11, and then as a much smaller fraction.

Table 2.3 was used as a guide in the brooding experiments in order to obtain broods in which the sperm sampled in successive broods had been treated at progressively earlier stages in their development. The time of appearance of the different stages in successive broods is not the same for all stocks and depends on the ability of the ♂♂ to mate, the speed of sperm release and on the concentration of chemical applied. Chemicals may act preferentially on different sperm stages

The germ cells are determined after 11 cell divisions (8 cleavage divisions plus 3 pole cell divisions), 18-26 cells giving rise to the germ line (in Lindsley and Tokuyasu, 1980).

Approximately 1½ hours after fertilisation there have been 8 nuclear divisions, thus DNA replication and cell

division proceed rapidly.

It is worth noting at this point that the gonads are unlike most larval organs which undergo histolysis and destruction or modification as the adult organs are formed. The gonads continue their growth and differentiate without interruption. The testes are fully formed and contain mature spermatozoa by the time of eclosion. Therefore any effects observed should not have been influenced by developmental changes within the organism.

B. Storage Technique

Chemical mutagens seem to produce "potential" lesions which require different periods of time to develop into "actual" lesions. This concept is discussed fully in the introduction (Chapter 1) and the discussion (Chapter 6). However, this means that storage experiments form an essential part of experimental protocol when dealing with chemical mutagens. Various methods have been used to facilitate storage - these are fully discussed in Chapter 4. It should be pointed out that regardless of the storage technique used, all sperm are "stored" until they are used to fertilise an egg, just prior to its being laid.

The storage technique can be used to study the effect of sperm storage on the types of genetic damage which have been described in section 2.3, i.e. slrl, translocations, small deletions, dominant lethals, recessive visibles.

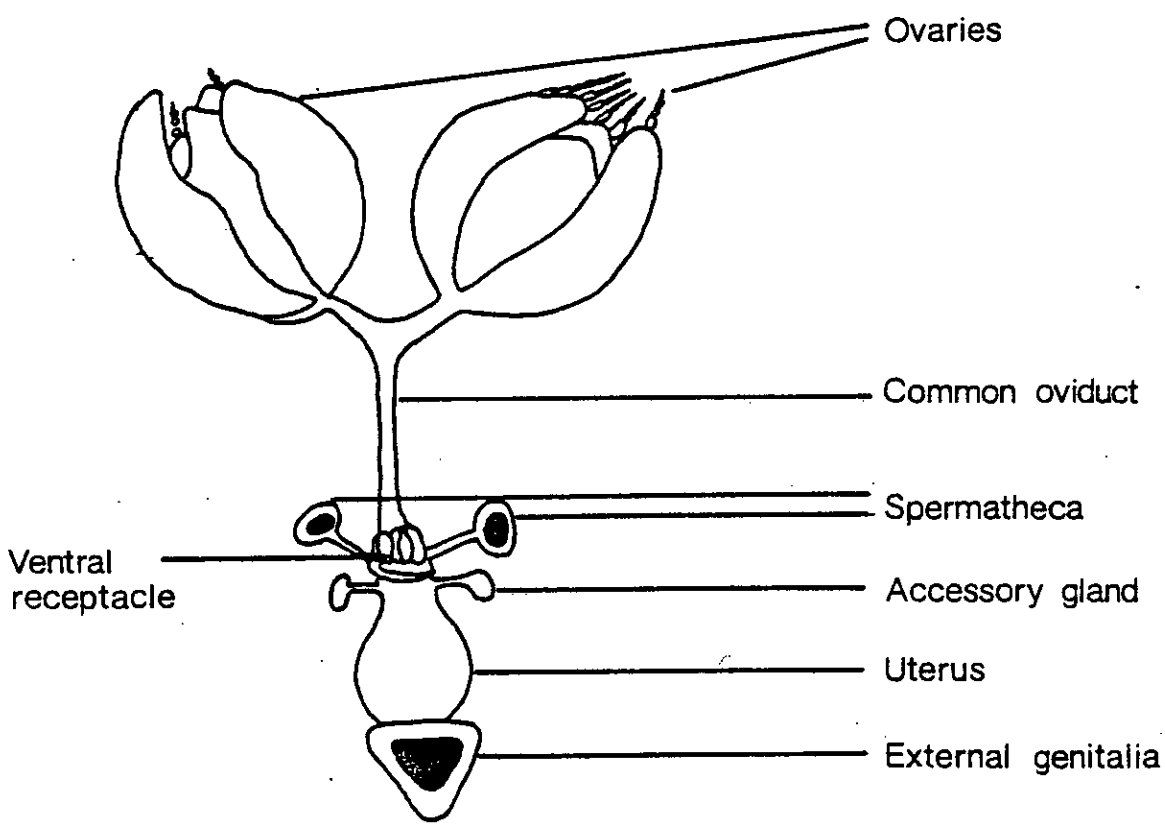
2.5 DISSECTION OF FEMALES TO LOOK FOR MOTILE SPERM

Drosophila ♀♀ have two organs in which they store sperm after insemination by a ♂.

(i) the spermathecae - a pair of mushroom shaped organs.

(ii) the ventral receptacle - a single, coiled, tube-like structure.

Fig.2.4. Female reproductive system in Drosophila



These organs can usually maintain large quantities of sperm for sufficiently long periods to fertilise many eggs. In the dissection, both storage organs were located in the posterior end of the abdomen near the external genitalia. The spermothecae were detected as a pair of mushroom-shaped organs attached to the upper end of the uterus and appear under the dissection microscope (x20 magnification) as two

tiny, dark, dot-sized structures. The ventral receptacle was seen as an opaque, single tube-like structure, usually coiled, which also inserted into the upper end of the uterus.

The dissection was easiest done by placing the etherised ♀ on its back on a microscope slide. The fly was viewed under the dissecting microscope. A bent dissecting needle was placed firmly on the thorax of the fly. With a second needle, the external genitalia was pulled out of the abdomen. Attached to the hairy, pigmented genitalia was seen the two, tiny, dot-sized spermathecae together with a number of translucent structures. The relative sizes of the structures can be seen in Figure 2.4. The structures anterior to the spermathecae (ovaries, intestines) were dissected away and moved together with the remainder of the fly, to one edge of the slide. A drop of Shen's* solution was added, the genitalia and spermothecae were centred in the drop of dissecting fluid and gently covered with a coverslip and examined immediately under low power in a compound microscope (100x with green or blue filter). The presence of the coiled ventral receptacle was noted. Once this had been detected, high power magnification (400x

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*Shen's solution: 0.9g NaCl
0.042g KCl
0.025g $CaCl_2$
100ml distilled H_2O

This solution should be freshly made. Chloroform may be added as an anti-bacterial agent if so desired.

or 1000x - no oil necessary) was used to look for masses or bundles of long, thin dark strands in the coil of the ventral receptacle. These strands were sperm and either all or some of them were actively vibrating. At times they could also be noted in the brown mushroom-shaped spermathecae, although absent in the ventral receptacle.

2.6 CHEMICALS TESTED

EMS	Ethyl methanesulphonate (Methanesulphonic acid ethyl ester)	Sigma
DES	Diethyl sulphate $(C_2H_5)_2\ SO_4$	B.D.H.

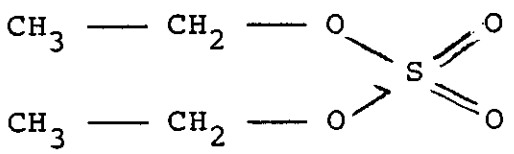
CHAPTER 3. DIETHYL SULPHATE

DIETHYL SULPHATE

3.1 INTRODUCTION

Diethyl sulphate (DES) also known as sulphuric acid, diethyl ester, has the following chemical structure:

$C_4H_{10}O_4S$



DES is a monofunctional alkylating agent which alkylates via an intermediate Sn_1/Sn_2 mechanism (Hoffmann,1980). It has been shown to be highly mutagenic in Drosophila in terms of inducing slrl (Rapoport, 1947; Pelecanos and Alderson, 1964). Pelecanos (1966) did not find any evidence to show that DES induced reciprocal translocations between the second and third chromosomes of Drosophila after either male or female larvae, or adult males, had been fed 0.5% DES. This dose of DES had been shown to induce slrl at frequencies of 11.6% when fed to male larvae, 15.77% when fed to female larvae, and 27.10% when fed to adult males. However, with prolonged storage of DES-treated sperm in the storage organs of the females, Muñoz and Barnett (1977) showed that translocations could be induced provided the initial frequency of slrl in the treated sperm was very high. When mature sperm were treated with a dose that yielded 27% slrl (0.5% DES fed to adult males for 3.30 hours), translocations were first detected after two weeks of storage. When a higher dose was made (0.75% DES fed to adult males for 2.30 hours - no data on slrl was

TABLE 3.1. Frequencies of II-III Translocations Induced by DES in Mature Sperm, Treated in 7-Day Old Males and Stored in Females (Muñoz & Barnett, 1977)

TREATMENTS / Sub-Culture	Days after Treatment	CONTROL: Chromosomes Tested	CONTROL: No.of Trans-locations	0.5% DES (3.30 hours): No.Trans. / No.chromosomes tested	0.5% DES (3.30 hours): %	0.75% DES (2.30 hours): No.Trans. / No.chromosomes tested	0.75% DES (2.30 hours): %
I	1-5	513	-	NT		$\frac{-}{628}$	0.00
II	6-8	155	-	NT		$\frac{1}{334}$	0.30
III	9-11	325	-	$\frac{-}{796}$	0.00	$\frac{10}{1160}$	0.86
IV	12-15	317	-	NT		$\frac{13}{461}$	2.82
V	16-18	301	-	$\frac{22}{969}$	2.27	$\frac{24}{674}$	3.56
VI	19-22	1008	-	$\frac{24}{807}$	2.97	$\frac{25}{594}$	4.21
VII	23-28	380	-	$\frac{7}{120}$	5.83	$\frac{14}{178}$	7.87

NT - Not tested.

included) one translocation in 334 tested sperm was detected as early as one week after storage, a frequency of 0.30%. As the storage time increased, the frequency of translocations also increased. Table 3.1 summarises these results.

In these experiments, Muñoz and Barnett did not use a special storage medium but simply kept the females on a standard Drosophila medium (SDM) and transferred the females to fresh bottles on days 5, 8, 11, 15, 18, 22 and 29. They reported that by day 29, the sperm supply had been exhausted therefore the storage time could not be prolonged further.

In a brood analysis of treated adult testes (Alderson and Pelecanos, 1964) it was shown that the post-meiotic germ cells, in particular mature spermatozoa, were the most sensitive to the mutagenic activity of DES as measured by sl-rl frequency. This mutational response showed a progressive decline as earlier germ cell stages were sampled. The adult males were newly emerged when treated. When larval testes were treated, it was shown that both primary spermatocytes and early spermatogonia were the most responsive to DES administered by larval feeding; however, the response was much lower than in the adult feeding method. Pelecanos (1966) also reported that no translocations were recovered from either male or female treated larvae or male adults in samples of 838, 902 and 775 gametes respectively. No further data was given in this paper but Shukla and Auerbach (1979) in a discussion on

the action of DES suggested that this showed that no translocations were detected in larval spermatogonia. With the exception of the first 24 hours of larval life, when only the highly sensitive spermatogonial stage (0-12h) and spermatogonia (12-24h) are present, spermatogonial and primary spermatocyte stages are present in the larval testis. These stages are at a pre-meiotic stage of development (Alderson and Pelecanos, 1964). By the time that larvae thus treated have hatched as adults, both these pre-meiotic stages will be represented in the mature spermatozoa. Therefore, the results of Pelecanos (1966) could be interpreted in two ways: either these cell stages (pre-meiotic) were not susceptible to the treatment; or alternatively, since a high frequency of lethals was induced, it seems possible that the reason for the absence of translocations was that the period between treatment and sperm utilisation, presumably 10-12 days, was too short for the primary lesions to develop into pre-breaks.

MMC produces a very unusual brood pattern. Mukherjee (1965) and Schewe et al. (1971) found that the frequency of sl rl decreased as earlier germ cell stages were sampled, while that of translocations, which was nil in mature sperm, increased. Shukla and Auerbach (1979) designed an experiment to examine this unusual brood pattern by looking at the effect of a very simple variable: the passage of time. This was done by determining whether the high frequency of translocations in spermatogonia was due simply to

TABLE 3.2. The frequencies of sex-linked recessive lethals (slrl) and II-III translocations (Tr) in mature stored and unstored spermatozoa and in late and early spermatogonia. (Shukla and Auerbach, 1979).

EXPERIMENT	METHOD	DAY	TREATED STAGE	slrl		Tr	
				n	%	n	%
I a		1	Spermatozoa	10/200	5.0	0/600	0
II a			"	12/200	6.0	0/600	0
I d	Broods	12-15	Spermatogonia	7/200	3.5	1/150	0.7
II d	"	"	"	10/400	2.5	5/600	0.8
I b	Storage	12	Spermatozoa			2/600	0.3
II b	"	"	"			1/600	0.2
III e	Broods	25-27	Spermatogonia	4/400	2.0	7/560	1.2
I c	Storage	25	Spermatozoa	12/200	6.0	2/175	1.1
II c	"	"	"	10/175	5.7	5/375	1.2

the long interval between treatment and the sampling of germ cells that had been treated as spermatogonia. The results of this experiment are summarised in Table 3.2.

The results of Shukla and Auerbach (1979), in agreement with those of Mukherjee (1965) and Schewe et al.(1971), confirmed that "the incomplete brood patterns for slrl and translocations show opposite trends". Additionally, they showed that stored sperm (treated as mature sperm but stored in the females) sampled at the same time after treatment as unstored sperm (which were treated at an earlier stage of development) yielded nearly identical frequencies of translocations. It was concluded that passage of time alone was responsible for the manifestation of translocations and not differences in sensitivity of germ cell stages to induction of translocations.

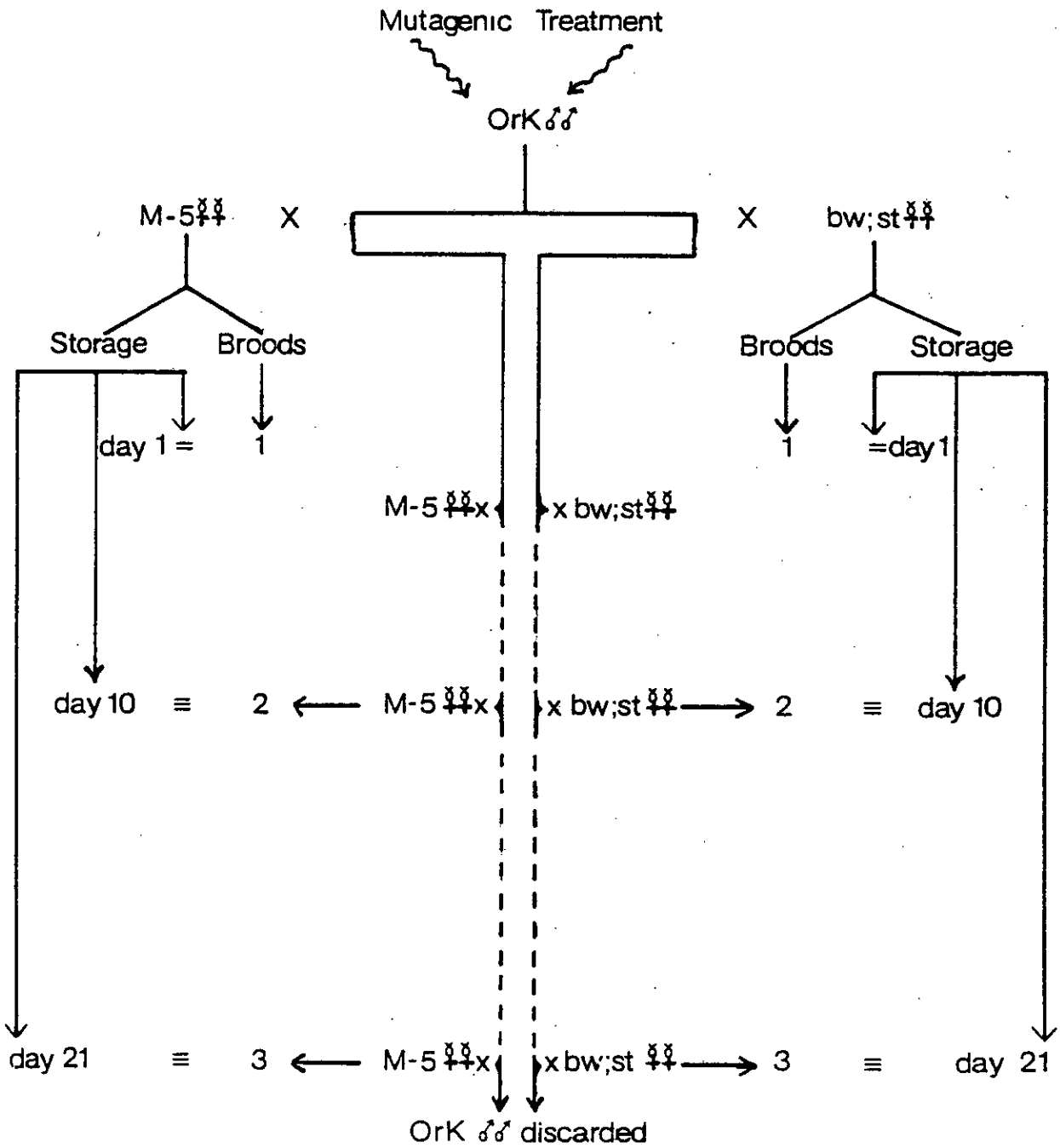
These results are of interest in relation to the results so far obtained by other workers for DES-induced translocations: translocations have not been detected without storage. A conventional brood pattern analysis should show whether or not DES also appears to produce translocations exclusively in germ cells treated at the spermatogonial stage.

3.2 AIM OF EXPERIMENTS and EXPERIMENTAL DESIGN

The aims of the initial experiments were:

(a) to repeat the experiment of Muñoz and Barnett (1977) by storing DES-treated spermatozoa for up to 30 days.

Fig. 3.1. Experimental design for DES matings: broods and storage



♂♂ mated to fresh ♀♀ every few days days in order to secure regular utilisation of germ cells

(b) to extend the observations of Pelecanos (1966) to determine if the failure to observe translocations in brooding experiments was simply a question of the time of observation being too short.

(c) to compare the results of conventional brood pattern analysis with those for MMC (Shukla and Auerbach, 1979).

This was to be attempted by carrying out simultaneous "brood" and "storage" experiments in the following manner: Treated males were mated to the appropriate virgin females for 3 days to give a D1-3 sample of sperm. The inseminated females were then transferred to storage media. The males were transferred to fresh SDM with a fresh supply of ♀♀. This was repeated every few days in order to keep the males sexually active and to secure regular utilisation of germ cells. Figure 3.1 visualises the experimental design.

When a batch of females was removed from storage to give, for example, a sample of sperm stored for 10 days, a brood would also be taken by mating the Ork ♂♂ to ♀♀ on day 10 and allowing those females to lay eggs for 3 days (see Fig. 3.1).

It was hoped to verify the results obtained by Muñoz and Barnett (1977) and to extend the work of Pelecanos (1966) who only sampled larval broods equivalent to 10-12 days of storage. Significant frequencies of translocations have only been detected in DES-treated sperm stored for twelve or more days. Larval development takes place over

a period of 4 days followed by a pupal period of 4.2 days (shown in Table 2.3). Therefore, it was not surprising that translocations were not detected in a larval brood sampled after an equivalent storage time of 10-12 days if the effect was due to time alone rather than to stage specificity.

3.3 DES FEEDING EXPERIMENTS: Toxicity Tests

Preliminary experiments were conducted to determine the optimal treatment conditions. Using the technique of Lewis and Bacher (1968), 3-day-old Ork ♂♂ were fed with a solution of 0.5% DES(w/v in 5% sucrose solution) for 4 hours, followed by a 24 hour recovery period. Matings were then set up to detect induced slrl and Tr. A storage experiment was set up simultaneously using Shukla's low-protein food recipe. All P_1 and F_1 matings were carried out in vials: P_1 matings consisted of 1 treated male mated to 2 tester virgin females, F_1 matings of 1 male mated to 1 female. For the storage series, vials of the low-protein medium were covered with gauze secured with rubber bands (the gauze maximises aeration within the vials to help prevent the food from becoming too sticky). The "stored" fertilised females were shaken into fresh food vials every three days. This method of storage resulted in the death of the inseminated females before the required period of storage time had elapsed. It was therefore not possible to carry out the required genetical tests on the stored sperm sample.

The results obtained in this preliminary test are shown in Table 3.3.

The slrl frequency obtained by treating males with a 20ml solution of 0.5% DES did not result in induction of a high frequency of lethals (i.e. 25-30%), it induced slrl at a frequency only 1/10 of that desired. This was the concentration used by Muñoz and Barnett (1977) although they had treated flies by saturating a piece of filter paper attached to the walls of empty 180ml bottles.

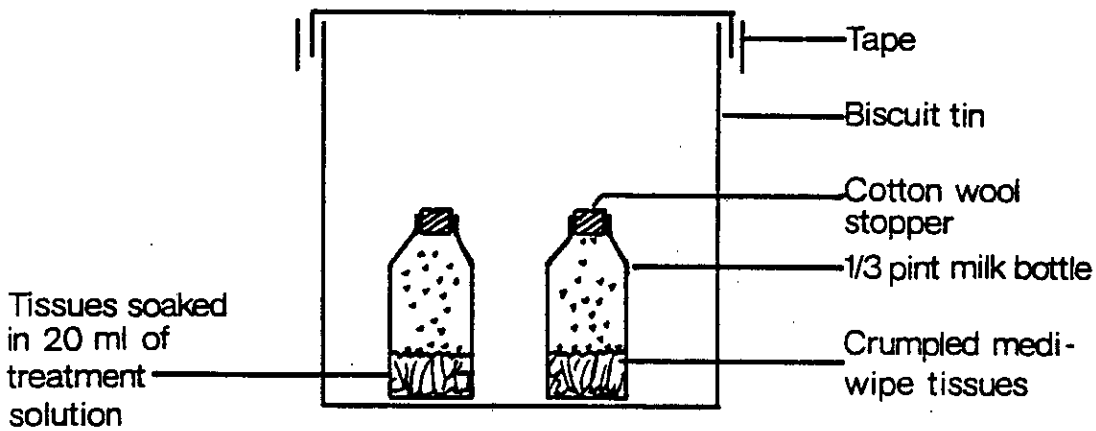


Fig. 3.2. TREATMENT VESSEL 'A'

Consecutive treatments using the above method yielded highly variable levels of survival; using an identical treatment procedure and dose of DES, it was found that

TABLE 3.3. Run FI Preliminary Feeding Experiment: 0.5% DES fed to 3 day old Ork♂♂ for 4 hours.

Sample	Days on which sample was taken	Sex-linked Recessive lethals		Translocations	
		n	%	n	%
Day 1= Brood 1	1-3	5/170	2.94	0/177	0
Brood 2	25-28	0/309	0	0/151	0
Day 25	25-28	-*	-*	-*	-*

* Very few P_1 females alive, no F_1 obtained.
FI First experimental run in the feeding experiments

TABLE 3.4a. Induction of slrl by 0.03% DES

FLASK	FREQUENCY OF SLRL		SURVIVAL
	n	%	(%)
A	4/162	2.47	100
B	6/163	3.68	100
A + B	10/325	3.08	100

TABLE 3.4b. Uptake of labelled 5% sucrose solution.

Treatment solution	Specific Activity of test solution (cpm/ml) (b)	1ml KOH (cpm)	100 flies (cpm)	Total cpm (KOH + flies) (a)	Solution taken up per fly (µl) (=a/b/100)
5% sucrose	724620	18508.75	32587.50	51096.25	0.71
+ 0.03% DES	724620	16245.00	42662.50	58907.50	0.80

survival could vary between 0% and 100% after 4 hours treatment. The survival levels tended to decrease with each successive treatment. This raised questions about the treatment procedure: was the mutagenic effect of DES due to the flies feeding on DES or to inhalation of DES vapour? (DES is highly volatile in solution); were the treatment bottles adequately sealed? (DES could evaporate out through the stopper into the tin); did the amount of time involved in handling a volatile agent such as DES, while making up a treatment solution and then decanting it into the treatment bottles, introduce an extra variable in the treatment procedure?

The following experiments were carried out to establish a suitable treatment procedure giving repeatable results, as determined by slrl frequency:

(i) ^{14}C glucose feeding, using four concentrations of DES, to establish if the flies were feeding on the DES solutions;

(ii) Vapour tests, using four concentrations of DES, to establish if this method of treatment was a viable alternative to feeding experiments.

(i) ^{14}C Glucose Feeding Test: Uptake of treatment solutions.

MacDonald and Luker (1980) have shown that the uptake of a radioactive labelled sucrose solution was considerably reduced by the addition of 12mM NaF. It was thus decided

to carry out a similar ^{14}C glucose-feeding experiment to determine whether or not flies were eating test solutions containing different concentrations of DES; and to determine whether or not the variable frequencies of slrl so far obtained in feeding tests was due to variable levels of feeding on the test solution.

The method was essentially that detailed in MacDonald and Luker (1980):

10 1-l flasks were set up, each containing 100 1-3 day-old Ork males. 4 different test solutions were administered:

0.5% DES)
0.3% ")
0.1% ") - in a 5% ^{14}C sucrose solution.
0.03% ")

plus a control which was an unlabelled 5% sucrose solution. These treatments were duplicated. 2ml of a test solution was placed at the bottom of a flask in a 2.5cm plastic dish containing a paper wick. The treatment vessel (Fig. 3.3) was stoppered and sealed with parafilm.

[U-^{14}C] sucrose (high specific activity, Radiochemical Centre, Amersham) was added to each test solution so as to give about 10^6 counts/min/ml (c.p. m/ml). The specific activity of each test solution was measured. The uptake of the test solution was determined by measuring the amounts of ^{14}C in the flies after treatment and the amount of ^{14}C expired as CO_2 during treatment. The total number of

counts per minute taken up by the flies was divided by the specific activity of the test solution to give the volume consumed.

The amount of ^{14}C in the flies was determined by homogenising them in water then solubilizing in 2 vol. of NCS tissue solubilizer for 5h. The solubilised flies were added to EM1 Micellar Scintillator ME 260 and counted in a scintillation counter. Expired CO_2 was absorbed in 1ml of 5M KOH contained in a vial suspended in the treatment flask. The ^{14}C in the KOH was counted in the same way as in the solubilised flies. Background counts were determined from the control. These control counts were subtracted from the values obtained in the feeding experiments.

Immediately after treatment had ceased, the males were divided and either mass-mated to M-5 females in a ratio of 1♂:2M-5♀♀, or used to determine the average intake of test solution per fly.

Results were obtained only for the lowest dose of DES and the control. Even after only 2-3 hours of treatment, flies at the higher concentrations of DES were immobile (0.1% DES), dead on the floor of the flask (0.3% DES) or dead in the vial (0.5% DES). There was 100% survival after 24 treatment for both 0.03% DES and the control solutions. Table 3.4a shows the results of the slrl tests.

It had been shown by MacDonald and Luker (1980) that the presence of small amounts of [U-^{14}C] did not lead to a detectable increase in recessive lethals in tests of 197

and 192 chromosomes. Therefore, it was concluded that the detection of slrl after feeding males with a 0.03% solution of DES plus [U-^{14}C] glucose, was due to the presence of the DES alone.

In Table 3.4b, a comparison of the uptake of radioactive labelled sucrose in the presence and absence of 0.03% shows that addition of DES did not reduce the uptake of the test solution. The results from replicate experiments were pooled because they were in close agreement. Thus, at least at this low dose of DES, the variable survival levels of treated males and induction of slrl which were observed

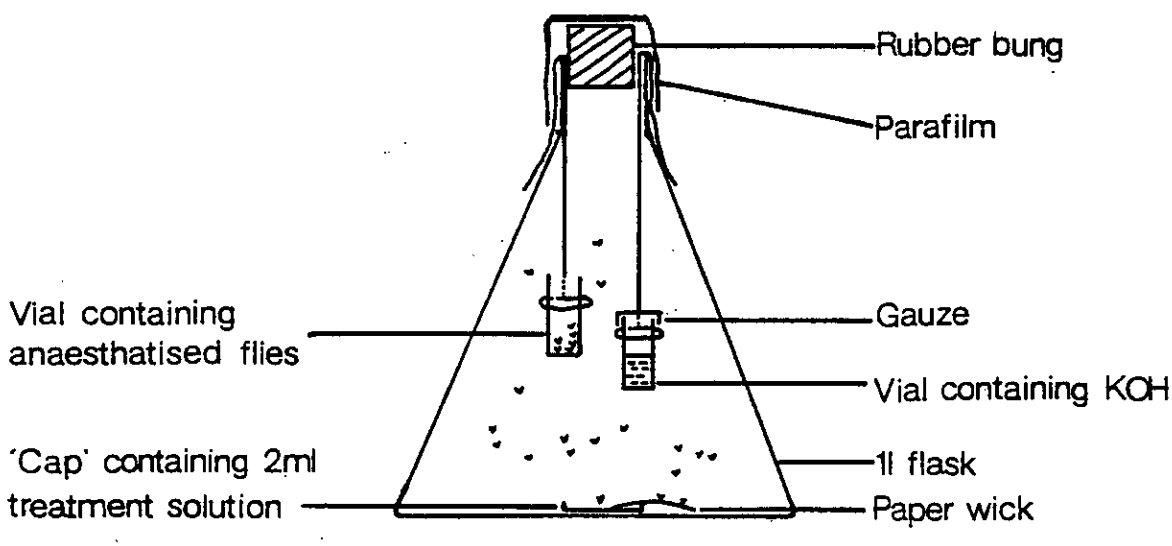


Fig. 3.3. TREATMENT VESSEL 'B'

Used for ^{14}C-glucose experiment

did not appear to be due to non-feeding. However, it could be inferred that using treatment vessel B, in which there was complete sealing of the vessel, was more satisfactory than treatment vessel A because a very low dose administered in 'B' gave the same frequency of slrl as a high dose administered in 'A' (compare Table 3.3 with Table 3.4a). Also, the greater toxicity of DES in vessel 'B' suggested that the vapour pressure, which would be higher than in treatment vessel 'A', may determine the level of toxicity and of mutagenicity of this compound.

(ii) Vapour Tests - Toxicity tests of vapour phase using four concentrations of DES

DES is a volatile liquid (Hoffmann,1980). This may have contributed to the variable survival levels obtained in the initial feeding experiments when the time taken to administer the treatment once DES was exposed to the air was dependent upon the dexterity of the operator. Although this time was only minutes, because DES is volatile, this would introduce a variable into the treatment procedure.

As stated earlier, the treatment vessels themselves also came under scrutiny. The treatment vessels were modified for use in these experiments, see Figure 3.4.

An added advantage was that the flies could be observed during treatment. Whereas in the feeding experiments, the flies come into direct contact with the treatment solution, this method permitted vapour contact only; however, a disadvantage was that the flies had to be

anaesthetised while they were placed in the chamber. This may result in lower survival rates if the anaesthetic (ether) and mutagen interact.

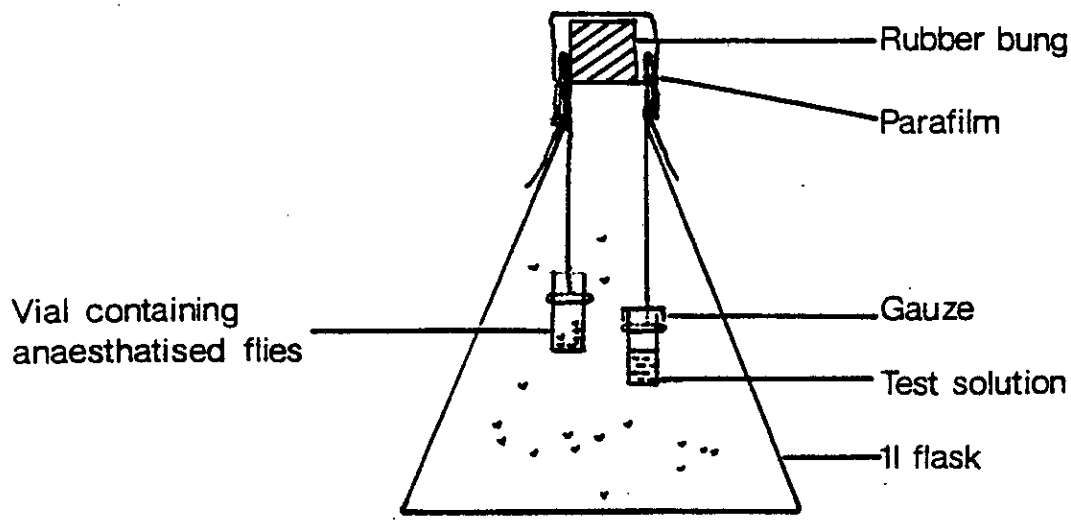


Fig. 3.4. TREATMENT VESSEL 'C'

There are reports in the literature of the use of Drosophila for vapour testing (Lee, 1976). Abraham, Goswami and Kesavan (1979) have shown, by the use of a more sophisticated system, that DES vapour is mutagenic. When 5ml DES was vapourised then pumped into a 250ml treatment bottle at a flow rate of 1l/min, the lethal exposure time at 30°C was less than 20 minutes. They determined the frequency of slrl obtained after exposure to the vapour for 5, 10 and 15 minutes; these results are shown in Table 3.5.

TABLE 3.5. Induction by DES-vapour of sex-linked recessive lethals in mature spermatozoa of two day-old adult Drosophila males. (Abraham et al., 1979).

DURATION OF EXPOSURE (Minutes)	CHROMOSOMES SCORED (n)	LETHALS (n)	LETHALS (%)
5	709	11	1.55
10	327	65	19.88
15	748	200	26.74
Control	1290	1	0.08

These results showed that the received dose of DES-vapour, as measured by slrl frequency, increased with time. Brood analysis gave a brood pattern consistent with the results obtained from feeding experiments (e.g. Alderson and Pelecanos, 1964). These results are shown in Table 3.6(a).

These results showed that the post-meiotic germ cells, particularly mature spermatozoa, were the most sensitive to mutagenic induction by DES-vapour as measured by slrl frequency. This response declined as earlier germ cell stages were sampled. The treatment was not observed to increase the rate of chromosome loss, as determined by the frequency of XO males, as shown by the results in Table 3.6(b). (The frequency of XO males is used as a measure of chromosome breakage). This work supported the earlier conclusion of Pelecanos (1966) and of Muñoz and Barnett (1977) that DES does not induce chromosome breakage events in unstored sperm.

TABLE 3.6(a). Induction of sex-linked recessive lethals by DES-vapour in the post-meiotic and pre-meiotic germ cells of adult Drosophila males. (Abraham et al., 1979)

Duration of exposure (mins)	Brood*	Stage at which germ cells treated	Time after treatment (days)	Chromosomes screened (n)	Lethals	
					n	%
15	I	Post-meiotic (spermatozoa and late spermatids)	0-3	541	141	26.06
	II		3-6	664	152	22.89
	III	Early spermatids and spermatocytes	6-9	83	14	16.86
	IV	Spermatogonia	9-12	304	8	2.63
	I-IV	Post- and pre-meiotic	0-12	1592	315	19.78
Control	I	Post-meiotic (spermatozoa and late spermatids)	0-3	1290	1	0.08
	II		3-6	1370	0	0.00
	III	Early spermatids and spermatocytes	6-9	948	0	0.00
	IV	Spermatogonia	9-12	889	2	0.22
	I-IV	Post- and pre-meiotic	0.-12	4497	3	0.07

*Brood 1 = mature spermatozoa
Brood 2 = mature spermatozoa & late spermatids
Brood 3 = early spermatids and 2° spermatocytes
Brood 4 = 2° and 1° spermatocytes
Brood 5 = 1° spermatocytes and spermatogonia
Brood 6 = spermatogonia

1-3 days after treatment
4-6 " " "
7-9 " " "
10-12 " " "
13-15 " " "
16-18 " " "

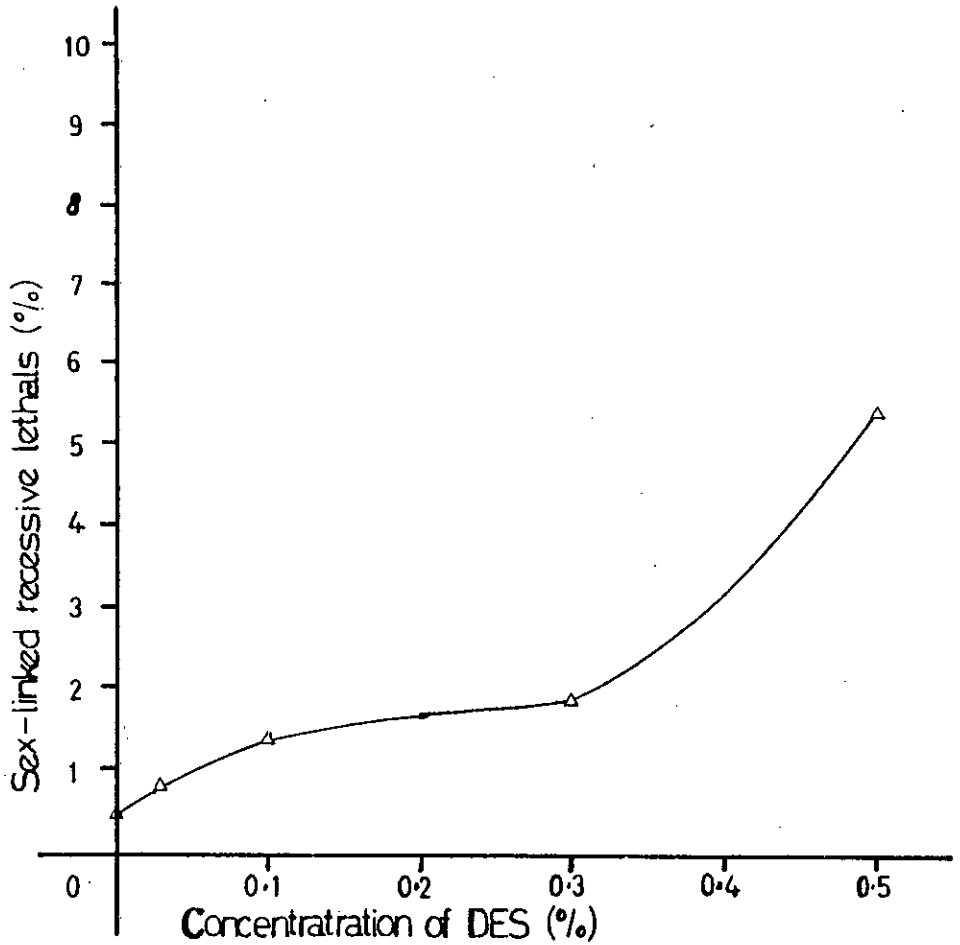
(Alderson & Pelecanos, 1964).

TABLE 3.6(b). Sex-chromosome loss in mature spermatozoa of *Drosophila* males exposed to DES-vapour.

Duration of Exposure (mins)	Brood*	Time after treatment (days)	Total Progeny	XO Males	
				n	%
15	I	0-3	1125	2	0.18
	II	3-6	1473	3	0.20
	III	6-9	197	1	0.50
	IV	9-12	596	1	0.17
	I-IV	0-12	3391	7	0.21
Control	I	0-3	2456	1	0.04
	II	3-6	2566	6	0.23
	III	6-9	1856	2	0.11
	IV	9-12	1614	2	0.12
	I-IV	0-12	8492	11	0.13

*For stage at which the germ cells were treated see Table 3.5(a). (Abraham *et al.*, 1979).

Fig. 3.5. Induction by DES-vapour of sex-linked recessive lethals in mature spermatozoa of three-day old adult Drosophila males : Effect of concentration of DES.



Using treatment vessel C, vapour tests were carried out with four concentrations of DES. The flies were observed until the highest concentration was seen to have slightly reduced the mobility of the flies without inactivating them. This time was 60 minutes: at this time all treatments were stopped. 24 hours later, the flies were counted and slrl tests were set up. The survival levels and lethals induced at each concentration of DES can be seen in Table 3.7, and Figure 3.5.

These results showed that the induction of slrl by DES-vapour was dependent on the received dose: as the dose increased so did the induction of slrl.

A second experiment was run using 0.5% DES with increasing exposure times. 0.5% DES was used because it was the dose used by Muñoz and Barnett (1977), and it should be possible to induce a high frequency of lethals using short exposure times. The survival levels and lethals induced by varying the time of exposure are shown in Table 3.8, Figure 3.6.

The number of males at each exposure time was small and lowered survival levels were obtained after 100 and 125 minutes; nevertheless an initial frequency of lethals could be determined. Although the number of gametes tested was small, the results obtained in the lethal tests were in line with those of, for example, Abraham et al. (1979): the received dose of DES-vapour, as measured by slrl frequency, increased with length of exposure. This shows that vapour treatments were an effective way of administering

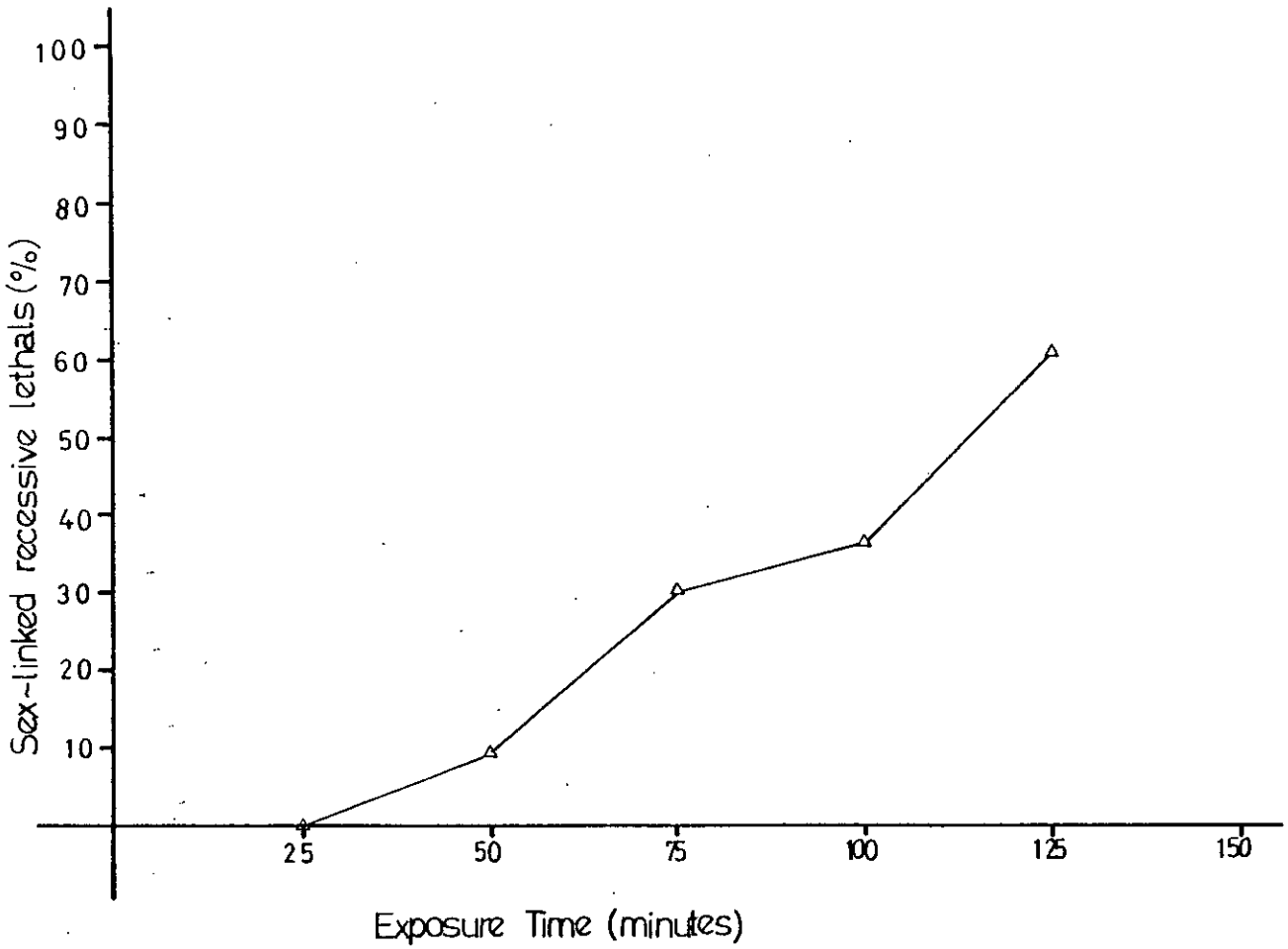
TABLE 3.7. Induction by DES-vapour of sex-linked recessive lethals in mature spermatozoa of three-day old adult Drosphila males: effect of concentration of DES.

Concentration of DES (%)	Survival (%)	Lethals (n)	Lethals (%)
Control	100	1/188	0.53
0.03	100	2/236	0.85
0.10	100	3/214	1.40
0.30	100	4/217	1.84
0.50	100	11/203	5.42

TABLE 3.8. Induction by 0.5% DES-vapour of sex-linked recessive lethals in mature spermatozoa of 3-day old adult Drosophila males: effect of length of exposure time.

No. of ♂♂ treated	Length of exposure (minutes)	Activity levels of ♂♂ at the end of treatment	Survival 24hrs after cessation of treatment (%)	Lethals	
				n	%
18	25	All flies active	100	0/94	0
18	50	All flies active	100	7/81	8.64
18	75	∿50% flies inactive	100	26/87	29.89
18	100	All flies inactive	33	5/14	35.71
18	125	All flies inactive	33	12/20	60.00

Fig. 3.6. Induction by 0·5% DES-vapour of sex-linked recessive lethals in mature spermatozoa of three-day old adult Drosophila males : Effect of length of exposure time.



a controlled dose of DES.

0.5% DES-vapour administered for 75 minutes was selected as an acceptable treatment because, at this dose, there was 100% survival of the males, and a frequency of 29.89% slrl was induced. Previous work by Muñoz and Barnett (1977) and Pelecanos (1966) had shown that a high frequency of slrl was a prerequisite for detecting DES II-III translocations.

The treatment vessel was modified further: this was done to minimise any interaction of anaesthetic with mutagen. In this procedure, flies were anaesthetised then placed in a treatment chamber and allowed to fully recover before the treatment proceeded. The modified treatment vessel (D) is shown in Figure 3.7. Treatment vessel D was used in the remaining DES experiments.

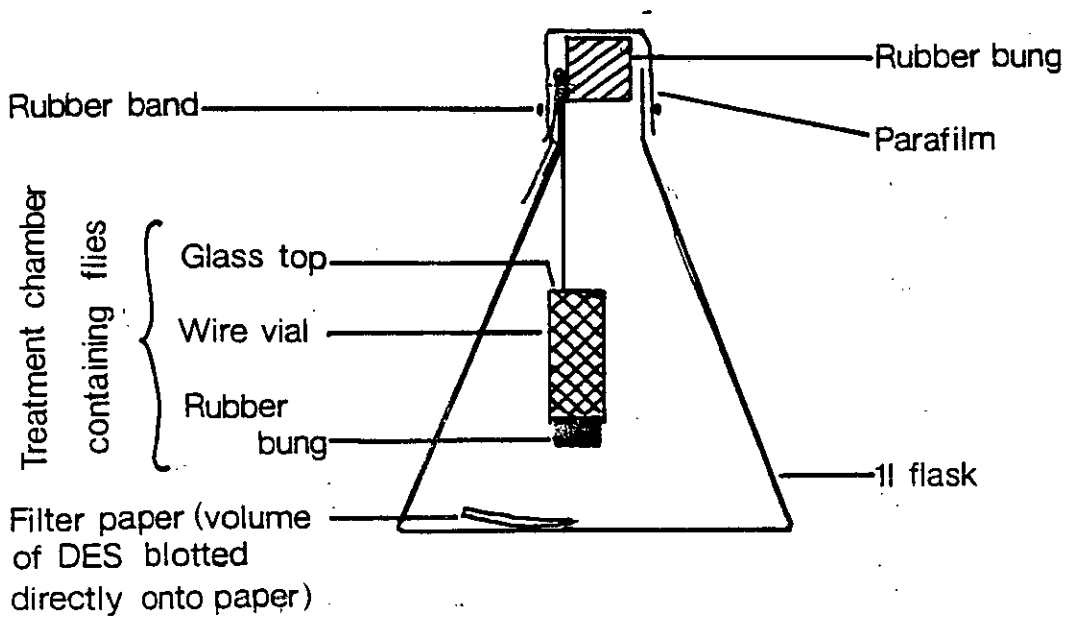


Fig.3.7. TREATMENT VESSEL 'D'

(Flies anæsthatised when placed in chamber but allowed to recover before treatment proceeded)

3.4 DES-VAPOUR INDUCED MUTATIONS:

(i) Extended brood analysis of DES-induced mutations

(ii) Effect of storage on DES-induced mutations.

These experiments were designed to obtain an extended brood analysis of DES-induced mutations and to attempt an extended storage experiment.

The general procedure for these experiments was to collect newly emerged Ork ♂♂ and keep them on SDM until they were 3-4 days old. Then they were treated with 0.5% DES for 75 minutes, allowed a 24 hour recovery period after treatment, then mated to the appropriate tester females. Matings were set up as single pair matings (1♂:2♀♀) in vials: for the detection of slrl, M-5 ♀♀ were used; for the detection of translocations, bw;st ♀♀ were used. The Day 1 = Brood 1 sample was collected over a period of 3 days like that of Pelecanos and Alderson (1964) and Abraham et al. (1979) but unlike that of Muñoz and Barnett (1977) who set up matings for 3-30 hours immediately after treatment had ceased. After the 3-day mating period the males were removed and each male given a fresh supply of 2♀♀ on days 4, 6, 8, 10, 12, 15, 18, 21, 25, 29; broods being secured at the desired times. In the storage series the inseminated females were transferred to fresh vials of low-protein food and shaken out every 3 days until the required storage time had been achieved (see Fig. 3.1). They were then returned to vials of SDM for egg-laying. Pair-matings were used, each male being identified by a number, so that a mutational event in both the brood and storage

series could be traced to the treated male and any clustering of mutational events could be detected. Due to the difficulties encountered with the storage series of experiments, the precaution of keeping the male lines separate and identifiable was discontinued in the storage series from experiment V3 onwards, the first brood was taken from individual matings but the females were massed in bottles for ease of storage. Successive broods remained as single-pair matings and thus the male line could still be traced.

The results obtained in the lethal and translocation tests are shown in Table 3.9 - 3.15 and in Figures 3.8 and 3.9.

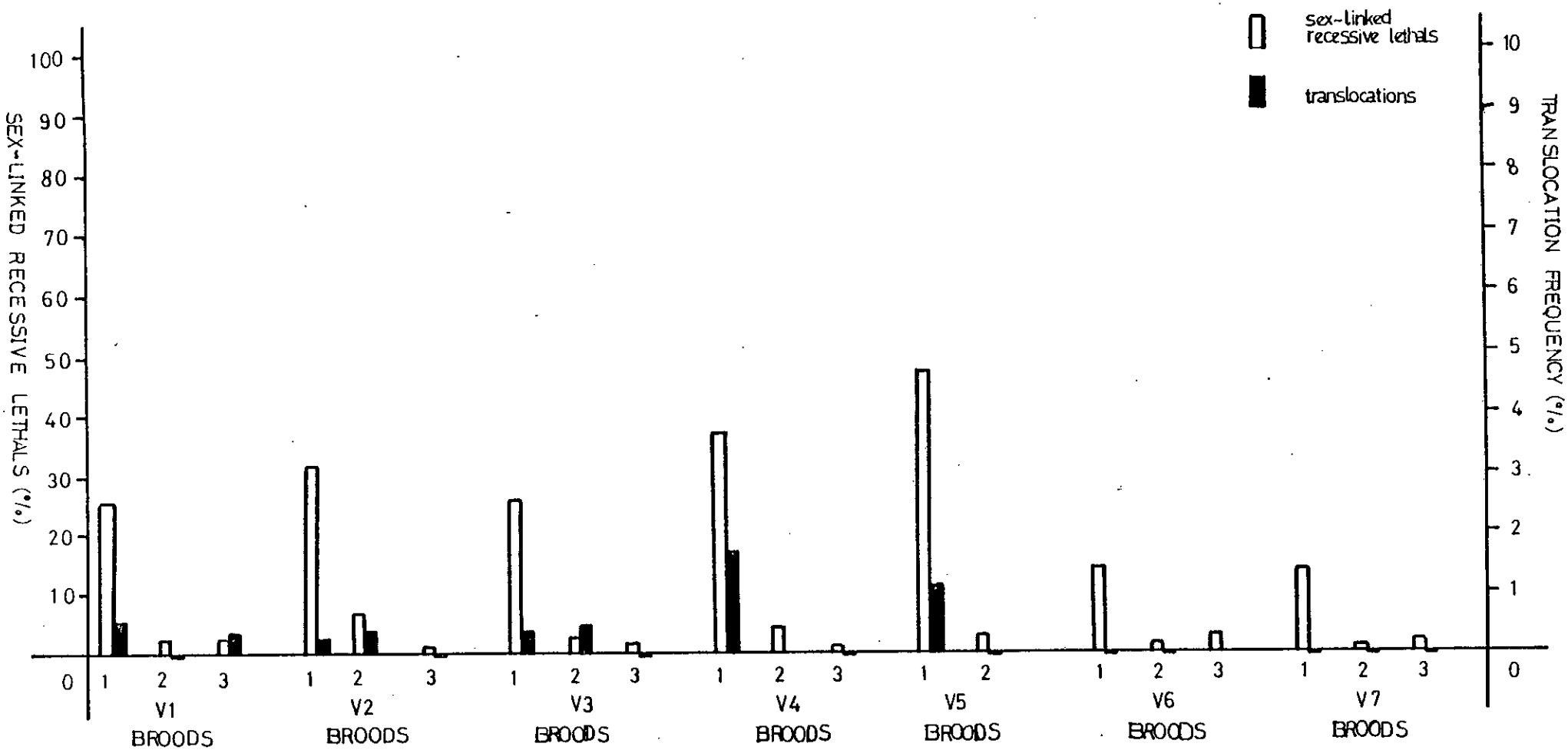
Results

(i) Brood Analysis

Figure 3.8 and Tables 3.9 - 3.13 and 3.15 show that the frequency of sex-linked recessive lethals decreases with successive broods, as earlier germ-cell stages are sampled. This observation, that post-meiotic germ cells were the most sensitive to the mutagenic activity of DES-vapour, agreed with the published data (Alderson and Pelecanos, 1964; Abraham et al., 1979).

Translocations were detected in Brood 1 samples, shown in Table 3.15, but only when the frequency of slrl was greater than 25%; none were detected at doses yielding up to 15% slrl. Since none of the administered doses gave a frequency of slrl in the range of 15-25%, it is possible

Fig. 3.8. Brood analysis of DES-induced sex-linked recessive lethals and translocations induced in three-day old adult Drosophila males



Tables 3.8.- 3.12 and 3.14 give the time at which each brood was taken; V1, 5, 6 and 7 brooded at identical times, as were V2, 3 and 4.

TABLE 3.9. Brood analysis of DES-induced mutations; 400 P_1 Ork♂♂ tested

Samples, Run v1	Days on which sample was taken	Sex-linked recessive lethals		Translocations	
		n	%	n	%
Day 1 = Brood 1	1-3	48/186	25.81	[1] 2/329	0.61
Brood 2	15-17	3/141	2.13	0/270	0.00
Brood 3	25-28	3/167	1.80	1/291	0.34

[1] No clustering.

TABLE 3.10. Brood analysis and effect of storage on DES-induced muations; 400 P_1 Ork♂♂ tested.

Samples, Run v2	Days on which sample was taken	Sex-linked recessive lethals		Translocations	
		n	%	n	%
Day 1 = Brood 1	1-3	122/378	32.28	1/360	0.28
Brood 2	10-11	2/26	7.69	1/227	0.44
Day 10	10-12	18/93	19.35	0/142	0.00
Brood 3	21-24	4/338	1.18	0/443	0.00
Day 22	22-24	-*	-*	-*	-*

* no F_1 flies were obtained from these samples.

TABLE 3.11. Brood analysis and effect of storage on DES-induced mutations; 425 P_1 Ork♂♂ tested.

Samples, Run v3	Days on which sample was taken	Sex-linked recessive lethals		Translocations	
		n	%	n	%
Day 1 = Brood 1	1-3	27/103	26.21	1/234	0.43
Brood 2	10-11	7/228	3.07	[1]2/388	0.52
Day 10	10-12	21/77	27.27	5/52	9.62+
Brood 3	21-24	5/216	2.31	0/431	0.00
Day 22	22-24	-*	-*	-*	-*

* no F_1 flies were obtained from this sample.
+ sample size is very small.
1 no clustering.

TABLE 3.12. Brood analysis and effect of storage on DES-induced mutations; 1,000 P_1 Ork♂♂ tested.

Samples, Run v4	Days on which sample was taken	Sex-linked recessive lethals		Translocations	
		n	%	n	%
Day 1 = Brood 1	1-3	45/123	36.59	[1]5/302	1.66
Brood 2	10-11	10/261	3.83	-	-
Brood 3	21-24	3/251	1.20	0/372	0.00

1 no clustering.

TABLE 3.13. Brood analysis and effect of storage on DES-induced mutations; 350 P_1 Ork♂♂ tested.

Samples, Run v5	Days on which sample was taken	Sex-linked recessive lethals		Translocations	
		n	%	n	%
Day 1 = Brood 1	1-3	65/138	47.10	[1] 3/271	1.11
Brood 2	16-17	10/294	3.40	0/365	0.00
Day 16	16-18	17/72	23.61	-*	-*
Day 25	25-28	3/56	5.36	-*	-*

* no F_1 flies obtained from this sample. 1 3 translocations from $2P_1$♂♂

TABLE 3.14. Survival of inseminated females kept on low-protein food.

Days after mating	Food type	Survival of M-5♀♀		Survival of bw;st♀♀	
		n	%	n	%
1 (Put on storage food on day 3)	SDM	300	100	400	100
12	storage	230	76.67	280	70
15	storage	200	66.67	75	18.75

These were the females used in Run v5.

TABLE 3.15. Effect of exposure time on induction of slrl and translocations in mature sperm treated with 0.5% DES-vapour in 3-day old adult Drosophila males.

Dose	Exposure Time (minutes)	Sample	Day on which sample was taken	Sex-linked recessive lethals		Translocations	
				n	%	n	%
0.5%	35 (Run v6)	Day 1 = Brood 1	1-3	58/417	13.91	0/590	0.00
		Brood 2	15-17	3/321	0.93	0/587	0.00
		Brood 3	21-24	11/363	3.03	-	-
0.5%	45 (Run v7)	Day 1 = Brood 1	1-3	59/417	14.15	0/658	0.00
		Day 16	16-18	32/267	11.99	2/91	2.20+
		Brood 2	15-17	2/366	0.55	0/801	0.00
		Day 22	22-24	15/327	4.59	-*	-*
		Brood 3	21-24	5/260	1.92	0/513	0.00
		Day 25	25-28	13/213	6.10	-*	-*
0.5%	50 (Run v8)	Day 1 = Brood 1	1-3	29/187	15.51	o/478	0.00

* no F_1 flies obtained from this sample.

+ sample size is small.

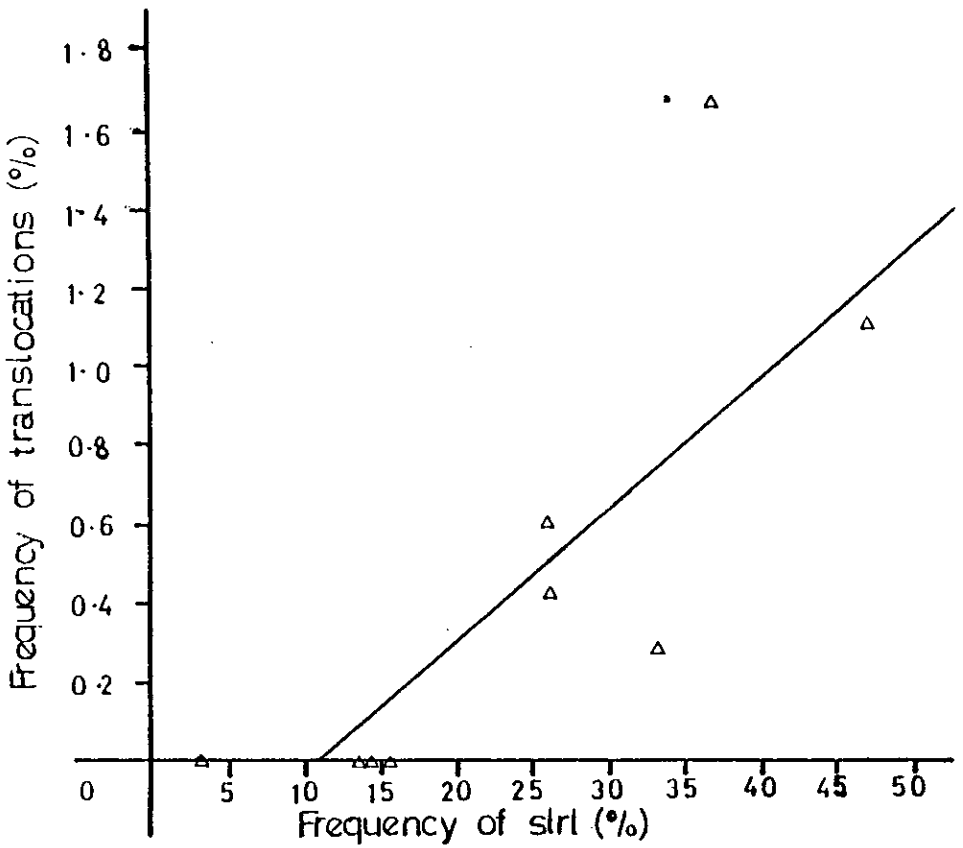
that the lowest dose at which translocations could be detected will lie within this range. However, the spontaneous frequency for translocations is reported to be ∿0.4% (Traut, 1963). Thus it may be that it is only above doses yielding ∿35% slrl (>1.0% translocations) that the detection of translocations is significant. Other workers have not reported the detection of DES-induced translocations in brooding experiments (Pelecanos, 1966; Muñoz and Barnett, 1977) or of chromosome breakage events (Abraham et al., 1979). Translocations were also detected in later broods, but only in the experiments in which translocations had been detected in brood 1. These results are shown in Figure 3.8 and Tables3.8 - 3.10: v1, brood 3, 1/291 (0.34%); v2, brood 2, 1/227 (0.44%); v3, brood 2, 2/388 (0.52%). These broods represent the equivalent of 10 - 11 days storage (v2 and v3 brood 2's) and 25 - 28 days storage (v1 brood 3). The brood 2 frequencies (v2 and v3) are slightly higher than the brood 1 frequencies (0.28% and 0.43% respectively, whereas the brood 3 frequencies is less than the brood 1 frequency (0.6%)(Table 3.17). Again, these results do not appear significantly different from the spontaneous frequency of induced translocations. Therefore, DES does not appear to produce translocations exclusively in germ cells treated at the spermatogonial stage.

An attempt was made to correlate the frequency of Brood 1 = Day 1 slrl with that of translocations (Fig. 3.9 and Table 3.16). There appears to be some correlation

TABLE 3.16. Relationship between Day 1 = Brood 1 sex-linked recessive lethal frequency and Day 1 = Brood 1 Translocation frequency induced in DES-treated Ork♂♂.

Sex-linked recessive lethals		Translocations	
n	%	n	%
5/170	2.94	0/177	0.00
58/417	13.91	0/590	0.00
59/417	14.15	0/658	0.00
29/187	15.51	0/478	0.00
48/186	25.81	2/329	0.61
27/103	26.21	1/234	0.43
122/378	32.28	1/360	0.28
45/123	36.59	5/302	1.66
65/138	47.10	3/271	1.11

Fig. 3.9 Relationship between Day 1 = Brood 1 sex-linked recessive lethal frequency and translocations induced by DES vapour in three-day old adult Drosophila males.



Regression line

$$Y = a + b(x - \bar{x})$$

$$Y = 0{\cdot}45 + 0{\cdot}03(x - 23{\cdot}83)$$

Sample sizes are given in table 3.16.

between these two parameters, as would be expected for an alkylating agent. Figure 3.9 suggests that there is a threshold of slrl frequency below which II-III translocations will not be detected.

(ii) Analysis of storage experiments

Table 3.18 shows that there is a trend for the frequency of slrl to decrease with increasing storage time.

Previous reports of the effect of storage on slrl induced by various alkylating agents show that the frequency of slrl remains fairly constant with storage (Lee, 1976). The slrl results obtained in this work were obtained from very small samples due to the problems encountered with the method of storage; they would need to be re-examined in order to determine if this decrease is real. A more detailed analysis of this class of mutations would also clarify this response by defining more precisely this heterogeneous class of mutations.

Translocations were detected in the Brood 1 = Day 1 sample in 5 of the 6 experimental runs. Storage experiments were carried out for 3 of these, but for only 1, v3, was storage time shown to increase the frequency of translocations. This result is shown in Table 3.11: after 10 days storage, the frequency of II-III translocations had increased from 0.43% (1/234) to 9.62% (5/52), although the stored sample was small. After 10 days storage in Run v2, the frequency of translocations dropped from 0.28% to 0.00%

TABLE 3.17. Frequency of II-III translocations detected in successive broods from 3-day old adult Drosophila males treated with 0.5% DES-vapour.

Experiment	Brood 1 (1-3 dat) Translocations	n	%	Brood 2 (10-11 dat) Translocations	n	%	Brood 3 (25-28 dat) Translocations	n	%
v1	2	329	0.61				1	291	0.34
v2	1	360	0.28	1	227	0.44			
v3	1	234	0.43	2	388	0.52			

dat = days after treatment.

TABLE 3.18. Frequency of slrl induced by DES- vapour in mature sperm, treated in 3-day old males and stored in females

Experimental Run	Sex-linked recessive lethal frequency (%) after the following storage times (Days)				
	Day 1	Day 10	Day 16	Day 22	Day 25
V2	32.28	19.35	NT	-*	NT
V3	26.21	27.27	NT	-*	NT
V5	47.10	NT	23.61	NT	5.36
V7	14.15	NT	11.99	4.59	6.10

NT Not tested, -* no F_1 flies obtained from this sample.

(Table 3.10); in Run v5, stored females failed to produce F_1 flies (Table 3.13). In Run v7, in which no translocations were detected in Brood 1 = Day 1, 16 days of storage resulted in an increase in the frequency of translocations to 2.20%; but again the sample size was small due to the problems with the storage method. However, a response to storage was expected, as shown by Muñoz and Barnett (1979).

The storage experiments were disappointing: using the low-protein food recipe of Shukla, it did not seem possible to keep females alive for much longer than 12 days of storage. Table 3.14 clearly shows that survival levels, particularly of the bw;st females used in the translocation test, were very low. M-5 females appeared to be much more resiliant. Females that survived up to or beyond 12 days did not lay adequate numbers of fertile eggs. In an attempt to overcome this problem, it was decided to embark on a series of food tests.

The difficulties experienced with the storage method, and the low numbers of F_1 flies obtained from stored females, suggest that these experiments have not yielded any meaningful results.

CHAPTER 4. FOOD TESTS

FOOD TESTS

4.1 INTRODUCTION

Inseminated females store sperm in special storage organs, the spermathecae and ventral receptacles; this store of non-replicating (Leigh and Sobels, 1970), non-transcribed DNA (Hennig, 1967) is gradually depleted as the sperm is used to fertilise eggs, just prior to their being deposited by the female. A sample of fertilised eggs laid on the xth day after mating is, therefore, fertised by sperm stored for x-days. In order to study the effect of storage time on mutation induction in mutagen treated sperm, it is useful to 'fractionate' samples of the sperm and look at the resulting damage in successive samples or 'fractions' of fertilised eggs. This can be done by simply transferring inseminated females to fresh food every few days (Schalet, 1955; Slizynska, 1957; Snyder, 1963; Sram, 1970a,b) but the store of sperm may be exhausted before the full effects of the mutagen are realised. Methods have therefore been devised whereby egg-laying by inseminated females is inhibited; thus the sperm store is not exhausted so quickly and, as a result, the time over which sperm can be stored is increased. One such method consists of collecting an initial (pre-stored) sample of fertilised eggs from inseminated females kept on SDM then transferring them to a semi-defined, protein deficient food for a pre-determined period of time before returning them to SDM in order to obtain a post-stored sample of fertilised eggs (Khishin, 1969; Matthew,

1964; Watson, 1964; Abrahamson et al., 1969; Khan, 1969; Slizynska, 1969; Brink, 1970; Ikebuchi and Nakao, 1979; Shukla and Auerbach, 1979). Other workers have collected a pre-stored sample of fertilised eggs from females mated to males on SDM. A post-stored sample of sperm was obtained by setting up matings for one-day on a reduced food, keeping the inseminated females on a storage food for two days more, then collecting 'fractionated' samples by transferring the females to fresh standard food every few days (Ryo, Ito and Kondo, 1981). Alternatively they (Ryo et al., 1981) set up matings on semi-enriched medium for 18 hours; the females were then transferred to sugar-agar medium for up to 15 days. Some workers have stored sperm by keeping inseminated females at a low temperature (Muller and Settles, 1927; Snyder, 1963; Muñoz and Barnett, 1977).

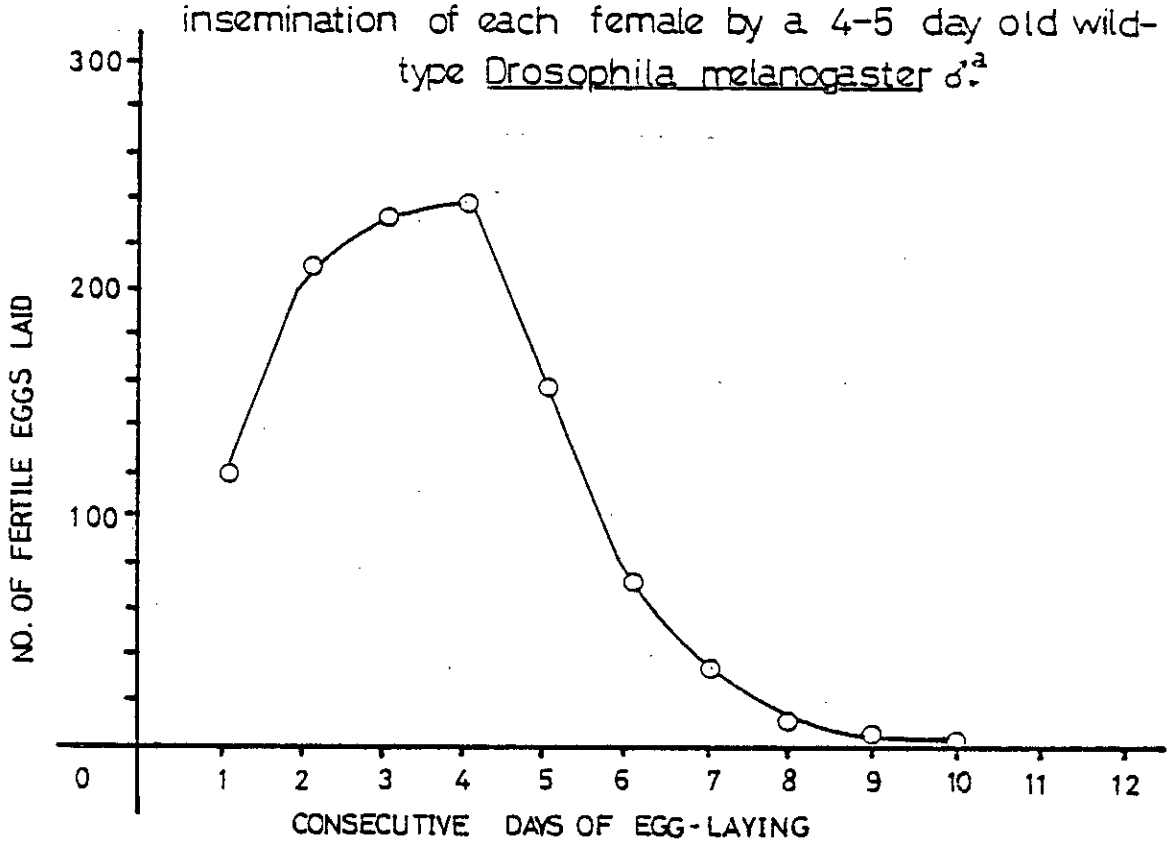
A brief discussion of the biology of Drosophila will provide some insight as to why these methods were adopted, and why they should, or should not, be effective.

4.2 METHODS OF STORAGE

A. Storage by transferring inseminated females to fresh SDM every few days

This method was used in early storage experiments as well as in more recent ones. It is a simple and logical method based on the knowledge that inseminated females store sperm in special storage organs until it is needed to fertilise eggs. The use of this method was satisfactory

Fig.4.1 Total number of F_1 laid by 11 females after a single insemination of each female by a 4-5 day old wild-type Drosophila melanogaster ♂.[a]



(Data from Kaufman and Demerec, 1942)

a. The maximum number of eggs laid by any one female was 322 ($\bar{x}$ = 96). Lefevre and Jonsson (1962) found that over a period of 14 days, the maximum number of offspring from one female was 530 ($\bar{x}$ = 419). Egg production by females kept on normal food medium reaches a peak between days 4 and 15 of adult life, when it may reach 100 eggs/day. The rate of egg production then falls gradually so that by day 20 egg production is ∿70 eggs/day, by day 30 it is ∿40 eggs/day, and by day 40 ∿20 eggs/day (David et al., 1974). This suggests that, provided females resume normal egg production when they are returned to SDM after a period on storage medium, it is not lack of eggs which will be the limiting factor in extended storage experiments. It has been shown that female Drosophila may lay as many as 3000 eggs in a lifetime although 700-1000 is a more usual number (Gowen and Johnson, 1946). Again this shows that sperm numbers are the limiting factor in the number of offspring produced by a female Drosophila from a single insemination.

because in those experiments studying the effect of sperm storage or the frequency of induced mutations, the storage time of interest was a period of several days rather than weeks (Schalet, 1955; Slizynska, 1969; Ratnayake, 1968; Sram, 1970b). This method has also been used for extended storage times of 10-12 days (Snyder, 1963), 12-14 days (Vogel and Natarajan, 1979a,b), 15-17 days (Ikebuchi and Nakao, 1979; Ikebuchi and Teranishin, 1981), 22-24 days (Sram, 1970a) and 25-28 days (Muñoz and Barnett, 1977).

A typical curve for the patterns of egg-laying by inseminted females on consecutive days is shown in Figure 4.1. The derivation of the curve and its meaning are explained in the legend.

Kaufman and Demerec (1942) found that a single insemination may provide a female with enough sperm to lay eggs for up to 12 days, but at a very low hatch rate (<10%) after day 8. Lefevre and Jonsson (1962) determined that inseminated females laid an average of 419.5 fertile eggs per female. No fertile eggs were obtained after 14 days on SDM. Manning (1962) gave a more conservative estimate of 6-8 days of fertile egg-laying from a single insemination. Snyder (1963) discarded females after 12 days due to utilisation/exhaustion of sperm; Vogel and Natarajan (1979a,b) discarded females after 14 days for the same reason, and found that, with increasing time, successive samples of F_1 offspring diminished in size; the same was true after 15 days in the experiments of Ikebuchi and

Nakao (1979). The sample size of F_1 flies obtained by Muñoz and Barnett (1977) during the day 23-29 period was also small (120 and 178 in two experimental tests).

These workers have shown that there is great variation in the longest possible time of fertile egg-laying from a single insemination. The differences in this time may be due to differences in food composition, in the number of females used in the initial mating period, in their age, in the density of females within the egg-laying vessel, in their survival with time, and their fecundity (which may differ according to genotype). This information is not given in any of the papers cited above.

Kaufman and Demerec (1942) also showed that an ejaculate from a single male may contain up to 4,000 sperm, far more than the estimates of 250-700 sperm (Kaplan et al., 1962), 650 sperm (Gugler et al., 1965) and 500 to 700 sperm (Zimmering and Fowler, 1966; Fowler et al., 1968) that any one female can store (Lefevre and Jonsson, 1962). Still, this must impose a limit on the number of offspring that any one female can produce from a single mating, even though stored sperm are used with a very high efficiency (Lefevre and Jonnson, 1962).

Since the number of stored sperm fixes the limit to the number of eggs fertilised, any method of storing which restricts egg-laying without destroying egg laying capacity will enable sperm to be studied in significant numbers after extended storage times.

B. Storage by keeping inseminated females on nutrient-deficient/low-protein food

The observation that a single insemination may provide a female with enough sperm to lay eggs for only 6-8 days (Manning, 1962) or up to 12 days (Kaufman and Demerc, 1942) points to a reason why a special food may be needed to prolong the time of storage. If egg-laying is continuous and not reduced then a single ejaculate, which will contain a finite number of sperm is insufficient to fertilise adequate numbers of progeny for more than 12 days. It was known as early as 1927 (Muller and Settles) that sperm stored in females remains viable for considerable periods of time - when females were kept at temperatures low enough to inhibit egg-laying, viable sperm persisted for up to 3 months. However, as will be discussed in more detail at a later stage, low temperatures are not suitable for storage experiments involving chemically treated sperm.

An effective storage food should meet the following criteria:

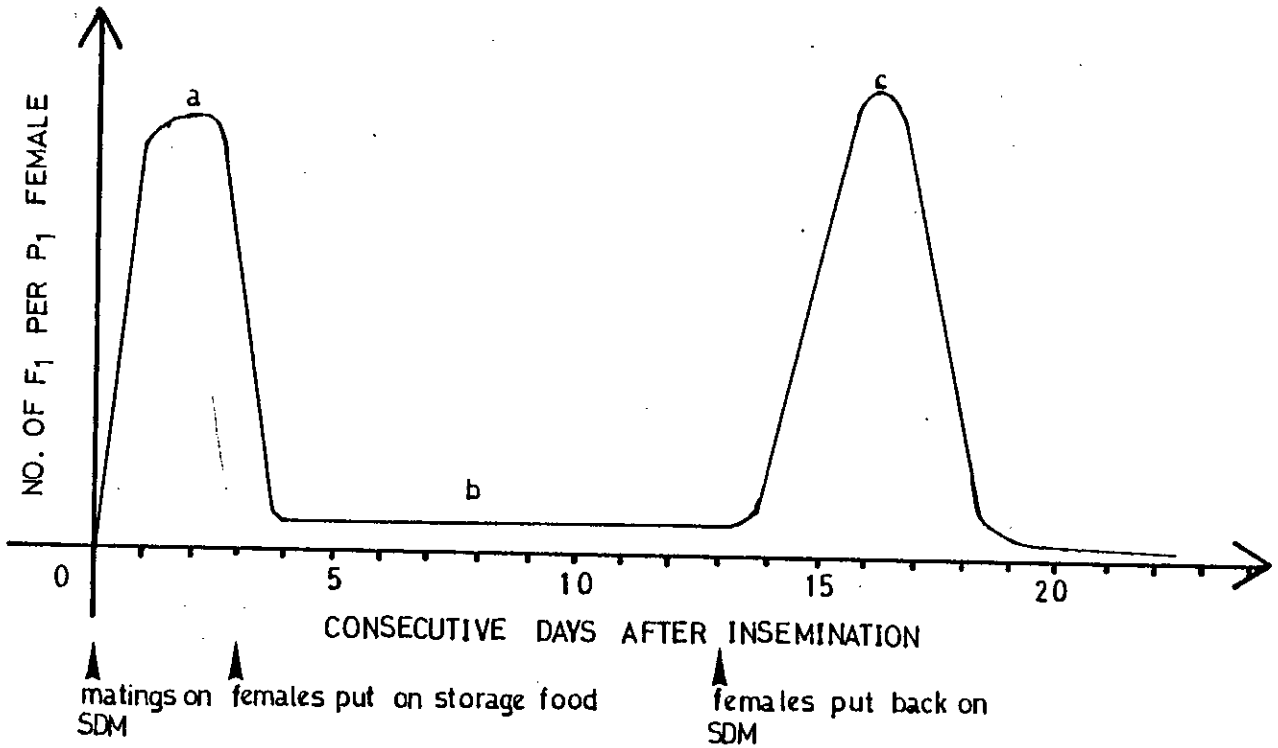
(i) the inseminated females must be kept alive.

(ii) the fertility of these females should not be so impaired that when they are returned to SDM they are unable to resume egg production.

(iii) egg-laying should be discouraged to minimise the utilisation of the stored sperm and thus prolong the storage time.

The composition of the storage food should take into account some general observations made about the nutritional requirements of Drosophila:

Fig. 4.2a Pattern of egg-laying by inseminated females kept for x days on low-protein food after an initial 3-day egg-laying period on SDM



a Initial "pre-stored" sample

b Period of storage (reduced egg-laying)

c Post-stored sample

(1) availability of food influences egg-laying, in particular:

(a) insufficient yeast will deter females from laying eggs (Robertson and Sang, 1944), and

(b) 10% sucrose inhibits egg laying (David, Herrewege and Fauillet, 1973).

(2) Drosophila do not need complex food media in order to survive: adult Drosophila can survive with sugar as the only food source, but such adults do not lay (Sang, 1978).

These observations have been taken into account by workers who use storage media. The composition of various media and the lengths of time for which females, and therefore the sperm in their storage organs, have been stored on them are listed in Table 4.1.

The ♀♀ used by all the named workers were kept on SDM until matings were set up. The age of the females at the time of mating was generally not reported. In all cases except one, the matings were carried out on SDM, males being left in with the females for 1 to 3 days. All females were left on SDM for 3 days to facilitate sampling of pre-stored sperm. The females were then transferred to storage food. This method of storage is visualised in Figure 4.2a. Ryo, Ito and Kondo (1981) are the one exception: they set up matings for 24 hours in bottles containing tissues soaked in 4ml of a 1% molasses solution before transferring females to a sugar-agar medium for one day followed by 4 hours on SDM then they were placed on standard rich medium for 2 days (Day 4-5) and from then they were transferred to standard yeast medium every 3

Table 4.1. Composition of Storage Media and the time of Storage for which they have been used

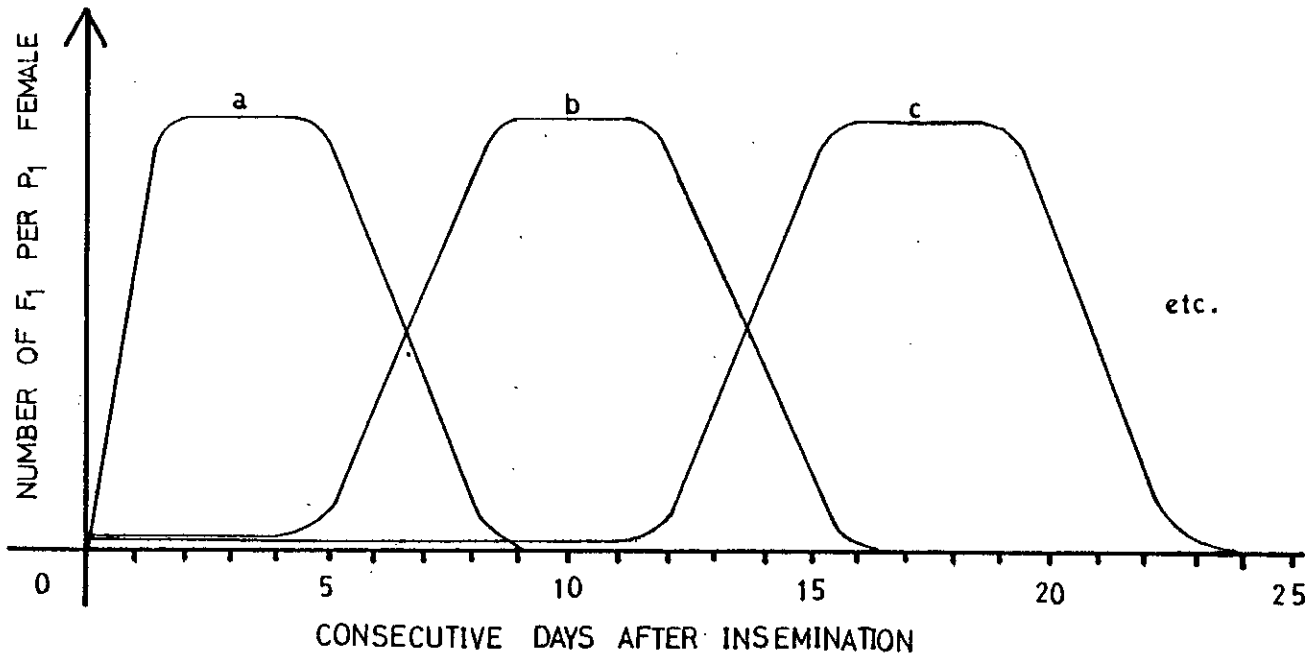
Reference	Storage Media	Storage Time (days)[a]
Watson, 1964 Ratnayake, 1968 Ikebuchi & Nakao,1979	4% agar: 2% sucrose	6 6 10[b]
Khan, 1969	4% agar: 2% glucose	6
Shukla & Auerbach, 1979 (P. Comm.)	2% agar: 2% sucrose	12-15 & 25-27
Brink, 1970	10% sucrose-agar	7-8
Abrahamson, 1969	non-yeasted	14-6
Khishin, 1963 Slizynska, 1969	protein deficient	6
Ryo, Ito and Kondo, 1981.	Day 0-1: 4ml of a 1% molasses solution Day 2: sugar-agar medium Day 3: 4hrs on SDM 20hrs on 2.5% agar: 3% sugar Day 4 onwards SDM	12-15

[a] The time given is storage time following a 3-day egg-laying period on SDM (unless stated otherwise). This time is not necessarily the maximum time for which the females can be maintained on this food, but only the time for which the females were 'stored' in that particular experiment.

[b] This storage time was claimed to be the maximum time for which this media would support viable, fertile females.

Fig. 4.2b Pattern of egg-laying by inseminated females

(See text for details)



a Peak 1 : matings set up on SDM

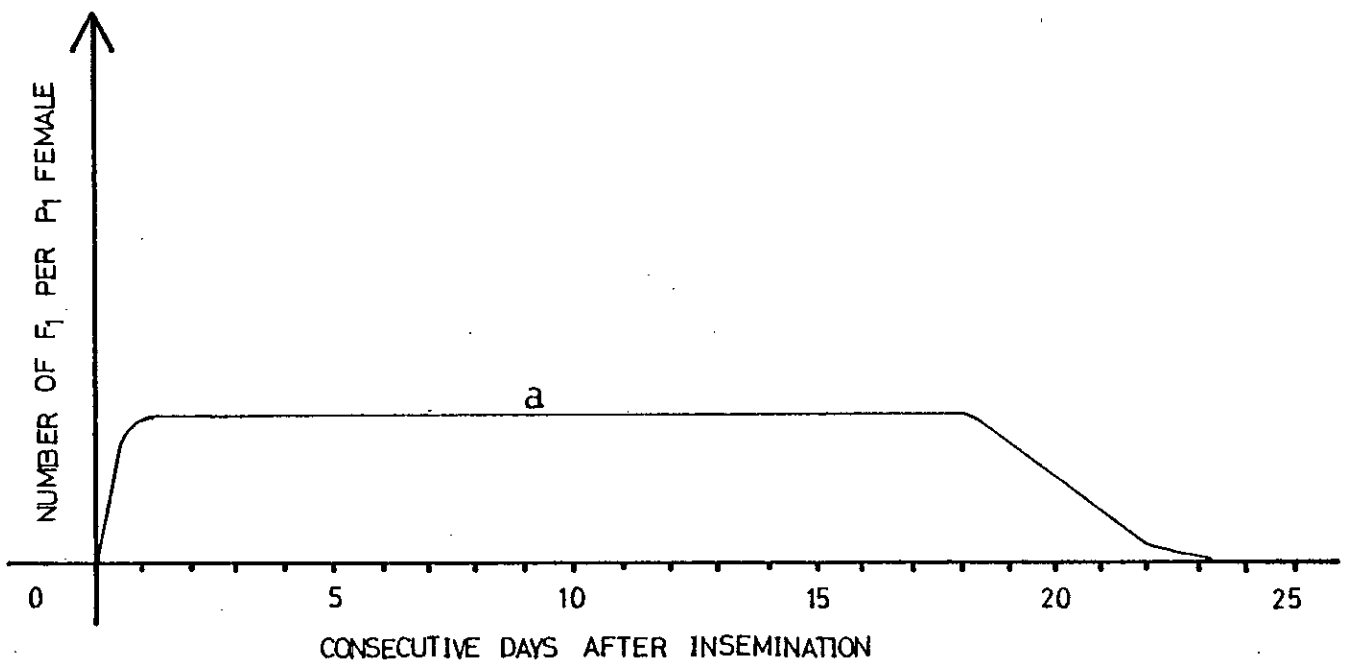
b Peak 2 : - - - , females stored for 4 days before being placed on SDM

c Peak 3 : - - - , - - - 10 - - - - - -

days. A pre-stored sample was collected on Day 1-3 from a batch of males mated to ♀♀ on SDM. This method of storage is visualised in Figure 4.2b, peaks a and b. Alternatively matings were set up for 18 hours in bottles containing semi-enriched medium to reduce egg-laying but still to provide an unstored sample. The females were then placed on a sugar-agar medium and changed to fresh bottles every 2-3 days for up to 15 days. This method of storage is visualised in Figure 4.2b, peaks a and c. They noted that with increasing storage time the number of surviving females decreased. Ryo et al. (1981) used these methods "because the egg-laying capacity had to be maintained for more than two weeks after mating". In order to obtain a complete picture of the rate of appearance of mutations, it may be necessary to combine different "methods" of storage to obtain a "complete" egg-laying pattern as shown in Figure 4.2b, or else to reduce egg-laying to obtain the pattern shown in Figure 4.2c.

Herskowitz (1963) reported that when females were starved before the irradiation of spermatozoa within them, then the frequency of translocations detected was greater than in females which had been continuously well fed. A similar difference was obtained when the females were starved after irradiation. These results may have been influenced by indirect maternal effects because the female germ cells would also have been irradiated. Herskowitz suggested that the effect was probably not on the frequency of chromosome breakage, but on the frequency of restitutional

Fig. 4.2c Pattern of egg-laying by inseminated females kept on a food medium capable of reducing but not totally suppressing oviposition



a Matings set up on and females kept on "semi-deficient" media

Effect of Storage on Two Food Media on the TEM-induced Dominant Lethal and Translocation Frequency in D. melanogaster

TABLE 4.2a. RDL Frequency

	Experiment Series	I	II	III	IV	V
Pre-stored	Food	RDL	RDL	RDL	RDL	RDL
	SDM	47.9	27.4	6.9	26.2	19.1
Post-stored	Agar Food	82.5	44.3	31.9	49.4	48.8
	SDM	NT	16.6	27.5	51.2	43.1

NT = not tested

TABLE 4.2b. Translocation Frequency

	Experiment Series	I	II	III	IV
Pre-stored	Food				
	SDM	3/274 1.09	3/488 0.60	3/607 0.49	6/1583 0.38
Post-stored	Agar Food	21/177 11.86	9/145 6.20	12/308 3.89	21/779 2.69
	SDM	35/397 8.82	16/231 6.93	11/288 3.81	33/935 3.52

Ratnayake (1968)

The results from the translocation test were open to less ambiguity than the RDL frequencies because factors other than chromosome breakage can cause dominant lethality, whereas translocations represent viable rearrangements resulting from two chromosome breakage events.

unions. Watson (1965) and Ferguson (1965) reported data which suggested that the storage effect on translocations induced by TEM treatment of adult Drosophila males was larger in starved females than in well-fed ones. They interpreted this as indicating that in Drosophila, as in bacteria, lesions in DNA induced by alkylating agents can be repaired and that the degree of repair can be influenced by maternal nutritional conditions.

Ratnayake (1968) carried out a series of experiments to determine the effect of storage on two semi-defined media (SDM and 4% agar: 2% sucrose). Females were stored for 6 days after a 3 day mating and laying period. F_1 and F_2 offspring were scored to determine RDL and translocation frequencies respectively. During storage, hardly any eggs were laid on the agar medium but quite a few were laid on the SDM. However, there was, in both cases, sufficient stored sperm post-storage for meaningful tests to be carried out, i.e. there was no sperm depletion. The results from the RDL and translocation tests are shown in Tables 4.2a and b. From these results Ratnayake concluded that the magnitude of storage depended little, or not at all, on the nutrition of females during the period of storage for both RDL and translocation frequencies.

Manning (1962) states that Bodenstein (1947) has shown that D. melanogaster females can mature a few eggs without access to protein as adults, but their ovaries remain very small and very few eggs are laid after insemination. Such females are not as receptive as normally

fed flies. If they are placed on a low-protein diet soon after eclosion they become, and remain, unreceptive to males. Their receptivity returns after a week of normal diet with the accompanying rapid growth of the ovaries and the onset of egg-laying. This point is of interest because preliminary tests were carried out to see if storage could be prolonged by placing ♀♀ immediately on to low-protein food and then allowing them to mate on low-protein food. The experiments were unsuccessful possibly because, as Bodenstein suggests, the females were unreceptive to the males and so did not become inseminated. That egg development is slowed down may explain a general observation: there is a delay in egg-laying once the starved females are returned to SDM - the longer the females are kept on storage food, the longer is the delay (Ratnayake, 1968). This effect was also noted by Slizynska (1969) - she noted a 2-day lag after 6 days of shortage.

C. Storage by keeping inseminated females at low temperatures

As a means of maintaining females for long periods of time while also suppressing egg-laying, this method would seem to be an ideal method of storage. However, in this thesis, the aim of storage was not simply to maintain a store of sperm within inseminated females but to examine these sperm for chemically induced alterations in the DNA.

Snyder (1963) and Slizynska (1969) stored TEM treated

sperm for 6 days at 25°C and 12.5 or 12°C respectively. They compared the frequencies of slrl and translocations obtained pre-storage and after storage at the two different temperatures.

Table 4.3. Effect of temperature of storage on induction of slrl and translocations in TEM-treated spermatozoa.

Storage Temperature	slrl frequency		Tr frequency	
	Pre-storage	Post-storage	Pre-storage	Post-storage
12.5°C	10.25%	12.24%	3.0%	9.6%
12.0°C	10.50%	13.00%		
25.0°C	10.03%	23.59%	3.0%	36.5%
25.0°C	10.50%	16.90%		

(Slizynska, 1969; Snyder, 1963).

The results obtained by Snyder and Slizynska are shown in Table 4.3. It can be seen that storage at a temperature lower than that used routinely (25°C) in Drosophila experiments can lead to a relative decrease in the observed frequencies of slrl and chromosome rearrangements. The purpose of carrying out storage experiments is to allow more time for chemical lesions to mature into breaks in order that they may be detected in genetic tests. A method of storage which inhibits this process is therefore unsuitable. The implications of storing at a low temperature will be discussed more fully in the next chapter.

4.3 PRELIMINARY TESTING OF STORAGE FOOD

Shukla and Auerbach (1979) were able to store sperm for periods of 12 and 25 days. They used a 2% agar : 2% sucrose medium (P.comm.). The inseminated females were changed to fresh bottles every 3 days. When this procedure was followed, it was found that females mated to DES treated males did not survive in sufficient numbers: the females showed a rapid decline in survival after 10-12 days of storage (see Table 4.4 and Figures 4.3a and b) and those which did survive did not lay fertile eggs, i.e. no F_1 flies obtained. The day 1-3 sample of eggs produced an abundance of F_1 flies showing that the females had been inseminated. Confirmation of the observation (see Chapter 3) that M-5 ♀♀ survived better than bw;st ♀♀ on the storage food was obtained and this is shown in Figures 4.3a and b and Table 4.4. The O-1; bw; st strain, a dual purpose stock originally intended to be used as the tester strain for both the M-5 and translocation tests had been discarded in favour of the two strains due to its poor survival during storage. Rahayake (1968) also reported problems with the O-1; bw; st stock: he discarded them in favour of other strains due to their low fertility, and the high frequency of unhatched eggs obtained for both pre- and post-stored control samples in tests for dominant lethality. This method was clearly unsatisfactory for storing DES treated sperm for longer than periods of about 10 days.

Storage experiments play an important role in determining the mutagenic potential of chemical agents. A series

TABLE 4.4. Survival of inseminated females on low-protein food[a]

Day Number	Expt. No.	bw:st females		M-5 Females	
		n	%	n	%
0-1	1	491	100	392	100
	2	634	100	378	100
	3	720	100	400	100
Σn		1845	100	1170	100
2-4	1	488	99.39	385	98.21
	2	595	93.85	371	98.15
	3	710	98.61	391	97.75
Σn		1793	97.18	1147	98.03
5-7	1	468	95.32	381	97.17
	2	559	88.17	353	93.39
	3	702	97.50	381	95.25
Σn		1729	93.71	1115	95.30
8-10	1	438	89.21	351	89.54
	2	525	82.81	330	87.30
	3	669	92.92	377	94.25
Σn		1632	88.46	1058	90.43
11-13	1	423	86.15	319	81.35
	2	491	77.44	322	85.19
	3	536	74.44	374	93.50
Σn		1450	79.59	1015	86.75
14-16	1	298	60.69	215	54.85
	2	433	68.30	302	79.89
	3	-	(66)[b]	-	(92.5)[b]
Σn		731	65.00[c]	517	75.75[c]
17-20	1	208	42.36	196	50.00
	2	243	38.33	264	69.84
	3	397	55.14	367	91.75
Σn		848	45.96	827	70.68
21-24	1	25	5.09	42	10.71
	2	36	5.68	177	46.83
	3	56	7.78	252	63.00
Σn		117	6.34	471	40.26

[a] Females were mated to DES treated males. The males were removed after 3 days mating on SDM; the females were then transferred to low-protein food and shaken over every 3 days.

[b] Values estimated from Figure 4.13. [c] These Σn include the estimated values

Fig.4.3a Survival of inseminated females on low-protein food (2°/₀agar; 2°/₀ sucrose; 0°/₀ yeast)

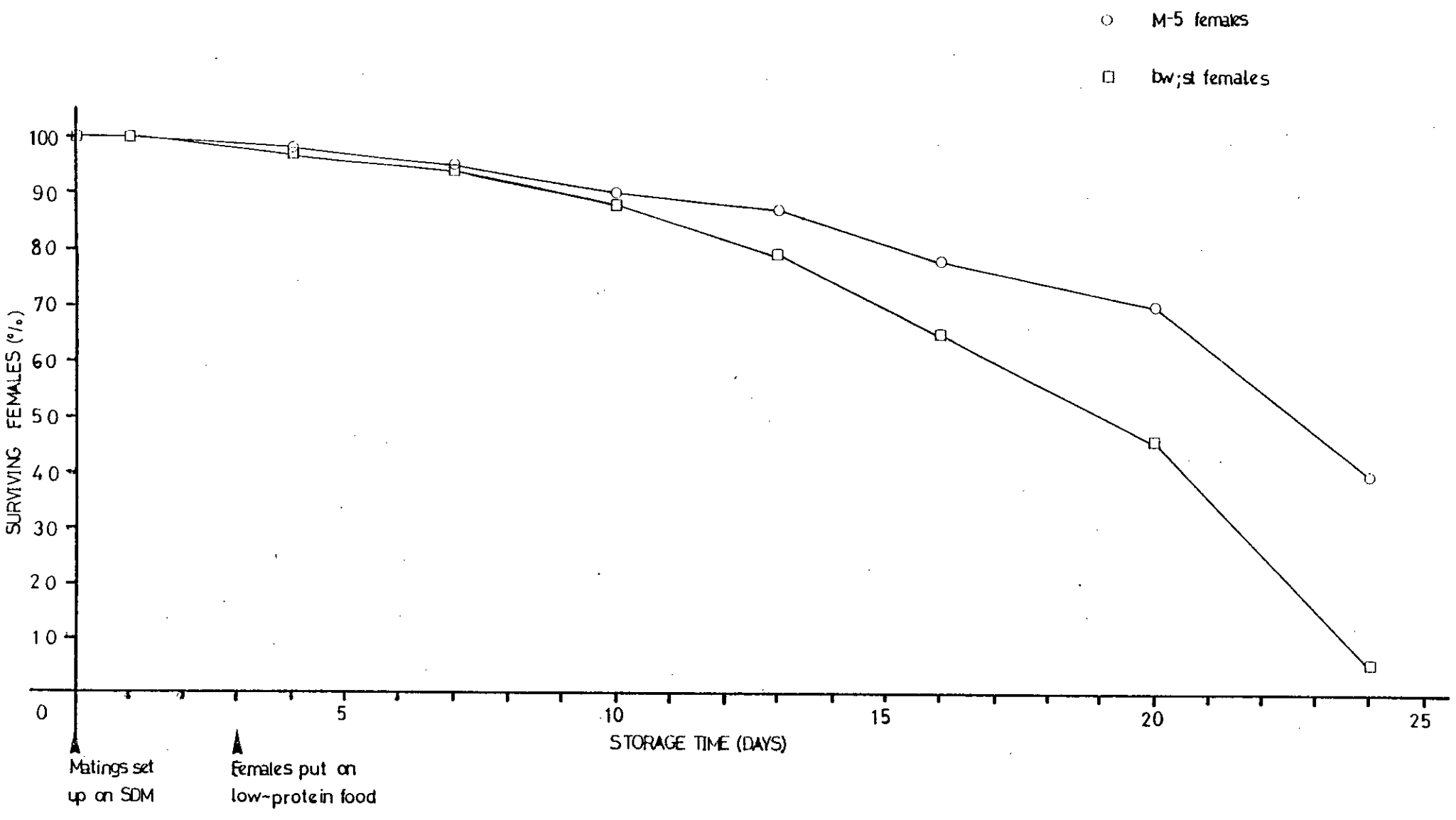
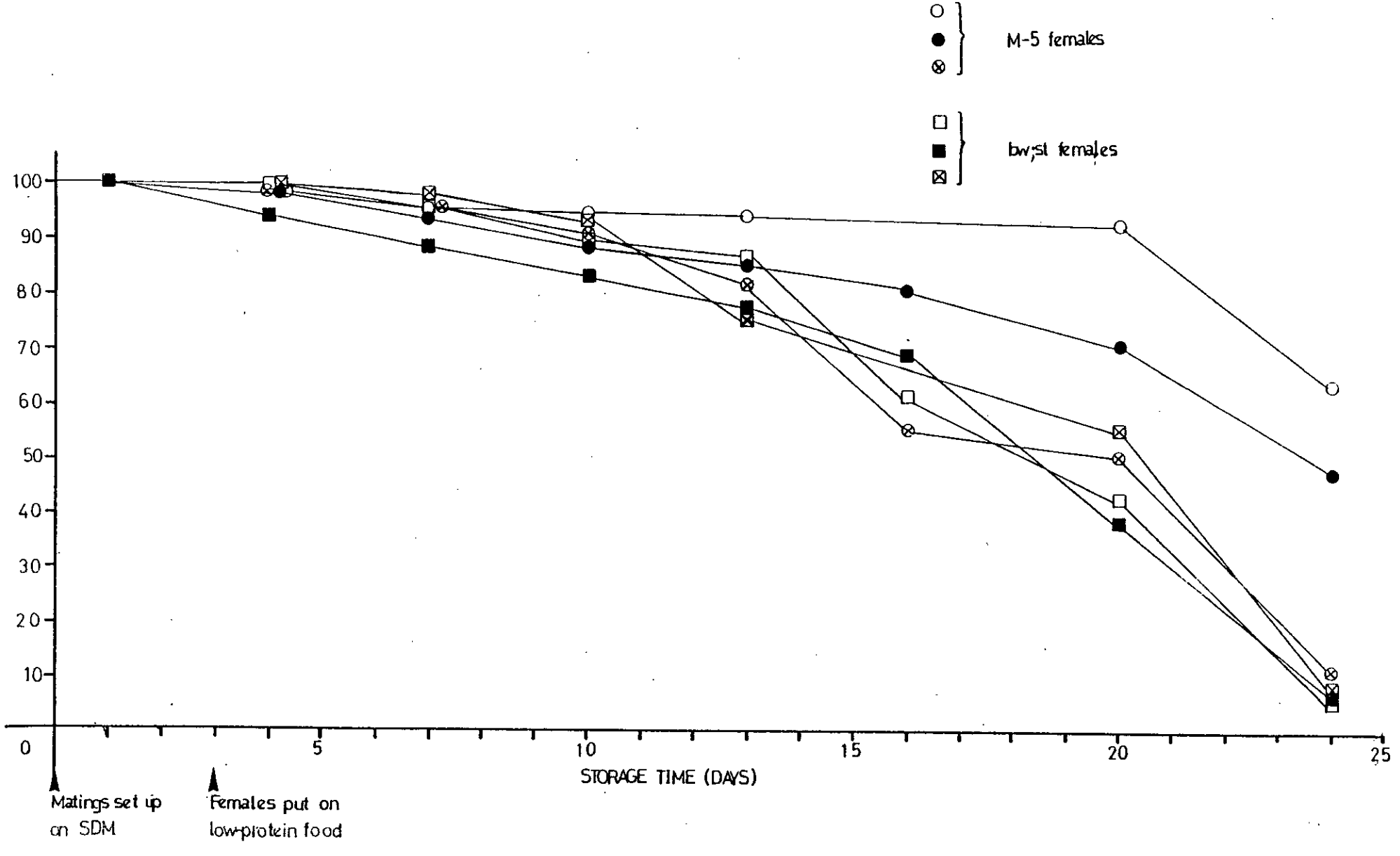


Fig. 4.3b Survival of inseminated females on low-protein food (2% agar; 2% sucrose; 0% yeast)



of food tests was therefore carried out with the aim of defining a food medium suitable for the lengthy storage experiments which may be necessary when investigating the mutagenic potential of chemicals such as DES. These media and their effectiveness at supporting healthy females while suppressing egg-laying are described in the following sections.

4.4 DETERMINATION OF A STORAGE MEDIUM

A. Comparison between SDM and protein deficient foods

1. The high mortality coupled with the low fertility levels of inseminated females kept for any length of time on the low-protein storage food (2% agar: 2% sucrose: 0% yeast) necessitated a series of tests to determine a set of conditions under which fertile females could be maintained for up to 25 days. The following media were tested:

(i) SDM - standard *Drosophila* media (1.5% yeast)

(ii) "SDM$^-$" - a standard food recipe with yeast added at 3 different concentrations, 0.25%, 0.5%, 1.0%.

(iii) a low-protein food - the basic storage food which had been previously used (2% agar; 2% sucrose) with yeast at 5 different concentrations: 0.0%, 0.25%, 0.5%, 1.0%, 1.5%.

In the preliminary food tests, the storage food had been autoclaved. Therefore the effect of autoclaving on the media was also determined - it is known that sugars interact with proteins during autoclaving (the sugar browning reaction) and this may affect the nutritive value of the

food (Sang, 1978).

Matings were always set up in bottles of SDM with 50♀♀ and 25♂♂ per bottle. The males were discarded after day 3 and the females were transferred to bottles of the storage medium, and thereafter transferred to fresh food bottles every 3 days. Prior to mating, both males and females were kept well fed. The males were 3-4 days old when matings were set up and the ♀♀ were 0-2 days old. The presence/absence of eggs and larvae in each bottle was noted. On days 10, 20, 25, a sample of 10 females was removed from each storage bottle and placed individually in vials on SDM to determine whether or not they were fertile.

From Table 4.5 and Figures 4.4a and b, it can be seen that:

(i) After 25 days on low-protein food, more females survived as the concentration of yeast in the food increased.

(ii) At higher concentrations of yeast, autoclaving had either less or no detrimental effect on the nutritive value of the food. At lower concentrations of yeast, autoclaving was detrimental. The presence or absence of eggs and larvae on the storage medium was monitored and it was seen that the numbers of both eggs and larvae decreased with increasing storage time. No eggs were detected after Day 20 at any of the yeast concentrations on any of the media used regardless of whether or not the media had been autoclaved.

TABLE 4.5. Survival of Inseminated Females kept on low-protein food until Day 25.

(2% agar: 2% sucrose: x% yeast) x = 0, 0.25, 0.50, 1.0, 1.50% bw;st females were used as these were the most difficult genotype to maintain on low-protein food, and because these were the females used in the Translocation test.

% yeast	Medium	No.of dead after 25 days (x/100)	% females alive	Fertility (n)			Fertility (%)		
				Day 10	Day 20	Day 25	Day 10	Day 20	Day 25
0.25%	LP	44	66	2	0	0	20	0	0
	LP-A	100	0	4	0	-	40	0	-
	SDM	20	80	6	7	0	60	70	0
	SDM-A	44	66	6	0	0	60	0	0
0.25%	ALL	208/400	48	18	7	0	45%	17.5	0
0.50%	LP	50	50	6	2	0	60	20	0
	LP-A	72	28	10	0	0	100	0	0
	SDM	16	84	4	6	0	40	60	0
	SDM-A	26	74	8	2	0	80	20	0
	ALL	164/400	59	28	10	0	70	25	0
1.00%	LP	24	76	8	2	5	80	20	50
	LP-A	26	74	8	4	0	80	40	0
	SDM	16	84	6	0	0	60	0	0
	SDM-A	16	84	6	1	5	60	10	50
	ALL	82/400	79.5	28	7	10	70	17.5	25
1.50%	LP	30	70	6	2	0	60	20	0
	LP-A	14	86	2	0	-	20	0	-
	SDM	16	84	6	0	5	60	0	50
	SDM-A	16	84	10	3	0	100	30	0
	ALL	76/400	81.0	24	5	5	60	12.5	12.5

LP = low protein food

LP-A = low protein food, autoclaved

SDM = standard Drosophila medium

SDM-A = standard Drosophila medium, autoclaved.

Fig. 4.4a. Graph to show effect of yeast concentration on survival of inseminated females after 25 days on low-protein food.

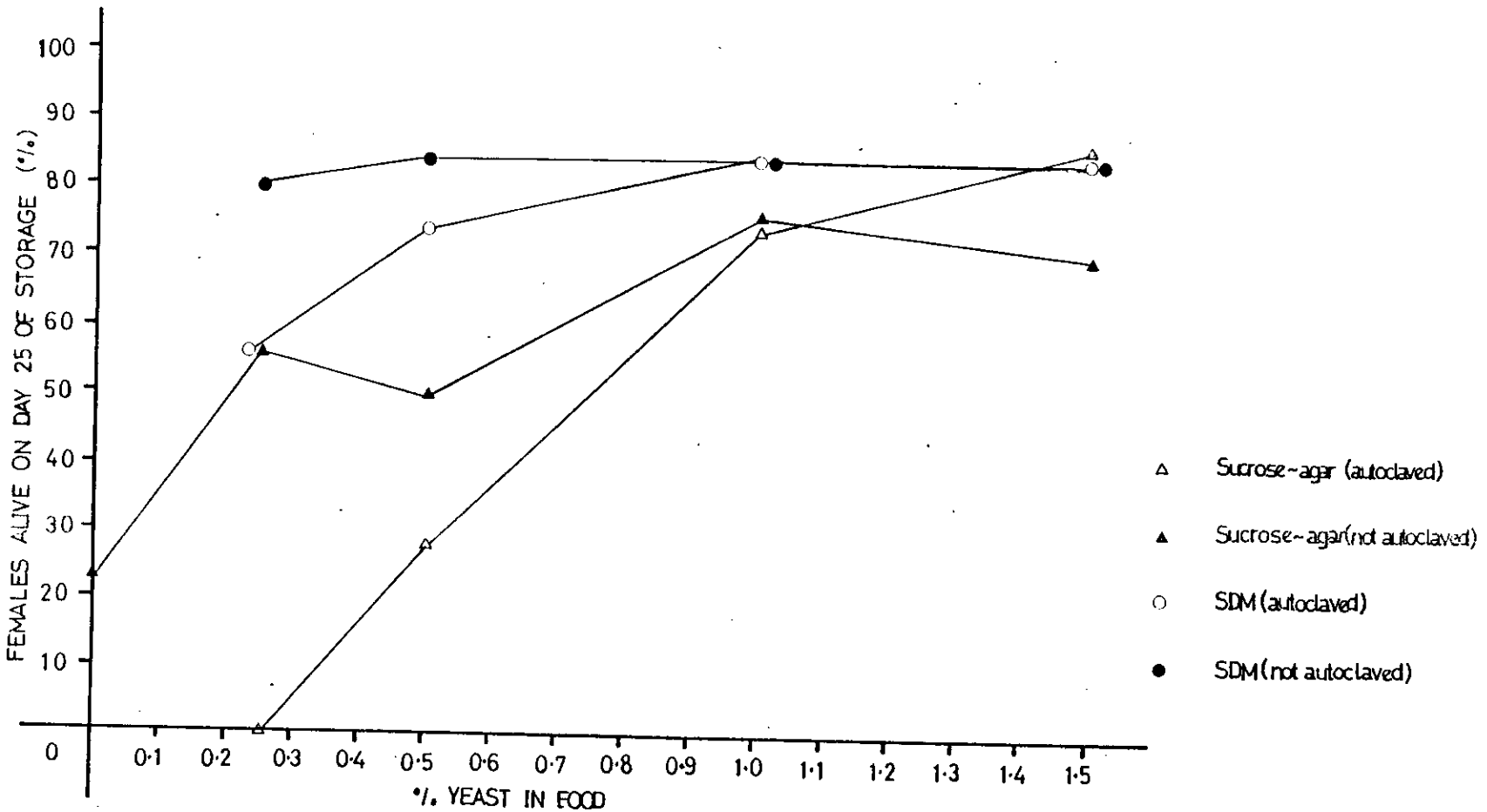
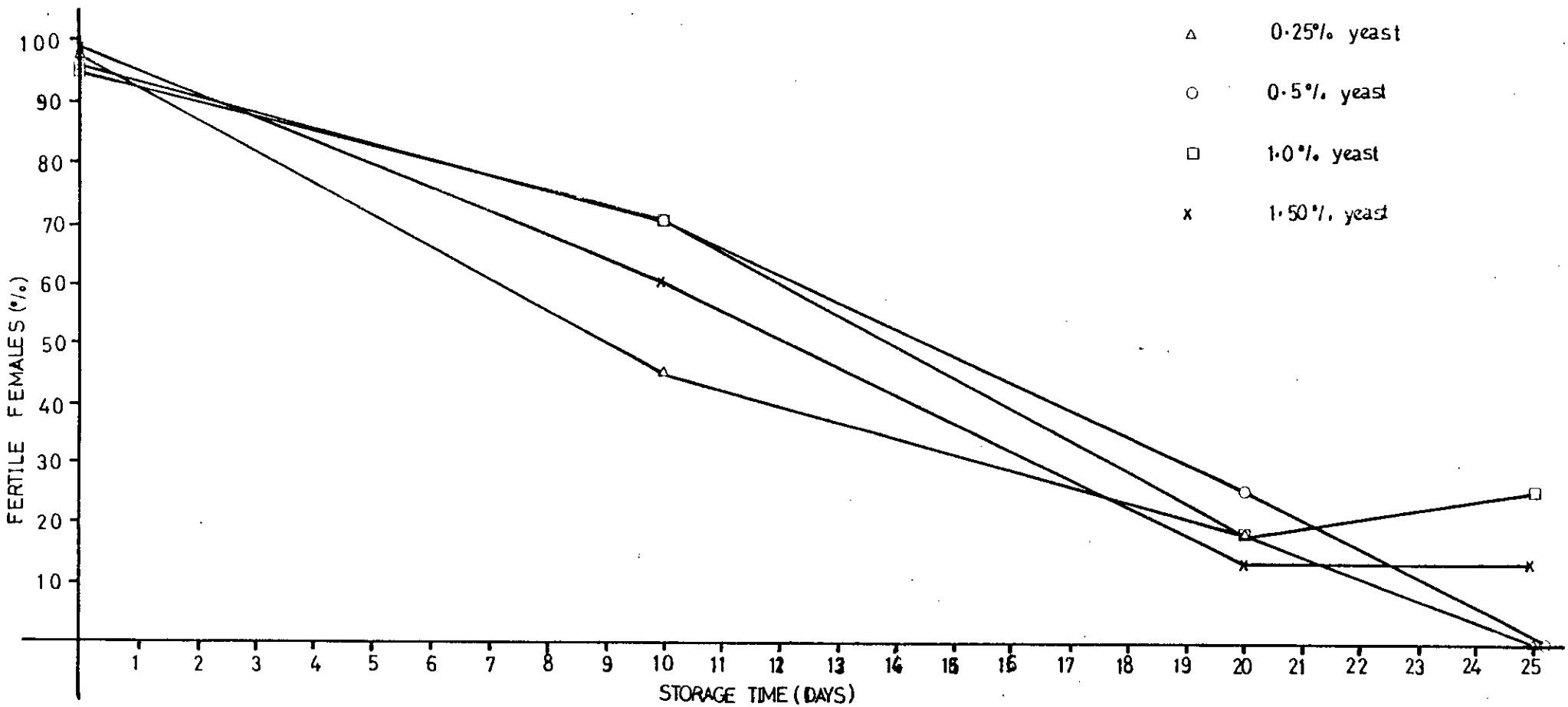


Fig. 4.4b. Graph to show the effect of storage time and the concentration of yeast in the storage media on female fertility.



(iii) The fertility of the 'stored' females was scored by noting whether or not they produced F_1 flies. This is not a quantitative results but merely gives an indication of their ability to lay fertile eggs on SDM after extended storage times. F_1 flies were obtained from females stored for up to day 10 but on day 20 there was a sharp decline in fertility levels. By day 25, 0.25% and 0.5% yeast did not yield fertile females, whereas 1.0% and 1.5% yeast did, but at very low fertility levels. The results for each concentration of yeast have been compiled by summing the results for each food-type; this was done because the samples of females was very small.

The lack of F_1 flies obtained from females, kept on low-protein food or SDM for extended storage times, could be due to sperm exhaustion. This possibility was investigated by dissecting inseminated females and monitoring the sperm content of the spermathecae and the ventral receptacle before storage and after 10 or 18 days on 2% agar; 2% sucrose.

2. Sperm hunt

To investigate the circumstances whereby females 'stored' for long periods of time yielded few offspring, inseminated females were dissected and their spermathecae and/or ventral receptacle examined for presence or absence of sperm. Females were mated to 3 day-old males which had been exposed to a low dose of EMS (0.04% for 4 hours).

Males and females were kept together for 3 days, the males were then removed and some females were transferred to 2% agar; 2% sucrose food, the others being kept on SDM. A sample of females was dissected on day 1 after insemination on day 13 ("stored" ♀♀ only) and on day 20 in order to establish if motile sperm were present in the storage organs. A sample of unmated males was also dissected in order to establish the appearance of motile sperm.

The results obtained from the dissections are given in Table 4.6. These results show that motile sperm was detected in females stored for up to 18 days on 2% agar; 2% sucrose food. No viable offspring had been obtained in experiments in which females had been 'stored' for this length of time. 18/56 of the females kept for up to 18 days on SDM yielded offspring just prior to dissection: on days 16-18 these 18 females produced 175 offspring, ∿10/♀. In none of the 56 ♀♀ could motile sperm be detected. This result was in agreement with those of Patterson (1954) who found that when females were dissected at intervals after mating, the sperm store may be observed to diminish progressively, so that by the time the females no longer lay fertile eggs, the sperm store is completely exhausted.

The lack of viable offspring obtained from females stored on 2% agar: 2% sucrose must therefore be due to some factor other than sperm depletion.

It was concluded that this medium was not satisfactory because females could not be 'stored' for 25 days. Therefore, additional experiments in which the effect of varying

yeast concentration and sugar source was examined were set up.

TABLE 4.6. Sperm content of storage organs of newly inseminated and stored females

Time after insemination (Days)	Sperm content in ventral receptacle and spermathecae of inseminated females.[a] bw;st ♀♀	M-5 ♀♀
1	+ + + + +	+ + + + +
13[b]	+ + +	+ + +
20[c]	+	+ +
20[d]	-	-

[a] The scoring system was devised as follows: the sperm content of the storage organs of newly inseminated females was designated + + + + +. The sperm content of the storage organs of these females stored for 10 or 18 days was ranked between + + + + + and - (no sperm detectable) by comparing the content with that of a newly inseminated female.

[b] Females kept on storage food (2% agar: 2% sucrose) from day 3-12 then returned to SDM. Females were dissected 24-48 hours later. A total of 20 ♀♀ were dissected.

[c] Females kept on storage food (2% agar: 2% sucrose) from day 3-20 then returned to SDM and females dissected the following day. The number of females surviving storage for this length of time was small, a total of 10 females being dissected.

[d] Females kept on SDM but otherwise treated and examined as 'stored' ♀♀. 56 females were dissected.

B. The Effect of Yeast Concentration and Sugar-Source on Survival of Inseminated Females and Suppression of Egg-Laying

(i) The aim of this series of experiments was to examine the effect of yeast concentration and sugar-source on survival of inseminated females and suppression of egg-laying in order to find a food medium capable of sustaining females for prolonged storage periods while decreasing the rate of utilisation of the sperm store. The storage medium should not be so detrimental to the health of the females that they do not resume high enough levels of egg-laying when they are returned to SDM. Three different types of media were tested:

(a) a sucrose low-protein food - 2% agar: 2% sucrose. Yeast was added to this at a concentration of either 1.5%, 1.0% or 0.5%. The food was not autoclaved.

(b) a sorbitol low-protein food - 2% agar: 2% or 15% sorbitol. It has been reported that sorbitol (15% w/v) is probably the best sugar for maintaining flies for long periods of time, i.e. weeks, in the absence of a more complete food (Kalmus, 1942). Four types of this medium were tested: 2% agar: 2% sorbitol: 1.5%, 1.0% or 0.5% yeast and 2% agar: 15% sorbitol: 0.5% yeast. The food was not autoclaved.

(c) a honey-agar (HA) food - 2% agar: 5%, 10% or 15% honey: 0% yeast. This food was recommended by Prof. A.M. Clarke. Using a 2% agar: 5% honey food he was able to store females for two weeks, changing the flies to fresh bottles only when necessary (i.e. if

the food became sticky or dried out). It was decided to include this food in the test and increase the concentration of honey to see if there was any effect on female survival. No yeast was added because honey is a more complete food than either of the two sugars.

In each test, bottles were set up with 100 bw;st ♀♀ (0-2 days old) and 50 Ork ♂♂ (4 days old). Matings were set up on the low protein foods, all flies having been kept well-fed prior to this. Every third day, the number of dead females was counted (these were then discarded) and the remaining females were shaken over into a fresh food bottle. Scoring was done in this way because it eliminated the need to use anaesthetic which may have had detrimental effects on the survival of the flies due to their low-nutritional status. The number of F_1 flies emerging from each 3-day period of storage was scored to give a measure of suppression of egg-laying and thus of suppression of utilisation of sperm.

(ii) (a) Effect of Yeast Concentration and Sugar Concentration on Survival of Inseminated Females

Tables 4.7 and 4.8, and Figures 4.5a and 4.5b show that regardless of sugar source, the concentration of yeast was important: more females survived as the concentration of yeast increases.

On the sucrose low-protein media, survival was the same at all three concentrations of yeast until day 9 (∿95%

TABLE 4.7. Effect of yeast concentration in sucrose low-protein food on the survival of inseminated females and suppression of egg-laying with storage time.

Day number	Concentration of yeast	0.5%[a]	1.0%[a]	1.5%[a]
1	n	400	400	400
	%	100	100	100
1-3	F_1/♀	1.51	1.72	0.48
4	n	392	397	396
	%	98	99	99
4-6	F_1/♀	1.44	1.15	0.73
7	n	381	393	383
	%	95	98	96
7-9	F_1/♀	0.94	1.52	1.17
10	n	371	380	378
	%	94	95	95
	(F_1/♀[b]	0.25	2.20	1.58)
10-12	F_1/♀	0.70	1.51	0.33
13	n	227	300	373
	%	57	75	93
13-15	F_1/♀	0.64	1.52	0.45
16	n	62	176	341
	%	16	44	85
16-18	F_1/♀	6.03	5.19	0.57
19	n	16	86	224
	%	4	22	56
19-21	F_1/♀	0.68	5.32	4.30
22	n	9	44	104
	%	2	11	26
22-24	F_1/♀	11.00	15.23	5.91
25	n	5	28	64
	%	1	7	16
	F_1/♀	NT	NT	NT

n = number of live females, 100 ♀♀ per bottle.
% = % of number of day 1 females, to the nearest whole number.
F_1/♀ = calculated by (i) determining the average number of P_1 ♀♀ alive during the 3-day egg-laying period, and (ii) dividing the total number of F_1 flies hatching from the same period by the average number of P_1♀♀.
a = medium also contained 2% sucrose: 2% agar
b = 1 set of day 10 ♀♀ were put into new bottles for days 11-12

TABLE 4.8. Effect of yeast concentration in sorbitol low-protein food on the survival of inseminated females and suppression of egg-laying with storage time.

Day Number	Concentration of yeast	0.5% a		1.0%a		1.5%a		0.5%b	
	Bottle No.	1	2	1	2	1	2	1	2
1	n	100	100	100	100	100	100	100	100
"	%	100		100		100		100	
1-3	F_1/♀	1.29		1.77		1.66		4.15	
4	n	92	100	100	100	96	98	64	100
"	%	96		100		97		82	
4-6	F_1/♀	0.95		2.76		4.43		4.48	
7	n	92	96	100	100	92	94	60	98
"	%	94		100		93		79	
7-9	F_1/♀	1.02		2.11		3.24		3.38	
10	n	92	88	100	100	92	94	56	96
"	%	90		100		1.93		76	
10-11	F_1/♀	2.57		2.17		2.83		1.12	
12	n	52	4	70	80	80	88	46	72
"	%	28		75		84		59	
12-15	F_1/♀	0.00		0.11		2.60		0.14	
16	n	24[c]		36[c]		80	84	24	20
"	%	12		18		82		22	
16-18	F_1/♀	8.20		17.00		8.25		0.00	
19	n	10[c]		34[c]		52	82	22[c]	
"	%	5		17		67		11	
19-21	F_1/♀	0.50		7.00		0.65		0.43	
22	n	4[c]		6[c]		40	68	14[c]	
"	%	2		3		54		7	
	F_1 ♀	NT		NT		NT		NT	

n = number of live females
% = % of number of day 1 females, to the nearest whole number.
F_1/♀ = calculated by (i) determining the average number of P_1♀♀ alive during the 3-day egg-laying period, and (ii) dividing the total number of F_1 flies hatching from the same period by the average number of P_1 ♀♀.
a = medium also contained 2% sorbitol: 2% agar
b = medium also contained 15% sorbitol: 2% agar
c = all surviving ♀♀ grouped together in one bottle.

NT - not tested

Fig.4.5a Graph to show effect of yeast concentration on♀ survival with time (sugar source = sucrose)

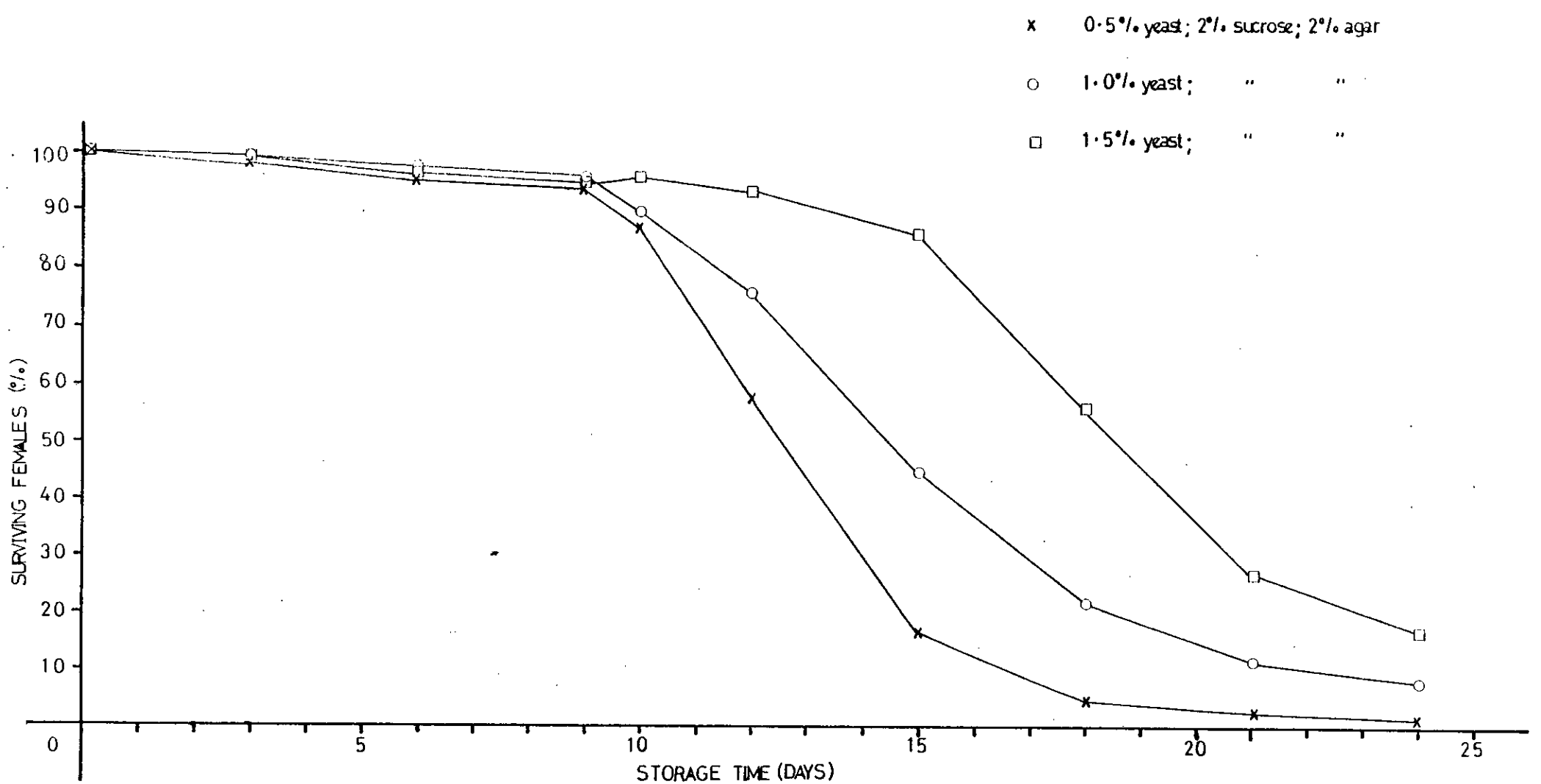
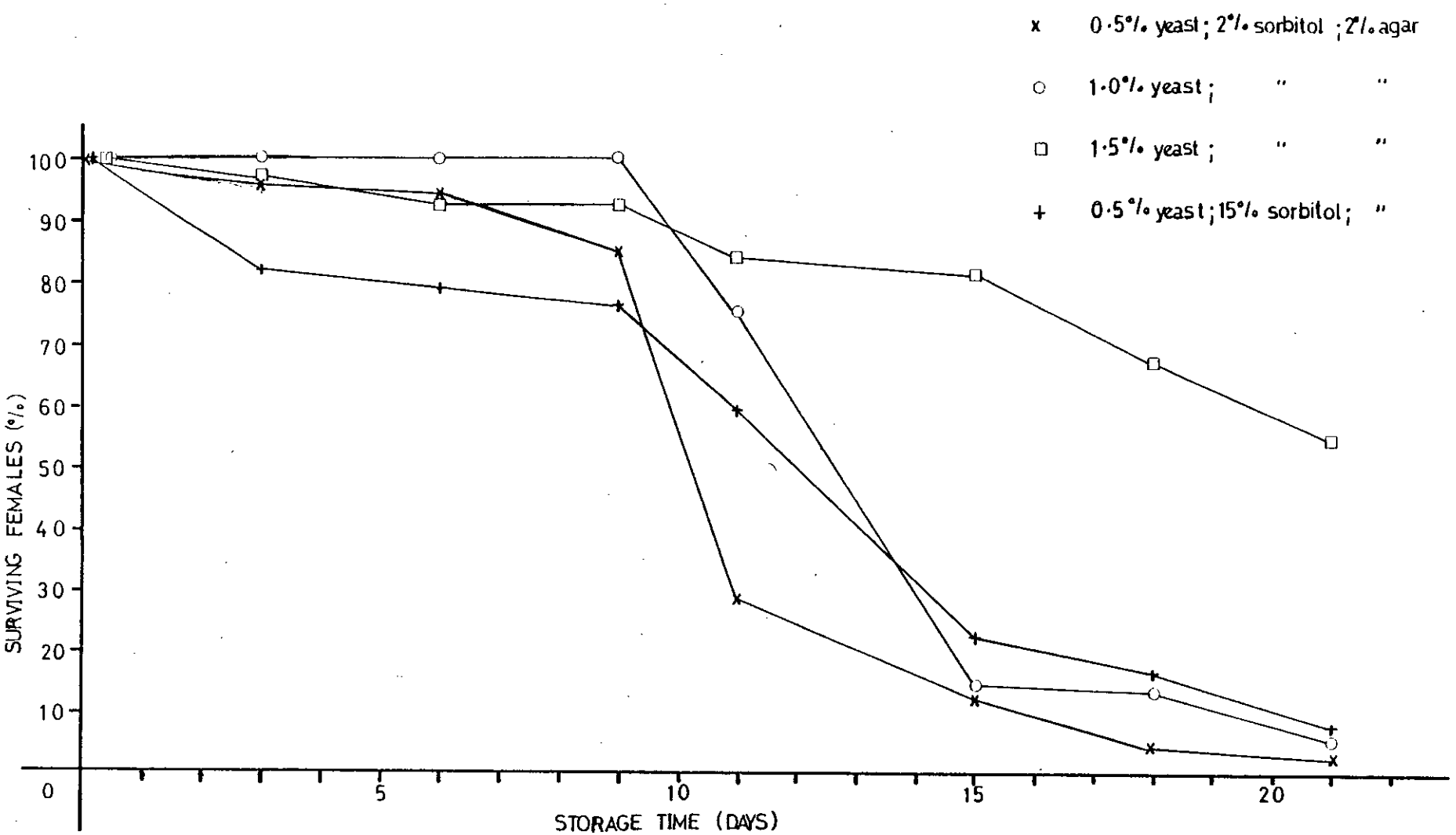


Fig. 4.5b Effect of yeast concentration on survival of inseminated females with time (sugar source = sorbitol)



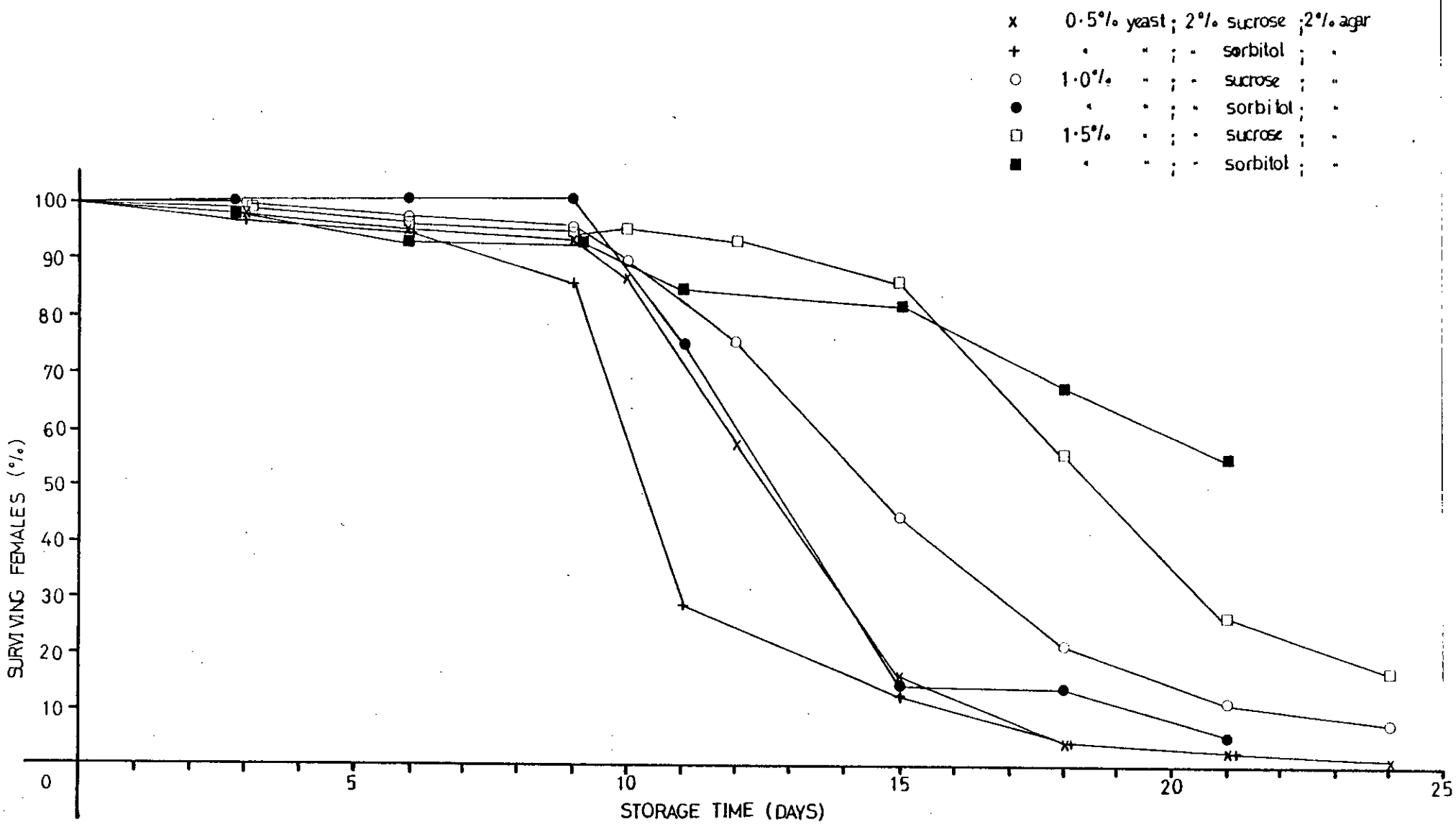
survival); but after this time the survival decreased at all three concentrations - the lower the concentration of yeast, the lower the survival of females at any given time. By day 21, only 2%, 11% and 26% of females were alive at 0.5%, 1.0% and 1.5% yeast respectively.

On the sorbitol low-protein media, differences in survival at the different yeast concentrations become apparent by day 3: for the first 9 days of storage, 1.0% yeast gave the best survival. After day 9 there was a sharp decrease in survival at all 3 yeast concentrations, 1.5% yeast now gave the best survival. 0.5% yeast: 15% sorbitol gave lower levels of survival than 0.5% yeast: 2% sorbitol from days 1-9, but better survival levels after day 9. By day 21, the levels of survival were 2%, 5% and 54% at 2% sorbitol plus 0.5%, 1.0% and 1.5% yeast respectively; and 7% at 0.5% yeast plus 15% sorbitol.

(b) Effect of Sugar Source on Survival of Inseminated Females

For ease of comparison the data from Tables 4.7 and 4.8 have been replotted in Figure 4.5c. When results for the two sugar sources were compared it could be seen that there was no great difference between survival levels: sucrose showed itself to be marginally more effective at maintaining females, except for the day 18-21 sample, when sorbitol plus 1.5% yeast gave the best survival.

Fig. 4.5c Effect of yeast concentration and sugar source on survival of inseminated females with time



(c) Effect of Yeast Concentration and Sugar Concentration on Suppression of Egg-Laying

Suppression of egg-laying, as shown in Tables 4.7 and 4.8, and Figures 4.6a and 4.6b, was greater the lower the concentration of yeast in the food medium.

On the sucrose low-protein food, suppression of egg-laying was equally effective at 0.5% and 1.0% yeast up to day 6 (3.8 F_1/♀), and more so than 1.5% yeast (5.2 F_1/♀). By day 10 it could be seen that as the concentration of yeast increased, the suppression of egg-laying decreased: 4.2 F_1/♀ (0.5% yeast), 6.4 F_1/♀ (1.0% yeast), 9.0 F_1/♀ (1.5%) whereas the survival levels were 52%, 75% and 92% respectively.

On the sorbitol low-protein food, suppression of egg-laying was more effective as the concentration of yeast decreased. By day 10 the following comparison could be made: 5.8 F_1/♀ (0.5% yeast), 7.8 F_1/♀ (1.0% yeast), 12 F_1/♀ (1.5% yeast) with survival levels of 24%, 60% and 83% respectively. 0.5% yeast plus 15% sorbitol appeared to be least effective of all at suppression of egg-laying, with 13.2 F_1/♀ at day 10 and 67% survival.

(d) Effect of Sugar Source on Suppression of Egg-laying

For ease of comparison, the data from Tables 4.7 and 4.8 have been replotted in Figure 4.6c. When suppression of egg-laying was compared for the two sugar sources there was little difference in results obtained at the lowest yeast concentration (0.5%) with either sucrose or sorbitol. However, at 1.0% and 1.5% yeast the sucrose low-protein food

Fig. 4.6a Effect of yeast concentration on fecundity of inseminated females with time
(sugar source = sucrose)

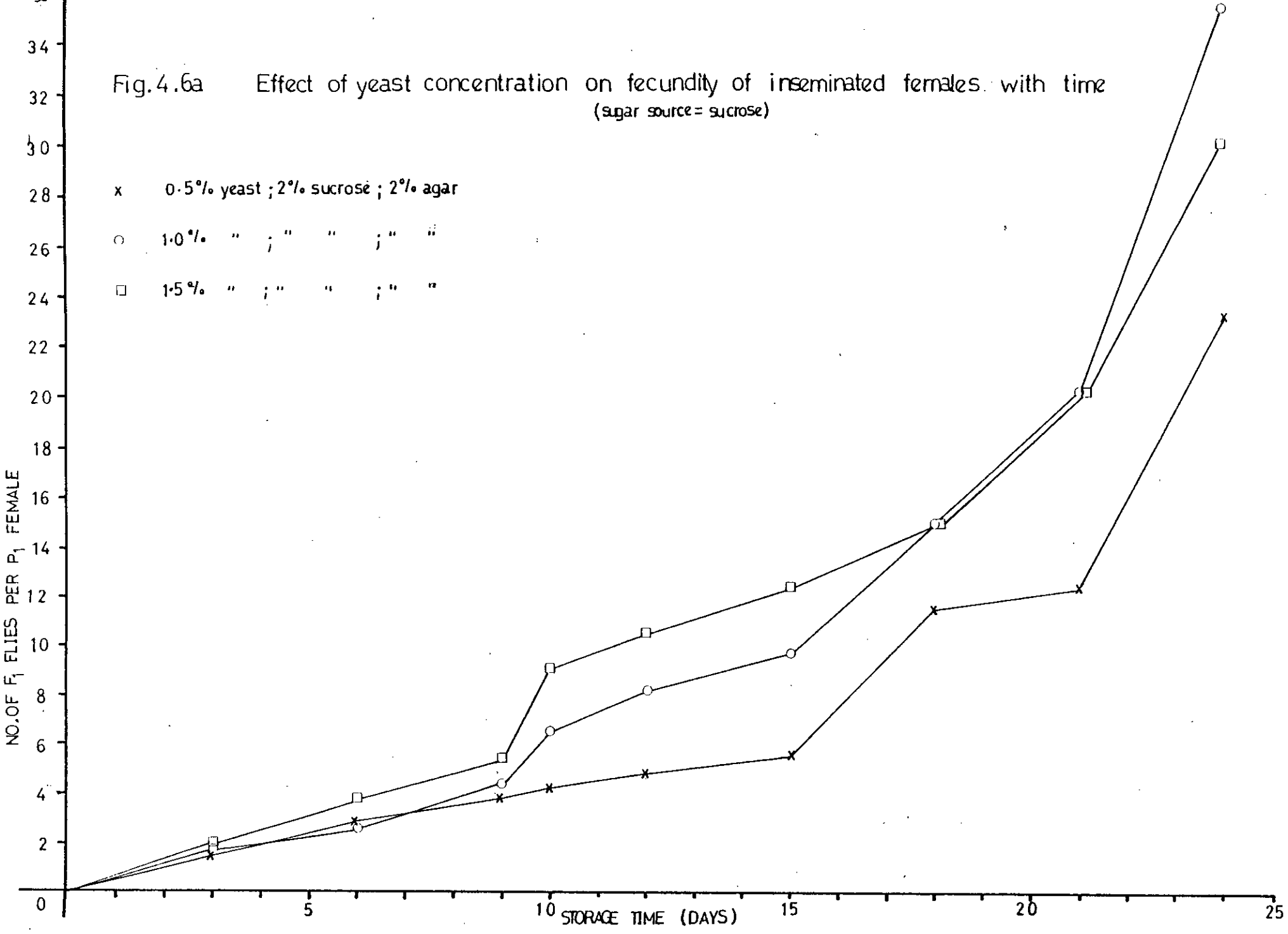


Fig. 4.6b Effect of yeast concentration on fecundity of inseminated females with time
(sugar source = sorbitol)

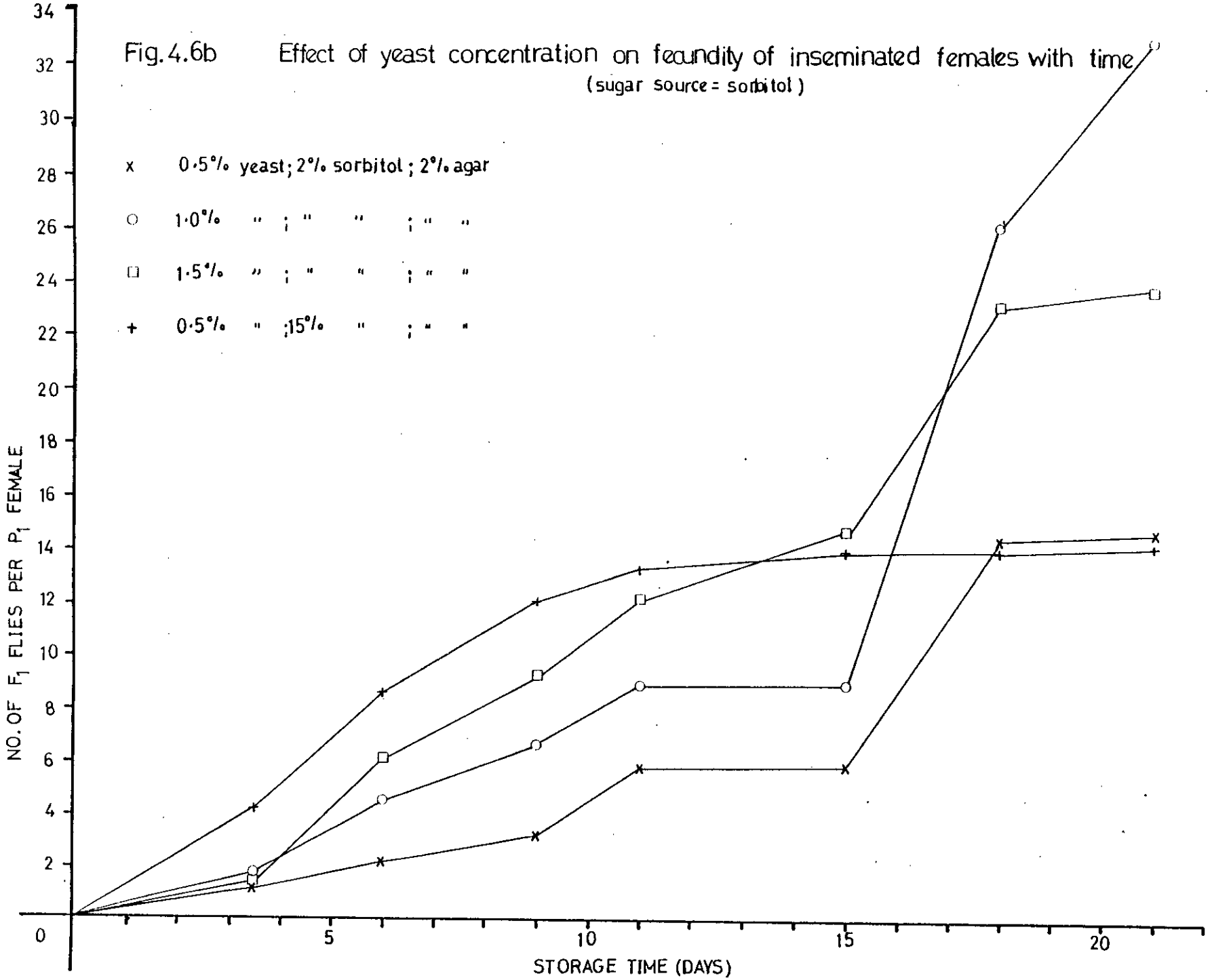
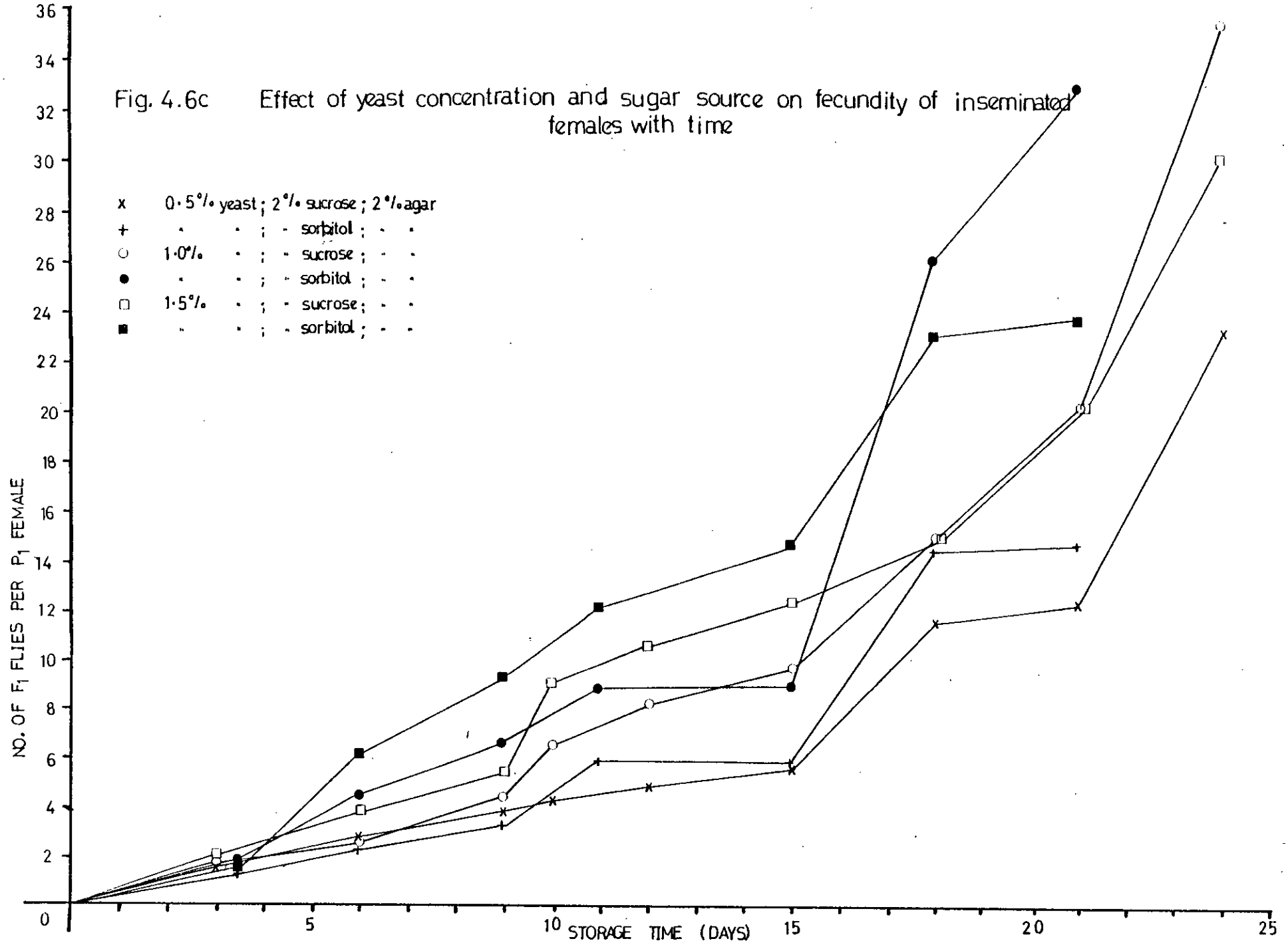


Fig. 4.6c Effect of yeast concentration and sugar source on fecundity of inseminated females with time



appeared to be more effective than the sorbitol low-protein food.

(e) Effectiveness of Sucrose Low-Protein Food and Sorbitol Low-Protein Food as a Storage Medium

Effectiveness as a storage medium would appear to require a compromise between survival of females and suppression of egg-laying. In order to determine which storage medium was best suited to this compromise, a comparison was made between the Day 9 levels of female survival together with suppression of egg-laying. Table 4.9 shows the relevant data. Day 9 values were used because, up to this time, there was little difference between levels of survival and therefore any effects of population density on egg-laying were minimised.

Table 4.9. Comparison of Sucrose Low-Protein Food and Sorbitol Low-Protein Food in terms of their Suitability as Storage Media

Composition of Food (All contain 2% agar)	Surviving Females (%) on Day 9	Cumulative Number of F_1 flies per female by Day 9
0.5% y: 2.0% s	93	3.8
1.0% y: 2.0% s	95	4.4
1.5% y: 2.0% s	94	5.4
0.5% y: 2.0% sor	90	3.25
1.0% y: 2.0% sor	100	6.75
1.5% y: 2.0% sor	92	9.25
0.5% y: 15% sor	76	12.00

y = yeast; s = sucrose; sor = sorbitol.

When the storage media were ranked according to how effective they were at ensuring survival then 2% sucrose plus yeast was more effective than 2% sorbitol plus yeast; 15% sorbitol plus yeast was the least effective. The same ranking was produced if the media were ordered for their effectiveness at suppressing egg-laying. Thus sucrose low-protein food was more suitable than sorbitol low-protein food as a storage medium.

(f) Effect of Honey Concentration on Survival of Inseminated Females

Table 4.10 shows that survival was equally good on 5% and 10% honey up to day 12, after which 5% honey showed a decline in its ability to maintain females. 10% honey showed a less steep decline, maintaining the highest number of females up to day 19. 15% honey, while being less good during the initial periods of storage showed a more constant rate of decline in its ability to maintain females; by day 22 it yielded highest survival levels, and maintained its superiority until the end of the experiment (Fig. 4.7).

(g) Effect of Honey Concentration on Suppression of Egg-Laying

Suppression of egg-laying, as shown by Table 4.10 and Figure 4.8 shows that the lower the concentration the greater the suppression of egg-laying (as measured by the number of F_1 flies). After day 12, none of the females on any of the three honey foods laid fertile eggs. The numbers of eggs laid were considerably lower than on either of the two food media tested before, reaching a total of

TABLE 4.10. Effect of honey concentration on survival of inseminated females and suppression of egg-laying with storage time.

Day Number	Concentration of honey	5%[a]		10%[a]		15%[a]	
	Bottle No.	1	2	1	2	1	2
1	n	115	115	115	115	115	115
	%	100		100		100	
1-3	F_1/♀	0.15		0.53		0.55	
4	n	113	112	113	111	102	108
	%	98		93		91	
4-6	F_1/♀	0.15		0.95		1.06	
7	n	109	112	109	108	93	101
	%	96		94		84	
7-9	F_1/♀	0.28		0.37		0.40	
10	n	106	109	104	103	89	94
	%	94		90		80	
10-12	F_1/♀	0.07		0.11		0.18	
13	n	94	104	98	101	84	81
	%	86		87		72	
13-15	F_1/♀	0.00		0.00		0.00	
17	n	66	80	79	84	69	72
	%	64		71		61	
17-19	F_1/♀	0.00		0.00		0.00	
20	n	28	37	56	60	57	64
	%	28		40		46	
20-22	F_1/♀	0.00		0.00		0.00	
23	n	11	22	35	50	44	54
	%	14		37		43	
26	n	4	11	18	26	24	37
	%	7		19		27	
29	n	2	5	9	16	9	24
	%	3		11		14	

n = number of live females
% = % of number of day 1 females, to the nearest whole number.
F_1/♀ = calculated by (i) determining the average number of P_1 ♀♀ alive during the 3-day egg-laying period, and (ii) dividing the total number of F_1 flies hatching from the same period by the average number of P_1 ♀♀.
a = medium also contained 2% agar.

Fig. 4.7. Survival of inseminated females on honey-agar food

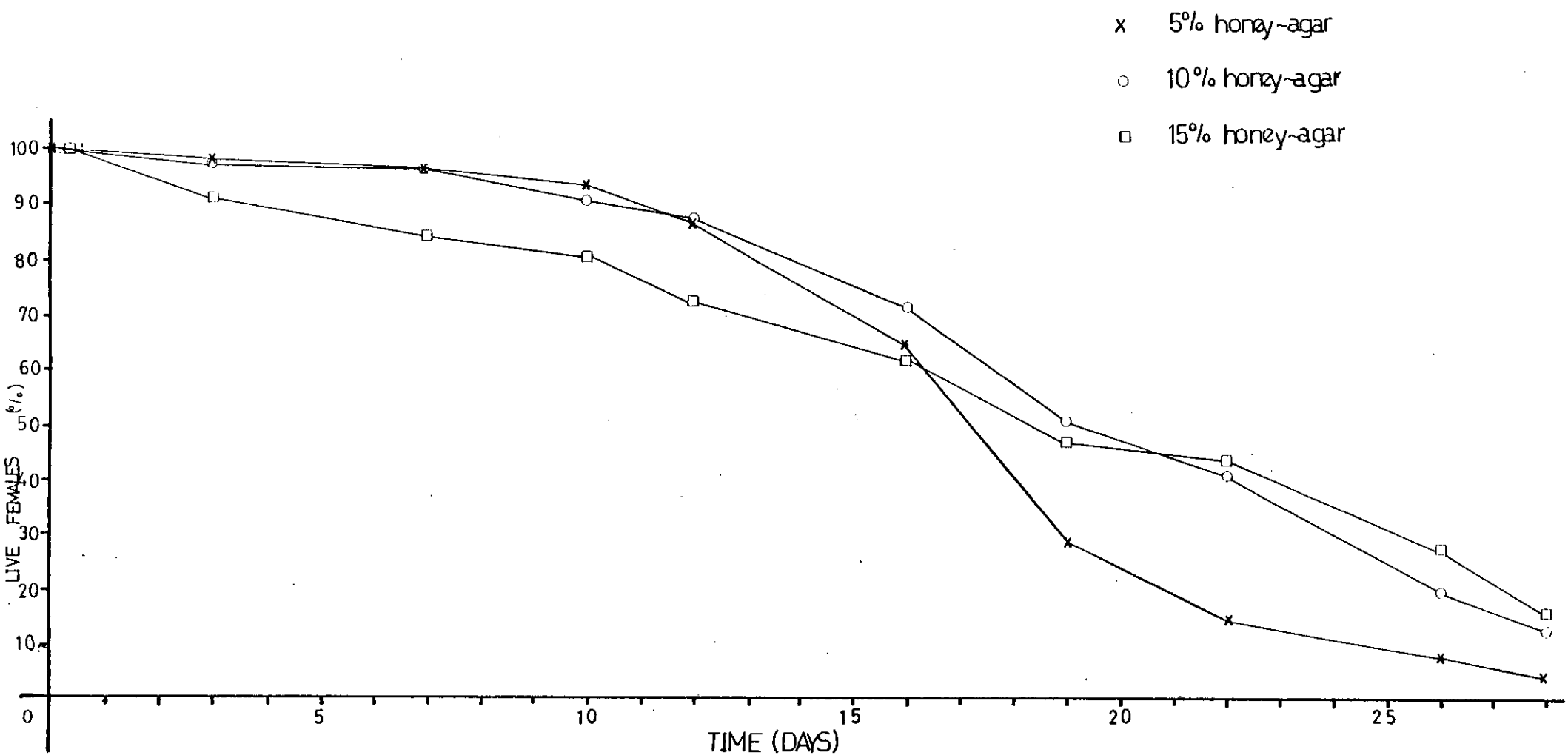
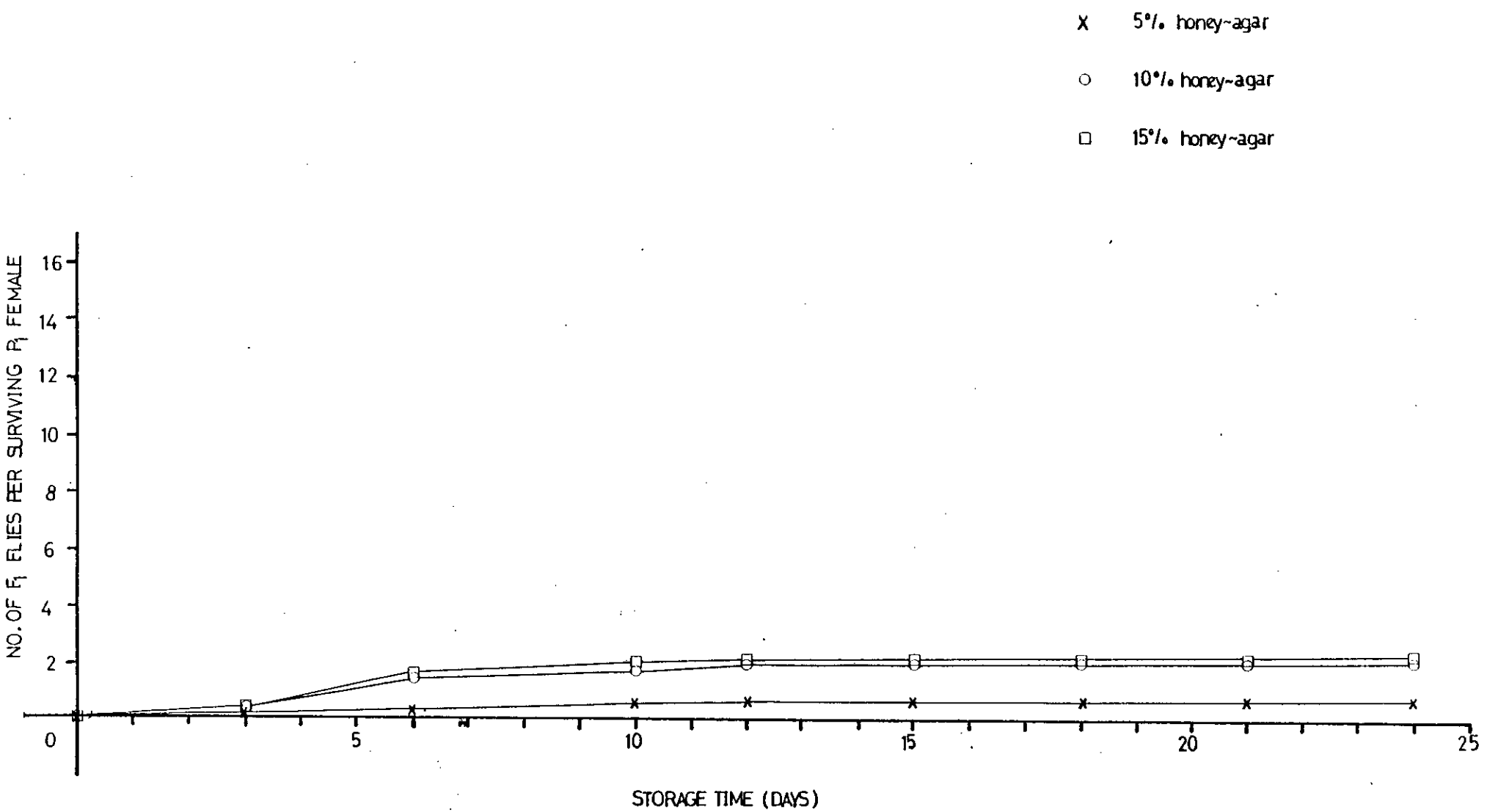


Fig. 4.8. Effect of honey concentration on fecundity of inseminated females with time



0.6, 2.0 and 2.2 F_1/♀ for 5%, 10% and 15% honey-agar respectively.

Table 4.11. Comparison of Sucrose Low-Protein Food with Honey-Agar Food in terms of their Suitability as Storage Media.

Composition of food	Surviving Female (%) on Day 9	Cumulative Number of F_1 Flies per female by Day 9
0.5% y: 2.0% s	93	3.8
1.0% y: 2.0% s	95	4.4
1.5% y: 2.0% s	94	5.4
5% honey-agar	94	0.4
10% honey-agar	92	1.7
15% honey-agar	81	2.0

y = yeast; s = sucrose.

The results of these food tests show that honey-agar medium was as good as both sucrose- and sorbitol-low protein foods in maintaining females for extended periods of storage. However, Table 4.11 shows that 5% of the honey-agar medium was the most effective at suppressing egg-laying. In order to establish whether or not such stored females would resume fertile egg-laying when the were returned to SDM, an experiment was set up in which ♀♀ were mated to Ork ♂♂ treated with EMS. The matings were set up on SDM, males being left with the females for Days 1-3. The males were then removed and the females placed on 5% honey-agar for 8 days, which was expected to give ∿95% survival of females, a percentage

high enough to give adequate ♀♀ to participate in genetic tests.

Table 4.12 shows the results obtained when inseminated females were stored for 8 days on 5% honey-agar medium.

Table 4.12. Effect of Storage on 5% Honey-agar on the Fertility of Inseminated Females

Time after Mating (Days)	Survival of Females (per bottle)	No. of F_1 flies hatching (per bottle)	No. of F_1 flies per female
0-3	95	729	7.5
3-11	Females 'stored' for 8 days on 5% HAM		
12-15	92	363	4[c]
16-20	<92[a]	776	>8.5[b,c]
21-26	<92[a]	700	>7.5[b,c]
27-33	<92[a]	215	>2.5[b]
Total F_1		2783	

[a] Survival levels were not recorded after the day 12-15 sample because it was preferred not to use anaesthetic in order to count the females. It is necessary to use anaesthetic to count flies kept on SDM because dead flies are not readily detected.

[b] These values are (under-) estimates based on the surviving number of females in the day 12-15 bottles.

[c] This value gives a measure of suppression of egg-laying, the higher the number of F_1/♀, the greater the suppression during storage.

These results showed that:

(a) Survival on 5% HA after 8 days was 96.84%.

(b) After 8 days storage on 5% HAM, the fertility of the

females was not impaired when they were returned to SDM.

(c) There was a "lag" phase when they were returned to SDM, but after 3 days, the fertility (and thus fecundity) of the females had returned to the level at which it was observed pre-storage.

(d) The numbers of F_1 flies obtained per bottle were sufficient to carry out meaningful genetical tests.

This method of storage, mating females on SDM for days 1-3 followed by 7-9 days on 5% honey-agar (depending on the survival of the females) to suppress egg-laying before returning the females to SDM to encourage egg-laying, was the method used in the remaining storage experiments. Although a storage food capable of keeping sufficient numbers of fecund females alive for up to 25 days had not been found, this method permitted sampling of significant numbers of stored sperm, as F_1 flies, for genetic tests.

5% honey-agar medium will be referred to as HAM in subsequent chapters of this thesis.

CHAPTER 5. ETHYLMETHANE SULPHONATE

ETHYL METHANE SULPHONATE

5.1 INTRODUCTION

A. Reaction with DNA

Ethyl methonesulphonate (EMS), a sulphonic acid ester has the chemical formula

$$CH_3 - CH_2 - O - SO_2 - CH_3.$$

A monofunctional alkylating agent, it has been shown to be mutagenically effective in a wide range of organisms, e.g. Drosophila (Fahmy and Fahmy, 1957; Alderson, 1964, 1965), barley (Heslot et al., 1959; Favret, 1960; Nilan, 1964), maize (Amano and Smith, 1965), Habrobracon (Lobbecke and von Borstel, 1962), yeast (Loprieno, 1966), Neurospora (Kølmark, 1956), bacteria (Loveless and Howarth, 1959; Schwartz, 1963), bacteriophage (Loveless,1955; Krieg, 1963) and mouse (Cattanach et al., 1966).

Its reaction with DNA has been well-studied. Like DES, EMS acts via an intermediate Sn1/Sn2 mechanism. This is reflected by the value of their Swain-Scott constants: for EMS, s = 0.67, while for DES, s = 0.65. The distribution of ethyl products formed in DNA treated with DES or EMS is similar, as shown in Table 5.1.

Typically sn2 agents yield two major products: 7 alkyl-guanine and 3 alkyl-adenine; plus two minor products: 3 alkyl-guanine and 7 alkyl-adenine. These lesions are not thought to be mutagenic per se. Trace quantities of several other species are also detectable: O^6 alkyl-thymine and O^4 alkyl-thymine. These two lesions have been

TABLE 5.1. Primary alkylation pattern of Hela DNA treated with either DES or EMS. (Sun and Singer, 1975).

Derivative	% ethylation (in vitro)		% ethylation (in vivo)	
	DES	EMS	DES	EMS
1-Et-A	2	2	0.3	0.1
3-Et-A[a]	10	4.4	4	2.2
7-Et-A[a]	1.5	1.4	0.5	0.7
3-Et-G[a]	0.9	0.7	0.2	0.4
O^6-Et-G	0.2	0.2	1.6	0.3
7-Et-G[a]	67	75	71	81
3-Et-C[a]	0.7	0.8	0.5	0.1
3-Et-T	ND	ND	0.2	0.1
ethyl phosphate	16	13	20	8
unidentified	1	2.5	2	7

ND not detected

a alkylation at these sites destabilises the glycosidic linkages

implicated in the production of point mutations. Sn_1 agents, on the other hand, also produce O^6 alkyl-guanine as a major reaction product. Vogel and Natarajan(1979a) found that EMS was more mutagenic (in terms of inducing s1r1) than a comparison of its s-value with other monofunctional alkylating agent would indicate. By the same means it was also found that it was less effective at breaking chromosomes

than its s-value would indicate (Vogel and Natarajan,1979a and b). This indicated that it was not merely reactivity of an agent, determined by its s-value, which determined the mutagenic response which it induced.

B. Dosimetry

EMS is the only chemical mutagen for which an exact dosimetry has been developed for Drosophila. Aaron and Lee (1978) used ^{3}H -labelled EMS to determine the dose of EMS received by mature sperm in terms of alkylations/nucleotide. They also determined the frequency of slrl induced by each dose. The results they obtained are shown in Table 5.2. These results provided good evidence for a linear relationship between alkylation/nucleotide and the frequency of slrl induced in mature sperm of Drosophila.

TABLE 5.2. Relationship between alkylations/nucleotide and sex-linked recessive lethal frequency.

Dose (ethylations/ sperm cell x 10^5)	Sex-linked recessive lethals	
	n	%
0.63	42/6296	0.55
2.33	113/5549	1.9
4.30	102/2540	3.9
43.00	1028/2390	43.00
Controls (accumulated)	34/27064	0.12

(Aaron & Lee, 1978)

The linear relationship held true for both low and high doses of EMS suggesting that there was no change in the mechanism of mutagenesis. The well established practice of using slrl as a "standard" for comparing administered doses of mutagens would thus appear an appropriate one to have selected. The frequency of slrl can be said to be a biological dosimeter of use in comparative studies of EMS mutagenesis.

Results obtained by Aaron and Lee (1978) also confirmed the finding of Fahmy and Fahmy (1957) that, in a brood analysis of EMS-treated Drosophila males, post-meiotic germ cells (sperm and spermatids) were more sensitive to mutation induction, as measured by the frequency of slrl, than meiotic and pre-meiotic germ cells (spermatocytes and spermatogonia). The earlier the germ cell stage at the time of treatment, the lower was the mutational response. Aaron and Lee (1978) found that mature sperm and late spermatids retained a high level of alkylation of DNA; much lower levels were retained in cells which had been treated as early spermatids or spermatogonia. No information exists on translocation induction in spermatids, meiotic and spermatocyte stages although these stages appear to be the most susceptible to dominant lethal induction by EMS (Vogel et al., 1981). However, this effect may be due to sterility rather than to chromosome breaks (Aaron and Lee, 1977; Lee, 1976).

The most effective way of administering EMS to adult

Drosophila ♂♂, in terms of inducing the highest frequency of slrl for a given administered dose, is by feeding rather than by injection (Lim and Snyder, 1968; Hotchkiss and Lim, 1968). Legator et al. (1976) suggested that this method results in penetration of the mutagen to the testes via the spiracular-tracheal system rather than via the digestive tract. An experiment by Vogel and Natarajan (1979) emphasises why care needs to be taken when equating dose with exposure: they found a non-linear response of exposed dose to slrl above a certain dose of EMS and showed that this was due to feeding-rejection behaviour.

C. Genetic Effects Induced by EMS

In terms of inducing slrl, EMS has been shown to be a potent mutagen in Drosophila (Alderson, 1964, 1965; Fahmy and Fahmy, 1957; Vogel and Natarajan, 1979a). In contrast, EMS seems less able to induce chromosome breakage, a high frequency of slrl being a prerequisite for detecting translocations, as shown by the data in Tables 5.3 and 5.4. An interaction of the effects of gamma rays with those of EMS has been demonstrated in Drosophila sperm (Sharma and Grover, 1970). The frequency of gamma-ray induced translocations was doubled by post-treatment with EMS, even thought the same dose of EMS on its own produced no translocations. The simplest explanation for this was that supermunery gamma-ray breaks formed additional translocations with single EMS breaks which otherwise would have been lost as dominant lethals, or repaired

TABLE 5.3 Induction of X-linked recessive lethal mutations and II-III translocations in spermatozoa exposed to EMS (Vogel and Natarajan, 1979a).

Sampling period (days)	slrl		Translocations		slrl		Translocations	
	n	%	n	%	n	%	n	%
0 - 2	32/499	6.4	1/396	(0.25)	109/453	24.1	0/508	0
2 - 5			0/360	0			0/523	0
5 - 7			0/415	0			6/538	1.12
7 - 10			1/517	(0.19)			1/184	0.54
10 - 13			0/200	0			3/43	6.95
2 - 13			1/1492	(0.07)			10/1288	0.78[d]
5 - 13			1/1132	0.09[a]			10/765	1.31[d]
0 - 1	114/536	21.3	0/383	0	98/561	17.5	0/309	0
1 - 4			1/361	(0.28)			0/316	0
4 - 6			1/111	(0.90)			0/92	0
6 - 8			0/25	0			0/56	0
8 - 11			0/10	0			2/41	4.9
1 - 11			2/507	0.39[b]			2/505	0.40[b]
4 - 11			1/146	(0.68)			2/189	1.06[c]
0 - 1	143/554	25.8	0/326		156/549	28.4	1/276	0.36
1 - 4			0/353				1/276	0.36
4 - 6			1/271	(0.37)			0/48	0
6 - 8			3/74	4.05			1/27	3.7
8 - 11			3/20	15.00			1/13	7.7
1 - 11			7/718	0.97[d]			3/431	0.7[d]
4 - 11			7/365	1.92			2/88	2.27

The results were significant at the following levels of probability:
a $p > 0.05$; b $0.01 \leq p < 0.05$; c $p \leq 0.01$; d $p < 0.01$

TABLE 5.4. Storage effect on translocations (induced by EMS)
(Ikebuchi and Nakao, 1979).

slrl		Translocations (%)				
n	%	D1-3	D4-6	D7-9	D10-12	D13-15
98/1324	7.06	0.00 (0/1250)	0.09	0.00 (0/870)	0.00 (0/789)	0.00 (0/447)
51/519	9.83	0.00 (0/812)	0.00 (0/899)	0.00 (0/662)	0.00 (0/319)	NT
179/1177	15.21	0.09 (1/1144)	0.12 (1/825)	0.00 (0/575)	NT	NT
286/1149	24.89	0.19 (2/1051)	1.03 (9/871)	1.93	NT	NT
380/1154	32.93	0.20 (2/992)	3.01 (23/763)	7.20 (27/375)	3.47 (5/144)	8.82 (3/34)

NT = not tested

in the zygote. Abrahamson et al. (1969) and Hotchkiss and Lim (1968) did not detect translocations in sperm which had received a dose of EMS inducing ∿20% slrl; Watson (1972), Ikebuchi and Nakao (1979) and Vogel and Natarajan (1979) did not detect translocations below a dose of EMS inducing ∿15% slrl. Evidence has also been obtained to show that mature spermatozoa of Drosophila are very resistant to the induction by EMS of dominant lethal and viable chromosome breakage (except after high doses of EMS) (e.g. Vogel et al.,1981). These data suggest that there is a threshold effect for the induction of translocations which is much higher than that for slrl.

In Drosophila, a storage effect has not yet been reported for the induction of slrl by a monofunctional alkylating agent. Pronounced storage effects on EMS-induced translocations have been demonstrated (Abrahamson et al., 1969) but only at very high doses: >24% slrl (Ikebuchi and Nakao, 1979), >17% slrl (Vogel and Natarajan, 1979). Tables 5.3 and 5.4 show the results of Vogel and Natarajan (1979) and Ikebuchi and Nakao (1979), respectively. No evidence has yet been obtained for storage effects at low doses of EMS - the question remains open as to what extent EMS is capable of producing breakage type damage at and below a dose of ∿6% slrl (∿2.3×10^{-3} alkylations/nucleotide).

Dominant lethals have not been shown to increase with storage after a dose of 6.1% slrl or 2.3×10^{-3} alkylations/nucleotide (Aaron and Lee, 1977). A dose of

38% slrl or 1.4×10^{-2} alkylations/nucleotide gave only a slight increase in dominant lethal frequency. Ikebuchi and Nakao (1979) found no storage effect on the relative dominant lethal (RDL) frequency at a dose of 8.79% slrl but a pronounced storage effect at doses of 24.89% and 25.85% slrl. Using high concentrations of EMS, Sram (1970b) for dominant lethals, Bishop and Lee (1969) and Schalet (1977) for sex-chromosome marker losses obtained evidence of a storage effect. It appears evident that both inviable (sex-chromosome marker losses and dominant lethals) and viable (translocations) chromosome breakage events are only induced when high doses of EMS are administered to the germ line.

This evidence lends further support to the hypothesis that EMS-induced breaks result from a mechanism distinctly different from that induced in the production of at least a major part of forward mutations, as measured by slrl.

Vogel and Natarajan (1979) found a positive relationship between the primary alkylation pattern of DNA and the ability of an agent to break the chromosomes of Drosophila. The implication made was that the ratio of O^6 to N7 alkylations may be of importance: O^6 lesions being implicated in base pair changes by mispairing, N7 lesions resulting in an increased rate of base hydrolysis which could lead to DNA strand breakage. However, the mechanisms by which each type of mutational event occurs are still unknown.

Although EMS is known to produce slrl with ease in the DNA of mature Drosophila sperm, this class of mutations is a heterogeneous one. EMS is very efficient in both multiple and specific locus tests, and there is evidence to believe that these mutations are predominantly the result of intralocus mutations. The evidence is provided by the following results. In complementation tests using EMS-induced recessive lethals in cytologically well-defined regions which had been "saturated" for lethal producing loci, Hochman (1971) found that 74/75 lethals on chromosome 4 affected only a single complementation unit. Lim and Snyder (1974) found that all 83 lethals in the zeste-white region of the X-chromosome affected only a single complementation unit. Similarly, Bishop and Lee (1974) found that 83 mutations on the X-chromosome at the yellow or white locus were intragenic charges. Shellenbarger (1972) found that 6 white recessive visible mutations were all intragenic. Lim and Snyder (1968) cytologically examined salivary gland chromosomes of Drosophila larva and found that of 45 EMS-induced lethals, none of them could be detected as band deletions. B.J. Judd (1974) found no deletions in 40 EMS-induced lethals. Rudkin (1965) determined that $\sim$5 kbp made up the smallest measurable band in Drosophila salivary gland chromosomes; this therefore represents the lower limit of resolution of salivary gland chromosome analysis.

On the other hand, Lifshytz and Falk (1969) detected

a low frequency of EMS-induced lethals affecting more than one complementation unit. Some of these were confirmed cytologically as interstitial deletions involving the loss of at least 10 salivary chromosome bands. There are also other reports that deletions of this size have been detected among recessive lethals induced by EMS in the proximal regions of the X-chromosome and the left arm of chromosome II (Schalet and Lefevre, 1973; Wright et al. 1976). In genetic tests carried out by Shukla and Auerbach (1981) they estimated that at least 60% of EMS-induced point mutations were small dele-tions extending from one locus into another. An alternative interpretation of their data was that EMS tended to produce clusters of linked mutations.

In the experiments of Lifshytz and Falk (1969) the production of deletions may have been influenced by the proximity of the centromere. Bishop and Lee (1973) found mutants detected as yellow, achaete in offspring of treated ♂♂ in which the tip of the X-chromosome (where these mutants were located) had been transposed to the Y chromosome or to the neighbourhood of the centromere. This suggested that neighbouring heterochromatin may enhance the ability of EMS to produce chromosome breaks which may favour the production of small deletions.

When all the results are considered together, most workers are of the opinion that deletions and gross structural changes are extremely rare among EMS-induced recessive lethal mutations.

5.2 THE AIMS OF THE EMS EXPERIMENTS

The aims of this work were fourfold:

i) Firstly to determine the kinetics of appearance of translocations between markers on chromosomes II (bw) and III (st) for different doses of EMS as determined by slrl frequency and thus by ethylations/nucleotide.

ii) To relate the frequency of translocations detected to an estimated number of depurinations of guanine bases which had been alkylated at the N7 position. It is known that the rate of depurination increases with an increase in temperature. Therefore it should be possible to determine if there is a temperature effect on the rate of appearance of both translocations and slrl: only those mutations caused by depurination should be enhanced by storage at a higher temperature.

iii) To determine the kinetics of appearance of single-breakage events as measured in dominant lethal tests.

iv) Finally, because slrl are a heterogeneous class of mutations, an attempt was made to examine this class of mutations and determine the proportion of lethals caused by small deletions. These small deletions, if due to a breakage event, should also be subject to a storage effect.

5.3 KINETICS OF APPEARANCE OF TRANSLOCATIONS

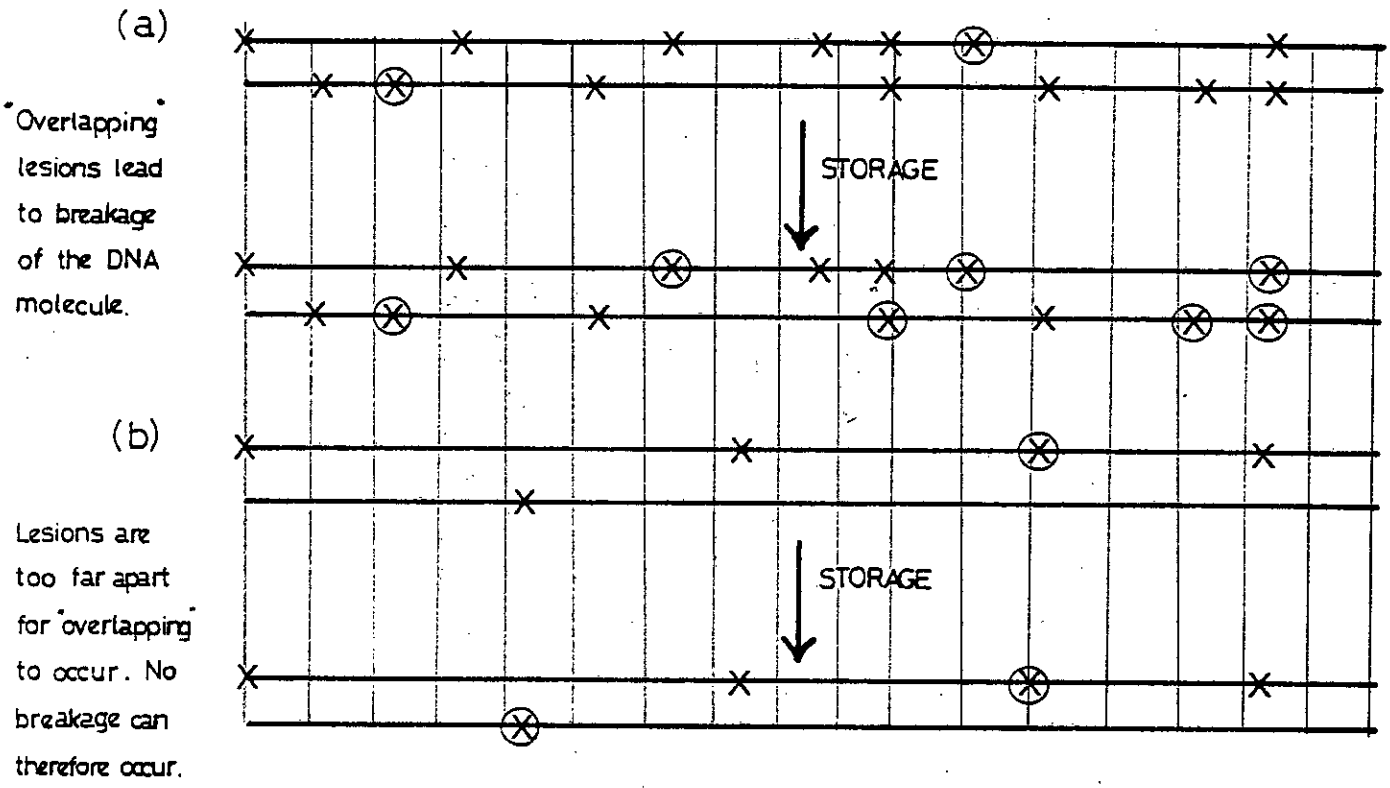
A. A Working Hypothesis

Translocations have not been detected at low doses of EMS, even after sperm storage. It was decided to extend the storage time for a low dose of EMS (inducing ~6% slrl) in order to maximise the likelihood of detecting breakage events. If single breaks are induced with a low frequency (2 single breaks are a pre-requisite for a translocation) then it may be possible to detect them as dominant lethals. Dominant lethals are considered to be unrejoined single breaks; translocations are caused by two breaks which interact. An unrejoined single break may stand a greater chance of detection than a translocation.

X-rays have been shown to elicit a linear response between slrl frequency, autosomal lethals and sex-linked visibles and dose of irradiation (Muller, 1940).

The production of chromosomal rearrangements such as translocations increases as the square of the dose of irradiation: each rearrangement requiring two independent hits, leading to two independent breakage events which can then interact (see page 4 ff). Single-hit dose response curves for slrl and two-hit response curves for translocations have also been established for mustard gas, a polyfunctional alkylating agent, in Drosophila. EMS has also been shown to elicit a linear dose-response curve (Aaron and Lee, 1978) for the production of slrl.

Fig. 5.1 Multi-hit hypothesis for chromosome breakage by EMS



(a) High dose of EMS

(b) Low dose of EMS

X Alkylated base

⊗ Apurinic site

Fine vertical lines represent regions of an unknown number of bases. Simultaneous depurination of two (or more) alkylated bases within the region may lead to chromosome breakage if the depurinations occur on opposite strands. The depurinations could interact if a repair mechanism excised a number of bases greater than half the distance between the two lesions and in the direction of the other lesion.

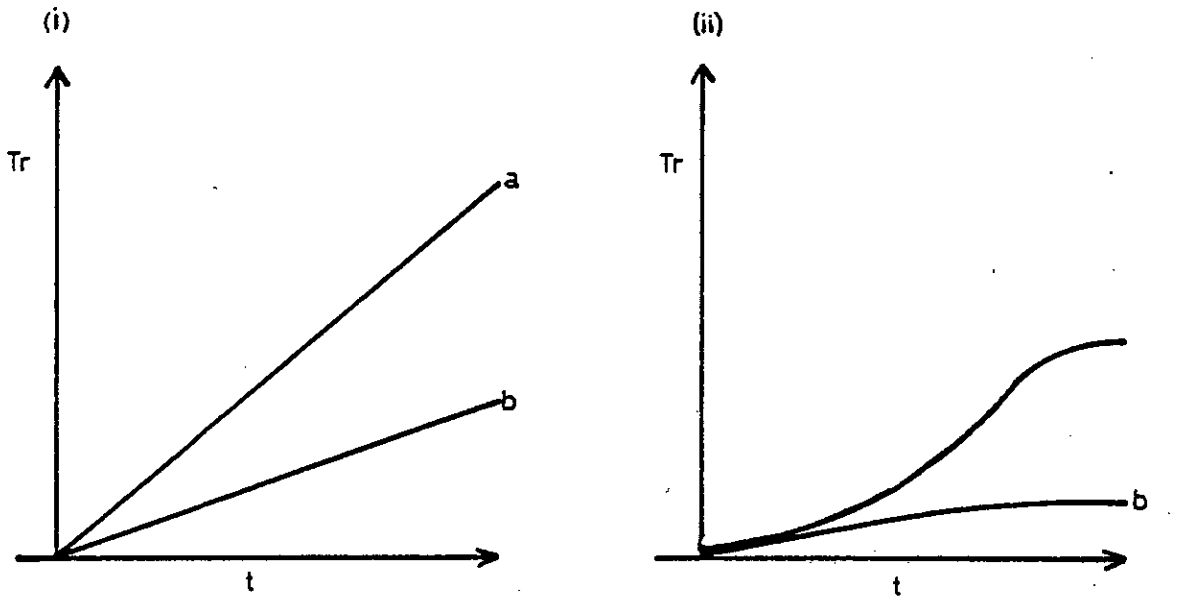
Using the multihit hypothesis it may be possible to explain why translocations are not detected at low doses of EMS.

The mechanism by which EMS causes chromosome breakage is not known. It has been suggested that for DNA with a high degree of alkylation, the sites of depurination may occur proximately enough in the twin strands of DNA to result in scission of the chain (Brookes and Lawley, 1961). This multi-hit theory was opposed by Ronen (1968) who found that a depurination event in one DNA strand of a phage particle with one alkylated and one non-alkylated strand of DNA, was sufficient to cause cell death. He interpreted this as showing that EMS acted via a single-hit mechanism, i.e. that one "hit" (alkylation followed by depurination) leads to strand breakage. This may be more likely for phage DNA which is much less structured than Drosophila DNA.

The multi-hit hypothesis for chromosome breakage is visualised in Figure 5.1. Multi-hit curves can be misleading if repair mechanisms are subject to damage (irrelevant for storage of mature spermatozoa) or to inhibition (of the maternal repair system ?) at high doses (Aaron and Lee, 1978).

An examination of the rate of appearance of translocations with time may reveal something about the mechanism of their formation. Two possible curves resulting from a plot of translocation frequency against time after treatment with either a high or low dose of EMS are shown in Figure 5.2.

Fig. 5.2. Possible curves from a plot of translocation frequency against time.



a High dose of EMS

b Low dose of EMS

i) Linear response - a linear relationship between storage time and induction of translocations. This response might be more difficult to explain since it would imply that some feature of the storage effect increased in direct proportion to the storage time: for both X-ray-induced and mustard-gas-induced translocations a dose-squared relationship has been demonstrated indicating that a translocation requires two breakage events.

ii) Non-linear response - if accumulation of "events" leading to a translocation occurs more rapidly with a higher dose of EMS then this should be reflected

by the slope of the curve and its height. The detection of a threshold would imply that a minimum number of events (depurinations ?) must accumulate before a translocation will be detected either because accumulation of events is slow or perhaps because a repair system can cope with a certain level of damage but above this level mis-repair may occur.

If translocations are detected after storage, the curve obtained from plotting their frequency against storage time may be a valuable indiction of the mechanism by which they arise.

B. Results

An analysis of the kinetics of appearance of translocations was carried out, using the initial frequency of slrl as a measure of dose. The frequency of slrl and translocations detected before and after storage was determined for each dose. A storage effect has already been observed for EMS-induced translocations in Drosophila (Abrahamson et al., 1969). At the chromosome level, this effect is assumed to be due to storage allowing more time for "potential" breaks to open up. The potential break hypothesis (Auerbach, 1949) has already been discussed in Chapter one. A storage effect has not been observed for gene mutations such as visibles (Brink, 1970) and slrl (Snyder, 1963).

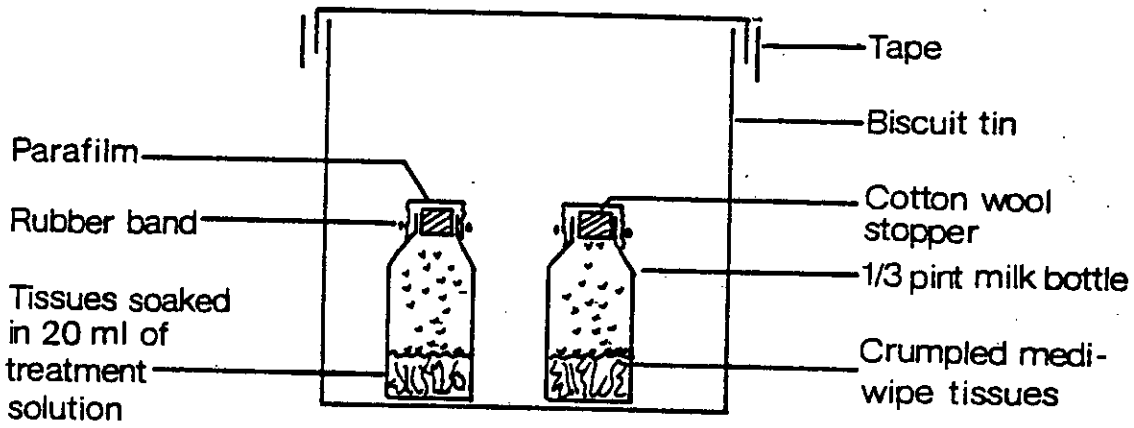


Fig. 5.3 EMS TREATMENT VESSEL

The experimental design for the storage experiments and brood analysis was essentially the same as the one detailed in Chapter 3 (Fig. 3.1) for the DES experiments. The only difference being that HAM was used as the storage medium. The method of treatment (modified from that of Lewis and Bacher, 1968) is visualised in Figure 5.3. The results obtained after EMS treatments are shown in Tables 5.5 - 5.8, and plotted in Figures 5.4 - 5.7.

1. Storage Experiments

(i) 0.1% EMS (Table 5.5, Figure 5.4)

This "dose" of EMS gave a slrl frequency between 7.03% and 8.76%. It can be seen from Figure 5.4 that the slrl frequency remained fairly constant with time, showing no storage effect. Translocations were detected, but not without storage, and then only at low frequencies. Translocations were not detected in more than one stored sample for any of the tests, i.e. all other samples within the test yielded no translocations, even when sperm had been stored for up to 24 days.

(ii) (a) 0.4% EMS (Table 5.6, Figure 5.5)

The dose range covered 13.85% slrl to 15.25% slrl. These results, plotted in Figure 5.5, show that, again, the slrl frequency remained fairly constant with time. No translocations were detected without storage. In both experiments, there was an increase in translocation frequency with time: the frequencies plateaued, suggesting

TABLE 5.5. 0.1% EMS: Storage of Treated Spermatozoa

Sample	Test	Experiment Number					
		EMS1.1	EMS1.2	EMS1.3	EMS1.4	EMS1.5	EMS1.6
D1-3 (Brood 1)	slrl	22/322 7.14%	21/267 7.87%	13/185 7.03%	22/274 8.76%	8/275 6.55%	18/140 12.86%
	Tr	0/556 0.00%	0/538 0.00%	0/406 0.00%	0/506 0.00%		
D4-6	slrl		20/249 8.03%				
	Tr		0/359 0.00%		0/605 0.00%		
D7-9	slrl			8/129 6.20%			
	Tr			0/222 0.00%	0/490 0.00%		
D10-12	slrl		28/222 12.61%				
	Tr		5/544 0.92%	4/420 0.95%	0/408 0.00%		
D13-15	slrl		33/303 10.89%				
	Tr		0/309 0.00%	0/503 0.00%	1/385 0.26%		
D16-18	slrl			7/65 10.77%			
	Tr			0/503 0.00%	0/468 0.00%		
D18-23	slrl					21/285 7.39%	
	Tr						
D23-26 *	slrl						
	Tr				0/392 0.00%		
D27-32 (EMS1.6)	slrl					27/293 9.22%	6/52 11.54%
D29-35 (EMS1.5)	Tr					0/139 0.00%	
Brood 2*	slrl		20/276 7.25%				
	Tr		0/534 0.00%		0/392 0.00%		

* Brood 2 sampled on day 8 (EMS1.2) or day 16 (EMS1.4) by mating EMS-treated ♂ to a succession of ♀ every few days until either 8 or 16 days after treatment. The frequency of slrl and translocations were then determined without storage of sperm.

Fig. 5.4

Storage of sperm treated with 0·1% EMS

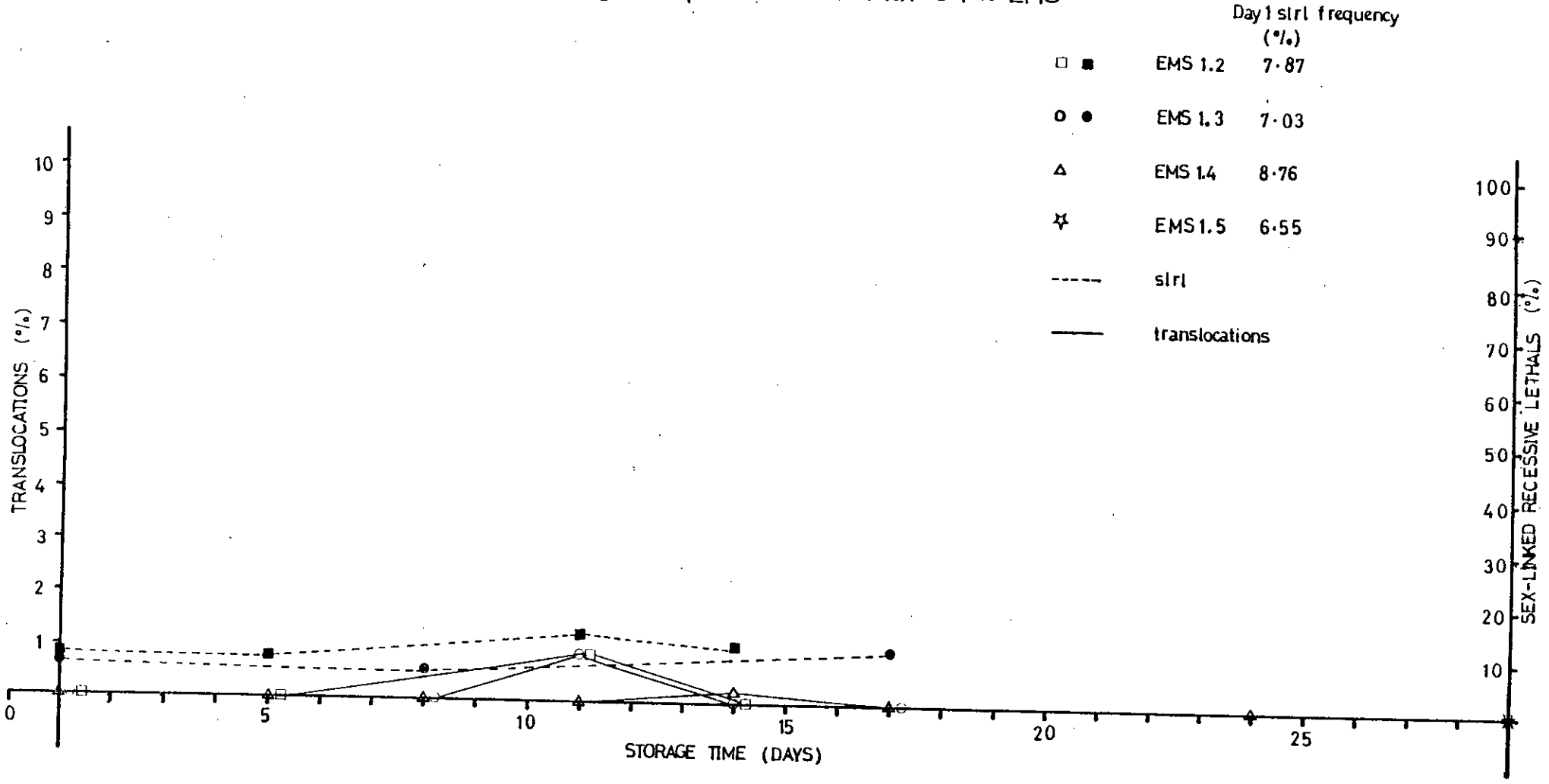
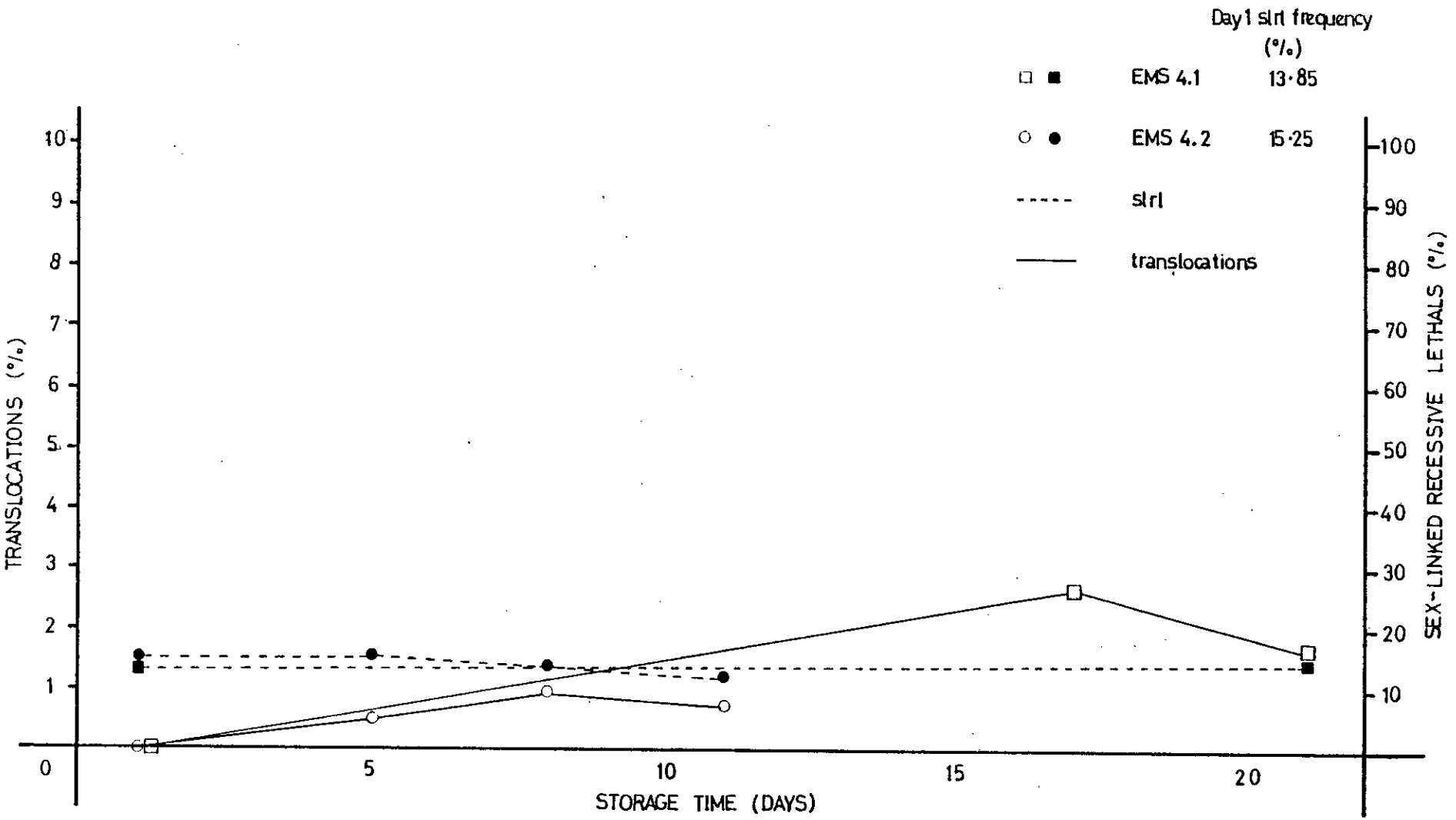


TABLE 5.6 0.4%EMS : Storage of Treated Spermatozoa

Sample	Test	Experiment Number	
		EMS4.1	EMS4.2
D1-3 (Brood 1)	s1r1	32/231 13.85%	39/256 15.25%
	Tr	0/535 0.00%	0/553 0.00%
D4-6	s1r1		40/257 15.56%
	Tr		2/420 0.48%
D7-9	s1r1		33/229 14.41%
	Tr		4/422 0.95%
D10-12	s1r1		26/213 12.21%
	Tr		2/283 0.71%
D16-18	s1r1		
	Tr	6/266 2.65%	
D19-22	s1r1	25/153 15.03%	
	Tr	2/120 1.66%	
Brood 2*	s1r1	2/223 0.90%	
	Tr		

*Brood 2 sampled on day 14 by mating EMS treated ♂♂ to a succession of ♀♀ every few days until 14 days after treatment. The frequency of s1r1 was then determined without storage of sperm.

Fig. 5.5 Storage of sperm treated with 0·4% EMS



that a maximum number of translocations had been obtained.

(b) 0.4% EMS (Table 5.7, Figure 5.6)

This dose range covered the slrl frequency of 18.29% to 24.00%. From the results, plotted in Figure 5.6 it can be seen that the slrl frequency shows no storage effect. The storage effect on the translocation frequency is more pronounced for this dose range.

For three of the doses (18.29%, 20.00% and 23.29%) the storage times were insufficient for the maximum number of translocations to have occurred (10 days, 17 days and 24 days respectively). At a dose of 23.29%, the frequency of translocations reached a maximum at day 24, and declined with further storage.

(iii) 0.75% EMS (Table 5.8, Figure 5.7)

The dose range covered by this dose was 28.66 - 35.40% slrl. The data plotted in Figure 5.7 show that slrl frequency declined slightly with storage. There was a striking increase in the frequency of translocations. It was difficult to obtain fertilised eggs beyond the times of storage shown. This was unlikely to be due to sperm exhaustion - this problem was not encountered at lower doses. One explanation may be that there was so much damage to the sperm DNA at this dose, that after storage, the pronucleus was unable to recover. The results obtained in hatchability tests (Section 5.5) indicate that this may be the explanation for the difficulties encountered both with high doses of EMS and DES.

TABLE 5.7 0.4% EMS: Storage of Treated Spermatozoa

		Experiment Number						
Sample	Test	EMS4.3	EMS4.4	EMS4.5	EMS4.6	EMS4.7	EMS4.8	EMS4.9
D1-2 (Brood 1)	slrl	54/243 22.22%	63/303 20.79%	96/400 24.00%	47/257 18.29%	42/210 20.00%	21/93 22.58%	58/249 23.29%
	Tr	3/384 0.78%	5/524 0.95%	0/413 0.00%	1/582 0.17%	1/555 0.18%	0/452 0.00%	2/384 0.52%
D3-5 (EMS4.6) (EMS4.9) D4-6 (EMS4.7)	slrl				38/266 14.29%			
	Tr				8/560 1.43%	3/472 0.64%		7/583 1.20%
D6-8 (EMS4.9)	slrl					48/187 24.06%		
D7-9 (EMS4.7)	Tr					6/258 2.33%		6/618 0.97%
D9-11	slrl				46/248 18.55%			
	Tr				17/517 3.29%			8/485 1.65%
D12-14 (EMS4.6) D11-15 (EMS4.8)	slrl				60/303 19.80%			
D13-15 (EMS4.7)	Tr					3/306 0.98%	1/52 1.92%	
D15-18	slrl							
	Tr					20/234 8.55%	25/679 3.68%	9/399 2.26%
D18-22	slrl							
	Tr			5/155 3.23%				
D23-26 (EMS4.9)	slrl							
D21-26 (EMS4.8)	Tr						31/689 4.50%	21/247 8.50%
D27-32	slrl							
	Tr						5/376 1.33%	
D33-35	slrl							
	Tr						0/22 0.00%	
Brood 2*	slrl		5/211 2.37%	3/110 2.73%	29/265 10.94%			6/240 2.50%
	Tr							1/440 0.23%

*Brood 2 sampled on day 8 (EMS4.6), day 15 (EMS4.4), day 16 (EMS4.9) and day 18 (EMS4.5) by mating EMS-treated ♂♂ to a succession of ♀♀ every few days until either 8, 15, 16 or 18 days after treatment. The frequency of slrl and translocations were then determined without storage of sperm.

Fig. 5.6.

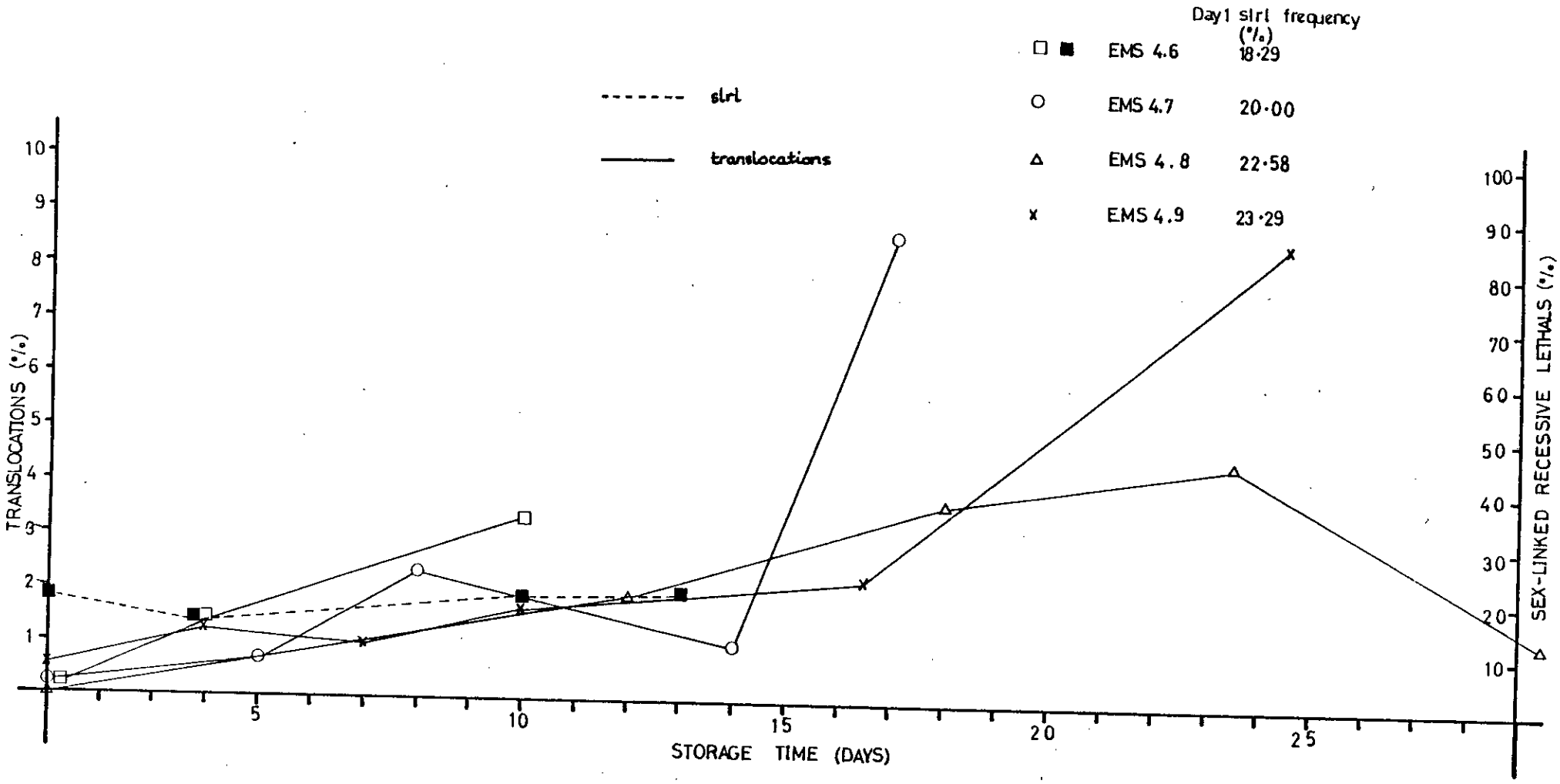

Storage of sperm treated with 0·4% EMS
Day1 slrl frequency (%)
EMS 4.6 18·29
EMS 4.7 20·00
EMS 4.8 22·58
EMS 4.9 23·29
slrl
translocations
TRANSLOCATIONS (%)
SEX-LINKED RECESSIVE LETHALS (%)
STORAGE TIME (DAYS)

TABLE 5.8. 0.75% EMS: Storage of Treated Spermatozoa; Effect of Temperature

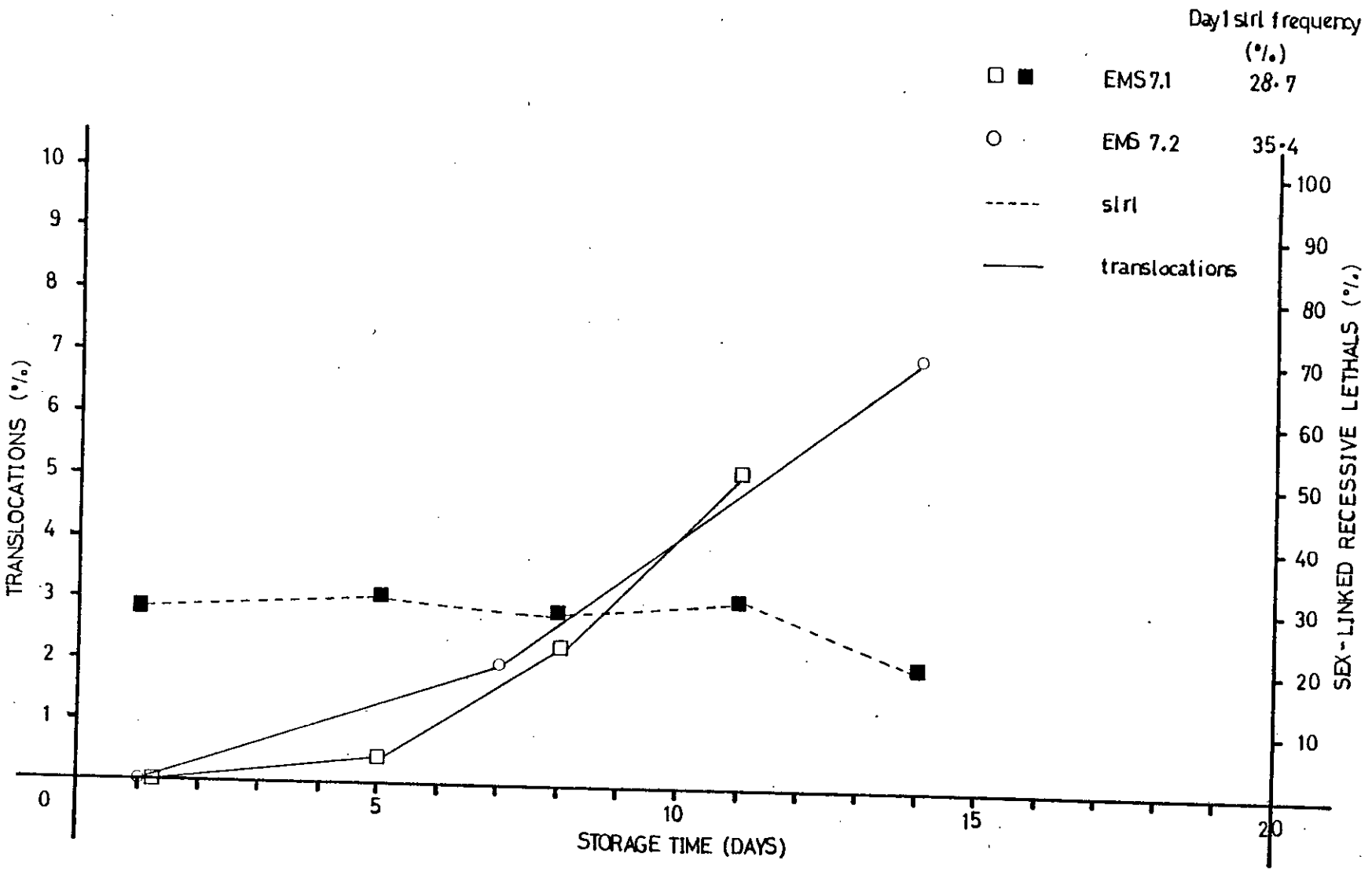
Temperature of storage*		25°C		18°C	29°C
		Experiment Number			
Sample	Test	EMS7.1	EMS7.2	EMS7.3	EMS7.4
Day 1-3 (Brood 1)	slrl	23/65 35.40%	88/307 28.66%	84/291 28.87%	73/227 32.16%
	Tr	0/55 0.00%	0/497 0.00%	4/464 0.86%	0/186 0.00%
D4-6	slrl	NT	72/231 31.17%	NT	32/131 24.43%
	Tr	NT	2/460 0.43%	NT	6/416 1.44%
D7-9	slrl	3/12 25.00%	57/200 28.50%	61/259 23.55%	37/127 29.13%
	Tr	1/50 2.00%	10/434 2.30%	7/482 1.45%	15/349 4.30%
D10-12	slrl	NT	65/211 30.81%	76/286 26.57%	30/103 29.13%
D11-13 (EMS7.4)	Tr	NT	21/408 5.15%	9/441 2.04%	15/276 5.44%
D13-15	slrl	5/9 55.6%	30/148 20.27%	64/251 25.50%	
D14-16 (EMS7.1)	Tr	2/30 7.00%		16/436 3.67%	
D16-18	slrl			47/165 24.48%	
	Tr			20/562 3.56%	
D20-22	slrl			39/132 29.55%	
	Tr			17/423 4.02%	
D23-26	slrl			NT	
	Tr			13/274 4.78%	

	ALL BROOD ANALYSIS CARRIED OUT AT 25°C			
Brood 1		88/307 28.66%	84/291 28.87%	EMS7.3 Brood 2 sperm stored for 14 days
		0/497 0.00%	4/464 0.86%	
Brood 2**		3/110 2.73%	6/244 2.46%	10/264 3.79%
			2/543 0.37%	0/484 0.00%

* All Day 1-3 samples collected at 25°C

** Brood 2 sampled on day 18 (EMS7.2) and day 21 (EMS7.3) by mating EMS-treated ♂♂ to a succession of ♀♀ every few days until either 18 or 21 days after treatment. The frequency of slrl and translocations were then determined without storage of sperm.

Fig. 5.7 Storage of sperm treated with 0·75 % EMS



2. Brood Analysis

The general trend at all doses was for slrl frequency to decrease with successive broods. The later the brood, the lower the frequency. This result was in agreement with the experiments of Fahmy and Fahmy (1957) and Aaron and Lee (1978).

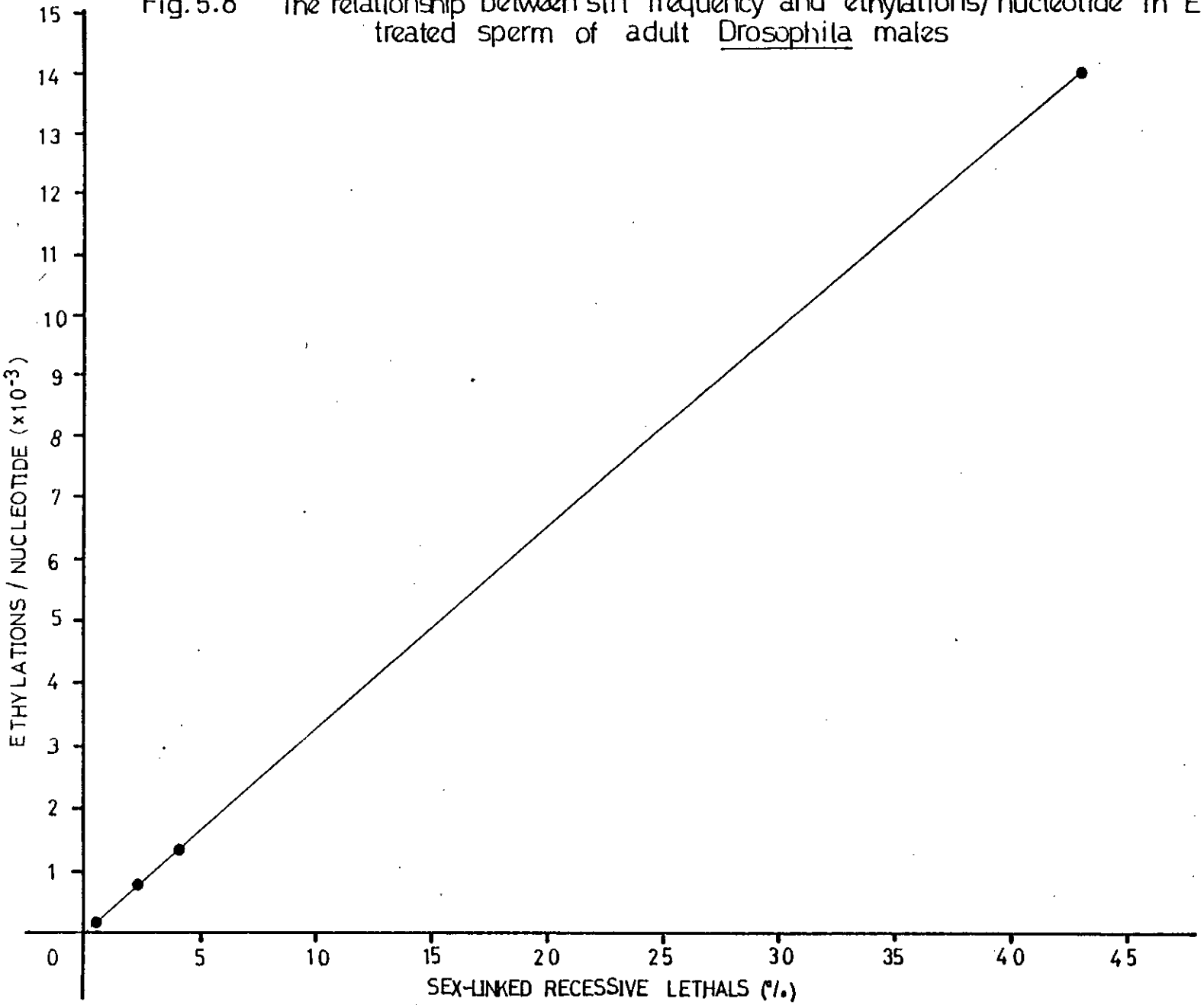
The picture was not so clear for translocations (Table 5.9). At 0.1% EMS (slrl = 6.55 - 8.76%) no translocations were detected in a brood sample on day 8 (EMS 1.2, 0/534) or on day 16 (EMS 1.4, 0/392). At 0.4% EMS (slrl = 18.29 - 24.00%) a frequency of 0.23% translocations was detected in a brood sampled on day 14 (EMS 4.9, 1/440), a frequency of 0.52% translocations (2/384) was detected in brood 1. At 0.75% EMS (slrl = 28.66 - 35.40%) a frequency of 0.37% translocations was detected in a brood sampled on day 18 (EMS 7.3, 2/543), a frequency of 0.86% (4/464) was detected in the brood 1. Apart from EMS 1.2 and 1.4, when no translocations were detected, the translocation frequencies detected were not significantly different from the brood 1 frequencies. They were much less than those detected in sperm stored for a length of time equivalent to the length of time over which the sperm sampled in the second brood had matured after treatment. It is likely that repair processes have removed the pre-mutagenic lesions by this time (repair is absent only in mature spermatozoa). A sample of sperm, which had been immature at the time of treatment but which had been allowed to mature for 21 days

TABLE 5.9. Comparison between the translocation frequencies obtained in the first and second broods of progeny of Ork ♂ treated with EMS.

Dose of EMS (slrl)	Experiment No.	Tr. frequency		
		Brood 1(%)	Brood 2(%)	in equivalent stored sample
0.1% (7.87%)	EMS 1.2	0.00	0.00	(0.00) (day8)
" (8.76%)	EMS 1.4	0.00	0.00	0.00 (day 16)
0.4% (23.29%)	EMS 4.9	0.52	0.23	(4.00) (day 18)
0.75%(28.87%)	EMS 7.3	0.86	0.37	(4.02) (day 21)

in the male, was stored for a further 14 days. The mutations scored in the brood 2 (sampled on day 21) were 2.46% (6/244) slrl and 0.37% (2/543) translocations. Brood 2 sperm, stored for 14 days in inseminated females yielded 3.79% (10/264) slrl and 0.00% (0/484) translocations which are not significantly different. This indicates that lesions present in Brood 2 sperm do not respond to storage either because they have already been fixed as mutational changes or because the lesions remaining do not constitute a level of damage high enough to produce translocations either with or without storage.

Fig. 5.8 The relationship between str frequency and ethylations/ nucleotide in EMS-treated sperm of adult Drosophila males



Plotted using the data of Aaron et al (1978)

C. Analysis of Results

The experiments of Aaron and Lee (1978) determined an exact dosimetry for EMS in Drosophila and found a linear relationship between the dose (as measured by ethylations/sperm cell) and sex-linked recessive lethals. Thus for a known frequency of sex-linked recessive lethals, the total number of ethylations per sperm cell can be determined. It has been estimated that the haploid sperm cell contains 3×10^8 nucleotides of DNA (Laird, 1973). From this the frequency of ethylations/nucleotide can be calculated and plotted against sex-linked recessive lethals, as shown in Figure 5.8. This graph was used to determine the frequency of ethylations/nucleotide over the doses used. The determined frequencies are shown in Table 5.10.

TABLE 5.10 Estimation of Ethylations/nucleotide for a range of doses of EMS

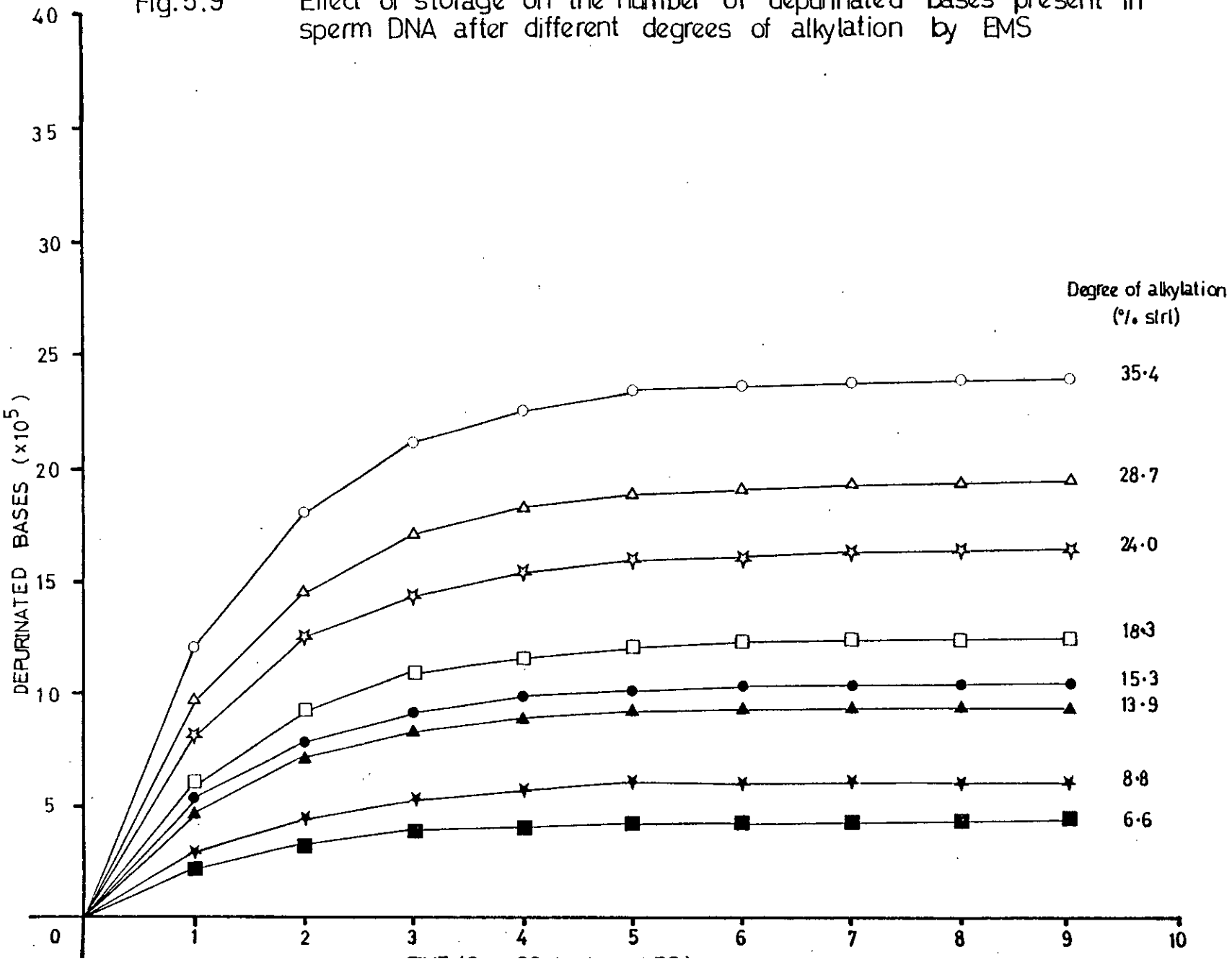
Dose of EMS	Induced slrl	Alkylation/ sperm cell ($\times 10^5$)	Ethylations/ nucleotide ($\times 10^{-3}$)	Probability that a base will be alkylated
0.1%	(6.55%	6.3	2.1	1/476
	(8.76%	8.7	2.9	1/345
0.4%	(13.85%	13.5	4.5	1/222
	(15.25%	15.0	5.0	1/200
0.4%	(18.29%	17.7	5.9	1/170
	(24.00%	23.4	7.8	1/128
0.75%	(28.66%	27.8	9.3	1/108
	(35.40%	34.5	11.5	1/87

Consider the lowest dose of EMS used, 6.55% slrl:

If alkylation along a DNA molecule were random, it might be expected that within a stretch of 476 base pairs one nucleotide on each DNA should be alkylated. The probability that two nucleotides directly opposite each would be alkylated simultaneously would then be $(1/476)^2$. If one alkylation must be within a certain distance (as measured by the number of base pairs) of a second opposing alkylation, and not necessarily directly opposite it, the probability of such an event occurring would increase according to this distance. However, alkylation along a DNA molecule is not random: 70% of alkylations of DNA occur at the N7 of guanine (see Table 5.1) the lesion implicated in chromosome breakage. There are known regions along a DNA molecule which are GC-rich: these regions should therefore be alkylated at a frequency higher than would be predicted if bases were randomly arranged along a DNA molecule. However, this probability may still be so low that translocations and chromosome breakage would be unlikely to occur at doses with a low probability of alkylating N7-G on opposite strands within the required distance of each other. The time dependent storage effect at higher doses could be explained by invoking depurination of N7-guanine, which is a time dependent event (Lindahl, 1972).

If two "hits" are required for each breakage event in the DNA and two breakage events are a prerequisite for a translocation, expressing the likelihood of such an event in terms of probability makes it easier to understand

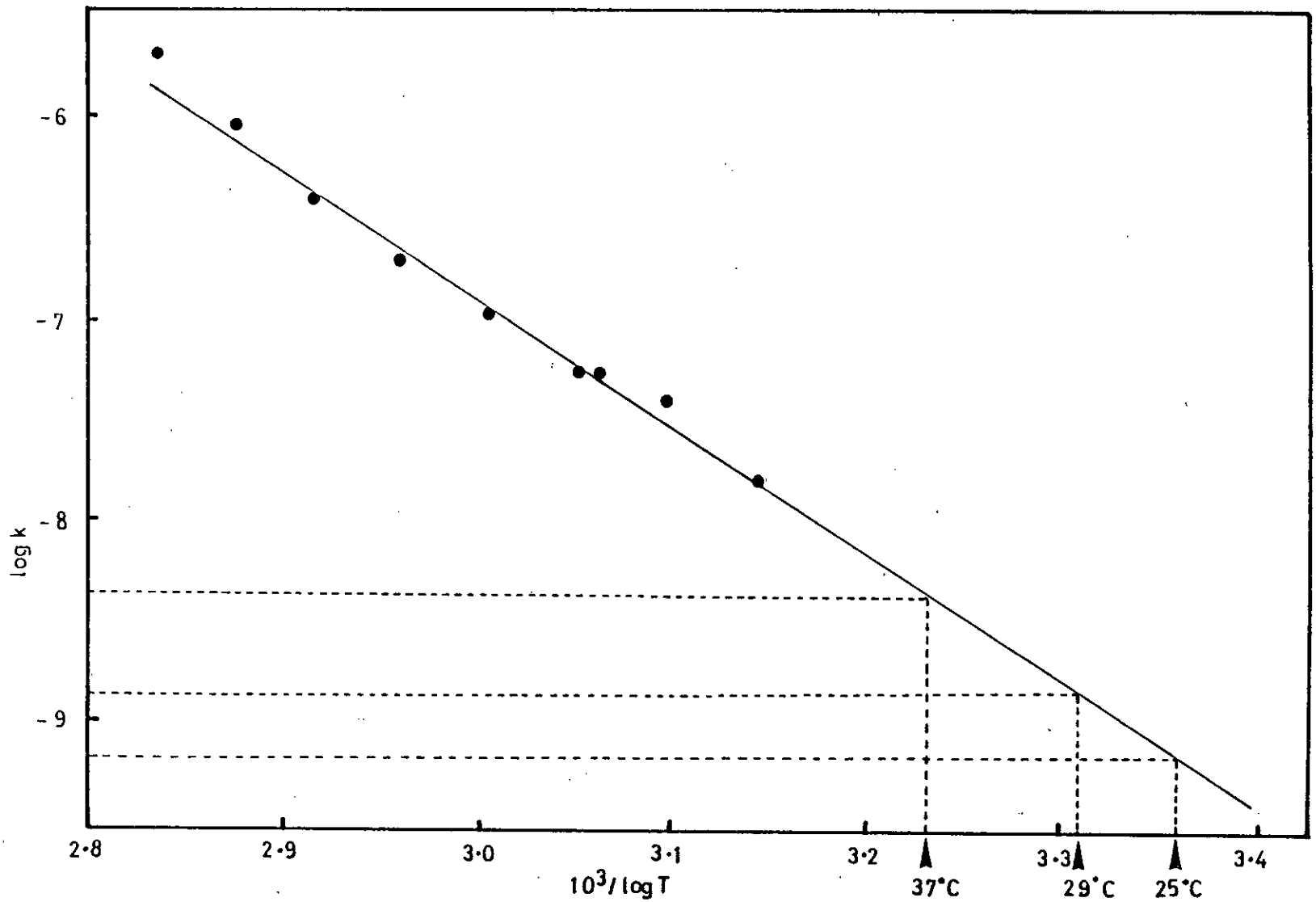
Fig. 5.9 Effect of storage on the number of depurinated bases present in sperm DNA after different degrees of alkylation by EMS



why a low dose of EMS does not induce translocations, even after prolonged storage. At higher doses of EMS there will be a higher probability that two alkylations will be within the required distance of each other but, without storage, the low rate of depurination will minimise the induction of a chromosome break. Storage will allow time for depurination to occur and so increase the probability of chromosome breakage and thus the frequency of translocations.

The rate of depurination depends on the nature of the alkylated base. At 37°C and neutral pH, N7-methyl guanine present in rat liver DNA was calculated to have a half-life of 6 days while O^6-methyl guanine was relatively stable (Margison and O'Connor, 1973). Ethylated bases are more stable than methylated ones. When the rate of depurination of alkylated DNA was determined, it was found that at 37°C, the half-life of alkylated N7 guanine was 16.4 hours for methyl products and 18.9 hours for ethyl products (Lawley and Brookes, 1963). This suggests that the rate of depurination in EMS treated sperm stored in inseminated females will be very slow, the alkylated bases having a half-life greater than 6 days. The rate of depurination of N7-ethyl guanine is visualised in Figure 5.9 from data calculated as shown in Table 5.10. The number of alkylated bases for each EMS dose used was estimated using the data of Aaron and Lee (1978) as plotted in Figure 5.8. It has been shown that ∿70% of bases

Fig. 5.10 Arrhenius plot : rate(sec^{-1}) of depurination of B.subtilis [^{14}C] native DNA in buffer(pH 5) as a function of temperature . Lindahl et al (1972)



alkylated by EMS at N7 guanine, the major lesion implicated in chromosome breakage, therefore depurination has been considered for only this lesion.

No translocations were detected at the low dose of EMS (0.1% EMS giving a frequency of slrl between 6.55% and 8.76%). If this lack of appearance of translocations at the low dose is considered to be due to insufficient depurination occurring, then at higher doses of EMS, translocations should only be detected when the level of depurination is greater than that reached after 30 days storage at the lowest dose of EMS (the maximum length of storage achieved in the experiments). In order to determine when this level of depurination is reached and thus when translocations should be first detected at the high doses of EMS it is necessary to know the rate of depurination of ethylated bases at 25°C.

No data is available for the rate of depurination at 25°C, which is the temperature at which Drosophila experiments are routinely carried out. It is possible to extrapolate the data obtained for depurination at higher temperatures in an Arrhenuis plot (Such a graph is shown in Figure 5.10) and from this to obtain an estimate of the half-life ($t_{\frac{1}{2}}$) of depurination at 25°C. The rate of depurination and the half-life are related in the following manner:

$$t_{\frac{1}{2}} = \frac{\ln 2}{K}$$

Thus it was possible to obtain a value for the half-life of depurination at 25°C from the estimated rate of reaction, as shown in Table 5.12.

TABLE 5.12 Effect of temperature on $t_{\frac{1}{2}}$ of spontaneous depurination of non-alkylated bases.

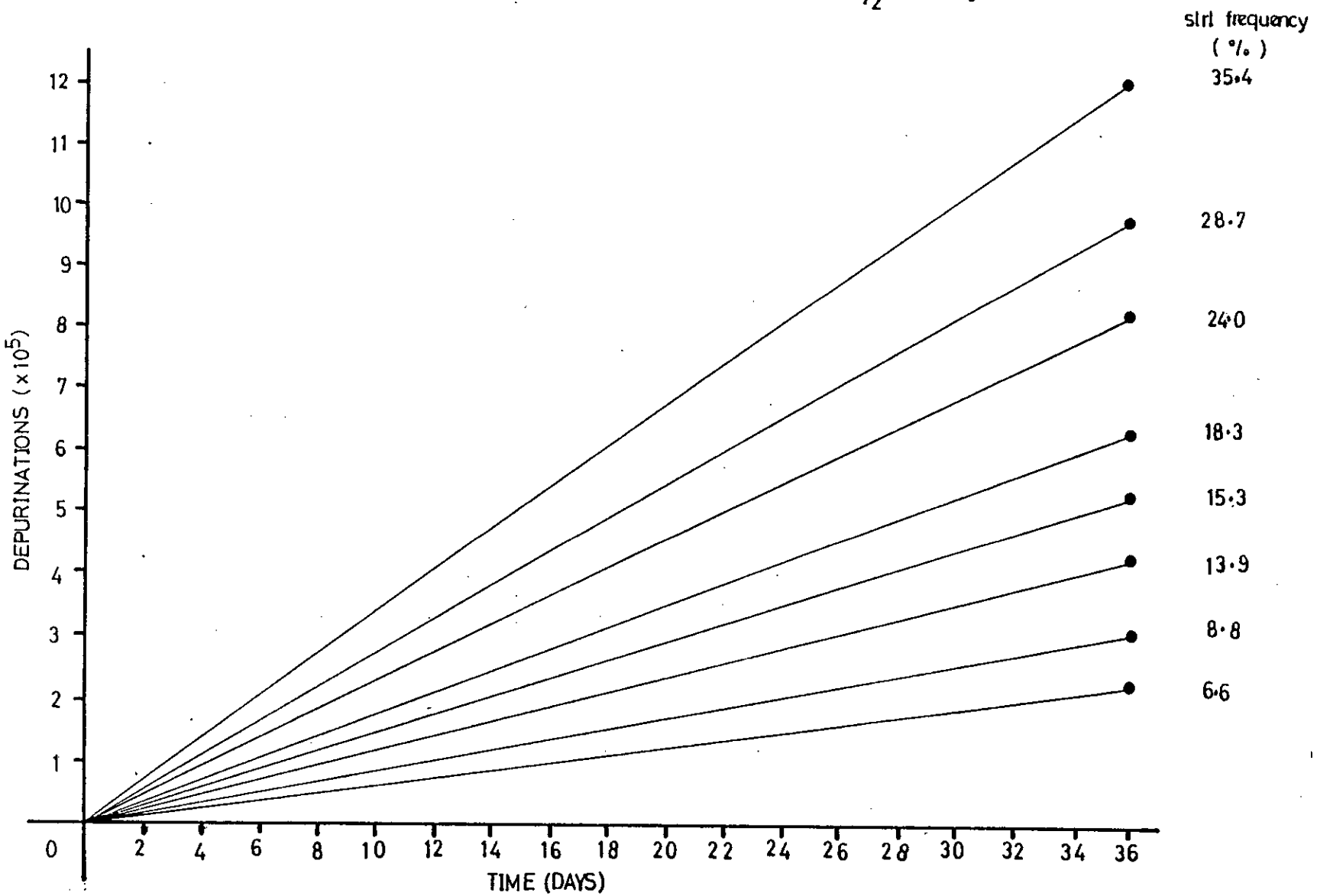
Temperature	log k	k (sec^{-1})	$t_{\frac{1}{2}}$ (hours)
37°C	-8.375	4.22×10^{-9}	45626 (1901 days)
25°C	-9.188	6.49×10^{-10}	296673(12361 days)
29°C	-8.863	1.37×10^{-9}	140541(5844 days)

The half-life of spontaneous depurination at 25°C (12361 days) can be calculated to be longer than that at 37°C (1901 days) by a factor of 6.5. If the half-life of depurination of N-7 methyl guanine is 144 hours (6 days) at 37°C then according to the calculations it will be ∿936 hours (39 days) at 25°C. The most common methylated bases can be ranked according to rate at which they are released. In a descending order of rate of release this ranking is:

7meA > 3meA > 7meG > 3meG >> O^6meG (Lawley & Warren, 1976).

This appears to hold true both <u>in vivo</u> and <u>in vitro</u>. The $t_{\frac{1}{2}}$ of 3meA <u>in vitro</u> at 37°C is 24 hours; at 25°C (according to the above calculations) the $t_{\frac{1}{2}}$ for 3meA will be ∿156 hours

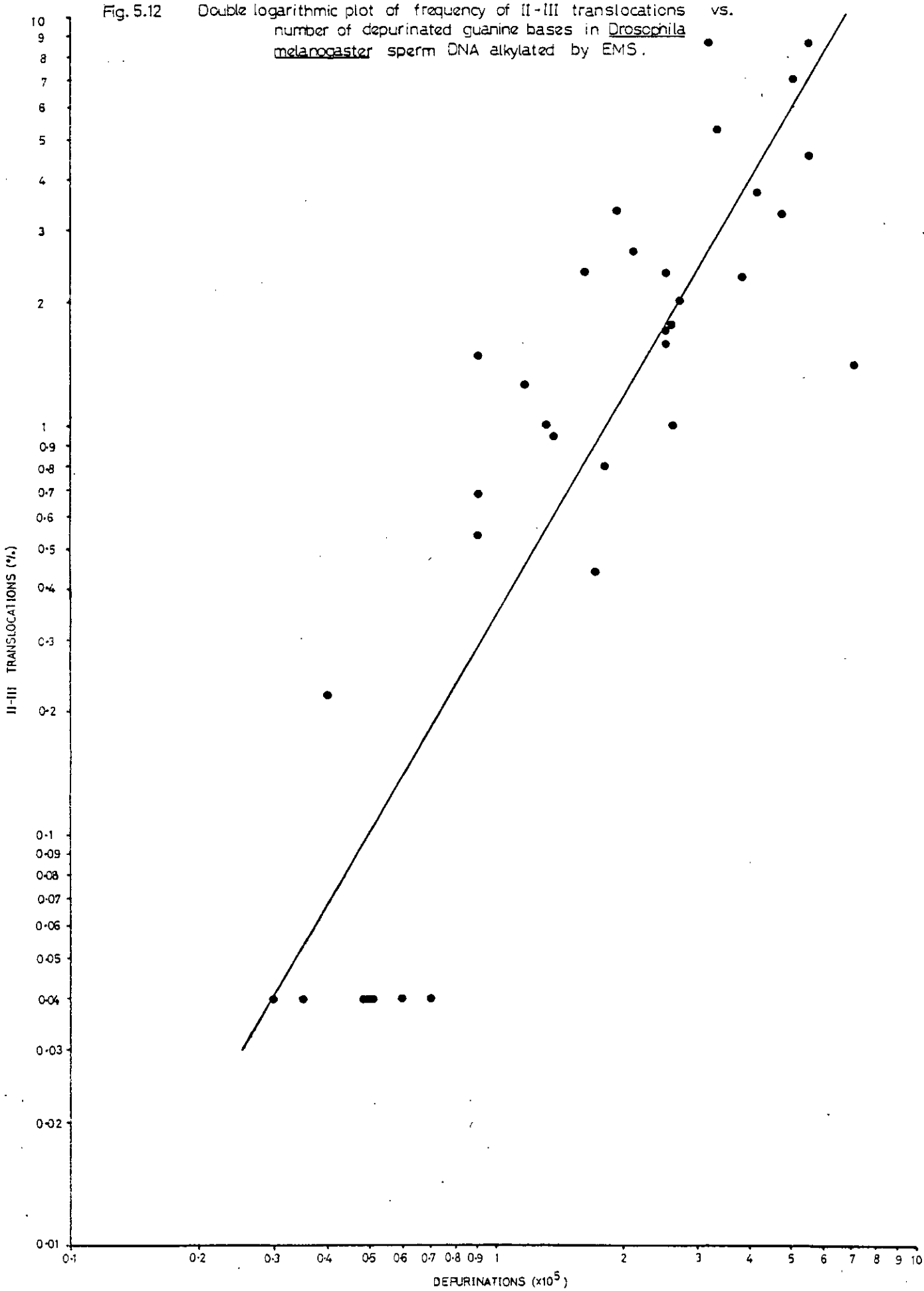
Fig. 5.11 Effect of storage on the number of depurinated bases in sperm DNA after different degrees of alkylation : assume $t_{1/2}$ = 36 days



(6.5 days). The $t_{\frac{1}{2}}$ of 7meA will therefore be less than 156 hours. Together, these two bases make up 3.5% alkylated bases in DNA *in vitro*, ∿7.0% *in vivo*. This will slightly increase the overall rate of depurination. The pH of the storage organs of *Drosophila* ♀♀ is ∿7. The rate of depurination decreases with an increase in pH. The slightly alkaline nature of the storage organs of the female will not affect the rate at which chain cleavage at apurinic sites would occur : histones and protamines (moreso) promote the rate of chain breakage at apurinic sites (McDonald and Kaufman, 1954). Protamine replaces histone as the nuclear protein component of DNA in mature sperm (Das *et al.*, 1964).

Using the data in Table 5.10, Figures 5.8 and 5.9, and that calculated from Figure 5.10, it was possible to estimate the number of depurinations which would occur with increasing time for each dose of EMS used. These estimates are given in Table 5.11 and plotted in Figure 5.11. For each frequency of translocations detected, at those doses showing a storage effect, the estimated number of depurinations at the time the translocations were detected was determined. These data, frequency of translocations vs the number of depurinations, were plotted on a double logarithmic scale. The slope of the regression line drawn to best fit the data gives an indication of the relationship between dose of "mutagen" (depurinations) and mutations (translocations) (see Figure 5.12).

Fig. 5.12 Double logarithmic plot of frequency of II-III translocations vs. number of depurinated guanine bases in Drosophila melanogaster sperm DNA alkylated by EMS.



The calculations have been executed according to the method used by Nasrat et al. (1954-55) to calculate the relationship between s1r1 and mustard gas induced translocations. This method uses the equation $T = kL^n$ where T = translocation frequency

k = constant

L = lethal frequency (in this case number of depurinations)

n = regression coefficient - this gives a measure of the relationship between T and L.

Substituting the logarithm values for the actual values of T and L, should result in a straight line

$\log T = \log k + n.\log L$

$(y = c + mx)$.

The value of n can be determined by fitting a regression line to the logarithmic data.

The value of n was estimated to be 1.86 ± 0.27 with 31 degrees of freedom with $p < 0.001$ which means that the likelihood of this result occurring by chance is less than 1 in 1000. This result can be said to be statistically significant from a linear response; it would appear that there is a "dose-squared" relationship between the number of depurinated bases and the frequency of II-III translocations. However, it should be remembered that this estimate relies heavily on the calculations on the effect of temperature, on rate of depurination and the level of alkylation of Drosophila sperm DNA.

This result is in agreement with that of Nasrat et

al. (1954-55) who (in unstored samples of mustard gas treated sperm) found a dose-squared relationship between slrl (a measure of dose) and II-III translocations. However, it differs from that of Ikebuchi (1982) who, using a different method of calculation, found a dose-cubed relationship between slrl frequency and II-III translocations in both stored and unstored samples of EMS-treated sperm over a range of doses from 7.06% - 48.10 slrl. Applying my method of analysis to the data of Ikebuchi, a dose-squared relationship between number of depurinated bases and thefrequency of II-III translocations was obtained. Auerbach (1976) advises caution when interpreting dose-effect curves as the curve will not only reflect the kinetics of the reaction between mutagen and DNA but also effects on enzymes involved in transcription, in repair and expression. Dose response curves would be modified by repair processes and bend steeply upward, as has been suggested to explain the dose-response curves for the chemically-inducedmutations in other organisms such as barley seeds (Ehrenberg et al., 1966; Mikaelson et al., 1968) and Neurospora (Auerbach and Ramsay, 1968; Kølmark and Kilbey, 1968).

No translocations were detected at the lowest dose (6.55% - 8.5% slrl) even after prolonged storage and at levels of depurination (up to 2.2×10^5 depurinated bases) which had resulted in frequencies of up to 3.29% translocations at higher doses of EMS. If a dose - to the power

4 relationship had been established, this could have been explained by invoking the probabilities of coincident hits within a small number of base pairs on opposite strands of a DNA molecule. Alternatively, it could be that at the higher doses of EMS, the alkylated bases which remain bonded to the DNA interfere with error-free repair mechanisms. Sega (1978) outlined a model involving ethylation of mouse sperm chromatin which led to chromosome breakage and dominant lethality. He proposed that the sulphydril groups of cysteine (present in the sperm protamine) are nucleophilic and as such could be alkylated by EMS. This would disrupt the protein-DNA structure and might lead to chromosome breakage.

5.4 EFFECT OF TEMPERATURE ON THE RATE OF APPEARANCE OF TRANSLOCATIONS AND slrl

Alkylated bases, produced by the treatment of DNA with alkylating agents, have been shown to be lost from DNA by depurination. The resulting apurinic sites can then be converted into single strand breaks (Lawley and Brooks, 1963; Verly et al., 1974). These processes proceed at a slow rate (Lindahl and Ljmquist, 1975) but they can be increased if the temperature is raised (Lindahl, 1979). Veleminsky et al. (1973) suggested that depurination and/or the resulting breakage of DNA was responsible for the enhancement of toxic and genetic effects after storage

of MMS and EMS treated barley seed. Ryo et al. (1981) have proposed that depurination is responsible for the storage effect on dominant lethals and recessive lethal mutations induced by MMS in Drosophila. This idea is supported by the finding of Janca et al. (1977) that tritium-labelled alkyl groups are lost from tritiated EMS-treated Drosophila sperm cells at a very slow rate, which would correspond to the rate for spontaneous hydrolysis.

It has been shown that, above a certain dose (slrl), the frequency of EMS-induced translocations increase with storage time. Using a dose which is known to produce a striking storage effect, what would be the effect on the rate of appearance of translocations and the slrl frequency if EMS-treated sperm were stored at different temperatures? The results of an experiment in which sperm were stored at 18°C, 25°C and 29°C are shown in Figures 5.13 and 5.14. It can be seen that there was no temperature effect on slrl frequency, but there was one on the induction of translocations. With a temperature difference of 10°C (± 1) it took twice as long (24 days as opposed to 12 days) to obtain a frequency of ∿5% translocations. The rate of spontaneous depurination of N7-akylated guanine is known to increase with an increase in temperature. This result agrees with the suggestion that it was the level of depurination of this lesion which contributed to the increase in translocation frequency at the higher temperature. The rate of spontaneous

Fig.5.13

Storage of sperm treated with 0·75% EMS: effect of temperature on frequency of slrl

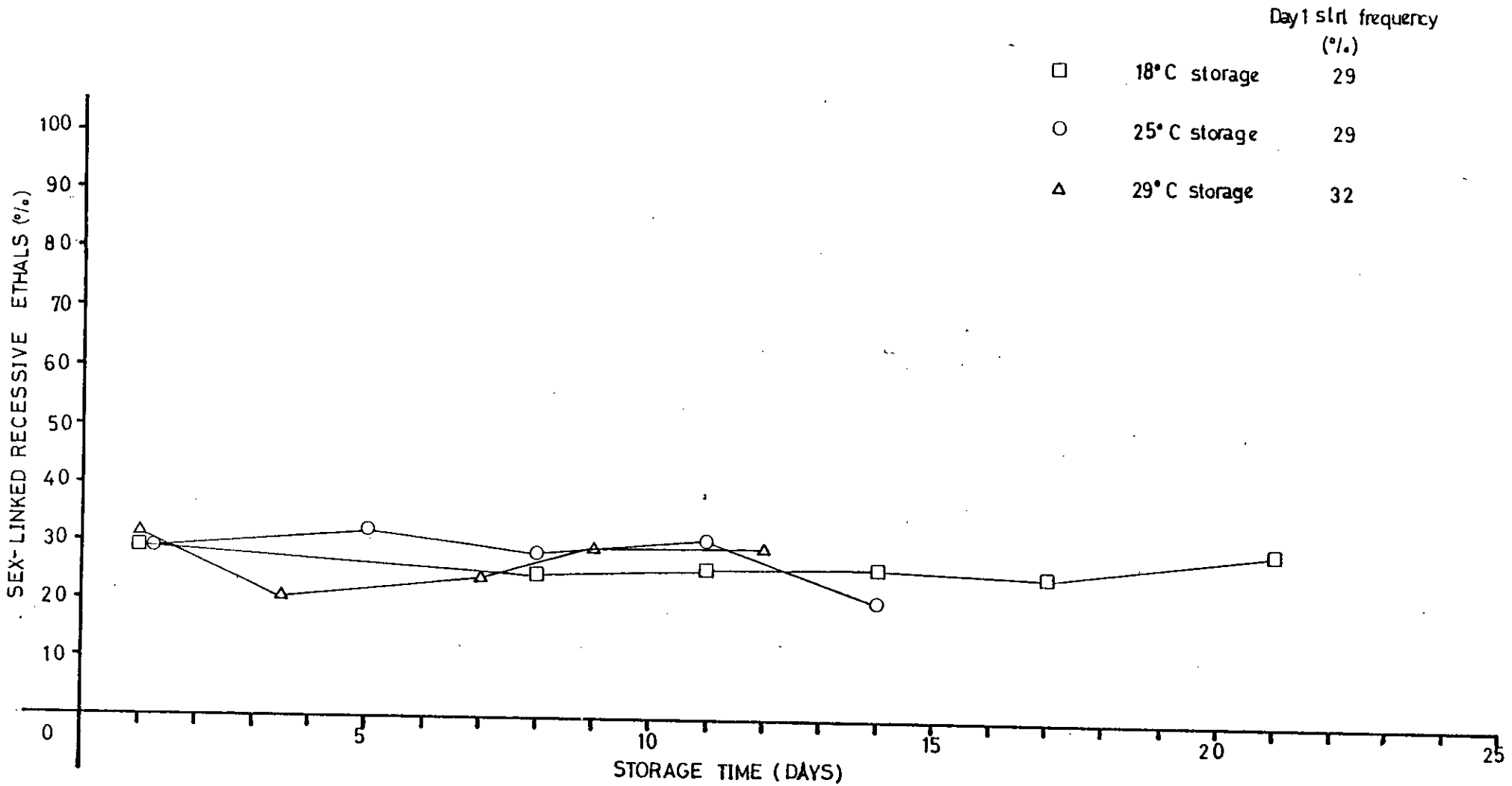
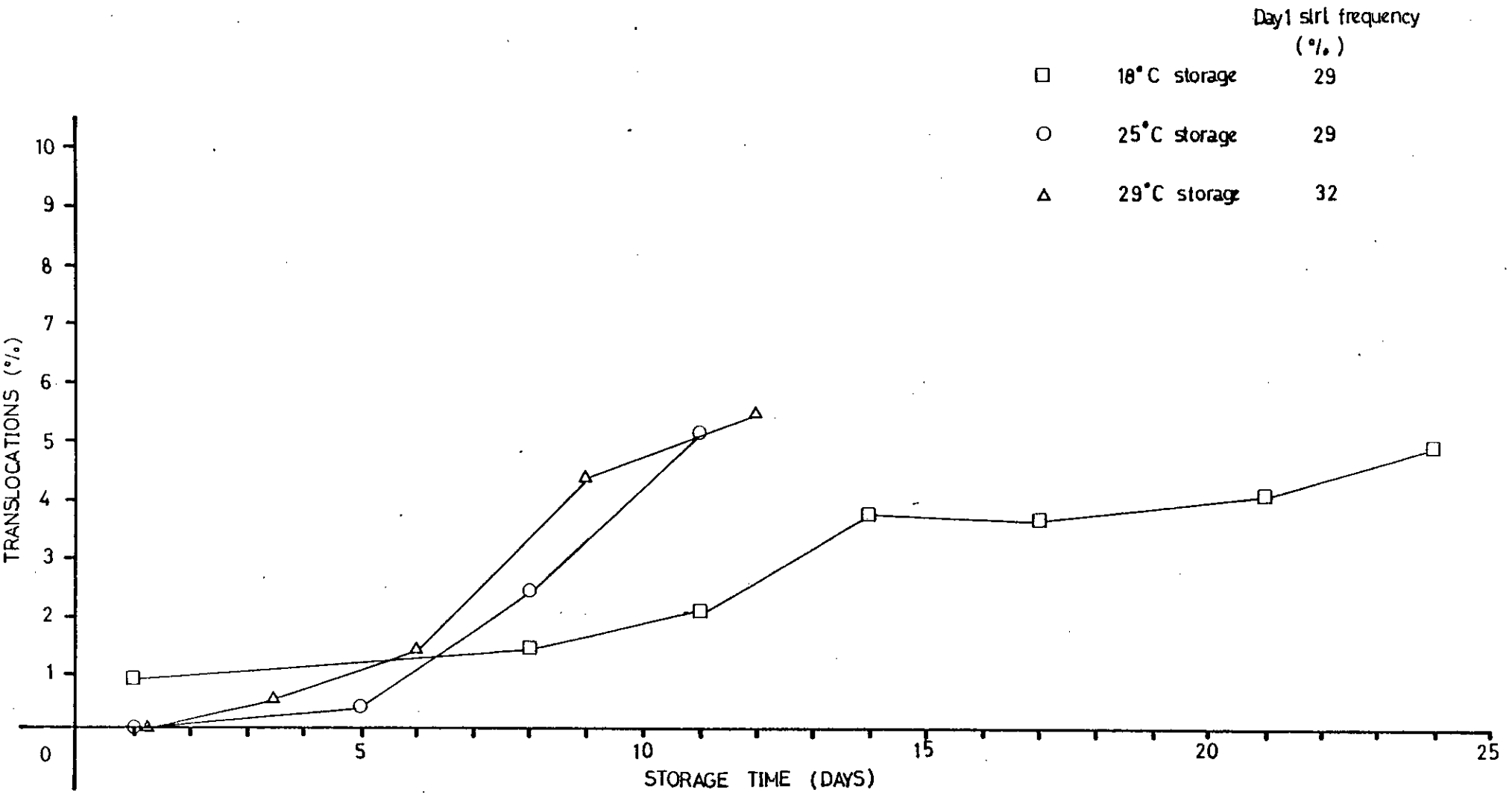


Fig 5.14

Storage of sperm treated with 0·75% EMS:
effect of temperature on frequency of translocations



depurination of O^6 alkyl-guanine is extremely low, thus if this is the lesion responsible for slrl in these tests, it is not surprising that there was no storage effect at any of the temperatures used.

5.5 HATCHABILITY TESTS - A TEST FOR DOMINANT LETHALS

A. Aim of Experiments

Hatchability tests are used to measure the frequency of dominant lethals. Dominant lethals are thought to be single unrejoined breaks in the chromosomes. However, hatchability tests will also include a fraction of eggs which have not been fertilised or else are lethal due to either non-genetic damage or genetic damage other than unrejoined breaks.

The hatchability tests were initially performed using only 0.1% EMS in order to provide additional information about breakage events at this dose both with and without prolonged storage. For completeness, tests were also carried out using the two higher doses of EMS. However, this data is less extensive and the results obtained must therefore be interpreted with caution.

It was also thought that a comparison between the storage effects on dominant lethals (single hit events) and translocations (double hit events which interact) would be useful: translocation tests rely on the detection

of breakage events in sperm which are still viable whereas dominant lethal tests, and to some extent sex-linked recessive lethal tests, will detect breakage events in sperm which have been made inviable by the treatment. Sperm of Drosophila melanogaster do not need to contain any viable genetic information in order to be able to complete spermiogenesis and fertilise an egg (Lindsley and Grell, 1969).

B. Preliminary Tests

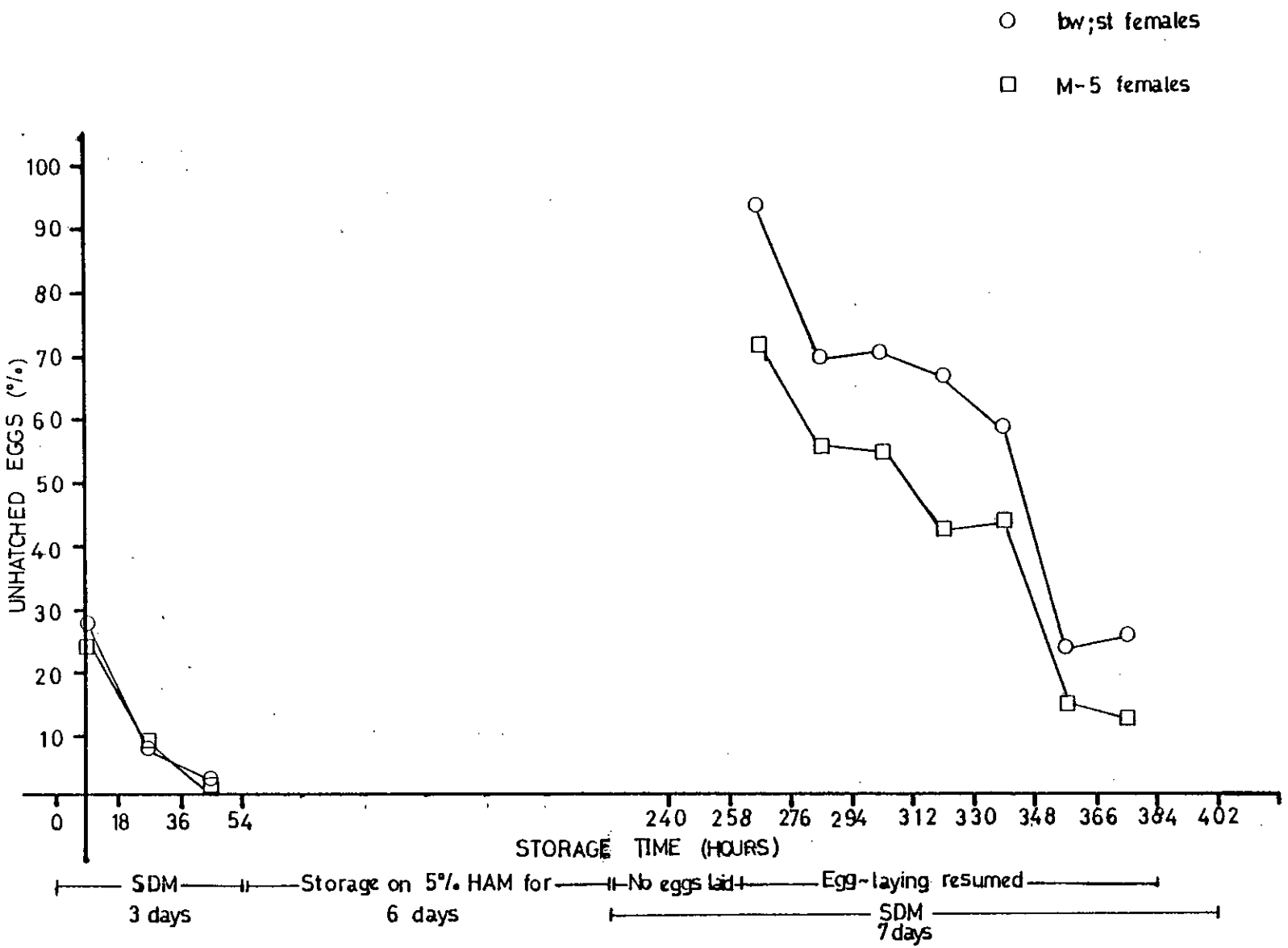
Both */M-5 and bw;st females were used in these tests: the results were pooled when it was found that both strains gave similar results in these tests, as shown by Table 5.13 and Figure 5.15. It was found that the first pre-stored sample showed a higher proportion of unhatched eggs than was expected. This was thought to be due to the fact that the females were newly emerged (0-1 day old) and to a possibility that not all of the females would have been inseminated within the first few hours of mating. The percentage of unhatched eggs decreased until it was ∿2%(average value) on day 3. This was within the expected range for a control series, i.e. <10%. After 6 days on HAM the females were returned to egg-laying chambers containing SDM. For the first two days the females did not lay; egg-laying resumed on the third day after the females had been removed from HAM but at a low level and with a high percentage of eggs unhatched. With

TABLE 5.13 Comparison of dominant lethals induced in two strains of Drosophila melanogaster

UNHATCHED EGGS (%) AT TIME (HOURS) AFTER MATING													
Time (hours)	18	36	54	72 - 102	180	198	216	234	252	270	288	306	324
*/M-5	4/17 23.53	17/189 8.99	5/340 1.47	Storage on 5% HAM	+	+	12/17 70.59	22/40 55.0	52/95 54.74	49/118 41.53	27/63 42.86	11/76 14.47	17/140 12.14
bw;st	15/54 27.78	39/482 8.09	16/560 2.86		+	+	13/14 92.86	42/61 68.85	90/128 70.31	95/149 65.77	59/103 57.28	33/143 23.05	60/245 24.49

+ no eggs laid.

Fig. 5.15 Preliminary results : hatchability test



each successive day the rate of egg-laying increased while the percentage of unhatched eggs decreased until by day 16 it was ∿12% for the */M-5 stock and ∿25% for the bw;st stock.

Care must also be taken not to confuse "dominant lethality" with physiological decreases in viability with time or aging effects on the females and eggs. When females resumed egg-laying after storage on HAM, the percentage of unhatched eggs was high and this declined over a period of a few days until it reached a frequency similar to that of the unstored sample of eggs. The initial high frequency was attributed to the detrimental effects of storage media on the fecundity of females, previously described in Chapter 4, page 85. However, once the females had resumed normal egg-laying the percentage of unhatched eggs began to increase due to normal aging effects on both the females and the sperm.

Full sperm storage organs stimulate ovulation and oviposition. It has been shown that poorly inseminated females tend to retain their eggs and only produce fertile eggs sporadically over a period of several days (Lefevre and Jonsson, 1962). A better experimental protocol would have used several batches of females, a separate batch being used for each sample of stored sperm so that all females had full storage organs at the times of egg-laying. A control series was thus an essential part of a test for dominant lethality and any results obtained at the three

doses of EMS was considered with reference to a control series of tests performed alongside the treated series.

C. Induction of Dominant Lethals by EMS: Results

These tests were carried out primarily to obtain a measure of breakage events induced by a dose of 0.1% EMS; at this dose no translocations had been detected. Results were obtained for all three doses previously used (0.1%, 0.4% and 0.75%) and these are given in Table 5.14 and plotted in Figure 5.16. They show the following:

(i) a low percentage of unhatched eggs over the day 1-4 period for the control series and for sperm treated with 0.1% and 0.4% EMS. At 0.75% EMS there was a much higher percentage of unhatched eggs; this level was maintained over the initial four day period.

(ii) when females inseminated by either treated, or untreated ♂♂ were returned to SDM after storage on HAM, they did not resume egg-laying immediately. It was usual to find a 'lag' of 2-4 days; sometimes the females did not resume egg-laying. Once they did resume egg-laying, the percentage of eggs which did not hatch was much higher than that obtained pre-storage. At the lowest two doses and the control series, the frequency of unhatched eggs decreased then increased with storage. At 0.75% EMS, the frequency of unhatched eggs continued to increase with storage.

TABLE 5.14 Comparison of dominant lethals induced in a control series and by three doses of EMS.

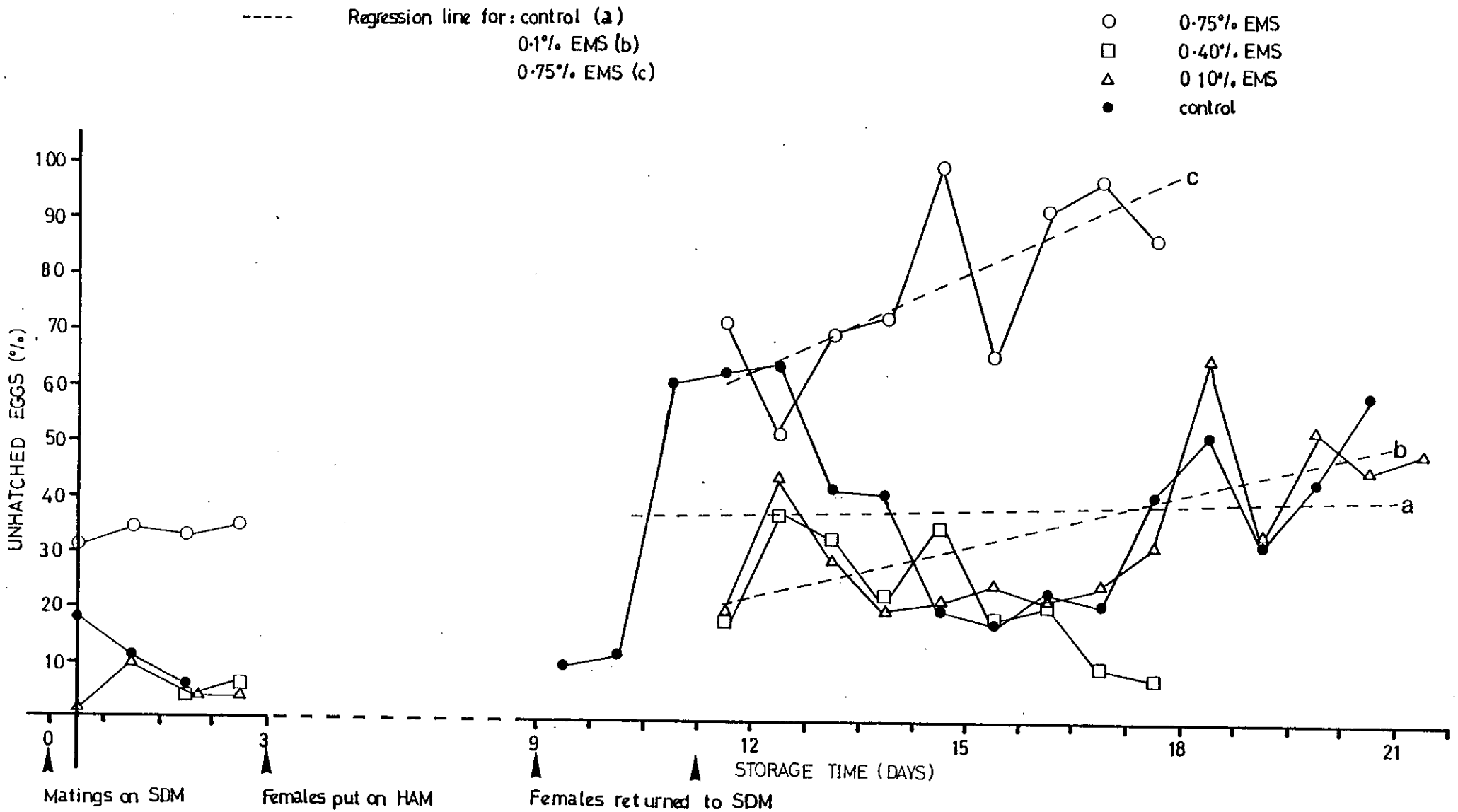
Day No.	Control Unhatched eggs		0.1% EMS Unhatched eggs		0.4% EMS Unhatched eggs		0.75% EMS Unhatched eggs	
	n	%	n	%	n	%	n	%
1	126/718	17.55	5/355	1.41	NT		31/99	31.31
2	241/2239	10.76	170/1724	9.86	NT		84/249	33.73
3	142/2237	6.35	45/1084	4.15	12/300	4.0	219/664	32.95
4	Storage on HAM		5/140	3.57	9/160	5.63	43/122	35.25
5	"		Storage on HAM		Storage on HAM		Storage on HAM	
6	"		"		"		"	
7	"		"		"		"	
8	"		"		"		"	
9	"		"		"		"	
10	6/61	9.84	"		"		"	
11	11/89	12.34	"		"		"	
12	25/31	80.65	71/354	20.06	18/101	17.80	85/118	72.03
13	64/101	63.37	28/64	43.80	25/67	37.30	54/104	51.92
14	142/223	63.48	124/433	28.64	90/272	33.10	103/148	69.60
15	170/403	42.18	70/348	20.11	40/175	22.90	80/110	72.73
16	208/509	40.86	60/278	21.58	28/80	35.00	84/84	100.00
17	137/681	20.12	149/602	24.75	30/160	18.75	40/61	65.57
18	181/1022	17.71	96/425	22.59	49/230	21.30	245/267	91.76
19	79/333	23.72	111/438	25.34	13/124	10.48	140/144	97.22
20	64/302	21.19	51/157	32.48	3/40	7.50	41/47	87.23
21	75/183	40.98	90/137	65.69				
22	146/281	51.96	96/281	34.16				
23	199/618	32.20	34/64	53.13				
24	73/168	43.45	408/880	46.36				
25	40/68	58.82	419/863	48.55				
26								
27								
28								
29			708/3664	19.32[a]				
30								

In all tests both bw;st and */M-5 ♀♀ were used; in general twice as many */M-5 ♀♀ were used for each test.

a. This value was obtained from */M-5 ♀♀ being used as the tester ♀♀ in the specific locus test; they had been tested for % of unhatched eggs in an unstored sample of eggs but were not tested again until day 29.

NT Not tested.

Fig. 5.16 Effect of storage on the percentage of unhatched eggs laid by females inseminated by EMS treated males



(iii) a dose of 0.4% EMS did not seem to induce dominant lethals at a higher frequency than that induced by 0.1%, even after prolonged storage. A dose of 0.75% EMS did induce a much higher frequency of dominant lethals both before and after storage. However, at all doses, an apparent storage effect was seen but such an effect was also present for the control series. In order to obtain a clear picture of what was happening, a regression analysis was carried out. This indicated that the control series of unhatched eggs did not show a significant increase with time whereas at 0.1% EMS there was a small but significant increase with storage time. At 0.4% EMS there was no apparent storage effect. The result obtained at 0.75% EMS was a significantly greater response to storage than that obtained with 0.1% EMS.

A storage effect for the induction of dominant lethals in EMS treated sperm had previously been demonstrated but only at high doses of EMS: 38% s1r1 (Aaron and Lee, 1977), (Sram, 1970b) and ~25% s1r1 (Ikebuchi and Nakao, 1979). A storage effect on dominant lethals had not been found when a dose as low as ~7% was used. In this work, a storage effect at such a low dose was found, the storage time being twice as long as that of previous experiments. Assuming that the majority of dominant lethals arise from chromosome breaks, it has been demonstrated that a low dose of EMS is capable of producing chromosome breakage, even though, at this dose, no translocations were detected.

5.6 GENETICAL TESTS FOR THE FREQUENCY OF SMALL DELETIONS AMONG EMS-INDUCED POINT MUTATIONS IN DROSOPHILA

A. Aim of Experiments; diagnostic criteria of tests.

Genetical tests were used to estimate the proportion of small deletions (chromosome breakage events) among EMS-induced recessive visible mutations; recessive visible mutations are generally assumed to be caused by intragenic changes in DNA (point mutations, frame-shifts, deletions of a small number of bases within the gene). Visible mutations were scored at 7 sex-linked loci either using a specific locus test, in which both small deletions and intragenic changes survived in heterozygous ♀♀, or an attached-X test, in which sons of attached-X ♀♀ did not survive if they had inherited a deletion-bearing X from their fathers. A comparison was made between the frequencies of the same visible mutations detected in the two tests. Deletions large enough to include a viability locus close to one of the selected visibles should not survive in the ♂♂ (attached-X test) but should be detected in the ♀♀ (specific locus test). The higher the proportion of deletions among visible mutations, the greater should be the excess of visibles detected in ♀♀ over those detected in ♂♂. A corresponding proportion of the visibles detected in ♀♀ should also be lethal in ♂♂, and this can be established by progeny tests on the mutant ♀♀.

The ratio of sex-linked lethals to male-viable visibles

should also increase with the proportion of deletions among visible mutations. Application of this criterion was made difficult and uncertain due to the high proportion of mosaics induced by EMS. The test was carried out using only the lowest dose of EMS (0.1% EMS giving a slrl frequency of 5.88 - 12.86%) in order to guard against the induction of advantitious lethals on the same chromosome. Shukla and Auerbach (1981) expected that even their highest dose (12.7% slrl) would not yield more than 1% advantitious sex-linked lethals among visibles.

B. Results

The results obtained in these tests are given in Tables 5.15 - 5.16. These can be summarised as follows:

(i) Pre-storage, equal frequencies of visibles were detected in both the attached-X and the specific locustest (1.02% and 0.89% respectively). When the transmission of visibles from ♀♀ (specific locus test) to ♂♂ was determined it was found that 3/15 (20%) of the visibles were transmitted and 1/3 (33.33%) of those were lethal to ♂♂. This provided an estimation that 1/3 of the transmitted visibles were due to small deletions (justification for preferentially using this estimate is given below in (iii)).

(ii) Post-storage, again similar frequencies of visibles were detected in both the attached-X and the specific locus test (0.87% and 1.0% respectively). This suggested that

TABLE 5.15 0.1% EMS : Results from Deletion Tests

Frequencies of visible mutations at 7 sex-linked loci (y w sn m g f car) in the progeny of EMS-treated ♂♂ mated either to attached-X ♀♀ (X̂X) or to multiply mated free-X ♀♀ (*/M-5). Analysis of mutants for mosaicism.

	Pre-storage		Post-storage	
(a) Test	X̂X	*/M-5	X̂X	*/M-5
(b) Day number of sample	1 - 3	1 - 3	10 - 35	10 - 35
(c) Frequencies of slrl for days 1-3	5.88 - 9.88%	6.55 - 12.86%		
(d) Frequency of observed visibles	41/4020 = 1.02%	24/2710 = 0.89%	105/12093 = 0.87%	68/6773 = 1.01%
(e) No. of visibles dead or sterile	17 (7c, 10m)	9 (1dmc, 7m)	29 (8c,10m,1dmm)	6 (1c, 5m)
(f) No. of visibles further analysed	24 (3c, 21m)	15 (1c, 1dmc,12m)	76 (21c, 55m)	62 (25c, 37m)
(g) Phenotype mosaic	21	12	55	37
(h) gonads wholly mutant	1 (v)	1 (v)	5 (21,1 s-l ,2v)	9 (71, 2v)
(i) gonads mosaic	0	1 (v)	1 (v)	1 (v)
(j) gonads not mutant	20	10	49	27
(k) Phenotype completely mutant	3	3	21	25
(l) gonads wholly mutant	0	1 (1)	8 (8v)	16 (81,8v)
(m) gonads mosaic	0	0	2 (2v)	0
(n) gonads not mutant	3	2 (dm)	11	9
(o) True completes (= l)	0	1	8	16
(p) Mosaics (=f-O)	24	14	68	46
(q) Transmitted visibles(=h+i+l+m)	1	3	16	26
(r) Proportion among (q) of male lethals	0 (0.00)	1 (C.33)	2.5 (0.16)	15 (0.58)
(s) Ratio of visibles in X/X:X̂X tests	(1.15) 1 :	0.87(1.00)	(0.86) 1 :	1.15(1.00)

c complete
m mosaic
dm double mutant
l lethal mutation
s-l semi-lethal mutation
v viable mutation

Experiment No.	slrl frequency X̂X	slrl frequency */M-5
1	6.55%	6.22%
2	12.86%	9.76%
3	9.57%	9.88%
4	12.69%	5.58%
5	12.86%	9.50%
$\bar{x}$	10.91%	8.19%

Fr.of visibles in X̂X = 1.02%
" " " " */M-5 = 0.89%
Ratio of 1:0.87
The difference between these two values is not significant at the 5 % level .'. estimate that none of the visibles induced are due to small deletions

By using the ratio of viable-: lethal-visibles, the following estimates are obtained:
*/M-5 1/3 =33.33%) of visibles induced are
X̂X 0/1 = 0.00%) due to small deletions and
) are lethal in ♂♂
This suggests that very few small deletions are induced in a pre-stored sample of EMS-treated spermatozoa.

Fr.of visibles in X̂X = 0.87%
" " " " */M-5 = 1.00%
Ratio of 1:1.15
The difference between these two values is not significant at the 5 % level .'. estimate that none of the visibles induced are due to small deletions

By using the ratio of viable-: lethal-visibles, the following estimates are obtained:
*/M-5 15/26 =57.69%) of visibles induced are due
X̂X 2.5/16 =15.63%) to small deletions are are
) lethal in ♂♂
This suggests that small deletions are subject to a storage effect

TABLE 5.16 Transmission of Visibles

Test	Trans-mission	Pre-Storage			Post-Storage		
		Completes	Mosaics	Both	Completes	Mosaics	Both
$\hat{XX}$	n	0/3	1/21	1/24	10/21	6/55	16/76
	%	0	4.76	4.17	47.62	10.91	21.05
*/M-S	n	1/3	2/12	3/15	16/25	10/37	26/22
	%	33.33	16.67	20.00	64.00	27.03	41.94
Both	n	1/6	3/33	4/39	24/46	16/92	42/138
	%	16.67	9.09	10.26	52.17	17.39	30.44

$\hat{XX}$ - attached-X test

*/M-S - specific locus test.

there was not a storage effect on the production of small deletions, none of which had, according to the same criteria, been detected in an unstored sample of sperm. When the transmission of visibles, detected in ♀♀, to the next generation of ♂♂ was determined, it was found that 26/62 (41.94%) visibles were transmitted (Table 5.16) and 15/26 (57.69%) of these were lethal to ♂♂ (line r, Table 5.15). The results suggested that post-storage, a higher proportion of visibles detected as somatic mutants were also gonadic mutants (i.e. completes) and that a higher proportion of these were lethal in ♂♂ as compared to the pre-stored sample.

(iii) When these results were considered in isolation, it was concluded that there was a storage effect on small deletions: storage increased the proportion of small deletions among point mutations. This supported the results obtained in the dominant lethal test where a storage effect was found on 'breakage events' induced by a low dose of EMS (the same dose range was used in these experiments). However, while these results agree with those of Shukla and Auerbach (1981) who found that a low dose of EMS (slrl frequency of 4.5 - 12.7%) could induce chromosome breakage events detectable in genetic tests, they disagree in that the frequency of small deletions they detected without storage is similar to that detected only with storage (this work).

Shukla and Auerbach (1981) found that twice as many visibles were detected in the specific locus test as in the attached-X test, suggesting that 50% of visibles detected in the specific locus test were due to small deletions. The proportions of transmitted visibles lethal in ♂♂ amongst those detected in their specific locus test (and therefore deletions) varied between 50% and 90%, depending on the dose of EMS used: 4.5-6.8% slrl, 90% deletion; 9.5% slrl, 50% deletions; 12.7% slrl, 62% deletions. They believed that the higher estimate, 50-90%, was closer to the true value for the following reasons. The majority of EMS-induced mutations occur first as mosaics, many of which do not include the gonads. An unknown proportion of these were presumed to be due to deletions which, if they had occurred as completes, would have prevented the male zygotes from developing into flies. Support for this assumption was provided by Ripoll and Garcia-Bellido (1973) who found that about 90% of EMS-induced zygotic lethals were not cell lethals. Mosaicism would result in an overestimate of the number of viable visibles in the ♂♂ and thus an underestimate of the ratio between mutant ♀♀ and mutant ♂♂. In the tests carried out by Shukla and Auerbach (1981) they found 85-100% mosaics in the attached-X test and 88-90% mosaics in the specific locus test. The values estimated from the data in Table 5.15 (this work) were 100% and 93% respectively. Such high frequencies of mosaics therefore justified using the second criterion

(transmitted visibles lethal in ♂♂) as opposed to the first (ratio of visibles detected in the specific locus test to those detected in the attached-X test).

A large proportion of the visibles was not transmitted, as shown by Table 5.16. The proportion transmitted was higher in the stored than the unstored sample. Visibles which could not therefore be tested for viability are problematic and, as pointed out by Shukla and Auerbach (1981) an estimate of the number of viable visibles would have to be based on two assumptions:

(i) all visibles detected in ♂♂ were viable - it was pointed out on page 139 that this will be an over-estimate. Evidence for this came from a post-stored sample in the attached-X test (line r, Table 5.15) when 16% of visibles detected in ♂♂ and transmitted to the next generation were lethal in ♂♂ offspring.

(ii) visibles detected in ♀♀ contain the same proportion of viables whether they are transmitted or not.

A further observation made from the data in Table 5.16 was that visible mutations were transmitted with a much higher frequency through the female line (specific locus test) than through the male line (attached-X test). This was also observed by Shukla and Auerbach (1981). They explained this in the following manner. It is the somatic distribution of mutant tissue which determines the degree to which the gonads are induced in mutation (Lee et al.,

1967). It is possible that in F_1 ♂♂ with both mutant and non-mutant gonads, spermatogonial lethals closely linked to the visible mutation may prevent the mutant spermatogonia from developing into spermatozoa. This may be the explanation for the finding (line r, Table 5.15) that visibles detected in ♂♂ could also be lethal to their ♂♂ offspring. Mosaic gonads are likely to be more frequent in ♂♂ than in ♀♀ because of the larger number of primordial germ cells that contribute to the testes (Sonnenblick, 1941).

In summary, the data published to date, as discussed in Section 5.1.C, favours the opinion that slrl mutations induced in unstored spermatocoa of EMS-treated Drosophila are predominantly the result of intralocus mutations. However, Shukla and Auerbach (1981) found that EMS-induced sex-linked recessive visible mutations included at least 60% small deletions which rendered the mutations lethal to ♂♂. Results obtained in this thesis agree that a low dose of EMS is capable of breaking chromosomes and inducing deletion-mutations, but only after storage. While the results obtained using genetical tests to establish the frequency of small deletions among EMS-induced point mutations in Drosophila do contrast with the published data, which find evidence to support the opinion that slrl mutations are predominantly 'point mutations', they may not be mutually exclusive conclusions. Inference has been made that if a large proportion of sex-linked visible mutations are due to

deletions, as detected by lethality of the mutation to ♂♂ (i.e. slrl), then the same is likely to hold true for sex-linked recessive lethal mutations. This need not necessarily be the case. Three diagnostic criteria were originally suggested for the use in genetic tests to detect small deletions (Shukla and Auerbach, 1980):

(i) this was used because it was the only criterion applicable to tests with EMS. It has been described in Section 5.6.A.

(ii) the ratio between the frequencies of recessive lethals and those visible mutations viable in ♂♂. The former but not the latter may include deletions large enough to cover at least one vital gene. This ratio should increase with the proportion of deletions among visible mutations. Application of this criterion was not possible due to the high proportion of mosaics induced by EMS.

(iii) the degree of germinal selection against sex-linked to autosomal lethals in spermatozoa and spermatogonia. EMS does not produce mutations in spermatogonia (Fahmy and Fahmy, 1957) therefore this criterion could not be applied.

Because the latter two criteria could not be used in these tests it was not possible to obtain direct data on the frequency of deletion events amongst sex-linked recessive lethals.

CHAPTER 6. DISCUSSION

DISCUSSION

Storage experiments have provided the strongest evidence that chromosomal aberrations result from a different mechanism to that which causes sex-linked recessive lethals: chromosome breaks but not point mutations have been shown to increase when chemically-treated mature spermatozoa are stored in the untreated female. The experiments of Vogel and Natarajan (1979a and b) suggested that two variables were of major importance in determining both the type and frequency of genetic changes induced in Drosophila by monofunctional alkylating agents. They identified these variables as:

(i) the sites along a DNA molecule which are alkylated by a particular agent

(ii) the dose or intensity of alkylation at those sites

A correlation was found between the reaction mechanism by which an agent alkylated the DNA (influenced by its s-value) and the genetic alteration observed. However a straightforward comparison of s-values is too simplistic. Two alkylating agents with a similar s-value (e.g. DES and EMS) will not necessarily elicit identical responses mutationally, although they should show the same trends. Other factors will be involved in determining the sites of alkylation by an agent: the lipid/water position co-efficient, the rate of hydrolysis of the alkylated bases, steric factors influencing the sites accessible to the alkylating agent.

Differences in the site of alkylation by sn_1 (low s-value) and sn_2 (high s-value) agents have been summarised in table 1.6. The main difference is the level of alkylation at O^6-guanine (favoured by sn_1 agents) and of N7-guanine (favoured by sn_2 agents). Miscoding by O^6 lesions has been implicated in gene mutations (Loveless, 1966; Veleminsky et al., 1970) whereas alkylation at N7 has been implicated in DNA strand breakage following depurination (Lawley and Brookes, 1963; Verly et al., 1963). The ratio of O^6 : N7 alkylation may be of use in determining an agent's predisposition towards induction of either gene mutations or chromosome breakage.

Evidence for the existence of a "lowest effective concentration" (lec) - one for gene mutations and one for chromosome breakage - has been demonstrated (Vogel and Natarajan, 1979a; Ikebuchi and Nakao, 1979; Vogel and Leigh, 1975). Results obtained in this thesis suggest that the lec will differ for different chromosome breakage events (e.g. dominant lethals and translocations).

A third factor to be considered is the rate of repair or misrepair of alkylation or alkylation-induced lesions. Alkylating agents with high s-values, as well as being more effective chromosome breakers, also have an enhanced affinity for proteins (Ross, 1962). This may lead to inhibition of repair processes. In Drosophila there is neither significant repair nor mis-repair of broken chromosomes before fertilisation, the relevant repair enzymes being

provided by the ovum. Rejoining of breaks is slowed down by all conditions which inhibit oxidative metabolism. Wurgler (1971) demonstrated that in Drosophila zygotes, breaks in paternally irradiated chromosomes rejoin only in the zygote, and they become incapable of doing so within the first 16 minutes after fertilisation. Under these conditions, the final yield of rearrangements depended on the speed of rejoining. This would explain the high degree of cytotoxicity observed after storage of sperm treated with 0.75% EMS (this work) which may contain a level of damage too great for repair enzymes to deal with in a limited period of time in an error-free manner or because the repair system is overwhelmed.

The nature of sex-linked recessive lethal mutations is still undetermined. The data obtained to date are not in total agreement but do show a general trend; deletions affecting more than one gene and gross structural rearrangements are extremely rare among EMS-induced lethals. In Drosophila a storage effect has not yet been detected for sex-linked recessive lethals induced by a monofunctional alkylating agent. This was confirmed in extended storage analysis with EMS over a range of doses, and at both low and high temperatures of storage (this work). An attempt was made to determine whether this non-response could be attributed to an increase in frequency of a proportion of lethals (caused by small deletions) with a postulated concomitant decrease in point mutation. Although the

results suggested that small deletions do increase with storage, this response would need to be verified by more experiments. Direct sequencing of the genome would yield this information but would be a lengthy process.

That chromosome breaks are produced by a mechanism distinct from that induced in the production of sex-linked recessive lethals was also provided by the work of Lim and Snyder (1968), Fahmy and Fahmy (1961) and Vogel and Natarajan (1979a and b) and many others. They attributed this to a unique set of reaction products in alkylated DNA leading to each mutagenic event (i.e. breakage or lethal event). This together with differences in the stability of the products of alkylation could explain the observed storage effect.

The evidence obtained to date for a variety of monofunctional alkylating agents emphasises the importance of storage experiments for the detection of translocations induced by monofunctional alkylating agents. For both DES and EMS, long periods of storage have proved to be necessary in order for the full effects of the mutagen to be realised. At high doses of DES it was possible to induce translocations in an unstored sample of treated spermatozoa (this work) - this may in fact reflect a storage period of up to 4 days because the experimental design was not stringent enough to totally eliminate storage of sperm within the male (1 day) and within the inseminated females (up to 3 days) in the initial sample of 'unstored' sperm. The translocations detected in unstored samples of EMS-treated sperm, although

lower than those detected with DES, may also be attributable to the same margin of error in sampling. With some agents, shortage or absence of rearrangements may be due to insufficient time lapsing between termination of treatment and usage of sperm. Results obtained with EMS (this work) suggest that even with extended periods of storage (up to 30 days) significant translocation-induction will not occur below a certain frequency of alkylated bases. The induction of translocations, and the rate at which they appear was shown to be dependant on dose (this work; Ikebuchi, 1982); the yield of translocations also being dependant on the temperature at which the sperm was stored (this work).

Brookes and Lawley (1960), Lawley (1966), Lawley and Brookes (1963) and Verly et al (1974) provided experimental evidence that spontaneous hydrolysis of alkylated DNA (depurination) led to cleavage of the sugar-phosphate backbone of DNA. Chromosome breakage could be accounted for by

(a) loss of an alkyl purine

(b) subsequent hydrolytic cleavage of the DNA.

This explanation was discounted by Lim and Snyder (1968) as being too improbable, particularly as chromosome breakage could be detected within 24 hours after treatment of mature spermatozoa. It should be possible to determine the likelihood of these two events occurring from the rates of hydrolysis of alkylated bases induced by

different alkylating agents. These have been calculated for a variety of agents: e.g. EMS alkylated bases have a ½-life of 39 days at 25°C. This slow rate would account for the delayed appearance to translocations induced by EMS and agrees with the finding of Janca (1977) that tritium-labelled alkyl groups following treatment of Drosophila sperm with tritiated-EMS were lost from sperm cell DNA at a very slow rate.

Ikebuchi (1982) explained the dose-response curves he obtained for EMS-induced translocations, both for unstored and stored samples, by invoking the multi-hit hypothesis (detailed on page 113 and in Figure 5.1) of chromosome breakage. He obtained a dose-cubed response for the induction of translocations at different lethal frequencies which suggested that each breakage event (two of which are needed to produce a translocation) was the result of two independent alkylations of the DNA within several neighbouring nucleotide-pairs, either in the same or opposite DNA strands. This differed from the dose-squared response calculated in this thesis for the induction of translocations at different "doses" as measured by number of depurinated guanines. While a dose-squared response for X-rays (Muller, 1940) and mustard gas (Nasrat et al. 1954-5) a polyfunctional alkylating agent, has been found and is easily explainable by the nature of their reactions with DNA, a $(dose)^4$ response would be more easily understood for a monofunctional agent. Ikebuchi's data

also shows a dose-squared response to the number of depurinated guanines. However, Auerbach (1976) advises caution when interpreting dose-response curves as the curve will not only reflect the kinetics of the reaction with DNA but also effects on enzymes involved in transcription, in repair and in expression.

The biochemical basis of repair of mutagen-damaged DNA in Drosophila remains to be elucidated. A number of mutagen-sensitive mutants, deficient in the repair of damaged DNA, and recombination-defective mutants have been isolated and characterised (Boyd and Setlow, 1976). These mutants survive in the absence of mutagen treatment. Both leaky and null alleles have been identified suggesting that the functions coded for by these alleles are dispensible. This approach, isolating and characterising mutagen-sensitive strains, has proved fruitful in examining the genetic control of DNA metabolism in unicellular organisms such as bacteria (Smith, 1978) and fungi (Haynes, 1975). Studies to date have revealed a complex interaction between genetic recombination, DNA replication, DNA repair and mutation in that they share common enzymatic steps and overlapping control systems. In particular, genetic studies of both prokaryotic and lower eukaryotic organisms suggest that there are many DNA repair systems which may effect the production of mutations. To determine if this relationship (between DNA repair and mutation production) exists in Drosophila, use of mutagen-sensitive strains to determine the number and functions of

genes controlling various aspects of DNA metabolism (replication, repair, recombination) and their role in mutation production has already begun in some laboratories.

The repair mutants could also be used to examine mutation production in each strain defective in a particular repair pathway. This should indicate the role of the pathway in providing error-free or error-prone repair. Genetic and cytological investigations of recombination-defective and repair-defective mutants have shown a substantial overlap in the functions used in various aspects of DNA metabolism in Drosophila. Biochemical characterisation of some repair-deficient mutants of Drosophila has shown them to be defective in one or more of the following: excision repair, post-replication repair, DNA synthesis. The pleiotropic effect of several mutants indicates that the wild-type allele normally participates in more than one of these processes. An ap-endonuclease activity has been identified suggesting that Drosophila may possess a base excision repair pathway. As a result of recent improvements in tissue culture (Boyd et al., 1980) it should now be possible to analyse each of the major forms of DNA-repair biochemically.

The highly sensitive alkaline elution procedure of Kohn et al. (1976), which to date has been used only to measure the accumulation of single-strand DNA breaks following UV-irradiation, might be usefully extended to

monitor the production of chemically-treated breaks in Drosophila sperm, both unstored and stored. It may establish if the breakage event occurs during the storage period or only after the sperm has fertilised an egg and the maternal repair systems begin to operate.

Maternal effects on the production of mutations have already been found (Proust et al., 1972). When sperm carrying pre-mutational lesions were introduced into repair deficient oocytes it was found that (a) alkylation damage led to increased frequencies of point mutations in an excision-repair deficient mutant and (b) another mutant was shown to be unable to process HN_2 (nitrogen-mustard) lesions into point mutations (slrl). It should therefore be possible to study the repair of different lesions and to determine which mutants influence the repair pathway(s) pertinent to the production of point mutations or to chromosome aberrations. Workers have already investigated the production of point mutations in repair deficient strains - an increase in frequency of slrl has been equated with lack of repair of premutationally altered DNA, possibly of O^6 lesions. This work could be extended to examine the production of chromosomal breakage and of both slrl and breakage events after storage.

Using molecular biological techniques, it is now possible to look in detail at the nature of mutagenic changes at the molecular level. These techniques have been used extensively to study spontaneous mutagenic

events in Drosophila: transposable elements and the hybrid dysgenic P-factors have been shown to cause site specific rearrangements at high frequencies. Some of these have been studied in detail, for example, the copia-like elements which comprise up to 10% of the Drosophila genome. A molecular analysis of these elements has shown them to be a highly but imperfectly conserved sequence 5-9 kb long, occurring with a frequency of 15-100 copies per haploid genome. They are dispersed and mobile throughout the genome. They show certain structural homologies with other families and vertebrate proviruses. In contrast to prokaryotes, eukaryote transposable elements seem capable of precise excision at high frequencies.

Perlman (1983) by using the technique of chromosome walking (Maniatis et al., 1982; Bender et al., 1983) isolated and cloned a deletion-fusion fragment which had arisen spontaneously. Using DNA hybridisation she found that DNA on the left side of the fusion contained sequences homolgous to the roo family of genetic elements. Restriction map data was consistent with the interpretation that the right end of a roo element was situated near the breakpoint. The implication from this finding was that the roo element had been involved in the deletion event. Further molecular analysis of rearrangements both spontaneous and mutagen-induced events, may reveal some consistent data on the role of transposible elements in mutagenesis. Evidence has

already been obtained for the involvment of transposable elements in spontaneous mutations at the white locus in Drosophila (Collins and Rubin, 1983). (The transposition mechanism has not yet been elucidated - it may be caused by excision and reinsertion of an element, or by duplication and excision).

An examination of mutagen-induced rearrangements, and slrl mutations, at a molecular level should reveal if transposable elements are also involved in producing these events. Although it is technically more difficult than comparable work in prokaryotes, mainly due to the size and complexity of eukaryotic chromosomes, it should be possible to employ molecular techniques to obtain a detailed picture of the mutagenic events. For example, the mutational specificity of depurination, a non-coding mutagenic lesion, has been studied in phage DNA (Kunkel, 1984). By selecting for the loss of a non-essential gene function (β-galactosidase) a wide range of mutagenic events was scored (base substitution, frameshifts, deletions, additions and rearrangements). The mutant gene was sequenced and compared with the WT gene in order to determine the nature of the mutational event. Similar work is being carried out in this laboratory using the am gene in Neurospora (P. Burns, unpublished). This technique should also be applicable to Drosophila, for example by using several marker genes along the X-chromosome (as in the specific locus test) and examining the sequence of flies mutant at one of the loci. The most difficult

part of this procedure would be the initial cloning and sequencing of the required marker genes. Once this had been achieved it would be a relatively easy step to use the WT clones to probe for the mutant genes. Visible mutations induced in either the specific locus of attached-X test could thus be sequenced and the proportion of small deletions amongst such events could be determined directly. Break-points could also be sequenced. Again it would be necessary to obtain probes, for example to the _bw_$^+$ and _st_$^+$ regions on chromosomes II and III. Reciprocal translocations induced between chromosomes II and III, detected by an exchange of segments carrying the _bw_ and _st_ markers could be examined. Using either the _bw_ or _st_ probes as starting points it would then be possible to "jump" from the markers normal location to its relocation site and "walk" towards the breakpoint using characterised chromosomal fragments from a _Drosophila_ library. By a combination of DNA hybridisation and restriction map analysis it would then be possible to locate the breakpoint and ultimately sequence it. This may provide information as to whether breakpoints are site-specific, whether rearrangements are accompanied by loss of genetic material, whether they are associated with transposable elements or share sequences in common with them.

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APPENDIX. STATISTICAL ANALYSIS

APPENDIX : STATISTICAL ANALYSIS

A. Regression Analysis

Regression analysis has been used to study the change in the mean value of one variable (the dependent variable) as the other variable (the independent variable) changes. Usually the investigator chooses the values of the independent variable (x) and asks what happens to the dependent variable (y) as x changes.

In this thesis it was used to answer the following two questions:

(i) What happened to dominant lethal frequency as the storage time increased?

(ii) What happened to the frequency of II-III reciprocal translocations as the number of depurinated guanine bases increased?

The method used was that of the least squares:

$$Y = a + bx$$
$$= \bar{y} + b(x - \bar{x})$$

the slope of the line = b

the intercept = $a = \bar{y} - b\bar{x}$

This equation can be used to fit a straight line with one observation at each x value. The values to calculate are

$\bar{x}$

$a = \bar{y}$

$$b = \frac{\Sigma (y-\bar{y})(x-\bar{x})}{\Sigma (x-\bar{x})^2} = \frac{\Sigma xy - \frac{\Sigma y . \Sigma x}{N}}{\Sigma x^2 - \frac{(\Sigma x)^2}{N}}$$

These can be substituted into the straight line equations to give values for Y at different x values.

B. Tests of significance and confidence limits on a slope

The test of significance used was

$$t_{n-2} = \frac{b - \beta_0}{\text{Est.SE}(b)}$$

The null hypothesis was assumed, thus $\beta_0 = 0$, i.e. there was no slope or relation between y and x in the underlying population.

The 95% confidence limits on the slope were obtained as

$$b \pm t_{n-2,\ 0.05}(\text{Est.SE}(b))$$

C. Data and Calculations

(i) Estimation of the relationship between storage time and percentage of unhatched eggs fertilised by non-treated or EMS-treated sperm, using regression analysis.

1. CONTROL

Day No. x	% Unhatched eggs y	xy
1	17.55	17.55
2	10.76	21.52
3	6.35	19.05
10	9.84	98.40
11	12.36	135.96
12	80.65	967.80
13	63.37	823.81
14	63.68	891.52
15	42.18	632.70
16	40.86	653.76
17	20.12	342.04
18	17.71	318.78
19	23.72	450.68
20	21.19	423.86
21	40.98	860.58
22	51.96	1143.12
23	32.20	740.60
24	43.45	1042.80
25	58.82	1470.50

$n = 19$

$\bar{x} = 15.05$ $\quad \bar{y} = 34.62$ $\quad \Sigma x.y = 11054.97$

$\Sigma x = 286$ $\quad \Sigma y = 657.75$ $\quad \Sigma x.\Sigma y = 188116.5$

$\Sigma x^2 = 5254$ $\quad \Sigma y^2 = 31231.56$

$(\Sigma x)^2 = 81796$ $\quad (\Sigma y)^2 = 432635.06$

$Y = \underline{a} + bx$ $\quad \bar{x} = 15.05; \quad a = 34.62; \quad b = 1.216$

$= \bar{y} + b(x - \bar{x})$

How different is this slope from 1?

$$t_{n-2} = \frac{b - 1}{SE(b)} \qquad t_{17} = \frac{1.216-1}{0.66} = 0.33$$

$p > 0.05$

∴ not significantly different from 1.

95% confidence limits -0.08 - 2.50.

2. 0.1% EMS

Day No. x	% Unhatched eggs y	xy
1	1.41	1.41
2	9.86	19.72
3	4.15	12.45
4	3.57	14.28
12	20.06	240.72
13	43.80	569.40
14	28.64	400.96
15	20.11	301.65
16	21.58	345.28
17	24.75	420.75
18	22.59	406.62
19	25.34	481.46
20	32.48	649.60
21	65.69	1379.49
22	34.16	751.52
23	53.13	1221.99
24	46.36	1112.64
25	48.55	1213.75

$n = 18$

$\bar{x} = 14.95$ $\quad \bar{y} = 28.12$ $\quad \Sigma xy = 9543.69$

$\Sigma x = 269$ $\quad \Sigma y = 506.23$ $\quad \Sigma x.\Sigma y = 136175.87$

$\Sigma x^2 = 5049$ $\quad \Sigma y^2 = 19771.54$

$(\Sigma x)^2 = 72361$ $\quad (\Sigma y)^2 = 256268.81$

$Y = a + bx$ $\quad \bar{x} = 14.95$

$= \bar{y} + b(x-\bar{x})$ $\quad a = 28.12$

$b = 1.92$

How different is this slope from 1?

$$t_{n-2} = \frac{b - 1}{SE(b)} \qquad t_{17} = \frac{1.92-1}{0.105} = 8.76$$

$p << 0.001$

$\therefore$ significantly different from 1.

95% confidence limits 1.71 - 2.13.

3. 0.4% EMS

Day No. x	% Unhatched Eggs y	xy
2	4.0	8.0
3	5.63	16.89
12	17.80	213.60
13	37.30	484.90
14	33.10	463.40
15	22.90	343.50
16	35.00	560.00
17	18.75	318.75
18	21.30	383.40
19	10.48	199.12
20	7.50	150.00

$n = 11$

$\bar{x} = 13.55$ $\quad \bar{y} = 19.43$ $\quad \Sigma xy = 3141.56$

$\Sigma x = 149$ $\quad \Sigma y = 213.76$ $\quad \Sigma x.\Sigma y = 31850.24$

$\Sigma x^2 = 2377$ $\quad \Sigma y^2 = 5572.18$

$(\Sigma x)^2 = 22201$ $\quad (\Sigma y)^2 = 45693.34$

$Y = a + bx$

$= \bar{y} + b(x-\bar{x})$

$\bar{x} = 13.55$

$a = 19.43$

$b = 0.69$

How different is this slope from 1?

$$t_{n-2} = \frac{b - 1}{SE(b)} \qquad t_9 = \frac{0.69-1}{0.663} = -0.47$$

$p >> 0.05$

$\therefore$ not significantly different from 1.

95% confidence limits -0.61 - 1.99.

4. 0.75% EMS

Day No. x	Unhatched eggs y	xy
1	31.31	31.31
2	33.73	67.46
3	32.98	98.94
4	35.25	141.00
12	72.03	864.36
13	51.92	674.96
14	69.60	974.40
15	72.73	1090.95
16	100.00	1600.00
17	65.57	1114.69
18	91.76	1651.68
19	97.22	1847.18
20	87.23	1744.60

$n = 13$

$\bar{x} = 11.85$ $\quad \bar{y} = 64.72$ $\quad \Sigma xy = 11901.53$

$\Sigma x = 154$ $\quad \Sigma y = 841.33$ $\quad \Sigma x.\Sigma y = 129564.82$

$\Sigma x^2 = 2394$ $\quad \Sigma y^2 = 62246.22$

$(\Sigma x)^2 = 23716$ $\quad (\Sigma y)^2 = 707836.17$

$Y = a + bx$

$= \bar{y} + b(x-\bar{x})$

$\bar{x} = 11.85$

$a = 64.72$

$b = 3.4$

How different is this slope from 1?

$$t_{n-2} = \frac{b - 1}{SE(b)} \qquad t_{11} = \frac{3.4 - 1}{0.196} = 12.25$$

$p << 0.001$ $\quad \therefore$ significantly different from 1.

95% confidence limits, **3.02 — 3.78**

How different is this slope from that produced by 0.1% EMS?

$$t_{n-2} = \frac{3.4 - 1.71}{0.196} = 8.63$$

$p << 0.001$ $\quad \therefore$ these two slopes are significantly different.

(ii) Estimation of the relationship between depurinated guanines ($t_{\frac{1}{2}}$ = 36 days) and translocation frequency, using regression analysis.

Depurinated guanines	Translocations (%)				
$x(x10^5)$	y	xy	log x	log y	log x.log y
0.30	0.04	1200	4.477	-1.398	-6.2589
0.35	0.04	1400	4.544	-1.398	-6.353
0.40	0.22	8800	4.602	-0.658	-3.028
0.50	0.04	2000	4.699	-1.398	-6.569
0.50	0.04	2000	4.699	-1.398	-6.569
0.50	0.04	2000	4.699	-1.398	-6.569
0.60	0.04	2400	4.778	-1.398	-6.680
0.70	0.04	2800	4.845	-1.398	-6.773
0.90	0.54	48600	4.954	-0.268	-1.328
0.90	0.68	61200	4.954	-0.168	-0.832
0.90	1.47	132300	4.954	0.167	0.827
1.15	1.23	142600	5.061	0.093	0.471
1.30	1.01	131300	5.114	0.004	0.021
1.35	0.94	126900	5.130	-0.027	-0.139
1.60	2.37	379200	5.204	0.375	1.952
1.70	0.44	74800	5.231	-0.357	-1.868
1.80	0.79	142200	5.255	-0.102	-0.536
1.90	3.33	632700	5.279	0.522	2.756
2.10	2.64	554400	5.322	0.422	2.246
2.50	1.69	422500	5.398	0.228	1.231
2.50	2.34	585000	5.398	0.370	1.997
2.55	1.74	443700	5.407	0.241	1.303
2.60	1.02	265200	5.415	0.008	0.043
2.70	2.04	550800	5.431	0.310	1.684
3.15	8.59	270590	5.498	0.934	5.135
3.30	5.24	1729200	5.519	0.720	3.974
3.80	2.30	874000	5.580	0.362	2.020
4.10	3.72	1525200	5.613	0.571	3.205
4.70	3.27	6301300	5.672	0.515	2.921
5.00	7.04	352000	5.699	0.848	4.833
5.40	4.54	2451600	5.732	0.657	3.766
5.40	8.54	4611600	5.732	0.932	5.342
7.00	1.37	959000	5.845	0.137	0.801

Log data.

$n = 33$

$\bar{x} = 5.2042424$

$\Sigma x = 171.74$

$\Sigma x^2 = 898.56231$

$(\Sigma x)^2 = 2.9494628 \times 10^4$

$\bar{y} = -0.0893939$

$\Sigma y = -2.952$

$\Sigma y^2 = 19.47451$

$(\Sigma y)^2 = 8.7025$

$\Sigma x . \Sigma y = -506.97648$

$\Sigma xy = -6.975935$

$Y = a + bx$

$= \bar{y} + b(x - \bar{x})$

$\bar{x} = 5.204$

$a = \bar{y} = -0.090$

$b = 1.75$

How different is this slope from 1?

$t_{n-2} = \frac{b - 1}{SE(b)}$

$t_{31} = \frac{1.75 - 1}{0.174} = 4.310$

$p << 0.001$

95% confidence limits 1.41-2.09.