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MATERNAL REPAIR SYSTEM—AN ANALYSIS IN RETROSPECT

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ABSTRACT

Influence of caffeine, cysteine, hydroxyurea and cycloheximide on maternal repair system operative in female *Drosophila* was studied using rod-X males and Oster females. The number of parental matings was kept constant. A new method of statistical analysis was employed to examine the frequency of sex-chromosome loss in these experiments. A re-analysis of published data was also done using this method. The existence of a maternal repair system was not upheld. The observed discrepancies between data reported herein and those published earlier and the possible reasons are discussed.

In an interesting study using *Drosophila* females treated with Actinomycin D, Proust, Sankaranarayanan and Sobels (1972) postulated that maternal repair processes acting at the stage of pronucleus formation are required for the repair (restitution) or misrepair (reunion giving rise to translocations) of chromosome breaks induced in matures permatozoa. Mendelson (1974) and Mendelson and Sobels 1974), in a follow-up study, using *Drosophila* females fed with caffeine and irradiated ring-X males obtained corroborative evidence for the existence of such a repair system operative in the oocytes (to be referred to as maternal repair system). Consequently, a decrease in repair would cause an enhancement in the frequency of dominant lethals and sex-chromosome loss while a concomitant decrease in misrepair—a reduction in the frequency of autosomal translocations, thus implying that both repair and misrepair have a common step (pathway) which is inhibited by caffeine.

Since caffeine is known to have many target sites of action, it is also possible to ascribe the observed phenomenon to an effect on DNA synthesis (Cleaver and Thomas, 1969), RNA and protein synthesis (Zuk and Swietlinska, 1973), repair enzymes (Wragg, Carr and Ross, 1967; Rajalakshmi *et al.*, 1971), c-AMP phosphodiesterase (Butcher and Sutherland, 1962), membranes (Weber, 1968) and various protein fractions (Galzigna, Maina and Rumney, 1971; Barry, Hill, Sadler and William, 1973). Since cysteine and cycloheximide also inhibit protein synthesis through different pathways (Kovacs, Kari, Nagy and Hernadi, 1968; Baliga, Pronczuk and Munro, 1969; McKeelian and Hardesty, 1969; Rajalakshmi *et al.*, 1971) and hydroxyurea inhibits semi-conservative DNA synthesis (Timson, 1975), a comparison of the effects of these chemicals with those of caffeine on the reported maternal repair system can provide vital clues to the mode of action involved.

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With these objectives in view, experiments using these chemicals were carried out and an evaluation of their results along with those reported earlier was made. Further, a chemical characterization of repair enzymes was attempted through gel electrophoresis of proteins. The results of these investigations are discussed in this paper.

Materials and Methods

The genetic constitution of the two stocks of flies used for all experiments was : 1. Oster females—*ysc*^{sl} *In*^{49} *sc*^{8} ; *bw* ; *st* $\bar{p}_{x}$; 2. Rod-X males—*yB*/*sc*^{8} p^{+} *Y* ; *f*. Cultures were maintained at 25° ± 2° C. 0-8hold virgin females were kept for three days on strips of tissue paper soaked with following solutions : 1. 10% honey + 0.2% caffeine (Sigma) ; 2. 10% honey + 0 2% cysteine (Merck) ; 3. 10% honey + 0.1% hydroxyurea (Sigma) ; 4. 10% honey + 120 µg/ml cycloheximide (Sigma) ; 5. 10% honey + 200 µg/ml cycloheximide (Sigma). Three day-old rod-X males were irradiated with 1,500 R of gamma- rays by means of a^{3680} Ci^{60}Co gamma cell 220 source (AEC Canada). The dose rate was 30R/sec. The treated females were mated singly to these males and the number of matings was kept constant for control and each of the different treatments. Sex-chromosome loss was scored in F_1 following Oster (1958). Whole body extracts of virgin Oster females fed either with honey alone or honey combined with caffeine were subjected to gel electrophoresis following the procedure for disc electrophoresis using anionic system due to Davis (1964).

Statistical analysis : The four combinations of radiation (R) and chemical (C) treatments—(i) T_{00}—Control i.e., no chemical, no radiation, (ii) T_{01}—no chemical, only radiation, (iii) T_{10}—only chemical, no radiation and (iv) T_{11}—both chemical and radiation—can be represented by a two-way contingency table.

	R		Marginal
C	0	1	frequency
0	T_{00}	T_{01}	C_0
1	T_{10}	T_{11}	C_1
Marginal frequency	R_0	R_1	

Results

For brevity, theses symbols will also be used to denote values of characters like the proportion of sex-chromosome loss (SL), dominant lethals (DL) and sex-linked recessive lethals (SLRL). The independence of the two factors C and R is tested by the contingency chi-square. Since the progeny sizes under each mating for various treatments would not be equal, it would be necessary to correct the observed proportions of the metric, e.g., SL or DL before attempting an analysis. In the published literature, the following variables due to Abbot (1925) have been used for correcting those proportions :

$$P^{a}x = T_{11}—T_{10}/1—T_{10} \qquad P^{b}x = T_{01}—T_{00}/1—T_{00}$$

P_{ax} measures proportionate effect of radiation when caffeine is applied and $P^{b}x$ that when caffeine is not applied. These values are standardized to vary between 0 and 1 so that a value 0 corresponds to nil effect and 1 to complete effect. The difference $I = P^{a}x—P^{b}x$ is another parameter which is tested statistically for its significance from zero, the details of which would be found in Mendelson (1974). The significance of I pooled over replica experiments was tested by a parameter T.

By a simple algebra it can be shown that

$$I=\frac{X-Y}{(1-T_{10})\ (1-T_{00})}, \text{ where}$$

$$X=(T_{11}-T_{10})-(T_{01}-T_{00}), \text{ and } Y=(T_{00}\ T_{11}-T_{01}\ T_{10})$$

When the number of matings was kept constant under each treatment, the differences in the size of the progeny under T_{00}, T_{10}, T_{01}, and T_{11} could be attributed to the viability and fecundity differences induced by R, C or both.

The effects of these differences would be felt in the values of SL, DL etc., since they were based on progeny size. By using the progeny size as weights to correct the observed proportions as detailed in Table 1 (using as an example, the data on SL of the experiment in Table 2), a relative evaluation of the treatment differences could be made unbiasedly. The relative proportion of progeny size under the treatments was used to calculate the corrected proportions in the same way as viability differences are nsed to estimate change in gene frequency in one generation of natural selection (see, for example, Li, 1955).

C_0, the effect of caffeine at zero level averaged over the two radiation levels or in other words, the average effect of non-application of caffeine and similarly the other effects, *i.e.*, C_1, R_0 and R_1 were estimated using cell frequencies in an unbiased manner as explained in Murthy *et al.* (1959*a*, b). In our example, the adjusted proportions were :

C	R 0	R 1
0	3.09/3583 =0.0008639	12.28/2585 0.0047514
1	1.28/2955 0.0004319	13.35/1931 0.0069112

For instance, an estimate of C_0 could be given by :

$$a=(3.09+12.28)\div(3583+2585)=0.002492$$

and also by $b=(0.0008639+0.0047514)\div 2=0.002808$

The unbiased estimate was given by $Z=2a-b=0.002176$

The variables T_{00}, T_{01}, T_{10} and T_{11}, C_0, C_1, R_0 and R_1 would all follow different binomial distributions; further, the distribution under treatment T_{00} could be considered independent of that under T_{01} and so on. Hence using the usual test for bionomial variates, all the eight possible differences (See Table 2) were statistically tested. As an example, let us consider the comparison $T_{00}-T_{10}$. The relevant statistic will be ;

$$t=\frac{T_{00}-T_{10}}{\text{s.e. } (T_{00}-T_{10})}$$

If the value of $T_{00}=P_1=0.0008639$ and of $T_{10}=P_2=0.0004319$, then

TABLE 1

Detailed analysis of data on SL of experiment 1, Table 2.

	T_{00}	T_{01}	T_{10}	T_{11}	Total
Frequency of SL	2	11	1	16	30 = F
Observed proportion of SL	2÷30	11÷30	1÷30	16÷30	
Progeny size	3583	2585	2955	1931	
Weighted non-normalized of SL	(Observed proportion of SL) × (Progeny size) 7166÷30	28435÷30	2955÷30	30896÷30	69452 ÷ 30 = S
Normalized proportion of SL	Weighted non-normalized proportion of SL)÷S 0.1031791	0.4094194	0.04254773	0.4448539	
Corrected frequency of SL	(Normalized proportion of SL) × F 3.09	12.28	1.28	13.35	30
Corrected proportion of SL	(Corrected frequency of SL) ÷ (Progeny size) 0.0008639	0.0047514	0.0004319	0.0069112	
Uncorrected proportion of SL	(Observed frequency of SL) ÷ (Progeny size) 0.0005581	0.0042553	0.0003384	0.0082858	

TABLE 2

Analysis of the frequency of sex-chromosome loss when females were treated with certain chemicals

Expt.	1			2			3			4			5		
	F	0	A	F	0	A	F	0	A	F	0	A	F	0	A
00	3583	2	3.09	3794	2	2.43	3478	1	1.32	2965	2	2.60	3195	2	2.11
01	2585	11	12.28	3162	14	14.20	2921	12	13.34	2199	10	9.64	2999	11	10.91
10	2955	1	1.28	3561	1	1.14	3494	2	2.66	2085	3	2.74	3648	5	6.03
11	1931	16	13.35	2913	12	11.21	2287	13	15.67	2283	9	9.01	2489	6	4.94
C_0-C_1	—NS		+NS	—NS			—NS			—NS			+NS		
R_0-R_1	—S		—S	—S			—S			—S			—S		
T_{00}-T_{01}	—S		—S	—S			—S			—S			—S		
T_{00}-T_{10}	+NS		+NS	—NS			—NS			—NS			—NS		
T_{00}-T_{11}	—S		—S	—S			—S			—S			—NS		
T_{01}-T_{10}	+S		+S	+S			+S			+NS			+NS		
T_{01}-T_{11}	—NS		+NS	—NS			—NS			+NS			+NS		
T_{10}-T_{11}	—S		—S	—S			—S			—NS			—NS		

F=Progeny size; 0=Observed frequency of SL; A=Corrected frequency of SL; NS= Not significant;
S=Significant; Sign of treatment comparison appended before S or NS.

$$\text{Var } (T_{00}) = P_1 (1-P_1) \div 3583, \quad \text{Var } (T_{10}) = P_2 (1-P_2) \div 2955$$

$$\text{and s.e. } (T_{00}-T_{10}) = \sqrt{(\text{Var } (T_{00}) + \text{Var } (T_{10}))}.$$

Thus in this example, $t = 0.694$ which is less than 1.96 and hence not significant at 5% level.

A joint analysis of the tests of significance of all such comparisons was used to draw possible inferences these experiments could provide.

We first evaluate our experiments through the new method of analysis against the conclusions drawn by earlier workers. The progeny sizes in Table 2 were comparable when the frequencies of parental matings in different treatments (including control) were kept constant. This, in turn, ensured a valid comparison of the corrected proportions of SL in any one experiment or across a number of experiments.

An analysis of SL (Table 2) unequivocally brought out the following results :

(a) The test of significance of C_0—C_1 showed that the average effect of administration of caffeine to females did not register significant increase in SL when compared to the average effect of non-administration. On the other hand, the average effect of radiation was to produce a significant increase in the frequency of SL as shown by the test of R_0-R_1.

(b) Corroborative evidence was present in all experiments without exception that it was only radiation that was responsible for bringing about a significant increase in SL. A significant difference in the frequency of SL occurred whenever there was a change in the radiation level regardless of a change in the chemical level; this was especially true for caffeine. This conclusion would be apparent by a critical examination of the significance of the following comparisons :

(i) T_{00}-T_{01} and T_{10}-T_{11} where radiation level alone changed, caffeine level being kept constant either at zero or at one.

(ii) T_{00}-T_{10} and T_{01}-T_{11} where caffeine level alone changed, radiation level being kept constant either at zero or at one.

(iii) T_{00}-T_{11} and T_{01}-T_{10} where both cafeine and radiation levels changed.

The results obtained above were identical whether caffeine, cysteine or hydroxyurea was administered to the females. When cycloheximide was used, the comparisons T_{01}-T_{10} and T_{10}-T_{11} were not significant; in addition T_{00}-T_{11} was not significant for the chemical level 200μg/ml. This would point out that the effect of radiation could be balanced by that of cycloheximide. Further, the results support the possibility of an optimum level of this chemical (probably higher than 200 μg/ml.) to counter the effects of radiation.

A comparison of protien bands in gels obtained from control and caffeine fed females indicated no difference between the two (Fig. 1).

It will now be appropriate to examine the published literature in the light of our results. An examination of the progeny size under the four treatments reported in a number of experiments published during 1974-1976 (Table 3) would clearly show that the frequency of parental matings under the treatments was not kept constant, control being obviously based on a fewer number of matings. After sorting the experiments

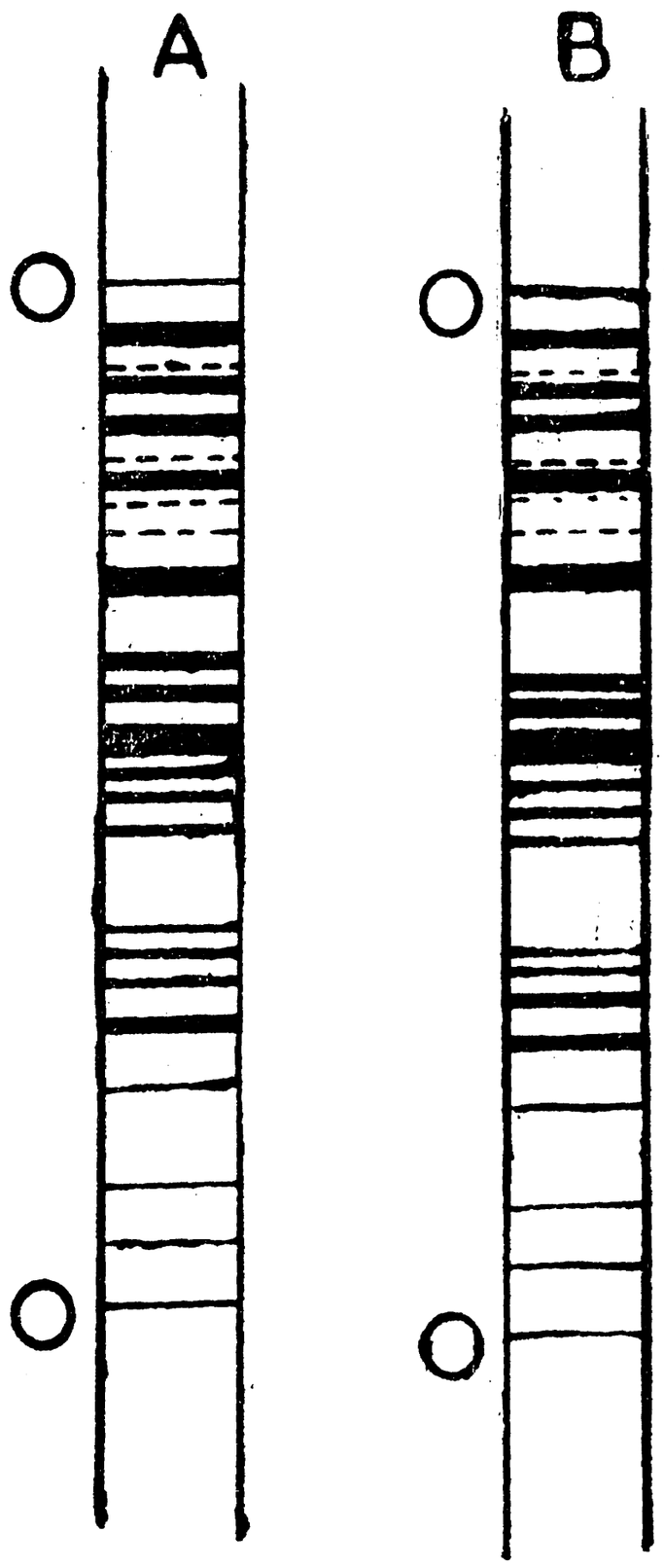

Fig. 1. Gel Electrophoresis of Proteins
(a) Control
(b) Caffeine-fed

where these discrepancies were less apparent, a reanalysis was done using the new method and the results compared with the published ones (Table 4). The following were the salient results :

(a) As tested by the statistic T advocated in the published papers, the proportionate effect of radiation on SL when caffeine was applied (P^a_x) was not significantly different from that when caffeine was not applied (P^b_x) in individual experiments; however, when pooled, this difference was found significant. Such a significant difference was not found even on pooled data in the case of DL when +60 females were used.

(b) On reanalysis by the new method, it became very clear that the results reported in Table 2, were confirmed in all respects execept for minor differences which could be attributed mainly to the unequal frequency of parental matings and consequent discrepancies in the progeny size, for e. g., T_{00}-T_{01} was not significant in the experiment 3B and T_{10}-T_{11} in experiment 3A in the case of SL. Similar was the result for T_{00}-T_{11} in experiments 1 A and 3A and T_{01}-T_{10} in experiments 1C and 3B. The underlying reasons for the differences and the implications of the confirmed results on maternal repair system would now be discussed.

TABLE 3

Discrepancies in the progeny size in the published data of various experiments

*Expt	00		01		10		11	
	F	0	F	0	F	0	F	0
1	567	75	1386	694	413	29	1172	695
2	511	3	818	23	329	3	480	19
3	343	5	706	19	625	6	1046	36
4	574	19	2124	77	1173	14	2651	123
5	1103	16	1975	80	697	5	2025	100
6	294	4	1318	80	379	4	1010	75

F=Progeny size; 0=Observed frequency; *1 Mendelson (1974) Table I, expt. 4B-DL; 2 Mendelson (1974) Table III, expt. 2 A-SL; 3 Mendelson (1974) Table VI, expt. 1A-SL; 4 Mendelson (1974) Table VI, expt. 1 B-SL; 5 Mendelson (1974) Table VI, expt. 2 B-SL;6 Mendelson (1976) Table III, expt. 3-SL.

TABLE 4

Reanalysis of data on SL and DL published in Mutat. Res. **22** : *145-156* (*Table III & IV*)

Expt.	1A			1C			3A			3B			3A(+60 females)			3B		
	F	0	A	F	0	A	F	0	A	F	0	A	F	0	A	F	0	A
00	726	6	7.9	1061	4	8.0	883	8	10.4	1322	11	12.4	254	35	16.3	450	60	30.1
01	656	18	21.3	488	14	13.0	823	18	21.8	1288	14	15.4	716	318	418.5	1164	546	709.1
10	336	1	0.6	683	6	7.8	497	5	3.6	932	12	9.6	227	27	11.3	557	83	51.6
11	291	10	5.2	401	20	15.2	446	14	9.2	1154	26	25.6	306	151	84.9	426	194	92.2
P_x[a]		3.1			4.1			2.1			1.0						38.2	
P_x[b]	1.9			2.5			1.3			0.3						37.2		
I	2.2			1.6			0.8			0.7						1.0		
T	0.917 NS			1.183 NS			0.769 NS			1.040 NS						1.007NS		
Pooled T							2.785											
C_0-C_1	S			NS			NS			NS			S			S		
R_0-R_1	S			S			S			S			S			S		
T_{00}-T_{01}	S			S			S			NS			S			S		
T_{00}-T_{10}	S			NS			NS			NS			NS			NS		
T_{00}-T_{11}	NS			S			NS			S			S			S		
T_{01}-T_{10}	S			NS			S			NS			S			S		
T_{01}-T_{11}	NS			NS			NS			NS			S			S		
T_{10}-T_{11}	S			S			NS			S			S			S		

F=Progeny size; 0=Observed frequency;=Corrected frequency; NS=Non signifcant; S=significant.

Discussion

The backbone of the concept of maternal repair system advanced by Mendelson (1974) and Mendelson and Sobels (1974) was a statistical evaluation of the differences observed in certain measurable characters like SL and DL. *A priori* considerations for a statistical validity of different comparisons among treatments would point out the following :

(a) The frequency of parental matings under each treatment should be kept constant.

(b) If some matings produced progeny of abnormal size (not attributable to the treatment), they should be discarded.

(c) If points (a) and (b) were taken into proper consideration, the differences observed among the progeny sizes, would by and large represent the associated viability/fecundity differences produced by the respective treatments and should be considered in evaluating the treatment differences.

The method of analysis adopted so far, using the proportions P^a_x and P^b_x were based on different denominators rendering the comparison $I = P^a_x - P_{hx}$ invalid. Further, I was a difference, X-Y, (See Materials and Methods) of quantities unamenable for interpretation. What a test of significance of I would indicate is hence a moot question.

The method of pooling data over a group of replica experiments, adopted by Mendelson, further weakened the interpretation. It was mentioned earlier that the starting point for the difficulties of an objective evaluation was the uncomparable progeny sizes between different treatments within an experiment. Obviously such progeny sizes could become more uncomparable when replica experiments were considered. For example, progeny sizes in an experiment by Mendelson (1974, Table 3) varied from 241 and 764 for the treatment T_{00} while the corresponding figures were 522 and 1498 for T_{01}, 69 and 413 for T_{10} and 333 and 1172 for T_{11} over four replica experiments consisting of observations on two days A and B. Thus the pooled statistic T would not help obviously to bring out the right conclusions. Normal criteria for pooling up of results over experiments would demand (1) comparable progeny sizes under a treatment between replicates and (2) comparable estimates of 'I' within each replicate.

We hence set up equal number of parental matings under the treatments including control so that the differences in the progeny sizes would provide an apt basis for correcting the observed SL.

Unlike the methods adopted so far, wherein the effect of caffeine was inferred only from the treatment comparison T_{01}-T_{11}, we obtained almost unbiased estimates of the 'main effects', C_0, C_1, R_0, and R_1. In this process, we defined : (i) a main effect say C_0 as an aggregate over the two possible levels of other factors which are in this case, T_{00} and T_{01} and (ii) a marginal mean to provide an almost unbiased estimator of the main effect.

We, therefore, believe, we have provided a reliable and repeatable statistical technique for evaluating experiments of this nature which would make comparisons between and within experiments most reliable provided the basic requirements of the

experimental procedure were assured.

An interestsng observation was made earlier (Mendelson, 1976; Mendelson and Sobles, 1974) that caffeine did not produce any effect on the frequency of sex-chromosome loss when specific female genotypes were used. A lack of effect of caffeine was reported especially when females of the genotype Ubx e^4/ Payne ca were used. This observation was in sharp contrast to those observed in experiments with Oster and ysn^3 females; obviously this would have led to the suggestion that Ubx females could be deficient for a repair enzyme that was susceptible to inhibitory action of caffeine. However, it would be necessary to establish that (a) the genetic constitution of the females had a certain influence in the repair process and (b) the lack of effect of caffeine in the repair process was due to such differences in the genetic constitution of females. Genetic analysis performed in Ubx e^4/Payne ca females has suggested that, at least, three third-chromosome factors may be involved in the control of maternal repair, the two most effective ones being located on the right arm of the third chromosome, one near the Ubx locus and the other near the ca locus (Mendelson, 1976). This, no doubt, strengthened the case for the existence of maternal repair system, but was, by no means, conclusive as the experiments involved only sex-chromosome loss (i.e. lack of repair only being detected, the occurrence or otherwise of misrepair being ignored) and the interpretations based on methodology and statistical analysis basically different from those used in this paper.

The assumption that the caffeine-sensitive repair factor could be an enzyme, would need verification. In the present study, whole body extracts of Oster females fed or not fed with caffeine were subjected to gel electrophoresis and it was found thas the protein profiles of caffeine-treated and control females were identical (Fig. 1). Although this was a negative evidence for the existence of a caffeine-sensitive maternal repair enzyme, it must be pointed out that it was, by no means, decisive. A more refined attempt would be to use only the oocytes recovered from the treated and control females for the analysis.

The use of cycloheximide on females indicated in our experiments interesting possibilities as brought out in 'Results'. It would seem logical to observe that an optimum level of concentration of this chemical possibly above 200 μg/ml applied to females could interact with the effects of radiation used on males to bring down the frequency of SL. The results on sex-linked recessive lethals and translocations (unpublished) appear to confirm these observations.

Encouraged by the consistency exhibited in the results of our experiments and in those published earlier on reanalysis, we can observe that it is radiation that is responsible for the increase in the frequency of SL regardless of the administration of caffeine to females. An inhibitory action of caffeine on the maternal repair system was not adequately supported by our data and those published earlier, even when experiments with different stocks of males like ring-X and rod-X were considered. Thus, published results and our experiments do not support the existence of a maternal repair system, as postulated by Mendelson and Sobles.

Finally, in experiments of this nature, the postulation and evaluation of any

experimental procedure were assured.

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Finally, in experiments of this nature, the postulation and evaluation of any

hypothesis pertaining to the interactions of the effects of chemicals and radiation were always done by an analysis of the data on a measurable character like sex-chromosome loss, dominant lethals, sex-linked recessive lethals, translocations etc. Interestingly, it is an inherent assumption in all these studies that the effect of inhibition of the so-called maternal repair system by a chemical, is directly proportional to the loss or gain in the frequency of the measurable trait. A need is strongly felt, therefore, to establish a link between the inherent mechanism, on the one hand and the observable character, on the other. It is therefore, imperative to pursue vigorous studies to establish the fact that these inherent genetic mechanisms manifest themselves on the measurable characters; or else, methods would have to be developed to parametrise those inherent genetic and biochemical mechanisms.

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