SOMATIC CROSSING OVER IN *GLYCINE MAX* (L.) MERRILL: EFFECT OF SOME INHIBITORS OF DNA SYNTHESIS ON THE INDUCTION OF SOMATIC CROSSING OVER AND POINT MUTATIONS*

B. K. VIG

Department of Biology, University of Nevada, Reno, Nevada 89507

Manuscript received August 14, 1972
Revised copy received December 4, 1972
Transmitted by J. H. TAYLOR

ABSTRACT

Glycine max (soybean) is the only known higher plant with a definitely established occurrence of somatic crossing over. This material lends itself to the analysis of somatic crossing over, gross chromosomal aberrations and mutations, all of which may be induced by the same treatment of the mutagen given to seeds. This is made possible because gene Y_{11} for chlorophyll development in the variety L65-1237 is incompletely dominant over its allele y_{11}, so that twin or double spots composed of a dark green ($Y_{11}Y_{11}$) and a yellow ($y_{11}y_{11}$) component can be observed adjacent to and as mirror images of each other on the light green $Y_{11}y_{11}$ leaves in the areas of complementary exchange for these genes. Lack of growth of either component of this double spot as well as several types of chromosomal disturbances give rise to single spots resembling phenotypes of $y_{11}y_{11}$ or $Y_{11}Y_{11}$ leaves. Point mutations can be studied by looking for green sectors originating from $Y_{11}y_{11}$ genotype on the $y_{11}y_{11}$ plants. Seeds obtained from heterozygous plants were treated with caffeine, cytosine arabinoside, actinomycin D and 5-fluoro-deoxyuridine, all known inhibitors of DNA synthesis, and puromycin, an inhibitor of synthesis of proteins. The treatments with caffeine and actinomycin D increased the frequency of somatic crossing over as measured by the frequency of double spots on $Y_{11}y_{11}$ leaves, but cytosine arabinoside, 5-fluorodeoxyuridine and puromycin did not. Thus somatic crossing over was induced only by those chemicals which are known to allow rejoining of chromosomes, thereby suggesting a correlation between the two phenomena. These observations indicate that it is not the mere inhibition of DNA synthesis, but some rather more specific event in DNA repair which is responsible for complementary exchanges. Some of these results differ from studies carried out with fungi. The main effect of all chemicals tested, except caffeine and actinomycin D, was inferred to be the production of deletions in $Y_{11}y_{11}$ plants which raised the frequency of single (dark green or yellow) spots relative to the doubles. Caffeine was the only chemical which constantly increased the frequency of specific point mutations. In the control material, the great majority of spots are found on the upper surface of the leaf. This picture could not be changed in any of the treated materials, thus indicating uniform resistance of spongy mesophyll tissue to the mutagens applied.

* Paper number 501 from the Department of Biology, University of Nevada, Reno.

OF all the higher plants claimed to have been analyzed for the occurrence of somatic crossing over, *Glycine max* (Soybean) appears to be the only one which upon critical studies meets all the requirements of the phenomenon in question (VIG 1972; VIG and PEACOCK 1968; NOTHIGER and DUBENDORFER 1971). Studies on somatic crossing over in *Drosophila melanogaster* initiated by STERN (1936) and recent studies using *Musca domestica* (NOTHIGER and DUBENDORFER 1971) are the only other cases among organisms higher than fungi wherein conclusive evidence for the occurrence of somatic crossing over has been found. In *Glycine max* this study is made possible because in varieties L65-1237 and T219, the gene Y_{11} for chlorophyll development expresses incomplete dominance over its allele y_{11}, so that $Y_{11}Y_{11}$ plants are dark green in color, $Y_{11}y_{11}$ are light green and $y_{11}y_{11}$ plants are golden yellow, as if almost lacking any chlorophyll. Often the two simple leaves and rarely the first compound leaf of the heterozygous plants have spots composed of a dark green sector (resembling the phenotype of $Y_{11}Y_{11}$ leaves) adjacent to and almost mirror image of a yellow colored sector (resembling the phenotype of $y_{11}y_{11}$ leaves). These so-called "double" or "twin" spots may lose, during development, one of the components, thus resulting in a "dark green" or "yellow" spot. The origin of the latter two type of spots could have been attributed to point mutations if it were not for the fact that $Y_{11}Y_{11}$ and $y_{11}y_{11}$ plants are almost totally devoid of any kind of spots. Application of mitomycin C to seeds of these two varieties (VIG and PADDOCK 1968, 1970; VIG 1972) results in an increase in the frequency of all three types of spots without disturbing their relative proportions as found in the control material. The induction of somatic crossing over by Mitomycin C has also been increased in certain fungi (HOLLIDAY 1964).

It is possible that some of the so-called single spots may result from chromosomal processes like nondisjunction, segmental deficiencies or gross numerical inequalities during mitosis (see VIG 1969). The origin of twin spots, however, can be explained best by the process of somatic crossing over, for the following reasons:

1) The quantitative pattern of inheritance makes nondisjunction an unlikely cause. The Y_{11}-$Y_{11}y_{11}y_{11}$ type of numerical inequality should produce only yellow spots, at best, since the sectors carrying only Y_{11} must be light green in color. This would also require the production of some light green spots on the $Y_{11}Y_{11}$ plants, but to date we have observed none. The alternate form of nondisjunction, *viz.*, y_{11}-$Y_{11}Y_{11}y_{11}$, may produce a twin spot on the $Y_{11}y_{11}$ background, but random failure of one or the other component to develop in the two types of nondisjunctions should produce at least twice as many yellow spots as dark greens. However, this stipulation is not validated by observations. Also, almost equal and parallel development of the complementary segments in population of twin spots demands equal rate of growth and division of the so produced monosomic and trisomic lines. Occasionally a leaf treated with radiation or a chemical may have as many as 50 to 60 spots, covering as much as 50–75% of the total surface area. This makes nondisjunction very unlikely on the basis of frequency alone. The chemicals used to induce double spots are not known to cause nondisjunction (at

least to this extent). One must also consider that only the chemicals known to permit reunions of broken ends of chromosomes are capable of bringing about double spots (present paper). Such a prerequisite would not be needed for induction of nondisjunction. Lastly, one should expect some aberrant progeny and segregation based on nondisjunction in premeiotic or meiotic cells. To the best of our knowledge, no such evidence has been found so far.

2) The loss of the chromosome segment carrying the gene in question may produce only yellow spots and the gain of the segment will also produce only single spots. A loss-gain situation in adjacent cell populations will lead to a discussion which is similar to (1) above, besides requiring a repeated perpetuation and inclusion of the acentric fragment into daughter cells.

3) The idea of gene conversion and point mutations can be dismissed on the grounds that the production of double spots requires two alternate events to take place simultaneously in time and space and that the high frequency of doubles in relation to the singles makes it unlikely. Neither of these ideas explains why untreated $y_{11}y_{11}$ leaves are almost always devoid of spots and why there is no parallel between any or all types of spots on $Y_{11}y_{11}$ leaves and light green spots on $y_{11}y_{11}$ leaves by the same treatment.

4) An attempt to explain the phenomenon of spotting based on alterations in the chloroplast DNA also suffers from the same general drawbacks as discussed above in (3).

5) The idea of some sort of controlling elements in *Glycine max*, similar to those discovered in *Zea mays*, may not explain the occurrence of double spots on the heterozygous background, especially in view of the fact that chemicals and radiation are required to stimulate such a system. Also, in contrast to $Y_{11}y_{11}$ leaves, the $y_{11}y_{11}$ leaves do not show any spots unless treated chemically or with radiations.

Inhibitors of DNA synthesis, e.g., mitomycin C and 5-fluorodeoxyuridine (FUdR), have been shown to increase the incidence of somatic crossing over in the fungus *Ustilago maydis* (Esposito and Holliday 1964; Holliday 1964). Similarly Wolff and deSerres (1967) have shown that FUdR induced impairment of DNA synthesis increases meiotic crossing over, as evidenced by analysis of second division segregation for ascospore color markers in Neurospora. In *Glycine max*, whereas mitomycin C is effective in increasing somatic crossing over, the effect is not limited to the period of DNA synthesis (Vig and Paddock 1970). In view of this information, we decided to study the effects of several inhibitors of DNA synthesis to find a common denominator which might be responsible for triggering the process of somatic crossing over and to understand the mechanism(s) underlying this rare phenomenon. Four known inhibitors of DNA synthesis, *viz.*, caffeine (1,3,7-trimethyl xanthine), cytosine arabinoside (1,β,D-arabinofurenosyl-cytosine), actinomycin D and FUdR, were used in this study. Since colchicine is known to increase the incidence of somatic crossing over (Vig 1971a), one inhibitor of protein synthesis, viz., puromycin, was also used.

Besides being of medicinal significance, caffeine has been reported to be mu-

tagenic in prokaryotes and a doubtful point mutagen in eukaryotes. However, there is no question about the chromosome breaking potential of this chemical (for review see KÜHLMAN, *et al.* 1968; FISHBEIN, FLAMM and FALK 1970). The chromosome breaking action of cytosine arabinoside is known for human leukocytes but negative results for plants have been reported (KIHLMAN 1966, p. 120). The breaks in cultures of hamster fibroblasts—clone Don C—and other systems tested indicate fragmentation but no rejoining of the chromosome fragments (BENEDICT, HARRIS and KARON 1970; KIHLMAN, NICHOLS and LEVAN 1963). Actinomycin D, however, does cause chromosome breaks in both plant and animal materials producing chromatid exchanges (OSTERTAG and KERSTEN 1965) or sub-chromatid exchanges induced in prophase (KIHLMAN 1966; p. 170–171). FUdR, on the other hand, causes chromosome breaks in plant (TAYLOR, HAUT and TUNG 1962) as well as in animal (HSU, HUMPHREY and SOMERS 1964) cells without producing rejoining.

Because of the similarities and differences in the mode of action of these chemicals and the end results in terms of chromosome aberrations, it was considered worthwhile to study their effect on somatic crossing over in *Glycine max*, with the hope of elucidating the phenomenon underlying this process.

EXPERIMENTAL SYSTEM

In the system under study, symmetric exchanges of chromatid type involving two homologous chromosomes at corresponding sites should result, basically, in the production of twin or double spots. Since non-homologous exchanges of chromatid-type usually result in genic imbalance in the daughter cells, there should be very few twin spots compared to what can be produced by exchanges involving homologous chromosomes at corresponding sites. The role of numerical aberrations (nondisjunction) and gain or loss of fragments has already been assessed. Nevertheless, most of the resulting spots should be of "single" type. The point mutations (which should produce single spots on the $Y_{11}Y_{11}$ leaves) should be immediately detected by analyzing $y_{11}y_{11}$ leaves for light green sectors. Thus this system should be suitable for an analysis of somatic segregation (somatic crossing over), chromosomal disturbances (e.g., deletions, exchanges, etc.) and point mutations, all at the same time. A comparison of numerical relationships between twin spots and single spots on the leaves of the treated v/s control materials should serve as a reliable index for determining the type of genetic change(s), preferably induced by a given mutagen.

MATERIALS AND METHODS

The seeds of variety L65-1237 were used for the present study. Samples of seeds in petri dishes without filter paper were soaked in 25 cc of aqueous solution of the chemical for periods and concentrations specified along with appropriate experiments. Controls were kept in distilled water. At the end of the treatment, seeds were thoroughly washed in water and then sown in galvanized metallic flats lined with a sheet of thick plastic and filled with white coarse sand of non-nutritive value. Since soybean cotyledons are able to supply food material to the growing seedlings and plants for approximately 4 weeks—the time at which data are collected—there was

no need for any exogenous supply of nutrients to the plants. At the end of this period, the two simple leaves and the first compound leaf from $Y_{11}y_{11}$ plants were separately analyzed for the three different kinds of spots on the upper and lower surfaces. The $y_{11}y_{11}$ plants were scored for light green spots on the upper surface of the leaf.

For the purpose of calculation, each trifoliate compound leaf was considered equal to 3 simple leaves, thus giving 5 leaf equivalents per plant. Where necessary, statistical comparisons were performed using chi-square.

RESULTS

The first experiment was performed using 25 cc of 0.5%, 0.25%, 0.125%, 0.0625% and 0.03125% of aqueous solutions of caffeine (0.01% = 5.2×10^{-4}M) for a period of 4 hr (0–4 hr) on 12 gm seed. Table 1 shows the significant increase in the frequency of spots in all materials treated with caffeine when compared to the control, except for the one treated with the lowest concentration. The double spots, which are the surest index of somatic crossing over's having occurred, show a dramatic increase in frequency, indicating that complementary exchanges had been induced by caffeine. The right part of Table 1 shows relative proportions between different types of spots, an index which confirms the disproportionate increase of the double spots as observed above. Another experiment was performed using six different concentrations ranging from 0.0125% to 0.125% for as long as 24 hr. The results of this study (Table 2) confirmed the general pattern observed in the first experiment—that is, caffeine is capable of inducing somatic crossing over. Even though there are some fluctuations in the relative proportions of spots of different kinds, increase in the frequency of double spots relative to the total spots is pretty constant around comparable figures from the control. The 24-hr treatment with 0.05% caffeine deviates from the general pattern in both the total spots observed as well as the relative proportions. This was attributed to rather drastic effects of caffeine on germination (only 60 leaves compared to 100 in control were available) and leaf expansion.

An interesting feature of somatic mosaicism on the leaves of the untreated materials is the disproportionate distribution of spots on the two surfaces of the leaf. The upper surface of the leaves in the untreated material generally has about

TABLE 1

Data on the type and frequency of spots induced on the leaves of $Y_{11}y_{11}$ *plants with the application of caffeine solution to seeds (0–4 hr)*

Concentration (percent)	Number of leaves analyzed	Type† and frequency of spots per leaf				Relative proportions between spots of different types			
		DG	Yl	Db	T	DG/Db	Yl/Db	DG/Yl	T/Db
000 (H_2O)	160	0.03	0.07	0.01	0.11	3.00	7.00	0.43	11.00
0.03125	125	0.03	0.04	0.01	0.08	3.00	4.00	0.75	8.00
0.0625	125	0.10	0.08	0.07	0.25*	1.43	1.14	1.25	3.57
0.125	115	0.34	0.23	0.19	0.76*	1.79	1.21	1.48	4.00
0.25	175	0.60	0.19	0.33	1.12*	1.81	0.58	3.16	3.40
0.5	130	0.83	0.40	0.71	1.94*	1.17	0.56	2.08	2.73

* Significantly different from the control (χ^2, $p \leqslant 0.05$).
† DG = dark green, Yl = yellow, Db = double or twin and T = total spots.

TABLE 2

Effectiveness of various doses of caffeine solutions applied to $Y_{11}y_{11}$ *seeds for 12 and 24 hr in the induction of somatic crossing over*

Time and concentration	Number of leaves analyzed	Type and frequency of spots per leaf DG	Yl	Db	T	Relative proportions of spots DG/Db	Yl/Db	DG/Yl	T/Db
000% (H_2O), 0–24 hr	100	0.07 (0.06/0.01)*	0.28 (0.27/0.01)	0.17 (0.15/0.02)	0.52 (0.48/0.04)	0.41	1.65	0.25	3.06
0.0125%, 0–24 hr	100	0.27 (0.19/0.08)	0.20 (0.19/0.01)	0.21 (0.20/0.01)	0.68 (0.58/0.10)	1.29	0.95	1.35	3.23
0.025%, 0–24 hr	100	0.37 (0.32/0.05)	0.48 (0.47/0.01)	0.61 (0.46/0.15)	1.46* (1.25/0.21)	0.61	0.79	0.77	2.39
0.05%, 0–24 hr	60	0.46 (0.43/0.03)	0.25 (0.25/0.00)	0.38 (0.28/0.10)	1.09* (0.96/0.13)	1.21	0.66	1.84	4.36
0.03125%, 0–12 hr	90	0.30 (0.23/0.07)	0.41 (0.41/0.00)	0.39 (0.36/0.03)	1.10* (1.00/0.10)	0.77	1.05	0.77	2.82
0.0625%, 0–12 hr	120	0.37 (0.34/0.03)	0.24 (0.24/0.00)	0.37 (0.34/0.03)	0.98* (0.92/0.06)	1.00	0.65	1.54	2.65
0.125%, 0–12 hr	120	1.78 (1.45/0.33)	0.74 (0.70/0.04)	1.08 (0.93/0.15)	3.60* (3.08/0.52)	1.65	0.68	2.40	3.33

* Spot frequency on the upper/lower surface of the leaves analyzed.

80% or more of the total spots. The reason for this variation is not known so far. To verify if this is true, even in the case of chemically induced mosaicism, the material used in the above experiments was analyzed for frequency of different kinds of spots on the upper and lower surfaces of the two simple leaves and the first compound leaf. The data presented in Table 2 (in brackets) under respective categories show, without exception, a very low frequency of spots on the lower surface.

The second DNA synthesis inhibitor tested was cytosine arabinoside. Concentrations in the range of 12.5 to 200 ppm were tried during the first 5 hr of germination. Results do not support the contention that inhibition of DNA synthesis by this compound causes any increase in the frequency of spots (Table 3A) at these doses or at higher doses ranging up to 1000 ppm when the treatment interval is increased to 12 hr (Table 3B). Similarly, no effect was found on plants obtained from the seed treated with 250 ppm for as long as 24 hr (Table 3B). The differences in the controls of the two sets are perhaps due to environmental factors such as the age of the seed, the season of growth (see VIG and PADDOCK 1970) and the temperature variations in the green house during the period of growth of these plants. The leaves of the plants receiving concentrations higher than 250 ppm were badly mutilated, ill-formed, notched and poorly developed, thus re-

TABLE 3

Spot frequency on the $Y_{11}y_{11}$ leaves after treatment with cytosine arabinoside

Concentration (percent)	Treatment interval	Number of leaves analyzed	Spot frequency per leaf DG	YI	Db	T
A:						
0.000 (H_2O)	0–5 hr	90	0.10	0.10	0.05	0.25
			(18.18)*	(0.00)	(25.00)	(13.64)
12.5	0–5 hr	120	0.08	0.17	0.05	0.30
			(0.00)	(9.52)	(0.00)	(5.56)
25	0–5 hr	70	0.13	0.16	0.04	0.33
			(11.11)	(9.09)	(0.00)	(8.69)
100	0–5 hr	120	0.04	0.25	0.03	0.32
			(40.00)	(10.00)	(0.00)	(13.14)
200	0–5 hr	170	0.10	0.14	0.09	0.33
			(5.81)	(0.00)	(6.25)	(3.39)
B:						
0.00 (H_2O))	0–24 hr	100	0.54	0.42	0.48	1.44
			(18.52)	(14.29)	(12.50)	(15.28)
250	0–24 hr	70	0.40	0.40	0.36	1.16
			(10.71)	(7.14)	(4.00)	(7.50)
250	0–12 hr	105	0.40	0.31	0.34	1.05
			(2.38)	(0.00)	(0.00)	(0.91)
500	0–12 hr	65	0.20	0.19	0.12	0.51
			(7.70)	(0.00)	(0.00)	(3.03)
1000	0–12 hr	40	0.18	0.12	0.15	0.45
			(0.00)	(0.00)	(0.00)	(0.00)

* Figures in parentheses represent percentage of spots on the lower surface.

ducing the surface area; hence the lowered spot frequency (Table 3B, last two treatments).

In order to study whether actinomycin D is capable of inducing somatic crossing over, the seeds were treated with 0.0005% for 6 hr (18–24 hr), 0.0005% and 0.001% for 12 hr and with 0.005% for 24 hr. The data (Table 4) point to the effectiveness of this chemical in increasing somatic mosaicism as indicated by an increase in total spot frequency as well as by the constancy of double spots relative to the total spots. However, there is a general increase in the proportion of dark green spots in all the concentrations tried in the two experiments; double spots declined in frequency in one treatment.

Several subsequent experiments using puromycin (an inhibitor of protein synthesis) and FUdR (inhibitor of DNA synthesis) showed their effectiveness in causing somatic crossing over. (In this case caffeine was used for purposes of comparison.) As seen in Tables 5 and 6, the increase in spot frequency, even with rather high doses of puromycin (up to 1.0% for 12 hr) and FUdR (10^{-3}M for 12 hr), is only moderate and, in the case of puromycin, it is primarily due to an increase in the frequency of dark green and yellow spots, which may not always result from the phenomenon of somatic crossing over. A similar impression is conveyed in the analysis of that part of Table 6 which deals with relative propor-

TABLE 4

Type and frequency of spots as induced by aqueous solutions of actinomycin D (10 gm of seed were soaked in 25 cc of the solution)

Concentration (percent)	Treatment duration (hr)	Number of leaves analyzed	Type and frequency of spots DG	Yl	Db	T	Relative proportions of spots DG/Db	Yl/Db	DG/Yl	T/Db
00 (water)	0–24	100	0.07	0.28	0.17	0.52	0.41	1.65	0.25	3.60
			(14.30)†	(3.57)	(11.76)	(7.70)				
0.0005	0–12	50	0.42	0.28	0.30	1.00*	1.40	0.93	1.50	3.33
			(33.33)	(0.00)	(13.33)	(18.00)				
0.001	0–12	100	0.72	0.51	0.48	1.71*	1.50	1.06	1.41	3.56
			(20.83)	(1.96)	(16.66)	(14.03)				
0.005	0–24	120	0.20	0.33	0.16	0.69	1.25	2.06	0.61	4.31
			(33.33)	(6.06)	(10.53)	(15.79)				
00 (H_2O)	0–24	110	0.44	0.35	0.46	1.25	0.95	0.76	1.26	2.72
0.0005	18–24	40	1.25	0.80	0.28	2.33*	4.44	2.86	1.56	8.33

* Significantly different from the control (χ^2, $p \leqslant 0.05$).
† Percentage of spots on the lower surface.

TABLE 5

Test of effectiveness of puromycin and FUdR in induction of somatic mosaicism in Glycine

Chemical and concentration	Treatment interval	Number of leaves analyzed	Type and frequency of spots per leaf DG	Yl	Db	T
H_2O (control)	0–6 hr	140	0.21	0.15	0.20	0.56
			(26.66)†	(4.80)	(25.00)	(19.80)
Caffeine—						
0.50%	0–6 hr	50	1.48	0.56	0.40	2.44*
			(2.70)	(0.00)	(0.00)	(1.64)
1.00%	0–6 hr	35	1.29	0.91	0.42	2.62*
			(2.22)	(0.00)	(0.00)	(1.09)
Puromycin—						
0.0125%	0–6 hr	120	0.37	0.16	0.21	0.74*
			(31.11)	(0.00)	(24.00)	(22.47)
0.025%	0–6 hr	110	0.28	0.25	0.07	0.60
			(12.90)	(10.71)	(25.00)	(13.43)
0.05%	0–6 hr	70	0.24	0.14	0.16	0.54
			(23.52)	(0.00)	(9.09)	(9.88)
FUdR—						
10^{-6}M	0–6 hr	130	0.34	0.14	0.15	0.63
			(11.36)	(0.00)	(15.79)	(9.88)
10^{-5}M	0–6 hr	150	0.25	0.17	0.32	0.74*
			(10.53)	(0.00)	(8.33)	(7.14)

* Significantly different compared to the control (χ^2; $p \leqslant 0.05$).
† Percent spots on the lower surface of the leaf.

TABLE 6

Data on the type and frequency of spots on the $Y_{11}y_{11}$ leaves after treatment with puromycin and FUdR for 12 hr

Chemical and concentration	Number of leaves analyzed	Type and frequency of spots per leaf DG	Yl	Db	T	Relative proportions of spots DG/Db	Yl/Db	DG/Yl	T/Db
H_2O	200	0.17	0.10	0.07	0.34	2.43	1.43	1.70	4.86
		(26.47)†	(4.76)	(7.69)	(16.17)				
Puromycin—									
0.125%	155	0.31	0.20	0.10	0.61*	3.1	2.10	1.55	6.1
		(12.50)	(3.23)	(18.75)	(10.53)				
0.25%	130	0.30	0.22	0.13	0.65*	2.31	1.69	1.36	5.00
		(7.69)	(0.00)	(0.00)	(3.53)				
0.50%	160	0.16	0.17	0.10	0.43*	1.60	1.70	0.94	4.30
		(3.85)	(0.00)	(6.25)	(2.90)				
1.0%	145	0.20	0.22	0.10	0.52*	2.00	2.20	0.91	5.20
		(34.48)	(6.25)	(13.33)	(18.42)				
FUdR—									
2.5×10^{-5}M	180	0.08	0.11	0.07	0.26	1.14	1.57	0.73	3.71
		(7.14)	(5.26)	(7.69)	(6.52)				
10^{-4}M	140	0.11	0.20	0.08	0.39	1.38	2.50	0.55	4.87
		(6.66)	(0.00)	(9.09)	(3.58)				
2.5×10^{-4}M	190	0.12	0.20	0.14	0.47*	0.87	1.57	0.60	3.36
		(13.04)	(2.50)	(3.85)	(5.62)				
1.5×10^{-3}M	180	0.08	0.20	0.11	0.40	0.73	1.82	0.40	3.64
		(20.00)	(0.00)	(0.00)	(4.17)				
10^{-2}M	140	0.17	0.23	0.10	0.50*	1.70	2.30	0.74	5.00
		(8.33)	(6.25)	(0.00)	(5.71)				

* Significant at 0.05% level (χ^2).
† Percentage of spots found on the lower surface.

tions of different types of spots. FUdR, on the other hand, appears to be only weakly effective in inducing somatic mosaicism, if at all.

Study of point mutations in materials treated with above chemicals

As discussed earlier, the appearance of a green sector on the leaves of $y_{11}y_{11}$ plants is most likely due to a mutation of the genes y_{11} to Y_{11}. To study the potentials of various chemicals in causing point mutations (specific locus mutations in this system), the frequency of light green sectors on the homozygous $y_{11}y_{11}$ leaves was determined. The data point to mutagenic effects of caffeine (Table 7). None of the other chemicals tried showed consistent increase in the frequency of mutant sectors. These data were based on analysis of 285 leaves from various treatments by cytosine arabinoside, 198 leaves from actinomycin D treatments, 264 leaves from FUdR treatments and 156 leaves from puromycin-treated materials.

DISCUSSION

The results summarized in Tables 1 and 2 leave no doubt that caffeine is not only capable of increasing the incidence of somatic mosaicism in the experimen-

TABLE 7

Relative frequency of $y_{11} \rightarrow Y_{11}$ *changes on the leaf primordia of* $y_{11}y_{11}$ *plants of* Glycine max *treated with caffeine*

Concentration (percent) and duration of treatment		Number of leaves analyzed	Number of mutant sectors
A. 0.00 (H_2O)	(0–24 hr)	20	0
0.0125	(0–24 hr)	30	1
0.025	(0–24 hr)	16	2
0.05	(0–24 hr)	20	15*
0.03125	(0–12 hr)	30	0
0.0625	(0–12 hr)	22	2
0.125	(0–12 hr)	26	9*
B. 0.00 (H_2O)	(0–4 hr)	22	2
0.03125	(0–4 hr)	18	0
0.0625	(0–4 hr)	30	11*
0.125	(0–4 hr)	28	12*
0.25	(0–4 hr)	32	13*
0.5	(0–4 hr)	16	6*
C. 0.00 (H_2O)	(0–6 hr)	16	1
0.25	(0–6 hr)	16	17*
0.50	(0–6 hr)	20	20*
1.00	(0–6 hr)	20	8*

* Significantly different from control (χ^2; $p \leqslant 0.05$).

tal system used, but as evidenced by increase in the frequency of double spots, can also increase the frequency of phenomenon responsible for somatic crossing over. Such complementary exchanges have not previously been reported to be induced by caffeine even though induction of chromosomal aberrations by this chemical is well known (see e.g., KIHLMAN *et al.* 1971b). Depending on the treatment temperature, the breaks resulting from this oxypurine may or may not reunite. In the present case, reunions between chromosomes are necessary in order to gain complementary exchanges of fragments. The temperatures at which treatments of the seeds were carried out ($\simeq$ 74°F) are known to permit reunions of broken ends of root tip chromosomes of *Allium cepa* (KIHLMAN *et al.* 1971a). This might mean that underlying processes for chromosomal reunions as well as somatic crossing over are the same or similar to each other. One essential difference may be the involvement of homologous chromosomes at specific corresponding loci in the latter case. Such observations have not been possible in *Glycine max* because of very small size and large number of chromosomes (VIG 1969, 1972). The molecular nature of reunions remains a matter of conjecture and direct involvement of DNA in the breakage-reunion process can be inferred if one can demonstrate that the underlying phenomena for crossing over are of intra- rather than inter-genic nature. The induction of point mutations by caffeine (Table 7), however, does require direct involvement of DNA molecule. It is interesting that both negative and positive results have been obtained in this regard with eukaryotes, whereas there appears little contradiction about the effectiveness

of caffeine on the genomes of prokaryotes (see, e.g., KÜHLMAN *et al.* 1968; OSTERTAG, DUISBERG and STURMANN 1965).

In view of the fact that cytosine arabinoside is inactive in *Vicia* and produces only fragments without rejoining in animal cell cultures (KIHLMAN 1966, p. 128; BENEDICT, HARRIS and KARON 1970), it is not surprising that this inhibitor of DNA synthesis does not cause an increase in frequency of spots on *Glycine* leaves. The decrease in spot frequency at higher concentrations (250 ppm and higher) is explainable on the basis that the drug caused mutilation, deformity and reduced expansion of the leaves. Thus, none of the mechanisms postulated for the action of cytosine arabinoside, *viz.*, reduction of cytidine diphosphate (CHU and FISCHER 1962), interference with DNA polymerase (KIMBALL and WILSON 1968; FURTH and COHEN 1968) and its incorporation into DNA and RNA polynucleotides (CHU and FISCHER 1962; SILAGI 1965), may be effective in the production of spots in soybean. It might mean that the mere inhibition of DNA synthesis by this chemical is not sufficient for the production of point mutations in the material studied.

The capacity of actinomycin D to break human chromosomes (OSTERTAG and KERSTEN 1965) and cause reunions (KIHLMAN 1966, p. 171) has been attributed to the binding of the molecule at position 7 in the guanine residue (WARING 1968), even though MILES (1970) has shown that the chromosome breakage sites do not coincide with the sites of accumulation of labelled actinomycin D on human chromosomes. The inhibition of DNA polymerase by this agent (KIHLMAN 1966, p. 170), as also noted for the foregoing cytosine arabinoside, may not provide a clue to the phenomenon resulting in complementary exchanges in *Glycine*. One might add that daunomycin, which also inhibits DNA synthesis at the DNA polymerase level, does not cause an increase in the incidence of somatic crossing over in this test material (VIG and PADDOCK 1970). The lack of increase in the incidence of point mutations in *Glycine* is of interest since there is little doubt about the effectiveness of actinomycin D at the level of DNA synthesis/replication.

There is some demonstrable effect of FUdR on the induction of double spots on the leaves of $Y_{11}y_{11}$ plants (Tables 5 and 6). The increase observed for dark green and yellow spots can be expected from what is known about the chromosome-breaking action of FUdR's producing fragments which may not rearrange themselves by reunions (TAYLOR, HAUT and TUNG 1962; HSU, HUMPHREY and SOMERS 1964). The results here may mean that FUdR-induced thymidylic acid deficiency is preventing rejoining (TAYLOR 1963) and subsequent exchange formation. Alternatively it is possible that the chromosomes carrying the locus Y_{11} are not susceptible to FUdR-induced breakage, since this agent has been shown in mammalian cell culture systems to affect only late-replicating parts of the genome (HSU, HUMPHREY and SOMERS 1964). Under any circumstances, there remains a difference of effectiveness of this agent in the induction of somatic crossing over in fungi (ESPOSITO and HOLLIDAY 1964) as well as in increasing meiotic recombination in Neurospora (WOLFF and DESERRES 1967) versus its ineffectiveness on the angiosperm under study. A small increase observed in the

incidence of somatic mosaicism (Table 6) induced by puromycin may be explained on the basis of chromosome aberrations or lack of reunions among fragments of spontaneous origin due to puromycin-induced inhibition of synthesis of various types of proteins (see VIG 1971b).

With the information from the present studies, one cannot make the generalization that all inhibitors of DNA synthesis increase somatic crossing over. Perhaps it is not DNA *per se* but a specific step in its synthesis, or in the synthesis of proteins or some other related phenomenon, which gives rise to somatic recombination in this organism. Caffeine (an inhibitor of oxidative phosphorylation), actinomycin D and mitomycin C do effect the recombination process, but other inhibitors of DNA synthesis studied, *viz.*, daunomycin, cytosine arabinoside, FUdR, do not. One rule which appears to be followed in this case is that only those chemicals which are known to cause exchange formation in somatic cells of plants are effective in inducing somatic crossing over in *Glycine*. This invokes the possibility of some relationship or similarities between the processes involved in somatic crossing over and chromosome rejoining in somatic cells.

The information about the effectiveness of caffeine is also of interest in relation to environmental mutagenesis. The doses which are used for the induction of somatic crossing over in *Glycine* are close to the common level of use in man (ALTMAN and DITTMER 1968). One then wonders about the possible genetic changes which caffeine might have brought about in the human system. Some other chemicals (e.g., cytosine arabinoside) are of interest as possible anticarcinogens.

The question about differential spot frequency on the upper versus lower surfaces of leaves remains unanswered. As seen in several tables, the spots on the lower surface of leaves constitute only a fraction of those observed on the upper surface. The treatment with various chemicals does not alter the picture, indicating that mesophyll parenchyma cells are uniformly resistant to all the chemicals tried, or to the natural mutagens present in the control material. It is difficult to believe that the differences between the two surfaces are due to variations in the cell number in the embryonic tissue. If anything, the total number of spongy mesophyll cells should be larger than the number of cells in the palisade. The approximate number, size and volume of the two types of cells, as well as their relationship to spotting, are under investigation at this time.

I am thankful to DRS. R. L. BERNARD, A. ESSA, H. N. MOZINGO and F. D. TIBBITTS for help in the different phases of this work.

LITERATURE CITED

ALTMAN, P. and D. S. DITTMER, 1968 Metabolism. Feder. Amer. Soc's. Exptl. Biol. Bethesda, Md.

BENEDICT, W. F., N. HARRIS and M. KARON, 1970 Kinetics of 1-β-D-arabino-furanosyl cytosine induced chromosome breaks. Cancer Res. **30**: 2477–2483.

CHU, M. Y., and G. A. FISCHER, 1962 A proposed mechanism of action of 1-β-D-arabinofuranosyl-cytosine as an inhibitor of the growth of leukemic cells. Biochem. Pharmacol. **11**: 423–430.

ESPOSITO, R. E. and R. HOLLIDAY, 1964 The effect of 5-fluorodeoxyuridine on genetic replication and mitotic crossing over in synchronized cultures of *Ustilago maydis*. Genetics **50**: 1009–1017.

FISHBEIN, F., W. G. FLAMM and H. L. FALK, 1970 Chemical mutagens—Environmental Effects on Biological Systems, Academic Press, N.Y.

FURTH, J. J. and S. S. COHEN, 1968 Inhibition of mammalian DNA polymerase by the 5′-triphosphate of 1-β-D-arabinofuranosyl-cytosine and the 5′-triphosphate of 9-β-D-arabinofuranosyl-adenine. Cancer Res. **28**: 2061–2067.

HOLLIDAY, R., 1964 The induction of mitotic recombination by mitomycin C in Ustilago and Saccharomyces. Genetics **50**: 323–335.

HSU, T. C., R. M. HUMPHREY and C. E. SOMERS, 1964 Responses of chinese hamster and L cells to 2′-deoxy-5-fluro-uridine and thymidine. J. Nat'l Cancer Inst. **32**: 838–855.

KIHLMAN, B. A., 1966 *Action of chemicals on dividing cells*. Prentice Hall, N. J.

KIHLMAN, B. A., W. W. NICHOLS and A. LEVAN, 1963 The effect of deoxyadenosine and cytosine arabinoside on the chromosomes of human leukocytes *in vitro*. Hereditas **50**: 139–143.

KIHLMAN, B. A., S. STURLID, K. NORDEN and D. TIDRIK, 1971a Caffeine, caffeine derivatives and chromosomal aberrations. II. Different response of *Allium* root tips and hamster cells to treatment with caffeine, 8 ethoxycaffeine and 6-methylcoumarin. Hereditas **69**: 35–50.

KIHLMAN, B. A., K. NORDEN, S. STURLID and G. ODMARK, 1971b Caffeine and 8-ethoxycaffeine produce different types of chromosome breaking effects depending on the treatment temperature. Mutation Res. **12**: 463–468.

KIMBALL, A. P. and M. J. WILSON, 1968 Inhibition of DNA polymerase by β-D-arabinosylcytosine and reversal of inhibition by deoxycytidine-5′-triphosphate. Proc. Soc. Exptl. Biol. Med. **127**: 429–432.

KÜHLMAN, W., H. G. FROMME, E. M. HAEGE and W. OSTERTAG, 1968 The mutagenic action of caffeine in higher organisms. Cancer Res. **28**: 2375–2389.

MILES, C., 1970 Labelling and other effects of actinomycin D on human chromosomes. Proc. Nat'l Acad. Sci. U. S. **65**: 585–592.

NOTHIGER, R. and A. DUBENDORFER, 1971 Somatic crossing over in the housefly. Molec. Gen. Genetics **112**: 9–13.

OSTERTAG, W., E. DUISBERG and M. STURMANN, 1965 The mutagenic activity of caffeine in man. Mutation Res. **2**: 293–296.

OSTERTAG, W. and W. KERSTEN, 1965 The action of proflavin and actinomycin D in causing chromatid breakage in human cells. Exptl. Cell Res. **39**: 296–301.

SILAGI, S., 1965 Metabolism of 1-β-D-arabinofuranosylcytosine in L-cells. Cancer Res. **25**: 1446–1453.

STERN, C., 1936 Somatic crossing over and segregation in *Drosophila melanogaster*. Genetics **21**: 625–730.

TAYLOR, J. H., 1963 DNA synthesis in relation to chromosome reproduction and the reunion of breaks. J. Cell. Comp. Physiol. (Sup. 1) **62**: 73–86.

TAYLOR, J. H., W. F. HAUT and J. TUNG, 1962 Effects of fluorodeoxyuridine on DNA replication, chromosome breakage, and reunion. Proc. Nat'l Acad. Sci. U.S. **48**: 190–198.

VIG, B. K., 1969 Relationship between mitotic events and leaf spotting in *Glycine max*. Canad. J. Gent. Cytol. **11**: 147–152. ——, 1971a Increase induced by colchicine in the incidence of somatic crossing over in *Glycine max*. Theorat. Appl. Genetics **41**: 145–149. ——, 1971b Nature of chrmosome aberrations induced in pre-DNA synthesis period in human leukocytes by daunomycin. Mutation Res. **12**: 441–452. ——, 1972 Suppression of so-

matic crossing over in *Glycine max* (L.) Merrill by deoxyribose cytidine. Molec. Gen. Genetics **116**: 158–165.

VIG, B. K. and E. F. PADDOCK, 1968 Alteration of spot frequencies in leaves of *Glycine max* (L.) Merrill by mitomycin. J. Hered. **59**: 225–229. ——, 1970 Studies on the expression of somatic crossing over in *Glycine max* L. Theoret. Appl. Genetics **40**: 316–321.

WARING, M. J., 1968 Drugs which affect the structure and function of DNA. Nature **219**: 1320–1325.

WOLFF, S., and F. J. DESERRES, 1967 Chemistry of crossing over. Nature **213**: 1091–1092.