Rev. Brasil. Genet. I, 4, 235-245 (1978)
(Brazil. J. Genetics)

PROTEIN AND LYSINE CONTENT IN DOUBLE MUTANT ENDOSPERMS OF MAIZE INVOLVING THE *floury*-2 GENE[1]

Hélio M. Barbosa[2] and D. V. Glover[3]

ABSTRACT

Twelve near-isogenic single mutant lines of the maize (*Zea mays* L.) inbred Oh43 homozygous for each of the genes *ae*, bt_1, bt_2, *du*, fl_1, fl_2, *h*, o_2, sh_1, su_1, su_2, and *wx*, their respective double mutant combinations involving the fl_2 gene, and the *normal* inbred were used in this study. The objective was to determine the effect of the interaction of the fl_2 gene in double mutant combinations with other endosperm mutants on protein and lysine content of the endosperm.

When combined with other endosperm mutants, fl_2 tended to increase protein percent and decrease protein yield in the endosperm but did not significantly affect lysine yield per endosperm. However, fl_2 increased lysine percent of sample and lysine percent of protein when combined with each of the other genes except o_2. With the exception of the o_2 fl_2 combination this effect was cumulative with all starch-modifying genes and this provides support favoring the hypothesis that there may exist different pathways leading to reduced zein synthesis in fl_2 and o_2 as compared to starch-modifying mutants with high lysine concentrations.

---

[1] Part of a thesis submitted by the senior author (H.M.B.) in partial fulfillment of the requirements for the Ph. D. degree, Purdue Univ. Journal Paper No. 7434, Purdue Univ. Agric. Exp. Stn., West Lafayette, IN 47907.

[2] Universidade Federal de Viçosa, 36570 Viçosa, MG, Brasil.

[3] Department of Agronomy, Purdue University, West Lafayette, IN 47907, U.S.A.

INTRODUCTION

There are many mutants in maize which produce striking changes in texture, form, and amount of endosperm. Some of these mutants affect the gelatinization temperature of starch (Kramer, Pfahler, and Whistler, 1958; Sandstedt, Hites, and Schroeder, 1968), the digestibility of starch (Sandstedt *et al.*, 1968; Sandstedt, Strahan, Ueda, and Abbot, 1962), and the synthesis of amylose, amylopectin, water-soluble polysaccharides, and sugars in the endosperm (Creech, 1965; Kramer *et al.*, 1958). The effects of several endosperm mutants, and of double and triple-mutant combinations on carbohydrate synthesis were summarized by Creech (1968). None of these combinations included the mutants *floury*-2 (fl_2) or *opaque*-2 (o_2). These two mutants improve protein quality in maize by increasing the concentrations of the amino acids lysine and tryptophan in the endosperm (Mertz, Bates, and Nelson, 1964; Nelson, Mertz, and Bates, 1965). Feeding tests have shown that fl_2 is superior to normal maize for rats (Veron, 1967), chicks (Cromwell, Rogler, Featherston, and Cline, 1968), and children (Harpstead, Pradilla, and Linares, 1969). The lysine content of fl_2 maize is usually intermediate between that of o_2 and *normal* (Dudley, Alexander, and Lambert, 1975; Glover, Crane, Misra, and Mertz, 1975; Misra, Jambunathan, Mertz, Glover, Barbosa, and McWhirter, 1972; Villegas, 1975). For this reason the work with fl_2 has been greatly reduced (Purdue University, 1974). However, unexpected interactions frequently occur between endosperm mutants in maize. Thus, it is of interest to explore possible genetic interactions between fl_2 and other endosperm mutants, as reported by Barbosa and Glover (1978 a, d) with the o_2 gene. This paper reports the effects of the incorporation of fl_2 in double mutant combinations with other endosperm mutants on protein and lysine content of the endosperm.

MATERIALS AND METHODS

Twelve near-isogenic lines established in the inbred 0h43 homozygous for each of the genes, *amylose-extender (ae), brittle*-1 (bt_1), *brittle*-2 (bt_2), *dull (du), floury*-1 (fl_1), *floury*-2 (fl_2), *soft-starch (h), opaque*-2 (o_2),

shrunken-1 (sh_1), *sugary*-1 (su_1), *sugary*-2 (su_2), and *waxy* (*wx*), and the 11 respective double mutant lines of each of these genes with fl_2, and the normal 0h43 inbred line were used in this study. The double mutant genotypes were isolated as described by Barbosa and Glover (1978b).

Kernels of each genotype were planted in rows 3 m long and 1 m wide. Spacing between plants was 33.3 cm and there were 10 plants in each row (plot). The experimental layout was a randomized complete-block design with two replications. All plants were self-pollinated.

Harvest was made at maturity and ears were forced-air dried. Well--filled ears from competitive plants were selected. A 30-kernel sample was taken from one ear in each plot and degermed. In this paper degermed kernel is referred to as endosperm due to the negligible contribution of the pericarp to the protein and lysine content as compared to that of the endosperm (Barbosa and Glover, unpublished results).

Endosperm samples were ground in a Wiley-mill prior to defatting. The defatted samples were further ground to a very fine powder in a Wig-L-Bug (Crescent Dental Mfg. Co.). Protein percent (N x 6.25) was determined by the micro-Kjeldahl procedure. Lysine analyses were made by ion-exchange column chromatography.

RESULTS AND DISCUSSION

The data on endosperm protein and lysine content of the single mutants, with the exception of sh_1 (Table I), were previously reported in a companion paper (Barbosa and Glover, 1978d). Therefore the discussion will be limited to the double mutants and the comparisons with the respective single mutants.

The double mutant bt_2 fl_2 (bt_2 bt_2; fl_2 fl_2) showed the highest protein percent (17.30), significantly different from that of the other double mutants, among which there was no significant difference (Table I). However, protein yield per endosperm was extremely low in bt_2 fl_2 as a consequence of its reduced kernel weight (Barbosa and Glover, 1978c). With the exception of bt_2 fl_2, o_2 fl_2, and su_1 fl_2, protein yield per endosperm of the other double mutants did not differ significantly. A comparison between double

mutants and their respective single mutants (Table II) showed that, in general, *fl*$_2$ increased protein percent when combined with the other endosperm mutants. The double mutants also tended to have higher protein percent than the single mutant *fl*$_2$. However, due to reduced kernel weight of the double mutants (Barbosa and Glover, 1978c) all of them yielded less protein per endosperm than the single mutant counterparts or *fl*$_2$.

Data on endosperm lysine percent of sample, lysine yield per endosperm, and lysine percent of protein (L/P) are presented in Table III. Lysine percent of sample of *fl*$_2$ was intermediate between that of *o*$_2$ and *normal* as observed by Dudley *et al.* (1975), Glover *et al.* (1975), Misra *et al.* (1972), and Villegas (1975). Lysine percent of sample in *bt*$_2$ *fl*$_2$ (0.583) was significantly higher than the other double mutants, and there were no significant differences among the other double mutants (Table III). As shown in Table IV, the double mutants had higher values for lysine percent of sample, many of which were significantly different, than the respective single mutants. One exception was the double mutant *fl*$_2$ *o*$_2$ whose lysine percent of sample was practically the same as that found in *o*$_2$ (Table III). With the exception of *o*$_2$ *fl*$_2$ combination, this cumulative effect of *fl*$_2$ in enhancing lysine percent of sample when combined with other endosperm mutants, was similar to, but less intense than, the effect of *o*$_2$ as shown by Barbosa and Glover (1978d). We have shown that the effect of the *o*$_2$ gene in increasing lysine percent of sample is cumulative with that of the other genes, except *fl*$_2$.

Lysine yield per endosperm (Table III) ranged from 0.400 mg in *bt*$_2$ *fl*$_2$ to 0.647 mg in *ae fl*$_2$. However, with the exception of *bt*$_2$ *fl*$_2$ and *su*$_1$ *fl*$_2$, there was no significant difference among the *fl*$_2$ double mutants. The high lysine yield of *ae fl*$_2$ was similar to that found in *ae o*$_2$ (Barbosa and Glover, 1978d). As shown in Table IV, there were no significant differences between double mutants and their single mutant counterparts or *fl*$_2$, with the exception of the comparison *wx* vs *wx fl*$_2$.

Few significant differences were found for lysine percent of protein among the double mutants (Table III). However, it is interesting to note that, with the exception of *o*$_2$ *fl*$_2$, all double mutants had higher values for lysine percent of protein than the respective single mutants (Table IV). These data give additional supporting evidence favoring the hypothesis of Misra *et al.* (1972, 1975) and Barbosa and Glover (1978d) that different pathways may exist leading to differential synthesis of the protein fractions in the

maize endosperm in fl_2 and o_2 as compared to starch-modifying mutants with high lysine concentrations.

ACKNOWLEDGMENTS

This research was supported in part by a Rockefeller Foundation Fellowship to the senior author (H.M.B.) and The Agency for International Development under contract Purdue University AID csd/2809 and AID/ta-C-1211, "Inheritance and Improvement of Protein Quality and Content in Maize".

REFERENCES

Barbosa, H.M. and Glover, D.V., (1978a). Isolamento e descrição de endospermas duplo-mutantes de milho (*Zea mays* L.) envolvendo o gene *opaco*-2. *Experientiae 24:* 43-54.

Barbosa, H.M. and Glover, D.V., (1978b). Isolamento e descrição fenotípica de endospermas duplo-mutantes de milho (*Zea mays* L.) envolvendo o gene *farinaceo*-2. *Experientiae 24:* 55-65.

Barbosa, H.M. and Glover, D.V., (1978c). Peso de sementes e porcentagem de germe em mutantes que afetam o endosperma do milho (*Zea mays* L.). *Experientiae 24:* 66-77.

Barbosa, H.M. and Glover, D.V., (1978d). Genes and gene interactions affecting protein and lysine content in the endosperm of maize. *Rev. Brasil. Genet. 1:* 29-39.

Creech, R.G., (1965). Genetic control of carbohydrate synthesis in maize endosperm. *Genetics 52:* 1175-1186.

Creech, R.G., (1968). Carbohydrate synthesis in maize. *Advan. Agron. 20:* 275-322.

Cromwell, G.L., Rogler, J.C., Featherstone, W.R., and Cline, T.R., (1968). A comparison of the nutritive value of opaque-2, floury-2 and normal corn for the chick. *Poult. Sci. 47:* 840-847.

Dudley, J.W., Alexander, D.E., and Lambert, R.J., (1975). Genetic improvement of modified protein maize, p. 120. In: *High Quality Protein Maize.* Dowden, Hutchinson, and Ross, Inc., Stroudsburg, Pa.

Glover, D.V., Crane, P.L., Misra, P.S., and Mertz, E.T., (1975). Genetics of endosperm mutants in maize as related to protein quality and quantity, p. 228. In: *High Quality Protein Maize.* Dowden, Hutchinson, and Ross, Inc., Stroudsburg, Pa.

Harpstead, D.D., Pradilla, A., and Linares, F., (1969). Response of malnourished children to diets of opaque-2, floury-2 and normal maize. *Agron. Abstr.* p. 59.

Kramer, H.H., Pfahler, P.L., and Whistler, R.L., (1958). Gene interactions in maize affecting endosperm properties. *Agron. J. 50:* 207-210.

Mertz, E.T., Bates, L.S., and Nelson, O.E., (1964). Mutant gene that changes protein composition and increases lysine content of maize endosperm. *Science 145:* 279-280.

Misra, P.S., Jambunathan, R., Mertz, E.T., Glover, D.V., Barbosa, H.M., and McWhirter, K.S., (1972). Endosperm protein synthesis in maize mutants with increased lysine content. *Science 176:* 1425-1427.

Misra, P.S., Mertz, E.T., and Glover, D.V., (1975). Studies on Corn Proteins. VI. Endosperm protein changes in single and double endosperm mutants of maize. *Cereal Chem. 52:* 161-166.

Nelson, O.E., Mertz, E.T., and Bates, L.S., (1956). Second mutant gene affecting the amino acid pattern of maize endosperm proteins. *Science 150:* 1469-1470.

Purdue University, West Lafayette. (1974). Annual report on the inheritance and improvement of protein quality and content in maize, U.S. Agency for International Development/Purdue Univ. Contract csd/2809. 1973--1974. West Lafayette, IN. P. 122.

Sandstedt, R.M., Hites, B.D., and Schroeder, H., (1968). Genetic variations in maize: Effects on the properties of the starches. *Cereal Sci. Today 13:* 82-94.

Sandstedt, R.M., Strahan, D., Ueda, S., and Abbot, R.C., (1962). The digestibility of high-amylose corn starches compared to that of other starches. The apparent effect of the *ae* gene on susceptibility to amylase action. *Cereal Chem. 39:* 123-131.

Veron, O.A., (1967). The determination of the protein quality of *opaque*-2,

floury-2 *opaque*-2, and high protein *opaque*-2 maize. Ph. D. Thesis, Purdue University. P. 75.

Villegas, E., (1975). An integral system for chemical screening of quality protein maize, p. 330. In: *High Quality Protein Maize.* Dowden, Hutchinson and Ross, Inc., Stroudsburg, Pa.

(Received December 11, 1978)

Table I - Protein percent and yield (mg/endosperm) in defatted endosperm of single and double mutant genotypes.

Genotype	Protein			
	%		mg/endosperm	
	Single mutant	Double mutant with fl_2	Single mutant	Double mutant with fl_2
ae	11.65 bcd*[1/]	12.05 b	24.02 abcd	21.42 a
bt_1	12.35 b	12.45 b	21.47 bcd	19.09 abc
bt_2	14.90 a	17.30 a	15.22 e	12.04 d
du	10.95 bcde	13.30 b	26.00 ab	20.55 ab
fl_1	10.90 bcde	10.85 b	24.06 abcd	20.51 ab
fl_2	11.45 bcd	–	23.76 abcd	–
h	11.35 bcd	10.90 b	28.53 a	23.08 a
o_2	9.40 e	11.25 b	18.69 de	16.49 bcd
sh_1	11.95 bc	12.60 b	23.43 abcd	20.87 ab
su_1	12.40 b	13.45 b	19.84 cde	15.54 cd
su_2	11.45 bcd	12.20 b	24.83 abc	22.57 a
wx	10.00 de	11.60 b	25.00 abc	21.94 a
Normal	11.45 bcd	–	27.75 a	–
C.V. (%)	6.43	8.95	10.40	9.82
$S_{\bar{X}}$	0.52	0.79	1.71	1.35

[1/] Means within each column followed by the same letter do not differ at the 0.05 level according to Duncan's Multiple-Range Test.

Table II - Difference in protein percent and yield (mg/endosperm) among several genotypes.

Single *vs.* double mutant			fl_2 *vs.* double mutant		
	Difference			Difference	
Genotypes compared	Protein percent	Protein yield	Genotypes compared	Protein percent	Protein yield
ae - ae fl_2	-0.40	2.60	fl_2 - ae fl_2	-0.60	2.34
bt_1 - bt_1 fl_2	-0.10	2.38	fl_2 - bt_1 fl_2	-1.00	4.67*
bt_2 - bt_2 fl_2	-2.40*	3.18	fl_2 - bt_2 fl_2	-5.85*	11.72**
du - du fl_2	-2.35*	5.45*	fl_2 - du fl_2	-1.85	3.21
fl_1 - fl_1 fl_2	0.05	3.55	fl_2 - fl_1 fl_2	0.60	3.25
h - h fl_2	0.45	5.45*	fl_2 - h fl_2	0.55	0.68
o_2 - o_2 fl_2	-1.85	2.20	fl_2 - o_2 fl_2	0.20	7.27
sh_1 - sh_1 fl_2	-0.65	2.56	fl_2 - sh_1 fl_2	-1.15	2.89
su_1 - su_1 fl_2	-1.05	4.30	fl_2 - su_1 fl_2	-2.00*	8.22**
su_2 - su_2 fl_2	-0.75	2.26	fl_2 - su_2 fl_2	-0.75	1.19
wx - wx fl_2	-1.60	3.06	fl_2 - wx fl_2	-0.15	1.82

* and ** indicate significant differences at the 0.05 and 0.01 levels, respectively, according to Least Significant Difference Test.

Table III - Lysine percent of sample, lysine yield (mg/endosperm), and lysine percent of protein in defatted endosperm of single and double mutant genotypes.

Genotype	Lysine					
	Percent of sample		mg/endosperm		Percent of protein	
	Single mutant	Double mutant with fl_2	Single mutant	Double mutant with fl_2	Single mutant	Double mutant with fl_2
ae	0.273 bc*[1/]	0.364 b	0.561 ab	0.647 a	2.34 bc	3.02 ab
bt_1	0.319 b	0.360 b	0.556 ab	0.552 abc	2.58 b	2.89 ab
bt_2	0.461 a	0.583 a	0.471 ab	0.400 c	3.09 a	3.35 a
du	0.250 bcd	0.338 b	0.596 ab	0.522 abc	2.27 bc	2.54 b
fl_1	0.221 cde	0.312 b	0.490 ab	0.592 abc	2.04 cde	2.88 ab
fl_2	0.261 bcd	–	0.543 ab	–	2.27 bc	–
h	0.192 e	0.282 b	0.483 ab	0.598 abc	1.68 e	2.58 b
o_2	0.310 b	0.315 b	0.615 a	0.461 abc	3.31 a	2.80 ab
sh_1	0.265 bcd	0.363 b	0.520 ab	0.601 ab	2.22 bcd	2.88 ab
su_1	0.278 bc	0.375 b	0.443 ab	0.434 bc	2.25 bc	2.79 ab
su_2	0.214 cde	0.297 b	0.464 ab	0.557 abc	1.87 cde	2.44 b
wx	0.170 e	0.308 b	0.425 b	0.582 abc	1.71 de	2.65 b
Normal	0.197 de	–	0.447 ab	–	1.72 de	–
C.V. (%)	10.84	15.81	14.47	14.97	9.62	9.89
$s_{\bar{x}}$	0.020	0.040	0.052	0.057	0.15	0.20

[1/] Means within each column followed by the same letter do not differ at the 0.05 level according to Duncan's Multiple Range-Test.

Table IV - Differences in lysine percent of sample, lysine yield (mg/endosperm), and lysine percent of protein among several genotypes.

Single *vs.* double mutant				fl_2 *vs.* double mutant			
Genotypes compared	Difference in lysine			Genotypes compared	Difference in lysine		
	% of sample	Yield	% of protein		% of sample	Yield	% of protein
ae - ae fl_2	-0.091*	-0.086	-0.68**	fl_2 - ae fl_2	-0.103**	-0.104	-0.75**
bt_1 - bt_1 fl_2	-0.041	0.004	-0.31	fl_2 - bt_1 fl_2	-0.099*	-0.009	-0.62*
bt_2 - bt_2 fl_2	-0.122**	0.071	-0.26	fl_2 - bt_2 fl_2	-0.322**	0.143	-1.08**
du - du fl_2	-0.088*	0.074	-0.27	fl_2 - du fl_2	-0.077	0.021	-0.27
fl_1 - fl_1 fl_2	-0.091*	-0.102	-0.84**	fl_2 - fl_1 fl_2	-0.051	-0.049	-0.61*
h - h fl_2	-0.090*	-0.115	-0.90**	fl_2 - h fl_2	-0.021	-0.055	-0.31
o_2 - o_2 fl_2	-0.005	0.154	0.51*	fl_2 - o_2 fl_2	-0.054	0.082	0.53*
sh_1 - sh_1 fl_2	-0.098*	-0.081	-0.66*	fl_2 - sh_1 fl_2	-0.102*	-0.058	-0.61*
su_1 - su_1 fl_2	-0.097*	0.009	-0.54*	fl_2 - su_1 fl_2	-0.114*	0.109	-0.52*
su_2 - su_2 fl_2	-0.083	-0.093	-0.57*	fl_2 - su_2 fl_2	-0.136	-0.014	-0.17
wx - wx fl_2	-0.138**	-0.157*	-0.94**	fl_2 - wx fl_2	-0.047	-0.039	-0.38

* and ** indicate significant differences at the 0.05 and 0.01 levels, respectively, according to Least Significant Difference Test.

Rev. Brasil. Genet. I, 4, 247-262 (1978)
(Brazil. J. Genetics)

ACRIFLAVINE AND ETHYL METHANESULFONATE-INDUCED MUTANTS OF *Aspergillus nidulans* STRAINS WITH CHROMOSOME DUPLICATION.

Yoko Bomura Rosato[1] and João Lúcio de Azevedo[2]

ABSTRACT

Two mutagenic agents, acriflavine and ethyl methanesulfonate (EMS), were used to induce mutations by treating conidia of a haploid stable strain and an unstable strain with a chromosome duplication and on morphological deteriorated strains derived from it. Different types of morphological variants were obtained, EMS being more effective than acriflavine. The production of mutants with acriflavine varied with the strain used, chromosome duplication appearing to lead to a greater susceptibility to this agent. Morphological variants isolated after mutagenic treatment were submitted to genetic analysis. Four of them induced by EMS from already deteriorated strains have shown suppression of the original determinants of deterioration in concomitance with the appearance of new ones at new locations and behaving as single gene mutations. This was explained either as the result of two events both induced by EMS, that is loss of the original determinant and new genetic alteration, or by transposition of the original

[1] Present address: Departamento de Genética e Evolução, Universidade Estadual de Campinas, S.P., Brasil.

[2] Setor de Genética de Microrganismos, Instituto de Genética, C.P. 83, 13.400 PIRACICABA, S. Paulo, Brasil.

determinant of deterioration from one region of the genome to another. Two other variants, also induced by EMS had the determinants of morphological changes in linkage group VIII and alleles of other determinants of deterioration spontaneously obtained from the same duplication strain. This suggested that EMS acts by favouring or selecting transpositions to this site of *A. nidulans* genome.

INTRODUCTION

The occurence of instability in strains of *Arpergillus nidulans* containing chromosome duplications was first demonstrated by Bainbridge and Roper (1966). Nga and Roper (1968, 1969) utilized strains with more favourable genetic markers. These strains had a duplication of part of linkage group I translocated to linkage group II, a characteristic "crinkled" morphology, reduced growth rate and were also unstable. They produced sectors showing various degrees of phenotypic improvement as well as sectors with deteriorated morphology. The origin of improved sectors was attributed to total or partial loss of the duplicate segment, while the deteriorated sectors were suggested to be due to tandem duplications. An unequal crossing-over or a crossing-over in an intrachromosomal loop may lead to the simultaneous formation of the two classes of sectors. The deteriorated sectors were later studied in more detail by Azevedo and Roper (1970), and their origin was explained tentatively as new duplications arising within one or another duplicate segment producing in this way, extra genetic material. This provokes enhanced instability; greater stability is achieved by transposition of all or part of this extra genetic material to another site in the non-duplicated part of the genome. In fact, in almost every case the determinants of morphological change segregated as single genes and they were designated "determinants of deterioration". It was also shown that instability may be altered by adding certain drugs to the culture medium (Cooke et al., 1970; Roper et al., 1972; Bonatelli Jr. and Azevedo, 1977; Majerfeld and Roper, 1978), as well as by genetic factors (Azevedo, 1975). However, little has been done to verify the action of drugs or mutagens on the instability of deteriorated variants derived from strains with chromosome duplication.

The objective of this research was to study the effect of two chemi-

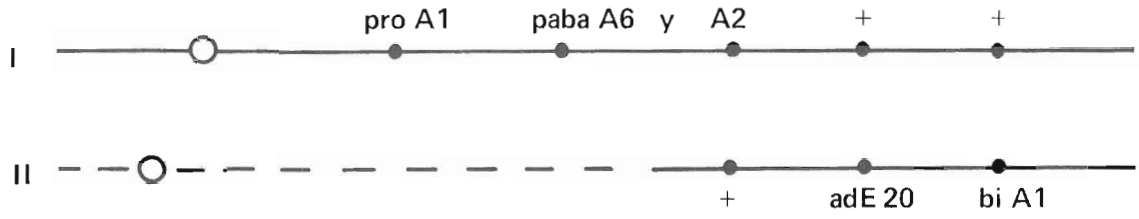

Figure 2 - Strain A, with chromosome duplication. Linkage groups I and II are shown by unbroken and broken lines respectively. Centromeres are designated by open circles. Strains V8 and V9 were derived from this strain. Strain V3 is derived from a similar strain (designated as B) with *y* A2 in linkage group II and *ad*E20 and *bi* A1 in linkage group I.

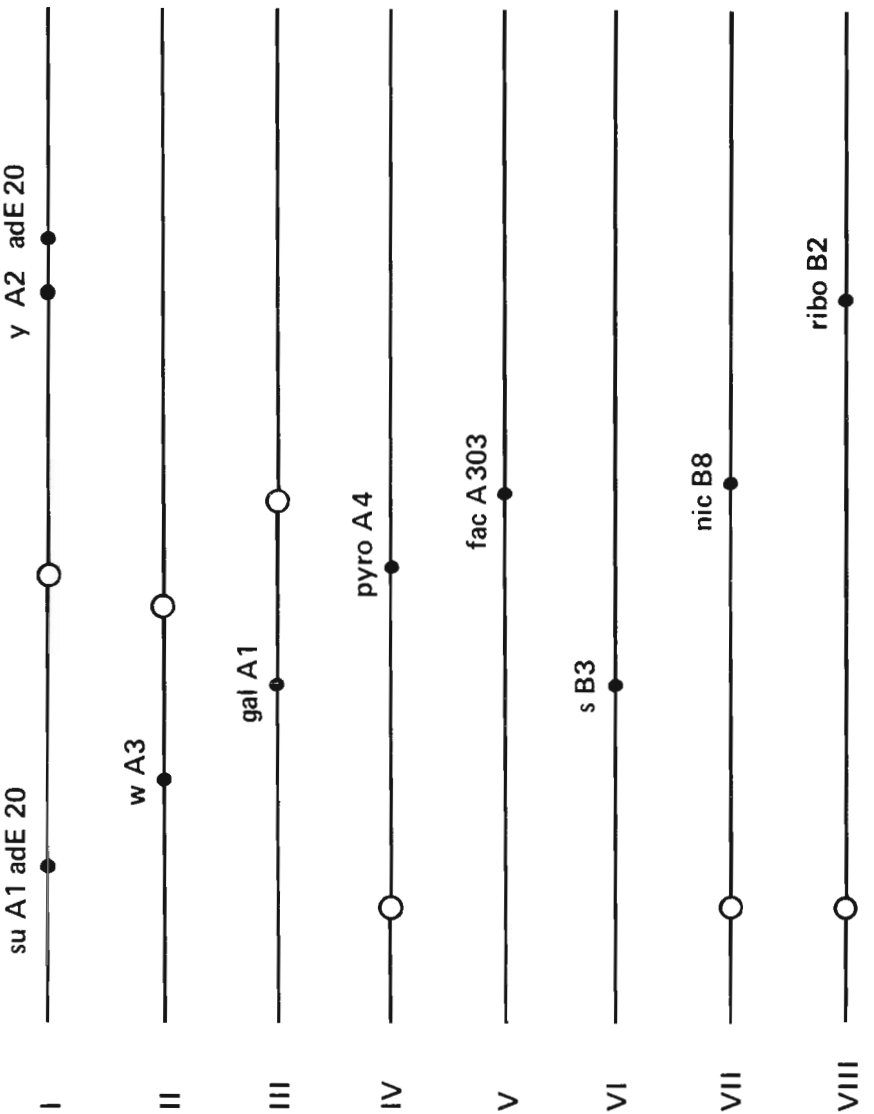

Figure 1 - Master strain E (MSE) with genetic markers in all eight linkage groups.

cal mutagens, acriflavine and ethyl methanesulfonate (EMS), on a strain with chromosome duplication and also on deteriorated variants derived from this or related strains and to observe the genetic alterations produced in mutants with morphological characteristics differing from those of the parent strains, obtained after mutagenic treatment.

MATERIAL AND METHODS

Culture Media

The general techniques and culture media used were those described by Pontecorvo et al. (1953). The minimal medium (MM) was Czapeck-Dox with 1% glucose, and the complete medium (CM) was a complex medium containing yeast extracts, hydrolyzed casein, yeast nucleic acids, vitamins, etc. The solid medium contained 1.5% agar.

Strains

The *Aspergillus nidulans* strains, derived from Glasgow stock, were kept in tubes with CM slopes at 5° C. The genetic markers used in this study, and designated according to Clutterbuck (1974), were: *w* A3; *y* A2, white and yellow conidia, respectively; *ad* E20, *nic* B8; *paba* A6; *pro* A1, *pyro* A4, *ribo* B2, and *s* B3, requirements for adenine, nicotinic acid, p-aminobenzoic acid, proline, pyridoxine, riboflavin, and sodium thiosulfate, respectively; *gal* A1; *fac* A303, inability to grow in medium containing galactose or acetate as the only sources of carbon, respectively; *su* A1 *ad* E20, suppressor of *ad* E20. Master strain E (MSE) carrying markers on all eight linkage groups (Figure 1) was that of McCully and Forbes (1965). The strain with chromosome duplication was the A strain (Nga and Roper, 1968), which has a duplication of linkage group I translocated to II (Figure 2). We also used strains V3, derived from strain B, and V8 and V9, derived from strain A, but having deteriorated phenotypes due to the presence of the determinants of deterioration *v*3, *v*8 and *v*9, respectively (Azevedo and Roper, 1970). Finally, strain

pro A1, *paba* A6, *y* A2 (a normal haploid) was also used.

Plating Methods and Incubation

Conidia were plated from suspensions prepared in 0.1% Tween 80 solution (v/v), or inoculated into CM-containing plates. Replication was done with a 26 point nichrome wire replicator (Azevedo et al., 1976). Incubation was carried out at 37° C, unless stated otherwise.

Treatment with Mutagens

Conidia suspensions in Tween 80 (0.1% v/v) were prepared with the following strains: *pro* A1, *paba* A6, *y* A2 (normal haploid), A (with chromosome duplication), and V3, V8 and V9 (deteriorated strains). Acriflavine was added to the suspension tubes at a final concentration of 12.5 μg/ml and EMS at a final concentration of 1.33% (v/v). After a 24 hour period of incubation at 37° C for acriflavine and 4 hour treatment at 28° C for EMS, the suspensions were diluted and plated on CM. Counts were carried out after 3 days incubation. Preliminary tests had already shown that these treatments produce about 10% survival for each mutagen. The control suspensions were processed in the same way, with no mutagens added.

Genetic Analysis

Genetic analysis was performed according to the techniques of Pontecorvo et al. (1953). The diploids were prepared by the technique of Roper (1952). Mitotic analysis was done by haploidization (Forbes, 1959), using *p*-fluorophenylalanine (Morpurgo, 1961).

RESULTS

Morphological Variants Obtained with Acriflavine

Different types of morphological variants were obtained by treating the strains with acriflavine (Table I). Some variants of deteriorated strains show improved phenotypes, i.e. more rapid growth and better conidiation than the original strain. However, most morphological variants include light, reduced-growth mycelial types which are easily lost in successive subcultures. No morphological mutants were obtained from the normal *pro* A1, *paba* A6 *y* A2 strain. Due to the difficulty in sustaining morphological variants, only one isolate (V3.4), derived from strain V3, was selected for further analysis. The new isolate was designated according to the nomenclature proposed by Azevedo and Roper (1970).

Table I - Percentage of morphological variants obtained with acriflavine and EMS treatment (10% survival) of *Aspergillus nidulans* strains with and without chromosome duplication.

Strain	Control	Acriflavine	EMS
pro A1, *paba* A6; *y* A2	0	0	11.43
A	0	4.87	24.92
V3	0	0.74	7.81
V8	10	27.34	39.33
V9	0	4.24	7.90

Morphological Variants Obtained with EMS

Treatment with EMS produced a higher percentage of variants than produced by acriflavine (Table I). The V8 strain shows a higher frequency of morphological variants than any other strain, consisting mainly of deteriorated phenotypes, but with improved characteristics when compared

to V8. The V8 strain also spontaneously produces mutants disomic for linkage group III with frequency (Azevedo and Roper, 1970). After treatment with EMS, the following isolates were selected: N1 and N2, obtained from strain *pro* A1, *paba* A6, *y* A1; V33 and V34 obtained from strain A; V8.2 and V8.3, obtained from strain V8; V9.6 and V9.7, obtained from strain V9.

Sector Production

The instability of the isolated mutants was verified by sector production. No instability was detected in isolates N1 and N2. Isolates V3.4, V9.6 and V9.7 exhibited colonies with irregular edges, so that no clear sector could be detected. Isolate V8.3 showed a frequency of 0.9 sectors/colony, while V8.2 turned out to be quite stable; V33 and V34 showed about 0.3 sectors per plate.

Genetic Analysis

The nine isolates obtained after treatment with acriflavine and EMS were submitted to genetic analysis. All nine retained the genetic markers *pro* A1, *paba* A6 and *y* A2 of the original strains. Diploids were synthetized between them and the MSE strain, and the results of haploidization showed the genetic alterations which had occurred. Table II shows the complete results of mitotic analysis for two of the nine isolates (V33 and V34). Table III summarizes the results for the mutants analyzed and the linkage groups involved. Meiotic analysis was also carried out for all cases, always using the MSE strain (Table III).

On the basis of the results of genetic analysis, the nine isolated mutants were divided into the following groups:

a) N1 and N2, EMS-induced morphological isolates derived from the normal haploid strain. Consequently, they are stable and have none of the characteristics observed in typical deteriorated isolates obtained from strains with chromosome duplication. Mitotic analysis of N1 shows association of linkage groups IV and VIII, since all normal segregants are pyro$^-$ribo$^-$,

Table II - Mitotic analysis: Haploids from V33//MSE and V34//MSE.

		Haploids from V33//MSE		Haploids from V34//MSE	
Linkage Group	Genetic Markers	Normal Sectors	Deteriorated Sectors	Normal Sectors	Deteriorated Sectors
I	pro^+ $paba^+$	11	7	19	2
	pro paba	6	0	4	0
II	w^+	0	*	2**	*
	w	17	*	21	*
III	gal^+	7	2	23	2
	gal	10	5	0	0
IV	$pyro^+$	6	3	16	0
	pyro	11	4	7	2
V	fac^+	8	4	9	1
	fac	9	3	14	1
VI	s^+	13	4	22	2
	s	4	3	1	0
VII	nic^+	13	4	11	2
	nic	4	3	12	0
	$ribo^+$	0	7	0	2
	ribo	17	0	23	0

* Colour not distinguishable

** The two sectors are yellow and exhibit normal morphology, having probably lost the duplicated segment in linkage group II. All other sectors were white (w), since sectors with duplications are selected against in *p*FA-containing media.

Table III - Meiotic segregation and mitotic analysis of the isolated variants.

Variant	Meiotic segregation Normal : Deteriorated	Linkage group Implicated by haploidization*
N1	237 : 118	IV-VIII
N2	65 : 62	I
V33	55 : 61	VIII
V34	143 : 179	VIII
V3.4	87 : 100	II
V8.2	97 : 50	VII
V8.3	104 : 97	I or VII
V9.6	101 : 83	VII
V9.7	110 : 193	III-VI

* All variants, except N1 and N2, have chromosome duplication in linkage group II, which is selectively eliminated in culture media containing *p*-FA.

while the morphological segregants are pyro$^+$ ribo$^+$, a result showing IV-VIII translocation. Meiotic segregation (2 normal: 1 morphological) confirms translocation. The absence of a class indicated that this translocation must be of the non reciprocal type, as already described by Bainbridge and Roper (1966), Ball (1967), and Clutterbuck (1970). Thus, the altered morphology may probably be associated with the breaking point is linkage group IV or VIII. Genetic analysis of N2 shows that the gene responsible for the morphological phenotypic changes is located in linkage group I.

b) V33 and V34, derived from the duplicated strain A after EMS treatment. In both cases, the determinants of deterioration (ν 33 and ν 34) were located in linkage group VIII (Table II) and segregated as single genes. A deteriorated segregant obtained by crossing V33 with the MSE strain was in turn crossed with V34, exclusively producing segregants of the deteriorated parental types. These data suggest allelism between ν 33 and ν 34. Crossing of V33 and V34 with a strain carrying the determinant of deterioration ν 20, spontaneously obtained from strain A, as described by Azevedo and Roper (1970), and located in linkage group VIII, also produced deteriorated

segregants of the parental types only, a fact which leads us to include *v* 20, *v* 33 and *v* 34 in a single allelic series.

c) V3.4. This was the only analyzed isolate obtained after acriflavine treatmente of strain V3. The determinant of deterioration for V3 (*v*3) is located in linkage group II (Azevedo and Roper, 1970). *v*3.4 is also located in linkage group II, and behaves as a single gene. Crossing of V3.4 with a meiotic segregant of V3 x MSE carrying *v*3, resulted in two types of segregants with deteriorated morphology (V3.4 and V3) and absence of normal colonies. Thus, these results indicate allelism between *v*3 and *v*3.4, with *v*3.4 representing an allelic form of *v*3 probably induced by acriflavine.

d) V8.2, V8.3, V9.6 and V9.7, obtained from already deteriorated strains after EMS treatment. Strain V8 (determinant of deterioration in linkage group IV) is quite stable although producing a large number of disomic conidia for linkage group III. However, after EMS-treatment, variants V8.2 and V8.3 were isolated. Both isolates V8.2 and V8.3 did not show the presence of *v*8 originally present in linkage group IV of V8. In V8.2 a new determinant of deterioration was located in linkage group VII and also a III-VI translocation was detected; in V8.3 there was involvement of linkage group I or VII in the morphological changes and a 1 normal: 1 deteriorated meiotic segregation was obtained (Table III). Finally, V9.6 and V9.7, two variants of V9 (determinant of deterioration in linkage group III) also showed absence of the original *v*9 marker and a new determinant of deterioration in linkage group VII (in V9.6) which segregates as a single gene, and the involvement of linkage groups III and VI in V9.7.

DISCUSSION

The effect of some drugs such as tripan-blue, caffein and coumarine has been studied on unstable strains of *A. nidulans* (Cooke et al., 1970; Parag and Roper, 1975; Majerfeld and Roper, 1978). In these cases, and increase in improved sectors from a strain with chromosome duplication was observed. These improved sectors were the result of variable size deletions of the duplicated segment; when tripan-blue or coumarine is used, there is preferential loss of the translocated segment. Other drugs, such as ethidium bromide

(Bonatelli, Jr. and Azevedo, 1977) and oxathiin (Azevedo et al., 1977) reduce the number of sectors. In all cases cited here, the effect of the drugs on mitotic instability was observed by adding the drug directly to the solid culture medium where the strain would be inoculated. In the present research, the two mutagens were added in suspensions of nongrowing vegetative cells, which after treatment were plated in a drugless solid medium, a method which differs considerably from that used by the other authors.

Production of morphological variants obtained with acriflavine is considerably different according to the strain used. The only mutant analyzed (V3.4) shows determinant of deterioration (v3.4) as an allele of v3. II-I duplication must produce a greater susceptibility to the action of acriflavine, since the standard haploid strain practically exhibits no morphological variants. The effect of acriflavine on *A. nidulans* is complex and its effective action involves interaction of the genotype with environmental conditions (Ball and Roper, 1966). Under the conditions used, it is difficult to distinguish whether the different response of the strains in the production of morphological variants reflects different permeability or metabolic processes. Acridins have been suggested to be acting as mutagens by inhibiting the repair system (Dulbecco, 1964) even in some case of *A. nidulans* (Ball and Roper, 1966). A weak mutagenic effect has been observed for *Escherichia coli* (Lerman, 1963), and even an antimutagenic action has already been described (Magni et al., 1964). The effect of some drugs on processes of mitotic nonconformity has been generally interpreted to be an interaction of these with the mutagens. The results obtained in the present case by plating treated conidia in a drugless culture medium show that the production of morphological variants is mainly, or exclusively, induced by acriflavine action, since the untreated conidia produce no morphological variants. Strain V8 represents an exception; however, there is a considerable increase in morphological variants which may be attributed to the action of acriflavine. A similar line of reasoning can be followed for EMS, adding that the action of this mutagen is more drastic than that of acriflavine, inducing a greater frequency of morphological variants. In the case of EMS, chromosome duplication does not appear to lead to a greater susceptibility when compared to the standard haploid strain, as was the case with acriflavine.

Genetic analysis of the mutants obtained by treating already deteriorated strains with EMS indicates the occurrence of a complex mutagenic

action, i.e. suppression of the original determinants of deterioration in concomitance with the appearance of new ones behaving as single genes at new locations, and in some cases the occurrence of chromosome breaks. Linkage group II seems to remain unaltered despite the replication errors of the duplicated segment (Cooke et al., 1970; Parag and Roper, 1975).

Suppression of the original determinant of deterioration in concomitance with the appearance of new factors at different locations is a phenomenon which occurs spontaneously, although to a lesser extent, in deteriorated strain. This phenomenon has been explained by transposition of genetic elements from one region of the genome to another, which creates the conditions for the appearance of a new phenotype (Azevedo and Roper, 1970). A similar phenomenon occurred in our study with the morphological variants obtained from strains V8 and V9. This explanation however, although plausible, is more difficult to accept in the case of treatment with mutagens such as EMS, since one could also hypothesize that alkylating agents which are powerful mutagens for *A. nidulans* (Duarte, 1971), may cause new mutations in the genome which, in turn, would lead to phenotypes with altered morphology, a fact which actually occurred in our study with the standard haploid strain (isolates N1 and N2). The effect of these new EMS-induced mutations, added to that of the already existing determinants of deterioration, must be very drastic and possibly lethal or selected against during the isolation phase. In fact, Azevedo and Roper (1970) have already demonstrated that certain determinants of deterioration produce extremely reduced viability when associated with others. Menezes and Azevedo (1978) have also shown that some of them are suppressed or even spontaneously lost with relatively high frequency. Thus, we may hypothesize that when, upon treatment with EMS the original determinant of deterioration is lost and new genetic alterations are observed, this may be the result of two events (EMS-induced new alterations and loss or suppression of the original factors), since the occurrence of these two events would give advantages to the colonies when they are selected by the method used. However, we must not discard the hypothesis that EMS acts by inducing transpositions, even at a very early phase, since all the variants analyzed here were obtained by plating conidia and not as sectors of duplicated strains. It should actually be pointed out that the two mutations *v*33 and *v*34 obtained with the use of EMS from strain A were alleles of *v*20, which was spontaneously obtained from the same strain. In this

case, we may assume that EMS acts by favouring or selecting transposition to this site in linkage group VIII.

ACKNOWLEDGMENT

The authors are indebted to FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo) for financial support.

REFERENCES

Azevedo, J.L. (1975). Altered instability due to genetic changes in a duplication strain of *Aspergillus nidulans. Genet. Res.*, Camb. *26:* 55-61.

Azevedo, J.L., Oliveira, A. and Rocha Campos, A.J. (1976). Replicador multifio para transferência de esporos de fungos filamentosos. *Summa Phytopathol. 2:* 237-241.

Azevedo, J.L. and Roper, J.A. (1970). Mitotic non-conformity in *Aspergillus:* successive and transposable genetic changes. *Genetic. Res.*, Camb. *16:* 79-93.

Azevedo, J.L. Santana, E.P. and Bonatelli Jr., R. (1977). Resistance and mitotic instability to chloroneb and 1,4 oxathiin in *Aspergillus nidulans. Mutat. Res., 48:* 163.172.

Bainbridge, B.W. and Roper, J.A. (1966). Observations on the effects of a chromosome duplication in *Aspergillus nidulans. J. Gen. Microbiol. 42:* 417-424.

Ball, C. (1967). Chromosome instability related to gene suppression in *Aspergillus nidulans. Genet. Res.*, Camb. *10:* 173-183.

Ball, C. and Roper, J.A. (1966). Studies on the inhibition and mutation of *Aspergillus nidulans* by acridines. *Genet. Res.*, Camb. *7:* 207-221.

Bonatelli Jr., R. and Azevedo, J.L. (1977). Effects of ethidium bromide in diploid and duplication strains of *Aspergillus nidulans. Experientia. 33:* 331-312.

Clutterbuck, A.J. (1970). A variegated position effect in *Aspergillus nidulans. Genet. Res,* Camb. *26:* 55-61.

Clutterbuck, A.J. (1970). A variegated position effect in *Aspergillus nidulans. Genet. Res.*, Camb., *16:* 303-316.

Clutterbuck, A.J. (1974). *Aspergillus nidulans,* in R.C. King (ed.). Handbook of Genetics, vol. I, Plenum Press, New York, pp. 447-510.

Cooke, P., Roper, J.A. and Watmough, W. (1970). Trypan blue-induced deletions in duplication strains of *Aspergillus nidulans. Nature* Lond., *226:* 276-277.

Duarte, F.A.M. (1971). Genetic and allied effects of certain esters of inorganic acids in *Aspergillus nidulans. Experientia, 27:* 966-967.

Dulbecco, R. (1964). Summary of 1964 Biological Research Conference. *J. Cell Comp. Physiol.*, 64 (Suppl. 1): 186.

Forbes, E. (1959). Use of mitotic segregation for assigning genes to linkage groups in *Aspergillus nidulans. Heredity, 13:* 67-80.

Lerman, L.S. (1963). The structure of the DNA-acridine complex. *Proc. Nat. Acad. Sci., 49:* 94-102.

Magni, G.E., von Borstel, R.C. and Sora, S. (1964). Mutagenic action during meiosis and antimutagenic action during mitosis by 5-amino acridine in yeast. *Mutat. Res., 1:* 227-230.

Majerfeld, J.H. and Roper, J.A. (1978). The effects of coumarin on the frequency of deletions in a duplication strain of *Aspergillus nidulans. Mol. Gen. Genet., 159:* 203-206.

McCully, K.S. and Forbes, E. (1965). The use of *p*-fluorophenylalanine with "master strains" of *Aspergillus nidulans* for assigning genes to linkage groups. *Genet. Res.*, Camb. *6:* 352-359.

Menezes, E.M. and Azevedo, J.L. (1978). Reversion in variants from a duplication strain of *Aspergillus nidulans. Mol. Gen. Genet., 164:* 255-258.

Morpurgo, G. (1961). Somatic segregation induced by *p*-fluorophenylalanine. *Asp. Newsletter, 2:* 10.

Nga, B.H. and Roper, J.A. (1968). Quantitative intrachromosomal changes arising at mitosis in *Aspergillus nidulans. Genetics, 58:* 193-209.

Nga, B.H. and Roper, J.A. (1969). A system generating spontaneous intrachromosomal changes at mitosis in *Aspergillus nidulans. Genet. Res.*, Camb. *14:* 63-70.

Parag, Y. and Roper, J.A. (1975). Genetic control of chromosome instability in *Aspergillus nidulans* as a mean for gene amplification in eukaryotic microorganisms. *Mol. Gen. Genet, 140:* 275-287.

Pontecorvo, G., Roper., Hemmons, L.M., McDonald, K.D. and Bufton, A.W.J. (1953). The genetics of *Aspergillus nidulans. Adv. Genet., 5:* 141-238.

Roper, J.A. (1952). Production of Heterozygous diploids in filamentous fungi. *Experientia, 8:* 14-15.

Roper, J.A., Palmer, H.M. and Watmough, W.A. (1972). Mitotic non-conformity in *Aspergillus nidulans:* the effects of caffeine. *Mol. Gen. Genet., 118:* 125-133.

(Received January 29,1979)

Rev. Brasil. Genet. I, 4, 263-278 (1978)
(Brazil. J. Genetics)

SEX CHROMATIN IN NORMAL NEWBORNS DURING THE FIRST TWO WEEKS OF LIFE: A BLIND STUDY*

Claudette Hajaj Gonzalez[1], Fernando Teixeira Mendes[2], and P.H. Saldanha[3]

ABSTRACT

X-chromatin bodies and drumstick counts were performed in 38 normal newborns, 19 males and 19 females, on the 1st, 5th and 15th day of life. Complete clinical and hematological examinations were conducted at the same time. In the female newborns, X-chromatin frequencies were 4.32%, 16.18% and 10.40%, and drumstick values were 9.96%, 7.60% and 5.65%, in the three successive counts. Variations in sex chromatin observed between periods were statistically significant. In the male newborns, drumsticks occurred very rarely, and X-chromatin formations were never found. No correlation between drumstick frequencies and neutrophil counts or between X-chro-

---

* This work was partially supported by a grant from "FAPESP" (Fundação de Amparo à Pesquisa do Estado de São Paulo).

[1] Instituto da Criança do Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo (Faculdade de Medicina da Universidade de São Paulo., São Paulo, Brasil).

[2] Formerly at the Laboratório Central do Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo, São Paulo, Brasil.

[3] Laboratório de Genética Médica, Faculdade de Medicina da Universidade de São Paulo, São Paulo, Brasil.

matin and drumstick values was statistically detected. Drumstick counts from enriched blood smears seem to represent a reliable and quick method for determining genetic sex in the early days of postnatal life.

INTRODUCTION

X-chromatin investigation represents an important screening method for detecting abnormalities of sexual differentiation (Barr, 1972). In population surveys, it has been studied in buccal smears and in amniotic cells (Mikamo, 1968; Robinson et al. 1969). In addition, Y-chromatin can also be studied in human interphase nuclei by fluorescence techniques (Dallapiccola, 1971; Lewin and Conen, 1971; Robinson, 1971; Golob and Wagenbichler, 1973).

Although neutrophil drumsticks are rarely used in clinical tests, their investigation should be complementary to ordinary blood smears (Davidson and Smith, 1954) or to concentrated blood smears (De La Chapelle, 1961).

Variations in X-chromatin frequency of female cells have been reported under different circumstances: during various hormone treatments (Taylor, 1963; Schetty et al. 1966; Dokumov and Spasov, 1968); in extensive burns (Weste et al., 1967); during menstrual cycles (Blanco de Del Campo and Garcia-Ramirez, 1965; Schmidt et al., 1966; Hagy and Brodrick, 1972) and during pregnancy (Smith et al., 1962; Wegman and Smith, 1964; Towsend et al., 1970; Chiantera, 1971). Alterations in Barr body size were also related to antibiotic use (Sohval and Casselman, 1961).

The frequency of drumsticks in normal females varies during the menstrual cycle (Caratzali, 1963), after the administration of ACTH and insulin (Caratzali and Phleps, 1958), with senility (Tonomura et al., 1962) and with cachexia (De Castro, 1963). In mammals there is evidence that X-chromatin bodies and drumsticks represent the heteropicnotic X-chromosome (Fraccaro et al., 1964; Bamford et al., 1964; Grimberg et al., 1966) and the fluorescence technique applied to blood smears has made it possible to correlate drumsticks with the number and size of Y chromosomes in normal males (Lamborot-Manzur et al., 1972).

Low frequencies of X-chromatin in buccal smears were found in the

first few days of life by Smith et al. (1962) and Taylor (1963) and were confirmed subsequently by Frasier et al. (1964) and Wegmann and Smith (1964). Eidenbenz (1964) and Méhes and Sulyok (1968) have also observed low chromatin frequency in full term newborns during the first days after birth but not in prematures. The study of Homma and Kajii (1968) has confirmed that low sex chromatin frequencies do not occur in true prematures and that, in small-for-date newborn infants, low X-chromatin frequencies were seen soon after birth.

Oral and vaginal smears of normal newborn females have revealed an increase in X-chromatin percentages from day 1 to day 4 of life, with some stabilization thereafter (Golob, 1969). Golob et al. (1968, 1969) have indicated a relationship between estrogen activity in the vagina and the frequency of X-chromatin in the buccal smear of female newborns. Reporting the absence of suppression of sex chromatin in prematures during the initial days of life, Eidenbenz (1964), Homma and Kajii (1968) and Méhes and Sulyok (1968) have reinforced the idea that the factors which cause a decrease in X-chromatin bodies in female newborns act at the end of gestation.

Data on drumstick variation in the neonatal period are scarce. Kosenov and Scupin (1956), Van Harnack and Strietzel (1956) and Peiper and Oehme (1956), have found much higher percentages of drumsticks in female newborns than in adult women. Peiper and Oehme's work with fetuses has shown that the number of drumsticks tends to increase progressively during gestation, reaching a peak at birth.

Resore-Quartino and Piccinini (1967) have performed time-variation studies of Barr chromatin and drumsticks in female newborns. From day 1 to 5, X-chromatin was found in increasing and drumsticks in decreasing frequencies. However, gestational age, Apgar rating, birth weight, clinical conditions and medication were not considered.

The purpose of the present study was to determine the frequency variation of X-chromatin and of drumsticks in normal newborn infants, during the first two weeks of life. Recognition of X-chromatin abnormalities can help newborn patients with genital ambiguity by earlier diagnosis.

MATERIAL AND METHODS

Thirthy-eight normal newborns, 19 males and 19 females, were investigated. Among them, 26% were white, 71% mulattoes and 2.6% of

mixed Indian and Negro ancestry ("cafuso"). Only full-term neonates born after 38-42 weeks of gestation (Battaglia and Lubchenco, 1967), weighing 3000-4000 g, and with Apgar scores of 8 or more at 5 minutes were included in the investigation. All subjects had normal cephalic deliveries, either spontaneous or assisted by low forceps. Newborns whose mothers had prolonged deliveries of more than 12 hours or had received general anesthesia, were excluded. Cases of early amniotic membrane rupture (Dorros et al., 1969) were also excluded. Newborns whose mothers had infections were excluded, as well as infants of diabetic mothers or born to mothers who received antibiotics or hormones during the last month of pregnancy (Sohval and Casselman, 1961; Schetty et al., 1966; Spasov and Dokumov, 1968). Weight and length were followed longitudinally. The examination was intended to exclude any infants with minor or major congenital malformations. Evidence of jaundice on first examination excluded the newborn from the study. At the second examination, only non-physiological jaundice caused exclusion. Any pathological condition or medication, especially antibiotics, preceding the second or third sampling resulted in exclusion. An intramuscular injection of 1 mg of vitamin K was given to all newborns soon after birth. The samples on the first day were obtained from 1 to 23 hours. Out of 38 newborns, 6 (2 females) received maternal milk before the first sampling.

The main anthropometric average values for the 38 newborns on the first day of life, classified by sex, appear in Table I. Other clinical-genetical data concerning individual hematological values, pediatric conditions, incidence of malformations in the sibship, parental conceptions, etc., were presented elsewhere (Gonzalez, 1972). Table II shows the hematological average values observed among 38 healthy newborns on the first, fifth and fifteeenth day of life, classified by sex.

Three counts of X-chromatin in bucco-mucosal cells and of drumsticks in polymorphonuclear neutrophils were performed for each newborn, on the first, fifth and fifteenth day of life, when clinical examinations and CBC were carried out.

X-chromatin studies of oral mucosa cells were done by the modified Feulgen technique. Four buccal smears were taken at the same time from each individual. Slides of many newborns were coded and mixed before "blind" examination. Counts of 500 nuclei were performed during each sampling time for each individual, by oil immersion objective. Only homo-

geneously stained nuclei were studied, with well defined membranes, without clumping of chromatin in not overlapping cells. Only clear chromatin bodies approximately 1 micron in diameter and located at the periphery of the nuclear membrane were counted.

Drumsticks were studied in enriched blood smears. Two cm^3 of venous blood were obtained and placed in a tube with EDTA. One cm^3 of the blood was centrifuged in Wintrobe tubes at 1500 r.p.m. for 15 minutes. The supernatant was removed with a Pasteur pipette and discarded. The fine and clear intermediary layer was removed with a second pipette. This leucocyte "cream" was then used to make at least 4 smears. After staining with Leishman dye, the slides were numbered. Drumstick counts were always done with oil immersion objectives in "blind" tests after mixing together many coded slides from several newborns with different days of life. Drumsticks were recorded according to the criteria of Kosenov and Scupin (1956). For each count, 500 neutrophiles with two or more segments were examined for the presence of drumsticks and the total number was the sum of pedunculated (type A) and sessile (type B) types.

A sample of 0.5 ml of blood was used for the hemogram determination in each of the samplings. Erythrocyte and leukocyte counts were made with an electronic Coulter counter, Model A. Two counts were made for each blood sample and the result was expressed by their arithmetic average. The differential leukocyte count was worked out by the usual methods.

Results concerning glucose-6-phosphate dehydrogenase activity investigated in the newborns will be reported elsewhere (Itskan, Gonzalez and Saldanha, 1978).

RESULTS

Table III shows the frequencies of Barr bodies in bucco-mucosal cells and drumsticks in segmented neutrophiles among 19 healthy female newborns, on the first, fifth and fifteenth day of life. While the percentage of drumsticks decreases steadily in three successive counts, Barr body frequencies increase rapidly up to the 5th day, but the values decrease on the 15th day.

Table I - Anthropometric mean values for 38 newborns, on the first day of life, classified by sex.

Parameter	Male newborns (19)		Female newborns (19)	
	Mean ± SD	Range	Mean ± SD	Range
Weight (g)	3430 ± 267	3000 - 3870	3371 ± 303	3030 - 3950
Length (cm)	50.07 ± 1.61	46.7 - 53.3	49.31 ± 1.38	47.5 - 52.8
Occipito-frontal circumference (cm)	34.81 ± 1.05	33.0 - 36.5	34.27 ± 1.08	32.5 - 36.0
Thoracic circumference (cm)	31.0 ± 1.15	31.0 - 36.0	33.62 ± 1.13	31.5 - 35.5

Table II - Hematologic mean values observed among 38 healthy newborns on the first, fifth and fifteenth day of life, classified by sex.

Sampling time		Erythrocyte (millions per mm^3)	Hemoglobin (g per ml)	Reticulocyte (%)	Leucocytes (per mm^3)	Segmented neutrophiles (%)
			Male newborns			
1st day	m ± sd	5.1 ± 0.4	16.9 ± 2.0	2.54 ± 1.18	15,352 ± 4074	48.8 ± 11.4
	range	4.6 - 5.9	14.4 - 21.4	0.8 - 5.0	8,300 - 21,500	19 - 71
5th day	m ± sd	5.0 ± 0.5	15.9 ± 1.2	1.18 ± 0.51	9,952 ± 2,590	32,2 ± 10.7
	range	3.6 - 5.9	13.8 - 18.0	0.4 - 2.3	6,200 - 16,400	5 - 60
15th day	m ± sd	4.5 ± 0.4	13.9 ± 1.4	0.61 ± 0.25	11,084 ± 3,029	30.0 ± 10.5
	range	3.9 - 5.7	10.7 - 17.2	0.2 - 1.1	4.400 - 17,000	11 - 55
			Female newborns			
1st day	m ± sd	5.4 ± 0.7	17.8 ± 1.9	3.12 ± 1.28	16,731 ± 3,775	54.8 ± 11.4
	range	4.4 - 7	14.9 - 21.8	1.5 - 7.0	10,600 - 23,000	27 - 78
5th day	m ± sd	5.1 ± 0.5	16.6 ± 1.8	1.08 ± 0.33	10,242 ± 2.603	37.0 ± 11.0
	range	4 - 6	13.2 - 21.4	0.4 - 1.7	5,900 - 16,200	22 - 59
15th day	m ± sd	4.7 ± 0.5	14.8 ± 1.6	0.76 ± 0.29	10,673 ± 3,178	28.3 ± 10.3
	range	4 - 64	12.2 - 18.5	0.3 - 1.1	5,400 - 20,000	9 - 52
			Both sexes			
1st day	m ± sd	5.3 ± 0.61	17.4 ± 2.0	2.83 ± 2.6	16,042 ± 3,937	51.89 ± 8.1
	range	4.4 - 7.0	14.4 - 21.8	0.8 - 7.0	8,300 - 23,000	19 - 78
5th day	m ± sd	5.11 ± 0.5	16.2 ± 1.6	1.13 ± 1.7	10,097 ± 2,565	34.61 ± 7.7
	range	3.6 - 6.1	13.2 - 21.4	0.4 - 2.3	6,400 - 16,400	5 - 60
15th day	m ± sd	4.6 ± 0.4	14.3 ± 1.5	0.69 ± 1.3	10,878 ± 3,069	29.1 ± 7.3
	range	3.9 - 6.4	10.7 - 18.5	0.2 - 1.5	4,400 - 20,000	9 - 55

The frequency of X-chromatin bodies was consistently low and drumsticks were appreciably high in females on the first day of life, as compared to adult values (cf. Saldanha, 1967).

No X-chromatin bodies were found in any of the male newborns. The number of drumsticks varied between 0 and 4 per 500 nuclei. In 26 counts one drumstick per 500 neutrophiles was found; in 6 counts, there were two; in 2 counts there were three drumsticks, and in one count there were four drumsticks. In 35 counts, there were no drumsticks. A great number of "small drumsticks" was found in many smears from newborns who were subsequently identified as males. They were observed with equal frequency on the 1st, 5th and 15th day.

Variances concerning absolute distributions per 500 nuclei of Barr bodies and drumsticks in females between the first, fifth and fifteenth day of life were analyzed (Table III). The heterogeneity of distributions of X-chromatin in each period was statistically significant (Turkey test). The data presented in Table III seem to indicate to some extent an association between variation in Barr bodies and drumsticks. Similarly, it is likely that the presence of drumsticks is related to the frequency of segmental neutrophiles (Table II).

Table V shows the linear correlation between frequencies of Barr bodies and of drumsticks as well as between values of drumsticks and of segmented neutrophiles in females on the first, fifth and fifteenth day of life. The coefficient of correlation varies between 0.10 and 0.20 but the differences are not statistically significant, probably due to the smallness of the samples.

Table III - Mean percentages of Barr bodies in bucco-mucosal cells and of drumsticks in segmented neutrophiles among 19 healthy female newborns on the first, fifth and fifteenth day of life.

	Barr bodies		Drumsticks	
Sampling time	Mean ± SE	Range	Mean ± SE	Range
1st day	4.32 ± 1.09	0.8 - 8.8	9.96 ± 1.58	6.2 - 15.8
5th day	16.18 ± 1.94	10.6 - 26.8	7.60 ± 1.39	4.2 - 14.2
15th day	10.40 ± 1.61	6.4 - 15.4	5.65 ± 1.21	3.8 - 8.4

Table IV - Analysis of variance of the distribution of Barr bodies (in 500 nuclei) and drumsticks (in 500 segmented neutrophiles) observed in female newborns between the first, fifth and fifteenth day of life.

Source of variation	Degrees of freedom	Sum of square	Mean square	F*
	Variation in Barr bodies			
Between sampling time	2	33,430.84	16,715.42	56.81
Between newborns	54	15,887.36	294.21	
Total	56	49,318.21		
	Variation in drumsticks			
Between sampling time	2	4,428.78	2,214.36	19.14
Between newborns	54	6,244.84	115.64	
Total	56	10,673.57		

* critical $F_{(0.05)} = 3.17$

Table V - Correlation between frequencies of Barr bodies and of drumsticks as well as between values of drumsticks and of segmented neutrophiles in female newborns on the first, fifth and fifteenth day of life.

Sampling time	Barr bodies vs Drumsticks		Drumsticks vs Segmented neutrophiles	
	r*	t	r*	t
1st day	0.20	0.82	0.13	0.55
5th day	0.14	0.56	0.22	0.92
15th day	0.10	0.40	0.08	0.31

* r = coefficient of correlation based on arc sen transformation; sample size = 19; critical $t_{(0.05)} = 2.11$

DISCUSSION

X-chromatin counts were consistently low in female newborns on the first day of life. Their frequencies increased by day 5 but by day 15 had decreased somewhat. The mean percentages of sex chromatin are slightly lower than those reported in the literature. This may be due to the standardization of the methods and procedures adopted. X-chromatin bodies in newborns were observed to be often faintly colored, especially on the first day of life.

Smith et al. (1962), Taylor (1963), Frasier et al. (1964), Businco and Vignetti (1964) and Rasore-Quartino and Piccinini (1967) have reported sex chromatin variations of 4 to 22% in their studies of newborns on the first day of life. Although different techniques and standardizations were used, all reports, regardless of the absolute count for day 1, indicate a consistent rise in the percentage of sex chromatin beginning on day 2. Highest frequencies are reported for days 3 and 4. However, these authors did not usually provide data for day 15. In the study presented here, day 15 data are similar to the day 15 data reported by Ciornea et al. (1971).

Van Harnack and Strietzel (1956), Peiper and Oehme (1956) and Rasore-Quartino and Piccinini (1957) have found a mean percentage of drumsticks of about 10% on the first day of life and of 3.57% on the fifth day of life. The value observed in the present study (9.96%) agrees with the day 1 observations of most reports, but our means for day 5 are higher (7.6%). There are no data in the literature concerning drumsticks in normal newborns on day 15. While the frequency of X-chromatin bodies rose by the fifth day, the frequencies of drumsticks decreased steadily from the first to the fifteenth day.

Total leukocyte counts vary considerably from child to child and from day to day in each child. Full term infants have from 9000 to 30,000 leukocytes at birth, generally with a mean of 15,000 to 20,000/mm^3. During the first few days of life there is a preponderance of polymorphonuclear neutrophiles, normally 61% of the total cell count. On day 14 the mean percentage of segmented neutrophiles is about 34% of the total cells (Oski and Naiman, 1966).

In our investigation all of the 38 newborns had a mean percentage of 51% polymorphonuclear neutrophiles or segmented neutrophiles on day 1; on day 5, 34%; and on day 15, 29% (Table II). Corresponding values for 19 female newborns appear also in Table II. The decrease in percentage of segmented neutrophiles and in the total number of neutrophiles is similar to the decrease observed in drumsticks. However, a significant positive correlation between the values of segmented neutrophiles and the values of the drumsticks in the three determinations could not be detected probably because of the sample size needed for the observed coefficient values. Higher frequencies of drumsticks are more likely to be found with higher percentages of segmented neutrophiles.

According to some authors (Taylor, 1963; Eidenbenz, 1964), the low frequency of sex chromatin on the first day of life could be explained by metabolic processes which include electrolyte alterations due to hormonal mechanisms. The same hormonal dynamics may explain the variations observed during mentrual cycle phases or during pregnancy, and the process was confirmed during various types of hormone treatments (Taylor, 1963; Schetty et al., 1966; Dokumov and Spasov, 1968). On the other hand, there are some observations of the variations of the frequency of drumsticks in normal females during the menstrual cycle (Caratzali, 1963) and after the adminis-

tration of ACTH and insulin (Caratzali and Phleps, 1958). The results observed in these investigations confirm the probable role of the hormones in the frequency of X-chromatin. To date, no explanations exist for the high drumstick percentages found in female newborns. It is likely that the significant variations in X-chromatin and drumstick counts for the three periods studied have common causes, that is, variations in neonatal hormone levels.

The findings described here confirm the data concerning low sex chromatin percentages at birth. However, our data draw attention to the high drumstick frequency at birth, previously not emphasized by others. From a clinical point of view, this would aid the cytological diagnosis of sex at birth. Frequencies of Barr chromatin as low as those observed in normal female newborns on the first day of life might, at least in part, have led to erroneous diagnosis or unnecessary doubts. An additional blood smear may facilitate the diagnosis. Leukocyte concentration allows quick, easy and precise determination of a full-term newborn's genetic sex. Drumstick percentages as high as those observed during the three periods of our study leave no doubt for diagnosis, even considering the possibility of X chromosome mosaicism. If these measurements were supplemented with fluorescent techniques for Y chromatin detection, few errors should occur in diagnosing gonosomal sex constitution at birth.

ACKNOWLEDGMENTS

The authors are most grateful to Dr. José Lauro Araujo Ramos and Dr. Hélcio Bahia Corradini of the Instituto da Criança do Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo for valuable suggestions.

REFERENCES

Blanco de Del Campo, M.S. and Garcia Ramirez, O.E. (1965). Fluctuations of the sex chromatin during the menstrual cycle. *Acta Cytol.* (Baltimore) *9:* 251-256.

Bamford, S.B., Cassin, C.M., Danute, D.L. and Mitchell, G.W. (1964). Neutrophil appendages as indicators of the sex chromosome aberrations. *Acta. Cytol.* (Baltimore) *8:* 323-331.

Barr, M.L. (1972). Some notes on the discovery of the sex chromatin and its clinical application. *Am. J. Obstet. Gynecol. 112:* 293-296.

Battaglia, F.C. and Lubchenco, L.O. (1967). A practical classification of newborn infants by weight and gestational age. *J. Pediatr. 71:* 159-163.

Businco, L. and Vignetti, P. (1967), apud: Rasore-Quartino, A. and Piccinini, A. (1967). Studio comparativo della cromatina sessuale nelle cellule di mucosa orale e nei granulociti durante il periodo neonatale. *Pathologica 59:* 129-135.

Caratzali, A. (1963). Sex appendages, Arneth formula and the menstrual cycle. *Cytogenetics, 2:* 15-23.

Caratzali, A. and Phleps, A. (1958). Considérations sur le déterminisme des corpuscules sexuels des leucocytes. C.R. Soc. Biol. (Paris) *152:* 1114--1115.

Chiantera, A. (1971). Il comportamento della cromatina sessuale nella gravidanza a termine e nella gravidanza protratta. *Minerva Ginecol. 23:* 654-655.

Ciornea, T., Trifan, N., Mihai, A. and Marian, L. (1971). Dinamica testului sexuale in primele zile de viata. *Rev. Med. Chir. Soc. Med. Nat. Iasi. 75:* 441-448, 1971.

Dallapiccola, B. (1971). Identification of the human sex chromosome complement in polymorphonuclear leukocytes: a new technique. *J. Lab. Clin. Med. 78:* 88-93.

Davidson, R.G. and Smith, D.R. (1954). A morphological sex difference in the polymorphonuclear neutrophil leucocytes. *Br. Med. J. 2:* 6-7.

De Castro, N.M. (1963). Frequency variations of "drumsticks" of peripheral blood neutrophiles in the rabbit in different alimentary conditions. *Acta Anat.* (Basel) *52:* 341-368.

De La Chapelle, A. (1961). A simple technique for concentrating granulocyte for the determination of chromatin sex. *Scand. J. Clin. Lab. Invest. 13:* 525-528.

Dokumov, S.I. and Spasov, S.A. (1968). Kern-sexchromatinkörper und östrogene aktivität. *Z. Geburtshilfe Gynaekol. 169:* 85-91.

Dorros, G., Kleiner, G.J. and Romney, S.L. (1969). Fetal leukocyte pattern

in premature rupture of amniotic membranes and in normal and abnormal labor. *Am. J. Obstet. Gynecol. 105:* 1269-1273.

Eidenbenz, R. (1964). Über das Geschlechtschromatinin des Neugeborenen. Biol. Neonat. *7:* 95-127.

Fraccaro, M., Lindsten, J., Mittwoch, U. and Zonta, L. (1964). Size of drumsticks in patients with abnormalities of the X-chromosome. *Lancet 2:* 43-44.

Frasier, S.D., Crudo, F.S. and Farell, F.J. (1964). Buccal smears in the newborn female. *J. Pediatr. 65:* 222-225.

Golob, E.K., Isracena, T. and Becker, K.L. (1969). Sex chromatin frequency and estrogenic activity in the newborn female. *J. Clin. Endocrinol. Metab. 29:* 116-118.

Golob, E.K., Oh, W.S. and Fischer, P. (1968). Zur Frage der Sexchromatin--frequenz in Vaginalabstrichen neugeborener Mädchen. *Wien. Klin. Wochenschr. 80:* 979-981.

Golob, E. and Wagenbichler, P. (1973). Geschlechtschromatin und Fluoreszens Körperchen in Mundschleimhautabstrichen von 1000 Neugeborenen. *Geburtsch u. Frauenheilk 33:* 86-92.

Gonzalez, C.H. (1972). Contribuição ao estudo da cromatina sexual em recém-nascidos normais nas duas primeiras semanas de vida. Doctoral thesis. Universidade de São Paulo, São Paulo, Brasil.

Greig, M., Coyle, M.G., Cooper, W. and Walker, J. (1962). Plasma progesterone in mother and foetus in the second half of human pregnancy. *J. Obstet. Gynaecol. Br. Commonw. 69:* 772-776.

Grimberg, M.A., Sullivan, M.M. and Benirschke, K. (1966). Investigations with tritiated thymidine of the relationship between the sex chromosomes, sex chromatin and the drumsticks in the cells of the female nine-banded armadillo (*Dansypus novemcitus*). *Cytogenetics: 5:* 64-74.

Hagy, G.W. and Brodrick, M.M. (1972). Variation of sex chromatin in human oral mucosa during the menstrual cycle. *Acta Cytol.: 16:* 314-321.

Homma, T. and Kajii, T. (1968). Absence of transient suppression of sex chromatin in female premature newborns. *Pediatrics 42:* 437-440.

Hsu, L.Y., Klinger, H.P. and Weiss, J. (1967). Influence of nuclear selection criteria on sex chromatin in frequency in oral mucosa cells of newborn females. *Cytogenetics 6:* 371-382.

Itskan, S.B., Gonzalez, C.H. and Saldanha, P.H. (1978). Association of

erythrocyte glucose-6-phosphate dehydrogenase activity with hematologic variables and X-chromatin among healthy newborns (Submitted for publication).

Kosenov, W. and Scupin, R. (1956). Die Bestimmung des Geschlechts Hilfe einer Kernanhangsformel der Leukocyten. *Acta Haematol.* (Basel) *15:* 349-363.

Lamborot-Manzur, M., Tishler, P.V. and Atkins, L. (1972). Quantitative studies of Y chromosomal fluorescence in human interphase nuclei. *Clin. Genet. 3:* 103-115.

Lewin, P.K. and Conen, P.E. (1971). Fluorescent Y screening of hospitalized newborns, *Nature* (Lond.) *233:* 344-335.

Méhes, K. and Sulyok, E, (1968). Sex chromatin in the premature. *Biol. Neonat. 12:* 239-245.

Mikamo, K. (1968). Sex chromosomal anomalies in newborn infants. A 3-year survey of fetal membranes. *Obstet. Gynecol. 32:* 688-699.

Mittwoch, U. (1964). Sex chromatin (Review article). *J. Med. Genet. 1:* 50-76.

Oski, F.A. and Naiman, J.L. (1966). *Hematologic problems in the newborn.* Chapter 1. Normal blood values in the newborn period. Philadelphia, W.B. Saunders, p. 1-21.

Peiper, U. and Oehme, J. (1956). Die Abhängigkeit geschlechtagelbundener Leukocytenmerknale bei Feten und Frühgeborenen vor der Reife. *Klin. Wochenschr. 34:* 1067-1068.

Rasore-Quartino, A. and Piccinini, A. (1967). Studio comparativo della cromatina sessuale nelle cellule di mucosa orale e nei granulociti durante il periodo neonatale. *Pathologica 59:* 129-135.

Robinson, A., Goad, W.B., Puck, T.T. and Harris, J.S. (1969). Studies on chromosomal non-disjunction in man. III. *Am. J. Hum. Genet. 21:* 466-485.

Robinson, J. (1971). Y chromosome fluorescence in buccal mucosa cells. A study on a newborn population. *Ann. Hum. Genet. 35:* 61-65.

Saldanha, P.H. (1966). Sexo nuclear. *Rev. Paul. Med. 71:* 243-256.

Schetty, K.T., Sharma, W.L. and Wahal, K.M. (1966). Sex chromatin in prednisone-treated children. *Br. Med. J. 2:* 84-86.

Schmidt, M.E., Miller, W.V., Peenen, H.J.V. and Lucas, F.V. (1966). Changes in sex chromatin pattern during the menstrual cycle. Amer. J. Obstet.

Gynecol. *94:* 422-424.

Smith, D.W., Marden, P.M., McDonald, M.J. and Speckhard, M. (1962). Lower incidence of sex chromatin in buccal smears of newborn females. *Pediatrics 30:* 707-711.

Sohval, A.R. and Casselman, W.G.B. (1961). Alteration in size of nuclear sex chromatin mass (Barr body) induced by antibiotics. *Lancet 2:* 1386-1388.

Spasov, S.A. and Dokumov, S.I. (1968). The effect of ACTH and prednisolone F on the nuclear sex chromatin pattern. *Probl. Endokrinol.* (Mosk.) *14:* 19-21.

Taylor, A.I. (1963). Sex chromatin in the newborn. *Lancet 1:* 912-914.

Tonomura, A., Toyofuko, Y. and Matsunuga, E. (1962), apud Mittwoch, U. (1964). Sex chromatin (Review article). *J. Med: Genet. 1:* 50-76.

Towsend, J.F., Case, M.S. and Lucas, F.V. (1970). The sex chromatin count in pregnancy. *Am. J. Obstet. Gynecol. 108:* 585-587.

Van Harnack, G.A. and Strietzel, H.N. (1956). Die Altersabhängigkeit der geschlechtsbedingten Leukocytenmerkmale. *Klin. Wochenschr. 34:* 401-402.

Wegmann, T.G. and Smith D.W. (1964). Lower incidence of sex chromatin in newborn females delivered by cesarean section. *Pediatrics 34:* 419-420.

Weste, S.M., Barnett, J.S., Garson, D.M. and Baikie, A.G. (1967). Variations in the frequency and form of sex chromatin in females with burns. *Lancet 1:* 745-746.

(Received July 28, 1978)

Rev. Brasil. Genet. I, 4, 279-287 (1978)
(Brazil. J. Genetics)

MUTAGENIC ACTION OF INTEGERRIMINE, AN ALKALOID PRESENT IN *SENECIO BRASILIENSIS* (SPRENGEL) LESS., IN *DROSOPHILA**

Ana Lígia de Paula Ramos and
Edmundo Kanan Marques[1]

ABSTRACT

The mutagenic activity of the pyrrolizidine alkaloid integerrimine, extracted from *Senecio brasiliensis* Less. var. *tripartitus*, a very common plant in the south-central region of Brazil, was tested on two strains of *Drosophila melanogaster*, Riverside, California (RC_1) and Columbia (CO_3). Integerrimine concentrations of 0.0025 M, 0.005 M, and 0.01 M produced 7.59, 16.23, and 16.38% sex-linked recessive lethals in the RC_1 strain, respectively, and 10.26, 9.89, and 17.14% in the CO_3 strain. Sex-linked recessive lethals in ultreated controls were 0.19% for RC_1 and 0.28% for CO_3, thus demonstrating the mutagenic activity of integerrimine. The RC_1 strain was more sensitive than the CO_3 strain to the induction of recessive lethals by integerrimine. In the concentration range studied, the dose-effect curve was not linear.

---

* This research was supported by the following grants: FINEP/CNPq/PIG/04/044, FAPERGS 19/78, and 154/78.

[1] Departamento de Genética, Universidade Federal do Rio Grande do Sul. Caixa Postal 1953, 90000 Porto Alegre, RS, Brasil.

INTRODUCTION

The genus *Senecio,* belonging to the family Compositae, contains pyrrolizidine alkaloids (Fig. 1) which are also known as the senecio alkaloids, since they were first isolated from plants of this genus. Later, their presence was demonstrated in plants of other families and genera such as *Crotalaria* and *Heliotropium*.

There are about 128 *Senecio* species in Brazil (Cabrera, 1957) which are mainly distributed in the south-central region of the country. *Senecio brasiliensis* (Sprengel) Less., popularly known as "Maria Mole" (= lazy Mary), is the most common. Although it is difficult to distinguish between its Brazilian varieties *tripartitus* and *brasiliensis* botanically, they can be differentiated on the basis of their alkaloid content. Both varieties contain two alkaloids. Retrorsine, the secondary alkaloid, is common to both. The major alkaloids, however, are different; senecionine is found in *brasiliensis* and integerrimine in *tripartitus* (Motidome and Ferreira, 1966).

The mutagenic activity of some pyrrolidizine alkaloids was first tested by Clark (1959, 1960) in *Drosophila melanogaster* by analysis of sex-linked recessive lethals. Later, further studies were made on *Drosophila* by Clark (1963), Brink (1963, 1966, 1969) and Cook and Holt (1966), and on other organisms by Hirchinson and Hill (1960), Avanzi (1961, 1962), Alderson and Clark (1966), Bick and Jackson (1968), Bick (1970), and Martin et al. (1972). The mutagenicity of some of these alkaloids was confirmed by these investigators.

MATERIAL AND METHODS

Due to the difficulties of botanical differentiation of the *Senecio brasiliensis* Less. varieties, all the collections were made at the same place, in the region called Depressão Central do Rio Grande do Sul (an area in the central part of the State of Rio Grande do Sul, in southern Brazil).

Alkaloids were extracted from the leaves according to the technique of Novelli and Varela (1945) as modified by Motidome and Ferreira (1966). After purification, they were identified on the basis of melting point and

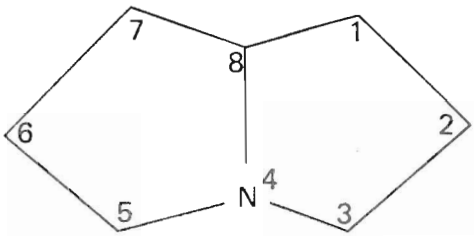

Figure 1 - Chemical structure of the pyrrolizidine ring.

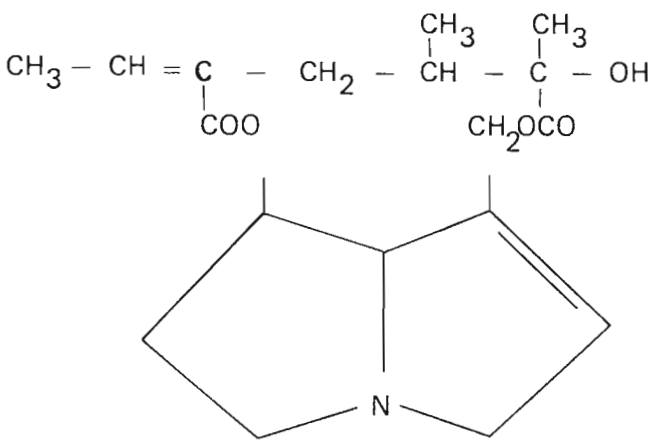

Figure 2 - Chemical structure of the pyrrolizidine alkaloid integerrimine.

infrared spectrum, and by thin-layer chromatography. The identification of the material as *tripartitus* was made on the basis of botanical and chemical data.

Isolated integerrimine (Fig. 2) was dissolved in a minimal volume of 0.1N HCl (Clark, 1963) and added to a sterile solution of 1% sucrose at final concentrations of 0.0025 M, 0.005 M, and 0.01 M. The solution was fed to males of two *Drosophila melanogaster* strains: Riverside, California (RC_1), and Columbia (CO_3). The insects, all of similar age, were allowed to feed on the solution over a period of 24 hours after 12 hours of fasting. The induction of sex-linked recessive lethals was determined by genetic analysis (Muller-5).

RESULTS

The alkaloids extracted from the *Senecio brasiliensis* Less. were chemically identified as retrorsine and integerrimine on the basis of melting point an infrared spectrum, and by thin-layer chromatography.

The percentages of sex-linked recessive lethals induced by 0.0025 M, and 0.01 M integerrimine were 7.59, 16.23, and 16.38% for the RC_1 strain, and 10.26, 9.89, and 17.14% for the CO_3 strain, respectively. The values for the RC_1 and CO_3 strain controls were 0.28 and 0.19%, respectively (Table I).

These data show that the RC_1 strain was more sensitive than the CO_3 strain to the induction of recessive lethals by integerrimine. The maximum effect was obtained with 0.005M in RC_1, while 0.01M was needed to obtain the maximum effect in CO_3. The data were analyzed by linear regression and the t-test was applied to determine if the dose-response curve was linear. The t values obtained for the RC_1 and CO_3 strains, 1.33 and 2.55, respectively, indicate that dose-response curves were non-linear.

DISCUSSION

Taxonomic determination and alkaloid content were used to identify the variety studied as *Senecio brasiliensis* Less. var. *tripartitus*.

Table I - Effect of pure integerrimine extracted from *Senecio brasiliensis* Less. var. *tripartitus* on the induction of sex-linked recessive lethals in two *Drosophila melanogaster* strains.

Strain	Concentration M	Analyzed Chromosomes	Lethals	
			nr.	%
RC_1	0	1568	3	0.19
CO_3		1820	5	0.28
RC_1	0.0025	158	12	7.59
CO_3		273	28	10.26
RC_1	0.005	154	25	16.23
CO_3		182	18	9.89
RC_1	0.01	177	29	16.38
CO_3		175	30	17.14

The comparison between sex-linked recessive lethal percentages induced by integerrimine and those of the controls demonstrated that this alkaloid is mutagenic. The pyrrolizidine alkaloids seem to act inside the cell through their metabolites dihydropyrrolizidine derivatives or "pyrrole derivatives" (Fig. 3) - by alkilation. According to this mechanism (Fahmy and Fahmy, 1959), DNA could be modified at phosphate groups to form semistable triesters, as well as at the nitrogen ring of the bases. The sex-linked recessive lethals observed may be due to single event mutations originating from the reaction of the alkaloids with DNA.

The fact that a linear relationship between dose of integerrimine and induction of lethals was not observed may be due to the selective elimination of the cells more sensitive to the mutagen due to the toxic effects of these

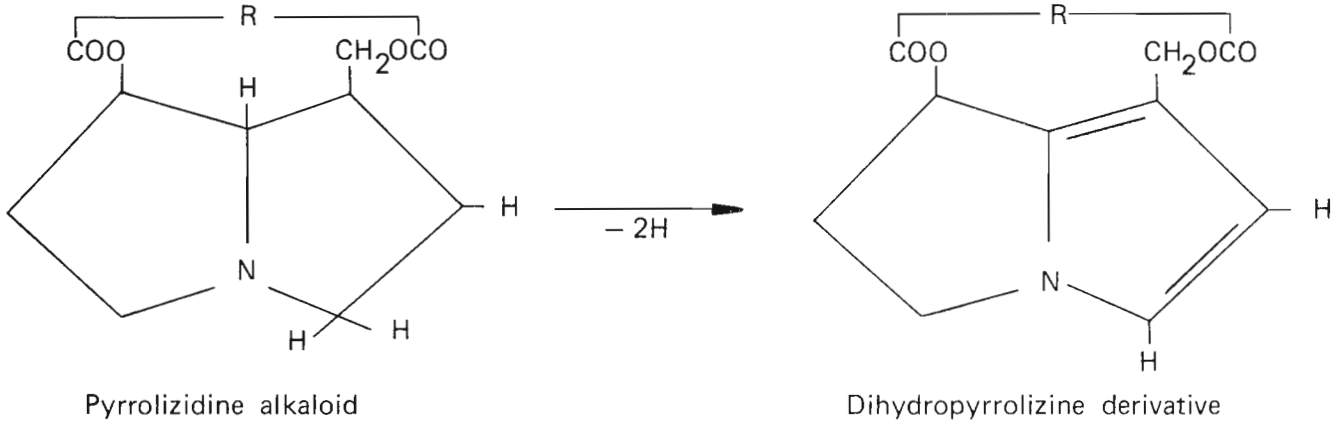

Figure 3 - Dehydrogenation of on unsaturated pyrrolizidine alkaloid to a dihydropyrrolizidine ("pyrrole") derivative (MATTOCKS, 1972).

agents (Fahmy and Fahmy, 1961). Indeed integerrimine satisfies the requirements of a toxic alkaloid suggested by Mattocks (1972).

The difference in sensitivity beyween the RC_1 and CO_3 strains to mutations induced by X-rays, ^{60}Co gamma radiation and alkilating agents such as DES and EMS has been observed by other authors (Marques and Wallace, 1971; Diehl, 1974; Rodrigues, Reguly and Marques, 1975; Andrade, 1976; Reguli and Marques, 1976). Andrade (1976) suggests that this difference in sensitivity is due to the repair mechanism in each strain and is not specific to the mutagen.

Reguly and Marques (1976), when analyzing the action of ^{60}Co gamma radiation and EMS on RC_1 and CO_3 strains pretreated with caffeine, suggested that the resistance of CO_3 as compared to RC_1 was due to a common repair mechanism which occurs at the level of the pre-mutational damage.

ACKNOWLEDGMENTS

We are thankful to Dr. Mario Motidome, Instituto de Química da USP, for the purification and identification of the alkaloids, and to Prof. Maria Luiza Porto, Departamento de Botânica da UFRGS, for the taxonomic determination of the collected variety of *Senecio brasiliensis*.

REFERENCES

Alderson, T., Clark, A.M. (1966). Interlocus specificity for chemical mutagens in *Aspergillus nidulans*. *Nature, 210 (5036):* 593-595.

Andrade, H.H.R. (1976). Efeito conjunto do etilmetanossulfonato e da radiação gama de ^{60}CO na indução de letais em *Drosophila melanogaster*. M. Sc. Dissertation, Curso de Pós-graduação em Genética, Universidade Federal do Rio Grande do Sul, Porto Alegre. 55 pp.

Avanzi, S. (1961). Chromosome breakage by pyrrolizidine alkaloids and modification of the effect by cysteine. *Caryologia, 14(2):* 251-261.

Avanzi, S. (1962). Chromosome breakage in *Vicia faba* by lasiocarpine and monocrotaline. *Caryologia, 15(2):* 351-356.

Bick, Y.A.E. (1970). Comparison of the effects of LSD, heliotrine and X-irradiation on chromosome breakage, and the effect of LSD on the rate of cell division. *Nature, 226:* 1165-1167.

Bick, Y.A.E., and Jackson, W.D. (1968). Effects of the pyrrolizidine alkaloid heliotrine on cell division and chromosome breakage in culture of leucocytes from the marsupial *Potorous tridactylus. Aust. J. Biol. Sci., 21:* 469-481.

Brink, N.G. (1963). The effect of cyanide and azide on the mutagenic activity of pyrrolizidine alkaloid heliotrine in *Drosophila melanogaster. Z. Vererbungsl., 94:* 331-335.

Brink, N.G. (1966). The mutagenic activity of heliotrine in *Drosophila.* I. Complete and mosaic sex-linked lethals. *Mutat. Res., 3:* 66-72.

Brink, N.G. (1969). The mutagenic activity of pyrrolizidine alkaloid heliotrine in *Drosophila melanogaster. Mutat. Res., 8:* 139-146.

Cabrera, A.L. (1957). El género *Senecio* (Compositae) en Brasil, Paraguay y Uruguay. *Arq. Jard. Bot., XV (163):* 227-230.

Clark, A.M. (1959). Mutagenic activity of the alkaloid heliotrine in *Drosophila. Nature, 183:* 731-732.

Clark, A.M. (1960). The mutagenic activity of some pyrrolizidine alkaloids in *Drosophila. Z. Vererbungel., 91 (4663):* 74-80.

Clark, A.M. (1963). The brood pattern of sensitivity of the *Drosophila* testis to the mutagenic action of heliotrine. *Z. Vererbungsl., 94:* 115-120.

Cook, L.M., and Holt, A.C.E. (1966). Mutagenic activity in Drosophila of two pyrrolizidine alkaloids. *J. Genet., 59:* 273-274.

Diehl, E.M. (1974). Estudo comparativo da mutabilidade induzida em linhagens radioresistentes de *Drosophila melanogaster.* M. Sc. Dissertation, Curso de Pós-graduação em Genética da Universidade Federal do Rio Grande do Sul, Porto Alegre. V + 52 pp.

Fahmy, O.G., and Fahmy, M.J. (1959). Differential gene response to mutagens in *Drosophila melanogaster. Genetics, 44:* 1149-1171.

Fahmy, O.G., and Fahmy, M.J. (1961). Cytogenetic analysis of the action of carcinogens and tumour inhibitors in *Drosophila melanogaster.* XI. Mutagenic efficiency of the mesiloxy esters on the sperm in relation to molecular structure. *Genetics, 46:* 1111-1123.

Hirchinson, V., and Hill, K.R. (1960). The effects of a *Senecio* alkaloid (monocrotaline) on human embryo liver in tissue culture. *Br. J. Cancer, 14:* 637-646.

Marques, E.K., and Wallace, B. (1971). Indução e seleção de linhagens de *Drosophila melanogaster* resistentes à ação dos raios X. *Cienc. Cult. (São Paulo), 23* (Suplemento): 108-109.

Martin, P.A., Thorburn, M.J., Hutchinson, S., Bras, G., and Miller, C.G. (1972). Preliminary findings of chromosomal studies on rats and humans with veno-occlusive disease. *Br. J. Exp. Pathol. 53(4):* 374-380.

Mattocks, A.R. (1972). Toxicity and metabolism of *Senecio* alkaloids. In: *Phytochemical Ecology* (Harborne, J.B., ed.), Academic Press, London and New York, pp. 179-200.

Motidome, M., and Ferreira, P.C. (1966). Alcalóides do *Senecio brasiliensis* Less. *Rev. Fac. Farm. Bioquím. Univ. São Paulo, 4(1):* 13-44.

Novelli, A., and Varela, A.P.G. (1945). Alcaloides de *Senecio* en la Argentina. *Anales Asoc. Quim. Argentina,* Argentina, *33:* 176-180.

Reguly, M.L., and Marques, E.K. (1976). Radio-resistência e radio-sensibilidade em *Drosophila melanogaster.* Efeito da cafeína. *Cienc. Cult. (São Paulo), 28* (Suplemento): 313.

Rodrigues, H.H.E., Reguly, M.L., and Marques, E.K. (1975). Efeito comparativo das radiações ionizantes e mutagênicos químicos no mecanismo da resistência à mutação. *Cienc. Cult. (São Paulo). 27* (Suplemento): 255.

(Received October 13, 1978)