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CYTOGENETIC STUDIES IN DIFFERENT TYPES OF APLASTIC ANEMIA*

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ABSTRACT

Chromosome studies were done in patients with aplastic anemias of primary (Fanconi and Blackfan-Diamond syndrome), secondary (chloramphenicol) and idiopathic types. Non-random distribution of chromosome breaks was found in the Fanconi and Blackfan-Diamond syndromes. Chromosomes were apparently normal in the secondary and idiopathic types. The chromatid breaks were found to be distributed in a non-random manner and with different patterns not only according to the condition but also according to the cell line, with groups A and B having more and the G group fewer breaks than expected in both conditions. In the Fanconi syndrome the D group has more and the E group fewer breaks in lymphocytes. In the Blackfan-Diamond syndrome the D group has fewer breaks than expected.

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INTRODUCTION

Chomosome studies have been performed most commonly in the "constitutional" aplastic anemias, i.e. the Fanconi and Blackfan-Diamond syndromes (Schmid, 1967; Bloom, 1966), less commonly in the secondary and idiopathic aplastic anemias (Cobo *et al.* , 1970). This paper presents results of cytogenetic studies in 10 patients with various forms of aplastic anemia.

MATERIALS AND METHODS

The Kiossoglou method (Kiossoglou *et al.*, 1964) was used to study bone marrow chromosomes. The marrow sample was incubated for 2 hours with colchicine at 37° C, then processed by the air drying technique. The Moorhead method (Moorhead *et al.*, 1960) was used for culturing peripheral lymphocytes. Fibroblasts from bone marrow particles were grown in T.C. 199 and subcultured (de la Chapelle *et al.*, 1973). In each case, the cells were incubated with colchicine 2 hours prior to the end of culture and chromosome preparations were obtained by hypotonic-air drying and staining with Giemsa in phosphate buffer 1:50. The number of cells per volume was adjusted to 10,000 per 0.1 ml. The preparations were scored for numerical chromosome aberrations and for chromosome breaks and gaps.

PATIENTS

All patients were studied at the Antioquia University Hospitals, Medellin, Colombia, upon their first return visit after diagnosis of aplastic anemia had been made; some of the clinical and therapeutic data have been previously published (Restrepo *et al.*, 1968).

Patient 1 (JEC, Nº 344590) was a 27-year-old, married but childless man with non-contributory family history who had been diagnosed as having the Fanconi anemia syndrome (FAS) on the basis of epistaxis, hematuria, petechiae, ecchymoses, and hematomata after minor injuries, shortness of stature (151 cm, $<$ 3rd percentile), brachycephaly, " widow's peak", small

nose, micrognathia, multiple café-au-lait spots, moderate pectus excavatum, hypogenitalism, hypoplastic left radius and ulna, and left equinovarus foot deformity; Hgb of 5.5 gm/100 ml, 3100 WBC/mm^3 with severe neutropenia and marked lymphocytosis, 85,000 platelets/mm^3; severe bone marrow failure, replacement of hematopoietic marrow elements by lymphocytes without evidence of myelofibrosis. Results of analysis of 36 marrow metaphases,70 lymphocytes and 23 fibroblasts are listed in Table I. This patient died after 2 months of hospitalization.

Patient 2 (EFG), a 7-year-old boy with unremarkable family history was diagnosed to have Blackfan-Diamond anemia (BDA) on the basis of apparently primary congenital anemia with secondary manifestations, a height less than the 3rd percentile, Hgb 6.5 gm/100 ml, 1,500,000 RBC, 310,000 platelets and 7,500 WBC/mm^3 and a differential white blood cell count of 49% neutrophils, 45% lymphocytes, 5% basophils and 1% eosinophils. The bone marrow manifested severe red cell failure but was otherwise normal. The patient did not respond to anti-anemia therapy and continued to require blood transfusions. The results of studies performed on 75 marrow cells and 100 lymphocytes are listed in Table I. The patient is alive and well.

Patients 3-5 were women who developed aplastic anemia 3 to 16 weeks after treatment with 2-8 gm of chloramphenicol. Metrorrhagia and purpura appeared one to 3 weeks before hospitalization at which time the women had developed anemia (mean Hbg 4.5 gm, range 3.5-5.6 gm/100 ml), neutropenia, thrombocytopenia with lymphocytosis, and bone marrow failure. Other physical and laboratory examinations were normal and the women were all fertile. The results of the cytogenetic studies on the bone marrow cells, lymphocytes and fibroblasts are cited in Table II.

Patients 6-10 were 2 men and 3 women (4 of them married with normal offspring), ranging in age from 22 to 48 years (mean age 33) with a negative history for use of or exposure to drugs or chemicals for at least two years before the onset of manifestations of the disease. These individuals had an otherwise normal physical and laboratory examination, a non-contributory family history and, thus, are considered to have "idiopathic" aplastic anemia. The results of the studies on their marrow cells are summarized in Table II. The death of all the patients with secondary and idiopathic aplastic anemia was due to hemorrhage and infection.The mean survival time was 7.8(s.d. 5.02) months after establishing the diagnosis.

Table 1 - Cytogenetic characteristics of three cell lines from patients with Fanconi and Blackfan-Diamond syndromes

	BONE MARROW									LYMPHOCYTES									FIBROBLASTS								
			Chromatid Aberrations per Chromosome Group									Chromatid Aberrations per Chromosome Group									Chromatid Aberrations per Chromosome Group								
	Karyotype	Total	A	B	C	D	E	F	G	Karyotype	Total	A	B	C	D	E	F	G	Karyotype	Total	A	B	C	D	E	F	G
Fanconi																											
2	47,XY (C+)		3	2	5	2	1	1	0	46,XY		120	65	142	37	10	10	1	46,XY		27	13	15	1	1	2	0
30	46,XY		32	18	20	1	3	1	0																		
1	46,XY,(C−)		3	2	9	3	1	1	0																		
1	45,XY,(D−)		4	3	8	2	1	1	0																		
Observed 36 cells		127	42	25	42	8	6	4	0	70	385	120	65	142	37	10	10	1	23	59	27	13	15	1	1	2	0
Expected 500 cells		13	*30	16	46	13	11	6	5	500	13	*91	46	139	39	34	18	18	500	13	*14	7	21	6	5	3	3
X^2 (d.f.6)		999	4.8	5	0.3	1.9	2.2	0.6	5		10.644	9.2	7.8	0.06	0.1	16	3.5	16		455.06	12	5.1	1.7	4.1	3.2	0.33	3
Blackfan-Diamond																											
5	47,XY,(C+)		1	0	2	0	0	0	0	46,XY		218	124	270	74	18	21	1									
66	46,XY		80	37	148	10	7	13	0																		
2	45,XY,(C−)		2	3	5	2	1	1	0																		
2	45,XY,(D−)		1	0	3	3	0	0	0																		
Observed 75 cells		319	84	40	158	15	8	14	0	100	726	218	124	270	74	18	21	1	58	196	67	40	58	15	8	8	0
Expected 500 cells		13	*75	40	115	33	28	15	11	500	13	*172	89	263	74	64	34	30	500	13	*46	24	71	20	17	9	9
X^2 (d.f.6)		7202	1	0	16	9.8	14	0.07	11		39.105	12.3	13	0.1	0	33	4.9	28		390.7	959	10.67	2.3	1.25	4.76	0.11	9

*The total observed breaks were distributed as if they occurred randomly per unit of length.

Table II - Cytogenetic observations in Aplastic Anemia secondary to chloramphenicol and in idiopathic aplastic anemia

Patient Number and Diagnosis	Number of Metaphases Analyzed			Total Chromatid Aberrations			Karyotypes			
	BM*	L*	F*	BM	L	F		BM %	L %	F %
Secondary to Chloramphenicol										
159	17	100	20	3	0	1	46,XX	95	100	100
							45,XX,(D–1)	5		
155	32	100	19	0	0	0	46,XX	94	100	100
							45,XX,(C–)	6		
151	32	100	23	1	1	1	46,XX	100	100	100
Idiopathic										
089	15	100	16	0	0	0	46,XX	100	100	100
094	35	100	25	1	3	1	46,XX	100	100	100
127	11	57	8	0	0	0	46,XY	91	100	100
							45,XY,(C–)	9		
134	7	28	9	0	0	1	46,XY	100	100	100
130	14	24	13	0	1	0	46,XY	100	100	100
Normal Mean Values/100 metaphases				3	7	3				
Secondary Anemia Chloramphenicol										
Mean Values	27		20.6	1.3	0.3	0.6	46,XX	96.3	100	100
Standard deviation	8.6		2.8	1.53	0.58	0.58		3.21	0	0
χ^2	197.37		306.4	2.22	149.	9.6		0.02	0.45	0.01
Idiopathic Anemia										
Mean Values							45,XX (D–C–)	4.7		
Females	20	100	16.3	0.5	1.5	0.5	46,XX	100	100	100
Males	14	36	10	0	0.3	0.3	46,XY	97	100	100
Standard Deviation M & F	10.85	37.12	6.83	0.45	1.3	0.55		5.2	0	0
χ^2										
Females-Normal	64	0	70	2.01	4.3	2.0				
Males-Normal	73	40	81	0	6.4	2.4				
Females-Males	25	113	3	0	4.8	0.13				

*Respectively, bone marrow, lymphocytes, fibroblasts

RESULTS

A. The *mitotic rate* was significantly reduced ($p < 0.005$) for all cell types in the FA and BDA syndromes – more so in the former than in the latter. Also it was lower ($p < 0.0005$) in the bone marrow-derived fibroblasts

of the patients who received chloramphenicol. It was normal in lymphocytes of females but not of males with idiopathic aplastic anemia.

B. *Chromatid breaks* were essentially absent in the 3 cell types from the patients with idiopathic and secondary aplastic anemia. The mean number of chromatid breaks in the FA patient was 169.8×10^{-4} which is significantly ($p < 0.0005$) increased above the normal of 1.127×10^{-4} per unit length (the total length is 211.75 and 207.75 for female and male karyotypes, respectively (Bergsma *et al.*, 1966). The number of chromatid breaks in the BDA patient was 204.7×10^{-4} which is also significantly increased ($p < 0.0005$).

Table III- Breaks per group recorded only when results were significant ($p < 0.05$) when compared with expected number of breaks occurring randomly per unit of length.

Diagnosis	Cell Type	A	B	C	D	E	F	G
Fanconi	Bone Marrow							
	Lymphocytes	M*	M		M	L		L
	Fibroblasts	M	M		M			
Blackfan Diamond	Bone Marrow			M	L	L		L
	Lymphocytes	M	M		L			L
	Fibroblasts	M	M		L			L

*M = increased above, or L, decreased below expected values.

Table III summarizes the significance calculations on the chromatid breaks according to chromosome group (A-G); these are cited as more (M) or less (L) frequent than expected breaks per unit length, and are recorded when the probability was $p < 0.05$. Note that the number of chromatid breaks per group was not significantly different in FA marrow cells, but is was significantly increased in FA and BDA lymphocytes and bone marrow fibroblasts for groups A and B, and significantly decreased for groups E and G. The distribution of chromatid breaks per group of FA fibroblasts differed from that of BDA fibroblasts: FA fibroblasts showed a greater than expected number of breaks in groups A,B and D, and BDA fibroblasts showed an increased number in group C and a decreased number in groups D,E and G.

C. *Chromosome breaks.* No chromosome breaks were observed in

the idiopathic or in the secondary aplastic anemia cases. The total number of chromosome breaks was significantly ($p < 0.0005$) increased in all 3 FA cell lines and in the BDA patient. In the FA cells the distribution of chromosome breaks by chromosome group was not significant. The mean number of breaks per cell was 4.01, 4.27 and 3.14 for FA bone marrow cells, lymphocytes, and fibroblasts, respectively; these differences are not significant. However, when we compared chromosome breaks in BDA bone marrow cells according to chromosome group, breakage was found with an increased frequency in group C, and a decreased frequency in groups D,E and G. BDA lymphocytes and fibroblasts showed significantly more ($p < 0.0005$) breaks in groups A and B, fewer ($p < 0.0005$) in groups E and G, and a non-significant distribution of breaks in the other groups. The number of chromosome aberrations in the secondary and idiopathic anemia groups was not significantly different from normal.

DISCUSSION

The various aplastic anemias have certain clinical factors in common, but our results and those of others suggest not only means of distinguishing them on subclinical levels but also differences in the pathogenetic events. We have performed cytogenetic and cell genetic studies on patients with genetic (Fanconi syndrome, Blackfan-Diamond syndrome), idiopathic and secondary (chloramphenicol) forms of aplastic anemias. Such studies are complicated by the relatively nonproliferative character of cells from these conditions. We like to discuss the aspects concerning each type of condition individually.

The Fanconi Anemia Syndrome: This is an autosomal recessive multiple malformation syndrome, characterized by absence or hypoplasia of thumbs and radii, short stature, eye anomalies, microcephaly with or without mental retardation, renal anomalies, cryptorchidism, hypogenitalism and a pigmentary dysplasia. Bone marrow failure with pancytopenia is thought to eventually occur in all homozygotes. Schroeder *et al.*, (1964) discovered a generally increased frequency of chromosome aberrations in this condition, an observation which has been amply confirmed. We studied direct preparations of bone marrow cells and proliferative cultures of lymphocytes and bone marrow fibroblasts and evaluated chromosome and chromatid aberrations by chromo-

some group (A-G) for each cell type. We confirmed the increased incidence of chromatid aberrations, ranging from prominent secondary constrictions to gaps and breaks. Counting of these abnormalities in cells from direct bone marrow preparations was difficult because of the reduced number of metaphases. In fibroblasts and lymphocytes we saw an increased number of chromatid breaks and gaps; rearrangements, although present, were not as frequent as they are reported in other "chromosome breakage syndromes". These observations in general confirm those of others. Recently, Bushkell *et al.*, (1976) found no significant difference in breakage rates in T and B lymphocytes of three FA patients. In cultured lymphocytes, Schroeder and German (1974) found the number of chromatid abnormalities per chromosome group increased in group A (chromosomes 1 and 2 only), B,C and D, and decreased in the other groups. Ayme *et al.*, (1976) studied by banding techniques the breakpoint distribution in lymphocytes from normal subjects and from patients with certain conditions, including the FA syndrome. They found the number of breaks increased for chromosomes 3, 7, 9, 11, 14, 16, X and decreased for all the other chromosomes. Von Koskull and Aula (1973) found a non random distribution of breaks in FA, with excess in chromosomes 1, 2, 3, 6 and 13 and deficit in the gonosomes. Our finding of an increased number of non random breaks in lymphocytes cultured from peripheral blood of FA patients is similar to that described by Schroeder and German (1974). Distribution of breaks in groups E and G showed an increased total number; their distribution was random in bone marrow and fibrolasts, but non-random and less than expected in lymphocytes.

Blackfan-Diamond Syndrome. This condition is also known as congenital hypoplastic anemia, chronic congenital aregenerative anemia, erythrogenesis imperfecta, or pure red cell hypoplasia. It is an autosomal recessive condition without associated congenital malformations. At times, the name, Blackfan Diamond Anemia, is also used to designate different and atypical conditions (Hoffman *et al.*, 1976). We believe that our patient has typical BDA. Hirschman *et al.*, (1969) previously reported for the BDA an increased number of chromatid breaks with a non-randon distribution pattern in some of the chromosome groups of the three cell lines studied.

In view of these results, we think that this type of BDA syndrome should also be counted among the chromosome breakage syndromes. The congenital hypoplastic anemias are etiologically and pathogenetically hetero-

geneous, and not all of them are associated with increased chromosome breakage. A similar observation had been made with respect to the FA syndrome and "related cases": collected data can be shown to consist of the autosomal recessive FA syndrome with increased chromosome breakage and an autosomal dominant entity, the *WT* syndrome (Gonzalez *et al.*, 1976) of spontaneously remitting aplastic anemia, radial *and* ulnar hypoplasia, without the other manifestations of the FA syndrome and without chromosome/chromatid breakage; *WT* heterozygotes seem to be at an increased risk of developing leukemia.

The distribution of the chromatid breaks in BDA lymphocytes and fibroblasts was similar to that of FA lymphocytes and fibroblasts in that groups A and B had more breaks than expected, but differed from that of FA bone marrow cells in that more breaks were seen in group C and fewer in groups D,E and G (Table III).

We think that the increased chromosome breakage in FA and BDA represents the accumulation of environmentally induced lesions which are inefficiently repaired. Variation in the monthly distribution of breaks in normal chromosomes has been shown (Ayme *et al.*, 1976). The preferential accumulation or absence is probably due to the non-randomness of the breakage which is related to the number of "clear bands" and probably to different types of hetero- and euchromatin (Ayme *et al.*, 1976; Nakagome and Chiyo, 1976). Most chromosome rearrangements are lethal to the cell during mitosis because of loss of chromosome material. However, some of these cells survive and give rise to new clones of abnormal cells, some of them gaining extra, normal chromosomes and some containing or gaining abnormal marker chromosomes arising from translocations, deletions, etc. The clonal proliferation described in these conditions (Berger *et al.*, 1975; Lisker and Cobo, 1974) may be related pathogenetically to the frequent occurrence of neoplasia in the chromosome breakage syndromes, most of which also share abnormalities of the repair mechanisms (ataxia telangiectasia, Bloom's syndrome and xeroderma pigmentosum).

An unusual susceptibility to chromosome breakage in FA syndrome cells exposed to radiation has been noted (Remsen and Cerutti, 1976; Sasaki, 1975). These observations suggest that the breakage we are observing in the cells of these patients probably represents the accumulation of environmentally produced chromatid lesions in a more susceptible type of cell, in

which the repair mechanism is deficient against X-rays, gamma and UV radiation. It is of interest that the accumulation (distribution) of the breaks does not occur in a random manner, but follows patterns more or less characteristic for the type of cell and the disease.

Idiopathic and Secondary Aplastic Anemia: This is an etiologically and pathogenetically heterogeneous group of conditions. Some are due to chemicals such as chloramphenicol, in others no distinct cause can be identified. There have been some reports (Cobo *et al.*, 1970) of chromosome gains and losses in differents types of acquired aplastic anemias involving different chromosome groups, but in our 5 patients with idiopathic aplastic anemia and 3 with a history of recent chloramphenicol treatment we found no significant chromosome abnormalities. The only significant finding in our patients was the significantly reduced mitotic rate of cells from direct bone marrow preparations and of cultured fibroblasts, but not of cultured lymphocytes. However, the patients died soon after these studies were initiated so that we cannot rule out the possibility of later evolution of cell lines with different chromosome abnormalities in some long-term survivors with the BDA syndrome.

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GENETIC COUSELING IN TWO CASES OF POSSIBLE ABO INCOMPATIBILITY

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ABSTRACT

The serum of two O Rh-positive women with a possible history of mother-fetus ABO incompatibility was tested for immune anti-A IgG for the purpose of genetic counseling. Immune anti-A IgG was demonstrated in the serum of both women by titration with IgG antibodies after the separation of IgM and IgG antibodies by treatment with 2-mercaptoethanol. The risk for the next child of each of these mothers to be affected by hemolytic disease of the newborn was calculated to be about 58% on the basis of A and O gene frequencies in the population of São Paulo.

INTRODUCTION

Maternal-fetal ABO incompatibility generally occurs between O-type mothers and A-type children (Rosenfield, 1955; Munk-Anderson, 1957) and

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is difficult to detect at birth because, contrary to what happens with Rh incompatibility, the indirect Coombs test on umbilical cord blood does not give positive results (McNeil *et al.*, 1955). It has been well established that damage to the fetus is not caused by natural antibodies of the IgM type, but rather by immune antibodies of the IgG type which can cross the placental barrier more easily. The mother's immunization may be due to soluble fetal antigens already present in six-week embryos, which cause early abortion (Zuelzer and Kaplan, 1954).

The presence of immune anti-A in the serum of a pregnant woman does not always indicate maternal-fetal incompatibility since the antibody may be caused by other stimuli such as anti-tetanus vaccine, or may even arise without any apparent stimuli (Mollison, 1972). Contrary to Rh incompatibility, damage to the fetus commonly occurs during the first incompatible pregnancy, which may lead to habitual abortion or even to sterility. Abortions due to ABO incompatibility tend to occur earlier (up to the twentieth week of gestation) than those provoked by Rh incompatibility (Newcombe, 1965). Studies by different authors such as Rosenfield (1955), Ames and Loyd (1964) and Voak and Bowley (1965), have shown that most cases of maternal-fetal ABO incompatibility in which the fetus reaches full term are mild. In Caucasian populations, 1 in 150 newborns develops jaundice, but only 1 in 3000 develops anemia which is serious enough to require treatment. Studies of South African Negro populations by Brink (1969), Ford (1969) and Farrell (1970) have demonstrated that hemolytic disease of the newborn due to ABO incompatibility is two to three times more common in black than in white newborns. These data were confirmed by Bucher *et al.* (1976) for Negro populations in North Carolina (USA). The seriousness of the disease, however, does not differ between the two groups.

In families where hemolytic disease of the newborn occurs due to ABO incompatibility, an affected child may be followed by another who, although ABO-incompatible, is not affected. This, however, applies only to mild cases since the subsequent children of a mother who gives birth to a severely affected child will also be affected if they belong to the same blood group (Mollison, 1972). As a confirmation of these data, both the size of the offspring an the number of group-A childrem have been found to be lower, on the average, in marriages between A men and O women than in marriages

between O men and A women (Allan, 1953). This lower number may be due to abortion, neonatal deaths or infertility.

In this paper we describe the use of anti-A IgG measurements as an aid in counseling two couples with a possible history of maternal-fetal ABO incompatibility.

MATERIAL AND METHODS

The following gestational histories describe two Caucasian couples who came to us seeking genetic counseling.

Couple Nº 1. The first child was born at term and exhibited early intense jaundice. Treatment by transfusion and phototherapy was successful. The child, now 11 years old, does not show any sequelae. The second pregnancy terminated in abortion at 3 months. The third pregnancy occurred only after 10 years even though no contraceptives were used during this period. The child, born by normal delivery, was a seven month premature boy with intense early jaundice which required phototherapy and three transfusions, and with development of hypertonia. The child died of bronchial pneumonia at 39 days of life.

Couple Nº 2. The first child died during the first week of life, remaining jaundiced despite specific treatment. The second pregnancy terminated in abortion at 5 months. The second surviving child also exhibited intense early jaundice which was treated. Now 2 years old, the child is severely mentally retarded, exhibiting hypertonia, hyperreflexia, stereotyped movements, especially of the face, and marked motor retardation. He does not walk or speak and has strabismus.

The following examinations were performed: the surviving child of couple Nº 1 was tested for inborn errors of carbohydrate metabolism, more specifically for G-6-PD enzyme deficiency and galactosemia, which may have caused her jaundice . Maternal-fetal ABO incompatibility was suspected since no abnormality was found and because jaundice had appeared during the first pregnancy although the mother had received no blood transfusions.

The surviving child of couple Nº 2 was karyotyped in addition to being tested for errors of carbohydrate metabolism. Since no abnormality was

found, and for the same reasons mentioned above, ABO incompatibility was suspected. The red cell ABO antigens were tested with anti-A and Anti-B sera (Johnson & Johnson) and with anti-A_1 lectin from *Dolichos biflorus*. The inverse tests, investigation of anti-A and anti-B agglutinins and determination of ABH substances in the saliva, were carried out to confirm blood groups. The Rh factor was analyzed with an anti-Rh serum (anti-D, 85 %. Johnson & Johnson). Qualitative investigation of anti-A immune antibodies in the maternal serum was carried out by the partial neutralization test with substance A, followed by the indirect Coombs test (Dunsford and Bowley, 1967, p. 377). The immune anti-A antibodies were titrated in the maternal sera with A_1 red cells in saline medium at 37° C, and titration was continued with the indirect Coombs test starting from the last tube in which no agglutination occurred. This procedure made it possible to observe the relative contents of immune antibodies with respect to saline or natural antibodies. The sera were also titrated in buffered saline medium at 37° C and by the indirect Coombs test after fractionation with 2-Me, according to Reesink *et al.*(1972). This technique makes it possible to estimate the drug-sensitive and drug-resistant fractions which correlate appreciably with IgM and IgG immunoglobulins, respectively.

RESULTS AND DISCUSSION

Couple Nº 1 consisted of a O, Rh-positive mother and an A_1, Rh-positive father. The child was A_1, Rh-positive. Couple Nº 2 consisted of a O, Rh-positive mother and an A_1, Rh-positive father. The child was A_1, Rh-negative. Thus the possibility of Rh incompatibility was discarded. However, the ABO blood groups and the high titres of maternal anti-A antibodies, both saline and non-saline (Table I) led us to assume the presence of maternal-fetal incompatiblity.

In view of the mother's gestational histories, the high titres of immune antibodies found in their sera, and because no data for weight, reticulocyte count, hemoglobin concentration, microspherocytosis or plasma bilirubin concentrations for the neonatal period were available to us, we opted for the most probable diagnosis. Voak and Bowley (1969) have stated

that, in the absence of serologic data for the newborn and when antibodies are present on the surface of the newborn's red cells, the presence of high IgG titres in the maternal serum (higher than 256) offers good evidence that the child suffers from hemolytic disease of the newborn.

Table I - Immune anti-H in the maternal sera and serum titration before and after 2-mercaptoethanol treatment.

Examinations performed	Mother (couple nº 1)	Mother (couple nº 2)
Indirect Coombs test after partial neutralization	++++	++++
Serum titration in saline medium (temperature 37°C)	1: 2048	1: 4096
Serum titration by the indirect Coombs test	1: 4096	1: 8192
Serum titration after treatment with 2-Me	1: 64	1: 64
Serum titration after treatment with 2-Me by the indirect Coombs test	1: 1024	1: 256

Saline and non-saline anti-A titres were very high in both cases. Stratton and Renton (1958) have stated that a titre higher than 1:512 in saline medium indicates presence of immune antibodies. According to Polley *et al.* (1965), IgG titres of 64 or less indicate maternal non-immunization, and titres higher than 256 indicate that the child was probably affected. Titres of more than 1000 are usually associated with moderate or severe disease. These

authors separated IgG from IgM by the method of partial neutralization with human substance A, followed by and indirect IgG antiglobulin test.

The non-saline antibody titres in the present study, significantly higher than the saline ones, and the presence of an elevated proportion of 2-mercaptoethanol-resistant agglutinins indicate that the hemolytic disease of the newborn was caused by maternal-fetal ABO incompatibility. The two couples were genetically counseled with an explanation for the cause of their unsuccessful reproductive attempts and of the risk to have new gestations terminate in abortion or produce brain-damaged children.

Since the blood groups of the couples' parents were not known, the risk was calculated by evaluating the probability of the father being homozygous (AA) or heterozygous (AO). The frequencies of A and O genes in the Caucasian São Paulo population are $p = 0.271$ and $r = 0.661$, respectively (Saldanha, 1976). Thus the frequency of AA homozygotes in the population is $p^2 = 7.3\%$, and of AO heterozygotes is $2pr = 35.8\%$. By normalizing, the probability of the father being homozygous AA is $0.073/(0.073 + 0.358)$, or 17%; and of being heterozygous AO is 1-0.17, or 83%. If the father is homozygous, the next child will have a probability of 1.0 (certainty) of having group-A blood, while if the father is heterozygous, this probability will be 0.5. By composing the two probabilities (using the 1.0 and 0.5 weights) we conclude that the total probability for the child to be group A is $1 \times 0.17 + 0.5 \times 0.83 = 0.58$. Since the mother is already immune, the probability of occurrence of hemolytic disease in the next child of A blood group is very high; thus the risk of having a new pregnancy terminate in abortion or produce an affected child is almost 58 %.

The couples were also advised that if, despite the risks, they should opt for a new pregnancy, the child should be delivered in a hospital equipped with the appropriate facilities to take care of the problem if it should arise.

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FACTORS AFFECTING MILK PRODUCTION OF PITANGUEIRAS CATTLE IN BRAZIL

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ABSTRACT

Lactation milk yields were recorded for 13 years (1962 to 1974) on 483 Pitangueiras cows with 1,723 lactations, maintained at Tres Barras farm, in the State of São Paulo, Brazil. The mean milk yield for the first lactation was 2556 ± 32 kg with a coefficient of variation of 27.6%. Maximum production occurred in lactation five with an increase of 22.6% over the first lactation. Statistical analysis was by method of least squares analysis of variance. Generation, age of cow and year and season of calving had significant effects ($P < .05$) on milk yields. There appeared to be some loss (362 to 610 kg, depending on age) of heterosis following *inter se* mating of first generation animals. Yields increased with advancing age of cow, reaching a maximum between 66 and 90 months. Cows freshening in the dry season (April to September) yielded 50 to 134 kg more than those freshening in the rainy season, doubtless due to concentrate supplementation during the dry season. Production fluctuated from year to year as expected, but slight declines occurred during 1972 to 1974. The results overall were very similar to those obtained from research with European breeds in temperate areas.

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INTRODUCTION

Milk yield is the most important single determinant of profit for the dairy cow. Effects of lactation number, age, and season and year of calving on yield are well known. However, little information is available about influences of these factors on new breeds developed from crossbred foundation *Bos taurus* x *Bos indicus*. The present investigation was undertaken to determine the effects of these factors on milk yield in a new tropical Brazilian Breed, the Pitangueiras.

MATERIALS AND METHODS

Animals and Management

Data were collected over 13 years (1962 through 1974) from records on 483 Pitangueiras cows (5/8 Red Poll x 3/8 Zebu) with 1,723 lactations, maintained at Tres Barras farm, in the State of São Paulo, Brazil. This farm has a cultivated area of 6,534 hectares, 2094 of which are planted with colonial grass (*Panicum maximum,* L.) and "pangola" (*Digitaria decumbens,* L.)

The farm is located at an altitude of 503m, 21°00' latitude South, and 48° 41' longitude West of Greenwich. The climate is tropical-humid, of the AW type in the Koeppen classification, with dry winters. The mean atmospheric temperature in this area varies from a maximum of 31.9° C to a minimum of 16.2° C, with an annual average of 24.0 ° C. Annual precipitation is about 1,346 mm, distributed over two different periods of the year, with 1,157 mm from October to March, and 189 mm from April to September (Table I).

The cows were maintained on pasture the year around. When pasture was plentiful, the herd did not receive any supplement; however, during the dry winter months (April to September), each lactating cow received daily 1 kg concentrate (16% crude protein) per 3 to 4 kg milk produced. In addition, from June to October, each cow also received daily 1 kg molasses enriched with urea. The health program of the herd involved systematic vaccination against symptomatic carbuncle, brucellosis and foot-and-mouth disease.

Diagnostic tests for brucellosis and tuberculosis were performed on the heifers once every 6 months, and the animals with positive reactions were eliminated. Calves were submitted to periodic analysis of the feces for parasites.

Statistical Analysis

Effects of generation (g_i), age of cow (a_j), season (s_k), and year (p_l) were estimated by least squares analysis of variance (Harvey, 1960), according to the following model:

$$Y_{ijklm} = u + g_i + a_j + s_k + p_l + e_{ijklm}$$

where Y_{ijklm} = lactation milk yield; u = least squares mean; g_i = effect of

Table I. Mean monthly temperatures and total monthly rainfall at Tres Barras farm, Pitangueiras.

Months	Temperature			Total Rainfall (mm)
	Minimum	Maximum	Mean	
January	19.9	32.2	26.1	241.7
February	20.1	32.8	26.4	188.4
March	19.2	33.4	26.3	145.5
April	16.7	32.8	24.7	46.7
May	13.3	30.8	22.0	28.3
June	11.7	29.5	20.6	28.7
July	10.7	29.6	20.1	29.5
August	12.1	31.6	21.8	15.3
September	14.9	33.7	24.3	40.5
October	17.5	32.4	25.0	142.2
November	18.4	32.7	25.5	141.0
December	19.4	32.0	25.07	298.2
General mean	16.2	31.9	24.0	1346.0

the ith generation (i = 1,2); a_j = effect of the jth age (j = 1,2, . . . , 4); s_k = effect of the kth season (k = 1, 2); p_l = effect of the lth year (1 = 1, 2, . . . 13); and e_{ijklm} is residual within-animal error variance. All effects except error were assumed to be fixed. The usual assumptions about the distribution of the e's were made.Months of calving were divided into seasons based on climate as follows: October to March (rainy season) , and April to September (dry season).

RESULTS AND DISCUSSION

Mean milk production for first lactation was 2556 ± 32 kg, with a coefficient of variation of 27.6% (Table II). These results demonstrate the high milk production potential of Pitangueiras cows when compared to data on crossbred cattle published by several authors in tropical countries. Wilson and Houghton (1962) found mean production of 2027 kg for Holstein-Zebu crossbred animals. Branton, *et al.* (1966) reported 1310 kg for 1/4 Jersey cows. Bhatnagar *et al.* (1970) found first lactation yields of 2009 kg for Schwyz Zebu crossbreds.

The Pitangueiras yields were higher than the yields reported for Zebu breeds utilized for milk production in the tropics . Tharparkar cows gave the next highest yields, with a mean production of 2167 kg (Reddy and Bhatnagar, 1971), whereas the Hariana breed was the lowest at 721 kg (Balaine, 1971).

The variation in milk yield with age shows some peculiarities in tropical cattle. Table II shows consecutive increases in production from the first to the fifth lactation. These increases, expressed as percentages of mean first lactation yields, were + 8.5% for the second lactation, +16.9% for the third, +18.4% for the fourth, and +22.6% for the fifth. Several investigators have observed maximum yields during the fifth lactation for Zebu breeds and European Zebu crossings (Asker et *al.*, 1962; Bayoumi and Danasoury, 1963; Dutt and Singh, 1961; Khanna and Bhat, 1972; Nagpal and Acharya, 1971). These results doubtless may be explained by older ages at first calving, long intervals between calvings, and the lower selective pressure applied to breeds raised in the tropics. However, Gill, *et al.* (1970), who found milk production

to be highest during the third lactation, concluded that the increase observed was due mainly to development of the mammary and endocrine systems caused by the successive gestations and lactations.

Table II. Mean and variability of milk yield (kg) in the first five lactations.

Lactation number	Number of records	Mean ± S.E.	% of the mean	C.V.(%)
First	483	2556 ± 32	100.0	27.6
Second	431	2774 ± 38	108.5	28.5
Third	357	2987 ± 43	116.9	27.2
Fourth	257	3027 ± 54	118.4	28.6
Fifth	195	3134 ± 71	122.6	31.5
Total or mean	1723	2835 ± 20	110.9	29.4

Mean milk yield for 1,723 lactations, 281 days in milk, was 2835 ± 20 kg, and the coefficient of variantion 29.4%. This value is quite satisfactory for cattle raised in the tropics on a semi-extensive basis. From a genetic viewpoint, an interesting feature common to all breeds of tropical dairy cattle is the relatively large coefficient of variation in milk yield (Alim, 1962; Balaine 1971; Bayoumi and Danasoury, 1963; Branton *et al.*, 1966; Nagpal and Acharya, 1971). Although in European cattle the usual coefficient of variation is 20 to 22%, coefficients for cattle populations in the tropics often are about twice as high.

The analyses of variance by method of least squares are presented in Table III. Generation effects were significant for all lactations. This effect possibly includes the combined effects of selection and crossing in this popula-

tion, since the genetic material analyzed is in segregation. Comparison of the productive performance of two generations shows an advantage of + 362 kg in milk yield at first lactation of first generation cows, a fact which may be explained in two ways: the selection to which Red Poll bulls utilized in mating with 1/4 Red Poll x 3/4 Zebu were submitted, since these sires belong to a breed which has been selected for a long time, and loss of heterosis by the P_2 ($= P_1 \times P_1$) cows, which, no matter how small, may contribute to decreased milk yield (Soller and Bar-Anan, 1964).

Table III. Analysis of variance (mean squares) for milk yield in the first five lactations.

Source of variation	Lactation order				
	First	Second	Third	Fourth	Fifth
Generation	13 287 700*	14 796 480*	13 294 150*	12 252 070*	10 600 890*
Age of cow	2 009 663*	897 369*	695 185*	1 368 759*	1 052 966*
Season	264 992	1 785 261*	356 711	555 751	730 764
Year	398 547*	708 807*	759 280*	754 515*	696 507*
Error	137 149	169 900	193 202	204 607	234 811

*$P < .05$

Least squares means for milk production at 281 days were 2476 kg for first lactation; 2718 kg for second; 2949 kg for third; 2942 kg for fourth; and 2830 kg for fifth (Table IV). It can be seen that, contrary to the arithmetic means of the data, highest production was reached at the third lactation, with a progressive increase of 19% from the first.

Effects of season were small but consistent, although only the second lactation effect was significant (Table III). Differences, favoring parturition in dry season, ranged from 50 kg (1st lactation) to 134 kg (2nd lactation), as shown in Table IV. Similar results have been obtained by several investigators (Bodisco *et al.*, 1968; Lobo, 1974; Nagpaul and Bhatnagar, 1972; Tomar *et al.*, 1971; Venkayya and Anantakrishnnan, 1958) for first lactations of Zebu and *Bos taurus* cows, and *Bos taurus* x *Bos indicus* crossings. A possible explanation of these results is the fact that the animals received concentrates

during the dry season. In any event, season effects were small, suggesting that the dairy farm management program was alleviating direct (climatological) and indirect (feed supply) effects upon production. This is a goal of many dairymen because of the quota system established by the Brazilian Government for the purchase of milk.

In much published research, season of calving is associated with 3 to 5% of the variability in milk production of different herds (Johansson and Hansson, 1942). In our study, however, this factor contributed only 0.25%. However, Nagpal and Acharya (1971) found that season of calving contributed only 0.09% to variation in milk production during first lactation. In general, the amount of variation seems directly related to variability in climate and food availability.

When dairy herds are studied over extended periods of time, increased milk production usually is observed. It is important to know how much of this increase is due to selection processes (genetic change) and how much reflects improved handling and feeding conditions (environmental change). In most cases it is difficult to separate these two components; the significant year effects detected (Tables III and IV) reflect a combination of environmental and genetic factors. This is due to the fact that, over a certain number of years, changes in handling and feeding occur, besides differences in climate. Furthermore, natural and/or artificial selection doubtless acted on the herd simultaneously. Similar results were described by several researchers in Zebu and European cattle and on their crossings in the tropics (Alim, 1962; Bodisco *et al.*, 1971; Khanna and Bhat, 1972; Lobo, 1974; Prasad and Prasad, 1972; Wilson and Houghton, 1962). Other workers, however, found no significant influence on milk yield (Bodisco *et al.*, 1968; Magofke and Bodisco, 1966; Singh *et al.*, 1973).

Effects of age among contemporary herdmates accounted for 10 to 30% of the variation in lactation milk yield. Correcting production according to cow age, however, is a complicated problem. When correction factors are calculated, the occurrence of genetic selection and changes in handling and feeding over the years are frequently not taken into account. Furthermore, high producing cows often show greater increases between first calving and maturity (highest production) than low producers. Thus, it is difficult to develop correction factors which can eliminate only the nongenetic causes of variation.

Table IV. Least squares constants for factors affecting milk yield during the first five lactations.

Source of variation		Constants (kg)				
		First	Second	Third	Fourth	Fifth
Least squares mean		2 476	2 718	2 949	2 942	2 830
Generation	G_1	181	208	218	254	310
	G_2	−181	− 208	−218	−254	−310
Age (months)	24	− 192	–	–	–	–
	36	3	− 201	–	–	–
	48	189	− 15	52	–	–
	60	–	134	− 3	−305	–
	72	–	82	118	− 6	−301
	84	–	–	−167	224	86
more than	96	–	–	–	87	215
Season	dry	25	67	34	51	63
	rainy	−25	− 67	−34	−51	−63
Year	1962	−8	–	–	–	–
	1963	− 125	− 189	–	–	–
	1964	131	356	62	–	–
	1965	184	150	348	546	–
	1966	43	273	173	160	396
	1967	−66	203	86	266	227
	1968	61	−76	33	− 196	− 47
	1969	62	− 119	− 229	−13	− 29
	1970	59	44	65	− 52	105
	1971	− 6	85	− 15	− 21	80
	1972	− 11	18	− 66	− 287	− 78
	1973	− 324	− 164	− 259	− 295	− 316
	1974	–	− 581	− 198	− 108	− 338

Int the present study, age contributed significantly to variation in

milk production for all lactations (Table III). Although results are presented on a within-lactation basis, examination of the entire set of least squares constants (Table IV) suggests an increase in yield with advancing age until about 66 months, a leveling off, and then perhaps a slight decline becoming apparent at 90 months. Similar results have been obtained by many other workers (Batra and Desai, 1964; Lobo, 1974; Nagpal and Acharya, 1971; Ronningen, 1967; Singh *et al.*, 1967; Tomar *et al.*, 1971).

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TISSUE DISTRIBUTION AND POPULATION VARIABILITY OF ESTERASES IN *CAVIA APEREA**

Luiz A.S. Monjeló[1] and A.R. Cordeiro[2]

ABSTRACT

The electrophoretic patterns of 17 esterase bands observed in starch gel were submitted to inhibition and activation tests, and their distribution in different *Cavia aperea* tissues was studied. It is suggested that at least 13 loci, five of which are polymorphic, could account for these zymogram patterns. The allelic frequencies of the five polymorphic loci were established in a sample of 96 animals from two sites in Dois Irmãos County, State of Rio Grande do Sul, Brazil. Six animals showed little or no expression of the major kidney esterases and enhancement of other bands, suggesting a compensatory change in regulation. The high frequency of homozygotes for the Est-7^0 and Est-9^0 silent alleles, especially in adult animals, may be due to differential selection or to regulatory phenomena.

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INTRODUCTION

Mammalian esterases are among the first enzymes reported to be heterogeneous (Aldridge, 1953 a, b, 1954; Hunter and Markert, 1957; Markert and Hunter, 1959). However, the wide range of substrates they can hydrolyze makes the specific classification of these enzymes difficult. The work of Augustinsson (1959 a, b, 1961), Bergmann *et al.*, (1957), Ecobichon and Kalow (1963), Holmes and Masters (1967), Chow and Ecobichon (1972), among others, established the basis for a more specific classification of these enzymes by using a variety of substrates, inhibitors and activators.

Esterase variability in rodents has already been studied in *Peromyscus* (Selander *et al.*, 1971) and *Mus musculus* (Wheeler and Selander, 1972). A review of genetic variation in vertebrates by Selander and Johnson (1973) discusses the main points of this subject. In this study we tried to characterize the esterases of *Cavia aperea* in an attempt to define the controlling loci and to determine the degree of polymorphism in a wild population of Rio Grande do Sul.

MATERIAL AND METHODS

Samples of 85 animals from Morro Reuter and 11 from Morro do Pedro, Dois Irmãos County, Rio Grande do Sul, Brazil, were analyzed. These two places form a continuum with no geographic barriers and with a similar environment even though they are 6.5 km apart. There is no significant difference ($P < 0.05$) between the two samples with respect to the observed number of esterase phenotypes. Consequently, the two samples are treated as a single population. Of the 96 animals captured between October and November, 1971, 29 were males and 67 females. The tissues were stored frozen at -25^0C until the time of use, when they were homogenized at 0^0C in Thomas tissue grinders with a teflon pestle at the following tissue: water (w/v) ratios: brain, 5:1, heart, 5:2, stomach, 1:2, duodenum, 1:3, liver, 1:5, kidney , 3:1. Plasma was used undiluted. All the homogenates were centrifuged for 10 minutes at 13,000 xg in a Misco refrigerated ultracentrifuge. The

supernatant was absorbed onto 4 x 2 mm rectangles of Whatman 3 MM paper which were then inserted into the gel starting slots just before connecting the electrodes.

Electrophoresis was carried out at 4° C on horizontal 13% hydrolyzed starch gel. We found Poulik's (1957) discontinuous buffer system to be best for this material when compared with the buffer systems used for esterases by Tashian (1961) and Grunder *et al.* (1965). A constant electric current of 10 V/cm measured in the gels was maintained until the buffer front advanced 10 cm from the sample starting line. To develop the zymograms, a mixture of alpha and beta-naphthyl acetate and 0.1% fast blue BB salt in 0.1 M phosphate, pH 6.0, was used for two hours at 38° C.

The inhibition and activation tests were performed as follows:

(a) – Temperature shock: incubation of gels at 60° C for periods of 0 to 45 minutes with 5 minute intervals prior to the development of the reactions.

(b) – The chemical agents were applied for 20 minutes at 38° C prior to incubation.

The chemicals tested as enzyme inhibitors were: 0.1 M and 0.05 M p-cloromercuribenzoate (PCMB), 0.1 M eserine sulphate (ES), 0.05 M ethylene diamine tetracetic acid dissodium salt (EDTA), and 5% N-propanol (P).

RESULTS

The general pattern of *Cavia aperea* esterases is presented in Fig. 1. The tissue distribution facilitates loci discrimination. Intestine and liver have by far the largest number of esterase bands. In this work no secondary bands were detected when the esterases were submitted to the range of temperature shown in Table I or when studied from samples of tissues stored for different periods of time (one day, one week, one month, three months). Tissues stored for three months showed small loss of activity but no changes in the general pattern of the isozymes were observed. Esterase-1, the fastest in the zymogram, is found exclusively in brain tissue and is characterized by two bands, the slower appearing together with the faster in only one of 96 individuals. We assume control for Esterase-1 is by one locus, Est-1, having two alleles.

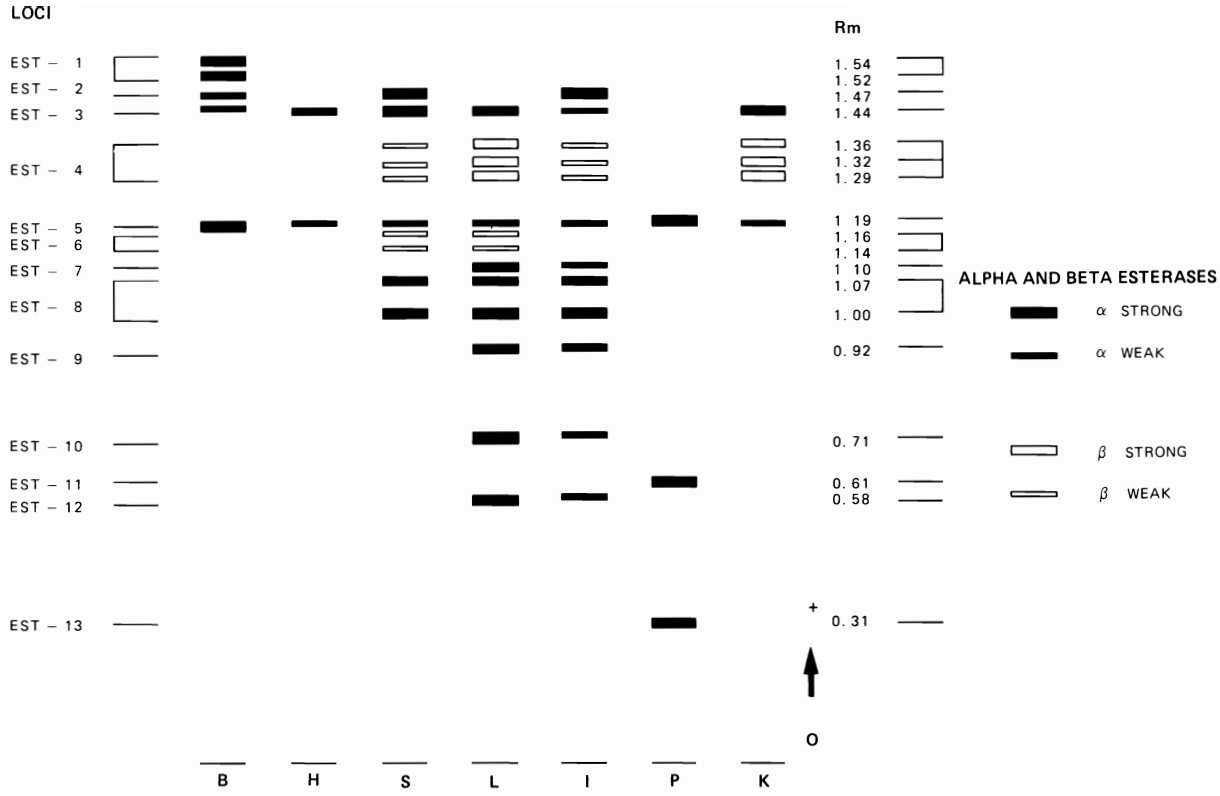

Figure 1 - Diagram of esterase phenotypes from different tissues of one population of *Cavia aperea*. B, brain; H, heart; S, stomach; L, liver; I, intestine; P, plasma; K, kidney

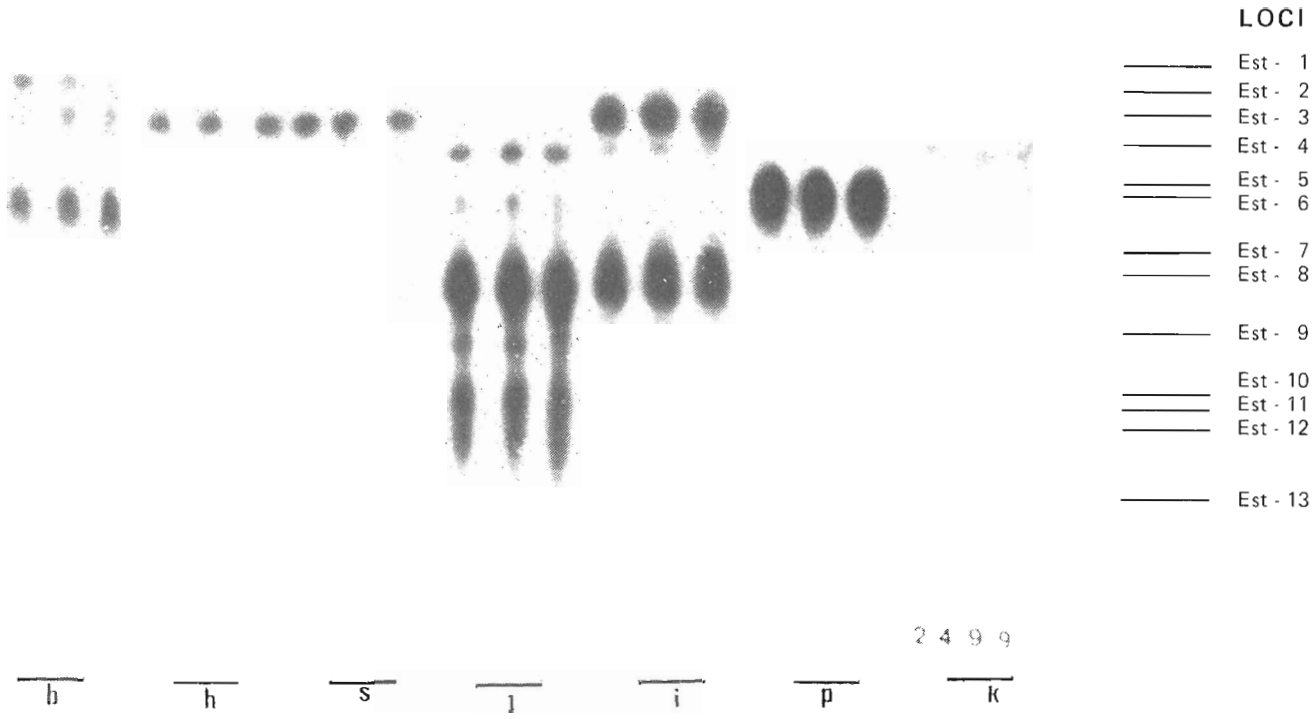

Figure 2 - Distribution of esterases in different tissues of one specimen of *Cavia aperea*. Three replications are shown for each tissue. B, brain; H, heart; S, stomach; L, liver; I, intestine; P, plasma, and K, kidney.

Esterase bands Nos 2, 3, 5, 10, 11, 12 and 13 are all alpha esterases and their tissue distribution is clearly variable as shown in Figs. 1 and 2.Esterases 2, 3 and 5 differ in their reactions to inhibitors and are classified as arylesterase, acetyl- or carboxylesterase, and cholinesterase, respectively. Esterases 10 to 13 have the same reaction pattern as acetyl- or carboxylesterase. However, esterases 10 and 12 are apparently restricted to liver and intestine and migrate apart from each other, while esterases 11 and 13 are better defined in plasma, although they can also be detected in kidney, liver and some other tissues. Esterase-5 is the most ubiquitous, for it was detected in all tissues studied in this work.

The beta-naphthyl-acetate specific bands, which are strong in kidney, were characterized as esterases-4. On the basis of the multiple electrophoretic forms of this enzyme we postulated an Est-4 locus with three alleles and a null or silent allele. The esterase-4 forms were absent in two out of six animals and showed little activity in the remaining four. Cholinesterase bands presumably controlled by the Est-6 locus (Fig. 3) were also atypically present. Esterase-6 with two electrophoretic alleles was detected in the stomach, liver and intestine. The activity distribution of other esterases is given in Figs. 1 and 2. We did not analyze the reproductive organs because we did not expect find any sex differences. Activation tests and substrate specificity

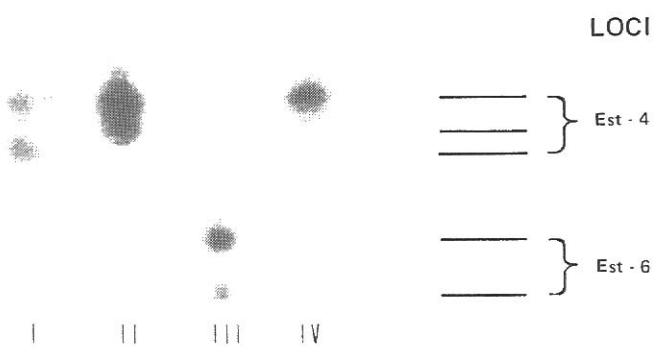

Figure 3 - Kidney esterases. I: heterozygote est-$4^{1.03}$/est-$4^{0.97}$; II: heterozygote est-$4^{1.03}$/est-$4^{1.0}$; III: atypical individual lacking Est-4 bands and with activity for Est-6 locus in heterozygote condition; IV: homozygote for est-$4^{1.03}$.

are shown in Table I. However, the inhibition and activation tests were used as an additional method to establish the homology of the esterase bands of the different tissues having the same position in the zymogram. The following evidence strongly suggests that esterase-4 is a carboxylesterase: 1) its greatest activity in kidney, 2) its substrate and inhibition patterns (Table I), and 3) its degree of polymorphism which is similar to that observed in other rodents.

The allelic frequencies for the loci that were classified as polymorphic at the 1% level are presented in Table II. The results of the Hardy-Weinberg equilibrium χ^2 test for the Est-4 and Est-8 loci are presented in the same table. It can be seen that these loci are at equilibrium. We found no significant differences in the frequency distributions of the two sexes. The high frequency of the null allele at the Est-7 and Est-9 loci are especially intriguing. Table III shows a comparison of young and adult animals considered to be homozygous for the silent allele with no bands for esterases 7 and 9 .

DISCUSSION

Due to technical difficulties, no crosses were performed in this investigation, however, locus characterization is suggested. We attempted to characterize the genetic control of the esterase bands by analysis of tissue distribution combined with the results of inhibition tests, and thermostability. The same procedures have been used by other authors in several mammalians including the *Cavia* genus (Holmes *et al.*, 1967, 1968a, b, and Kingsbury and Masters, 1970). The genetic control of mammalian esterases is believed to be due to single genes as shown by the work of Tashian (1961), Popp and Popp (1962), Grunder *et al.* (1965), Pelzer (1969), and others.

The 13 esterase loci and their characteristics in *Cavia aperea* do not differ greatly from the 12 loci of *Cavia porcellus* reported by Holmes and Masters (1967) and Holmes *et al.* (1968). However, some differences cannot be ascribed to variations in technical procedures. The fast-moving strong Est-5, very conspicuous in the plasma of *C. porcellus*, is classified as a carboxylesterase by Holmes and Masters (1967) and Chow and Ecobichon (1972). It was strongly inhibited by eserine in our material and was classified as a cholinesterase. On the other hand, Holmes and Masters (1967), in a study on *C. porcellus*, identified slow-moving zones of the serum as carboxylesterases,

Table I - Inhibition and activation tests on *Cavia aperea* esterases, with a preliminary classification.

AGENTS	0.01M p-CNB	0.05M p-CMB	0.01M Eser Sulph	0.05M EDTA	Propanol 5%	Substrates (alpha and beta naphtyl acetate)	Temperature (0-45 min) at 60º C	Type
ESTERASE - 1	+++	+++	+++	+	—	º	+	Ar
ESTERASE - 2	+++	+++	++	+	—	º	+	Ar
ESTERASE - 3	+	+	+	+	—	º	+	Ac or C
ESTERASE - 4	+	+	A	+	A	§	+	C
ESTERASE - 5	++	++	+++	+	—	º	+	Ch
ESTERASE - 6	+	+	+++	+	—	§	+	Ch
ESTERASE - 7	+	++	+	+	—	º	+	Ac or C
ESTERASE - 8	++	A	+	+	—	º	+	Ac or C
ESTERASE - 9	+	++	++	+	—	º	+	Ch
ESTERASE -10	+	A	+	+	—	º	+	Ac or C
ESTERASE -11	+	A	+	+	—	º	+	Ac or C
ESTERASE -12	+	A	+	+	—	º	+	Ac or C
ESTERASE- 13	+	A	+	+	—	º	+	Ac or C

+++, strong inhibition; ++, partial inhibition; +, weak inhibition; —, no effect; A, activated; º , preferential hydrolysis of α-naphthyl acetate; §, preferential hidrolysis of β-naphthyl acetate; Ar, arylesterase; Ac, acetylesterase; C, carboxylesterase; Ch, cholinesterase.

Table II - Allelic frequencies for esterase genes in a sample of 192 genomes from a natural population of *Cavia aperea* from Rio Grande do Sul, Brazil.

LOCI	ALLELES	ALLELIC FREQUENCY
Est-1	1.0	0.9948
	0.98	0.0052
Est-4*	1.03	0.2081
	1.0	0.4239
	0.97	0.1948
	0	0.1732
Est-7	1.0	0.4227
	0	0.5773
	1.07	0.4375
	1.0	0.5625
Est-8*	1.07	0.4375
	1.0	0.5625
Est-9	1.0	0.3953
	0	0.6047

χ^2 values for Est-4 and Est-8 are 1.919 and 0.454, respectively.

Table III - χ^2 test comparing the number of young and adult homozygotes for est-7^0 and est-9^0, in *Cavia aperea.*

LOCI	STAGE	0	E	χ^2
Est-7	young	05	16	13.78*
	adult	27	16	
Est-9	young	06	7.5	0.27
	adult	09	7.5	

*Significant at the 5 % level.

while Chow and Ecobichon (1972), working on the same species, defined these zones as cholinesterases. On the basis of our tests, these are acetyl- or carboxylesterases. It is expected that *Cavia aperea* would differ from the domestic guinea pig at many loci. We would even anticipate more allelic differences between domestic and wild species, especially if these structural genes are kept as a mutational load, since natural selection in domestic guinea pigs was certainly partially reduced and its direction changed for more than 2000 years in South America.

The main arylesterase detected in the brain of *C. aperea* may be homologous to the same type of enzymes that predominate in the brain of *C. porcellus* (Holmes and Masters, 1967).

Di-isopropylfluorophosphate (DFP) was not available to us and consequently we could not fully characterize the carboxylesterases, but the Est-4 reaction with PCMB and ES does not exclude the possibility of it being a carboxylesterase. Furthermore, its electrophoretic mobility, polymorphism and tissue distribution parallels the carboxylesterase described by other authors for rodents.

The Est-6 locus activation in the kidney of six individuals with absence (two animals) or low activity (four animals) of the esterase controlled by the Est-4 locus is probably due to a regulatory phenomenon, suggesting the occurrence of a compensatory change. In the postulated regulatory process, Est-4 is repressed while a simultaneous Est-6 locus activation develops in the Kidney. This compensatory activation might result from another level of regulation if a deficient or a nonfunctional enzyme (Est-4) is produced due to a structural mutation. These alternative hypotheses will be tested in the near future as soon as other cases are detected in our laboratory.

The absence of Est-7 bands in adults (Table III) may have two alternative explanations: differential selection pressure at young and adult stages, favoring null alleles in adults, or an ontogenetic regulatory difference. If so, we would expect all adults to show no activity for these loci, unless this regulatory mechanism depends on an environmental trigger.

Our results on the polymorphism of esterases from this population sample of *Cavia aperea* are very preliminary ones. However, we can say that five out of 13 loci are probably polymorphic. Esterases are usually highly polymorphic in all organisms so far studied.

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MORPHOLOGICAL ALTERATIONS IN THE COMPOUND EYES OF EYE-COLOR MUTANTS OF *APIS MELLIFERA* L. (HYMENOPTERA - APIDAE). I*

Carminda da Cruz-Landim[1], José Chaud-Netto[1] and Lionel Segui Gonçalves[2]

ABSTRACT

This study describes morphologic features of the compound eyes of four genotypes of *Apis mellifera*: homozygous and heterozygous normal workers, and "laranja" and "chartreuse red" mutant workers. The most striking difference among groups is in the amount and distribution of eye pigment. Degenerative changes were also observed in the cone and retinular cells of mutant bees, with the "chartreuse" genotype being the most severely altered.

INTRODUCTION

One of the important means of comunication between domestic honeybee

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workers is a dance with code movements indicating the location of a food source in relation to the position of the sun and its distance from the hive.

Eye-color mutant honeybees appear to be defective in transmitting information to their companions because they are unable to dance correctly or to perceive the information contained in the dance performed by normal workers. Gonçalves and Sénéchal (1972) found that "laranja" mutants are unable to receive and/or utilize the information transmitted by the dance of normal bees, while the "chartreuse red" mutants retain this ability. The activity of the drones is also impaired by eye-color mutations, so that certain mutations in the "chartreuse" locus cause difficulties in orientation (Witherell, 1971 and 1972). Generally, these mutations affect the amount of eye pigmentation especially in young individuals, a fact that, according to Tilson (1968), may lead to blindness in very luminous environments. This conclusion is based on the fact that the drones with the darkest pigments have the highest rate of return to the hive after a flight (Witherell, 1972). Thus, the problem involved in the behavioral alterations observed in mutants appears to be a visual one. However, other functions may also be affected by this mutation. Lopatina *et al.* (1976) have reported that the genes which influence tryptophan metabolism, especially those which alter the initial stages of kinurenin metabolism, also have an effect on the behavior by depressing motor activity.

The present study was carried out on normal workers (homozygotes and heterozygotes) with brown-black eyes, and on "laranja" and "chartreuse red" mutants in order to detect morphologic alterations in the compound eye which may be attributed to the action of the mutated genes.

MATERIAL AND METHODS

The bees utilized in this study were newly emerged or middle-aged (10-15 days old) domestic *Apis mellifera* workers with brown-black eyes, both homozygous and heterozygous *(Ch/ch^2)*, and "laranja" *(s^{la}/s^{la})* and "chartreuse red" *(ch^r/ch^r)* mutant workers. The compound eyes were dissected, fixed in 3% glutaraldehyde for 12 hours, and embedded in Epon 812. Sections 0.5 to 1 μ thick were obtained with the glass knives of a Porter Blum MT2

microtome, mounted on glass slides, stained with a mixture of methylene blue and azure II, and photographed .

RESULTS

The "laranja" and "chartreuse red" eye-color mutations are known to affect the amount and distribution of pigmentation in bees' eyes. The eyes of normal bees are light brown in newly emerged individuals and practically black in older insects. The "laranja" mutants have cream-colored eyes at emergence, while the "chartreuse red" mutants have pink-reddish eyes. With age, the eye color of these mutants also darkens, becoming orange for the "laranja" mutants and reddish-brown for the "chartreuse red" mutants.

Even though the pigmentation of the compound eyes increases with age, in the four genotypes studied the amount of pigmentation present is different, i. e. even the bees with normal eye color, but heterozygous, show a lower amount of pigmentation than the homozygous ones (Fig. 1). The strongest pigmentation of the compound eye is observed at the level of the pigment cells enveloping the crystalline cone and the initial portion of the retinules, and in the layer formed by the basal pigment cells at the distal end of the retinules on the basal membrane (Figs. 1 and 4).

Additional differences were found in the structure of the compound eye such as vacuoles inside the crystalline cones (Fig. 2) of "laranja" (Fig. 2c) and "chartreuse red" (Fig. 2e) mutants. This vacuolization reached large proportions in the "chartreuse" mutants, damaging more than one cone cell and affecting a relatively larger number of ommatidia. The vacuoles are not found in all ommatidia. The retinular cells of these mutants also appeared to be affected, with the peripheral portion of the cell appearing to be entirely occupied by a large vacuole (Fig. 3). Some alterations of this type were also found in the normal heterozygotes (Fig. 3b) and in the "laranja" mutants (Fig. 3c). However, in the "chartreuse"eye-color mutants, as many as three retinular cells per ommatidium may be affected, and the number of altered ommatidia in this zone is very large (Fig. 3e).

Several other alterations were observed in the compound eye of the

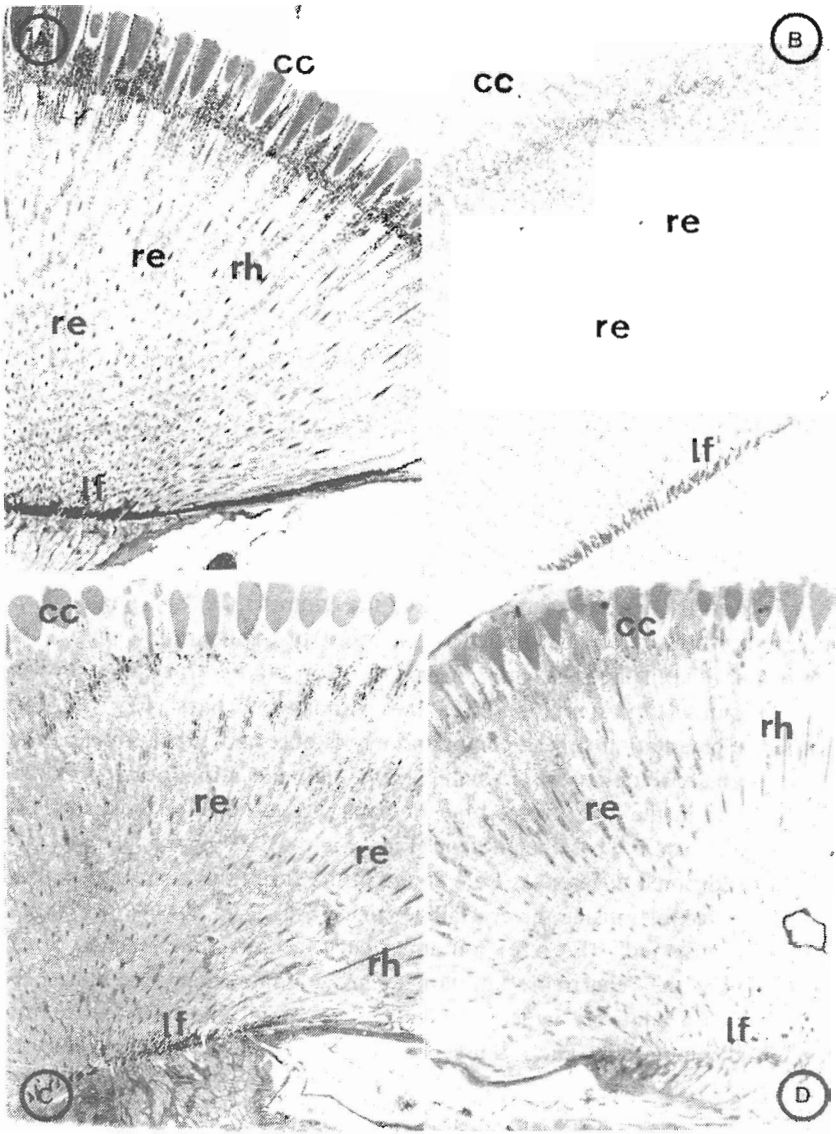

Figure 1 - General aspect of the compound eye of *Apis mellifera* showing pigment distribution throughout the ommatidia. a) normal homozygote; b) normal heterozygote; c) laranja mutant; d) "chartreuse" mutant. Cc = crystalline cone; re = retinules; rh = rhabdom; lf = microslide etched on one side. 80 X

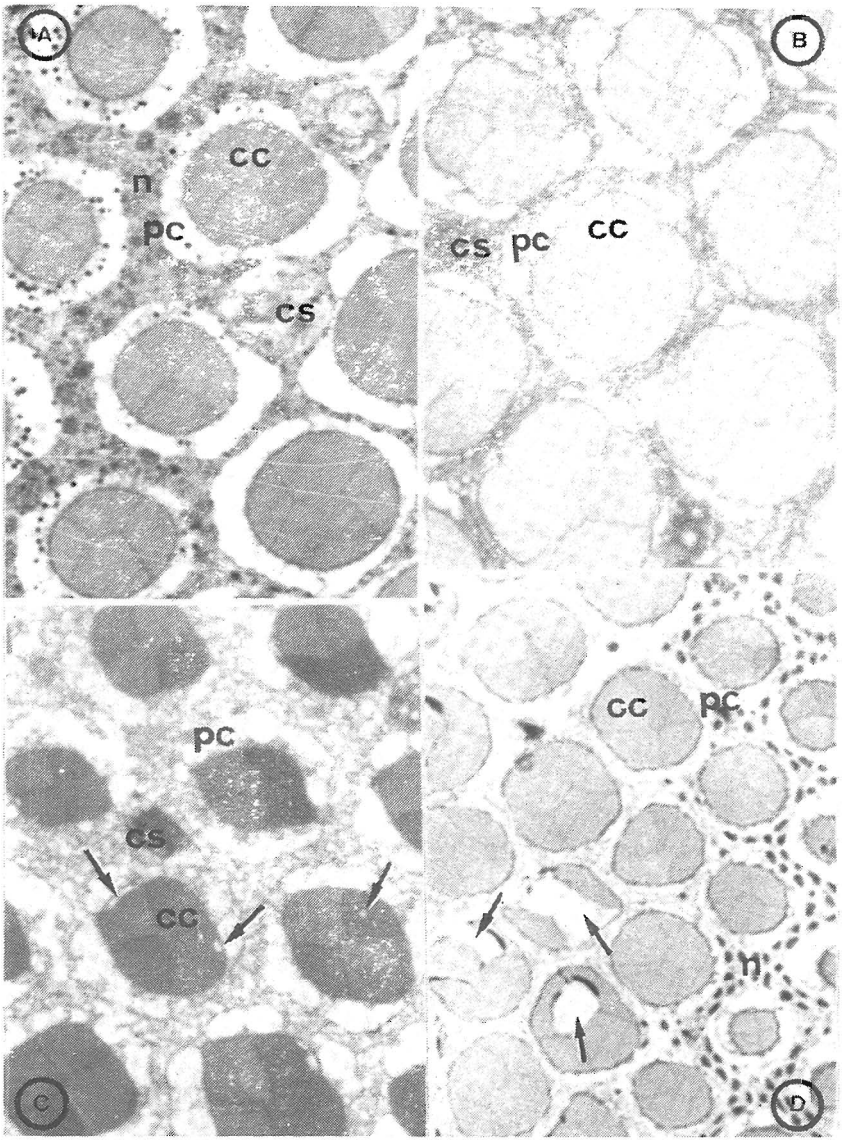

Figure 2- Cross section of the crystalline cone region with differences in shape and degenerative alterations in the mutants. a) normal homozygote, b) normal heterozygote; c) laranja mutant; d) "chartreuse" mutant. Cc = crystalline cone pc = pigment cells; cs = neurons of the sensory hairs of the eye; n = nuclei. The arrows indicate degeneration of the crystalline cone cells. 250 X

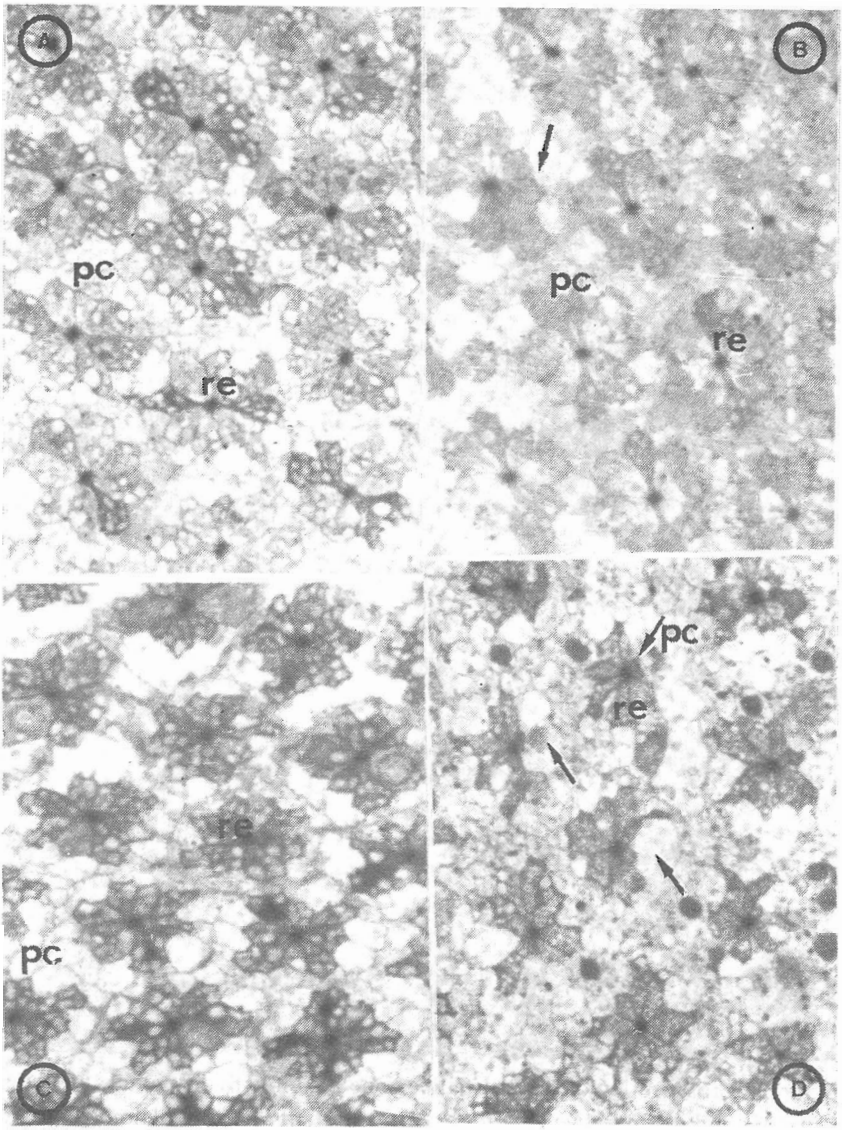

Figure 3 - Cross section in the retinule region (re) with alterations in some retinular cells (arrows). cp = pigment cells. a) normal homozygote; b) normal heterozygote; c) laranja mutant; d) "chartreuse" mutant. 250 X

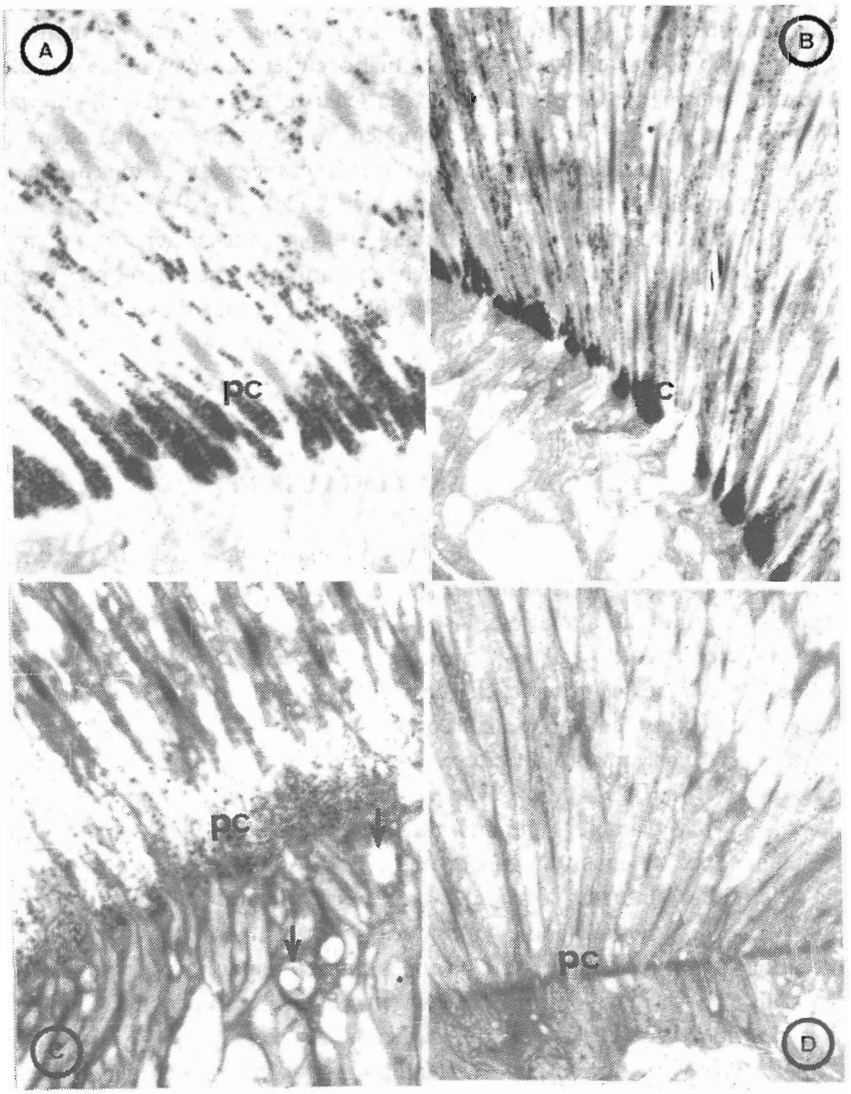

Figure 4 - Longitudinal section of the basal region of the ommatidia showing the difference in the amount of pigment in the basal pigment cells (pc). a) normal homozygote; b) normal heterozygote; c) laranja mutant; d) "chartreuse" mutant. The arrows indicate axon degeneration. 250 X

four honeybee genotypes studied but their appearance was less well defined, so that some doubts exist as to their actual occurrence. An example was the sinuosity shown by the rhabdom in the "chartreuse" mutants, which was observed in one third of the eyes examined. Another case was the changed shape of the crystalline cone, which can be seen in the cross sections in Fig.2, occurring mainly in normal heterozygotes (Fig. 2c). This altered shape was also observed in the other genotypes studied, although much more infrequently, thus leaving some doubts as to the genetic nature of the alteration. Some changes were also observed in several axons, such as that shown in Fig. 4c, belonging to a"laranja" mutant.

DISCUSSION AND CONCLUSIONS

The purpose of this study was to verify the possible structural differences between the compound eyes with normal and mutant colors, keeping in mind the alterations in behavior observed in the mutants. The normal heterozygotes *(Ch/ch^r)* were included because of the observed decrease in number of dance cycles carried out by the workers and the lower percentage of recruited bees (Küzima and Lopatina, 1975).

The most obvious difference observed is in the amount of pigmentation, as shown in Figs. 1 and 4. The pigment distribution also appeared to be altered: in the "laranja" mutants the pigmented apical cells around the crystalline cone were almost pigment-free (Fig. 1c). The same was observed in the "chartreuse" mutants (Fig. 1c), whose basal cells were also thus affected (Fig. 4 c). The other oustanding difference is represented by the degenerative alterations observed in the crystalline cones and retinular cells of the mutants, especially the "chartreuse" ones. No degenerative alterations were found in newly emerged bees. Pupae were not studied in this paper.

In view of the fact that these morphological alterations were present with considerably high frequency, it seems justified to assume that they are produced by the action of the mutated genes.It also seems obvious that such extensive alterations would affect the vision of the carriers with consequent behavioral effects. Our observations also show that among the genotypes

studied even the normal heterozygotes exhibit small alterations and that the eyes most severely affected belong to the "chartreuse" mutants.

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