Contrasted, a steel allele in the mouse with intermediate effects

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SUMMARY

The steel allele, contrasted (Sl^{con}), arose in a neutron irradiation experiment. Sl^{con} is fully penetrant and heterozygotes can be recognized at or soon after birth by darkly pigmented external genitalia in both sexes, while the adult coat tends to be a little lighter than normal. Homozygotes also have dark genitalia and a markedly diluted coat. Both eumelanin and phaeomelanin are affected, with reduced numbers of cortical and medullary pigment granules in the hairs. Contrasted also affects the haematopoietic system, causing slight macrocytic anaemia in the homozygote. Sl^{con} homozygous males are fertile but testes weigh on average 20 % less than in their heterozygous litter-mates. Homozygous females are usually sterile although if mated early ($4\frac{1}{2}$–6 weeks) they occasionally have a single litter. Ovarian sections showed a gradual degeneration of oocytes in Graafian follicles so that most had gone by 2 months. Similarly, vaginal smears indicated that after about three normal cycles homozygous females lapsed into a state of persistent dioestrus; injections with gonadotrophins did not prolong their period of fertility or cause a resumption of their oestrous cycles. The effects on fertility, pigmentation and haematology of contrasted when combined with other steel alleles are also described.

1. INTRODUCTION

The dominant spotting (W) and steel (Sl) series of alleles (Silvers, 1979; Green, 1981) are of particular interest because they have remarkably similar phenotypic effects of medical importance (Russell, 1979) but a different mode of gene action. The characteristic syndrome in both series combines severe macrocytic anaemia in the homozygote with sterility and a black-eyed white phenotype in survivors. Heterozygotes usually show low-grade white-spotting, perhaps with slight dilution of coat-colour, combined sometimes with slight macrocytic anaemia, but fertility and viability are unaffected. However, W alleles appear to act directly on the cells concerned while Sl alleles act *via* the tissue environment (Mayer & Green, 1968; Mintz, 1970; Mayer, 1973).

Steel alleles with the above characteristics are Sl, Sl^d (steel-Dickie), Sl^{gb} (grizzle-belly) Sl^{so} (sooty) and Sl^m (steel-m). Two other alleles with less severe effects are Sl^{cg} (cloud-gray), in which homozygotes are neither white nor sterile and Sl^{du} (dusty), with no noticeable anaemia in the homozygote. In this paper we describe another milder allele, contrasted (Sl^{con}), so called because it changes the

normal pattern of pigmentation rather than abolishing it. In other respects, also, it contrasts with previously described alleles and thus throws fresh light on the normal function of the *Sl* locus. Brief descriptions of contrasted, based on the present authors' preliminary findings, have been given by Silvers (1979) and Green (1981).

Table 1. *Segregation of contrasted in original (O) and recent (R) matings*

| Type of mating	Progeny			Total	Per cent Sl^{con}/Sl^{con}
	Sl^{con}/Sl^{con}	$Sl^{con}/+$	$+/+$		
Outcross (O)	—	487	458	945	—
Intercross (O)	124	530	308	962	$12 \cdot 9 \pm 1 \cdot 1$
Backcross (O)	437	712	—	1149	$38 \cdot 0 \pm 1 \cdot 4$
Backcross (R)	126	133	—	259	$48 \cdot 6 \pm 3 \cdot 1$

2. GENETICS

(i) *Origin and phenotype*

(C3H/HeH × 101/H)F$_1$ hybrid males were given 2·14 Gy (214 rad) fission neutron irradiation (+γ-contamination) over 12 weeks, then mated to a specific locus tester stock (Batchelor, Phillips & Searle, 1966). One F$_1$ male from litter SN/83C·5 had an agouti coat which was slightly lighter than normal. This was associated with marked darkening of the external genitalia, first visible within a day or two of birth. Such localized hyperpigmentation showed autosomal dominant inheritance and is a constant characteristic of the heterozygote, while hypo-pigmentation of the coat (noted in both agouti and non-agouti mice) is seen only occasionally. Homozygotes, however, have a very light coat on agouti, non-agouti and yellow (A^y) backgrounds, which is diluted to about the same extent as that in c/c^{ch} mice (Plate 1). However, external genitalia are again dark in both sexes (with eumelanin pigmentation even in A^y mice), as are the nipples in adult females. This contrast between pigment intensification in some areas but reduction in others led to the name contrasted, with Sl^{con} as eventual symbol.

(ii) *Segregation and linkage*

On outcrossing, contrasted behaves like a fully penetrant dominant mutation (Table 1). Intercross and backcross progeny originally showed a marked deficiency of the homozygous phenotype, although there was heterogeneity between matings. More recent backcross matings have failed to show the deficiency in homozygotes found earlier (Table 1). Thus Woolf's G-test for heterogeneity on results for a series of ten intercross matings gave a χ^2_{18} value of 34·1 ($P = 0.012$). Later, Sl^{con} heterozygotes were intercrossed or outcrossed and the amount of intrauterine lethality was compared with that for wild type crosses (Table 2). There was significant heterogeneity ($\chi^2_3 = 9.9$, $P = 0.02$) with respect to the amount of post-implantation lethality in the four types of cross. This seems to be largely the result of excess intrauterine lethality in the intercross matings, presumably connected with production of the homozygote. This, together with the heterogeneity
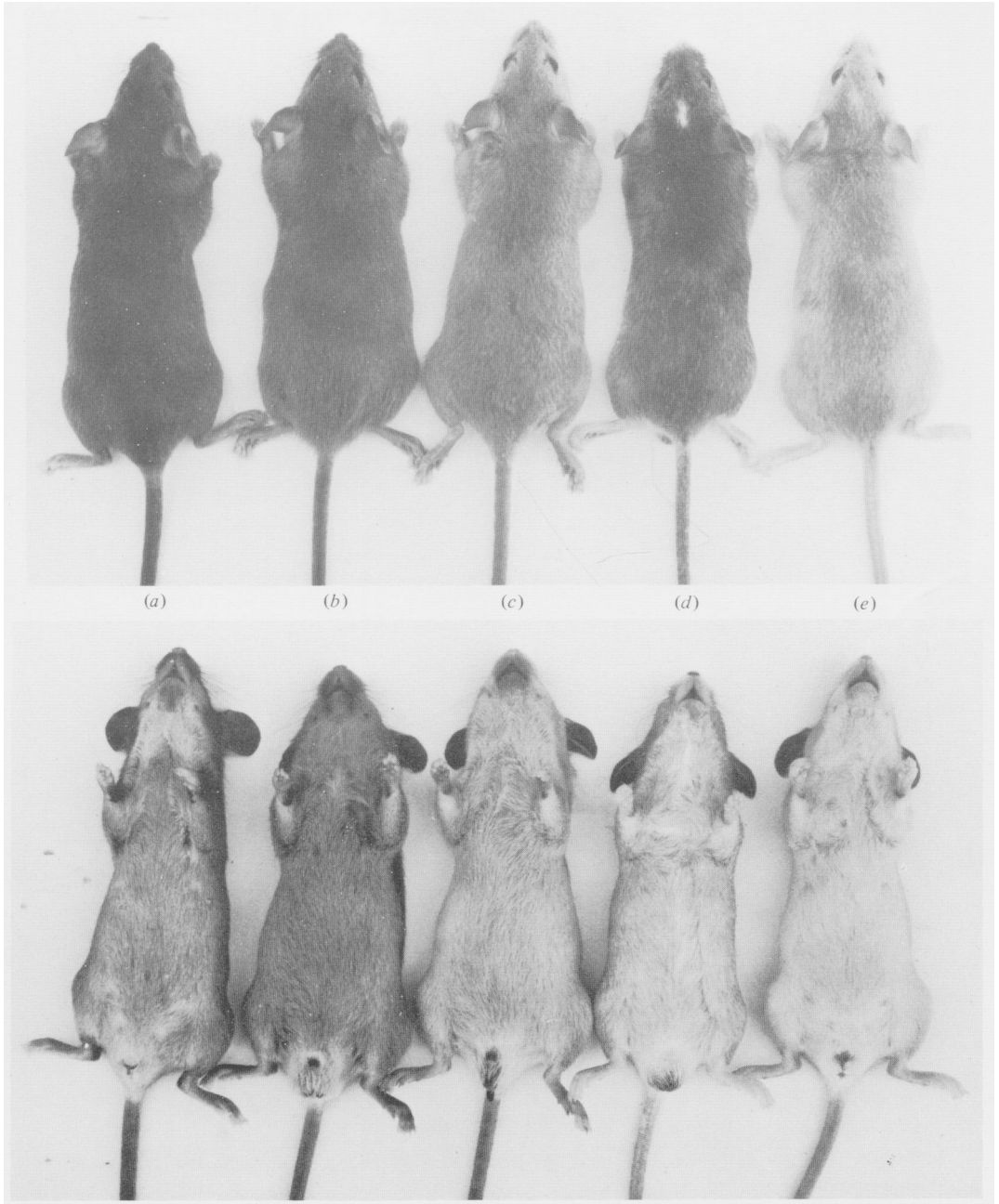
Dorsal and ventral views of (a) $+/+$ ♀, (b) $Sl^{con}/+$ ♂, (c) Sl^{con}/Sl^{con} ♂, (d) $Sl^{gbH}/+$ ♂, (e) Sl^{con}/Sl^{gbH} ♀. Note darkened genitalia in $Sl^{con}/+$, Sl^{con}/Sl^{con} and Sl^{con}/Sl^{gbH}.

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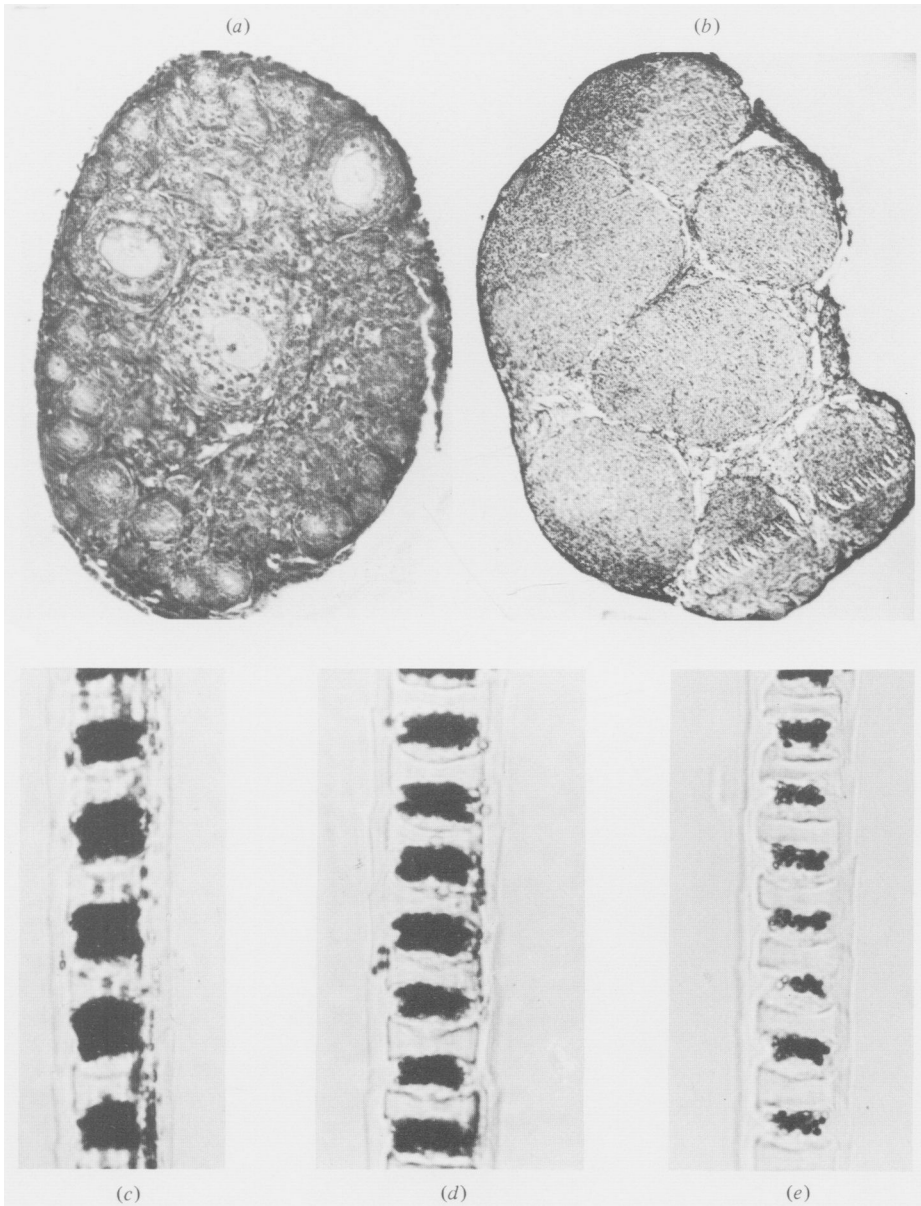
(a) Section of Sl^{con}/Sl^{con} ovary ($\times 157$) at 19 days, to show numerous follicles at different stages of maturation; (b) ovarian section ($\times 44$) of the same genotype at 54 days to show the absence of follicles with only corpora lutea remaining; (c)–(e) parts of mounted hairs ($\times 1250$) from wild type, $Sl^{con}/+$ and Sl^{con}/Sl^{con} respectively, to show some reduction in cortical and medullary pigmentation in the heterozygote and severe reduction in the homozygote.

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between matings already mentioned, suggests that the lethality was not a property of contrasted itself, but of a separate linked factor which perhaps was induced at the same time as contrasted.

Crosses to various linkage-testing stocks gave negative results except with a steel allele Sl^H. This steel-Harwell mutant (formerly called Sl^{dH}) is now extinct, but it resembled Sl^{gb} in the time of lethality of homozygotes soon after birth. A marked interaction was found between contrasted and Sl^H, the compound's coat being very light with a large head-spot and white patches on the body. Moreover, female compounds were completely sterile. Males were fertile, however, and on outcrossing produced 91 contrasted, 105 Sl^H but no recombinant phenotypes. Thus contrasted was given the symbol Sl^{con}, since it was regarded as an allele of steel. In addition, linkage tests with downless (dl), known to be linked to Sl on chromosome 10, gave the following phenotypes in the progeny from backcrosses of male and female heterozygotes: 44 Sl^{con} +, 38 + dl, 12 Sl^{con} dl, 12 +, total 106. The recombination frequency between Sl^{con} and dl is $22{\cdot}6 \pm 4{\cdot}1\%$, in good agreement with expectation from the linkage map (Roderick & Davisson, 1981). Linkage tests have also been carried out with grizzled (gr) and waltzer (v), known to be on chromosome 10. Backcrosses of Sl^{con} + +/+ gr v females gave offspring of the following phenotypes: 35 Sl^{con}, 1 gr v, 2 Sl^{con} gr v, 6 +, 5 Sl^{con} v, 2 gr, 1 v, total 52. Analysis of results for non-gr progeny because of the greatly reduced viability of gr (Bloom & Falconer, 1966) gives an RF of $16{\cdot}3 \pm 5{\cdot}6\%$ between the Sl and gr loci and $25{\cdot}6 \pm 6{\cdot}7\%$ between Sl and v, with most probable order of loci v–gr–Sl. Translocation T(10;18)18H was used to show that the centromere was at the v end of this linkage group (Searle & Beechey, 1978).

(iii) *Genetic interactions*

Sl^{con} has been combined with the following mutants, described by Green (1981):

(a) piebald (s). Sl^{con}/+ s/s mice did not show the dark external genitalia typical of contrasted. However Sl^{con}/Sl^{con} s/s mice, which were lighter than non-piebald Sl^{con} homozygotes, did have dark genitalia.

(b) viable allele of dominant spotting (W^v). Sl^{con}/+ W^v/+ mice also do not show dark genitalia while head and body spotting is more pronounced than in W^v. Sl^{con}/Sl^{con} W^v/+ mice are as grey as contrasted homozygotes but the spotting is even more extensive.

(c) rump-white (Rw). Sl^{con}/+ Rw/+ mice have little or no depigmentation in the rump region (unlike Rw/+ alone) but often have white areas on the tail, feet and head. External genitalia are dark except where included in an area of depigmentation. Sl^{con}/Sl^{con} Rw/+ mice are similar, apart from lighter coats, while Sl^{con}/Sl^{con} Rw +/+ W^v mice are almost completely white, and thus more affected than Rw +/+ W^v alone.

(d) grizzle-belly-Harwell (Sl^{gbH}). This is a steel-Harwell mutant resembling Sl^{gb}. Sl^{con}/Sl^{gbH} mice have dark genitalia but a lighter coat than Sl^{con}/Sl^{con}, with white head-dots and a very light belly (Plate 1). Males are fertile but females are sterile, with very small white ovaries devoid of oocytes.

Table 2. *Uterine contents at 12½–17½ days of gestation in $St^{con}/+$ intercrosses and outcrosses*

Mating type: female × male	No. of uteri	Corpora lutea	Total implants	Live embryos	Small moles	Large moles	Dead embryos	Preimplantation loss (%)	Postimplantation loss (%)
$St^{con}/+$ $St^{con}/+$	13	131	116	84	30	2	0	11·5	27·6
$+/+$ $St^{con}/+$	10	90	86	70	12	1	3	4·4	18·6
$St^{con}/+$ $+/+$	10	93	87	77	7	2	1	6·5	11·5
$+/+$ $+/+$	12	106	99	84	10	2	3	6·6	15·2

Table 3. *Comparative haematology of month-old female mice from litters segregating to give various genotypes*

Genotype	No. of animals	Hb g/100 ml mean ± S.E.M.	Erythrocytes per mm³ × 10⁻⁶ mean ± S.E.M.	Haematocrit (%) mean ± S.E.M.	Mean cell volume (μm^3) range and (median)
St^{con}/St^H	9	12·58 ± 0·49	6·06 ± 0·25	40·10 ± 1·07	60–76 (65·0)
St^{con}/St^{con}	5	14·00 ± 0·66	6·29 ± 0·67	42·74 ± 2·72	59–87 (61·5)
$St^{con}/+$	11	14·32 ± 0·89	7·20 ± 0·29	42·28 ± 1·66	48–79 (58·7)
$St^H/+$	5	13·74 ± 1·16	7·56 ± 0·40	45·70 ± 0·77	55–70 (60·1)
$+/+$	8	14·47 ± 1·09	7·92 ± 0·49	45·94 ± 0·98	49–84 (56·5)

3. HAEMATOLOGY

Possible effects of Sl^{con} on haematopoiesis were tested in females aged 1 month from litters segregating in Sl^{con} and Sl^H, by determination of haemoglobin content, packed cell volumes (haematocrit) and erythrocyte counts, with calculations of mean cell volumes from the last two (Table 3). There were fewer erythrocytes in the mutant genotypes, especially in those homozygous for Sl alleles. Within-litter

Table 4. *Statistical results of within-litter comparisons of testis weights in 3-week male mice segregating for Sl^{con} and Sl^H*

(Values of t_{30} are given with probabilities in parentheses. The plus and minus signs show whether mean testis weight is higher or lower in mice of the upper genotype.)

Genotype of litter-mate	$Sl^{con}/+$	Genotype Sl^{con}/Sl^H	$+/+$
$Sl^{con}/+$	—	-4.93 (0·00003)	$+1.28$ (0·21)
$Sl^H/+$	$+1.30$ (0·20)	-3.70 (0·0009)	$+2.27$ (0·031)
Sl^{con}/Sl^H	—	—	$+4.97$ (0·000025)

comparisons were made of values obtained for Sl^{con}/Sl^H *vs.* $Sl^H/+$, $Sl^{con}/+$ *vs.* $+/+$ and Sl^{con}/Sl^{con} vs $Sl^{con}/+$, by a two-way analysis of variance. The only marked differences found were for erythrocyte counts, as follows: Sl^{con}/Sl^H *vs.* $Sl^H/+$, $t_{24} = 5.21$ $(P = 0.000024)$; $Sl^{con}/+$ *vs.* $+/+$, $t_{24} = 2.81$ $(P = 0.0097)$; Sl^{con}/Sl^{con} *vs.* $Sl^{con}/+$, $t_{24} = 1.76$ $(P = 0.092)$. Derived mean cell volumes suggest that Sl^{con}, like Sl^H and other Sl alleles (Silvers, 1979) leads to slight macrocytosis.

4. REPRODUCTION

(i) *Males*

As mentioned previously, Sl^{con} males are fertile even as homozygotes. Bennett (1956) found that the testis weights of adult $Sl/+$ mice were about 25 % less than normal despite undiminished fertility. We therefore compared 3-week testis weights in (a) $Sl^H/+$, $Sl^{con}/+$, Sl^H/Sl^{con} and wild type litter-mates (Table 4), and (b) heterozygous and homozygous Sl^{con} litter-mates.

In the first set of comparisons, differences in mean testis weight between the four genotypes were very highly significant, since $F(3, 30) = 11.72$, $P = 0.00003$, without significant variance between litters in the size of these differences. As Table 4 shows, the Sl^H/Sl^{con} mice were mainly responsible for the variance, having significantly lighter testes than mice in all the other three genotypes. Average reductions in Sl^H/Sl^{con} testis-weight were about 40 % with respect to wild type and $Sl^{con}/+$ and 30 % with respect to $Sl^H/+$. Body weights of Sl^H/Sl^{con} mice also averaged about 10 % less than those of Sl^H ($t_{31} = 2.52$, $P = 0.02$) and $Sl^{con}/+$ ($t_{31} = 2.17$, $P = 0.04$) although differences between the four genotypes were only on the margin of significance on an overall analysis of variance [$F(3, 31) = 2.57$, $P = 0.07$].

In the second set of comparisons, which comprised five litters segregating for Sl^{con}, testis weights in Sl^{con}/Sl^{con} males ranged from 4 to 32 % less than in $Sl^{con}/+$ litter-mates, with an average of 20 % less. The mean difference was very highly

significant ($t_{10} = 7\cdot82$, $P = 0\cdot000014$) with significant variation between litters in the size of the difference [$F(4, 10) = 7\cdot63, P = 0\cdot00444$]. This reduction in testis weight was accompanied by a significant reduction in body weight in the contrasted homozygotes ($t_{14} = 2\cdot82$, $P = 0\cdot014$), which averaged 9%, about half the testis-weight reduction.

Table 5. *Sperm counts and testis weights from 8-week Sl^{con}/Sl^{con} and $Sl^{con}/+$ litter-mates*

Genotypes	No. of males	Mean sperm count $\times 10^6$	Testis weight (mg) mean $\pm$ S.E.M.
Sl^{con}/Sl^{con}	10	$8\cdot32 \pm 0\cdot41$	$78\cdot43 \pm 1\cdot67$
$Sl^{con}/+$	14	$9\cdot16 \pm 0\cdot38$	$93\cdot81 \pm 2\cdot45$

Sperm counts using the method of Searle & Beechey (1974) and testis weights at 8 weeks of age were compared in Sl^{con}/Sl^{con} and $Sl^{con}/+$ litter-mates from stock matings. Results (Table 5) show a highly significant ($t_{43\cdot34} = 5\cdot19$) reduction of 16% in Sl^{con}/Sl^{con} testis weight. The Sl^{con}/Sl^{con} sperm count showed the same trend (9% reduction) though the difference was not significant ($t_{46} = 1\cdot48$).

(ii) *Females*

The sterility of Sl^{con}/Sl^{con} females was not complete, as females that mated early ($4\frac{1}{2}$–6 weeks) occasionally had a single litter or were pregnant when autopsied. Ovary sections made at 19 days of age showed that they were smaller than $Sl^{con}+$ ovaries of the same age and contained approximately half as many maturing follicles, while at 54 days only corpora lutea were seen in Sl^{con}/Sl^{con} ovary sections (Plate 2). When the oestrous cycles from isolated females were followed from the age of 4–5 weeks (when the vagina opened) we found that Sl^{con}/Sl^{con} females only had about three fairly normal cycles before lapsing into a state of persistent dioestrus. Injections with gonadotrophins (PMS + HCG) failed to bring them into oestrus or prolong the period of fertility.

Five Sl^{con}/Sl^H females were completely sterile, even though exposed to a male from 3 weeks of age, and ovary sections at 54 days looked similar to those from Sl^{con}/Sl^{con} females.

5. PIGMENTATION

The light coat colour of Sl^{con}/Sl^{con} mice is not due to abnormal hair types or to any change in frequency of the four hair types of the mouse. This was shown by hair counts and examination of hair from 18 day Sl^{con} heterozygotes, homozygotes and normal litter-mates. Hair mounts (prepared by the method of Grüneberg, 1969) showed that there was a marked reduction in numbers of cortical and medullary pigment granules in all Sl^{con}/Sl^{con} hair types when compared to heterozygotes or normals (Plate 2). Thus, on a wild type background, numbers of medullary pigment granules in Sl^{con}/Sl^{con} were less than half those in $Sl^{con}/+$. The shape and size of the granules seemed unchanged. Reduced numbers of cortical and medullary pigment granules were also found in Sl^{con}/Sl^H hairs, although the effect was not so extreme as in Sl^{con}/Sl^{con}. In the latter, eumelanin pigmentation

was found in various unusual places besides the external genitalia. These included the duct of the preputial gland and epididymis in males and the uterine horns, Fallopian tube, ovarian capsule and the ovary itself in females. Microscopic examination of $Sl^{con}/+$ and $+/+$ external genitalia soon after birth showed many more pigmented melanocytes in the former, often grouped around circular unpigmented areas.

Skins of hairless (hr/hr) mice homozygous for Sl^{con} or $+$ were compared. The contrasted skin showed pigmentation of nipples and genitalia but in other areas was pinker than wild type, with a less even distribution of dendritic melanocytes in the epidermis. Skins of older Sl^{con}/Sl^{con} mice showed small dark patches of eumelanin pigmentation between the hairs.

6. DISCUSSION

The particular interest of Sl^{con} lies in the intermediate nature of its effects, which act on the same systems as the more extreme steel alleles but give greater opportunities for the underlying mechanisms to be studied than when the homozygote is lethal, completely sterile, or completely devoid of dendritic melanocytes. In its effects on coat colour Sl^{con} resembles cloud-gray Sl^{cg} (see Silvers. 1979), except that homozygotes for the latter have white blotches on their light grey coats. Little else in known about Sl^{cg}, except that the fertility of homozygotes is very poor, contrasting with the high fertility of Sl^{con} homozygous males.

The effect of Sl^{con} on coat colour is much clearer in homozygotes than in heterozygotes, but there is marked darkening of external genitalia in both genotypes. This is detectable soon after birth, when many more pigmented melanocytes can be seen in these areas of contrasted mice than in wild type. This might suggest that there was some defect of melanoblast migration from the neural crest, so that excessive numbers of pigment cells were reaching these regions at the expense of other parts of the skin. However, it seems just as likely that total number of melanocytes in these areas are unchanged but that some change in receptor or stimulus is causing more of these cells to start melanogenesis in the external genitalia and fewer to do so in hair follicles. It may be relevant to note that increased pigmentation of nipples and external genitalia is found in pregnant women and after oestrogen therapy (Riley, 1974). This is associated with a significant increase in melanocyte count, thought to be the result of stimulation of previously amelanotic melanocytes into melanogenic activity (Snell & Bischitz, 1963). Clearly, oestrogen can hardly be involved in stimulating any early post-natal melanogenesis in Sl^{con} but possibly the melanocyte-stimulating hormone MSH is. Although the functions in mammals of this ACTH-related pituitary hormone are still not clearly understood (Wallis, 1975) it can have a marked effect on coat colour in some mammals and is known to act in the mouse (Geschwind, Huseby & Nishioka, 1972). These authors injected adult viable yellow (A^y) mice with MSH after shaving areas to stimulate new hair growth. The new hairs were dark in colour and pigmented with eumelanin, as opposed to the phaeomelanin of the original hair. The authors did not look at the response of newborn mice to MSH but in a limited study of 4 day olds they obtained a response similar to that in adults.

They also showed that in adults given a lengthy series of MSH injections the number of pigment granules in dorsal skin increased. In addition microscopic examination of skin from the nipples, tail and scrotum, which tended to darken after a series of injections, showed an increased number of melanocytes.

Thus it could be postulated that the contrasted mutant either alters regional patterns of response to a hormonal stimulus or changes the pattern of melanoblast migration, or possibly both. In this connexion, is is interesting to note that steel (Sl) germ-cells seem to migrate normally to the gonadal ridges but fail to proliferate once they have reached them (McCoshen & McCallion, 1975). In contrasted homozygotes, germ-cells do proliferate but in females the number of resultant oocytes is less than normal, as shown by reduced follicle counts at 19 days. This is followed by follicular atresia, unaffected by gonadotrophins, so that one litter at most is produced. The reduced testis weights in Sl^{con} homozygotes and compounds with another steel allele, Sl^H, together with the evidence for a sperm-count reduction in the former, suggest that male mutants also suffer from some defect in proliferation and/or maturation of germ-cells but to a lesser extent. This is in line with Bennett's (1956) findings in Sl heterozygotes. The concomitant reduction in body weight may be connected with effects on the haematopoietic system.

In common with other Sl alleles (Silvers, 1979), contrasted leads to macrocytic anaemia in the homozygote, although it is much less extreme than in Sl, where it is lethal.

McCulloch *et al.* (1965) by transplantation experiments with Sl and W alleles showed that the defect in haematopoiesis is related to deficiency in the process of colony formation. In the W series the defect is in the colony-forming cells themselves while in the Sl series the cells seem normal but do not function because the tissues of these mice are incapable of supporting their proliferation and differentiation.

Thus Sl^{con} would appear to be less severe in its effects on the haematopoietic tissue environment than several other alleles in the series (such as Sl, Sl^d, Sl^{gb}, Sl^{so} and Sl^m). This is in line with its milder effects on fertility and pigmentation, but the exact nature of the underlying defect in the microenvironment of the three groups of affected cells remains unknown.

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