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Non-random X-chromosome inactivation in the mouse: difference of reaction to imprinting

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SUMMARY

Selection for increased and for decreased expression of the sex-linked gene brindled (Mo^{br}) in heterozygous females produced two lines with non-random *X* chromosome inactivation. In the High line the *X* chromosome marked by brindled was active in about 60% of cells, while in the Low line it was active in about 25% of cells. The whole of the difference was caused by the chromosomes carrying brindled: neither the unmarked *X* chromosome nor the autosomes were differentiated. There was a positive correlation between the expression of brindled in daughters and mothers. This was probably not caused by residual genetic variation, but was more probably a maternal effect similar to that described by Cattanach & Papworth (1981). On this assumption the daughters' scores were adjusted to a standard maternal score. Enzyme assays on females doubly heterozygous for brindled and for the sex-linked *Pgk-1* locus proved that the percentage of brindled in the coat provided an accurate measure of the *X*-inactivation proportions in the blood, liver and kidney. The accuracy was improved by adjustment for maternal score. In the selection lines, brindled was always inherited from the mother. When brindled was transmitted by male parents the probability of activation of its chromosome was increased by 8 percentage points in the High line and 18 in the Low line. This effect of the parental source is much greater than has previously been reported. The responses to selection can be interpreted in terms of the *Xce* locus controlling the activation probability, different alleles on the chromosomes carrying brindled being selected in the two lines. If this interpretation is correct, the alleles on one or both of the chromosomes carrying brindled were different from any of the three known alleles. The different effects of male transmission in the two lines can be described as a difference between the two chromosomes in their reactions to imprinting. This difference might possibly also be due to the *Xce* locus.

1. INTRODUCTION

When the inactivation of one *X* chromosome in female mammals was first proposed as the mechanism of dosage compensation (Lyon, 1961) it was supposed that the inactivation was random in each cell. That is to say, the maternally and

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paternally derived *X* chromosomes in any cell were equally likely to be inactivated. Random inactivation has subsequently been proved to be the general rule, but several situations have been found in which the inactivation is not random, for example in marsupials (Sharman, 1971) and in the extra-embryonic membranes of the mouse (Papaioannou & West, 1981). In both these cases it is the maternally derived chromosome that is predominantly the active one. Non-random inactivation of this sort implies that one or other of the *X* chromosome is 'marked' in some way according to the sex of the parent from which it came. This 'marking' of a chromosome is referred to as 'imprinting' (Chandra & Brown, 1975).

After inactivation, the same *X* chromosome remains inactive in the clonally descendant cells. Consequently, when one of the *X* chromosomes in a heterozygous female carries a gene whose effects can be seen in the coat pigmentation, a variegated pattern results, with patches of mutant and patches of normal colour according to which of the two chromosomes is active. Individuals vary in the proportion of mutant and normal pigmentation, the variation being presumably due to binomial sampling of progenitor cells of the coat melanocytes, (see Nesbitt, 1971). On average, however, the two colours usually occupy roughly equal areas, which is what would be expected from random inactivation. However, the proportions of the two colours in mice can be altered and made non-random by selection, which would not be expected if inactivation were always random. This was shown by Cattanach & Isaacson (1965) with Cattanach's translocation *Is*(*X*; 7)*Ct* as a marker, and by Falconer & Isaacson (1972) with structurally normal *X* chromosomes and the gene brindled (Mo^{br}) as a marker. Other evidence of non-random inactivation is cited by Cattanach & Papworth (1981).

There is now much evidence to show that the inactivation probability of an *X* chromosome is controlled by a locus on the *X* chromosome called the *X chromosome controlling element*, *Xce* (see Cattanach & Papworth, 1981). Three alleles of *Xce* are known, conferring different inactivation probabilities on the chromosome carrying them.

The selection experiment with brindled as a marker, cited above (Falconer & Isaacson, 1972), left several questions that could not be answered at the time. The selected lines, however, were continued and it subsequently became possible to answer these questions. The present paper describes this new work. In explanation of the objectives of the new work we shall first summarize the previous conclusions.

2. PREVIOUS CONCLUSIONS AND NEW OBJECTIVES

Individual heterozygous brindled females were scored visually on a percentage scale for the area of the dorsal coat showing the brindled phenotype, i.e. for the 'expression' of brindled. Two-way selection was applied for increased expression in the High line and for decreased expression in the Low line. Both lines responded to selection up to generation 5 or 6, but after that there was very little, if any, further response. The generation means are shown in Fig. 1. The previous paper reported the results up to generation 12. After the responses had ceased the High

line had a score of about 60 % brindled and the Low line about 30 %. At that time there was no way of finding out if the changed expression was due to non-random *X*-inactivation or to different rates of proliferation of cells according to which *X* chromosome was active. Subsequently, however, the discovery of a variant of the sex-linked enzyme phosphoglycerate kinase, PGK-1 (Nielson & Chapman, 1977) makes it possible to discriminate. Johnston & Cattanach (1981) have shown by

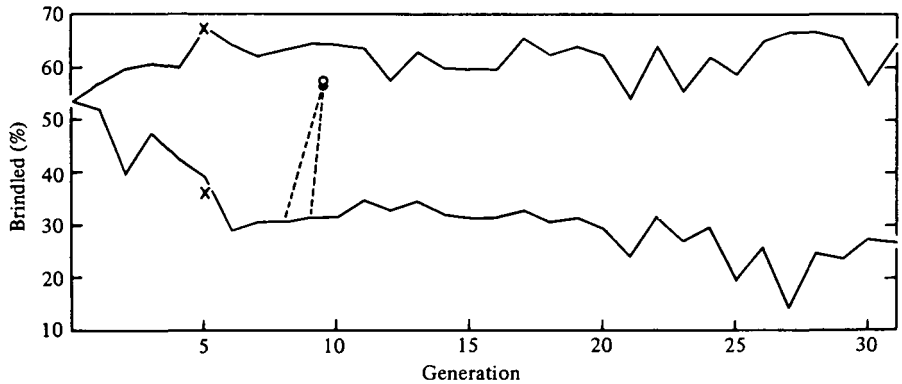

Fig. 1. Generation means of the selected lines. The two crosses at generation 5 mark the means of reciprocal crosses as explained in the text. Circles mark the means of the daughters of brindled males from the Low line mated to normal females from the Low line (full circle) and from the High line (open circle).

this means that differences of expression of Cattanach's translocation are due to non-random *X*-inactivation and not to cell selection. West & Chapman (1978) also found no evidence of cell selection in their material. There are therefore good grounds for assuming, as we shall do, that non-random *X*-inactivation was the cause of the non-random phenotypes of our lines.

The main conclusion from the previous work was that the difference between the selected lines resided in the *X* chromosome marked by brindled, which had become differentiated in the two lines as a result of the selection; neither the unmarked *X* chromosomes nor the autosomes had been affected. The evidence for this conclusion came mainly from reciprocal crosses made at generation 5. Brindled females of one line were mated to normal males of the other. The mean scores of the cross-bred daughters, marked in Fig. 1 by crosses, were equal to those of the maternal line transmitting the brindled chromosome; the normal chromosomes did not differ in their effect on the phenotype. The crosses did not rule out the possibility that a maternal effect of some kind, such as cytoplasmic determinants, was responsible for the difference between the lines. Though other evidence made this unlikely, one of the objects of the present work was to test more critically for a maternal effect. The matings required for this test also provided a further comparison of the normal *X* chromosomes and of the autosomes of the lines.

Brindled males and homozygous females normally die at about two weeks after

birth. Consequently the brindled gene must normally be transmitted through heterozygous brindled females mated to normal males. All the matings in the selected lines were of this sort. Occasionally, however, brindled males survive and breed, and three such survivors in the Low line were described in the previous paper. Their daughters, however, did not have the low expression characteristic of the Low line, where brindled was inherited from the mother. The males were mated to normal (i.e. non-brindled) females from both the Low and the High lines. The mean scores of the daughters are shown in Fig. 1 connected by broken lines to the generations of the Low line from which the brindled males came. The line of the mother made no difference and both sets of daughters had scores about equal to the original mean before selection. Thus, the effect of the selection in the Low line was entirely lost when *Br* was transmitted by these males. The inactivation probability of Cattanach's translocation was known to be influenced in a similar way by the sex of the parent transmitting it (Cattanach & Perez, 1970), but this parental-source effect seemed to be very much greater in our brindled line. Since then, the discovery that brindled mice have a defect of copper uptake (Hunt, 1974) has made it possible to 'rescue' brindled males and homozygous females by injection of copper (Hunt, 1976). The main purpose of the work described here was to explore further the effects of the sex and the genotype of the parents by breeding from (i) brindled males, (ii) heterozygous daughters of brindled males, (iii) brindled *XO* females, and (iv) homozygous brindled females. It was particularly important to breed from brindled males of the High line to find out if the scores of their daughters would, like those of the Low-line males, be changed toward the unselected level, or would be increased above the level of the line, as would be expected from the observations of Cattanach & Perez (1970), who found that the marked *X* chromosome in their material always had a higher probability of activation when derived from the father than when derived from the mother.

In discussing non-random inactivation it is important to know precisely what phenotype corresponds to random inactivation. The visual score of brindled could be biased either way so that, for example, the 60 % score of the High line might in reality represent random inactivation. Another purpose of the present work was therefore to find out, by means of the PGK-1 variant, how the visual score was related to the inactivation proportions.

Finally, a maternal effect of a kind different from the cytoplasmic factors mentioned above has to be considered following the observations of Cattanach & Papworth (1981). These authors worked with a different allele, viable brindled (Mo^{vbr}), or *Vbr* for short. This allele differs from *Br* in being less extreme in its effects on viability and on pigmentation. They found that the scores of heterozygous females were positively correlated with the maternal scores. For several reasons they concluded that this resemblance between mothers and daughters was not genetic and did not represent a correlation of inactivation proportions. They attributed it to a maternal effect dependent on the degree of copper deficiency in the mother. Higher-scoring mothers have a greater copper deficiency, in consequence of which the daughters suffer more from copper deficiency in early life. This results

in reduced pigmentation in the *Vbr* patches, which leads to a higher score being assigned. This maternal effect thus leads to a discrepancy between the visual score and the inactivation proportions. We therefore examined the relationship between the scores of daughters and mothers in our material to see if a similar maternal effect was present, and if any adjustments would have to be made to allow for it.

3. MATERIAL AND METHODS

(i) *Terminology*

The following terminology will be used to symbolize genotypes. The brindled gene will be symbolized by *Br*, instead of Mo^{br}, and its normal allele by +. A chromosome derived from the High line will be symbolized by *H* and one from the Low line by *L*. A chromosome inherited from the mother (maternally derived) will be symbolized by *m*, and one inherited from the father (paternally derived) by *p*. Thus an individual with genotype

$$\frac{Br\ H\ m}{+\ L\ p}$$

is a heterozygous female who got her brindled chromosome from a High-line mother and her normal chromosome from a Low-line father.

(ii) *The continued selection lines*

The previous paper described the selected lines up to generation 12, and their generation means up to generation 31 are shown in Fig 1. The lines were maintained throughout by about 10 matings of brindled females by normal males, the females being selected for their brindled expression. Despite the continued selection the means remained constant, at least up to generation 20. The genotypes of the patents and progeny in all generations of the lines were as follows:

$$\text{parents}: \frac{Br}{+} \text{♀} \times + \text{♂},$$

$$\text{progeny}: \frac{Br\ m}{+\ p} : \frac{+\ m}{+\ p} : Br\ m : +\ m.$$

The new work concerns generations 22 to 31. The numbers of females scored in these generations ranged from 31 to 98, with a mean of 59 in the High line and 72 in the Low line. The standard errors of the generation-means ranged from 1·2 to 2·5 percentage points.

Loss of fertility, presumably due to inbreeding, made it necessary to outcross the lines. This was done at generation 21, when brindled females were mated to normal males of a genetically heterogeneous strain. The foreign normal *X* chromosomes introduced by the males proved to have a higher activation probability than the normal chromosomes of the lines, and in consequence the

mean brindled score of both lines went down. In the generations after the outcross the normal chromosome in brindled heterozygotes alternated between the 'old' and the 'new' and the effect of this is seen in the zig-zag pattern of the means in Fig. 1. Brindled females with the 'new' *X* chromosomes are in odd-numbered generations from 21 to 27. Unfortunately the effect of the new chromosome was not realized until the regularity of the zig-zags became apparent. The new chromosome was then eliminated by backcrossing to males of the previous generation, and from generation 28 on only the original chromosome was present. Much of the new work on the parental-source effect was done between generations 23 and 27. Valid comparisons with the lines required adjustments of the line means to take account of the different effects of the two normal chromosomes. The adjustments made will be described with the results.

The graph of the means in Fig. 1 suggests that the introduction of the new chromosome may have resulted in some further response of the Low line, particularly in the generations with the new chromosome. When tested by the regression of mean on generation number, however, the change was not significant.

The low expression of brindled in the Low line had the result that some heterozygous females had no brindled visible in their coats. These were classified as non-brindled and could not be recognized as brindled with scores of 0%, as they should have been. The existence of these 'normal-overlaps' was implied by the significant deficiency of brindled females classified in the Low line. The segregation of brindled:normal was very close to the expected 1:1 in females of the High line and in males of both lines. The numbers of misclassified brindled females were estimated from the segregation ratios to see what adjustments might be made to the observed means in the Low line. In the generations with the 'old' normal *X* chromosome about 9% were misclassified and the mean scores should be adjusted downwards by about 2 percentage points; in the generations with the 'new' chromosome about 15% were misclassified and the means should be adjusted downwards by about 3 percentage points. These adjustments, however, will not be applied in the comparisons to be made because they are crudely estimated and it will be sufficient to remember that the real means of the low line are a little below the values given. Another source of bias, particularly in the Low line, is the skewed distribution of scores expressed as percentages. Transformation to angles, however, did not affect any of the conclusions and so the results are given in untransformed percentage scores.

(iii) *Treatment with copper*

In order to obtain brindled males that survived to breeding age, brindled males were identified at birth by their tightly curled vibrissae, and were injected intraperitoneally with 0·04% solution of copper chloride ($CuCl_2$) in distilled water. The doses given were 0·05 ml on alternate days from day 2 to day 12 inclusive. It was more difficult to get High line males to survive than Low line males, and a smaller proportion of survivors proved to be fertile among High than the Low

line males: 45% of High-line and 83% of Low-line surviving males were fertile ($\chi^2 = 13{\cdot}2, P < 0{\cdot}01$). The difference in survival and fertility is probably attributable to prenatal maternal effects, High-line males having mothers with higher expression of brindled and therefore more severe copper deficiency.

Br O and *Br/Br* females were also obtained by the same treatment. The *Br O* females proved more difficult to rear than the *Br* males, but all of the ten obtained proved to be fertile. All but one of the 36 *Br/Br* females were fertile, some producing up to 12 litters. Their litters, however, had to be fostered, because the heterozygous *Br/+* young did not survive when reared by *Br/Br* mothers. It seems that, while *Br/+* mothers provide enough copper for their *Br/+* young, *Br/Br* and *Br O* mothers do not, though *Br O* provide enough for their non-brindled *XO* young, and *Br/Br* enough for fostered *+/+* young.

(iv) *Enzyme assays*

In order to find out how the visual brindled score was related to the actual inactivation proportions, the PGK allozyme proportions were determined in 32 females heterozygous for *Br* and for the *a* and *b* alleles of *Pgk-1*, the *b* allele being carried on the *Br* chromosomes. These females were produced by mating brindled females of both lines to *Pgk-1*^{a} males from a PGK stock kindly provided by Dr Anne McLaren. The doubly heterozygous daughters were chosen, 24 from the High line and 8 from the Low line, to cover a wide range of brindled scores, the range obtained being from 5% to 65%.

The allozymes were separated on starch gels as described by Beutler (1969) with modifications suggested by Dr J. D. West (personal communication). The relative proportions of the allozymes were determined by the serial dilution technique (Klebe, 1975) with details as described for GPI by Falconer *et al.* (1981). The two allozymes were known to have equal specific activities (West & Chapman, 1978). Tests with known mixtures from homozygotes were made and confirmed the reliability of the method. Samples of blood, liver and kidney from all the females were assayed in this way. The samples were coded so that the brindle scores were not known when the gels were read.

RESULTS

(i) *Inactivation proportions*

The enzyme assays showed that the brindled score was a very good measure of the inactivation proportions. In agreement with West and Chapman's (1978) observations, we found the three tissues – blood, liver and kidney – to be highly correlated in respect of their enzyme proportions; the three pair-wise correlations were 0·95 or 0·96. For each individual the mean of the three organs was taken as the inactivation proportion of that individual for comparison with its brindled score. The relationship between inactivation proportion (as the independent

variate) and brindled score is shown in Fig. 2. The relationship is linear and very close, the correlation being 0·98 and the regression of inactivation proportion on brindled score 0·918 ± 0·034. From this we can conclude that mean brindled scores differing from 50 % by more than a few percentage points represent real deviations from random inactivation. The mean score of the High line was about 60 %. The lower 95 % confidence limit of the PGK proportion for a brindled score of 60 % is

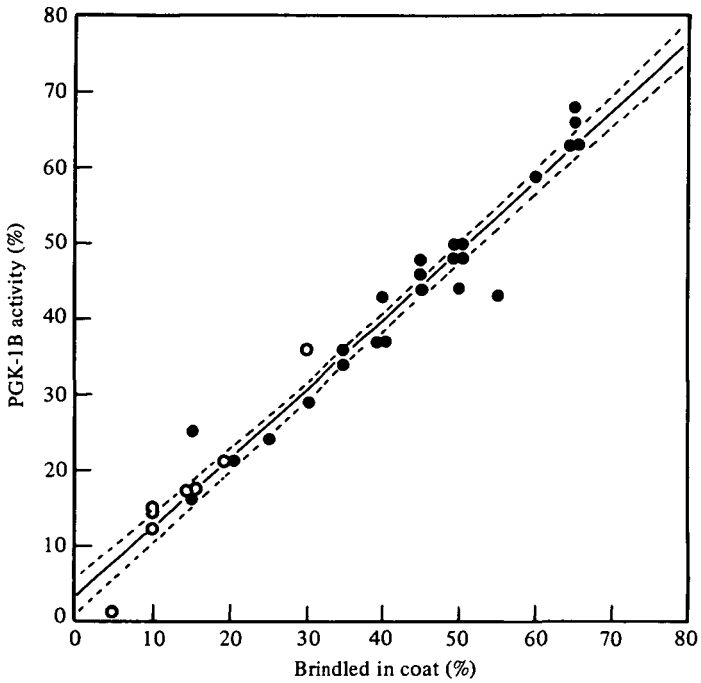

Fig. 2. Relation between *X*-inactivation proportions and brindled score. The vertical axis shows the proportion of cells in which the brindled chromosome is active as measured by the relative amount of PGK-1 B allozyme activity in blood, liver and kidney. The horizontal axis is the brindled score.

56·5 %. Inactivation was therefore non-random in both selection lines, the brindled chromosome being preferentially activated in the High line and preferentially inactivated in the Low line.

We should note here that the *X* chromosome from the PGK stock carrying *Pgk-1*^{a} substantially reduced the brindled scores. The crosses with the two lines were made at generations 27 to 31. Among 95 F_1 daughters from the High line the mean score was reduced from about 60 % to 43·6 ± 1·6, and among 124 from the Low line it was reduced from about 25 % to 17·7 ± 1·0, which would be further reduced if allowance were made for normal overlaps. This reducing effect of the *Pgk-1*^{a} chromosome has been noted by Johnston & Cattanach (1981).

(ii) *Regression of daughters' on mothers' scores*

In the next section we shall be comparing the *Br*/+ daughters of *Br* males with those of *Br*/+ females. These two groups differ not only in the sex of the parent transmitting *Br* but also in the phenotype of the mother, the daughters of *Br* males having non-brindled mothers with brindled scores of zero. Before these comparisons can be properly made we must therefore see if the scores of *Br*/+ daughters were

Table 1. *Regressions (within generations) of daughters' on mothers' brindled score*

Line	Generations	Regression ± S.E.	*t*	D.F.	*P*
High	10–21	−0·0572 ± 0·1038	0·551	37	
	22–31	+0·1960 ± 0·0958	2·046	57	< 0·05
Low	10–21	+0·0264 ± 0·0905	0·292	34	
	22–31	+0·1597 ± 0·0598	2·672	46	< 0·01
Both	10–21	−0·0125 ± 0·0683	0·183	72	
	22–31	+0·1801 ± 0·0611	2·949	104	< 0·01
	Differences between periods (22–31) − (10–21)				
High		0·2532 ± 0·1412	1·793	94	
Low		0·1333 ± 0·1085	1·229	80	
Both		0·1926 ± 0·0916	2·102	176	< 0·05

influenced by their mothers' phenotype through a maternal effect of the sort described for the *Vbr* allele by Cattanach & Papworth (1981). Accordingly we took the *Br*/+ daughters of *Br*/+ mothers and calculated the regression of daughters' *Br*-scores on their mothers' *Br*-scores. This was done within generations and the mean regression calculated from the total sums of squares and products within generations. In each generation all mothers with the same score were combined and the mean of their daughters calculated. The sums of squares and products were then calculated for the daughter-mean (Y) and the maternal score (X), each pair of X–Y values being weighted by the number of daughters. These weights, however, were scaled to have a mean of 1, so that the degrees of freedom for error were 2 less than the number of X–Y pairs. The generations were divided into two periods, from 10 to 21 and from 22 to 31, i.e. before and after the introduction of the new normal X chromosome. Generation 11 of both lines was omitted because there were only two maternal classes. In addition to the line matings, all other groups where the mothers were *Br*/+ were included.

The results are given in Table 1. The High and Low lines are consistent in showing no regression in the first period but a significant positive regression in the second period. This is 0·196 in the High line, 0·160 in the Low line and 0·180 ± 0·061 in the two lines combined. The generations with old and new normal chromosomes did not differ. The difference between the periods is significant ($P < 0·05$) when the two lines are combined. The regression of 0·18 in the second period is close to the value of 0·14 found with the *Vbr* allele by Cattanach & Papworth (1981).

The fact that this regression appeared only after the outcross that introduced

the new normal *X* chromosome suggests that its cause was not a maternal effect, but the introduction of some new genetic variation. It is important to decide which was the cause of the regression because the adjustments to be made are very different. If it is a maternal effect then all groups of both lines should be adjusted to a standard maternal score – e.g. 50 % – and non-brindled mothers should be assigned a score of 0. On the other hand, if the cause is genetic variation then the only adjustment needed is for the selection of parents in the lines, and non-brindled mothers, being unselected, should be assigned a score equal to the mean of their generation.

The maternal effect envisaged affects the score assigned but not the inactivation proportions. The results of the PGK enzyme assays described in the previous section seem to argue strongly against such a maternal effect. On closer examination, however, this is not so. We adjusted the *Br*-scores of all the assayed animals by the regression of 0·18 to a standard maternal score of 50 %. The regression of PGK proportion on the adjusted score was $1{\cdot}015 \pm 0{\cdot}044$. This is closer to unity than the regression on the unadjusted score, which was $0{\cdot}918 \pm 0{\cdot}034$. (The difference is $0{\cdot}097 \pm 0{\cdot}055$, $t = 1{\cdot}76$, $P = 0{\cdot}08$.) In other words the adjusted score gives a better prediction of inactivation proportion than the unadjusted score, and the improvement is not far from being significant. This does not prove conclusively that the cause of the daughter–mother regression was a maternal effect but on balance we think it is more likely than genetic variation and we shall proceed on this assumption. The reasons for preferring the maternal effect will be considered in the Discussion.

All generations and groups discussed in the next section were therefore adjusted by the regression 0·18 to a standard maternal score of 50 %, non-brindled mothers being assigned a score of 0 and *Br* 0 mothers a score of 100. The unadjusted means will, however, also be given. In addition, we made the adjustments appropriate to a genetic causation of the daughter–mother regression to see how different they would be, but these results will not be given in full. It should be noted that the regression of 0·18 by which the adjustments were made was not precisely estimated, having 95 % confidence limits of 0·058 and 0·302. For this reason no attempt will be made to test the significance of any differences between adjusted means.

(iii) *Effect of parental source*

In this section we describe the phenotypes resulting from transmission of brindled by *Br* males, by the daughters of these *Br* males, and by *Br O* females. Comparisons have to be made with the lines themselves, where transmission was continuously by *Br*/+ females.

Brindled males of both lines, surviving after treatment with copper, were mated to females of their own lines to produce *Br*/+ daughters contemporaneous with generations 23, 24, 26, 28 and 30 of the lines. For generations 23, 24 and 26 the males were mated to +/+ females, for generation 28 to *XO* females whose normal

X came from the lines, and for generation 30 to *Br*/+ females. The mean scores of these daughters are shown in Figs. 3 connected by broken lines to the generation of the selection line from which the *Br* males came. The unadjusted means are in Fig. 3(*a*) and the means adjusted from the maternal effect as described in the previous section are in Fig. 3(*b*). The unadjusted means of all groups to be compared in this section are given in Table 2. Before proceeding we must explain

Table 2. *Mean* Br*-scores in selection lines and contemporary groups with different sources of the* Br *chromosome*

(Transmission in the lines was always from the mother. Means not adjusted for maternal score.)

Gen.	n*	Mean ± S.E.	m*	n*	Mean ± S.E.	*Br* source	Normal *X*†
	High line		Contemporary groups				
23	45	59·0 ± 0·9	1	5	60·0 ± 10·0	Father	½ O:½ N
24	67	59·3 ± 1·3	2	55	55·5 ± 2·9	Father	½ O:½ N
25	43	58·5 ± 2·5		51	60·6 ± 2·6	Grandfather	N
26	55	63·7 ± 1·1	7	33	52·9 ± 3·3	Father	½ O:½ N
27	69	66·5 ± 1·6		63	61·6 ± 1·9	Grandfather	N
			3	24	67·1 ± 3·4	*Br O* mother	N
28	31	66·5 ± 2·4	1	6	55·8 ± 10·6	Father	O
			1	11	64·1 ± 5·4	Father	N
29	65	65·2 ± 1·4		—	—	—	—
30	60	56·3 ± 1·7	4	11	66·4 ± 3·3	Father	O
	Low line		Contemporary groups				
23	57	28·7 ± 1·1	3	8	40·6 ± 7·8	Father	½ O:½ N
24	78	26·2 ± 1·2	3	35	48·1 ± 3·2	Father	½ O:½ N
25	53	19·3 ± 1·7		68	28·1 ± 1·6	Grandfather	N
26	77	21·3 ± 1·0	19	94	40·2 ± 2·0	Father	½ O:½ N
27	70	14·0 ± 1·2		70	23·2 ± 2·1	Grandfather	N
			7	53	29·7 ± 2·8	*Br O* mother	N
28	45	24·4 ± 2·3	4	38	44·5 ± 2·2	Father	O
			10	58	14·0 ± 1·5	Father	N
29	90	23·6 ± 1·4		—	—	—	—
30	75	27·3 ± 1·6	19	56	45·1 ± 2·3	Father	O

* m = number of *Br* fathers or *Br O* mothers; n = number of daughters measured.

† Identity of normal *X* chromosome in contemporary groups: O = original; N = new. The lines have the original in generations 28–30, and the new in generations 25 and 27; generations 23, 24 and 26 are adjusted as explained in the text for comparison with the contemporary group.

the adjustments to the line means in some generations needed to allow for the different effects of the two normal *X* chromosomes whose presence was explained in Section 3(ii).

The females to which the brindled males were mated for generations 23, 24 and 26 were all non-brindled and were heterozygous for the original and the new normal chromosome. The daughters of the brindled males in these generations were therefore of two sorts, in equal proportions, one with the original and the other with the new normal chromosome. The values of the lines with which they should

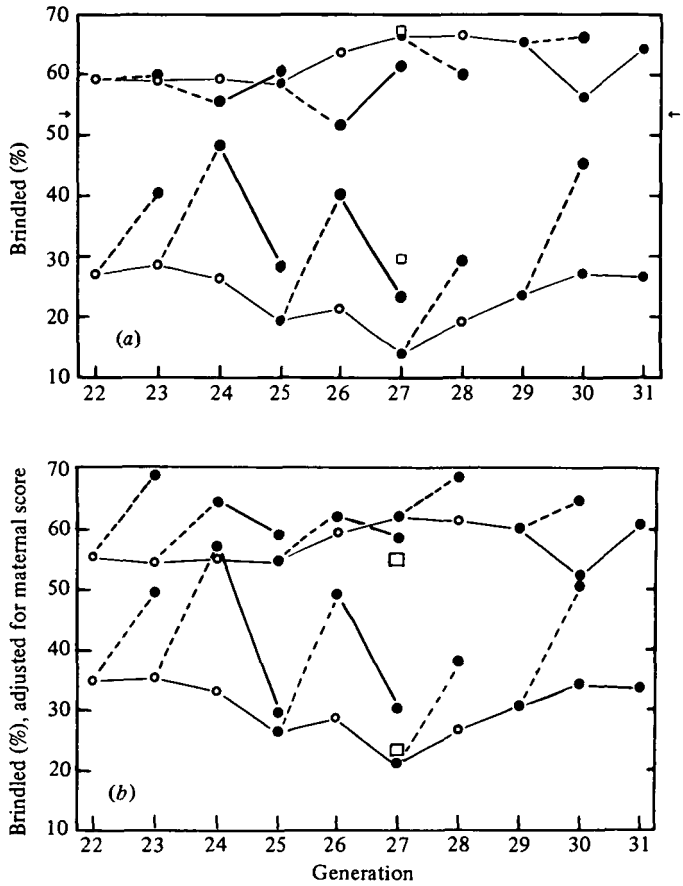

Fig. 3. Effects of parental source on brindled scores. The points are the mean scores of heterozygous brindled daughters. Transmission from female parents is indicated by continuous lines, and transmission from male parents by broken lines, connected to the generation from which the parents came. The squares are the daughters of *Br O* females. The generation means of the selection lines are shown by open circles where they are adjusted as explained in section 4(ii) and by filled circles where they are not adjusted. (*a*) Means not adjusted for maternal score. (*b*) Means adjusted by regression, $b = 0{\cdot}18$, to a standard maternal score of 50%.

be compared are therefore the averages of generations with original and with new chromosomes. The adjusted means were calculated as follows, where m is the observed mean and t signifies the generation to be adjusted:

$$\text{adjusted } m_t = \tfrac{1}{2}m_t + \tfrac{1}{4}m_{t-1} + \tfrac{1}{4}m_{t+1}.$$

The sampling variances of the adjusted means were calculated in the same way, with coefficients $\frac{1}{4}$ and $\frac{1}{16}$ in place of $\frac{1}{2}$ and $\frac{1}{4}$. The generation means that have been adjusted in this way are shown as open circles in Figs. 3, the unadjusted

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generations as closed circles. For generation 28 the brindled males were mated to *XO* females in which the *X* chromosome was one of the normal chromosomes from the line, and its identity was known from the pedigrees. Daughters with the original *X* may be compared with generation 28 of the lines, and daughters with the new *X* with generation 27. The points in Figs. 3 are unweighted means of the two groups and generations. Finally, in generation 30 only the original *X* was present and no adjustment is needed. It should be mentioned, however, that the females to which the brindled males were mated for generation 30 were brindled heterozygotes. The heterozygous daughters, with which we are concerned here, can only have got their brindled chromosome from their fathers.

(*a*) *Transmission by males.* The *Br* males of the Low line amply confirm the previous result: much of the low expression of the line was lost when the brindled chromosome was transmitted by males. Or, to put this in another way, the activation probability of the *Br*-chromosome was much greater when paternally than when maternally derived. The picture in the High line is less clear. The unadjusted means show little or no difference between paternal and maternal transmission. The adjusted means, however, show the paternally derived *Br*-chromosome to have a higher probability of activation, as in the Low line and in agreement with Cattanach & Perez (1970). The parental source, i.e. the difference between male and female transmission, was considerably greater in the Low line than in the High. Table 3 summarizes the comparisons of adjusted means in the five generations where male and female transmission was compared. The weighted mean difference between male and female transmission is 8·4 percentage points in the High line and 18·0 in the Low line. These are larger than the differences reported by Cattanach & Perez (1970). The mean difference between the selected lines in these five generations was 25·8 percentage points with transmission by females and 16·2 with transmission by males.

It is worth noting the effect of adjustments made on the supposition that the daughter–mother regression was caused by genetic variation. After adjustment to a maternal score equal to that of the contemporary line generations, the daughters of *Br* males were raised in the High line and lowered in the Low line by amounts averaging 2 percentage points in both lines. This adjustment would therefore have very little effect on the conclusions that might be drawn from the unadjusted means.

(*b*) *Transmission by daughters of* Br *males.* If the brindled chromosome is transmitted by females after having been transmitted by males, will any of the effect of male transmission persist in the grand-daughters? In other words, will the chromosome 'remember' its grandparental source? To answer this question we mated the daughters of the brindled males described above to normal males of their own lines. The grand-daughters of the brindled males were contemporaneous with generations 25 and 27. Their means are shown in Figs. 3, connected by solid lines with the daughters of the brindled males. All these grand-daughters were known from the pedigrees to have the new normal chromosome, so they are directly comparable with the line-means in generations 25 and 27.

The conclusions are not completely clear. In the High line there was no carry-over of the effect of male transmission, the grand-daughters having scores not differing from the line itself. In the Low line, however, the grand-daughters were above the line in both generations. In generation 27 the maternal scores of the two groups were identical so a valid comparison can be made between the unadjusted means. The difference is $9{\cdot}2 \pm 2{\cdot}4$, $t = 3{\cdot}82$, $P < 0{\cdot}001$. In view of the difficulty in imagining

Table 3. *Effect of parental source after adjustment for the maternal effect and for the different normal chromosomes*

(Columns headed *m* are the line means with maternal transmission of the *Br* chromosome. Columns headed *p* are the contemporary daughters of *Br* males, with paternal transmission.)

	High line			Low line		
Generation	*m*	*p*	(*p*−*m*)	*m*	*p*	(*p*−*m*)
23	54·6	69·0	14·4	35·2	49·6	14·4
24	55·4	64·5	9·1	33·0	57·1	24·1
26	59·4	61·9	2·5	28·5	49·2	20·7
28*	62·0	69·0	7·0	26·6	38·2	11·6
30	52·4	64·8	12·4	34·2	50·9	16·7
Means†			8·4			18·0
Mean† line-difference (H − L)						
Maternal transmission				24·4		
Paternal transmission				15·2		

* Generation 28: under *p* are the unweighted means of daughters with old and new normal *X* chromosomes; under *m* are the unweighted means of generations 27 (new *X*) and 28 (old *X*) of the lines.

† All means weighted by $n_1 n_2/(n_1+n_2)$, *n* being the number of daughters scored.

how the parental source effect could be carried over to the next generation, this difference, though highly significant, cannot be accepted as sufficient proof of a carry-over.

(*c*) *Transmission by* Br O *females*. The reason for the inactivation of a chromosome differing according to whether it comes from the mother or the father might possibly be connected with the number of *X* chromosomes in females and males. In order to test this possibility we made *Br O* females, i.e. *XO* females with the brindled chromosome from the lines as their single *X* chromosome. If the number of *X* chromosomes is implicated in the parental-source effect, *Br O* females should behave like males in respect of inactivation in their daughters; if not, they should behave like females. To make the *Br O* females, brindled males from each line were crossed to an *XO* stock. Owing to the difficulty of rearing *Br O* females, only three were obtained from the High line and seven from the Low line. The *Br O* females were mated to normal males of their own line to produce heterozygous brindled daughters contemporaneous with generation 27. The mean scores of these daughters are shown by squares in Figs. 3. In the High line they were almost identical with the line itself, but in the Low line they were substantially above the line-mean.

It is not clear whether the adjustment for the maternal effect was correctly made by assigning a score of 100 to *Br O* females, because the *Br*/+ daughters were fostered at birth. On the basis of adjusted means, transmission by *Br O* females did not differ from transmission by *Br*/+ females. On the basis of unadjusted means, transmission by *Br O* females of the Low line resulted in scores significantly higher than transmission by *Br*/+ females (difference = $15{\cdot}7 \pm 2{\cdot}8$, $t = 5{\cdot}6$, $P < 0{\cdot}001$). This single test, however, cannot be accepted as conclusive and we regard the results of transmission by *Br O* females as ambiguous.

(iv) *Line-crosses and test of cytoplasmic factors*

The final experiment to be described was designed to test the possibility that the differences between the lines in their inactivation probabilities might be the result of a maternal effect, a possibility that was not fully excluded by the earlier work. The maternal effect considered in the preceding sections was one affecting the brindled phenotype but not the inactivation proportion. Here we are concerned with one causing non-random inactivation. Such a maternal effect might be mediated by factors in the egg cytoplasm and, to avoid confusion, it will be referred to as a cytoplasmic effect.

The experiment also provided tests of autosomal effects and of whether the normal *X* chromosomes of the two lines differed in their inactivation properties; the previous results had proved them to be the same in the earlier generations. The plan of the experiment was to obtain *Br*/+ daughters bred from *Br*/*Br* homozygotes of four sorts, produced by matings within the lines and by reciprocal crosses. Two generations were required for the test, the first to produce the *Br*/*Br* females and the second to produce their daughters. The second generation provided the test of a cytoplasmic effect. The first generation, which involved line crosses, tested the effects of the autosomes and of the normal *X* chromosomes. We shall start by describing this first generation.

(*a*) *Line crosses.* To produce *Br*/*Br* females, matings were made between *Br*/+ ♀♀ and *Br* ♂♂. The matings were of four sorts: within the two lines and reciprocal crosses, i.e. $H \times H$, $L \times L$, $H \times L$ and $L \times H$. We are concerned here with the heterozygous *Br*/+ progeny. They got their *Br* chromosomes from their fathers and their normal chromosomes from their mothers. So in the comparisons to be made we are dealing with transmission by brindled males. The $H \times H$ and $L \times L$ matings provided part of the data on transmission by males already considered; they are the groups contemporaneous with generation 30. Here we are concerned with comparing the effects of the chromosomes from the two lines. Table 4 gives the mean scores of the progeny of the four types of mating, both unadjusted and adjusted for maternal scores. The unadjusted means suggest some difference, averaging 6·3, between the two normal chromosomes maternally transmitted. This difference, however, disappears in the adjusted scores, and the conclusion must be that the normal *X* chromosomes of the two lines did not differ in their inactivation probabilities. The difference between the two *Br* chromosomes paternally trans-

mitted is 15·4. The average obtained from all generations with male transmission was 15·2 (Table 3). Evidence about autosomal effects comes from comparing the lines with the crosses. The crosses differed very little from the lines so there is no evidence of autosomal effects. Thus, this generation confirms the two conclusions from their early generations of the experiment: that neither the normal *X* chromosome nor the autosomes were altered by the selection.

Table 4. *Means of lines and reciprocal crosses, all with the* Br *chromosome transmitted by males*

(Numbers of *Br*/+ progeny classified are in parentheses.)

Maternal chromosome	Paternal chromosome: *Br H p*	Paternal chromosome: *Br L p*	Difference (*H* − *L*)	
	Unadjusted means			
+*H m*	(11) 66·4 ± 3·3	(48) 53·2 ± 2·2	13·2	15·0
+*L m*	(16) 61·9 ± 3·3	(56) 45·1 ± 2·3	16·8	
Difference (*H* − *L*)	4·5	8·1		
	6·3			
	Adjusted means			
+*H m*	64·8	50·8	14·0	15·4
+*L m*	67·7	50·9	16·8	
Difference (*H* − *L*)	−2·9	−0·1		
	−1·5			

Table 5. *Mean scores* (± S.E.) *of* Br/+ *daughters of* Br/Br *mothers of four types, all mated to normal males of the Low line*

Group ...	H/H	L/L	H/L	L/H
Maternal genotype	Br H m / Br H p	Br L m / Br L p	Br H m / Br L p	Br L m / Br H p
Paternal genotype	+ *L*	+ *L*	+ *L*	+ *L*
Egg cytoplasm	*H*	*L*	F_1	F_1
No. scored	107	162	164	141
Mean	57·6 ± 1·2	31·2 ± 1·3	43·2 ± 1·5	42·7 ± 1·4
Variance	167·1	276·3	356·1	290·6

(*b*) *Test of cytoplasmic effect*. Four sorts of brindled homozygotes were produced as explained above, two being homozygous for chromosomes from their own lines and two being reciprocal F_1's of crosses, and therefore heterozygous for *Br H* and *Br L* chromosomes. All these *Br*/*Br* females were mated to normal males from the Low line so that the daughters were all *Br*/+ with the same normal chromosome, and all got their brindled chromosome from the mother. No adjustment for maternal score is needed because all mothers were the same in phenotype. The genotypes of the parents and the mean scores of the daughters are given in Table

5. The four progeny groups will be referred to by the abbreviated symbols for the genotypes of the mothers, as shown at the heads of the columns. The H/H and L/L groups of daughters had egg cytoplasm characteristic of their own lines, but the H/L and L/H groups had the egg cytoplasm from the F_1 of the cross. Thus comparisons of groups H/H and L/L with H/L and L/H tests for a cytoplasmic effect. If there is no cytoplasmic effect, the H/L and L/H mothers should produce half their daughters with High-line scores and half with Low-line scores. The

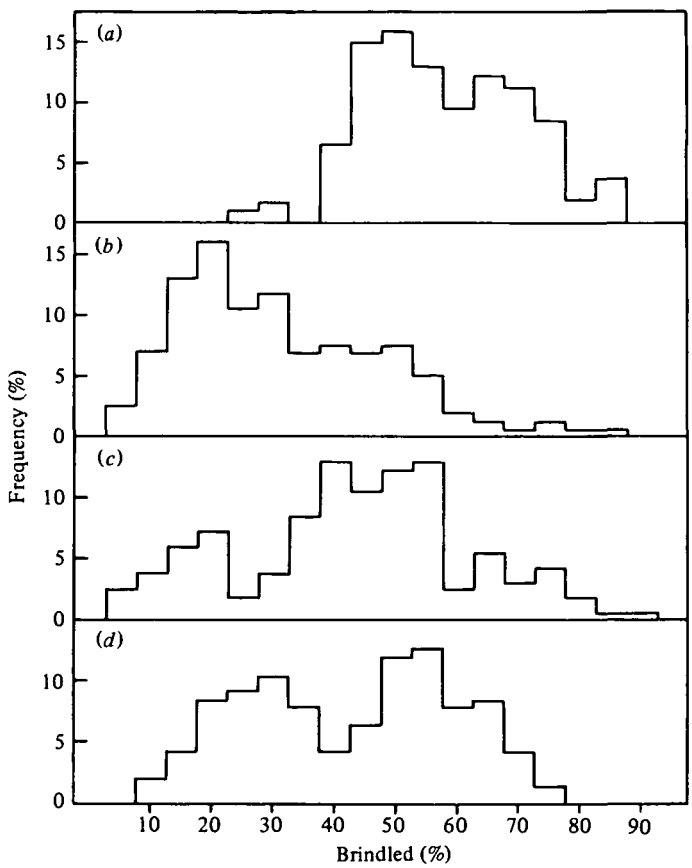

Fig. 4. Frequency distributions of brindled scores in the progeny of brindled homozygotes all mated to + L males. The mothers are (*a*) *Br H/Br H*, (*b*) *Br L/Br L*, (*c*) *Br H m/Br L p*, (*d*) *Br L m/Br H p*.

distribution of the scores should therefore be the same as those of the H/H and L/L groups together. If, on the other hand, the inactivation probability is determined by the egg cytoplasm, the daughters in the H/L and L/H groups should form a single distribution, with scores centred round some new value intermediate between the H/H and L/L groups. The observed distributions are clearly in accordance with the first but not the second expectation. The distributions of the

four groups are shown separately in Fig. 4. The comparison is made more clearly in Fig. 5. Here the H/H and L/L groups are added together in a combined distribution for comparison with the H/L and L/H distributions, also combined. The two combined distributions have the same range and are very similar in shape, which is what would be expected in the absence of a cytoplasmic effect.

To find out how alike the two combined distributions are, a $2 \times n$ heterogeneity χ^2 was calculated as follows. The combined distribution of H/L and L/H was got simply by adding the numbers of the two groups in each phenotypic class. The

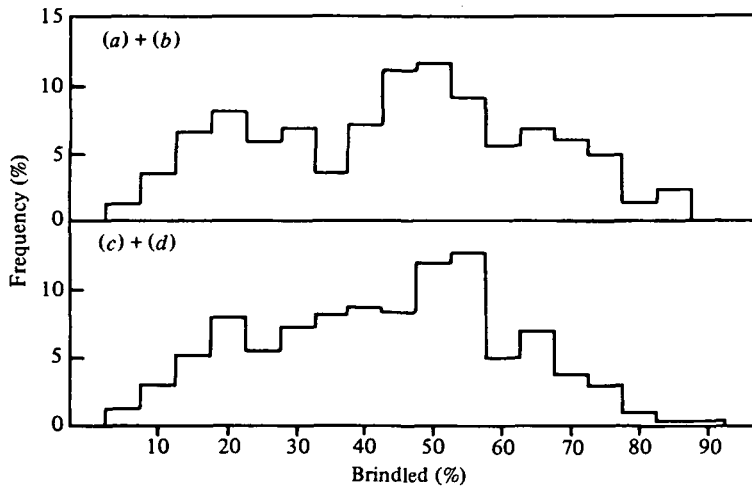

Fig. 5. Frequency distributions of Fig. 4 combined as explained in the text.

combined distribution of H/H and L/L was the distribution expected if the total number of 269 individuals classified had been equally divided between the two groups. Comparison of these two combined distributions gave $\chi^2 = 13{\cdot}8$, D.F. $= 16$, $P \sim 0{\cdot}6$. Thus the two distributions resemble each other very closely, and there is no evidence that the cytoplasm, or any other form of maternal effect, contributes to the different inactivation probabilities in the two selected lines.

5. DISCUSSION

(*a*) *Conclusions.* The main conclusions are the following.

(1) *X* chromosome inactivation was non-random in both selected lines.

(2) The difference between the lines in their inactivation proportions was entirely due to altered properties of the chromosomes marked by *Br*. The unmarked, normal, chromosomes in the two lines did not differ. The autosomes had no effect on the inactivation proportions and nor did any maternal effect such as might be transmitted in the egg cytoplasm.

(3) The visually assigned scores of *Br*/+ females were correlated (within lines

and generations) with the scores of their mothers. The adjustments needed to allow for this correlation depend critically on its cause which was not firmly established. In consequence some of the conclusions are conditional on the right adjustments having been made.

(4) The inactivation proportions in the Low line were strongly affected by the sex of the parent transmitting the *Br*-chromosome, which had a higher probability of activation when transmitted by males than when transmitted by females. This was true also of the High line if the adjustment made for maternal scores was correct, though the differences between male and female transmission was less marked.

(5) The effect of the sex of the transmitting parent was not carried over the the next generation in the High line, i.e. there was no effect of the sex of the grand parent. The evidence on this point from the Low line was inconclusive.

(6) *Br O* females of the High line did not differ from *Br*/+ females in the activiation probability of the transmitted *Br*-chromosome. On this point also the evidence from the Low line was inconclusive.

(*b*) *Maternal effect mediated by copper deficiency*. The evidence concerning the cause of the correlation of mothers and daughters in respect of their *Br*-scores was conflicting. The facts that the correlation was not present before the outcrosses that introduced the new normal *X* chromosome, and that the Low line appeared to respond to selection after that time, point to genetic variation as the cause. We chose, however, to attribute it to a maternal effect of the sort known to affect the *Vbr* allele (Cattanach & Papworth, 1981): the greater copper deficiency in high-scoring mothers leads to reduced pigmentation in the mutant patches of the daughters, and so to higher scores, but without affecting the inactivation proportions. The reasons for choosing this interpretation were (1) that, when the appropriate adjustment for maternal score was made, the adjusted score gave a better prediction of the inactivation proportions as estimated from the PGK allozyme proportions; (2) it is hard to see how genetic variation within the lines introduced by the crosses could be confined to the *Br* chromosomes; (3) the precedent of the maternal effect on *Vbr* scores; and (4) on this interpretation transmission by males of the High line falls better into line with what was previously known of male transmission (Cattanach & Perez, 1970).

The uncertainty about the cause of the daughter–mother correlation, and consequently about the appropriate adjustment for maternal scores, puts the following conclusions in some doubt: that male transmission in the High line increased the activation probability, that the effect of male transmission was not carried over to the next generation, and that *X O* females transmitted like *Br*/+ females. In what follows, however, we shall assume these conclusions to be valid.

(*c*) *Relative 'strength' of chromosomes*. If one *X* chromosome is preferentially activated the other must be preferentially inactivated. It is therefore meaningless to speak of the activation probability of a chromosome without specifying the other chromosome with which it is in 'competition'. For want of better terms, we shall refer to the chromosomes of a pair as being 'strong' and 'weak' in respect of their

probabilities of being active. The High line therefore has a strong *Br*-chromosome and a weak normal chromosome, while the Low line has a weak *Br*-chromosome and a strong normal chromosome. The normal chromosomes of the two lines, however, were shown to be of equal strength when transmitted by females (this paper) or by males (Falconer & Isaacson, 1972). Therefore the *Br*-chromosome of the High line must be stronger than that of the Low line and this is the cause of the difference between the lines.

(*d*) *The selection.* The original selection was, in effect, selection between families. It resulted in all but one of the original brindled chromosomes being eliminated from the Low line and all but two from the High line (Falconer & Isaacson, 1972), one of which was subsequently lost at generation 21. After generation 5 selection was within families on individual scores. Selection within full-sib families cannot discriminate between whole chromosomes because all $Br/+$ females within a family have the same *Br* chromosome from the mother and the same + chromosome from the father. So it can produce a response only if useful recombinants can occur. Selection for high scores selects simultaneously for a stronger brindled chromosome and a weaker normal chromosome. If the strength of a chromosome were determined by several loci on it, recombination could produce stronger or weaker chromosomes. But if the strength is determined by a single locus no useful recombination can occur. In the High line for example, recombination between this locus and the locus of *Br* would produce a weaker brindled chromosome and a stronger normal chromosome. The only effect of the continued within-family selection would then be to eliminate such recombinants. The fact that no further response occurred, at least up to generation 20, argues for a single locus controlling the strength.

(*e*) Xce *locus.* The rapid initial response and the subsequent stability of the lines are consistent with the inactivation probabilities being controlled by the *Xce* locus (Cattanach & Isaacson, 1967; Cattanach, 1975). The original stock must have contained two different alleles of *Xce* on the *Br*-chromosomes, with the stronger one at a higher frequency than the weaker. Fixation of the stronger one led to a small response in the High line; elimination of the stronger one led to a larger response in the Low line. The normal chromosomes must have all had the same *Xce* allele since their strenght remained the same in both lines. *Xce* is closely linked to *Br* (Cattanach & Papworth, 1981) so after the brindled chromosomes had been fixed, the lines would be expected to remain stable, with any rare recombinants being eliminated by the continued selection.

If the *Xce* locus is responsible for the non-random *X*-inactivation in the lines, one or perhaps two additional alleles are needed. There are three known alleles (Cattanach & Papworth, 1981). Xce^a is present in the CBA/H and four other inbred strains, Xce^b in C57BL/GoH and one other inbred strain, and Xce^c is associated with the $Pgk\text{-}1^a$ allele in the PGK stock. In what follows we shall refer to these *Xce* alleles as *a*, *b* and *c*. Their relative strengths are in the order $c > b > a$. Can we determine the relative strengths of the alleles in the selection lines and the strengths of these relative to the known alleles? Four chromosomes are involved

in the selection lines: the two *Br*-chromosomes, whose *Xce* alleles will be referred to as *H* and *L* for the High line and Low line *Br*-chromosomes respectively; the original normal chromosome (symbolized by *N*); the new normal chromosome introduced at generation 21 (symbolized by *n*). In addition there are data on the *c* allele following crosses to the PGK stock. Table 6 summarizes the adjusted *Br*-scores of the *Br H* and *Br L* chromosomes when maternally derived, in combination with the three paternally derived normal chromosomes. The order of

Table 6. *Summary of the activation probabilities of the two* Br*-chromosomes in combination with the three normal chromosomes*

(All *Br*-scores adjusted for maternal score. The putative *Xce* alleles are symbolized by: *H* and *L* on *Br*-chromosomes in High and Low lines; *N* and *n* on original and 'new' normal chromosomes in the lines; *c* from PGK-A stock designated Xce^c by Johnston & Cattanach (1981).)

	Maternal	
Paternal*	*H*	*L*
c	42	23
n	54	27
N	59	34

* Sources of data: *c*, from the unadjusted means given in Section 4(i); *n*, unweighted means of line-generations 21, 23, 25, 27; *N*, unweighted means of line-generations 20, 22, 24, 26, 28–31.

the strengths of the alleles on the three normal chromosomes is $c > n > N$. The original normal chromosome (*N*) was derived from the CBA and RIII inbred strains (Falconer & Isaacson, 1972), and CBA, at least in another sub-line, is known to carry the *a* allele (Cattanach & Papworth, 1981). It is therefore reasonable to equate *N* with *a* and *n* with *b*. We then have the three known alleles, *a*, *b* and *c* represented by *N*, *n* and *c* on the three normal chromosomes. The question then is whether one or both of the *Br* chromosomes carry alleles different from these three. The large parental source effect makes this question difficult to answer. The *Br H* chromosome gave non-random inactivation in combination with all three normal chromosomes both when maternally and when paternally transmitted. *H* therefore seems to be different from all three known alleles, with strength between that of *c* and *b*. The *Br L* chromosome gave very non-random inactivation when maternally transmitted, but when paternally transmitted the inactivation could have been random in combination with *N* and *n* (see Fig. 3 and Table 3). The *Br L* chromosome, therefore, might possibly have carried the *a* allele, but it seems more probable that it carried an allele weaker than *a*. To sum up, if the three normal chromosomes involved in these experiments carried the three known alleles of *Xce*, then one of the *Br* chromosomes, and more probably both, carried alleles different from any of the three known ones.

(*f*) *Imprinting*. The effect that the sex of the parent has on the chromosome it transmits has been described as 'imprinting' (Chandra & Brown, 1975). Preferential activation due to imprinting is the normal situation in marsupials, where the

maternal *X* chromosome is preferentially activated in most tissues (Sharman, 1971), and in the extra-embryonic membranes of the mouse, where again the maternal chromosome is the active one in most or all of the cells (Papaioannou & West, 1981). Imprinting in the mouse is thus a normal occurrence, though a strongly preferential activation is normally seen only in the extra-embryonic membranes. Imprinting has an opposite effect in the somatic tissues, where it is the paternally derived chromosome that has an increased probability of being the active one. Our results agree with those of Cattanach & Perez (1970) in showing that paternal transmission increased the probability of activation. The effect of imprinting was much greater in our lines than in previous reports. The probability of activation of the *Br* chromosome in the High line was increased by 8·4 percentage points by male transmission, and the *Br* chromosome in the Low line was increased by 18·0 percentage points. The increases in the probability of activation with male transmission reported by Cattanach & Perez (1970) were about 3·8 percentage points in a line scoring 61 % with female transmission and 6·4 % in a line scoring 52 %. Johnston & Cattanach (1981) estimated the activation proportions of the Xce^c allele by means of the PGK-1 allozymes. In combination with Xce^b, male transmission increased it from 63 to 64 % and in combination with Xce^a from 71 to 74 %.

The large parental source effect in our results raises the question of whether the lines could have differed in the strength of their imprinting. The answer to this must be no, because only the *Br*-chromosomes showed effects that might be ascribed to differences of imprinting, the normal chromosomes being unaffected. The two *Br*-chromosomes differed not only in their strength but also in their reaction to imprinting, the difference between them being 40 % less when transmitted by males than when transmitted by females. If the difference in strength is attributable to the *Xce* locus, the possibility that the reaction to imprinting is also a property of the *Xce* locus should be considered.

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