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The quantitative relations between variation in red eye pigment and related pteridine compounds in *Drosophila melanogaster*

BY ILSE B. BARTHELMESS AND FORBES W. ROBERTSON*
Department of Genetics, University of Edinburgh

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SUMMARY

The relations between the quantity of red eye pigment and related pteridine compounds of *Drosophila melanogaster* have been studied in a variety of genotypes, which include strains selected for high or low pigment content, various derivatives of these lines and also lines in which one or other of the major autosome pairs were represented by homozygous chromosome pairs, derived by random sampling from the base population and also inbred lines. The quantity of red pigment was defined by the optical density when whole heads were extracted in a suitable solvent, while the pteridines were separated by chromatography and their amounts estimated by means of their characteristic fluorescence.

The evidence from selection, inbreeding and chromosome sampling from the base population demonstrated the presence of substantial genetic variation for pigment content and amounts of related pteridines.

The genetic and biochemical properties of the selected lines differed according to the direction of selection. High lines remained heterozygous after many generations of selection and displayed dominance and epistasis in favour of higher pigment content in crosses to the unselected stock. Selection for low pigment content led to fixation of recessive effects, attributable to particular chromosomes. The dominance-recessive relationship in red pigment differences was also applicable to the associated pteridines.

The metabolic pattern in all lines with reduced pigment content is compatible with the assumption of reduced enzyme activity at particular steps of the pathway leading to the drosopterins (red eye pigments). The two steps accessible to study are subject to genetic variation in the base population, while inbreeding or selection for low pigment content leads to genetically fixed alterations at one or other of these steps. The genetic analysis was consistent with the biochemical evidence.

Increase in pigment content above the normal level, either by selection or chance fixation, is accompanied by correlated increase in all the precursors. Several alternatives are possible but it is suggested that this may be due to an increase in early precursors, before the stages which have been altered in the low pigment lines.

Attention is drawn to the similarity in genetic behaviour between

* Member of the Agricultural Research Council Unit of Animal Genetics.

pigment content and body size. Particular emphasis is laid on the value of selection as a means of creating biochemical differences which offer a basis for relating biochemical function and genetic behaviour.

1. INTRODUCTION

The red pigments in the eye of the individual adult *Drosophila* can be extracted with an appropriate solvent and the quantity estimated spectrophotometrically. Hence variation in this 'character' may be handled by the methods used in biometrical genetics. A survey of the general properties of such variation, both within and between populations, by means of selection, inbreeding, etc., has been described in an earlier publication (Chauhan & Robertson, 1966), which showed that there is abundant genetic variation within populations and that selection in either direction leads to substantial differences in the quantity of eye pigments. The trait has the attributes of a quantitative character, which varies about an intermediate optimum.

Quantitative characters are highly complex in the developmental and biochemical processes which are involved in their phenotypic expression, although some are less complex than others. Since the red pigments are composed of a few closely related drosopterins, and since some information already exists about the biochemical steps which precede their formation (Ziegler, 1961*a*, 1965), this character might allow more insight into the underlying complexity than most of those which have been studied in higher organisms. A number of the associated pteridines, which are related to pigment precursors, can be separated by chromatography, as first shown by Hadorn & Mitchell (1951; see Ziegler, 1961, for a literature review), eluted and quantitatively estimated by their degree of fluorescence at particular wavelengths.

Major obstacles, however, hinder our understanding of the biosynthesis of the pigment compounds. The exact sequence of antecedent steps is still uncertain; the enzymes which mediate the successive conversions are mostly unknown and a number of the pteridines, which are separated by chromatography, are breakdown products, according to Ziegler (1961*a*, 1965). Furthermore, variation in the 'quantity of red pigments', as defined by the optical density of extracts of *Drosophila* heads, may depend on a number of physiological factors, such as eye size, number of pigment granules in the pigment cells, tissue specific distributions of precursors, etc., apart from the biochemical steps involved in the synthesis of end products.

This paper attempts to analyse the contributions of the biochemical variation which can be exposed by selection and other means. It deals with lines of *Drosophila melanogaster* selected for high or low pigment quantity, as well as various inbred lines. A study of the pattern of correlated biochemical changes, when the quantity of pigments has been selected for, narrows the range of alternative biosynthetic explanations. Pleiotropic effects of different red eye-colour mutants have been used pragmatically to identify them, ever since Hadorn & Mitchell (1951) discovered this

possibility (e.g. Hadorn, 1956; Gregg & Smucker, 1965). As these compounds occur in a metabolically interrelated system, we are using pleiotropy in the present study not only to obtain general information about the effects of selection but also to deduce primary effects in the metabolic pathway which involves the red eye pigments and related pteridines. We wish to stress the largely unexploited value of selection in successive generations as a tool for creating substantial biochemical differences, since this is often the quickest way to create material for genetic and biochemical analysis. Such a study may provide a basis for comparisons with other characters, which are subject to genetic variation within populations and indicate profitable areas for future study. It is to be hoped that further progress along these lines will help to bridge the gap between population and biochemical genetics.

2. MATERIALS AND METHODS

Selected and inbred lines were derived from the long-maintained Pacific cage population of *D. melanogaster*; for details of selection procedure, etc., see Chauhan & Robertson (1966). Two pairs of lines were studied, each of which included a line selected for high (H) or low (L) pigment content, but which differed according to whether family (H_1 and L_1) or phenotypic selection (H_2 and L_2) had been applied.

A variety of alternative genotypes was created by combining particular chromosomes from the selected lines and/or the base population or by making one or the other or both major autosomes homozygous in different genetic backgrounds. This was achieved by making use of marked inversions, which suppress recombination in the chromosome concerned and an appropriate series of crosses; see Robertson (1954) for general procedure. Either Curly (*Cy*) or Curly Lobe-4 (CyL^4) was used for chromosome II, Moiré (*Mé*), Ultra-bithorax (*Ubx*) or Stubble (*Sb*) for III and cubitus interruptus (ci^D) for IV. *Sb* was used only as a marker, not as a cross-over suppressor.

The various categories included: (i) lines isogenic for chromosomes II and III, which were extracted from the selected lines; (ii) derivatives of the selected lines in which the mutant *vermilion* had been introduced; (iii) lines in which markers for each autosome were incorporated in the background of the selected lines, to produce all possible combinations of either homozygous or heterozygous autosomes; (iv) sub-lines in which individual pairs of chromosomes from selected lines H_2 and L_2 were introduced into the genetic background of the Pacific population; (v) partially isogenic lines in which individual chromosomes II or III from the Pacific population were made homozyous in the otherwise heterozygous Pacific background.

Flies were grown at 25 °C on the usual cornmeal molasses medium, fortified with dried yeast, at densities per vial which minimized variation in body size. Whenever possible, eye width was scored on the flies from which pigment was extracted. Red pigment extraction followed the procedure recommended by Ephrussi & Herold (1944). The heads, from which the proboscis and clypeus were removed, were sub-

merged for 24 h at 25 °C in 30 % ethanol, acidified to pH 2 with hydrochloric acid (A.E.A.), generally with 0·5 ml A.E.A. per head. Absorption was recorded in a spectrophotometer at 480 mμ, the wavelength of maximum absorption (Nolte, 1952*a*, *b*). Pigment content is generally expressed in terms of optical density. Pigment was extracted from flies of comparable age, usually between 4 and 7 days when pigment content reaches a constant level (Chauhan & Robertson, 1966). There is variation in optical density score between experiments, as for example in the values of 0·085 and 0·077 for the controls in Tables 1 and 4. This could be due to minor environmental differences and so each set of experiments included a Pacific control group as a reference.

The fluorescing compounds in the heads of flies were separated by descending paper chromatography. Heads of male or female flies, singly or in groups of three, were squashed on Whatman no. 1 paper with the 'erlenmayer shape' exactly as described by Gregg & Smucker (1965). This and any further handling of the papers was carried out in dim electric light, since most of the fluorescing compounds are light sensitive. Papers were equilibrated in the dark for 24 h in an atmosphere of ammonia and then developed for 17–19 h with a propanol ammonia solvent (*n*-propanol:distilled water: 1 N ammonium hydroxide in the ratio 8:3:1). After drying the papers were viewed under a u.v. mineral light (Hanovia 'Chromatolite' with 90 % of the output at 2537 Å) and the fluorescing areas were outlined. The marked spots were cut from the paper, placed individually in 1·5 ml of 0·02 N ammonium hydroxide and stored for 20 h at 25 °C. Blanks were prepared by cutting similar areas of paper from non-fluorescing regions and extracted in the same way. Fluorescence determinations were carried out with a Locarte fluorimeter with filter combinations rating 254–400 mμ at the primary and 436 mμ (monochromatic) at the secondary position.

The same filter combination was used for all compounds and readings are given in relative fluoresence units. The fluorimeter was adjusted to give maximum scale deflexion at position 14 with 0·50 μg/ml anthranilic acid; the scale was shown to be linear by comparing the fluoresence values which were obtained with different concentrations of anthranilic acid. Before each set of determinations the machine was adjusted to give the maximum deflexion with the same concentration of anthranilic acid.

The following substances were separated by paper chromatography, listed in order of increasing R_F values:

(i) Red and yellow coloured compounds, comprising the red (RD) and yellow (YD) drosopterins.

(ii) A blue-green compound, xanthopterin (X), confirmed by running the pure compound on the same paper. The same area is probably also occupied by isoxanthopterin, but as the amount of this compound, especially in female flies which have been mostly used, is small, no attempt was made to estimate this compound separately.

(iii) A yellow-green fluorescing compound, probably identical with xanthopterin-like (X-L) (Hadorn & Ziegler, 1958).

(iv) A pale yellow fluorescing compound, sepiapterin (S), confirmed by comparison with the mutant sepia which accumulates this compound.

(v) Two blue fluorescing compounds, 2-amino-4-hydroxypteridine (AH) and biopterin (B). These were confirmed by running pure AH and the mutants *rosy* and *maroon-like* which accumulate AH and B.

The conditions for paper chromatography were such that repeated runs varied in the degree of separation of some of the compounds. In some runs the yellow drosopterins were separated into three distinct spots, in others they ran as a single spot. Similarly AH and B, which run close together, were separated in some runs but not in others, while S could not be detected in some runs. Such differences have been taken account of in the various comparisons which we shall now consider.

3. RESULTS

(i) *The selected lines*

Figure 1 shows the differences produced by selection for high or low pigment quantity in terms of the units used in family or phenotypic selection. The average pigment content varied considerably between successive generations and the effects

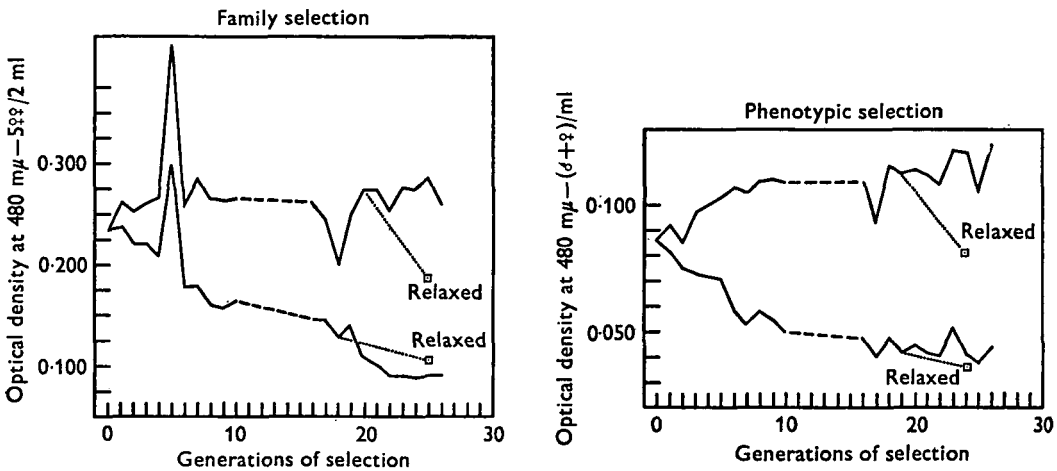

Fig. 1. Selection lines: optical density values for red eye pigment during the course of selection. The scales differ; 5 female heads/2ml A.E.A. were scored for family selection, and one male plus one female head/ml A.E.A. were scored for phenotypic selection.

of selection are best expressed by differences between strains cultured at the same time. Between generations 10 and 15 the number of flies scored per generation was variable and selection intensity was lower, hence the disregard of averages over this period. For purposes of direct comparison the pigment content per female per 0·5 ml A.E.A. has been estimated for generations 22–26 and these values are shown in Table 1. Small differences in eye width are also noted in the table: one of the high lines (H_1) has a slightly larger eye width and both the small lines have slightly

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smaller eyes. These differences were already apparent at generation eight and did not increase with further selection. If the effect of differences in eye width is adjusted by dividing by eye width the effect of selection is still clearly apparent.

Selection led to some 90% difference in pigment content relative to the Pacific stock; most of this difference was attained in the first eight generations of selection. The deviations in either direction are roughly the same for the lines selected by family selection, while the lines selected for phenotypic differences show a relatively greater response in favour of the higher pigment content. An analysis of variance for generations 22–26 indicated statistically significant differences between the two high and also the two low lines.

Table 1. *Pigment content* (O.D.) *and eye width measurements of the selected lines*

Line	O.D.	Eye width ($\frac{1}{100}$ mm)	(O.D./eye width) × 100	Deviation from Pacific (%)
H_1	0·127 ± 0·004	45·7 ± 0·2	0·278	43
H_2	0·139 ± 0·002	43·1 ± 0·3	0·323	66
L_1	0·042 ± 0·001	39·8 ± 0·3	0·106	−46
L_2	0·052 ± 0·001	39·8 ± 0·2	0·131	−33
Pacific	0·085 ± 0·002	43·6 ± 0·2	0·195	.

Average of generations 22–26; 1 ♀ head/0·5 ml A.E.A.

Such large differences offer favourable material for comparing correlated differences in the associated pteridines. But before we do so we must examine the possibility that pteridines other than the red and yellow drosopterins may have contributed to the score based on extracts of whole heads and thereby been selected for. However, tests showed that the drosopterins were almost exclusively extracted and also that the optical density of the combined fluorescing compounds, which were separated by chromatography and eluted, was negligible. Hence there is little doubt that the combined quantity of red and yellow drosopterins were selected for almost exclusively and that changes in the other compounds are a secondary consequence of changes in these pigments.

Figure 2 is a composite diagram in which the scores for the different fluorescing compounds are expressed as percentage deviations from the unselected controls; it displays the characteristic pattern of differences between the lines and indicates the level of significance. This figure is based on two experiments carried out at generations 17 and 22 between which times the pigment content in L_1, at least, declined further. Possible differences between the generations have been ignored since we are not concerned here with sequential changes. Variance analysis showed highly significant differences between lines in the level of each compound.

Although the absolute quantities may vary, the pattern of deviations as shown in Fig. 2 occurs with high regularity. In the high lines all the associated pteridines show a statistically significant increase over the control level. Except for statistically significant differences in the content of X and X-L the two high lines are very similar in the proportional changes of the various compounds. Between the low

lines the pattern is strikingly different. Line L_1 shows a correlated decline in all compounds except B, in which there is an increase which is quite as large as that recorded for the high lines while the negative deviations from the control levels are relatively less than the corresponding positive deviations in the high lines. Line L_2 is clearly and consistently different from L_1 in showing a high increase in X, X-L and AH, while S and B have hardly changed.

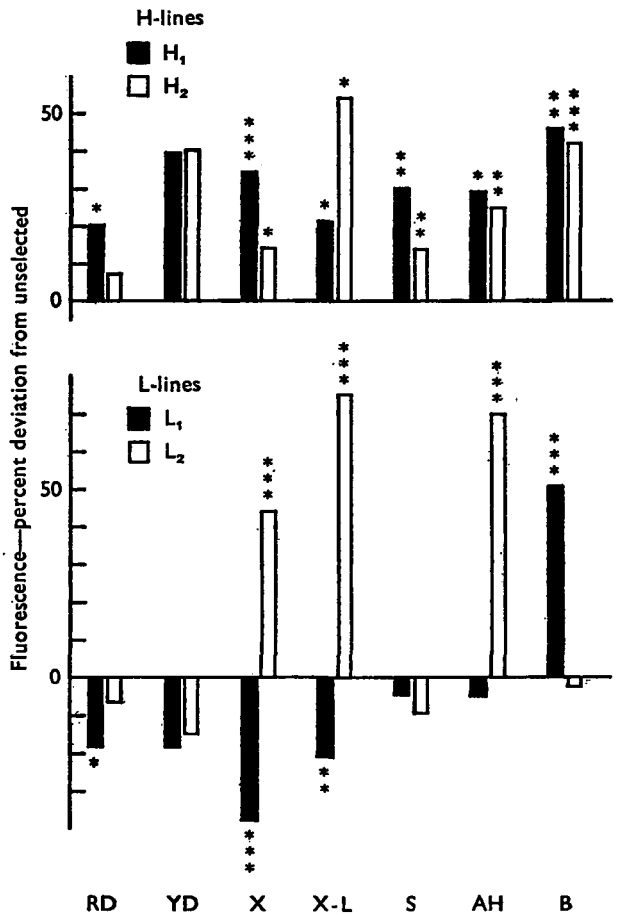

Fig. 2. Selection lines: percentage deviation of fluorescing compounds from the Pacific control level. This is a composite diagram; the AH and B values were derived from a different experiment. * $P < 0{\cdot}05$, ** $P < 0{\cdot}01$, *** $P < 0{\cdot}001$.

The characteristic differences created by selection were further compared in a series of derivatives from the selected lines. With the aid of marked inversions on the second and third chromosomes, several haploid sets of autosomes were derived randomly from the selected lines at generations 19–20 and the major pairs of autosomes were made homozygous; the *X*-chromosome, derived from the selected line, was not made homozygous. Table 2 shows the optical density readings for the various lines and also their deviations from the original selection lines. The

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numbers following the line designations, e.g. H_1-1, H_2-1, etc., refer to the different sub-lines derived from the selection lines H_1 or H_2, each with a homozygous second and third pair of chromosomes. The deviations from the original selection lines are comparatively small in the low pigment lines, while H_2 sub-lines show an average decrease of 25% in optical density. Yet the gross differences between selection lines have been maintained. This applies also to the pteridine patterns, so that these lines provide further evidence for persistent differences between the selection lines. It is worth noticing that the differences in eye width between the original selection lines, as shown in Table 1, disappeared in the homozygous sub-lines.

Table 2. *Pigment content* (O.D.) *in homozygous derivatives of the selection lines and the* F_1 *of crosses to Pacific*

	Optical density			
Line	Homozygous lines	Deviation from parent line	F_1	F_1-MP
H_1-1	0·108 ± 0·001	− 0·007	0·101 ± 0·003	0·008
H_2-1	0·100 ± 0·005	− 0·029	0·111 ± 0·003	0·022
H_2-2	0·110 ± 0·005	− 0·019	0·103 ± 0·006	0·009
H_2-3	0·104 ± 0·002	− 0·025	0·101 ± 0·001	0·010
H_2-4	0·101 ± 0·004	− 0·028	0·099 ± 0·003	0·010
L_1-1	0·051 ± 0·003	− 0·009	0·079 ± 0·002	0·015
L_1-2	0·063 ± 0·001	+ 0·003	0·080 ± 0·000	0·010*
L_1-3	0·063 ± 0·001	+ 0·003	0·081 ± 0·003	0·011
L_2-1	0·055 ± 0·000	+ 0·003	0·075 ± 0·001	0·011*
L_2-2	0·056 ± 0·001	+ 0·004	0·077 ± 0·001	0·010
L_2-3	0·050 ± 0·002	− 0·002	0·065 ± 0·001	0·001
L_2-4	0·057 ± 0·002	+ 0·005	0·080 ± 0·001	0·013*
Pacific	0·077 ± 0·001	.	.	.

Each O.D. value represents the average of three measurements of 1 ♀/0·5 ml A.E.A. MP represents mid-parent. * $P < 0·05$.

The sub-lines were crossed to Pacific to examine the dominance relations. Table 2 gives the optical density values of the F_1 generation as well as its deviation from the mid-parent value. All the crosses between the high lines and the controls showed a marked positive deviation from the mid-parent value, suggesting a general tendency for dominance in the direction of higher pigment content. The dominance deviation is greater in the crosses which involve Pacific and the low lines. Apart from one cross (Pacific × L_2-3), in which the F_1 was intermediate, the F_1 values suggest that the differences which lead to low pigment content behave as recessive when compared with the alleles of the Pacific population.

However, it is likely that epistasis plays a part in the apparent dominance-recessive relationship since, when H_1 was crossed with L_1 and H_2 with L_2, the F_1 was almost exactly intermediate as shown in Table 3.

The fluorescence patterns were examined for a few individuals of the partly homozygous sub-lines and each of the crosses to Pacific. Too few individuals were scored to give precise average values. However, the typical patterns were noticed

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and there was a distinct tendency in the crosses for the associated pteridines to show a dominance deviation which paralleled the deviation for pigment content. Thus, in crosses between high lines and Pacific, the pteridine patterns were generally like the high line parent while, in crosses between control and low sub-lines, the F_1 generally resembled the former. There was one consistent exception in the case of the L_2 sub-lines where the content of xanthopterin and AH + B was intermediate in all the F_1 progenies. A similar situation has been reported by Ziegler-Günder & Hadorn (1958) for the same compounds in the F_1 of crosses between wild type and *sepia* strains.

Table 3. *Pigment content* (O.D.) *in reciprocal crosses between selected lines*

Parent lines	Selection lines	F_1	F_1-MP
H_1	$0{\cdot}127 \pm 0{\cdot}004$	$0{\cdot}085 \pm 0{\cdot}001$	0·000
L_1	$0{\cdot}042 \pm 0{\cdot}001$		
H_2	$0{\cdot}139 \pm 0{\cdot}002$	$0{\cdot}090 \pm 0{\cdot}001$	−0·006 N.S.
L_2	$0{\cdot}052 \pm 0{\cdot}001$		
Pacific	$0{\cdot}085 \pm 0{\cdot}002$		

Each O.D. value represents the average of at least eight measurements of 4-day-old flies at generation 25, 1 ♀/0·5 ml A.E.A.

The data from reciprocal crosses are pooled. MP represents mid-parent; N.S. indicates statistical insignificance of deviation from mid-parent value.

Table 4. *Percentage deviation, after relaxation of selection from the original lines, in pigment content* (O.D.) *and fluorescing compounds*

Relaxed line	Eye pigments O.D.	Fluorescing compounds RD + YD	X	X-L	S	AH + B
H_1	−32**	−44**	−63***	−67***	−31**	−27***
H_2	−29**	−29	−25**	−17	− 5	−19**
L_1	− 8	− 6	1	2	−26*	−21**
L_2	−16**	− 5	3	−12	8	4

O.D. readings for 10 flies per/line; fluorescence readings in standard units are the average of four estimates of 3 ♀ heads of 4-day-old flies. Comparisons are made with generation 25 of the selection lines.

* $P < 0{\cdot}05$, ** $P < 0{\cdot}01$, *** $P < 0{\cdot}001$.

Further evidence of stability in the pattern of fluorescing compounds was provided by substituting an *X*-chromosome carrying the mutant *vermilion*, which blocks brown pigment synthesis, in the backgrounds of the selected lines and the unselected stock. The substitution led to a substantial reduction in O.D.—ranging from 24 to 48 %—with an average of 37 % but the general pattern of drosopterins and pteridines was qualitatively the same as in Fig. 2. The reduction in absolute amounts, the origin of which is unknown, was also observed by Clancy (1942) and Hadorn & Mitchell (1951).

So far the evidence points to a direct association between changes in the drosopterins and the associated pteridines. To provide further information about correlated change, selection was relaxed at either generation 19 or 20 (see Fig. 1). After

five generations of relaxed selection optical densities and fluoresecnce patterns were scored. Table 4 shows the effects of relaxation, in terms of deviation from the generation in which selection was first relaxed. The relaxed low lines showed no increase in optical density, and this suggests that, at least partial fixation may have occurred. Their apparent decline in pigment content is probably due to environmental variation. In the high lines, however, there was a distinct decline of about 30 % in optical density. The failure to achieve fixation in the high lines is probably due to the apparent dominance in favour of higher pigment content.

Apart from a few statistically insignificant differences, the fluorescence patterns remained unchanged in L_2 while in L_1 there was evidence of a statistically significant decrease in S and in AH and B. This fact, together with the evidence that the line L_1 declined in pigment content with further selection after generation 18, implies that this line had not yet become completely homozygous for the loci concerned. In both high lines there was evidence of decline in all the fluorescing compounds, especially in H_1, indicating correlations with the changes in pigment content. Thus the effects of relaxation of selection provide further support for causal relations between pigment content and the associated pteridines.

(ii) *Individual chromosome effects*

The distribution of activity on different chromosomes and dominance relations were studied by introducing the marked inversion chromosomes, *Cy* (II), *Sb* (III) and ci^D (IV) singly or jointly into the various selection lines or the unselected background; the *X* chromosomes were always derived from the same strain as the

Table 5. *Individual and joint effects of substituting chromosomes from the selected lines on pigment content* (O.D.)

	Optical density			
		Deviation from multiple heterozygote		
Line	Multiple heterozygote *Cy*/II; *Sb*/III	II/II; *Sb*/III	*Cy*/II; III/III	II/II; III/III
Pacific	0·078 ± 0·002	−0·001	+0·003	+0·008**
H_1	0·084 ± 0·004	+0·009*	+0·009*	+0·015***
H_2	0·100 ± 0·004	+0·003	+0·000	+0·002
L_1	0·069 ± 0·001	−0·017***	−0·023***	−0·036***
L_2	0·069 ± 0·001	−0·003*	−0·022***	−0·023***

1 ♀ head/0·5 ml. A.E.A., 5- to 7-day-old flies, *N* at least 13.
* $P < 0·5$, ** $P < 0·01$, *** $P < 0·001$.

autosomes which were replaced by markers. The fully heterozygous type, with all three markers present, was taken as a basis for comparing the various genotypes in which marked chromosomes were replaced by one or more homologues from the appropriate selected line. No certain effect of replacing the fourth chromosome could be detected and hence this category was combined with the other relevant types. Table 5 shows the deviations from the fully heterozygous type with both *Cy* and *Sb* present.

There is little evidence of deviation in pigment content from the level in the multiple heterozygote when individual marked chromosomes are replaced by Pacific homologues, but when both marked chromosomes are replaced the pigment content is significantly increased, suggesting the presence of interaction between unlinked genes. H_1 chromosomes are not fully dominant over the marker chromosomes in the maximally heterozygous type. When the markers are replaced by homologues from the H_1 selection line, each such substitution increases the pigment content slightly and the joint effect is about additive. H_2 autosomes are almost completely dominant over the marked homologues and no significant increase occurs when either marked second, third or both chromosomes are replaced by selected homologues. Thus, in these comparisons, as well as the effects of

Table 6. *The effect on pigment content* (O.D.) *of substituting pairs of homologous chromosomes from selection lines* H_2 *and* L_2 *in the Pacific background*

Original lines			O.D.	Eye width ($\frac{1}{100}$ mm)
Pacific			0·071	35·4
H_2			0·097	35·4
L_2			0·045	33·8
Substitution lines				
I	II	III		
H_2	P	P	0·093**	36·1
P	P	H_2	0·092**	39·1
H_2	P	H_2	0·092**	38·0
H_2	H_2	P	0·093**	36·4
P	H_2	H_2	0·092**	36·1
L_2	P	P	0·070	34·8
L_2	L_2	P	0·071	37·7
P	P	L_2	0·055**	31·9
L_2	P	L_2	0·054**	32·8

Optical density (O.D.): average of 10 flies, 1 ♀ head/0·5 ml A.E.A.
** Deviation from Pacific at 0·01 level of probability.

relaxing selection, we have evidence that the two high lines are genetically different, and it is possible that this difference arises from the use of family selection in H_1 and phenotypic selection in H_2.

In L_1, substitution of a marked second or third chromosome by a selected homologue leads to a distinct reduction in pigment content (25 % for the second, 33 % for the third) while, in line L_2, there is a striking effect of substituting the selected third (32 %) but little effect of substituting the second chromosome, all consistent with the earlier evidence of recessive behaviour. Table 5 also shows that the effects of the separate substitutions in L_1 combine additively in the joint substitutions (total reduction 52 %).

The chief interest in these comparisons lies in the evidence for recessive behaviour of gene combinations which reduce pigment content, the different levels of

activity displayed by the second chromosomes and the similarity in activity of the third chromosomes. When chromatograms of genotypes homozygous for either third chromosome were prepared, striking differences were revealed. Whenever the third chromosome pair of L_2 was homozygous the typical L_2-line pattern of fluorescence appeared while a homozygous third chromosome of L_1 displayed a pattern very similar to the original L_1-line. Further evidence of the recessive nature of the differences was provided by the F_1 of a cross between L_1 and L_2 which resembled the unselected controls both in pigment content and in the fluorescence pattern and thereby demonstrated also the non-allelic nature of the differences responsible for the contrast in fluorescence.

Further evidence of the genetic behaviour of individual chromosomes was provided by making use of marked inversions and appropriate crosses to substitute particular homologous pairs of chromosomes from either L_2 or H_2 in the genetic background of the unselected Pacific stock. The fourth chromosome was ignored and not all the possible comparisons were available. The available sets are listed along with their average scores for optical density in Table 6.

When chromosomes of H_2 were substituted in a Pacific background either the first or third pair alone or the first and third, first and second or second and third pair of chromosomes led to virtually identical average scores which closely resembled the level in the selected H_2 line. In this particular background there is thus striking evidence of epistasis and a complete departure from additive combinations of the effects of different homozygous combinations.

In the comparisons which include chromosomes from L_2, neither the substitution of a pair of L_2 *X*-chromosomes alone or accompanied by substitution of the second pair causes any difference from the level in the controls. Only when the third pair of L_2 chromosomes is substituted is there a striking reduction in optical density. This test confirms that selection in L_2 has acted almost entirely on genes in chromosome III.

Sex-linked differences were tested for in reciprocal crosses between H_1 and L_1 and H_2 and L_2. There was no difference between the scores of females, but male flies had a higher pigment content when the *X*-chromosome was derived from a high as opposed to a low line. The relative contributions of the parent lines to this difference is unknown. In the substitution experiment there was no evidence of a sex-linked effect in L_2, but the substitution of an H_2 *X*-chromosome pair into Pacific background raised the pigment content to the level of the H_2 line.

(iii) *Backcrosses*

When it became clear that most of the reduction in eye pigment in L_2 could be ascribed to the third chromosome, the possibility arose that this might be due, in part or entirely, to the fixation of a recessive gene on that chromosome. To test that possibility, the partly homozygous sub-line L_2-1 (Table 2), in which the second and third chromosomes from L_2 were isogenic, was crossed to a line derived from the Pacific population, in which the second pair of chromosomes was isogenic. The Pacific line had been shown, in several tests, to be virtually indistinguishable

from the average of the Pacific population both in pigment content and fluoresence pattern. It was hoped that the lack of variation in the second pair of chromosomes would simplify the analysis. The first cross was followed by two successive backcrosses to L_2-1 for the following reasons: as this experiment was designed to check segregation not only in pigment content but in the fluoresence pattern as well, both of which traits cannot be scored on the same individual, the flies of the first backcross generation were checked only for pigment content, while their progeny, produced by a further backcross to the L_2 sub-line, were used to score the pigment content of 10 flies per family, while a further 6 flies per family were scored for fluorescence patterns. Thus the individual B_1 flies, of known pigment content, were subjected to a progeny test. To allow recombination, females were used in the F_1 and B_1 generations to cross to the L_2 sub-line males.

Out of a total of 25 first backcross females, scored for optical density and crossed individually to the L_2 sub-line, nine produced offspring with an average optical density of eye pigment close to that of the low line (0·062 units), while the remaining 16 produced offspring whose score exceeded 0·065 units and averaged 0·073, which is very close to half-way between the optical density scores of L_2 sub-line and the unselected Pacific stock. The variation was much higher in the latter group, suggesting segregation for which there was no evidence whatever in the progeny from the first group of nine females.

Inspection of the chromatograms of single heads of B_2 females allowed us to score them as either 'like the L_2 sub-line' or 'like Pacific'. It turned out that all the progenies of the nine females with low optical density had a chromatographic pattern which was like that of L_2 while, in the progeny of flies with a higher pigment content, there was striking evidence of a 1:1 segregation. A total of 43 were like the Pacific controls and 53 were like the L_2 line. Thus the evidence suggests that a single locus may have been responsible for the characteristic fluorescence pattern as well as the low pigment content in L_2. However, the situation is not quite as simple as that, since both in B_1 and B_2 the number of flies which fell within the range of the Pacific controls was low while flies with optical density scores half-way between L_2 and Pacific values were abundant, whereas we should expect, on the simple hypothesis of only one locus, that the former would comprise about half the flies in the families which show segregation. This discrepancy could be due to segregation at an additional locus which influences the pigment content, but is not easily detected by an effect on the fluoresence pattern, or to chance fixation in the test Pacific sub-line of an allele which is only partly dominant to its L_2 allele. We cannot discriminate between these alternatives, but, it is worth noting that the intermediate pigment score for the F_1 of the cross between the L_2 sub-line, L_2-3 and the Pacific control (Table 2), shows that the second alternative cannot be ignored.

(iv) *Inbred lines*

Further evidence on the relation between drosopterin levels and pteridines was sought in comparisons between unselected inbred lines. Three lines were available,

each inbred by brother–sister mating for some 40 generations before their fluorescence patterns were studied. Table 7 shows that in each case the optical density of the eye pigment fell below that of the Pacific parent population and also that they differed strikingly among themselves. One line, P_4, had a pigment content even lower than that of the low selected lines.

Table 7. *Pigment content* (O.D.) *in inbred lines*

Line	O.D.	Eye width ($\frac{1}{100}$ mm)	O.D. of Pacific (%)
P 7	0·065 ± 0·001	39·3 ± 0·11	85·5
P 11	0·061 ± 0·005	40·6 ± 0·26	80·3
P 4	0·020 ± 0·003	40·3 ± 0·34	23·3
Pacific	0·076 ± 0·002	39·8 ± 0·24	

The O.D. values refer to the average of 11 measurements of 1 ♀ head/0·5 ml A.E.A.

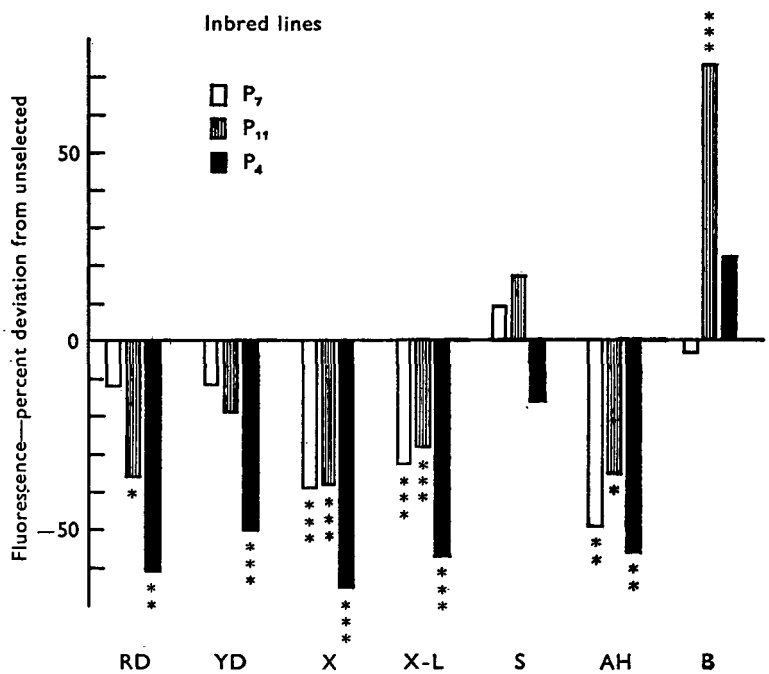

Fig. 3. Inbred lines: percentage deviation of fluorescing compounds from the Pacific control level. * $P < 0{\cdot}05$, ** $P < 0{\cdot}01$, *** $P < 0{\cdot}001$.

Variance analysis showed highly significant differences in the quantity of all the pteridines except sepiapterin. Figure 3 shows the pattern of values, expressed as percentage deviations from the parent Pacific population, and this may be compared with Fig. 2 which shows similar comparisons for the selected lines. Obviously the pattern in the inbred lines differs from that in the selected lines. Correlated with the reduction in the drosopterins, all the significant deviations from the Pacific

controls are negative, except for B, which shows a highly significant increase in P_{11}, while in P_4 the deviation is also positive, although not significantly so. Line P_{11} shows some features in common with the pattern in the selected line L_1, apart from the statistically significant reduction in AH and the even more well defined increase in B. These results underline the considerable variability which must exist in the Pacific population.

(v) *Variability in the base population*

So far then, we have evidence that at least several loci, concerned with the level of the eye pigment, were heterozygous in the unselected population. This prompted a search for direct evidence of such variation.

Table 8. *Percentage deviation from controls in the sub-lines homozygous for Pacific chromosomes II or III with atypical fluorescence patterns*

Chromosome	Pattern type	RD	YD	X	X-L	S	AH+B
III-1	.	26*	17*	55**	39**	34*	53**
III-2	.	21	8	45**	50**	−2	35**
II-1	*c*	9	5	8	9	15*	18*
II-2	*c*	8	2	−14*	−9*	32**	17**
II-3	*a*	−4	−4	8	20*	12	9
II-4	*b*	−20	−1	−12*	−19**	5	−6
II-5	*b*	−9	−8	−22*	−24**	−4	−12
II-6	*b*	−14	5	−13*	−15**	−3	32**

The values, in standard units, are the average of three estimates of 3 ♀ and of three estimates of 3 ♂ heads each of 4-day-old flies.

* $P < 0{\cdot}05$, ** $P < 0{\cdot}01$.

Again with the aid of marked inversions on chromosomes II and III, 16 second and six third chromosomes were drawn at random from the Pacific population and each was made homozygous, while the rest of the genetic background was made up of a random sample of chromosomes derived from the Pacific population.

There was considerable variation in average pigment content between the synthetic strains but in no case did the deviation from the Pacific control reach the levels attained by the selected lines. Chromatograms were prepared from a few individuals from each of these sub-lines and were inspected for fluorescence patterns. Eight out of the total of 22 clearly differed from Pacific and among these about four different patterns can be distinguished at this level of discrimination. Table 8 gives the value of the percentage deviations of the eight strains grouped according to their pattern differences; these will be considered later. Thus out of 22 lines, homozygous for either the second or third pair of chromosomes, about a third differed significantly from Pacific and at least four different patterns are represented. Both third chromosome lines resemble the lines selected for high pigment since all compounds are increased and this seems also to be true, to a lesser degree, for one (II-1) of the second chromosome lines. One second chromo-

some line (II-3) showed a pattern similar to L_2, namely a decrease in drosopterins but an increase in related compounds, while the third group of lines homozygous for chromosome II is characterized by a decrease in almost all compounds, except AH + B in sub-line II-6. Thus this rather limited test provided ample evidence of variation in the base population on the part of compounds related to the red pigment pathway. As we shall see later, this kind of variation resembles in several respects the changes detected in inbred and selected lines.

4. DISCUSSION

Most of the correlated changes in pigment content and fluorescing compounds can be accounted for in terms of a biochemical pathway, bearing in mind that the relations between the various compounds in the pathway are not yet unequivocally established. We have also to recall Ziegler's (1961*a*, 1965) claim that the eye is not the site of synthesis of the compounds, except for the last step which gives rise to the drosopterins and the consequent possibility that differences in the translocation of compounds could give rise to differences in the eye. For reasons of simplicity we shall ignore this particular alternative.

It is generally accepted (Ziegler, 1961*a*, *b*, 1965) that the drosopterins which make up the eye pigments of *Drosophila* are end-products. According to Ziegler the antecedent steps in the pathway are:

precursor/s → tetrahydro-compound → sepiapterin → drosopterins.

An alternative possibility cannot yet be excluded, in which the last two categories are derived directly rather than sequentially from the tetrahydro-compound, but we shall adopt the sequential hypothesis.

One difficulty for a metabolic interpretation arises from Ziegler's (1961*a*, 1965) claim, that the compounds which are identified by standard propanol-ammonia chromatography are breakdown products resulting from various degrees of decomposition. Thus xanthopterin (X) and xanthopterin-like (X-L) represent the breakdown products of sepiapterin (S), a dihydro-compound, while 2-amino-4-hydroxypteridine (AH) and biopterin (B) are believed to be derived from a tetrahydropterin.

If the relative breakdown of the dihydro- and the tetrahydro-compounds is independent of genotype, then our approach should not be seriously handicapped by the fact that we are recognizing pleiotropic effects by derivatives rather than the genuine metabolic intermediates. The rather small amount of S among the different genotypes may indicate that our conditions of chromatography were such as to decompose the bulk of this substance into X and X-L and the fairly similar quantities of the latter could be accounted for by their derivation from the same compound. On the other hand, we find a rather complex situation for AH and B, which are the alleged breakdown products of the tetrahydro-compound, but show almost opposed behaviour in particular low and inbred lines. We assume that this may be due to the fact that AH is not only a breakdown product, but

occurs as the natural precursor of isoxanthopterin (Hubby & Forrest, 1960), which is not directly involved in the pigment pathway.

To analyse the quantitative relations, we may therefore take the average sum of the red and yellow drosopterins (RD + YD) as proportional to the amount of red pigment (D). The preceding step in the pathway (S) may be represented by the sum of the amounts of xanthopterin, xanthopterin-like and sepiapterin (X + X − L + S), while the quantity of the non-fluorescing tetra-hydro-compound (T) may be represented solely by the amount of biopterin (B). This procedure is not entirely free of difficulties, witness the increase in S in II-1 and II-2 and the decrease in X and X-L in II-2, noted in Table 8. Nevertheless the proposed procedure offers the most rational approach, and, leads to some rather interesting and suggestive conclusions.

We thus obtain values for the hypothetical synthetic sequence:

$$\text{precursor/s} \rightarrow \text{T} \rightarrow \text{S} \rightarrow \text{D}.$$

As long as we compare the values in different lines in terms of the ratios of the quantities listed above, the relative concentrations are independent of their absolute molarities. Hence all our comparisons will be based, for each compound in turn, on the ratio of the difference between the amount in the appropriate line

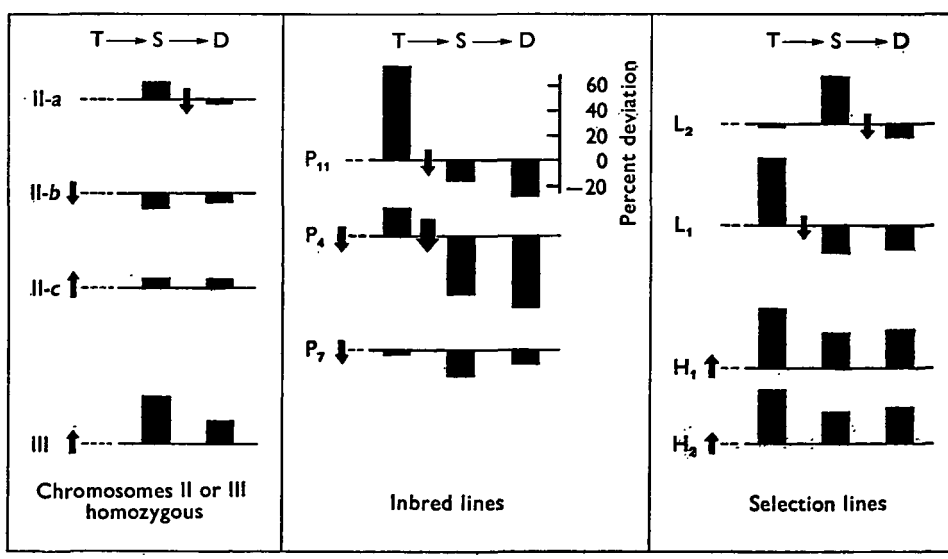

Fig. 4. The percentage deviation from the Pacific level, in different genotypes, of the metabolic pools in the final steps of the pathway leading to formation of the drosopterins. The values for the precursors T and S and of the end-product, D, are estimated by the procedure which is described in the text. The arrows indicate an increase (upward) or a decrease (downward) of enzyme activity at steps after T or of enzyme activity and/or precursors before T. The proposed degree of impairment of enzyme activity is indicated by the width of the arrows. In the series with chromosomes II or III homozygous, the categories II-*a*, II-*b*, II-*c* or III represent average values for those genotypes whose chromatograms show similar deviations from the Pacific pattern.

and the Pacific control strain to the latter value, multiplied by 100 [(difference/Pacific) × 100]. This gives us the percentage deviation from the base line, represented by the Pacific controls. The only difference between the present procedure and that adopted for the representation of separate differences in Figs. 2 and 3 is that here we are dealing with the combined amounts of particular compounds.

We are now in a position to re-examine the precursor patterns for the sub-lines with homozygous second or third chromosomes, the inbred and the selection lines. The distribution of deviations and the interpretation are displayed in Fig. 4.

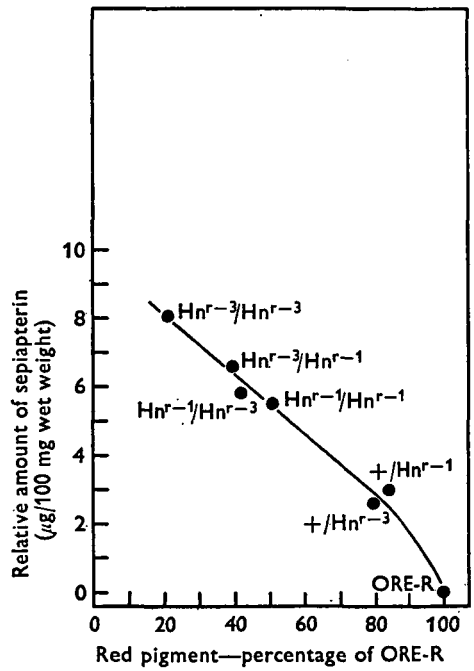

Fig. 5

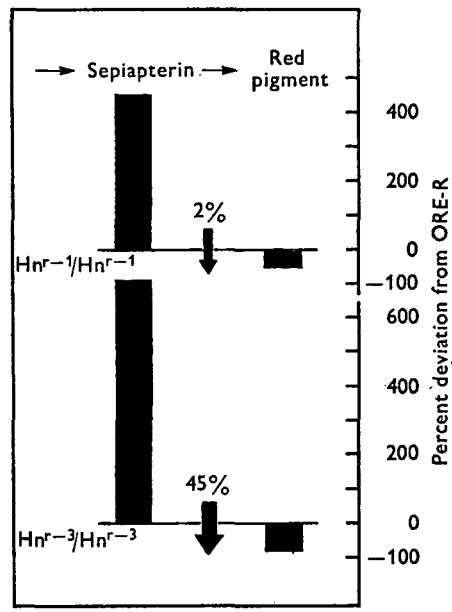

Fig. 6

Fig. 5. Graphical representation of data reported by Taira (1960) which show the relations between red pigment content in different allelic combinations at the *Henna* locus and the amount of sepiapterin. The values refer to male heads and are shown on a scale relative to the score in the wild-type Oregon-R strain.

Fig. 6. Graphical representation of data reported by Taira (1960) which shows the relations between quantity of red pigment, sepiapterin and the level of sepiapterin reductase activity.

It is important for our argument to recall that ever since the metabolic implications of mutants, which block steps in a pathway, were first studied in *Drosophila melanogaster* (Beadle & Ephrussi, 1937), an accumulation of precursors or their derivatives has been found associated with the impairment of particular steps in a biochemical sequence. Mutants of *Neurospora crassa* which cause reduced enzyme activity provide evidence that accumulation of precursors may occur even under these conditions (Davis, 1968; Donachie, 1962), while observations on the same species (I. B. Barthelmess, unpublished) have demonstrated a quantitative relation

between the amount of accumulated precursor and product along with different levels of enzyme activity.

A similar situation has been encountered in *Drosophila* and this is demonstrated in Fig. 5, which is based on data published by Taira (1960) who investigated two different alleles at the *Henna* locus which differ in the activity of the enzyme sepiapterin reductase when compared with the Oregon-R strain. Strains homozygous for the allele Hn^{r-1} showed, in dialysed extracts, 2 % reduction while strains homozygous for Hn^{r-3} showed 45 % reduction in enzyme activity. Figure 5 demonstrates the strong negative correlation, in percentage terms, between the amount of red pigment and its precursor sepiapterin in all possible combinations between the two *Henna* and the wild-type alleles. In Fig. 6 the patterns of the two homozygous genotypes for which the enzyme activities are known are plotted in the same manner as our own data in Fig. 4. The greater the loss in enzyme activity the greater the accumulation of precursors and the lower the amount of product. It is evident that the change in enzyme activity is quantitatively reflected in the relative pool levels. In the following discussion we shall adopt such an inverse precursor–product relation as a tentative diagnostic criterion for a decrease in enzyme activity. Of course, such patterns may also be generated by more complex changes, if there are many factors which cause the deviation from the average Pacific pattern, but this possibility will be ignored since we are opting for the minimum hypothesis.

If we consider first the lines with low pigment content, namely the various sub-lines with homozygous second chromosomes, the inbred and the selected low lines, we find (Fig. 4) that an increase of a particular precursor is associated with a decline in the postulated succeeding metabolite. Each such instance has been marked by a downward pointing arrow to indicate that, operationally, we assume that the enzyme, which is involved in the particular step, has undergone an unknown change to diminished efficiency. Until these enzymes can be assayed in homogeneous genetic background we cannot infer whether the postulated changes are primary or merely of a regulatory nature due to inhibition or repression. The work of Glassman (1965 and earlier) on the enzyme xanthine dehydrogenase demonstrates how manifold are the influences of the genetic background on enzyme activity.

Thus we may infer that there has been a decrease in enzyme activity in the last step, leading to D, in one of the second chromosome patterns, II-*a*, while in another, II-*b*, a similar event has taken place at an earlier stage of the sequence. In two of the inbred lines, P_{11} and P_4, there is an inverse relation between T and S. If we consider the relative reduction of the later pools in these lines, we might infer a more extreme impairment of the enzyme concerned in P_4, leading to even lower levels of S and D, which should cause an even greater accumulation of T in P_4 compared with the situation in P_{11} than we actually observe. This suggests that an additional impairment of enzyme activity may have occurred at an earlier step, to the left of T in our diagram. The P_7 pattern can be accounted for by an impairment at a step before the formation of T.

The patterns of the two selected lines L_1 and L_2 are compatible with the assumption of decreased enzyme activity in the last step in L_2 and in the previous step in L_1. The assumption of only one change in the sequence in L_2 is in fair agreement with the genetical investigation, which indicated that the main effect on the pigment quantity in this line is probably due to a single locus. The diagram also indicates a minimum of one change only in line L_1, although the genetic analysis proved the existence of influences on both the second and the third chromosomes of this line. The additivity of these effects leads to the assumption that they probably impair the same step, as it is unlikely that effects on consecutive steps in a particular biochemical sequence will show additivity, judging from recent evidence in *Neurospora crassa* (I. B. Barthelmess, unpublished). The postulated lack of additivity between loci which affect different steps in a sequence will often appear as epistatic interaction between genes for low pigment content, when they are homozygous, since the final outcome will depend chiefly on the most stringent impairment in the sequence. This type of epistasis could account for the low pigment content in all the inbred lines. Given the occurrence of deleterious alleles which influence different steps of the same biochemical pathway, there is a likelihood, depending on the gene frequency, that under conditions which favour fixation at least one negative allele controlling one or other of the steps will be fixed and thereby lead to a reduction of pigment content. Our inference that there has been an impairment of enzyme activity in all the low pigment lines is consistent with the genetic evidence of recessive behaviour.

Thus, by applying the hypothesis which was defined earlier, by looking for the least number of changes to account for the deviations from the Pacific pattern and by assuming that, in other respects, the metabolic situation does not differ from that in the base population, we emerge with a consistent biochemical interpretation of the origin of low pigment content, which is also compatible with the genetic evidence.

Figure 4 shows that when the pigment level is increased by selection or by chance fixation, in the sub-lines made homozygous for one or other of the major autosomes, the content of all the detectible precursors is also increased. A possible explanation for this behaviour would postulate an increase in the amount of some early precursor/s or enzyme/s which act before the sequences which have been considered in the low pigment lines. Alternatively, the same result could be due to alteration of a common signal which would de-repress the activities of all the enzymes in the pathway. But it is clear that the pool patterns provide no support for a reduction of activity in any of the synthetic steps in the high lines. This is consistent with expectation according to our model and thereby provides a further argument in its favour.

The genetic behaviour of the high lines poses a few problems. When selection is relaxed the quantity of pigment declines in a few generations, showing that the lines remained heterozygous and carried, at relatively high frequency, genes which reduce pigment content but whose presence is concealed by the partially dominant behaviour of alleles which favour higher pigment content. It is improbable that the

high pigment content is an attribute of heterozygosity *per se* since derived lines, homozygous for the second and third pair of chromosomes have, without exception, almost as high a pigment content as the parent lines. The construction of homozygous pairs of chromosomes will fix genes which tend to reduce pigment content, but their presence is effectively concealed by epistatic effects of genes and/or gene combinations which favour high pigment content, and this is most evident in the substitution of H_2 chromosomes in the Pacific background. Such epistasis could depend on more than one locus per chromosome pair. When selection is relaxed the favourable combinations created by selection are dissipated by recombination and segregation. How selection maintains such epistatic effects on each chromosome pair, when the full effect can apparently be achieved by changes on each pair alone, is not clear. Finally we have no plausible explanation for the observation that the F_1 of crosses between high pigment lines and Pacific shows partial dominance in favour of higher pigment content while the cross between high and low lines is almost exactly intermediate.

A similar result has been reported by Robertson & Reeve (1955) for crosses between inbred lines selected for large or small body size. Indeed the general genetic behaviour of the two characters, pigment content and body size, shows many parallels in their response to selection, reaction to inbreeding and in the evidence derived from chromosome substitution experiments (Robertson, 1955). In spite of the intrinsic developmental differences between the two characters, they may share certain general properties of growth which are adjusted to stabilize the mean of either above a certain minimum level. Unidirectional dominance and epistasis will serve to limit the expression of segregating differences, which would otherwise increase the phenotypic variance, especially at the lower end of the scale.

The resemblance in genetic behaviour between pigment content and body size suggests that the kind of biochemical explanations proposed for the former may also be applicable to a number of other quantitative characters. Clearly, progress in this field must depend on a joint approach in which the concepts and experimental techniques of quantitative inheritance are combined with biochemical analysis in a single attack.

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