THE GENETIC CONTROL OF RIBOFLAVIN

IN <u>TRIBOLIUM</u> <u>CONFUSUM</u>

by

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ABSTRACT

The identity of a yellow fluorescent substance
isolated from Tribolium confusum, with riboflavin was
shown by the independent methods of spectrophotometry,
chromatography and electrophoresis. Two decomposition
products of riboflavin as well as two conjugated flavines
FMN and FAD, were identified. The genetic control of
riboflavin and its relationship to some mutant eye color
genes in the pleiotropic hierarchy of gene effects was
investigated in a series of experiments involving more
than 8000 Tribolium from 11 genetically different lines.
All developmental stages of individuals homozygous for a
recessive pearl eye color gene, p^r (formerly called p,
but renamed) were associated with a significant, heritable
reduction in riboflavin content in the body. This
reduction was principally in the Malpighian tubules.
In all of the other lines tested the vitamin was
accumulated in the tubules in relatively high concentrations.
The normal allele of this gene was found to be incompletely
dominant with respect to the riboflavin phene. Changing
the residual genotype of p^r by means of two generations
of recurrent selection did not detectably alter its
expression. Two alleles of pearl (p and p^s) were
phenotypically identical to p^r, except at the level of

the riboflavin phene. Crosses of these alleles with $\underline{p}^r$ were shown to complement. Genetic differences in riboflavin content and the pigmentation of Malpighian tubules were shown. Individuals of the pearl phenotype ($\underline{p}^r$, $\underline{p}$, $\underline{p}^s$) always had colorless Malpighian tubules. Sexual dimorphism for riboflavin content was observed in all lines tested. The quantitative parameters of variation of the riboflavin phene were examined and its heritability was found to be high by two different comparisons.

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INTRODUCTION

In recent years <u>Tribolium</u> <u>confusum</u> has increasingly been used for population and quantitative genetic studies. Through these studies a large number of mutations have been found and categorized. Stocks of these mutants are readily available throughout Canada and the United States, however, only a few have been investigated in detail. A number of mutations which affect the pigmentation of the eye have been found. Preliminary investigations by the author using these eye mutants led to the demonstration of qualitative and quantitative differences in a yellow fluorescent pigment that was subsequently identified as riboflavin. Since this pigment is a vitamin and essential for the survival of <u>T</u>. <u>confusum</u>, its investigation might clarify the nature of the genetic interrelationships between those genes responsible for riboflavin differences and those genes associated with eye pigment differences.

Workers have observed the association of riboflavin with the genetics of pteridines particularly in <u>Drosophila</u> and <u>Ephestia</u>, yet the genetics of riboflavin

has received very little direct attention. Therefore
this thesis was concerned with attempts to define
the genetic control of this pigment, with the
description of the changes in its concentration
during development, and with quantitative strain
differences of this phene.

REVIEW OF THE LITERATURE

Mutations are usually classified according
to their most conspicuous effects. In the case of
eye color genes, initially only the directly visible
pigments can be observed. As a rule these easily
recognizable features are preceded and accompanied
by an additional set of characters which become
apparent only by the use of special methods. The
totality of all phenes, in which a mutant deviates
from a "normal" or "standard" or wild type (W.T.),
constitutes the "pleiotropic pattern of manifestation"
(Hadorn, 1954) of the respective gene or factor.
Such pleiotropy of genic action, conditions bio-
chemical and physiological, as well as morphological
phenes. Eye color genes, studied in Drosophila and
Ephestia, have provided much information concerning
the pleiotropic action of genes (Hadorn and Mitchell,
1951; Hadorn, 1956; Ziegler, 1961; Hubby, 1962).

Flavines are often isolated with pteridines, and
in some of the earlier literature these two classes
of compounds may have been confused (Viscontini, et al.,
1955). Viscontini (1956) isolated riboflavin along
with pteridines from the heads of Drosophila and Ephestia.

A close relationship exists between pterins
and riboflavin (Ziegler, 1961). Both have the
pyrimidine-pyrazine nucleus and share possibly some
common steps in biosynthesis (Weygand and Waldschmidt,
1955).

The disappearance of riboflavin from the eggs
of insects and the coincident appearance of other
fluorescent compounds which seem to have the properties
of pteridines also suggests the possible biological
derivation of pteridines from flavines (Bodine and
Fitzgerald, 1947; Burgess, 1949). The function of
flavines as prosthetic groups of several dehydrogenases
has been well established. Whether they have an additional
function in insects, which their accumulation in such
large quantities might suggest, is not known (Gilmour,
1961).

Using the bacteriological assay, Caspari and
Blomstrand (1958) pointed out that the pigment
deposited in rodlets in the outer layer of the testis
sheath of Ephestia was partly riboflavin. In the
W.T. riboflavin gradually appears during the last
larval instar, reaches a plateau in the early pupa
and disappears in the late pupa. Subsequently, they
suggested that riboflavin might then be transferred

to the Malpighian tubules since the rate of disappearance
from the testis was exactly balanced by its appearance
in the Malpighian tubules. In addition they demonstrated
that gene a, which blocks the oxidation of tryptophan
to kynurenin, causes the appearance of considerably
higher amounts of riboflavin in the testis sheaths,
but the riboflavin seems to disappear somewhat earlier
than in the W.T. The pleiotropic action of this gene
includes not only tryptophan metabolism of proteins, but
also metabolic processes such as the effect on pterins
and riboflavin which cannot be explained in detail
at the moment (Ziegler, 1961). In mutant weiss-augig
(wa), which is characterized by the suppression of
many fluorescent substances (Hadorn and Kuhn, 1953;
Kuhn and Berg, 1956) the appearance of riboflavin in
the testis sheaths was inhibited at all times. The
total amount of riboflavin in these individuals (wa)
was reduced compared to a individuals by the amount
present in a testes.

In addition to effects due to single genes,
Caspari and Blomstrand (1958) demonstrated significant
strain differences in the amount of riboflavin and
the pattern of its disappearance in Ephestia.

Eye color genes which affect the amount of

riboflavin have also been found in <u>Drosophila</u>.
Hadorn and Mitchell's (1951) spot Fl 4/5 contained
riboflavin and three other substances all of which
have been shown to be pteridines. This spot was
missing in the mutants brown (<u>bw</u>) and white (<u>w</u>).
Riboflavin as well as the other substances are
lacking in animals homozygous for these genes. There
are many other color mutants in which the intensity
of Fl 4/5 is increased (clot, <u>cl</u>; sepia, <u>se</u>; sepiaoid,
<u>sed</u>; rosy^2, <u>ry</u>^{2} and also ebony, <u>l</u>; claret, <u>ca</u>; prune,
<u>pn</u>^{2}) or reduced (white-apricot, <u>w</u>^{a}; white-eosin, <u>w</u>^{e} ;
garnet, <u>g</u>^{2}; pink-peach, <u>p</u>^{p}; light, <u>lt</u>; carnation, <u>car</u>;
purple, <u>pr</u>) as compared to the wild type (Hadorn and
Mitchell, 1951; Hadorn and Schwinck, 1956).

In some of these cases it is known that a yellow
pteridine is accumulated; but the literature does not
clarify whether the amount of riboflavin present was
also affected by these genes.

Taira and Nawa (1958), using the bacteriological
method of assay, found that mutant <u>se</u> of <u>D. melanogaster</u>
contains much more flavin than vermilion (<u>v</u>) or <u>bw</u>.
Hubby (1961) showed significantly higher levels of a
"riboflavin-like" pigment in the testis of light
isoxanthopterin (<u>lix</u>) compared to <u>ry</u>^{2} and Oregon-R.

Evidence for the genetic control of free riboflavin among the higher forms has been found in the domestic fowl. Bernier and Cooney (1954) obtained a highly significant difference in the incidence of riboflavin deficiency between black ($\underline{E}$) and non-black ($\underline{e}$) embryos as well as in chicks from a cross involving the segregation of these alleles. They suggested that riboflavin may be involved in melanization. Boucher and his co-workers (1959; Buss et al., 1959; Cowan et al., 1961; Cowan et al., 1961 a) found physiological characteristics associated with a recessive gene causing riboflavin deficiency ($\underline{rd}$). Their data showed that free riboflavin was retained more effectively in the normal ($\underline{Rd}$-) than in the recessive ($\underline{rdrd}$) laying hen, whereas, riboflavin retention in non-layers was independent of the $\underline{rd}$ gene. The normal allele $\underline{Rd}$, was furthermore shown to be incompletely dominant.

Although $\underline{Tribolium}$ has extensively been used in ecological, and more recently in genetical studies (McDonald and Peer, 1960; Schlager, 1963; Dawson, 1964a, b; 1965; Crenshaw and Lerner, 1964; Sokoloff, 1964 a, b) there appears to be no literature concerned with the genetics of physiological traits.

MATERIALS AND METHODS

All cultures, parents and progeny were reared
in a constant environment of 32° C., and 72% relative
humidity. The medium was prepared from sifted (No. 30)
Meota whole wheat flour mixed with 10% Torula yeast
(Doty, 1965). Sterilization of the flour (60° C. for
24 hours) was performed prior to adding the yeast, since
the latter was found to clump upon heating.

Virgin females were obtained for all matings
by sexing in the pupal stage and checking the
phenotype upon eclosion. Males were sexed similarly,
with the exception of some which were removed from
stock cultures in the adult stage and sexed by the
method of Pfadt (1962). Progeny were usually sexed
in the pupal stage, however, some were sexed as
adults. Etherization was employed for immobilization.
Small matings were carried out in 20 ml. creamers,
larger ones in 110 ml. cellulose nitrate tubes.

All of the stocks were obtained from the
Tribolium Stock Center, University of California,
Berkeley. They were maintained in mass breeding
370 ml. containers. All crosses were reciprocal and

at least in duplicate. In general crosses were
made by mating five males to five females. In
accordance with Tribolium convention males are put
first in the description of a cross. The description
of the stocks used is as follows:

pearl ($\underline{p}$), an autosomal recessive, eliminates the

pigment from the ommatidia but not from the

ocular diaphragm resulting in a bicolored or

spectacled appearing eye (Graham, 1957). This

gene is referred to in the text as "Graham's

pearl" ($\underline{p}'$) and will subsequently be redesignated

to $\underline{p}^r$.

pearl ($\underline{p}^S$), spontaneous in the Berkeley synthetic

strain; allelic to pearl described by Graham

(Sokoloff, 1963). $\underline{p}'$ and $\underline{p}^S$ are phenotypically

indistinquishable.

light ocular diaphragm ($\underline{lod}$), is not strictly an eye

mutation but it can be observed when eye color

mutants such as pearl (which modifies the black

pigment of the ommatidia) are present. $\underline{lod}$ is

an autosomal recessive which blocks the synthesis

of melanin pigment from the ocular diaphragm,

an endoskeletal structure which helps to support

the compound eye. Since the diaphragm is not

affected by genes affecting the color of the

ommatidia, it appears as a dark band under the

colorless or lightly pigmented marginal
ommatidia, giving the beetle a spectacled
appearance (Sokoloff, 1962).

ruby spot (rus), produces a pinkish eye either in
the late pupa or in the teneral adult, but with
aging the eye darkens, leaving only a reddish
spot which can be identified by casting a
shadow on the beetle's eye as it lies on the
stage of a dissecting microscope. The spot is
not stationary for, if the beetle is rotated,
it moves in the direction opposite to the
movement of the beetle. This indicated that,
in reality, the ommatidia are all dark red
but special intense lighting is required to
detect the pigment (Sokoloff, 1961).

dirty pearl eye (dpe), is distinquished from rus
during a brief period in the developmental
cycle, in the pupa and sometimes in the
teneral adult the compound eyes appear
uniformly pinkish in rus whereas in dpe the
eyes of the late pupa or early imago appear
pearl-like except that the central ommatidia
appear dirty. When fully colored the eyes of
the imago become dark red, perhaps darker
than rus, somewhat resembling the chestnut

eye mutation in _T_. _castaneum_ (Sokoloff, 1964).

eye spot (_es_), is a sex-linked recessive characterized by a clear spot in the center of the eye which can be readily seen in young adults, but becomes increasingly difficult to identify as the insect ages, however it never disappears completely (McDonald, 1961).

red (_r_), is a sex-linked recessive producing a light-red area in the eye comparable in the early stages with pearl; with age the spot becomes reduced in size intermediate between _p_ and _es_ (Ho, 1962).

pearl, light ocular diaphragm (_p_,_lod_); pearl, ruby spot (_p_,_rus_); pearl, droopy elytra, creased abdominal sternites (_p_,_dre_,_cas_), stocks were obtained from the Berkeley stock center along with the above mutant stocks.

CFI-1, is an inbred line, brother-sister mated for 43 generations and free (as far as is known) of eye color mutations. This line is used as the W.T. control.

CFI-7, is also an inbred line, obtained from the same base population as CFI-1, and brother-sister mated for 43 generations. Both CFI-1 and CFI-7 were kept as random mating closed populations since their arrival from Berkeley (about 10 generations).

A modification of the procedure described
by Hadorn and Mitchell (1951) was used for most
of the chromatographic separations. All chroma-
tograms which were to be measured fluorometrically
were prepared from beetles ten days after eclosion.
The beetles were boiled for two minutes in distilled
water in order to coagulate proteins and to improve
the subsequent chromatographic resolution of
fluorescent substances. The boiling was done
directly with the larvae and the pupae, but the
adults were immersed briefly in 95% ethanol prior
to boiling. Excess water was removed from the
boiled animals by blotting them on filter paper.
They were then transferred immediately onto Whatman
No. 1.paper. Beetles were firmly mashed onto the
paper with a glass rod and allowed to dry at room
temperature in the dark. Residual tissues were not
removed from the paper since the author's pre-
liminary experiments demonstrated that the chitinous
exoskelton does not interfere with chromatography.

Internal organs were dissected in an insect
Ringer (Patton and Craig, 1939) and transferred
with as little adhering liquid as possible onto the
chromatography paper.

The dried chromatography sheets were rolled into cylinders, stapled and placed upright into a pyrex tank containing the developing solvent. Unless otherwise stated all experiments were carried out in the ascending direction in dark with tert-butanol, pyridine and water (10:3:7) as the solvent (Caspari and Blomstrand, 1958). When the solvent front reached 14 cm. (about five hours) the cylinders were removed and dried in a darkened fume hood.

Developed chromatograms were observed by means of two ultraviolet lamps with principle emission at 253 and 366 millimicrons respectively. Spots were cut out and extracted overnight in four ml. of double distilled water at room temperature in a dark room. The samples were read in a Turner photofluorometer, Model 111, using 436 (filters 47B and 2A) and 570 (filter 23A) millimicrons as primary and secondary wavelengths and with slit arrangement 30X (AOAC, 1960). For quantitation a standard curve was prepared from pure riboflavin using the above methods.

RESULTS AND DISCUSSION

PART I. CHEMICAL ANALYSIS

A. Preliminary observations

The results of paper chromatography of the
W.T. is shown in Table I. In total eight fluorescent
and four non-fluorescent spots appeared on the
chromatograms. The yellow fluorescent spots 1, 2
and 5 appeared on all the chromatograms. Spot 3
seldom appeared; when it was present the yellow
spot 2 with the identical R_f value was invariably
absent. The violet fluorescent spots 4 and 6 varied
considerably in intensity of fluorescence. The brick-
red spot 7 appeared well defined on fresh chromatograms,
but gradually changed to a dirty brown color after a
few days. The fluorescent spot 8 was only visible while
the chromatogram was still wet. Spot 9 weakly absorbed
when illuminated with the 253 mu principle emission
lamp; it also disappeared shortly after drying the
chromatogram. Spot 10 migrated with the solvent front
and was either brown colored or blue fluoresent, but
never both.

The results obtained by visual estimation of

TABLE I

CHROMATOGRAPHIC DISTRIBUTION OF SUBSTANCES AND THEIR R_f VALUES IN W.T. ADULT TRIBOLIUM CONFUSUM

Spot No.	visible color	Fluorescent color	R_f	intensity
1	-	Yellow	.05	medium
2	Yellow	Yellow	.27	strong
3	-	Violet	.27	medium*
4	-	Violet	.50	variable
5	Yellow	Yellow	.60	strong
6	-	Violet	.64	variable
7	Red	-	.72	weak
8	-	Blue	.81	trace
9	-	Absorbs**	.91	trace
10	Brown	Blue***	.97	medium

* Seldom appears; in its presence the yellow spot is usually absent.

** Maximum absorbance at 253 mu.

*** This spot is either brown, or blue and fluorescent.

fluorescence of the yellow fluorescent spots 1, 2
and 5 in 11 lines of T. confusum are shown in
Table II. The intensely yellow fluorescent spot 5
shows considerable visual variation between different
eye color mutants and the inbred lines. Although
a large number of p' individuals were chromatographed,
spot 5 was never present in this mutant. Comparison
of the R_f values of this spot with those in the
literature for similar solvents (Hais and Pecakova,
1949; Caspari and Blomstrand, 1958; Schmidt and
Viscontini, 1962; Harkness et al., 1964) gave a
good indication that it might be riboflavin.

B. Isolation, purification and characterization
of spot 5

1. Identification by spectrophotometry

For the purpose of spectrophotometric,
electrophoretic and chromatographic identification,
the substance in spot 5, suspected to be riboflavin,
was extracted and purified by a modified method
based on that of Schmidt and Viscontini (1962).

TABLE II

VISUAL ESTIMATION OF THE YELLOW FLUORESCENT SUBSTANCES IN

11 LINES OF T. CONFUSUM

		Lines										
Spot	R_f	p dre cas	p lod	p rus	p^S	p'	es	r	dpe	rus	CFI-1	CFI-7
1	.05	+	+	+	+	+	+	+	+	+	+	+
2	.48	++	+	++	+	+	+	+	+	++	+	+
5	.60	+++	+++	+++	++	-	++	+	+	++	++	+
number chromatographed*		68	68	68	68	375	68	68	68	68	68	20

* Equal numbers of males and females

About one gm. portions of beetles were
homogenised in an ice-cooled Potter-type tissue
homogeniser. A 95% ethanol extraction was followed
by evaporation of the excess alcohol, and chromato-
graphy on Whatman No. 1 paper in tert-butanol:
pyridine: water (10:3:7). The faster moving
fraction was eluted in distilled water and
concentrated by vacuum evaporation at 60° C., then
chromatographed in n-butanol: acetic acid: water
(4:1:1). The faster fraction was eluted again and
neutralised in an icebath. The solvent was evaporated
under vacuum and the residue redissolved in a small
amount of water.

Salts were removed by subsequent chromatography
in water. Following elution the concentration of
the solute was brought to equality by dilution with
that of a similarly prepared pure sample of riboflavin.
Using a Unicam (SP. 500) spectrophotometer, an ultra-
violet absorption curve was prepared (Figure 1).
The identity of standard and sample appears evident.
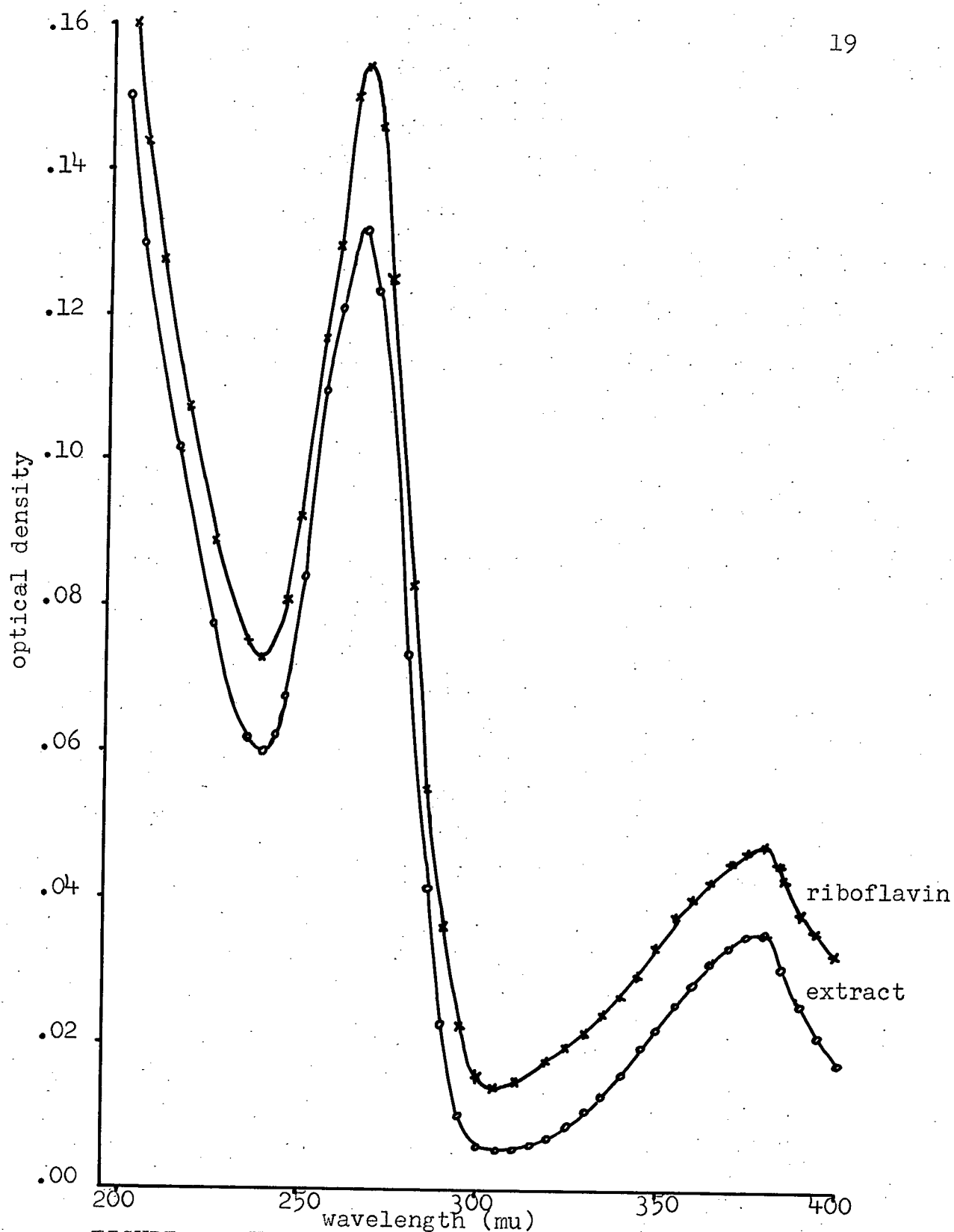
FIGURE 1. ULTRAVIOLET ABSORPTION-SPECTRUM OF PURE

RIBOFLAVIN AND RIBOFLAVIN EXTRACT, AT pH 7

2. Identification by means of chromatography

Whole beetle squashes were made on two
different grades of filter paper and chromatographed
in five different solvents using pure riboflavin
with each run for comparison. The solvents and the
results for each filter paper are shown in Table III.
Each value was the mean of ten or more determinations.
The migration properties of sample and standard appear
nearly identical under all conditions.

The use of decomposition products for the
identification of riboflavin has frequently been
employed in the past (Yagi, 1957: Cerletti and
Siliprandi, 1955). Using the methods of Yagi (1957)
lumiflavin and lumichrome were prepared by photolysis
of pure riboflavin as well as the Tribolium extract.
These, along with flavin-mononucleotide (FMN),
flavin-adenine-dinucleotide (FAD) and squashes of
whole beetles were chromatographed on Whatman No. 1.
filter paper using n-butanol: acetic acid: water
(4:1:5) for solvent. The results are shown in
Table IV. The R_f values of reference nucleotides
and decomposition products agree well with those of
the corresponding substance in the extract and whole
squashes of beetles.

TABLE III

MEAN R_f VALUES OF SAMPLE AND STANDARD IN VARIOUS

CHROMATOGRAPHIC SOLVENTS

paper	spot	solvents*				
		A	B	C	D	E
Whatman No. 1	spot 5	.32	.60	.37	.25	.32
	riboflavin	.31	.64	.36	.25	.33
Whatman No. 4	spot 5	.39	.64	.45	.27	.37
	riboflavin	.41	.66	.46	.27	.39

* A n-propanol; 1N. acetic acid (3:1)

  B tert-butanol; pyridine; water (10:3:7)

  C n-propanol; 1% aqueous ammnonia (2:1)

  D n-butanol; acetic acid; water (4:1:1)

  E n-butanol; acetic acid; water (4:1:5)

TABLE IV

R_f VALUES OF REFERENCE COMPOUNDS AND

SAMPLES

compound	reference cpd.	Tribolium extract	whole squashes	color of fluorescence
FAD	.05	-	.05	yellow
FMN	.13	-	.12	yellow
riboflavin	.32	.32	.31	yellow
lumiflavin	.46	.46	-	yellow
lumichrome	.63	.63	-	blue

3. Identification by means of electrophoresis

The method of Yagi (1957) was used for the electrophoretic separation of flavines. Sample squashes of different genotypes were run concurrently with FMN, FAD riboflavin and a mixture of these standards. Phosphate buffer of pH 8, and .05 M was used with constant current at 2.44 mA/cm. giving 400 volts. Whatman No. 3MM paper was found most suitable for supporting medium. Satisfactory separations were obtained during a two hour run. The results are shown diagrammatically in Figure 2.

$(+)$

$(-)$

riboflavin   FMN   FAD   FAD   +/+   p'/p' p^S/p^S p/p; dre/dre; cas/cas
                            FMN
                            riboflavin
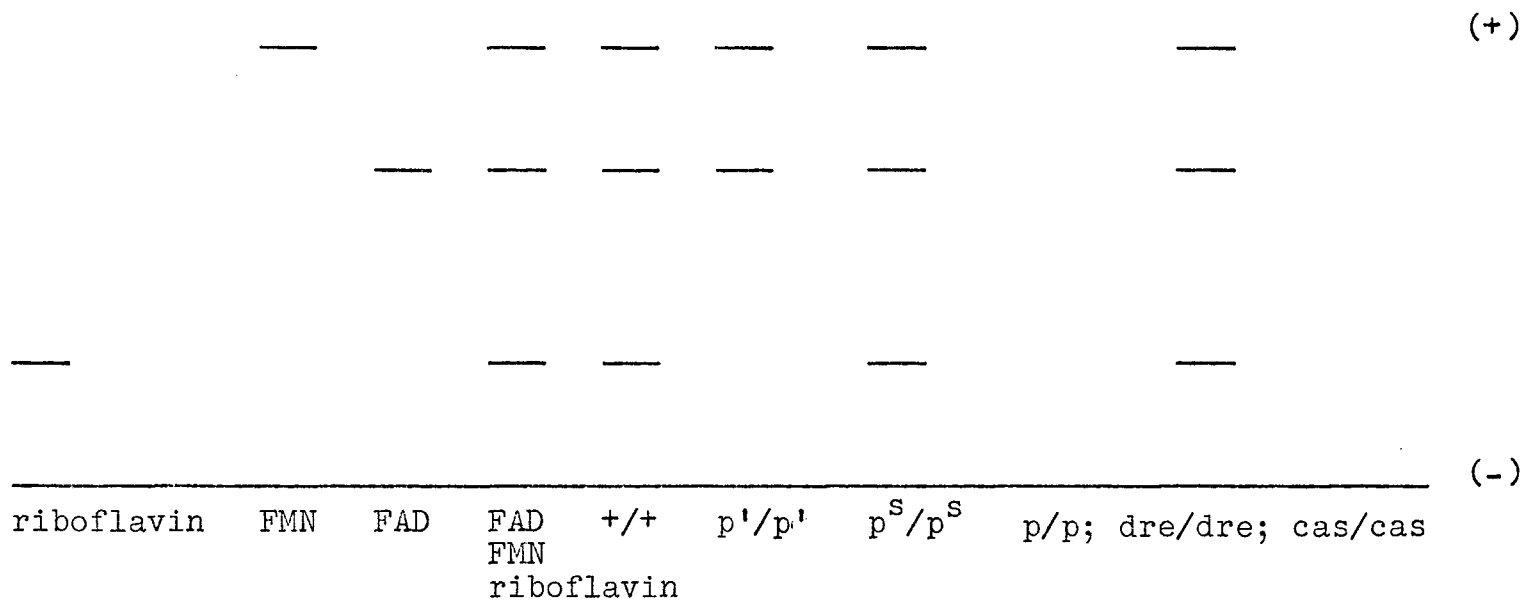
FIGURE 2.   PAPER ELECTROPHEROGRAM OF FLAVINE-MONONUCLEOTIDE (FMN),

FLAVIN-ADENINE-DINUCLEOTIDE (FAD), RIBOFLAVIN, A MIXTURE OF THESE,

AND WHOLE ANIMALS OF DIFFERENT GENOTYPES.

DISCUSSION

The identity of the yellow fluorescent substance, spot 5, isolated from _Tribolium confusum_ adults, with riboflavin was shown by the independent methods of spectrophotometry, chromatography and electrophoresis. In addition, the decomposition products of sample and standard riboflavin were also shown to be identical. It appears that none of the fluorescent spots obtained in chromatography (Table I and Table II) were decomposition products of riboflavin. Therefore, the riboflavin values subsequently obtained by the foregoing method are assumed to be representative of the actual amount of riboflavin present.

FMN and FAD were also identified by their R_f values and electrophoretic migration in order to distinguish them from free riboflavin and to observe any possible variation in their levels due to variation in the level of free riboflavin. Such concomitant variation might be expected on theoretical grounds since FMN and FAD derive the flavin part of the molecules from free riboflavin.

FMN and FAD are present in all six genotypes (Figure 2). Visual inspection indicated (Table II) that free riboflavin was present in varying quantities

in all lines except $\underline{p}'$ in which it was not detectable
by visual observation under ultraviolet light. Since
the eyes of the five mutant genotypes tested are
phenotypically pearl and $\underline{p}^S$ has been designated an allele
of $\underline{p}'$ (Sokoloff, 1963) it was of interest to note this
aberration on the part of the $\underline{p}'$ stock.

PART II. GENETIC ANALYSIS

A. Analysis of pearl

In the previous section it was shown by chromato-
graphy and electrophoresis that the p' mutant line had
no visible amount of riboflavin, whereas the p lod,
p rus, and p dre cas mutant lines possessed clearly
visible quantities (Table II., and Figure 2).

The question arose, was this riboflavinless phene
heritable, and if so, was it dependent or independent of
the p' gene. To answer this question the following cross
was made:

$$P_1 \qquad p'/p' \times +/+$$

$$F_1 \qquad \text{selfed}$$

The results of this cross showing the presence or
absence of riboflavin in the F_1 and F_2 progeny are
shown in Table V. The F_1 progeny were all phenotypically
W.T. and possessed riboflavin. In the F_2 progeny a ratio
of 3:1 (W.T. to pearl) would be expected. The riboflavinless
phene should only be present in pearls, if these two
characters are associated with the same gene. Analysis
of the progeny in Table V indicated the results are

TABLE V

THE DISTRIBUTION OF RIBOFLAVIN IN THE F_1 AND F_2 PROGENY*

FROM THE CROSS p'/p' x +/+

generation	phenotype	males	females	total	number chromatographed	number with riboflavin
F_1	wild	317	409	726	100	100
	pearl	-	-	-	-	-
F_2	wild	653	664	1317	100	100
	pearl	143	164	307	307	-

* Progeny from reciprocal crosses were pooled as they were not appreciably different.

significantly different from the expected 3:1 ratio
(x^2 = 32.18, d.f. = 1, P = .005). However, this was not
unexpected since Graham (1957) and Park (1937) showed
a loss of fitness of the pearl homozygotes. With over
300 progeny tested, the riboflavinless phene was always
associated with the p' gene. The possibility of linkage
was somewhat reduced by the fact that there were no wild
types which lacked riboflavin, nor were there any pearls
which had riboflavin. Recombination would be expected
to have produced such reciprocal phenotypes if linkage
was as little as .3.

Since p' and p lod have been separate breeding
populations presumably since they were first discovered,
it was considered possible that the accumulation of
different modifier backgrounds could have caused the
observed difference in the riboflavin trait. To check
this alternative, recurrent selection for pearl was
practiced for two generations in the cross p'/p' x +/+.
Twenty progeny were chromatographed from each generation
and the riboflavin was measured fluorometrically. These
values are compared in Table VI with those obtained from
the p' base population (P_1), and F_1 heterozygotes (p'/+)
and the W.T.

TABLE VI

COMPARISON OF TOTAL RIBOFLAVIN (ug/mg FRESH WEIGHT)

OF TWO GENERATIONS OF RECURRENTLY

SELECTED p' FROM p'/p' x +/+, WITH THE PARENTS,

THE HETEROZYGOTES AND THE W.T.*

generation	genotype	sex	riboflavin ug/mg fresh weight	+ standard − error
P_1	p'/p'	male	.0050	.0005
		female	.0060	.0004
F_1	p'/+	male	.091	.004
		female	.063	.0009
F_2	p'/p'	male	.0057	.0006
		female	.0062	.0004
F_4	p'/p'	male	.0060	.0008
		female	.0059	.0004
−	+/+	male	.110	.004
		female	.078	.004

* Ten observations per sex

The values obtained for p' homozygotes must be
viewed with some caution as these measurements were
made at the limits of sensitivity of the fluorometer.
The results show that changing of the residual genotype
does not detectably alter the apparently riboflavinless
condition of p'/p' individuals. The W.T. heterozygote
p'/+, shows that at the level of the riboflavin phene,
the normal allele is incompletely dominant to p'.

B. Analysis of p lod.

p lod, although phenotypically similar to p',
has riboflavin (Table II, Figure 2). Since the p and p'
genes are supposedly identical alleles (Sokoloff, 1964)
the integrity of the positive riboflavin phenotype of
the p lod mutants was tested. The following cross was
made to test if a riboflavinless p lod mutant could be
obtained:

$$P_1 \quad p/p \; lod/lod \; x \; +/+$$

$$F_1 \quad\quad selfed$$

The results of this cross showing the presence or absence
of riboflavin in the F_1 and F_2 progeny are shown in Table
VII. The F_1 progeny were all W.T. and had riboflavin. In
the F_2 the expected phenotypic ratio of W.T. to pearl, to pearl

TABLE VII

THE DISTRIBUTION OF RIBOFLAVIN IN THE F_1 AND F_2

PROGENY* FROM THE CROSS p/p lod/lod x ++

generation	phenotype	males	females	total	expected ratio	number chromatographed	number with riboflavin
F_1	wild	202	189	391	–	50	50
F_2	wild	163	167	330	12	100	100
	pearl	34	56	90	3	90	90
	pearl, lod	8	10	18	1	18	18

* Progeny from reciprocal crosses were pooled as they were not appreciably different.

light ocular diaphragm is 12:3:1 since the p/+;lod/lod
and +/+;lod/lod genotypes are indistinguishable from
the W.T. The deviation from the expected ratio was not
significant (x^2 = 3.79, d.f. = 2, 0.1 P 0.25), and
all phenotypes were positive for riboflavin.

From these results it can tentatively be
concluded that the pearl gene in the p lod stock may
not be identical to the pearl gene in the p' stock as
suggested by Sokoloff (1964) and more recently by
Ackerman (1966). Therefore, it is suggested that the
apparently riboflavinless p' be re-designated "p^r".

C. Test for allelism between p^r and p

The differential response of the two pearl genes raised the question, what is the relationship between them if they are not alleles.

To answer this question, the following crosses were made;

1. P_1 p^r/p^r x p/p,lod/lod

    F_1 a. selfed

                 b. crossed with p^r/p^r

2. P_1 p^r/p^r x p/p,rus/rus

    F_1 as in cross 1. a. and b.

3. P_1 p^r/p^r x p/p,dre/dre,cas/cas

    F_1 as in cross 1. a. and b.

The results and analysis of these crosses are shown in Tables IX, X and XI. The F_1 progeny from all three crosses were wild type and positive for riboflavin. Therefore, p^r and p are not true alleles. None of the F_2 and backcross phenotypic ratios deviate significantly from expectations applicable in the case of segregation at a single locus. Hence it may be concluded that p^r and p are either closely linked loci with similar genetic functions, or heteroalleles capable (as described for Drosophila

TABLE IX

THE DISTRIBUTION OF RIBOFLAVIN IN THE F_1, F_2 AND BACKCROSS (BC_1)
PROGENY OF p^r/p^r x $p/p,lod/lod$

cross		phenotype	riboflavin present	absent	total	expected ratio	x^2	$<P<$
p^r/p^r x $p/p,lod/lod$	F_1	wild	100	–	1899*	–	–	– –
$p^r/p,lod/+$ selfed	F_2	wild	95** 99	– –	194	8		
		pearl	39 45	34 41	159	6	0.721	.5 .75
		pearl,lod	13 11	10 15	49	2		
p^r/p^r x $p^r/p,lod/+$	BC_1	wild	84 87	– –	171	1		
		pearl	– –	82 90	172	1	0.003	.9 .95
reciprocal	BC_1	wild	98 88	– –	186	1		
		pearl	– –	86 92	178	1	0.176	.5 .75

* Total progeny, out of which a sample of 100 was assayed for riboflavin.
** The top value refers to males, the bottom to females.

TABLE X

THE DISTRIBUTION OF RIBOFLAVIN IN THE F_1, F_2 AND BACKCROSS (BC_1)

PROGENY OF p^r/p^r x p/p,rus/rus

cross		phenotype	riboflavin present	absent	total	expected ratio	x^2	< P <
p^r/p^r x p/p,rus/rus	F_1	wild	100	-	777*	-	-	- -
p'/p,rus/+ selfed	F_2	non-pearl**	206*** 214	- -	420	1		
		pearl***	105 101	97 92	202 193	1	0.77	.25 .5
p^r/p^r x p^r/p,rus/+	BC_1	wild	60 71	- -	131	1		
		pearl	- -	59 63	122	1	0.320	.5 .75
reciprocal	BC_1	wild	103 106	- -	209	1		
		pearl	- -	98 115	213	1	0.038	.75 .9

* Total progeny, out of which a sample of 100 was assayed for riboflavin
** Wild types and <u>rus</u> homozygotes.
*** The top value refers to males, the bottom to females.

TABLE XI

THE DISTRIBUTION OF RIBOFLAVIN IN THE F_1, F_2 AND BACKCROSS (BC_1) PROGENY OF p^r/p^r x $p/p,dre/dre,cas/cas$

cross	phenotype	riboflavin present	absent	total	expected ratio	x^2	$<P<$	
p^r/p^r x F_1 $p/p,dre/dre,cas/cas$	wild	100	–	670*	–	–	–	–
$p^r/p,dre/+,cas/+$ F_2 selfed	non-pearl**	122*** 110	– –	232	1	0.021	.75	.9
	pearl	56 64	58 57	114 121	1			
p^r/p^r x BC_1 $p^r/p,dre/+,cas/+$	wild	71 68	– –	139	1			
	pearl	– –	65 72	137	1	0.014	.9	.95
reciprocal BC_1	wild	81 93	– –	174	1			
	pearl	– –	82 87	169	1	0.073	.75	.9

* Total progeny, out of which a sample of 100 was assayed for riboflavin
** Wild types and dre and cas homozygotes.
*** The top value refers to males, the bottom to females.

by Carlson, 1959) of apparently complete complementation
when heterozygous.

D. The synthesis of a double mutant; pearl, ruby spot

In an experiment unrelated to the subject of this
thesis, and prior to the results described in the fore-
going, the following crosses were made to synthesize the
double mutant p^r rus from p^r and rus :

$$P_1 \quad p^r/p^r \quad x \quad rus/rus$$

$$F_1 \qquad selfed$$

$$F_2 \quad rus/rus \text{ progeny were selected} \\ \text{and selfed}$$

$$F_3 \quad pearl \text{ progeny were selected}$$

Since p^r is epistatic to rus, it was necessary to select
for rus homozygotes (F_2) one generation prior to selecting
for p^r . In this way all pearl progeny in the F_3 ought
to be homozygous for p^r rus. Table XII. shows the results
of these crosses. The F_1 progeny were discarded after
a few days of mating, hence no data is available from them.
The F_2 progeny were counted but not sexed, however it was
noted that both sexes were present in each of the three
phenotypes. This generation gave the unexpected pheno-
typic ratio of 9 pearl: 6 ruby spot: 1 W.T., instead of

TABLE XII

THE F_1 AND F_2 PROGENY FROM p'/p' x rus/rus AND THE DISTRIBUTION

OF RIBOFLAVIN IN THE F_3 PROGENY FROM THE SELF OF THE F_2 rus/rus

generation	phenotype	males	females	total	expected ratio	number chromatographed	number with riboflavin
F_1	wild	*	*	*	-	-	-
F_2**	wild		11	11	9	-	-
	ruby spot		73	73	3	-	-
	pearl		118	118	4	-	-
F_3	ruby spot	361	373	734	-	20	20
	pearl	88	102	190	-	20	20

* Data unavailable.

** Progeny were not sexed.

the expected 9 W.T.: 3 ruby spot: 4 pearl. In an effort to explain the overabundance of pearl progeny the <u>rus</u> stock culture was checked for possible contamination with pearl, but no homozygotes were found among 209 adults. However, this does not entirely exclude the possibility of pearl genes existing in heterozygous combination. In the F_3 progeny ten male and ten female pearl and ruby spot adults were chromatographed to check for the presence of riboflavin. All possessed high levels. In the next generation an additional 100 individuals were checked. All were found to be positive for riboflavin. Apparently in terms of the riboflavin phene, "$\underline{p}^r$ <u>rus</u>" now appeared to behave as $\underline{p}$ <u>rus</u> which was imported from Berkeley. Since in section C. it was shown that $\underline{p}$ <u>rus</u> crossed with $\underline{p}^r$ produced all W.T. progeny and the riboflavinless phene appeared in half of the F_2 pearl progeny, it was of interest to know the phenotype of the following crosses;

1. "p^r/p^r rus/rus" x p^r/p^r

2. "p^r/p^r rus/rus" x p/p rus/rus

The results of these crosses are shown in Table XIII.

Contrary to expectation all F_1 progeny from cross 1. were W.T. and positive for riboflavin. The progeny

TABLE XIII

THE PHENOTYPE AND DISTRIBUTION OF RIBOFLAVIN OF

THE F_1 PROGENY* FROM THE CROSSES

"p^r/p^r rus/rus x p^r/p^r (1.)

AND "p^r/p^r rus/rus" x p/p rus/rus (2.)

cross	phenotype	males	females	total	number chromatographed	number with riboflavin
1.	wild	241	263	504	100	100
	pearl	-	-	-	-	-
2.	wild	-	-	-	-	-
	pearl	82	77	159	159	159

* Progeny from reciprocal crosses were pooled as they were not appreciably different.

from cross 2. however, were all pearl and had riboflavin.

The unusual results of these experiments indicated that there may be genetic factors operating reminiscent of a conversion-type system such as paramutation (Coe, 1966), which cannot at the present and from these results alone be explained.

E. Partial analysis of a possible third allele; $\underline{p}^S$

Sokoloff (1963) reported another pearl mutant which occurred spontaneously in the Berkeley synthetic W.T. strain. He found it to be allelic to the pearl described by Graham (1957). This new pearl he designated $\underline{p}^S$, after the pest infestation laboratory at Slough, England.

This pearl mutant only recently became available to this laboratory, consequently its analysis was limited However, it was thought of interest to include here what data is available.

The eye phenotype of $\underline{p}^S$ is indistinguishable from either $\underline{p}^r$ or $\underline{p}$. The riboflavin content of 10 day old male and female imagoes were visually

estimated from the chromatograms to be about the same as the W.T. (Table II.).

The following crosses were made in order to test p^s against p and p^r :

1. p^s/p^s x p^r/p^r

2. p^s/p^s x p/p rus/rus

Cross 1. produced 137 F_1 progeny; they were all W.T. and positive for riboflavin. Cross 2. largely failed as it only produced three progeny. It is suspected that a mite infection may have been the cause. The three progeny, however, were all phenotypically pearl and riboflavin positive.

The results of cross 1. show that p^r and p^s are not homoalleles. The results of cross 2. indicate that p and p^s are allelic, which has already been shown (Ackermann, 1966).

F.   Distribution and developmental variation of riboflavin

1.   Distribution of riboflavin

Based on the report of Drilhon and Busnel (1939), who demonstrated the presence of riboflavin in high concentrations in the Malpighian tubules of many insects, the pupae and adults were dissected in insect Ringer and the Malpighian tubules were chromatographed separately from the rest of the body. The results obtained from $\underline{p}^r$, $\underline{p}^S$ and W.T. control are given in Table XIV. These values were not corrected for weight as the Malpighian tubules were difficult to weigh.

The greatest concentration of riboflavin in W.T. and $\underline{p}^S$ pupae and adults was in the Malpighian tubules. In W.T. the riboflavin content of the Malpighian tubules and the body increased proportionally from pupae to adults, being about 85% and 15% of the total in both respectively. In $\underline{p}^S$ the total increase in riboflavin content from pupae to adults was mostly due to increase in the Malpighian tubules. Thus the two genotypes only differed in the level of riboflavin present in the Malpighian tubules, while the level in the body when the Malpighian tubules are removed was essentially the same.

TABLE XIV

DISTRIBUTION OF RIBOFLAVIN IN ug* IN THE PUPAE AND ADULTS OF

W.T. $\underline{p}^s$ AND $\underline{p}^r$ LINES

lines	number assayed	source	pupae ± S.E.		adults ± S.E.	
W.T.	10	Malpighian tubule	.106	.009	.136	.014
		body	.019	.002	.025	.003
$\underline{p}^s$	10	Malpighian tubule	.091	.007	.192	.014
		body	.020	.003	.023	.003
$\underline{p}^r$	10	Malpighian tubule	.011	.002	.003	.0006
		body	.029	.002	.006	.0006

* These values were not corrected for weight.

The pattern of distribution of riboflavin in
the p^r line was completely reversed compared to W.T.
and p^s. The Malpighian tubules of the pupae contained
only 28% of the total riboflavin although the concent-
ration in the body was slightly higher compared to W.T.
and p^s In the Malpighian tubules and body of adults,
however, the concentration of riboflavin was reduced
to near zero.

2. Developmental variation of riboflavin

Developmental differences in riboflavin concentration
among mutant lines of _Ephestia_ have been shown by
Caspari and Blomstrand (1958). Visually detectable
differences in the amount of riboflavin among mutant
lines in adult _Tribolium_ have already been shown in
this thesis (Table II.).

The following methods were used to examine and
compare the developmental stages of p^r and p^s to W.T.
Four samples each containing 50 eggs on the basis of
equivalence to average imagonal body weight, were
assayed for riboflavin. Since the sexes can only be
distinguished after pupation, a mean for the larvae
was obtained from ten individual determinations.
Separate determinations based on ten individuals of

each sex were obtained from the early and late pupae
and the adults, 1 day, 10 days and 30 days after
eclosion. The means of the developmental stages and their
standard errors are given in Table XV. In order to
better visualize the developmental and sex differences
the means are graphed in Figure 3.

The eggs of p^s and W.T. contained very nearly the
same amount of riboflavin but were significantly
different from the eggs of p^r in which riboflavin was
very much reduced. In the p^r and p^s genotypes
riboflavin was accumulated during the larval stages
at about the same rate. In the W.T. a slight reduction
during the larval stage was followed by a rapid rise
which took place during the short time interval prior
to pupation.

In the main the pupal stages of all genotypes
showed a general reduction in riboflavin concentration.
Since the Tribolium do not eat during this stage, this
decline agrees with the findings of Fraenkel and Blewett
(1947) that Tribolium cannot synthesize riboflavin.
The slight riboflavin increase in the p^s female pupae
was not significant.

In both W.T. and p^s the males had higher values
of riboflavin than the females. The greatest sexual

TABLE XV

DEVELOPMENTAL VARIATION OF RIBOFLAVIN IN W.T., $\underline{p}^s$ AND $\underline{p}^r$

LINES MEASURED IN ug/mg FRESH WEIGHT

	lines					
	W.T. $\pm$ S.E.		$\underline{p}^s \pm$ S.E.		$\underline{p}^r \pm$ S.E.	
eggs*	.044	.002**	.041	.001	.014	.002
last instar larvae	.043	.004	.051	.003	.022	.002
early pupae	.059	.004***	.051	.004	.017	.002
	.047	.003	.042	.004	.023	.002
late pupae	.054	.004	.048	.004	.017	.002
	.047	.004	.044	.005	.023	.002
day old adults	.078	.008	.050	.004	.015	.002
	.065	.005	.045	.004	.019	.003
10 day old adults	.110	.004	.093	.004	.005	.0005
	.078	.004	.081	.002	.006	.0004
30 day old adults	.131	.007	.109	.008	.005	.0007
	.101	.007	.096	.006	.006	.0007

* Four determinations, each of 50 eggs.
** Standard error for ten observations each.
***Upper values refer to males, lower ones to females.
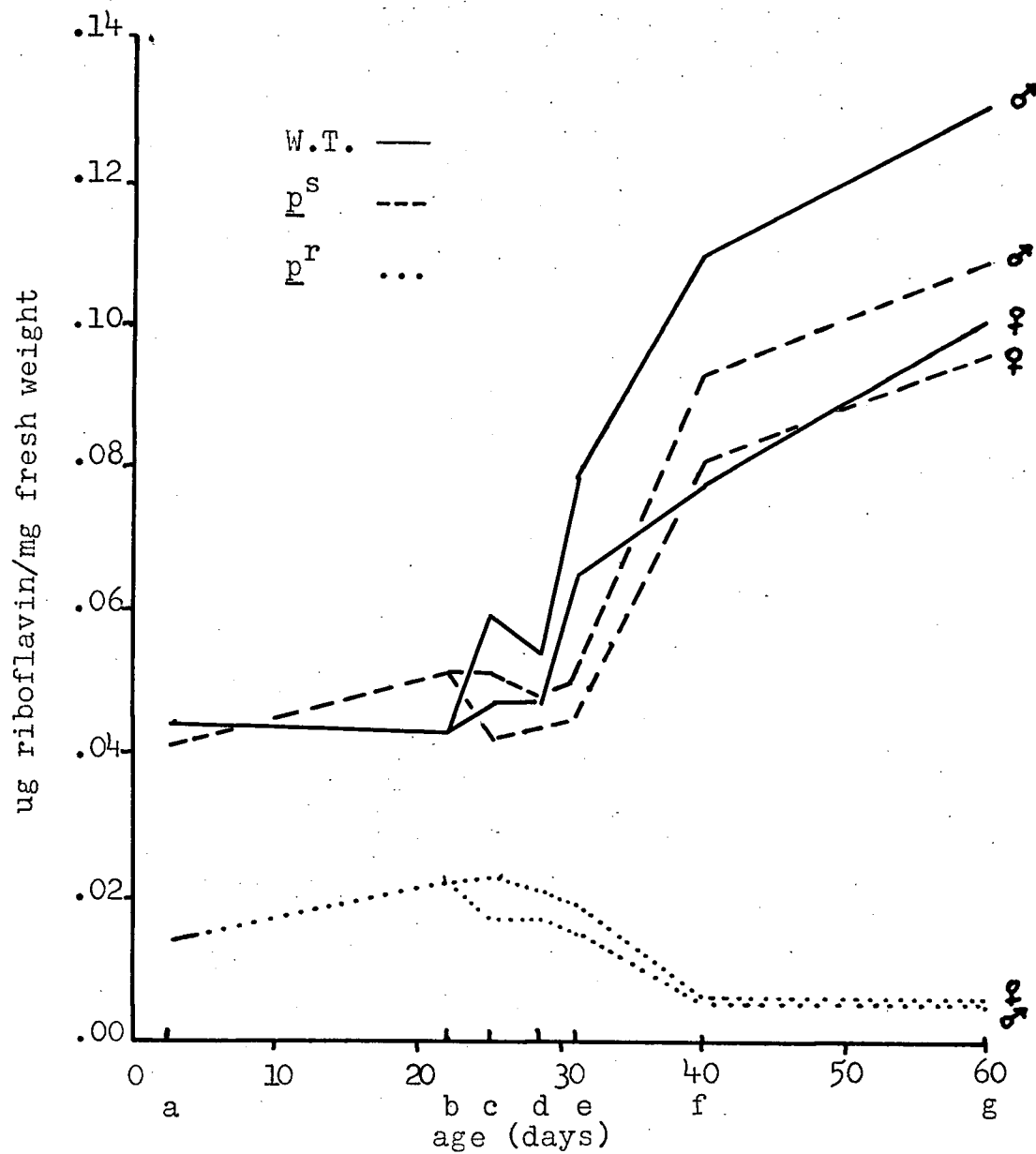
FIGURE 3. DEVELOPMENTAL VARIATION OF RIBOFLAVIN IN
$\underline{p}^r$, $\underline{p}^s$ AND W.T. MALES AND FEMALES. THE DEVELOPMENTAL
STAGES ARE: a, EGGS; b, LAST INSTAR LARVAE; c, EARLY,
d, LATE PUPAE; e, DAY OLD, f, 10 DAY OLD, g, 30 DAY
OLD ADULTS

dimorphism was noted in the 10 day old adults (t = 5.71, $P \leq .001$) and the 30 day old adults (t = 3.03, $P \leq .01$) of W.T. In $\underline{p}^S$ only the 10 day old adults showed significant sexual dimorphism for riboflavin (t = 2.86, $P \leq .02$).

The post-pupal increase in the level of riboflavin was slightly delayed in $\underline{p}^S$ compared to W.T. In $\underline{p}^r$ the downward trend of the pupal stage was preserved until 10 days after eclosion, following that only traces of riboflavin remained at a constant level. However, as previously noted, such low concentrations of riboflavin were measured at the limits of sensitivity of the fluorometer. Thus again the developmental effects of $\underline{p}^r$ were markedly different from those of $\underline{p}^S$ and W.T., while only smaller differences existed between the latter two.

G. Genetic differences in riboflavin concentration
and coloration of Malpighian tubules.

Genetic differences for concentration of riboflavin
in *Ephestia* have been shown by Caspari and Blomstrand
(1958) and in *Drosophila* by Beadle (1937) and Hubby (1962).
Kikkawa (1953) attributed changes in the coloration of
Malpighian tubules in *Bombyx* to the pleiotropic effects
of genes causing mutant eye color pigments.

This thesis has already shown that visually
detectable differences existed in the amount of riboflavin
between mutant lines (Table II.). These visual
differences were quantitated in nine eye color mutant
lines (p dre cas, p rus, p lod, p^s, p^r, es, rus,
r, dpe) and in two W.T. inbred lines (CFI-1 and CFI-7).
Concurrently the pigmentation of the Malpighian tubules
was also examined. The results are given in Table XVI.

Individuals of the pearl phenotype always had
colorless Malpighian tubules. The pigmentation of the
Malpighian tubules of non-pearl eye color mutants
ranged from brown in dpe and es, through speckled brown
and black in r and rus, to pronounced black in the two
W.T. inbred lines.

With the exception of p^r, the riboflavin concent-
ration was always higher in the males than in the

TABLE XVI

THE MEANS* OF RIBOFLAVIN CONCENTRATION IN ug/mg FRESH

WEIGHT FOR MALES AND FEMALES OF 11 LINES

(THEIR AVERAGE COMPARED BY DUNCAN'S NEW MULTIPLE RANGE

TEST) AND THE COLOR OF MALPIGHIAN TUBULES

line	males	females	average	color of Malpighian tubules
p dre cas	.133	.097	.115^{a**}	colorless
p rus	.105	.085	.095^{b}	colorless
p lod	.112	.086	.099^{b}	colorless
p^{s}	.093	.081	.087^{b}	colorless
p^{r} ***	.005	.006	.006	colorless
es	.098	.081	.089^{b}	brown
rus	.083	.074	.078^{b}	speckled black
r	.059	.047	.053^{d}	speckled brown to black
dpe	.051	.042	.046^{d}	brown
CFI-1	.110	.078	.094^{b}	black
CFI-7	.077	.054	.065^{c}	black

* Means are based on ten individuals per sex.
** Any two means having the same letter are not significantly different at P ≤ .05.
*** Not included in analysis of variance; its low riboflavin concentration has been demonstrated.

females. The p^r females had slightly more riboflavin
than the males, although as discussed earlier, these
values must be viewed with some caution.

The riboflavin content of non-pearl eye color
mutants was roughly proportional to the degree of
pigmentation of the eyes and the Malpighian tubules.
This relationship did not exist in the pearl eyed mutant
lines.

The extent of variation between the lines and
between the sexes, as well as the possibility of a
line by sex interaction was determined via the analysis
of variance. The p^r line was excluded from the analysis
of variance as it appeared to have no reliably measurable
amounts of riboflavin and its significant difference from
the rest of the lines has been demonstrated. The
results of the analysis of variance is given in Table
XVII. Highly significant differences existed between
lines. The difference between the sexes was also highly
significant, and there was no indication of a line by
sex interaction.

Duncan's New Multiple Range Test was used to
demonstrate the extent of line differences. The results
are shown in Table XVI. Any two means having the same
letter are not significantly different at the probability
level of .05. With the exception of the p dre cas line

TABLE XVII

ANALYSIS OF VARIANCE OF TOTAL RIBOFLAVIN

IN ug/mg FRESH WEIGHT IN

10 LINES OF T. confusum

source of variance	d.f.	mean squares
between lines	9	.00868**
between sexes	1	.01802**
line x sex	9	.00044
individuals/line x sex	180	.00031
total	199	

** Highly significant (P ≤ .01)

the means of the riboflavin positive pearl lines were
not significantly different from each other and from
the means of es, rus and CFI-1. It was of interest
to note that the means of the two inbred lines, which
originated from the same parents, were significantly
different. Genetic differences between, as well as
within highly inbred lines, have been shown before
for red blood cell antigens in the domestic fowl
(Briles, 1949).

H. Estimation of heritability of the riboflavin trait.

Prior to conducting genetic studies on populations
of a species, particularly with respect to a single
quantitative character, it is important to know of the
extent of different sources of phenotypic variation
(Lerner, 1958). Therefore, the relationship between an
individual's total riboflavin content and its body
weight was assessed. In addition the heritability of
the riboflavin trait was used to determine the pro-
portion of the total variance that was attributable to
the average effects of the genes which influenced this
trait.

The simple correlation coefficient (r) between individual body weight and riboflavin content was assessed using 90 males and 90 females. The male and female r values as well as the coefficient of determination (r^2) are shown in Table XVIII.

TABLE XVIII

CORRELATION OF 10 DAY ADULT WEIGHT

WITH RIBOFLAVIN CONCENTRATION IN THE p lod

MARKED STOCK

	males	females
simple correlation coefficient[1] (r)	.32**	.44**
coefficient of determination (r^2)	10%	23%

1 89 degrees of freedom.
** Highly significant ($P \leq .01$)

Only a small percentage of the variability of riboflavin concentration can be accounted for by the variability in body weight (r^2 = 10% in males; 23% in females). However, a highly significant association exists between the traits for both sexes. Consequently

the measurement of riboflavin on a per body weight basis was not imperative for males, but provides more accurate data in the case of females.

The progeny of p lod (random bred for over 36 generations) were used to estimate the heritability of the riboflavin trait. Thirteen males were each mated to four females. After five days, the males were removed and the females were each distributed into vials containing fresh medium. After 10 days the females were removed and discarded. Three sire groups each having three dams were chosen from the successful matings. While it was realized that the number of sires tested was quite low, it was assumed that the heritability estimates subsequently obtained would not be unduly affected. Ten days after eclosion the progeny were individually weighed, chromatographed and riboflavin was measured by the fluorometric method. Ten male and ten female progeny from each sire/dam mating were analyzed. The general method of Cockerham (1956; after Becker, 1964) was used for the statistical analysis. Standard errors of heritability estimates were calculated using the method of Dickerson (1960).

The statistical model used was as follows;

$$Y_{ijk} = u + s_i + d_{ij} + e_{ijk}$$

where Y_{ijk} : the record of the kth progeny of the jth dam mated to the ith sire; u : the common mean; s_i : the effect of the ith sire; d_{ij} : the effect of the jth dam mated to the ith sire, and e_{ijk} : the unexplained environmental and genetic deviations attributable to the kth progeny from the jth dam mated to the ith sire.

The average effects of the riboflavin trait for the sires, the dams and the sexes, can be assessed from the means which are presented in Table XIX. The means of the male progeny were consistently higher than those of the female progeny. The average contribution to the progeny was the same for sire A and sire B. With the exception of the progeny from the sire A - dam 1 family, the progeny of the dams within sire C had on the average the lowest riboflavin values. Hence the progeny of sire C were also much lower compared to the progeny of sires A and B.

To determine whether the sexual dimorphism exhibited by the means was statistically significant, the analysis of variance was performed (Table XX.).

TABLE XIX

MEANS OF TOTAL RIBOFLAVIN IN ug/mg FRESH WEIGHT OF THE PROGENY

FROM THE MATING OF THREE DAMS WITHIN EACH OF THREE SIRES

dams	sex	sire A 1	2	3	sire mean	sire B 1	2	3	sire mean	sire C 1	2	3	sire mean
progeny	M*	.108	.127	.147	.127	.111	.115	.134	.120	.091	.093	.105	.096
mean	F	.072	.098	.096	.089	.085	.108	.096	.096	.062	.070	.089	.074
dam mean		.090	.112	.121		.098	.111	.115		.076	.081	.097	
sire mean					.108				.108				.085

* M = males, F = females.

The F-test for the sex effect was highly significant (P $\leq$ .01), confirming the above observations.

TABLE XX

ANALYSIS OF VARIANCE BY SEX OF THE TOTAL

RIBOFLAVIN IN ug/mg FRESH WEIGHT IN THE

p/p lod/lod MARKED LINE

source of variance	d.f.	mean squares
sex	1	0.03606**
error	178	0.00061
total	179	

** P $\leq$ .01

Due to the highly significant sexual dimorphism heritability was estimated separately for each sex, as well as making a combined estimate. The analysis of variance of the male progeny, female progeny, combined sexes as well as the expected mean squares are presented in Table XXI. The F-test showed the dam contribution was highly significant in each of the three analyses. The sire contribution was not

TABLE XXI

ANALYSIS OF VARIANCE AND EXPECTED MEAN SQUARES OF TOTAL RIBOFLAVIN

IN ug/mg FRESH WEIGHT IN THE p/p lod/lod MARKED LINE

| source of variance | d.f. | mean squares | | | expected mean squares[1] |
		male progeny	female progeny	combined	
sires	2	0.00785	0.00398	0.01070	$\sigma^2 W + K_1 \sigma^2 D + K_2 \sigma^2 S$
dams/sires	6	0.00196**	0.00180**	0.00308**	$\sigma^2 W + K_1 \sigma^2 D$
individuals/ dams/sires	81[A]	0.00043	0.00034	0.00061	$\sigma^2 W$
total	89[B]				

1 When the sexes are analyzed separately, $K_1 = 10$, $K_2 = 30$.

  When the sexes are analyzed together, $K_1 = 20$, $K_2 = 60$.

** $P \leqq .01$.

A For the combined analysis it is 171; and B, 179.

significant in any of the three analyses. The

great amount of variation contributed by the dams may

have masked the sire effect.

The estimates of the components of variance are

presented in Table XXII. The sire component, σ_S^2

estimates $\frac{1}{4}\sigma_A^2$ (additive genetic variance) plus

decreasing fractions of higher order additive x

additive epistatic interactions. In the absence of

the latter effects, $4\sigma_S^2$ provides an estimate of

the additive genetic variance. The dam component σ_D^2

estimates $\frac{1}{4}\sigma_A^2 + \frac{1}{4}\sigma_{Dom}^2$ (dominance variance) plus

fractional higher order epistatic terms, plus σ_M^2

(variance due to maternal effects). Thus again

ignoring the epistatic interactions, $4\sigma_D^2$ includes σ_A^2,

σ_{Dom}^2 and $4\sigma_M^2$ (Falconer, 1960).

For each analysis the sire component of variance

(σ_S^2) contributed 25% in the male, 13% in the female

and 71% in the combined sexes towards the total variance

for the trait. Since the mean squares from which

these were estimated were not significant, the percentage

values indicated differences that were more easily

resolvable in terms of the total variation in the

three groups of progeny. The high estimate obtained

when the sexes were combined (71%) was probably due to

TABLE XXII

COMPONENTS OF VARIANCE ESTIMATED FROM THE ANALYSIS OF

VARIANCE OF TOTAL RIBOFLAVIN IN THE p/p lod/lod

MARKED   LINE

component	male progeny		female progeny		combined	
	value	% total	value	% total	value	% total
σ^2_S	0.000196	25.16	0.000073	13.06	0.000615	71.10
σ^2_D	0.000153	19.64	0.000146	26.12	0.000123	14.22
σ^2_W	0.000430	55.20	0.000340	60.82	0.000127	14.68
σ^2_T	0.000779	100.00	0.000559	100.00	0.000865	100.00

the marked sexual dimorphism. Differences among the dams contributed 20%, 26% and 14% in the male, female and combined analyses, respectively. The greater uniformity of these components indicated that fairly reliable estimates of heritability may be obtained from them.

Since epistatic interactions can usually be ignored without introducing any serious error (Falconer, 1960), an estimate of heritability in the narrow - sense from the sire component is given by $4\sigma_s^2/\sigma_T^2$ (where σ_T^2 is the total phenotypic variance, or $\sigma_s^2+\sigma_D^2+\sigma_w^2$) (Cockerham, 1956). A second estimate, from the dam component, $4\sigma_D^2/\sigma_T^2$ is biased by dominance and maternal effects, although as discussed above it may be more reliable in the present experiment, due to the greater number of dams tested.

For each analysis the estimates of heritability and their standard errors for the male, female and the combined sexes are given in Table XXIII. In general the heritability estimates were higher when the sexes were analysed separately, although the combined sex analysis gave more comparable estimates for the sire and dam components; 0.59 and 0.57 respectively. Maternal and dominance effects in the dam component

TABLE XXIII

ESTIMATES OF HERITABILITIES AND STANDARD ERRORS OF THE

RIBOFLAVIN TRAIT IN THE p/p lod/lod MARKED LINE

component	heritability ± standard error		
	male progeny	female progeny	combined
sire	1.00* ± 1.35	0.51 ± 0.34	0.59 ± 0.83
dam	0.78 ± 0.20	1.00 ± 0.26	0.57 ± 0.41
sire + dam	0.89 ± 0.06	0.77 ± 0.31	0.58 ± 0.63

* Estimates greater than 1.00 were assumed to be equal to 1.00

probably caused the estimate from the female progeny
to be elevated. The consistently higher levels of
riboflavin in the males and the low number of sires
used provides a possible explanation for the high
heritability based on the sire component which was
obtained in the analysis of the male progeny. Since
the heritability estimates based on the sire and the
dam components can only be considered equal in the
combined analysis, it is only here that the assumption
of $\sigma_S^2 = \sigma_D^2$ (Becker, 1964) was met for validly
combining the heritability estimates obtained
separately from the sires and the dams. Therefore
from the combined analysis the heritability estimate
of 0.58 may be taken as the best estimate.

Broadly interpreted the heritability estimates
in the male progeny indicated mostly additive genetic
variance, whereas the heritability estimates in the
female progeny indicated additive genetic variance as
well as dominance effects and possibly maternal effects.
The heritability estimates obtained from the combined
analysis were essentially the same and there was no
evidence for dominance effects or maternal effects.
A valid comparison of the heritabilities between the
sexes may be difficult to interpret on account of the
extensive sexual dimorphism for this trait.

DISCUSSION

It has been assumed in this thesis that the yellow fluorescent substance separated by chromatography was largely riboflavin. This assumption was based on identification via spectrophotometric, chromatographic and electrophoretic methods.

All developmental stages of individuals homozygous for a recessive pearl eye color gene, p^r (formerly called p, but renamed) were associated with a significant, heritable reduction in riboflavin content in the body and in the Malpighian tubules. The difference in riboflavin content of W.T. and p^r adults established by the chromatographic method was so large that it may be regarded as real.

The cross between p^r and W.T. gave a greatly distorted 3:1 ratio in the F_2 progeny. This suggested a reduction in fitness associated with the p^r gene. Since this effect was not observed in crosses involving the p gene, it may be postulated that the reduction in fitness was associated with the riboflavinless phene.

The genes, p and p^s appeared to be identical alleles as noted by Ackermann (1966). The complementation

demonstrated between $\underline{p}^r$ and $\underline{p}$, and between $\underline{p}^r$ and $\underline{p}^s$

was only surprising insofar as it appeared to contradict

an earlier technical note by Sokoloff in Tribolium

Information Bulletin, vol. 6, 1963. However, it may

be that Sokoloff was using only $\underline{p}$ and $\underline{p}^s$, being

unaware of the existence of the $\underline{p}^r$ line. The effect

of different environments could provide a possible

alternative explanation. Environment dependent

complementation has been demonstrated in microorganisms

(Garen and Garen, 1963; Schlesinger and Levinthal, 1963;

Fincham and Stadler, 1965; Fan, et al., 1966). The

rearing conditions of Sokoloff were 30° C and 70%

relative humidity compared to 32° C and 73% relative

humidity in this investigation. In addition his flour

was enriched with brewer's yeast, rather than Torula

yeast.

Fundamentally two different types of complementation

may be distinguished (Schlesinger and Levinthal, 1963).

Firstly, if the two mutations are in functionally

different regions of the genetic map (inter-cistronic)

then they will always complement (Benzer, 1957). This

situation can be explained if it is assumed that each

cistron determines the structure of a separate polypeptide

chain. The "normal" effect would be produced if each polypeptide chain was made in its normal configuration by at least one of the two chromosomes. Complete complementation would then be expected if the different polypeptide chains were part of a single enzyme or determined different enzymes in the same biochemical pathway. In the second type of complementation (intra-cistronic) the two mutations are in the same cistron and affect the same polypeptide chain. In this case, the "normal" effect would only be produced between some pairs of mutants. On the basis of the data presented here for the p^r gene versus the p and p^s genes, it is not possible to distinguish between inter- and intra-cistronic complementation. However, it was certain that p^r was complementary to p and p^s.

Brink (1956) demonstrated that regular heritable changes in the function of a gene can be directed by an allele. In 1958 he designated this change as a paramutation. Paramutation denotes the heritable nature of the change and the origination through heterozygous association of a sensitive (paramutable) allele with an inducing (paramutagenic) allele. This subject was recently reviewed by Coe (1966). The results, involving the apparent conversion of p^r to p in the synthesis of

the double mutant "p^r $\underline{rus}$" are indicative of this type of behaviour. Time did not permit repeating the lengthy mating scheme to verify these results. While paramutation has not yet been demonstrated in animals, it is not unreasonable to assume it exists.

The relationship between the time of disappearance of riboflavin in $\underline{p}^r$ and the time of deposition of pigments in the W.T. Malpighian tubules and eyes was of interest. A relationship was suggested by the fact that the time during which riboflavin disappears corresponds approximately to the period during which pigment synthesis would normally take place. Furthermore, mutants $\underline{p}^r$, $\underline{p}$ and $\underline{p}^S$ affect the amount of pigment in the Malpighian tubules. The $\underline{p}^r$ gene reduced the riboflavin content of the tubules, while $\underline{p}$ and $\underline{p}^S$ did not have the same effect. A similar situation exists in $\underline{Ephestia}$ where the gene $\underline{wa}$ functions in an analogous manner (Caspari, 1955).

The pleiotropic effects of the gene causing the pearl eye and the colorless Malpighian tubules may be resolved by the data of Wolsky and Zamora (1960). They suggested that the dark pigment in the eyes and Malpighian tubules of $\underline{Latheticus}$ $\underline{oryzae}$, a closely related species to $\underline{Tribolium}$, are probably ommochromes.

The genetic control of the synthesis of this group
of pigments has been partly documented in Ephestia
(Hadorn and Kuhn, 1953; Caspari, 1955; Viscontini, et
al., 1956; Reisener-Glasewald, 1956; Ziegler, 1961)
and Bombyx (Kikkawa, 1953; Ziegler, 1961). In
addition in nearly every case mutants affecting the
amount of ommochrome pigment have also been shown to
affect the amount of riboflavin.

The possibility that riboflavin may be involved
in one of the steps leading to ommochrome formation,
was suggested by the experiments of Charconnet-Harding,
et al., (1953) and by Mason (1953) with the rat. If
riboflavin deficient rats are fed tryptophan, urinary
excretion of anthranilic acid, kynurenic acid, xanthurenic
acid and occassionally of kynurenin and 3-hydroxykynurenin
is observed. These author suggested that riboflavin
deficiency may affect the steps leading from kynurenin
to 3-hydroxykynurenin. According to a summary by
Gilmour (1961), these steps occur in the chain of
reactions leading to ommochrome pigment. The riboflavin
content and the degree of pigmentation of the Malpighian
tubules and eyes appeared to be related in the non-pearl
eye color mutant lines. However in the p^r versus the p
and p^s lines no such relationship was noted.

The question arises whether the lack of
riboflavin in the $\underline{p}^r$ adults was due to an inhibition
of its formation, to its destruction or to an interference
with its absorption. Nutritional experiments have
shown that <u>Tribolium</u> <u>confusum</u> cannot survive in the
absence of riboflavin, suggesting that they are
unable to synthesize this vitamin (Fraenkel and Blewett,
1947). The chromatographic pattern of fluorescent
substances of $\underline{p}^r$ did not contain detectable amounts
of the usual degradation products, lumiflavin and
lumichrome. Therefore it was unlikely that these
products were formed if riboflavin was indeed degraded.
However, the possibility of bacterial degradation of
riboflavin to non-fluorescent compounds by a symbiotic
bacteria, such as <u>Pseudomonas</u> <u>riboflavinus</u>, so far
not demonstrated in <u>Tribolium</u>, may exist (Harkness,
1964). This possibility would have to be predicated on
an incompatibility system of <u>P</u>. <u>riboflavinus</u> with all
other strains used.

The possibility that the lack of riboflavin
storage in the Malpighian tubules and the reduced amount
of riboflavin in the body of $\underline{p}^r$ mutants may be due to
an inability to retain this vitamin. This was suggested
by the findings of Cowan, et al., 1964 , 1966, 1966a)
in the domestic fowl. The concentration·of free

riboflavin in the blood of a mutant strain of chickens homozygous for rd, failed to increase as it did in normal chickens at the onset of egg production (Maw, 1954; Boucher et al., 1959). Isotopic tracer studies have shown that free riboflavin was absorbed normally from the digestive tract (Cowan et al., 1964). It thus appeared that the gene conditioned event which prevented accumulation of riboflavin in the blood must take place after absorption. The possibility that riboflavin was destroyed in the tissues of the recessive hen was ruled out by subsequent evidence (Cowan et al., 1966) Recent data (Cowan et al., 1966a) showed that in the mutant laying hen, the recessive gene expressed itself by altering the renal reabsorptive mechanism for free riboflavin. As a result of this alteration, the intact vitamin was rapidly excreted via the urine, hence it failed to accumulate in the blood.

In the estimation of heritability of the amount of stored riboflavin in the p lod line it was assumed that the epistatic contributions to variance were relatively unimportant (Falconer, 1960). As far as it can be determined the strain used for the study had been kept as a closed population for a number of years. As Mayr (1963) points out, epistatic effects would be less

important and overdominant effects more important as sources of genetic variation in such populations. In natural populations which are continually subject to gene flow in the form of migration, a greater amount of epistasis would interact in many combinations. In closed populations there would be less need for such genes, and in fact, as Dobzhansky and Spassky (1962) have shown, closed populations can evolve overdominance.

The design used for the estimation of heritability is one of a number of methods available for this purpose (see Falconer, 1960). The hierarchical design (a full and half-sib relationship) provides a more accurate estimate of heritability than the parent-offspring regression methods. In addition an indication of the non-additive genetic variance and maternal effects can be noted.

The differences in the heritability estimates based on the combined sexes from that of the separate estimates from the male and female progeny can not be resolved using the available genetic models. In general, the significant differences shown between the mutant lines and especially between the two W.T. inbred lines and the high heritability estimates suggest that the amount of riboflavin present in _T. confusum_ is probably controlled by a small number of genes.

Unusual sex ratios were obtained in many of the crosses in that more females than males were produced. From a total of 8451 _Tribolium_, 4061 were males and 4390 were females. This was a highly significant deviation from the expected 1:1 ratio (P ≤ .005). There was no indication that the pearl eye mutation was a sex-linked character, nor was there any indication that the riboflavinless trait was associated with this divergent sex ratio. Similar sex-ratio differences (in pearl eyed stocks) in favour of females were evident in the data of Graham (1957) and were also noted in _T_. _castaneum_ by Park (1937). The possibility of genetic distortion of the sex ratio, as reported for other insects, is not unlikely (Peacock and Erickson, 1965; Hickey and Craig, 1966).

CONCLUSIONS AND SUMMARY

The identity of a yellow fluorescent substance
from *Tribolium confusum*, with riboflavin was shown by
the independent methods of spectrophotometry,
chromatography and electrophoresis. Two decomposition
products of riboflavin as well as two conjugated flavines
FMN and FAD, were identified.

The genetic control of riboflavin and its relation-
ship to some mutant eye color genes in the pleiotropic
hierarchy of gene effects was investigated in a series
of experiments involving more than 8000 *Tribolium*
from 11 genetically different lines. All developmental
stages of individuals homozygous for a recessive pearl
eye color gene, p^r (formerly called p, but renamed)
were associated with a significant, heritable reduction in
riboflavin content in the body. This reduction was
principally in the Malpighian tubules. In all of the
other lines tested the vitamin was accumulated in the
tubules in relatively high concentrations. The normal
allele of this gene was found to be incompletely dominant
with respect to the riboflavin phene. Changing the
residual genotype of p^r by means of two generations of
recurrent selection did not detectably alter its expression.

Two alleles of pearl ($\underline{p}$ and $\underline{p}^S$) were phenotypically identical to $\underline{p}^r$, except at the level of the riboflavin phene. Crosses of these alleles with $\underline{p}^r$ were shown to complement. The results, involving the apparent conversion of $\underline{p}^r$ to $\underline{p}$ in the synthesis of the double mutant "$\underline{p}^r$ $\underline{rus}$" were indicative of a paramutation-type mechanism.

Genetic differences in riboflavin content and the pigmentation of Malpighian tubules were shown. The riboflavin content of non-pearl eye color mutants was roughly proportional to the degree of pigmentation of the eyes and the Malpighian tubules. Individuals of the pearl phenotype ($\underline{p}^r$, $\underline{p}^S$, $\underline{p}$) always had colorless Malpighian tubules. The depth of pigmentation of adult eyes in the mutant lines was roughly proportional to the degree of pigmentation of the Malpighian tubules.

Sexual dimorphism for riboflavin content was observed in all lines tested. The quantitative parameters of variation of the riboflavin phene were examined and its heritability was found to be high by two different comparisons. In general an excess of female progeny from all crosses was observed.

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