GENETICS AND BIOCHEMISTRY OF "RED CELLS" IN *DROSOPHILA MELANOGASTER*

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ABSTRACT

Studies were made of the "red cells" strain of Drosophila melanogaster. "Red cells" flies are characterized by the presence of reddish pigment in certain fat cells.

A genetic analysis revealed that the fat cells are pigmented only when flies are homozygous for two mutant genes. The two genes are 4.4 crossover units apart on the second chromosome. They were separated, maintained in separate stocks and recombined. When they were recombined the "red cells" phenotype was again produced as it is in the original stock. The two mutant genes have been named red cells (rc) and lysine (lys).

With or without rc, the mutant gene, lys, causes flies which are homozygous for it to contain a greater quantity of the amino acid, lysine, than normal flies. In experiments with injection of radioactive lysine, over a period of eight hours normal flies converted thirteen times more lysine into carbon dioxide than did lys flies.

The hypothesis was offered that lys is a mutation of a gene which is important in the degradation of lysine. The mutation causes an impairment in the processes by which lysine is degraded, therefore lysine tends to accumulate in the flies if they ingest more lysine than is required in protein synthesis.

Attempts were made to localize the position of the step at which lys impairs the degradation of lysine.

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PART I

GENETICS OF "RED CELLS" IN DROSOPHILA MELANOGASTER

1. Introduction

A. Multifactorial Characters in Drosophila

While most mutant characters of Drosophila melanogaster have been found to be due to a single mutant gene, not a few involve a complex of genes. By selection, modifiers of many phenotypes may be accumulated. For example, Morgan (1) selected a stock of Notch until the notching in the wings, characteristic of this mutant, was almost eliminated. Tests showed that one or more third chromosome modifiers were responsible for the alteration in phenotype. In the course of the experiment an extreme Notch was discovered. It was shown to be due to a modifier in the X-chromosome.

Beaded, a dominant in chromosome III was also subject to alteration of phenotype as a result of selection. Muller (2) out-crossed Beaded to other strains and extracted factors which tended to intensify or suppress the Beaded phenotype.

Bridges (4) studied eight modifiers of eosin eye color. Seven tended to lighten the color of eosin eyes and one darkened it.

Several modifying genes like those mentioned above have been studied and listed in Bridges and Brehme (3). Among them are enhancers of Minutes, Star and Notch and suppressors of

black, deltex, forked, Hairless, Hairy-wing, purple, Star, vermilion and veinlet.

True digenic characters are more rare in Drosophila. The vortex phenotype is a character which is primarily digenic, but is modified by other genes (5). The principal gene is dumpy-vortex ($\underline{dp^v}$) in chromosome II and the second gene vortex-3 ($\underline{vo\text{-}3}$) is in chromosome III. Neither gene has a phenotype without the other. When both genes are homozygous nearly all flies show funnel-like vortices on the dorsal thorax. The microchaetae are arranged in whorls around the funnels. The gene, $\underline{vo\text{-}3}$, is semi-dominant and 20% of the females heterozygous for it and homozygous for $\underline{dp^v}$ have vortices. A third mutant was isolated which when heterozygous increased the expression of vortex so that nearly all $\underline{dp^v}$ $\underline{vo\text{-}3}$ / $\underline{dp^v}$ + females have vortices. The mutant Streak suppresses the vortex character so that even when flies are homozygous for $\underline{dp^v}$ and $\underline{vo\text{-}3}$ they do not develop vortices. A fifth factor near Streak reverses the inhibition of Streak to a considerable extent.

The sex-linked recessive mutant, divers-2, is dependent upon combination with yellow for expression (3). Divers-2 by itself appears wild type. The combination with yellow gives spirally curled wings. Divers-1 also has strongly curled wings when with yellow, but it has a phenotype without yellow. The wings are shorter and darker, the postscutellar bristles are curved in, the body is smaller than normal and fertility and viability are low. Divers-1 is modified by

mutants other than yellow. With forked the wings are crumpled and flies with both divers-1 and scute are extremely inviable.

Ski is a true digenic character (6). It is the result of an interaction between a recessive gene on the third chromosome and a dominant on the second chromosome. Neither gene produces a detectable phenotype when it is without the other. In the mutant phenotype of ski the wings are bent upward at the tips. The appearance closely resembles Curly or jaunty.

A very clear-cut and highly specific interaction between genes is that between prune and Prune-killer (7). Prune is a sex-linked mutant with good viability. The eye color of prune is dark translucent brown. In terms of its effects on the pigments, most of the red pigment is removed; but the other pterins are unaffected and the brown pigment is unaffected. Prune-killer is on chromosome III. It has no detectable phenotype when by itself. It is fully viable in the homozygous state and was originally discovered homozygous in a stock of S / Ins (2L + 2R)Cy, Cy E-S.

Prune-killer acts as a dominant and kills larvae that are homozygous or hemizygous for prune. They die in the middle of the second instar. Several alleles of prune were tested and all are killed by Prune-killer. No other eye color mutant is known to be killed by Prune-killer and no combination of prune with another eye color will protect it from the action of Prune-killer.

B. The Red Cells Phenotype

Drosophila melanogaster with red cells were first discovered by Lewis (8) in the stock of the mutant, spineless, kept in the stock collection of the California Institute of Technology. Individuals with red cells were selected and mated to each other to establish a pure breeding red cells strain. Jones and Lewis (9) investigated the location, histology and cytology of the red pigmented cells.

Adults and late pupae have red cells under the cuticle of the head, thorax, abdomen, and legs. They are most numerous and conspicuous in the head and thorax. In the head they are distributed rather randomly. In the thorax most are arranged in irregular rows along the dorsal midline of the mesonotum and scutellum. Less prominent rows occur on either side of the main rows, and a few individual cells and small clusters are scattered throughout the thorax. Even though they often are present in groups they are not connected to each other nor are they attached to surrounding tissue.

The red cells are round or ovoid and have a diameter of about 20 microns. They have a single round nucleus. The pigment is not distributed uniformly throughout the cell, but is confined to granules. The granules range in size from 0.5 to 4 microns in diameter. A cell may have from one to as many as fifty granules.

The pigmented cells also contain non-refringent droplets and other cytoplasmic inclusions. Staining with Sudan III

indicates that the droplets are fat containing. Some deposits are Bauer-positive, indicating that polysaccharide is present.

The same type of cell with cytoplasmic inclusions, including fat droplets and polysaccharide but without granules containing red pigment, can be identified in wild-type flies. Apparently the red cells are abnormal only in that the pigment is present. Miller (10) suggests that the vacuoles found in these cells may also contain waste products accumulated during pupation and correspond to the urate cells described in other insects. These cells are formed in the pupa and disappear in adult flies a few days after eclosion like urate cells. When the cells contain red pigment their disappearance may be followed. The cells do not disintegrate, but rather they shrink. In old adults the red cell is only a pinpoint of red and barely discernible under the dissecting microscope.

The pigment seems to be related to or perhaps identical to the brown eye pigment. When the genetic factors for "red cells" are combined with a mutant like vermilion, cinnabar, or scarlet which prevents the formation of the brown eye pigment, the fat cells do not become pigmented. When formation of the red pteridine pigment is prevented by the mutant brown, the brown eye pigment is formed and the red cells are present in the other parts of the body. The fact that the pigment appears red may be due to the different location rather than a chemical difference between it and

the brown eye pigment.

The cells do not become pigmented by contact with the eyes or from material from the eyes. Flies which are eyeless as the result of combination with the mutant, Microcephalus, possess the same pattern of pigmented fat cells as the strain with normal eyes.

Prior to the study described in this report some of the genetic aspects of "red cells" were known. It was known to be a recessive character. The factor (now known to be factors) was assigned a position on the left arm of the second chromosome between Sternoplural and Jammed.

At the start of this work on "red cells," the main interest was in the reasons for the occurrence of what appears to be brown eye pigment in the fat cells. It was thought that this phenomenon might have bearing on one of the problems of differentiation. That problem is the one of the relationship of genes to differentiation. From what we know about the mitotic process in cell division, all of the many kinds of cells in an organism have the identical gene content. It appears that the cells which make up the eye contain the same genes as those in a fat cell or any cell in the body. Yet in a normal animal only the eyes become pigmented with the tryptophan-derived brown pigment. In "red cells" we apparently have a situation in which the rigid control normally exercised over the site of pigmentation has

been loosened. For these reasons a study of "red cells" was begun. As will be reported in the following pages obstacles to investigation of that problem are present in this strain.

2. Experiments, Results and Discussion

A. Injection of Larval Fat Bodies

The first experiments performed were transplantation of larval fat bodies from "red cells" donors to wild-type hosts. The adults of the host larvae were examined for pigmented cells. The experiment was intended to see if these cells are derived from larval fat bodies and if the ability to become pigmented is autonomous.

Larval fat bodies are known to degenerate during pupation and the fat cells which become pigmented make their appearance at that time. For the cells to be pigmented even if they were derived from larval fat bodies the process would have to be autonomous, that is, not affected by the host.

The technique for fat body transplantation used in these experiments is essentially that described by Beadle and Ephrussi (11). Both donor and host are migrating larvae. The donor is dissected in insect Ringer's solution and fat bodies of the proper size isolated. The fat body to be transplanted is drawn up into a glass needle attached by a length of polyethylene catheter tubing to a hypodermic syringe.

In preparation for the operation the host is first etherized. The host is held down firmly and the glass needle inserted into the posterior half of the larva. It may be held down in one of three ways: holding it down with a forceps, drying it with an air jet so that it adheres to a slide, or placing it on the sticky side of Scotch tape. The fat body is expelled into the body cavity by exertion of pressure on the plunger of the syringe.

Of forty larvae satisfactorily injected with fat bodies, seventeen survived pupation and became adults. The resulting adults were carefully examined for signs of red cells through the cuticle and then dissected. In no case were any of the fat cells found to be pigmented.

The reciprocal experiment was performed in which wild-type fat bodies were injected into "red cells" larvae. The resulting adults had fewer pigmented fat cells than uninjected "red cells" animals. "Red cells" adults, however, which had been injected with "red cells" fat bodies as larvae also have fewer pigmented cells than uninjected animals. The operation itself appears to be responsible for a decrease in pigmented cells in an animal which is genetically "red cells." Food conditions also affect the number of red cells. The last flies from a culture of "red cells" often overlap wild type and addition of extra brewers yeast to a culture enhances the expression of "red cells."

B. Mosaics

When "red cells" is combined with eye color mutants which inhibit the formation of the brown pigment the fat cells are devoid of pigment. The sex-linked factor, white, is such a mutant. In hope of learning something about the origin or movement of the red pigmented cells, flies were produced which were homozygous for "red cells" but mosaic for white eye color. Such animals are obtained in the following way: Females that are homozygous for "red cells" and claret-non-disjunction are crossed to males that are yellow, white, split "red cells"; Claret-non-disjunction has peculiar effects on chromosome disjunction in the female (12, 13). In addition to non-disjunctional progeny, about five percent gynandromorphs are produced. Most of the gynandromorphs appear to be caused by the loss of a maternal X-chromosome in part of the fly. Gynandromorphs resulting from the above cross have a wild-type eye color on the female side and white eyes on the male side. The linkage of yellow andwhite makes it possible to determine the genotype of the hypodermis.

Many lateral gynandromorphs of the proper type were examined. In all cases the red pigmented cells were observable under the yellow hypodermis as well as under the wild-type hypodermis. Brightly pigmented fat cells even surround the white eye of the mosaic. The amount of pigmentation of fat cells was as great on the genetically white side as on

the wild side, and was as great as in flies wholly wild type for eye color.

Five posterior-anterior gynandromorphs were produced. The head, wings and thorax were yellow white split and male. The abdomen was female and normally colored. Again the yellow thorax and the head with white eyes were filled with red fat cells.

The results are clear-cut but do not have a clear-cut interpretation. The internal tissues in such mosaics are not necessarily identical genotypically to the hypoderm or the eyes. Cell lineage from an early cleavage is not predictable in Drosophila. The eyes and hypoderm are of ectodermal origin and would more likely be of the identical genotype on one side of the fly. The fat cells and other tissues of mesodermal origin are likely not to be of the same genotype as the hypoderm and eyes. In other words, the pigmented fat cells may not be genotypically white just because they lie under hypoderm that is genotypically white.

C. The Digenic Nature of "red cells"

A second phenotype of the "red cells" strain was discovered. These flies contain an abnormally high concentration of lysine. The lysine character was used in the genetic analysis of this strain. It will be more fully discussed in Part II.

A genetic factor, red cells (rc), responsible for "red cells" and located on the second chromosome between Sterno-

pleural and Jammed was described by Jones and Lewis (9). In order to check the location, more crossover tests were made. A chromosome carrying Sternopleural, "red cells" and Jammed was obtained and crossing over between it and an unmarked chromosome was studied.

Region 1 2

Cross 1 $\frac{\underline{Sp} \quad \text{"red cells"} \quad \underline{J}}{+ \qquad + \qquad +}$ X "red cells"

Table 1. Progeny of Cross 1.

Parentals	Recombinants	
	Region 1	Region 2
Sp "red cells" J	Sp + +	Sp "red cells" +
1711	103	102
+ + +	+ "red cells" J	+ + J
1879	21	197

Total 4031 Crossing-over percentage between Sp and J: 10.5%

These data are consistent with the interpretation that "red cells" lies between Sp and J. The best indication of this is that there are no flies in the classes, + "red cells" + and Sp + J, even though there is a number of recombinants between "red cells" and the markers. If "red cells" is in the

middle, those classes should be quite rare because it would require infrequent double cross overs to produce them.

The data look very strange because reciprocal cross-over classes should be equal, but they are not. There are 103 Sp + + compared to only 21 + "red cells" J and 102 Sp "red cells" + compared to 197 + + J. There is a deficiency of animals in the "red cells" classes. There are 123 "red cells" to 300 +.

The explanation cannot be one of viability because the viability of the parental triple mutant is only about 5% less than the viability of the parental wild type. Also a viability factor, unless it were exactly between them, would disturb the equality of the outside markers in the cross-over classes. The difference between 205 Sp J^+ and 218 Sp^+ J is not statistically significant.

Another experiment was performed using the Sp "red cells" J chromosome over either a Canton-S chromosome or one marked with dachs. Instead of making the testcross by crossing to "red cells," the heterozygous females were crossed to Canton-S males. The progeny could not be scored for "red cells" because the paternal chromosomes were wild type.

$$\text{Cross 2: } \frac{\text{Sp} \quad \text{"red cells"} \quad \text{J}}{+ \qquad + \qquad +} \text{ X Canton-S}$$

$$\text{Cross 3: } \frac{\text{Sp} \quad \text{"red cells"} \quad \text{J}}{+ \qquad \text{d} \qquad +} \text{ X Canton-S}$$

Table 2. Progeny of crosses 2 and 3

Parentals	Recombinants
Sp J	Sp +
2441	259
+ +	+ J
2532	263

Total 5495 Percentage crossing over: 9.5%

The male cross overs between Sp and J were selected and progeny tested by mating to y f:=; "red cells" virgin females in order to determine their genotype with respect to "red cells." Yellow attached-X females were used because the red cells are easier to see through a yellow cuticle. The female cross overs were discarded because they were not collected as virgins and the results of a progeny test would be uncertain unless special stocks had been synthesized.

This method involving a progeny test was used because the crossovers are not subjected to selection against "red cells" or other recessive viability factors and classification of "red cells" is more certain because in the progeny test many individuals carrying the same cross-over chromosome are examined. Sometimes "red cells" overlaps wild type, especially in late counts.

In addition to scoring for "red cells" a sample of three

flies bearing each crossover was squashed on filter paper and chromatographed in n-butyl alcohol: acetic acid: water (11:3:4). The chromatograms were sprayed with ninhydrin solution and heated to make the spots visible, and each cross over was scored for the accumulation of lysine.

Cross 4: y f:=; "red cells" X $\frac{\text{Recombinant from cross 2 or 3}}{+}$

Table 3. Results of progeny tests on samples of recombinants from crosses 2 and 3.
Data obtained from cross 4.

+ + + J 109	Sp lysine "red cells" + 52
+ lysine "red cells" J 10	Sp lysine + + 54
+ lysine + J 0	Sp + + + 11

Total 236

From the data in Table 3 it is evident that more than one factor is involved in the "red cells"-lysine phenotype. Fifty-four of the crossovers accumulate lysine and do not have red cells in their bodies. The lysine character appears to be due to a single factor; the difference between 116 lysine accumulators and 120 wild types is not statistically significant. The difference between 62 "red cells" flies and 174 wild-type flies is very significant. That difference may

mean that "red cells" is digenic. The influence of viability and penetrance on the data was eliminated by the use of the progeny test method and cannot be the cause of the discrepancy. No flies showed "red cells" without also having the lysine accumulation. It appears that the presence of lysine is necessary for the "red cells" phenotype.

The following hypothesis is consistent with the data: The accumulation of lysine is dependent upon a single gene, lysine (lys). The red cells phenotype is produced by an interaction between lysine and another gene, red cells (rc). For the fat cells to become pigmented, both lys and rc must be homozygous. If lys is not also homozygous, rc has no mutant phenotype.

From the data the relative location of the four loci in terms of crossover units may be calculated. Region 1 is between Sp and lys, region 2 is between lys and rc and region 3 is between rc and J.

Region 1: $\frac{11 + 10}{236}$ X 9.5 = .85 units

Region 2: $\frac{54}{54 + 52 + 11}$ X 9.5 = 4.4 units

Region 3: $\frac{52}{54 + 52 + 11}$ X 9.5 = 4.2 units

The following map of the four loci may be constructed:

Sp .85 lys 4.4 rc 4.2 J

The crossover chromosomes bearing J cannot be used for calculating the size of regions 2 and 3 because rc has no phenotype without lys and therefore rc J cannot be distinguished from + J.

Of the 236 crossovers tested in cross 4, 174 were derived from cross 3 and arose in a female that was heterozygous for dachs. To test for the presence of d in these chromosomes, sons of cross 4 which carried the recombinant chromosome were mated to females carrying d.

Cross 5: $\frac{d}{\text{Ins (2L + 2R)Cy, dp}} \quad \frac{}{\text{Cy Bl L}^4}$ X

$$\frac{\text{Recombinant from cross 3}}{\text{lys rc}}$$

The distribution of crossovers are listed in Table 4.

Table 4: Genotypes of crossovers from $\frac{\text{Sp}^1\ \text{lys}^2\ +^3\ \text{rc}^4\ \text{J}}{+\quad +\quad d\quad +\quad +}$ females.

Data from crosses 4 and 5

Region 1	Region 2	Region 3	Region 4
Sp + d + + (6)	Sp lys d + + (16)	Sp lys + + + (26)	Sp lys + rc + (37)
+ lys + rc J (8)	+ + + rc J (21)	+ + d rc J and (59)	+ + d + J

Dachs was placed between lys and rc because Sp lys occurs with and without d. The relative size of regions 2 and 3 may be calculated, but the crossovers bearing J may not be used because only the sum of the recombinants in regions 3 and 4 can be determined. Based on the recombinants bearing Sp, region 2 is .38 of the lys-rc region and region 3 is .62 of the lys-rc region. From the data in Table 3 it was calculated that the lys-rc region is 4.4 units. Therefore region 2 is 1.7 units and region 3 is 2.7 units.

The following map of the five loci may be constructed:

Sp .85 lys 1.7 d 2.7 rc 4.2 J

The map distance between Sp and J listed in Bridges and Brehme (4) is 19 units. In the above experiments the distances were 10.5 units (Cross 1) and 9.5 units (Crosses 2 and 3). To test the possibility that lyc, rc or another factor in that region was reducing crossing over another crossover test was performed in which neither lys nor rc was present. The sources of Sp and J^{34e} were the stocks of those mutants kept in the California Institute of Technology collection of stocks.

Cross 6: $\frac{Sp \quad +}{+ \quad J^{34e}}$ X Canton-S

Table 5. Progeny of Cross 6.

Parentals	Crossovers
Sp + 1167	Sp J 135
+ J^{34e} 1083	+ + 155

Total: 2540 Percentage of crossing over: 11.4%

Using Sternopleural and Jammed from another source the crossing over is again less than the standard map distance would indicate. Combining the data from crosses 1, 2, 3 and 6, the crossover value is 10.2% from a sample of 12,048 flies.

D. Establishment of homozygous $\underline{lys}^{+}$ $\underline{rc}$ and $\underline{lys}$ $\underline{rc}^{+}$ stocks.

Because J is viable as a homozygote, the formation of a $\underline{lys}^{+}$ $\underline{rc}$ stock was not difficult. Crossovers from cross 3 carrying J but not d were mated to Ins (2L + 2R)Cy, $\underline{dp}^{txI}$ Cy Bl $\underline{L}^{4}$ females. Males and females from that cross which were phenotypically J Bl L were selected and mated to each other. Among the progeny were individuals which were J $\underline{Bl}^{+}$ $\underline{L}^{+}$. These must be homozygous for rc as well as J. The chromosome did not carry d, therefore the crossover had to be between lys and d and the $\underline{d}^{+}$ to J region with rc in it remained intact.

Sp is lethal when homozygous and therefore the above technique could not be used. Crossovers from Cross 2 which carried Sp lys and the Canton-S allele of rc^+ were crossed to females which were d / SM5, al^2 Cy lt^v sp^2 to make females of the constitution Sp lys + / + + d. These females were crossed to males from a stock of Sp lys d / SM1, al^2 Cy sp^2. The non-Cy flies were examined and those that were d^+ were selected for further testing. They were made up of crossovers in two regions. A crossover between lys and d would result in a completely wild-type chromosome. The desired crossover is between Sp and lys. That chromosome would have lost Sp but still carry lys. Male crossovers of the type + (lys ?)+ / Sp lys d were mated to females which were dp Sp lys rc pr / Cy O, dp^{txI} Cy pr cn^2. Flies from this mating which were (lys ?) / dp Sp lys rc pr were chromatographed to test for the presence of the accumulation of lysine. If they did accumulate lysine, their brothers and sisters which were lys / Cy O, dp^{txI} Cy pr cn^2 were mated to each other and the lys homozygotes selected from among their progeny. Three lines of lys homozygotes were established.

E. Appearance of lys^+ rc J and lys rc^+ Stocks.

The mutant J is a rather extreme mutant and not fully viable. J also reduces fertility. The stock of rc J while not vigorous does survive.

Except for the Jammed phenotype the flies from the

stock appear to be completely wild type. Many flies were examined and no pigmented cells were ever found in the body cavity. They give a wild-type pattern of amino acids when chromatographed.

The lys homozygotes also appear entirely wild type by all criteria of a visible mutant. No red cells have been observed in these flies. Only when they are chromatographed is there any evidence that they are not normal. The lysine accumulation in lys flies does not appear to differ from the accumulation in the double recessive, lys rc, homozygotes.

F. The Origin of rc.

Apparently lys is due to a mutation which occurred in one of the stocks of spineless that are kept in the California Institute of Technology collection of Drosophila stocks. Two bottles of each stock are kept and only one of the two contained some "red cells" flies. Flies from the line free of "red cells" do not accumulate lysine and therefore do not possess lys.

Since rc has no phenotype when lys is not present it could not be determined if they carried rc except by combining the locus from that stock with lys.

Females of the constitution Sp lys d rc^{+} J / SM1, al^{2} Cy sp^{2} were crossed to males of the spineless stock whose rc locus will be designated (rc ?). Females that were

Sp lys d rc^{+} J / (rc ?) were crossed to d / Ins (2L + 2R)Cy, dp^{txI} Cy Bl L^{4}. The non-Bl non-L offspring were examined and the crossover males which were phenotypically only Sp were selected for further testing. The crossover could have been in one of two regions. If it had been between Sp and lys, the chromosome would carry (rc ?) but not lys, and without lys the presence of rc could not be determined. If the crossover had been between lys and d the crossover chromosome would carry Sp lys (rc ?) and that is the combination in which rc could be identified.

Forty-three males of the constitution Sp lys? (rc ?) / d were mated to yf:=; lys rc virgin females. Samples of the flies carrying Sp lys? (rc ?) / lys rc were chromatogramed and tested for the accumulation of lysine. Twenty-five of the forty-three crossovers did carry lys. Sp lys (rc ?) / lys rc flies were examined for the presence of red pigmented cells in their bodies. No red cells were found. It appears that the normal spineless stock does not carry rc and that rc is confined to the "red cells" stock of spineless. A mutation of rc may have occurred in the "red cells" stock or it may have been heterozygous when spineless was put into stock and it persisted in one line and not the other. Since Bridges found the mutant, spineless, in 1914 there has been a large number of fly generations and time for mutations to occur and be fixed in the stock.

G. Recombination of lys and rc.

If lys and rc are two separate loci and correctly located, they should be subject to recombination so that they are on the same chromosome again. When they are together and homozygous, the "red cells" phenotype should be present.

Females of the constitution, Sp lys d / rc J were synthesized. They were mated to Canton-S wild-type males. From this mating fifty-two crossover males, carrying Sp and J were mated to y f:=; lys rc females. Reconstitution of a chromosome carrying lys and rc would only result from crossovers in regions 2 and 3. These regions make up 47 per cent (4.4 / 9.4) of the total map distance between Sp and J. The expected number of crossovers of the lys rc type based on this distance is .47 x 52 or 24. Of the 52 tested males 20 were positive for "red cells." In these twenty pigmented cells were visible in the body to the same extent that are in the original "red cells" strain.

The fact that the recombination test was positive supports the digenic hypothesis of "red cells." The two genes can be separated, kept in isolation and recombined to give the original "red cells" phenotype.

H. Red cells-2

Drosophila from the stock of singed-4 kept in the California Institute of Technology collection of stocks also possess red pigmented cells in their bodies. This mutant discovered in the stock by R. Grell has tentatively been named red cells-2.

The pigment is located in the same cells as in lys rc and resembles that phenotype. There are some differences, however. Red cells-2 is temperature sensitive. At 17°C the pigmented cells are easily visible. At 25°C no red cells can be observed in this strain. It does not accumulate lysine nor any other amino acid. No biochemical difference of any kind has been found between rc-2 and wild type, except for the pigmentation of the fat cells.

The gene (or genes) responsible for this phenotype is on the second chromosome. Its exact location on the chromosome has not been determined, but cross-over tests indicate that it is between Sternopleural (22.0) and Lobe (72.0).

The F_1 generation of crosses between rc-2 and lys rc are wild type. On that basis rc and rc-2 are presumably not alleles.

PART II

BIOCHEMISTRY OF "RED CELLS"

1. Introduction

A. Biochemical Genetics of Drosophila melanogaster

The genetics of no other organism has been so thoroughly studied as that of Drosophila. Hundreds of mutants are known and located on linkage maps. The mutants are either "visible," affecting morphology or eye or body color, or they have lethal effects and kill the animal before it becomes an imago. Few of the many mutants effects known in Drosophila, however, can be defined in chemical terms.

In other organisms, fungi, bacteria, man and higher plants, a much larger body of knowledge has been accumulated about inherited chemical differences. In fungi and bacteria biochemical mutants which otherwise would be lethal can be made to survive by the addition of substances they require but are unable to make. The knowledge about man has been uncovered in medical work. Many of the inherited chemical differences in man involve excretion of abnormal substances in the urine. Much early work was done on plants because the plant pigments, particularly the anthocyanins and anthoxanthins are abundant, easily identified, easily brought into solution and within some species many differences can be detected simply by looking at flower color.

In Drosophila the pigments are convenient indicators of biochemical differences and most of the work with Drosophila on the biochemical level has been with mutants which affect eye color. The eye pigments are not simple to work with however. The chemical structure of the brown pigment is unknown. The red pigment is a pteridine, but the substituents on the rings are not known. Structural formulae have been assigned to four of the less highly colored pteridines of Drosophila (14,15).

Most eye color mutants have effects that cannot easily be explained in simple chemical terms. The mutants usually cause quantitative changes in both brown and red pigments, despite the fact that it seems unlikely that they have a common precursor.

The value of Drosophila to biochemical genetics should not be measured by the number of mutants that give clear-cut chemical differences. The result of biochemical work on the vermilion and cinnabar mutants was of the greatest theoretical importance and undoubtedly gave workers insight which led to the use of Neurospora to investigate biochemical steps by using induced mutations to block steps in synthetic pathways.

The work on the phenotypically identical mutants, vermilion and cinnabar, was started by Beadle and Ephrussi in 1935 (11). They developed a technique by which the optic disc of one larva could be implanted into another. After metamorphosis of the host the transplanted eye lies in the

abdominal cavity.

Eye discs of vermilion and cinnabar larvae transplanted into wild-type hosts developed eyes which were pigmented like wild type. This is in contrast to more than twenty other mutants which pigmented autonomously, that is the genotype of the disc and not the host determined the color of the eye (16). In reciprocal transplants, wild-type eyes developed autonomously in vermilion or cinnabar hosts.

Substances were postulated which diffuse into the transplanted v or cn eye from the hemolymph of the wild-type host and are responsible for the pigmentation in the transplant. In order to see if the effect on vermilion and cinnabar implants was caused by the same substance or two different substances, reciprocal transplantation were made between vermilion and cinnabar. Vermilion transplants in cinnabar hosts are wild type, but cinnabar eyes in vermilion are phenotypically mutant. There must be two substances. The hemolymph of vermilion contains neither substance, the hemolymph of cinnabar contains one substance and the wild type contains both. They were called the v^+ hormone and the cn^+ hormone, indicating their presence in flies carrying the wild allele of vermilion and cinnabar.

Hemolymph, malpighian tubes, and fat bodies of cinnabar or wild type when injected into vermilion larvae caused a modification of eye color (17,18). The lymph of several insects was also active (19). Characterization of the hormones from Calliphora pupae indicated that they had an amino

acid-like nature and were rather small and stable molecules (20).

Feeding experiments showed that tryptophan had an effect on vermilion eye color, but when it was injected into larvae the results were negative (21). Tryptophan was thought to be a precursor of the $\underline{v^+}$ hormone and tryptophan derivatives were tested to see if they were active. Among the substances tested by Butenandt et al. (22) was kynurenine. It gave positive results by both feeding and injection. An active substance produced by the action of a Bacillus on tryptophan was isolated by Tatum (23). It proved to be the sucrose ester of kynurenine. Thus the $\underline{v^+}$ substance was defined in chemical terms.

Butenandt and coworkers (24) went on to identify 3-hydroxykynurenine as the $\underline{cn^+}$ substance. It was isolated from extracts of Calliphora and later synthesized (25).

The following series of reactions may be written:

Tryptophan → kynurenine → 3-hydroxykynurenine → brown pigment

Vermilion prevents the formation of kynurenine from tryptophan and cinnabar prevents the conversion of kynurenine to 3-hydroxykynurenine. In both vermilion and cinnabar, accumulation of the substances preceding the blocks have been reported. Kikkawa (26) reports that in cinnabar flies there is an accumulation of a compound which reacts with a diazotizing reagent. He believes that compound to be kynurenine.

Green (27) reports that vermilion flies contain about five times more non-protein tryptophan than wild type or cinnabar flies. He finds the amount of protein-bound tryptophan to be equal in those genotypes. Those substances accumulate because the pathway through kynurenine is probably also the degradation pathway for tryptophan in Drosophila as it is in mammalian liver, Neurospora and some bacteria (28). The fly can neither make pigment nor degrade the substance to carbon dioxide, therefore the substance accumulates.

The eye color mutant, rosy-2, was found by Hadorn and Schwinck (29) to lack the pteridine, isoxanthopterin, and to have reduced amounts of the red pigment. On the other hand, there are abnormally large amounts of the "HB" pterins including 2-amino-4-hydroxypteridine.

Rosy-2 eyes were found to be non-autonomous when transplanted into wild-type hosts. This is like vermilion and cinnabar. It was postulated that there exists a $\underline{ry^+}$ hormone analogous to the $\underline{v^+}$ and the $\underline{cn^+}$ hormone. However the reciprocal transplants of wild-type eyes in rosy hosts were also non-autonomous and the hypothesis about a $\underline{ry^+}$ substance had to be discarded.

Transplantation of fat bodies, malpighian tubes, eyes or testes from wild type into a rosy host altered the phenotype of the hosts. The eyes of the hosts have more red pigment and also isoxanthopterin spots may be observed on chromatograms of them.

The biochemical basis of this mutant was made clear by the work of Forrest et al. (30). They homogenized wild-type pupae and found that extracts could oxidize 2-amino-4-hydroxypteridine to isoxanthopterin. Working without knowledge of the results obtained with rosy, they discovered two other mutants, maroon and maroon-like, which lack isoxanthopterin. The mutants which contain no isoxanthopterin do not contain the enzyme which is capable of forming it from its precursor, 2-amino-4-hydroxypteridine. Xanthine dehydrogenase from milk also carries out the transformation and the enzyme from Drosophila can oxidize hypoxanthine to form uric acid. The missing enzyme in these mutants is almost certainly xanthine dehydrogenase.

Rosy was also verified to be deficient in xanthine dehydrogenase activity. An accumulated substance in the malpighian tubes is hypoxanthine. It cannot be converted to uric acid in this mutant. The reason for the reduction in red pigment cannot be explained as yet.

Hadorn and his collaborators (31) have investigated the amino acid phenotype of several lethals of Drosophila melanogaster. These mutants allow the larvae to live to a certain point, but die before metamorphosis is complete.

Lethal-meander causes slow growing larvae that never enter pupation. They characteristically have trunks of the tracheal system which do not run straight along the length of the larva but tend to meander. Phenocopies of lethal-meander can be produced by starving genetically normal larvae.

The chromatographic patterns of the amino acids showed large differences between lme and wild type. Many of the essential amino acids such as valine, leucine, histidine, and lysine are completely absent from the hemolymph of lme. The non-essential ones, glycine, alanine, glutamic acid, glutamine, and serine are present in lme larvae. Glycine is present in a higher concentration than in wild type. The chromatographic as well as the morphological phenocopy is produced by starvation. However after feeding with caseine, starved wild-type larvae had much amino acid in their hemolymph while lme had none.

If free amino acids rather than caseine are fed to lme the hemolymph does become rich in amino acids as does wild type. Amino acids can be resorbed from the gut in lme as well as the normal. Injected valine and leucine is removed from the hemolymph of lme.

To test the hypothesis that the lme animals cannot digest proteins intestines of normal and lme larvae were homogenized. Extracts from normal animals released large amounts of amino acids from caseine but extracts of lme did not. Hadorn concludes that the deficiency of a proteolytic digestive enzyme is largely responsible for the phenotype of the mutant.

No report was made of one experiment that would be a decisive test of the proposed action of lme. If lme larvae develop any more normally on food which has been supplemented

with all essential amino acids than they do when the amino acids are bound in protein, it would indicate that it is the proteolytic enzyme that is the limiting factor.

B. The Degradation of Lysine

The degradative pathway of lysine has not been easily determined for any organism, and several postulated steps have not been verified experimentally. Lysine metabolism for a long time was an enigma and refactory to experimental attack.

There is no evidence to indicate that lysine is either glycogenic or ketogenic (32). In the rat amino groups from lysine could be found in glutamic acid, aspartic acid and arginine, but the reverse was not true (33). Amino groups from no other amino acid could be found in lysine nor could the nitrogen from ammonia be found in lysine. That would indicate that lysine can be deaminated, but its deamination product cannot be reaminated.

The first identification of a catabolite of lysine was by Borsook and his collaborators (34). They showed that in guinea pig liver homogenates α-aminoadipic acid is formed from lysine. They postulated that lysine is deaminated and oxidized in the -position to form that dicarboxylic acid. Borsook et al. (35) studied the fate of labeled α-amino-adipic acid and concluded that it is oxidatively deaminated to α-ketoadipic acid and the latter is decarboxylated to glutaric acid.

Rothstein and Miller (36) used the metabolite overloading technique and confirmed the sequence in the whole rat. They reported conversions of lysine to α-aminoadipic acid, glutaric acid, α-ketoglutaric acid and acetate.

In plants pipecolic acid-C^{14} was shown to be derived from lysine-C^{14} (37). Rothstein and Miller (38) demonstrated that lysine is converted to pipecolic acid in the rat. Using the metabolic overloading technique they recovered 20% of the radioactivity of the injected lysine in pipecolic acid. It was discovered that nitrogen of the -amino group of lysine is present in pipecolic acid. Therefore lysine must loose the α-amino group in the formation of pipecolic acid.

In the rat the formation of α-aminoadipic acid and glutaric acid from pipecolic acid was demonstrated (38). Glutaric acid is converted to α-ketoglutaric acid by way of α-hydroxyglutaric acid (39).

In the rat the step between lysine and pipecolic does not appear to be reversible to any large extent. Pipecolic acid will not substitute for lysine in the diet (40), nor will α-aminoadipic acid (41).

Schweet et al. (42) prepared dehydropipecolic acid from lysine using the L-amino acid oxidase present in Neurospora medium. When it is fed to Neurospora it was converted to pipecolic acid, but no evidence was obtained for the conversion of pipecolic acid to α-aminoadipic acid. In Neurospora dehydropipecolic acid can be converted back into lysine.

2. Experiments, Results and Discussion

A. Lysine Accumulation in "red cells."

In addition to the red pigment in the fat cells, the "red cells" strain has another phenotype. One dimensional chromatographs clearly show that "red cells" homozygotes possess a larger amount of a ninhydrin-positive substance than the wild type, Canton-S. F_1 hybrids between "red cells" and Canton-S appear wild type chromatographically as well as visibly.

The technique for chromatography used is similar to that employed by Hadorn and Mitchell (43). In preparation for chromatography, adult flies are first dipped into 95% ethanol and then boiled in water for one minute. If adults are not first dipped in ethanol, they tend to float on top of the boiling water. Pupae and larvae do not require the ethanol treatment but also must be boiled. The heat treatment coagulates proteins which cause streaking of the amino acids on the chromatograms. At the origin on Whatman #1 filter paper two or three animals are squashed at each point with the rounded end of a glass rod. The squashed animals are allowed to dry and the sheet of paper is rolled into a cylinder. The cylinder is placed in 1/4 inch of solvent on the bottom of a chromatography jar. About twelve hours are required for the solvent to ascend to 11-1/4 inches. After the solvent has reached the top of the paper, the chromatogram is removed from the jar and allowed to dry.

The chromatogram is sprayed with 0.25% ninhydrin and 2% pyridine in ethanol. After spraying it is heated for a few minutes in an oven at 100°C until the spots are clearly visible.

In the "red cells" strain the accumulation of an amino acid is evidenced by the dark color and large size of the spot resulting from the reaction of the amino acid with ninhydrin. It is present in larvae, pupae and adults of that strain. Several solvent systems were employed for chromotography and in all cases the Rf values of the dark ninhydrin spot are close to those in lysine. Amino acids run from squashed flies usually have a slightly lower Rf value than if the pure amino acid is spotted on the paper. Therefore the Rf's of amino acids from squashed flies do not exactly equal those of the standard. The identity of a substance can never be proven by chromatography, so other methods of identifying the accumulated substance were attempted.

An apparatus was assembled to do paper electrophoresis. The general design of the apparatus and the method was devised by Dr. H. K. Mitchell.

The flies are squashed mid-way between the ends of Whatmann 3 MM filter paper that previously has been dipped in buffer and allowed to equilibrate for one hour. The sheet of filter paper is sandwiched between two sheets of parafilm. (Several alternate layers of filter paper with samples and parafilm may be used.) The center portion of the paper where

the samples are is supported by a pane of glass and the ends of the paper are allowed to hang down into troughs of buffer. A pane of glass is placed on top of the sandwich and a jar filled with ice and water placed on top of the upper pane of glass to hold the sandwich flat and at the same time keep the system cool.

The electric field is supplied by a Lambda Model 28 power supply. It is operated at 325 volts and supplies a current of about 100 milliamperes. The electrodes are simply platinum wires. The cathode is submerged in one trough of buffer and the anode in the other.

The buffer that has consistently given the best results is a mixture of pyridine and acetic acid in water with a pH of 5.5. It is made by mixing 85 ml. pyridine and 40 ml. acetic acid with one liter of distilled water. After about two runs, the buffer acquires a yellow color and gives poor results. Distillation removes the coloring substance and restores the buffer to its original quality. The time for good separation of the basic and acidic from the neutral amino acids is about four hours.

During electrophoresis the substance accumulated in "red cells" flies, moves toward the cathode. It has the same mobility as lysine. The other basic amino acids, arginine, histidine and ornithine also have the same mobility. They are also the most difficult to distinguish from lysine chromatographically.

A test for lysine using a biological indicator is possible by using a lysineless mutant of Neurospora crassa. Lysineless Neurospora will grow only if lysine or a lysine precursor that it can use is present in the medium.

Twenty adult Canton-S females and twenty adult "red cells" females were squashed in lines along the origin of a chromatogram. On both ends of the sheet of paper three "red cells" females were squashed. The chromatograms were run in n-butyl alcohol: acetic acid: water (11:3:4).

After drying, vertical strips containing the patterns of the three "red cells" females were cut from both ends, sprayed with ninhydrin solution and heated to bring out the spots. The region of the accumulated substance was located on these test strips and the corresponding region marked on the main body of the chromatogram. Horizontal strips containing the desired region were cut from the chromatogram. The strips were eluted in hot water and the eluate added to Fries minimal medium. After autoclaving and cooling, flasks containing the solutions were inoculated with conidia of a lysineless mutant of Neurospora obtained from Mary Mitchell.

Growth on the "red cells" eluate was slight, but it was clearly a positive result. This rest indicates that there is more lysine in "red cells" animals than in Canton-S. The chromatography would tend to separate lysine from precursors which might give a false positive result. Considering

this and the other evidence, the accumulated amino acid seems to be lysine.

Table 6: Results of test of accumulation from "red cells" with lysineless Neurospora

Flask no.	Contents	Growth response
1.	10 ml. 2X minimal medium 10 ml. distilled water	no growth
2.	10 ml. 2X minimal medium 10 ml. distilled water 1 mg. L-lysine·HCl	abundant growth
3.	10 ml. 2X minimal medium 10 ml. eluate from Canton-S	no growth
4.	10 ml. 2X minimal medium 10 ml. eluate from "red cells"	growth (1 mg., dry wt.)

B. The Reason for the Accumulation of Lysine in Drosophila which Carry the Mutation, lys

Lysine is an essential amino acid. For Drosophila growth and development, lysine must be supplied in the diet (44). This means that Drosophila cannot synthesize the lysine that they require for the construction of proteins. Flies with the mutation, lys, which accumulate lysine must acquire it from an external source. It is not accumulated because they produce too much of it.

Flies grown on the culture medium used at the California Institute of Technology probably ingest more lysine than is

required in the synthesis of proteins. In normal flies the excess lysine is broken down and eliminated from the fly. All animals possess pathways by which excess amino acids are degraded, the nitrogen excreted and the carbon eliminated as carbon dioxide or as part of nitrogenous compounds such as urea or uric acid.

To explain the accumulation of lysine by Drosophila homozygous for the mutation, lys, the following hypothesis is offered: lys^+, the normal allele of lys, is necessary for the degradation of lysine. It may be responsible for an enzyme which catalyzes one of the steps in the degradation or it may have some other role. When lys^+ is mutated to lys, it cannot perform its function in the degradation of lysine. Lysine cannot be broken down and eliminated; therefore it accumulates in the body of the fly.

The test of the hypothesis that the mutation lys blocks lysine degradation was based on the following reasoning: When amino acids are degraded many of their carbon atoms are ultimately respired as carbon dioxide. If the degradation of an amino acid is blocked, its carbon atoms will not be converted into carbon dioxide. To perform the test, randomly labeled L-lysine C^{14} was injected into the body cavity of adult Drosophila and the amounts of radioactivity in the carbon dioxide produced by Canton-S wild type and lys rc compared.

Injection technique

The method for injection of liquids into Drosophila larvae and adults is a very simple one. Needles were made from one millimeter capillary tubing on the needle pulling device constructed for making needles for dissection of Neurospora asci. The needles may also be drawn by hand after heating the capillary tubing in a microflame, but the machine made needles have finer points. The blunt end of the needle is inserted into the end of a length of 1 mm I.D. polyethylene catheter tubing. The other end of the polyethylene tubing is attached to a hypodermic syringe.

The fluid to be injected is drawn up into the needle, the needle inserted into the body of the fly and the fluid forced into the body cavity through the needle by the exertion of pressure on the plunger of the syringe. Fluid is injected until the animal swells slightly. When the needle is withdrawn very little fluid leaks out of the animal if the needle has a sharp point which makes a clean hole.

This method does not deliver a measured amount of liquid but the average volume of liquid injected into an adult female is 0.47 microliters. This was determined by weighing a needle and filling it with water and weighing again. This gave the amount of water in the needle. The water was injected into Canton-S female flies and the total volume of the water was divided by the number of flies to give the volume per fly.

Collection of carbon dioxide

The carbon dioxide respired by flies was collected by passing air over the flies and bubbling it through barium hydroxide.

The injected flies were placed in a test tube with a small amount of glucose agar on the bottom. A two-hole stopper was placed in the mouth of the tube. In one hole was a glass tube connected to the air line and in the other hole was a glass tube leading to the bottom of a test tube containing half-saturated barium hydroxide.

As air is passed through the system, the carbon dioxide was precipitated in the tube of barium hydroxide as barium carbonate. At timed intervals the tube of barium hydroxide was replaced with a fresh one. The precipitated barium carbonate was carefully rinsed out of the tube and on to a copper planchet. The amount of radioactivity was determined with a gas flow counter.

Other fractions

At the end of the collection of carbon dioxide, usually eight hours, the flies were ground with sand and extracted. In Experiment II they were first extracted with ether. The water was frozen out of the ether with dry ice and the ether solution placed on a copper planchet.

The water soluble fraction is made up of the substances that are soluble in hot water. The residue is the material which was not soluble in or in the case of proteins precipitated

Table 7: Results of Experiment I

Twenty-five Canton-S and twenty-five lys rc males were injected with randomly labeled 0.1 M L-lysine-C^{14} with an activity of 1 mc/mM.

		Counts per minute	
Fraction	Time (minutes)	Canton-S	lys rc
CO_2	0 - 75	680	51
CO_2	75 - 170	460	52
CO_2	170 - 325	1400	150
CO_2	325 - 480	1500	23
Total CO_2	0 - 480	4040	276
water soluble	0 - 480	5300	15000
residue	0 - 480	1900	3400
Total	0 - 480	11240	18676

Table 8: Results of Experiment II

Twenty-five Canton-S and twenty-five lys rc males were injected with randomly labeled 0.1 M L-lysine-C^{14} with activity of 1 mc/mM.

		Counts per minute	
Fraction	Time (minutes)	Canton-S	lys rc
CO_2	0 - 120	1800	120
CO_2	120 - 270	2200	230
CO_2	270 - 480	1500	240
Total CO_2	0 - 480	5500	590
ether soluble	0 - 480	1300	326
water soluble	0 - 480	3200	330
residue	0 - 480	2600	1700
excretion	0 - 480	1100	2663
Total	0 - 480	13700	13620

Table 9: Fractions from Experiments I and II expressed as percentages of recovered counts

	Canton-S		lys rc	
Fraction 0 - 480 min.	Expt. I	Expt. II	Expt. I	Expt. II
CO_2	36%	40%	2%	4%
Water soluble	47	23	80	61
Residue	17	19	18	13
Ether soluble		10		2
Excretion		8		20

by boiling water. The excretion products were collected simply by washing out the tube which had contained the flies for the eight hours.

Discussion of results of Experiments I and II

The distribution of radioactivity recovered from Canton-S flies eight hours after injection of labeled lysine is quite different from the distribution from lys rc flies. Canton-S animals convert on the average 37.9% of the recovered carbon atoms from L-lysine into carbon dioxide, but lys rc convert only 3.0% of the lysine carbon atoms into carbon dioxide. In other terms, Canton-S flies produce 12.6 times more carbon dioxide in an eight hour period than do lys rc flies.

Most of the radioactivity not put into carbon dioxide by lys rc flies is in the water soluble fraction. Chromatography of this material indicates that by far the greatest amount of the radioactivity in this fraction is free lysine.

The radioactivity in the residue is probably an index of the lysine incorporated into protein. In Canton-S animals 18.2% and in lys rc animals 15.6% of the recovered counts are in the residue. Taking into account the variability of the other numbers, this difference is not significant. The amount of radioactive lysine incorporated into protein might be expected to be slightly lower in lys rc flies, for at the time of injection there is a larger pool of unlabeled lysine in lys rc flies and it would dilute the radioactive material with stable lysine.

The difference in the amount of radioactivity in the ether soluble fraction is not great but evidence which will be presented later suggests that the difference is real and the ether extract of Canton-S animals does contain more radioactive material.

The excretion product category is subject to error. If injected lysine were to leak out of the needle hole it would be included in this category. It may reflect a real difference nevertheless. Since free lysine remains at a high concentration in lys rc animals more of it might be excreted as lysine.

These experiments substantiate the hypothesis that lys is a mutation which prevents the degradation of lysine and therefore causes its accumulation. This argument would be much more convincing if the step at which the block occurs could be identified. The difficulty with the identification of a step is the fact that the pathway of lysine degradation is not known in Drosophila. It is not safe to extrapolate from rats to Drosophila and assume that the same pathway is in operation.

C. Transamination of Lysine

Since lysine itself is accumulated, it seems reasonable that the lys block is early in the pathway; perhaps in the first step. In the scheme worked out for the rat and Neurospora, the first step would be the deamination of the α-carbon of lysine. Transamination is one process by which other amino acids are deaminated and would be a logical way

to deaminate lysine, but only very small amounts of transamination of lysine with any α-keto acid have been reported (45). Attempts were made to detect transamination of lysine by extracts of Drosophila.

The method used to detect transamination was described by Feldman and Gunsalus (45). In transamination the acceptor of the amino group is usually α-ketoglutaric acid and sometimes pyruvic acid or oxaloacetic acid. The detection depends on the chromatographic identification of their respective amino acids, glutamic acid, alanine or aspartic acid. If keto acids are aminated only in the presence of lysine, the α-amino group must have been transferred from the lysine by a transaminase.

The Drosophila used in these experiments were from an inbred strain of Canton-S wild type. It is the same strain that was used for Experiments I and II.

Each test for transamination was made up of seven test tubes containing:

1. 1 ml. fly extract, 0.1 ml. .125M L-lysine, 0.1 ml. .25M keto glutarate
2. 1 ml. fly extract, 0.1 ml. .125M L-lysine,
3. 1 ml. fly extract, 0.1 ml. .25M keto glutarate
4. 1 ml. fly extract, 0.1 ml. .125M L-lysine, 0.1 ml. .25M pyruvate
5. 1 ml. fly extract, 0.1 ml. .25M pyruvate
6. 0.1 ml. .125M L-lysine, 0.1 ml. .25M keto glutarate
7. 0.1 ml. .125M L-lysine 0.1 ml. .25M pyruvate

Each tube also contained 0.1 ml. .5M phosphate buffer of the desired pH and 50 pyridoxal phosphate. Water was added to each tube to bring the final volume to 1.5 ml.

The fly extracts were either soluble proteins or particulates. The preparations were made by starting with approximately 10 grams of adult flies and homogenizing them with 15 ml. of buffer in a blender. Soluble proteins were obtained as the supernatant after centrifugation of the homogenates in a Spinco centrifuge at 105,000 x g. Particulates were prepared by first centrifuging down cells and debris and sedimenting the particulates at 105,000 x g.

The following preparations were examined for transaminase activity:

1. Soluble proteins in .1M phosphate buffer, pH 7.5
2. Soluble proteins in .1M phosphate buffer, pH 7.5, dialysed for 12 hours against 4 liters of .1M phosphate buffer, pH 7.5
3. Soluble proteins in .05M phosphate buffer, pH 5.0
4. Soluble proteins in .05M phosphate buffer, pH 6.0
5. Soluble proteins in .05M phosphate buffer, pH 7.0
6. Soluble proteins in .05M phosphate buffer, pH 8.3
7. Soluble proteins in .05M phosphate buffer, pH 7.5, proteins precipitated with saturated ammonium sulfate, dialysed against 4 liters of .05M phosphate buffer, pH 7.5
8. Particulates in .05M phosphate buffer, pH 5.0
9. Particulates in .05M phosphate buffer, pH 6.0
10. Particulates in .05M phosphate buffer, pH 7.0
11. Particulates in .05M phosphate buffer, pH 7.5
12. Particulates in .05M phosphate buffer, pH 8.3

Results of transamination experiments

The results of all of the experiments were negative. No evidence of a transaminase which transfers amino groups from lysine to either pyruvic acid or α-keto glutaric acid could be found.

A transaminase which catalyses the transfer of amino groups from alanine to α-keto glutaric acid was detected.

Activity was especially high in particulates.

D. L-Amino Acid Oxidase

Another way in which lysine could be oxidatively deaminated is by the action of L-amino acid oxidase. This enzyme brings about the formation of the α-keto acid and ammonia from an amino acid. In the process 1/2 mole of oxygen is taken up for every mole of ammonia that is produced. The uptake of oxygen is a convenient way to detect such a reaction. The reaction can be followed in a Warburg apparatus. An attempt was made to detect such an enzyme in a Drosophila preparation. It was found that the presence of lysine inhibited the background uptake of oxygen. Thus it is unlikely that such an enzyme is active in Drosophila.

E. Substituted Lysine

Since lysine does not transaminate nor undergo oxidative transamination readily it has been suggested that before deamination can occur the -nitrogen must be substituted. Neuberger and Sanger (46) found that -N substituted derivatives of lysine are more susceptible to deamination by L-amino acid oxidases than unsubstituted lysine. Meisters (47) found that the -N substituted α-keto derivatives of lysine will undergo transamination with glutamine in the presence of a rat liver preparation.

The decision to try the N acetyl derivative of lysine was based on several scraps of evidence. Schweet et al. (42)

found an acylated derivative of lysine, α-hydroxy ϵ -N acetylaminocaproic acid, in Neurospora cultures to which had been added dehydropipecolic acid. In the synthesis of ornithine the amino group of glutamic acid is masked to prevent cyclization of glutamicsemialdehyde. Ring closure would lead to proline instead of ornithine.

Synthesis of ϵ-N acetyllysine

ϵ-N acetyllysine was synthesized by the method described by Neuberger and Sanger (46). Excess basic $CuCO_3$ was added to a boiling solution of 2 g. of L-lysine HCl. To the solution was added 1.7 g. $Ba(OH)_2$ and the mixture was cooled in ice. Added alternately in small portions with shaking were 1.2 g. acetic anhydride and 2.2 g. $Ba(OH)_2$. To this was added 0.34 ml. H_2SO_4 and H_2S passed through the solution. The $BaSo_4$ and CuS were filtered off and care exercised so that there was no excess of Ba^{++} or SO_4^{--}. The solution was evaporated to dryness and the residue taken up in hot water. Hot absolute ethanol was added until the solution became cloudy. On cooling the N-acetyllysine crystallized out. The yield was 0.5 g.

Injection of -N acetyllysine into Drosophila

Injection of .1N solution of this material was made into Canton-S and lys rc adult females. It is ninhydrin positive and its disappearance could be followed by chromatography.

The flies were squashed on paper at 24 hour intervals and chromatographed. The N-acetyllysine persisted in the flies

for at least six days. It disappears much more slowly than lysine which is gone in 18 hours. No difference between Canton-S and lys rc flies could be detected with respect to the rate at which the -N-acetyllysine disappeared. The animals appeared to have more lysine than normal. Probably it is slowly deacylated and lysine is formed. -N acetyllysine does not seem to be an important intermediate in lysine degradation in Drosophila.

F. Decarboxylation of Lysine

In certain bacteria, for example Bacterium cadaveris, lysine is decarboxylated to form the diamine, cadaverine. The cadaverine is apparently excreted into the medium and is not further degraded. Amino acid decarboxylases have been reported for other amino acids in higher animals but not for lysine.

Injected into adult Drosophila, cadaverine is quite toxic in concentrations at which the flies tolerate amino acids. Injection of .01 M cadaverine is tolerated. It is retained in the animal for several days and not easily removed from the hemolymph. It is detected by first separating it from the amino acids by paper electrophoresis and spraying with ninhydrin. Cadaverine has a charge of +2 at pH 5.5 and therefore migrates toward the cathode at a faster rate than any amino acid.

Verylittle radioactivity from labeled lysine appears in the +2 spot. Five Canton-S and five lys rc adult female flies

were injected with randomly labeled L-lysine-C^{14}. After four hours they were crushed on paper and subjected to paper electrophoresis. The +1 and +2 spots were cut out and the material placed on a planchet. The counts were distributed in the following way:

	Counts per minute	
Spot	Canton-S	lys rc
+1 (includes lysine)	543	1210
+2 (includes cadaverine)	21	19

Lysine does not appear to be decarboxylated in Drosophila.

G. Injection of Pipecolic Acid and α-Aminoadipic Acid into Drosophila

In the rat both pipecolic acid and α-aminoadipic acid are catabolites of lysine. To see if they are metabolized in Drosophila, solutions of .05 M DL-pipecolic acid and .05 M L-α-aminoadipic acid were injected into adult Drosophila. Their disappearance was followed by killing the flies at intervals and observing their ninhydrin spots on paper chromatograms. Both compounds disappeared rapidly from flies and are not detectable twelve hours after injection. No difference between Canton-S and lys rc in the disappearance of these compounds was observed.

Drosophila apparently has the ability to rapidly degrade these compounds in contrast to -N-acetyllysine and cadaverine.

They could be catabolites of lysine, but it was not possible to demonstrate that they are actually formed from lysine by Drosophila.

H. Other Catabolites of Lysine

Attempts were made to find catabolites of lysine after injection of radioactive lysine into flies. At intervals after injection the flies were squashed on filter paper and chromatogrammed. The developed chromatogram was cut into vertical strips and the strips placed in a strip counter. Except for free lysine and protein, the only radioactivity was at the solvent front. It was not identified.

After separation by paper electrophoresis a radioactive spot can be detected at the position where the ions with a charge of -2 have migrated. Canton-S flies produce more radioactivity in that spot than lys rc flies. Two hours after injection of 15,000 counts per minute of lysine, 423 cpm were recovered in the -2 spot from Canton-S and 118 cpm in that spot from lys rc flies.

Attempts to identify the constituents of this spot by chromatography have failed. It probably is not a single substance. The dicarboxylic acids of the Krebs cycle would all migrate to that position, and some peptides may also be there. These dicarboxylic acids would be ether soluble and they could account for the difference in labeling between Canton-S and lys rc in Experiment II.

I. Hypothesis of the Action of Lethal Translucida

The mutant, lethal translucida, accumulates a large amount of hemolymph (48). The larvae and pupae are greatly swollen with liquid. They undergo only a partial metamorphosis.

On the average the amount of free amino acids is very high in ltr. The greatest increases are in serine-glycine, lysine-ornithine, threonine, glutamine, cystine, and hydroxyproline. Glutamic acid and aspartic acid are not detectable in ltr animals, but are present in normal. Alanine, proline, and tyrosine concentrations are also decreased in ltr larvae.

The amount of protein is reduced in ltr larvae. The total nitrogen content is about the same for both ltr and normal animals. Hadorn suggests that the reason for the accumulation of amino acids is due to a defect in protein synthesis. The amino acids cannot be used for protein synthesis and therefore accumulate in the hemolymph.

The writer, however, wishes to present an alternate explanation for the amino acid accumulation. If protein synthesis in general were disturbed, it seems doubtful that the animals would live as long as they do and enter into metamorphosis. Hadorn finds that imaginal discs of ltr develop normally if they are transplanted into normal animals. The organs can synthesize their own proteins if they are in the proper environment. The reduction in protein synthesis could well be a secondary effect which is the

indirect result of some other metabolic disturbance.

Amino acids can be injected into larvae or adults and they do not remain in the hemolymph at the initial high concentration but disappear rather rapidly. They can be introduced in quantities that must be greater than can be used for protein synthesis, yet they are removed from the system. It is obvious that they undergo degradation. Injection of radioactive lysine has proven that lysine is degraded in normal Drosophila. Much of the lysine is converted into labeled carbon dioxide.

It is interesting to notice that with the exception of glutamine, the amino acids which accumulate are those amino acids which are known not to participate readily in transamination. On the other hand, glutamic acid, aspartic acid, and alanine, the amino acids that are in a lower concentration in ltr, are the ones that are most active in transaminations.

It is proposed that the primary disturbance is in one of the processes that lead to the excretion of nitrogen. It may be a block in uric acid synthesis or a block in the formation of a compound which is necessary for uric acid synthesis. Glycine and glutamine, two of the compounds involved in uric acid synthesis are accumulated in ltr animals.

If the synthesis of uric acid were blocked it would also prevent the formation of the other purine, adenine, and guanine. They are constituents of nucleic acids. Adenine is

also a constituent of ATP and enzyme co-factors. Since Drosophila are normally reared on medium containing dried yeast, enough purines are probably supplied in the diet so that they need not be synthesized by the fly.

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